

Research Paper

Common Freshwater Cyanobacteria Grow in 100% CO₂

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ABSTRACT

Cyanobacteria and similar organisms produced most of the oxygen found in Earth's atmosphere, which implies that early photosynthetic organisms would have lived in an atmosphere that was rich in CO₂ and poor in O₂. We investigated the tolerance of several cyanobacteria to very high (>20 kPa) concentrations of atmospheric CO₂. Cultures of *Synechococcus* PCC7942, *Synechocystis* PCC7942, *Plectonema boryanum*, and *Anabaena* sp. were grown in liquid culture sparged with CO₂-enriched air. All four strains grew when transferred from ambient CO₂ to 20 kPa partial pressure of CO₂ (pCO₂), but none of them tolerated direct transfer to 40 kPa pCO₂. *Synechococcus* and *Anabaena* survived 101 kPa (100%) pCO₂ when pressure was gradually increased by 15 kPa per day, and *Plectonema* actively grew under these conditions. All four strains grew in an anoxic atmosphere of 5 kPa pCO₂ in N₂. Strains that were sensitive to high CO₂ were also sensitive to low initial pH (pH 5–6). However, low pH in itself was not sufficient to prevent growth. Although mechanisms of damage and survival are still under investigation, we have shown that modern cyanobacteria can survive under Earth's primordial conditions and that cyanobacteria-like organisms could have flourished under conditions on early Mars, which probably had an atmosphere similar to early Earth's. **Key Words:** Early Earth—Mars—Photosynthesis—Planetary engineering—Primordial atmospheres. *Astrobiology* 5, 66–74.

INTRODUCTION

ACCORDING TO CURRENT EVIDENCE, life on Earth appeared 3.8–3.4 billion years ago (Ga) (Des Marais, 1998; Nisbet and Sleep, 2001). Several models of Earth's early atmosphere have been proposed, but in all of them O₂ was essentially absent, and the concentration of CO₂ was orders of magnitude larger than in the present-day atmosphere (Pollack, 1991; Kasting, 1993). The frac-

tion of CO₂ varies in these models, depending upon the total atmospheric pressure, but 3.5 billion years ago, Earth's atmosphere may have contained partial pressure (p) of CO₂ levels of 10–500 kPa (compared with present-day pCO₂ of approximately 0.035 kPa).

Photosynthetic organisms are responsible for the appearance and maintenance of free O₂ in the atmosphere. Although the timing of the origin of photosynthesis is unclear, significant amounts of

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atmospheric O₂ do not appear until 2.3–2.0 Ga (Kasting, 1993; Bekker *et al.*, 2004), which suggests that photosynthesis evolved before then. Fossilized structures that resemble modern cyanobacteria suggest that photosynthetic bacteria may have evolved as early as 3.5 Ga, but it has not been universally accepted that these structures are cyanobacterial, or even biological, in origin (Schopf and Packer, 1987; Schopf, 1993; Kudryavtsev *et al.*, 2001; Brasier *et al.*, 2002; Schopf *et al.*, 2002). Based upon microfossil and stromatolite evidence, and upon the oxidized state of ancient shallow seas, a conservative estimate places the evolution of oxygenic photosynthetic organisms no later than the end of the Late Archaean Eon (2.5 Ga) (Buick, 1992; Des Marais, 1998). After this period, eukaryotic algal fossils (Han and Runnegar, 1992) and significant increases in atmospheric O₂ (Holland, 1994; Knoll and Holland, 1995) indicate the existence of oxygenic photosynthesis.

Regardless of exactly when they appeared, the first oxygenic photosynthetic organisms would have originated in an atmosphere containing very high concentrations of CO₂ and low concentrations of O₂. Cyanobacteria are the direct descendants of these first photosynthesizers, and the chloroplasts of eukaryotic photoautotrophs (plants and algae) most likely evolved from endosymbiotic cyanobacteria (Margulis and Bermudes, 1985). Given the biochemical similarity of oxygenic photosynthesis across cyanobacteria and eukaryotes, it is likely that the fundamental photosynthetic apparatus has changed very little since the last common ancestor of extant cyanobacteria, and perhaps over the past 2.5 billion years. One might expect, then, that some photosynthetic organisms could still operate under conditions similar to those under which photosynthesis evolved.

