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CHROOCOCCIDIOPSIS FROM DESERT TO MARS

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1. Introduction

In 1986 R.M. Powers (1986), a master space writer, stated: "We will go to Mars – that is taken for granted. Our children, or our children's children, may not only go there, they may settle down and carry on as pioneers of the first really New World. The famous canals, the deserts, the immense canyons and looming volcanoes – and the dream of Mars – are all waiting for us on the Red Planet." Powers hypothesized that the first Mars habitants should be bacteria, cyanobacteria, fungi.

At that time we knew very little about Mars surface compared to what we know today, thanks to the numerous and successful explorations by means of the Surveyor USA, 1996; Orbiter and Lander, 1998–1999; Internazionale Mars, 1996; Mars Pathfinder 1996, USA; Planet B, 1998–1999, Japan; Mars 01, USA, Orbiter and Lander; Mars 01, 2001, Russia; Mars 03, USA, Orbiter and Lander; Mars surveyor 05, 2005 (Sawyer, 2001). In 2003 the European Space Agency's Mars Express orbiter and NASA's rovers, Spirit and Opportunity, went to Mars: they are still acquiring data sets revealing new information about the history of Martian water and the possibility that the planet once harboured habitable conditions (Bibring et al., 2006).

Fundamental problems in investigating on extinct or extant life on Mars, concern the presence of water and finding out to what extent living organisms may survive at its very low temperatures and atmosphere conditions. The collected data transmitted to Earth from the above-mentioned missions and the fascinating images of Mars, allowed a detailed reconstruction of the Red Planet surface; conjunctures were made about water as not an unknown element, at least in the past, on the planet. Scientists have now to ascertain if the astronomic and meteorological data about the actual conditions of Mars are compatible with the concept of life that we have on Earth (McKay et al., 1996). Once established that on Mars the actual conditions of water, temperature and atmosphere might be compatible with extreme life forms, which earthly organisms could colonize the Red Planet? About that, many other questions arise, such as life ever existed on Mars in the past or if some of the pre-existing organisms are still present somewhere; if terrestrial organisms could adapt to Mars conditions (Friedmann, 1986; Friedmann and Ocampo-Friedmann, 1995; Beaty et al., 2005).

This review is dedicated to the memory of Roseli Ocampo-Friedmann. She with E. Imre Friedmann pioneered researches on Chroococcidiopsis and life in extreme environments.

In fact, only organisms which are capable of surviving at very low temperatures and water shortage on Earth, could represent hypothetical habitants of Mars. Biologists and NASA researchers conjectured about life outside our planet: in order to provide answers to this question, they focused their interest on terrestrial organisms, living in extreme environments, such as hot deserts and Antarctic regions, originating a new research field called "exobiology" (Friedmann, 1993).

2. The Life in Hot and Cold Deserts

Friedmann (1971) along with Ocampo-Friedmann (1985) started the study of life conditions in the hot and dry deserts of Asia and Antarctica, and then extended their researches to other continents. They found out that *Chroococcidiopsis* constantly appears in extreme dry environments and wherever it survives, it is often the only photosynthetic organism present.

Life in the desert is interesting from a biological point of view: there are numerous ways by which organisms can settle down in these environments. Bell (1993) reported that the lithobionts are mainly microbionts, which live inhabiting rocky environments. They are distinguished by location in: *epilithics* dwelling on the rock surface; *hypolithics* forming biofilms at the stone–soil interface; *endolithics* colonizing microscopic fissures (*chasmoendoliths*) and structural cavities (*cryptoendoliths*) of rocks, and *euendoliths* actively boring into rocks. Cryptoendolithic microorganisms living inside rocks can provide a terrestrial model explaining what may have happened to life forms on Mars, when the planet became dry and cold. There are evidences of fossil traces of microbial rock colonization in Antarctica: similar structures might exist on Mars and may represent an easier target for life-detection systems, other than fossils of cellular structures. A paramount feature of the biology of Antarctic cryptoendoliths is their extraordinary slow growth, with exfoliation occurring when the microbial biomass reaches the carrying capacity of the cryptoendolithic habitat (Sun and Friedmann, 1999). Hypolithic communities containing a single *Chroococcidiopsis* morphospecies with heterotrophic associates were reported to colonize translucent stones along a gradient of aridity in the Atacama Desert in Chile (Warren-Rhodes et al., 2006). In the hyper-arid core of this desert, endolithic growth of *Chroococcidiopsis* and heterotrophic bacteria has been detected within halite rocks and representing the only life form present (Wierzchos et al., 2006). Among microorganisms able to withstand extreme conditions, the so called extremophiles (Rothschild and Mancinelli, 2001), *Chroococcidiopsis* become landmark for possible Mars inhabitants.