Published research concerning this question is surprisingly scarce. In higher plants, the problem becomes more complex since 50% or more of the organism is not photosynthetic (roots, woody stems, flowers). Though few have studied plant growth in primordial CO₂ levels, considerable work has been done on the effects of moderate pCO₂ (up to 5 kPa) and low pO₂ on vascular plants. Primary production in terrestrial C₃ plants is inhibited by pO₂ as low as 2 kPa (Bjorkman, 1966), but most plants require at least 5 kPa pO₂ for proper overall development (Siegel *et al.*, 1962; Atwell *et al.*, 1982; Bar-

clay and Crawford, 1982; Alpi and Beever, 1983; Jackson and Drew, 1984).

A few species of green algae that survive in 101 kPa (100%) pCO₂ have been described (Seckbach and Libby, 1970; Seckbach *et al.*, 1970; Negoro *et al.*, 1991). All were thermoacidophiles, and were isolated from acidic hot springs and similar environments. Few studies of cyanobacteria grown at very high pCO₂ have been reported. *Synechocystis* and *Synechococcus* (among others) have been grown in 3 kPa pCO₂ (Thomas *et al.*, 1998, 1999, 2001), *Anacystis* has been grown at 33 kPa pCO₂ (Mizutani and Wada, 1982), and a preliminary study indicated slow growth of a marine cyanobacterium, *Synechococcus* sp. 7002, in 101 kPa pCO₂ (Hall *et al.*, 1998). The purpose of this study was to determine whether any common, mesotrophic cyanobacteria have retained the presumed ancestral ability to grow in a primordial-like (high-CO₂) atmosphere. We found three species of cyanobacteria that survive in 101 kPa pCO₂.

MATERIALS AND METHODS

Stock cultures of *Anabaena* sp. (Carolina Biological Supply, Burlington, NC), *Plectonema boryanum* UTEX485, *Synechococcus* sp. PCC7942, and *Synechocystis* sp. PCC6803 were maintained on pH 8 BG-11 agar plates and slants supplemented with 5 mM NaCO₃ and 20 µg/L vitamin B₁₂, and grown at 25°C under cool-white fluorescent illumination of 10–25 µmol of photons m⁻² s⁻¹. Working cultures were grown at 25°C in pH 8 liquid BG-11 medium (supplemented with 20 µg/L vitamin B₁₂) in 50-ml culture tubes bubbled with filtered air (Thomas *et al.*, 1998, 1999) through custom-made positive-pressure culture tube closures (Thomas and Herbert, 2005). The cultures were continuously illuminated at 25–35 µmol of photons m⁻² s⁻¹ with cool-white fluorescent tubes.

Experimental samples were provided with mixtures of CO₂ in either air or N₂. Gas mixtures were obtained with correlated rotameters (Cole-Parmer Instruments, Vernon Hills, IL) at a flow rate of approximately 100 mL min⁻¹ at each culture tube. When different pH values in the media were needed, 250 mM K₂HPO₄ buffers of the appropriate pH were added to the media for a final concentration of 10 mM. Growth was monitored spectrophotometrically by measuring ap-

parent absorbance (scattering) at 750 nm over 7–10 days (Thomas *et al.*, 1998, 1999, 2001). At 24–48-h intervals, 2-ml aliquots of cyanobacterial suspension were aseptically transferred from the culture tubes to spectrophotometer cuvettes. Measurements of media pH were made concurrently with growth measurements by dipping a pH probe into the cuvettes. Unless otherwise stated, all experiments were performed in quadruplicate. Statistical analyses were performed with Microsoft Excel[®] XP (*t* test, $\alpha = 0.05$).

RESULTS

In our initial experiments, we transferred late-exponential phase cultures (diluted to $A_{750} = 0.1$) directly from air to 20 kPa or 40 kPa pCO₂ in air. Uniformly, all of the cultures survived and grew in 20 kPa pCO₂, but died when transferred directly to 40 kPa pCO₂ (data not shown). After these initial attempts, we transferred cultures into 10 kPa pCO₂ in air and then increased the pCO₂ by 15 kPa (15%) every 24 h (Fig. 1). Under these conditions, *Synechocystis* cultures died when the CO₂ concentration rose above 40 kPa pCO₂. *Anabaena* and *Synechococcus* continued to grow until the pCO₂ reached approximately 55 kPa, after which the cultures remained viable but showed no significant growth. *Plectonema* survived the entire treatment and continued to grow (slowly) in 101 kPa pCO₂. As one would expect, the pH of the media in the high CO₂ samples dropped quickly to around 6.0.

To differentiate between the effects of carboxia and anoxia produced in the previous experiment, cultures were grown in 100% N₂. After 24 h, all cultures ceased growing, and the media pH rose to 10.0–11.5 (data not shown). The rise in pH appeared to be due to the removal of dissolved CO₂ by the constant flow of N₂. BG-11 contains some Na₂CO₃, which, in an aqueous solution or in cells, would dissociate into Na⁺, CO₂, and OH⁻. As the CO₂ is purged by the flowing N₂, the pH would increase because of the accumulation of hydroxides. We interpreted the lack of growth as due to a complete lack of available inorganic carbon, and so a similar experiment was performed in 5 kPa pCO₂ in N₂. Under these conditions, all four strains grew, although *Synechococcus* and *Synechocystis* grew poorly (Fig. 2). During the course of the experiment, the pH of the cultures in CO₂ + N₂ dropped to approximately 7.0.

Finally, to attempt to isolate a possible pH effect, cultures were bubbled with air for 5 days in media buffered to pH 5, 6, 7, and 8 (Fig. 3). *Plectonema* grew over the pH range of 5–8, with optimal growth at pH 6. *Anabaena* and *Synechococcus* also showed optimal growth at pH 6, but did not grow well at pH 5 or pH 8. *Synechocystis* grew optimally at pH 7 and, among the species tested, exhibited the best growth at pH 8.

DISCUSSION

We have shown that three common cyanobacterial species can grow under conditions that resemble those thought to have existed on early Earth: very high pCO₂ and low pO₂. *Anabaena*, *Plectonema*, and *Synechococcus* all grew at 40 kPa pCO₂. *Plectonema* was extremely CO₂-hardy and capable of surviving and growing in an atmosphere of pure CO₂. In addition, all of the cyanobacteria tested grew in an anoxic atmosphere as long as CO₂ was provided—results that are consistent with previous findings in *Oscillatoria limnetica* (Cohen *et al.*, 1975; Oren and Padan, 1978). These findings are consistent with traits one would expect to find in organisms that evolved in an anoxic, mildly reducing atmosphere of CO₂ and N₂, such as that proposed for early Earth. Our results support the idea that oxygenic photosynthesis evolved relatively early during Earth's history when O₂ was absent from the atmosphere.

All four of the species tested were capable of growth in an anoxic atmosphere, but they did not grow equally well under these conditions. Presumably, modern cyanobacteria need some O₂ for normal metabolism. *Synechococcus* and *Synechocystis*, unicellular cyanobacteria, grew poorly in the CO₂ + N₂ treatment. Conversely, the filamentous forms, *Anabaena* and *Plectonema*, grew relatively well in the CO₂ + N₂ mixture. The unicellular cyanobacteria, having a larger surface area to volume ratio, should lose O₂ by diffusion faster than the filamentous cyanobacteria. Perhaps the better growth of the two filamentous forms was due to the formation of aerobic microenvironments within and/or around their cells. At present we are unable to determine the existence or extent of aerobic cellular microenvironments. The existence of such microenvironments, however, could have influenced the evolution of two other major meta-