3. *Chroococcidiopsis* Cell Organization and Survival in Nature

Cryptoendoliths were first detected by Friedmann et al. (1967) in the Negev Desert. Later they were reported from the Middle East (Negev, Sinai), Central Asia (Gobi), North America (Sonora), South America (Chile), South Africa

(Natal) and McMurdo Dry Valleys (Antarctica). *Chroococcidiopsis* is a cyanobacterium that constantly appears in extremely dry, hot and cold places and wherever it survives, it is often the only photosynthetic organism present. This coccoid cyanobacterium was first reported from hot deserts together with *Gloeocapsa* (Friedmann, 1971). Rock-inhabiting communities in McMurdo Dry Valleys are wetted and metabolically active only for a total of 500–800 h per year (Ocampo-Friedmann et al., 1988); in the most arid areas of hot deserts, such as the Atacama Desert (Chile), instead, the number of metabolically active hours per year is considerably inferior to this (Warren-Rhodes et al., 2006). In these environments, biological and geological processes overlap, for this reason, the desert *Chroococcidiopsis* are thought to be extant representatives of "eoanhydrobiotes", the old desiccation-tolerant cells (Billi and Potts, 2002), able to enter a dormancy state at the desiccation onset and resume metabolic activities when water becomes available, a phenomenon known as anhydrobiosis (life without water).

Lithic *Chroococcidiopsis* has a complicated and variable life cycle with vegetative cells baeocytes (nanocytes) and resting cells. A single vegetative cell undergoes extensive binary fission in three planes to produce cell aggregates of about 10 μ diameter, within a fibrous envelope. Then, multiple fission occurs in almost all of the cells within an aggregate, followed by the release of small numerous baeocytes, non-motile cells of about 3 μ diameter and enveloped by fibrous materials. When released, the baeocytes enlarge and undergo binary fission to produce vegetative cells. These cells can modify their envelope and cytoplasm under the form of resting cells, able to survive in adverse life conditions.

4. Chroococcidiopsis in Culture

The cytology and life cycle of *Chroococcidiopsis* were studied in cultures obtained from strains CCMEE 34(N6909b) and CCMEE 29(N6904) isolated by Friedmann in 1969, from cryptoendolithic and hypolithic growth, in the Negev Desert (Israel). Cultures were stored in Tallahassee (Florida, USA) for a long period (over 66 months) on 1.5% agarized BG11 medium at 30°C and 20°C on a 16:8 h LD cycle under a photon flux of 25–35 μmol m^{-2} s^{-1}. Samples of these cultures were studied at the University of Rome "Tor Vergata". Here, the strain VRUC 176 was isolated from a sandstone sample collected in the Negev Desert and further investigated. Cultures of different age and period of desiccation allowed the investigations of the cytology of *Chroococcidiopsis* after long-term desiccation (Grilli Caiola et al., 1993); about the effects of desiccation on its envelope composition (Grilli Caiola et al., 1996a); the effects of nitrogen and phosphorus deprivation on its cellular organization (Billi and Grilli Caiola, 1996a); the physiological and ultrastructural effects of nitrogen limitation and starvation (Billi and Grilli Caiola, 1996b); of the subcellular distribution of iron superoxide dismutase (Fe-SOD) (Grilli Caiola et al., 1996b) and of calcium distribution as revealed by ESI and EELS techniques (Grilli Caiola M. and Canini

A., unpublished data). In addition, *Chroococcidiopsis* was tested to evaluate the resistance to ionizing-radiation (Billi et al., 2000a) and to simulated Martian UV flux (Cockell et al., 2005).