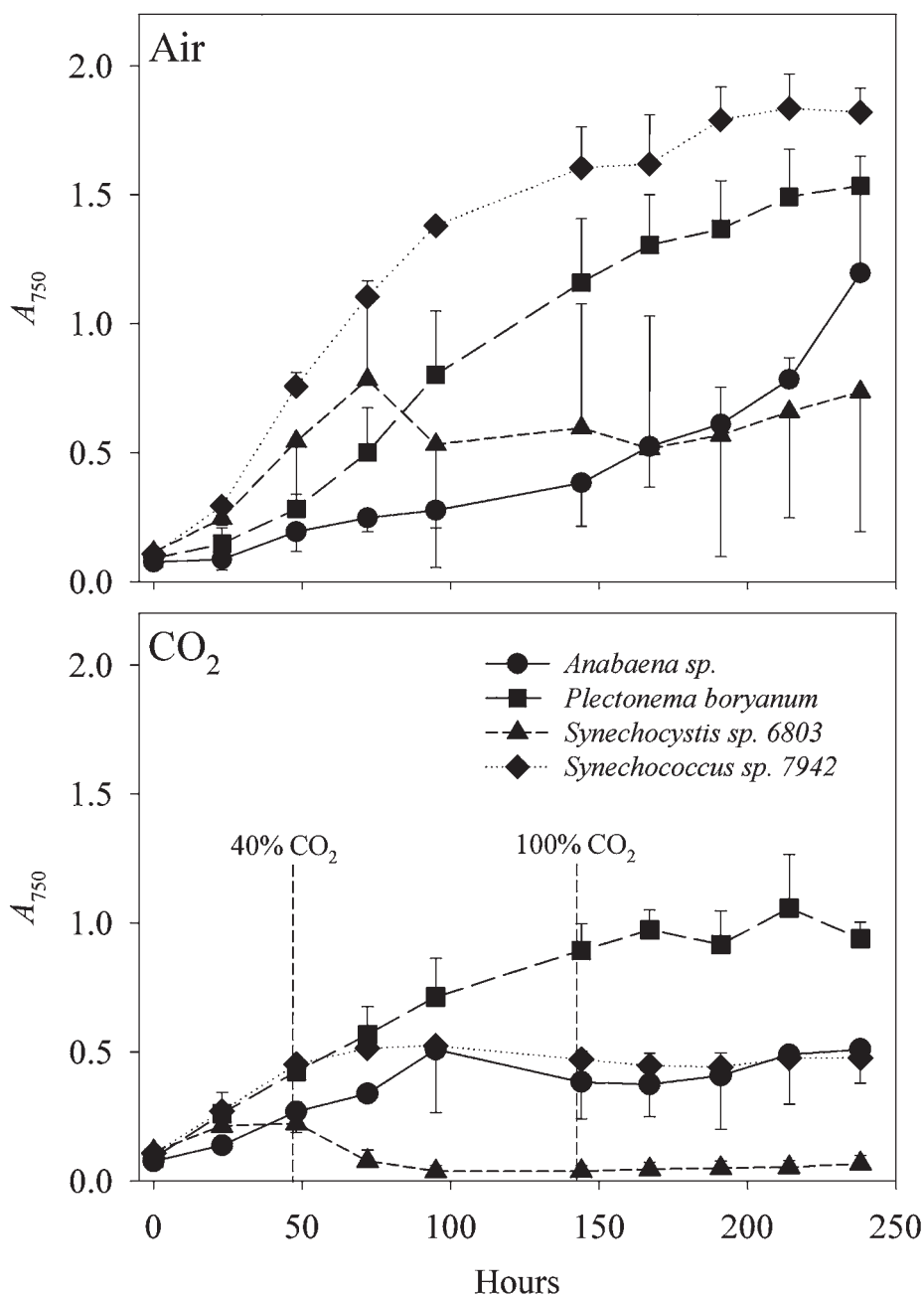


FIG. 1. Growth of cyanobacteria in air (top panel) versus CO₂ (bottom panel). All cultures were initiated at 10 kPa pCO₂ in air. The pCO₂ was increased by 15 kPa every 24 h to reach 101 kPa (100%) pCO₂ at 144 h ($n = 4$, bars = SD). At 101 kPa pCO₂, the media pH in all cultures was 5.9–6.1.

bolic pathways: the antioxidant system and aerobic respiration.

Modern photosynthetic organisms have well-developed antioxidant systems (Niki, 1991; Reddy, 1991; Simic, 1991; Tsang *et al.*, 1991). Without antioxidants, photosynthetic organisms are damaged or killed by the reactive oxygen species that are produced as by-products of photosynthetic electron transport (Allen, 1977; Asada and

Takahashi, 1987; Scandalios, 1993; Foyer *et al.*, 1994). This results in an evolutionary paradox. Which evolved first: oxygenic photosynthesis or antioxidants? If oxygen diffused freely from early photosynthetic cells, and aerobic microenvironments did *not* form, then oxygenic photosynthesis could have preceded and provided the selective pressure for the evolution of antioxidants. If aerobic microenvironments did form within early

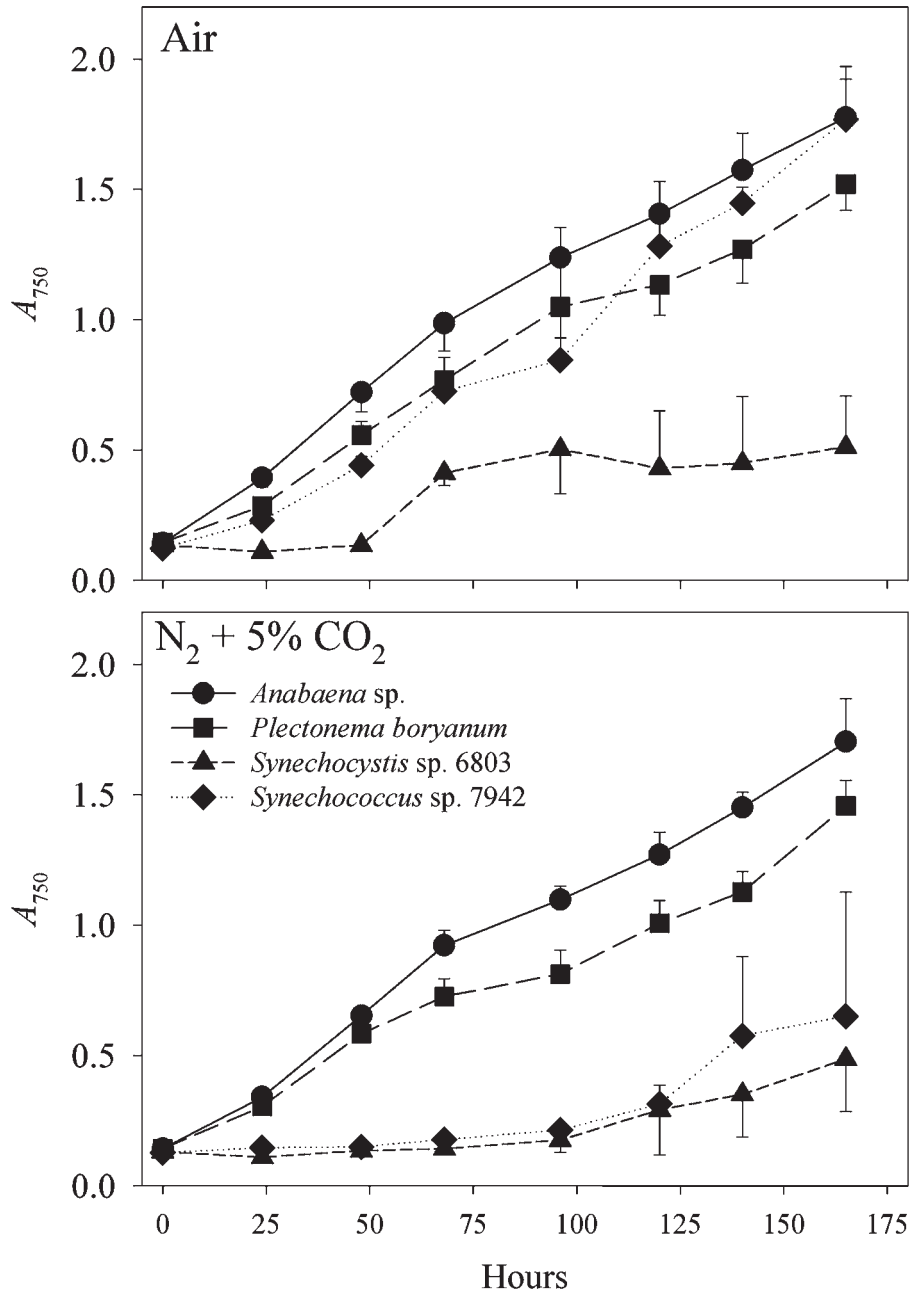


FIG. 2. Growth of cyanobacteria in air (top panel) versus 5% CO₂/95% N₂ (bottom panel). To differentiate the effects of anoxia from the effects of carboxia, cultures were grown in 5 kPa pCO₂ in N₂. All of the cultures grew (albeit *Synechocystis* grew poorly), indicating that anoxia by itself probably was not a major factor in growth in high CO₂ ($n = 4$, bars = SD). The media pH in all cultures was ~ 7.0 .