Previous studies supposed the existence of different *Chroococcidiopsis* species based on morphological, ecological and cultures studies (Dor et al., 1991). A method set up for the extraction and purification of genomic DNA from *Chroococcidiopsis* was first employed to identify the cell division gene *ftsZ* (Billi et al., 1998), then to investigate the genetic diversity among desert isolates of *Chroococcidiopsis* (Billi et al., 2001). Preliminary results based upon 16S rRNA gene sequencing, suggest that hot and cold isolates of *Chroococcidiopsis* are closely related and divergent from other Pleurocapsalean representatives (Billi et al., 2001). Due to the high sequence divergences present within the *Chroococcidiopsis* lineage (Fewer et al., 2002), including isolates from hot and cold deserts, it is still unclear whether some forms should be regarded as different species or genera.

Studies on old (Fig. 1) and young (Fig. 2) cultures of *Chroococcidiopsis* indicated that cell viability in aged cultures (40–66 months) was lower compared to that of young ones (2–5 months), being the first as low as 0.5–10% compared to 90–100% survival rate of the youngest ones (Fig. 3). Other significant differences

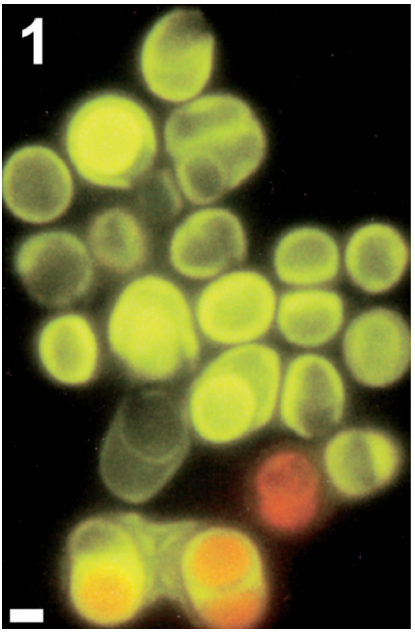

Figure 1. *Chroococcidiopsis* under light microscope. In 41-months-old culture of strain CCMEE 29(N6904), most of cells are unviable while few show red autofluorescence due to pigments. Bar = 1.5 μm.

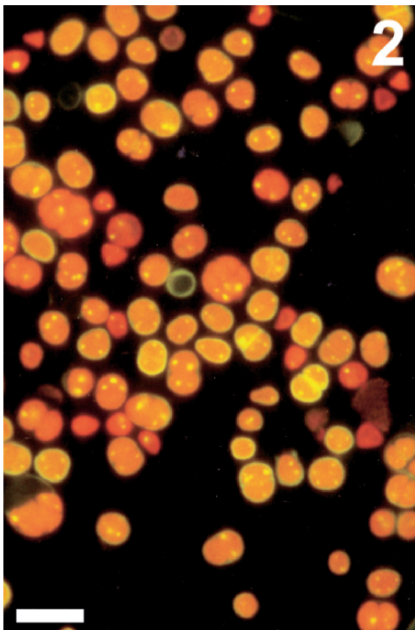

Figure 2. In 1-month-old culture of strain CCMEE 29(N6904), cells show a white fluorescence of the DAPI-stained nucleoids and red pigment autofluorescence. Bar = 10 µm.

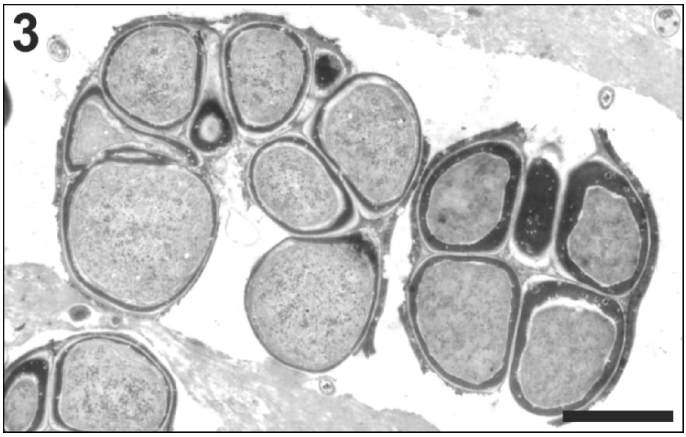

Figure 3. *Chroococcidiopsis* under transmission electron microscope. Mother cells with baeocytes in 1-month-old culture of strain VRUC 176. Bar = 1 µm.

concern the organization and composition of both the sheath and the ultrastructure of the cells adapted to survive to desiccation.

The sheath of aged cells was enriched with polysaccharides both in the baeocyte mother cells and the released baeocytes. The phycobiliproteins content

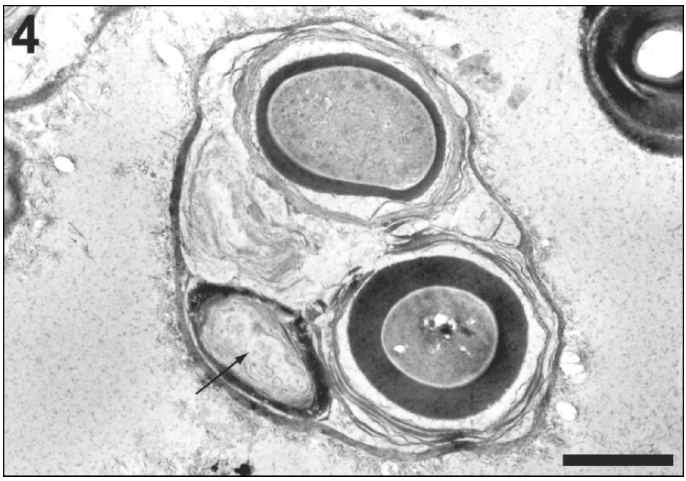

Figure 4. A cell aggregate with cells in different developmental stages and one dead cell (arrow) in 6-months-old culture of strain CCMEE 34(N6909b). Bar = 1 μm.

decreased with aging. In most of the aged cultures, up to 66 months, cells occurred in various degenerating stages and among the dead cells, single living cells were scattered. These last cells (Fig. 4), showed an envelope organization similar to that peculiar of spores and akinetes, previously described in some *Nostoc* strains (Grilli Caiola and De Vecchi, 1980). The living cells showed a thick sheath, but also a cytoplasm with ribosomes and glycogen. Thus a selective process like a programmed cell death seems to occur in the old and desiccated cultures, although the occurrence within the same aggregate, of cells in different physiological states might influence their survival. Under these conditions, inside a single cell aggregate, single cells keep their usual structure pattern, while the others gradually deteriorate. The latter, generally contain carbohydrates in the envelope, fragmented thylakoids and carboxysomes, but no glycogen granules. Cells with multilayered and thick envelopes show intact cytoplasmic structures. The presence of pores (Fig. 5) crossed by membranes on the cell walls of the resting cells is peculiar; it is perhaps useful for intercellular or environmental exchanges. The presence of pores across the cell walls was previously reported in cyanobacteria (Grilli Caiola and De Vecchi, 1985). When old liquid cultures were transferred into fresh nutritional medium, aged cells created new aggregates by means of multiple divisions and the new aggregates released baeocytes, originating new young aggregates. When agarized cultures were allowed to dry, almost all the cells were organized in single cells with a thick envelope and cytoplasmic structures, similar to those observed in the aged cultures kept for many months in liquid culture (Fig. 6). So, the modification of the envelope and cytoplasm result to prove the main structural mechanism to survive to desiccation.