photosynthetic cells, then the evolution of antioxidants would have been a necessary pre-adaptation for oxygenic photosynthesis.

Because of the lack of free O₂ in Earth's early atmosphere, aerobic respiration is generally thought to have evolved after the Archean Eon (reviewed in Des Marais, 1998). However, localized aerobic environments would likely have

been produced by early cyanobacteria-like organisms, and early aerobic organisms may have evolved alongside the photosynthesizers in such environments. Additionally, molecular phylogenetic evidence indicates the close co-evolution of photosynthesis and respiration (Tomiki and Saitou, 2004). If the 3.5–3.3 Ga structures described by Schopf and co-workers (Schopf and

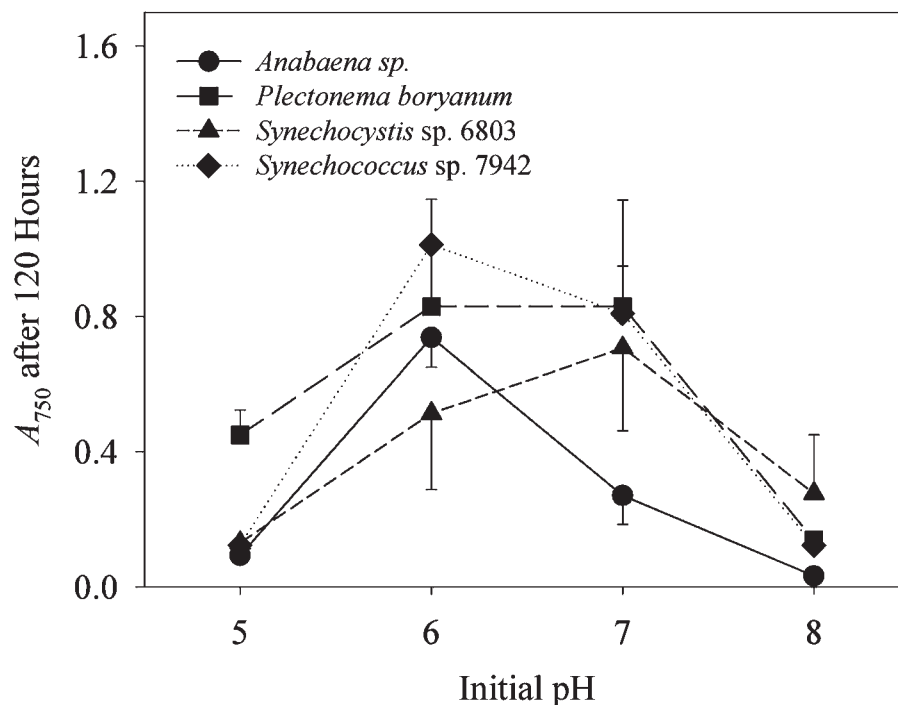


FIG. 3. Optimal pH for cyanobacterial growth. Cyanobacteria were grown in phosphate-buffered media, and growth was recorded after 120 h. All four cyanobacteria grew over the range of pH 5–8, but had different optima. Mild acid tolerance appears to correlate with CO₂ tolerance ($n = 4$, bars = SD).