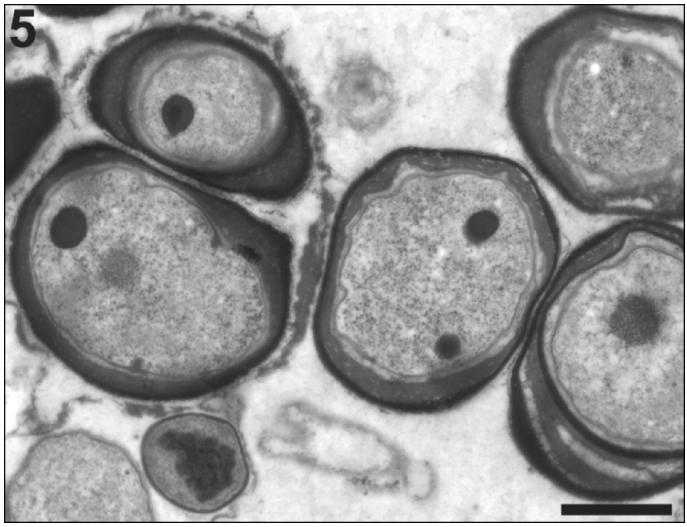

Figure 5. *Chroococcidiopsis* under transmission electron microscope. Resistant cells with thickened envelope from 1-year-old and desiccated culture of strain VRUC 176. Bar = 1 μm.

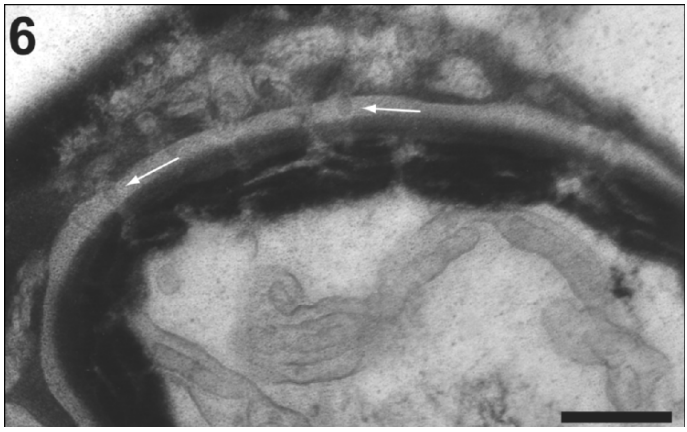

Figure 6. Envelope and cell wall of 1-year-old cell of strain VRUC 176. Pores crossed by membranes-like structures are visible (*arrows*) within the electron transparent wall. Bar = 0.5 μm.

Such a mechanism was confirmed also by experiments made with cultures deprived of nitrogen and phosphate. In cultures grown for 3 months in nitrogen-free medium only isolated viable forms were detected. In these cells a decreased content in chlorophyll and phycocyanin paralleled undetectable O_2 evolution and

depressed O_2 uptake. In addition, such isolated cells showed a multilayered envelope and cytoplasm with vesiculation of thylakoids, but no glycogen granules. N-limited and N-starved cells were able to recover their cellular organization and physiological activity upon N-repletion. These surviving cells had a spore-like feature comparable, from a functional point of view, to akinetes (Grilli Caiola and De Vecchi, 1980).

In cultures grown for one month in phosphate-free medium, cells were still dividing, though their pigment content was reduced compared to that of N-deprived cells. Viable cells were characterized by a complex envelope and vesiculation of thylakoid, undetectable oxygen evolution and oxygen uptake rates. In addition, spore-like forms occurred, suggesting that single cells might allow the survival under P- and N-depletion as well. After one month, P- and N-depleted cells were able to recover upon nutrient repletion.

In order to get information about the capacity of *Chroococcidiopsis* to survive to desiccation, the sheath composition was analyzed with an electron transmission microscopy (TEM) by means of cytochemical reactions, ESI and EELS techniques. The studied samples were obtained both from quartz flint collected in the Negev Desert (Israel) and wet- and laboratory-desiccated cultures.

The envelope of cells from rock as well from desiccated cultures, showed the presence of sporopollenin-like compounds, acid sulphate and beta-linked polysaccharides, positively charged glycoproteins, other than lipids and proteins. These compounds form a very elaborate structure in the envelope of the cells present in both stones and desiccated cultures. In addition, the thickness of the envelope increased with the age and desiccation, suggesting that the envelope represents a crucial mechanism in preventing water loss. However, such a mechanism might cause the cell death by reducing exchanges between the cells and the environment. Isolated living cells occurring in desiccated cultures, might be considered as resting forms allowing the survival of the cyanobacterium. Looking at a cell aggregate, it is difficult to establish which cells will die in order to assure the survival of the others. A similar process recalls the "programmed cell death", occurring in eukaryotic organisms, both animal and plant.

In compliance with the aims above reported, the cell sheath was analysed by ESI and EELS techniques in order to ascertain the presence of calcium and nitrogen in the envelope. By ESI it was possible to localize the different elements on the ultrathin sections of a cell previously identified by TEM. It was possible to detect on the same section different elements and thus to evaluate their amount in a target part of the cell. These techniques indicated that in cells from one-month-old cultures, the envelope contains calcium as well as nitrogen. However, EELS spectra indicated that the calcium amount was very high compared to that of nitrogen. In fact, nitrogen was present in such a low rate that was almost undetectable in the envelope, whereas it resulted more abundantly in the cell wall. The composition of the envelope was similar in both cell aggregates and single cells. Inside the cell, nitrogen was mainly present in the thylakoids, whereas the calcium amount was low. The EELS spectra acquired on the envelope of cells from one-year

old cultures revealed a very high peak of calcium, higher than that detected in the young ones, whereas nitrogen was present in a very low amount. In the old cells, when calcium and nitrogen were checked in cyanophycin storage granules, both elements resulted present.

The oxidative stress is also an important parameter to take into account, in order to highlight the survival strategies in *Chroococcidiopsis*. As term of comparison, the presence and distribution of iron Fe-SOD was measured by means of immunogold technique, using an antibody produced against Fe-SOD purified from *Anabaena cylindrica* (Grilli Caiola et al., 1993). In one-month-old cultures, the enzyme was localized mainly in the nucleoplasm and in the cell envelope. In one-year-old cultures, where mainly single cells occurred, Fe-SOD was localized in the peripheral cytoplasm and in the envelope, but it was absent in the nucleoplasmic areas (Fig. 7). This finding suggests that Fe-SOD could work as a barrier against oxygen radicals both in the cytoplasm and in the extracytoplasmic area. The presence of Fe-SOD reinforces the idea that *Chroococcidiopsis* is an ancient cyanobacterium, and that this enzyme originated very early on Earth, when oxygenic photosynthesis arose. The presence of Fe-SOD in periplasmic and apoplastic compartments was recently reported in other prokaryotes and eukaryotes. In the desiccation-tolerant cyanobacterium *Nostoc commune* the presence of a highly stable and active Fe-SOD in the extracellular matrix was reported after prolonged desiccation (Shirkey et al., 2000).

The great similarity between fossil and modern cyanobacteria indicates that cyanobacteria changed very little, if not at all, since their appearance on Earth billions of years ago. This accounts for their evolution low rate. The fossil taxa show slight or not evident morphological changes over hundreds of millions of years. This morphological evolutionary preservation is well documented for both coccoid and filamentous forms by numerous researchers (Kremer, 2006).

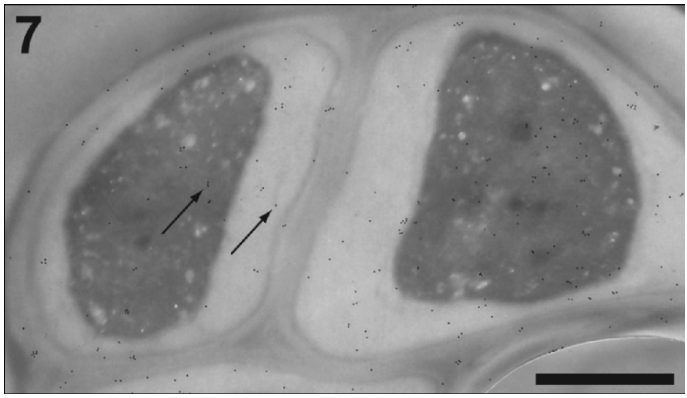

Figure 7. Gold particles indicating Fe-SOD localization in the periplasm and cell wall of *Chroococcidiopsis* cells from 1-year-desiccated culture of strain VRUC 176. Bar = 1 μm.

Chroococcidiopsis is not only capable to survive to extreme desiccation, but it shows also a remarkable resistance to high doses of ultraviolet radiation (Martian UV-flux; Cockell et al., 2005) and ionizing radiation (up to 15 KGy; Billi et al., 2000a). In addition, since it is the only desiccation-tolerant cyanobacterium fit to genetic manipulation (Billi et al., 2001), the molecular analysis of its desiccation tolerance and radioresistance might reveal how to enhance its survival potential and obtain an organism better suited to extraterrestrial conditions.

5. Molecular Contributions to Decipher Drought Tolerance in *Chroococcidiopsis*

The capability to face the physiological constraints imposed by the complete removal of water, the storage in the dried state and subsequent rewetting, is a complex phenomenon, which involves every level of the cellular organization, through mechanisms not yet completely understood (Billi and Potts, 2000, 2002).

In order to survive desiccation, *Chroococcidiopsis* must avoid and/or repair several damages, spanning from those mediated by the oxidative stress to those due to the transition phase of the phospholipid bilayers (Potts, 2001). All these events generate injuries to proteins, lipids, nucleic acids and membranes and lead to the death of the majority of the organisms. It was speculated that the extraordinary radioresistance of hot and cold desert strains of *Chroococcidiopsis* is due to their ability to repair DNA damages (Billi and Potts, 2002). Indeed, the exposure to high doses of X-rays radiation (up to 15 KGy) induces the complete fragmentation of the genome of desert strains of *Chroococcidiopsis*, which is repaired within few hours (Billi et al., 2000a). This capability makes the resistance to ionizing radiation of *Chroococcidiopsis* comparable to that reported for the non-spore–forming bacterium *Deinococcus radiodurans* (Cox and Battista, 2005). At the moment, there are no data available on the effects of desiccation on the genome of *Chroococcidiopsis*. While, it was reported that after prolonged desiccation, the genome of the desiccation-tolerant cyanobacterium *Nostoc commune* is covalently modified, but protected from oxidative damage and degradation (Shirkey et al., 2003). In order to understand how *Chroococcidiopsis* reconstructs its genome from the remaining fragments, it must be pointed out that DNA repair pathways other than nucleotide excision repair should be employed. In fact, under circumstances of complete DNA fragmentation, even the search for the lost information via homologous would be necessarily useless, even in microorganisms containing multiple genome copies. In *D. radiodurans* the numerous genome copies represents only a passive contributor to ionizing resistance and biochemical mechanisms are employed to limit DNA degradation and restrict the fragment diffusion (Cox and Battista, 2005). For this bacterium a previously unknown mechanisms for fragments reassembly requiring at least two genome copies was reported (Zahradka et al., 2006). When approaching the DNA repair mechanisms in *Chroococcidiopsis*, it might be also relevant to undertake ultra-

structural to investigate whether the genome of this cyanobacterium is arranged in toroids, as reported for *D. radiodurans* (Cox and Battista, 2005).

However, in addition to DNA repair, molecules and enzymes with antioxidant activity are likely to play a central role in the desiccation and radiation tolerance of *Chroococcidiopsis*, taking into account that the oxidative stress is exacerbated by the photosynthetic process (Potts, 2001). The role of trehalose and sucrose in desiccation tolerance and cryoprotection has been widely documented (Crowe et al., 1997). These disaccharides prevent in vivo and in vitro the phase transition of cellular membranes and stabilize dried proteins. The expression of cyanobacterial sucrose-6-phosphate synthase in the desiccation-susceptible bacterium *Escherichia coli*, increased its survival after both freeze-drying and air-drying (Billi et al., 2000b). Trehalose was also reported to stabilize purified and dried plasmid DNA (Shirkey et al., 2003). Desert strains of *Chroococcidiopsis* accumulate trehalose and sucrose in response to osmotic stress, but no data are available about their presence and abundance upon desiccation. In deciphering the molecular basis of *Chroococcidiopsis* survival under extreme conditions, it might be important to investigate the role played by molecules known to contribute to the desiccation tolerance of anhydrobiotes, such as the Late Embryogenesis Abundant Proteins and Heat Shock Proteins. Finally, the possibility to genetically manipulate desert strains of *Chroococcidiopsis* provides the challenge to decipher the molecular basis of its desiccation tolerance (Billi et. al., 2001).

Compared to UV radiation, drought and nutrient depletion resistance studies dealing with chilling and interplanetary space resistance of cyanobacteria are very few. Experiments carried out with the purpose to test the symbiotic system *Azolla-Anabaena azollae* resistance by means of exposing it to space weightlessness for 6 days, indicated that microgravity does not affect the main biological characteristics of both fern and cyanobacterium (Carrapico, 2001). Concerning the chilling effects on cyanobacteria, Nishida and Murata (1996) have revealed that changes in membrane fluidity could be involved in the initial event leading to the expression of genes for desaturases. But the extent of cyanobacterial resistance to chilling is still unknown. There is no information available about the behaviour of cyanobacterial cultured cells after storage at –196°C (Whitton, 1987). Laboratory experiments on *Hemicloris Antarctica* – a free living alga often present in Antarctic cryptoendolithic communities – at temperature oscillations in the range 5°C and –5°C or –10°C did not damage the cells. In addition, *H. antartica* appeared to be undamaged after slow or rapid cooling at –50°C (Meyer et al., 1988).

6. *Chroococcidiopsis* Towards Mars?

As we have forwarded in the introduction, Mars dream goes on. But the researches on the actual meteorological and atmosphere conditions on the Red Planet indicate that they are limiting life to terrestrial organisms exclusively. However, studies on *Chroococcidiopsis* offer a frame of information useful for

future researches about life on it as well as in the interplanetary spaces. In addition, they allow the planning of new missions to the Red Planet. Among the numerous terrestrial organisms, *Chroococcidiopsis* show some features worthy of attention such as its capacity to survive to long-term desiccation and deprivation conditions of basic nutritional elements, such as nitrogen and phosphorous, and then to recover when water and nutritional elements are available. It can survive to ionizing radiation by repairing DNA damages and it may be genetically modified in order to enhance its resistance to extraterrestrial conditions.

Chroococcidiopsis is able to live in conditions proximal to the limits of life on Earth. At the moment it is impossible to establish whether or not it will be able to survive to the limiting life conditions present on Mars. However, today we have accumulated a great deal of information on Mars and its possible past and future inhabitants. Opinions for and against the real chance of colonizing Mars will continue to be debated (Beaty et al., 2005). Other studies and missions will be needed to monitoring the conditions on it compatible with the green world, such as the soil composition, the water content, the absence of the gravity effects, and the details about its atmosphere. With these data, perhaps, also *Chroococcidiopsis* could become a pioneer paving the way for the advent and settling down of more evolved organisms on the Red Planet.

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