Packer, 1987; Schopf, 1993; Schopf *et al.*, 2002) are fossilized cyanobacteria, then aerobic metabolism could have evolved much earlier than is currently thought.

Our results also provide insight into the possibility of life on early Mars. Mars' present-day atmosphere consists of approximately 95% CO₂ with most of the balance consisting of N₂ and Ar. Currently, Mars' atmospheric pressure is <1% of Earth's, resulting in a pCO₂ of approximately 0.95 kPa. However, Earth and Mars probably evolved similarly during the first billion years or so of their existence (reviewed in Pollack *et al.*, 1987; McKay and Stoker, 1989; Helfer, 1990; Pollack, 1991). Therefore, Mars' atmosphere probably was much thicker during its early history, and the proportion of gases in Mars' early atmosphere may have been similar to its current state. Thus, Mars may have had a thick, predominantly CO₂ atmosphere when life was first evolving on Earth. Could life have originated on Mars at 3.5 Ga? Assuming that liquid water was present, an organism like *Plectonema* could have grown on early Mars, and would be an ideal pioneering organism for planetary ecopoiesis (Thomas, 1995).

Our results beg other questions: Why did *Plectonema* perform so much better than the other cyanobacteria when the pCO₂ rose above 55 kPa? Why did *Synechocystis* grow poorly at pCO₂ greater than 25 kPa?

The answer to the first question appears to be due, in part, to pH tolerance. Under high CO₂ atmospheres, the water-based growth medium becomes acidic (pH ~6.0). As Fig. 3 shows, all four species grew over the pH range of 5–8, but *Plectonema* grew better under acidic conditions, whereas *Synechocystis* grew better under neutral to basic conditions. These results correlate with relative CO₂ tolerance—high tolerance in *Plectonema* and low tolerance in *Synechocystis*. The other two species, *Anabaena* and *Synechococcus*, had pH optima and ranges between those of *Plectonema* and *Synechocystis*, which correspond to moderate CO₂ tolerance. However, medium pH does not appear to be the sole impediment to growth in high CO₂. At 101 kPa pCO₂, the pH of the medium was 5.9, well within the growth range of all of the cyanobacteria tested, including *Synechocystis*. Acid tolerance appears to be correlated to CO₂ tolerance but does not fully account for it.

Anoxia tolerance may be another trait related to CO₂ tolerance. When the cyanobacteria were grown in 5 kPa pCO₂/96 kPa pN₂, *Synechocystis*, the most CO₂-sensitive form, was also the most anoxia-sensitive form (Fig. 2). Likewise, *Plectonema*, the most CO₂-tolerant form, was also the most anoxia-tolerant form. Notably, *Plectonema* grew much faster in 5 kPa pCO₂/96 kPa pN₂ than it did in air. During these experiments, the medium pH of the experimental group remained in the range of 6.8–7.2—close to optimum for *Synechocystis*. These results indicate an even smaller role for pH in the retardation of growth under non-ambient atmospheric conditions; however, the role of pH cannot be ruled out entirely.

In the pH buffer experiments (Fig. 3), the pH of the medium tended to increase as the cyanobacteria grew. The change in pH probably has two major components: (1) Excess CO₂ is driven off as air bubbles through the medium, and (2) cellular metabolism changes the pH via utilization of H⁺ and secretion of alkalis. Thus during the buffer experiments, the cyanobacteria may have altered their environments, allowing more growth. However, during the CO₂ experiments, the constant influx of CO₂ held the pH relatively constant, which would have prevented organism-induced changes to the environment. Thus, pH tolerance may still have an important role in CO₂ tolerance, but it does not correlate with anoxia tolerance.

The next step in our research will be to find the intracellular components that are damaged during exposure to high pCO₂. During many forms of environmental stress (e.g., chilling stress, oxidative stress, light stress), the photosynthetic electron transport system of proteins is often an early site of damage (Wise, 1995; Sonoike, 1996; Martin *et al.*, 1997; Asada *et al.*, 1998; Thomas *et al.*, 1998, 1999; Tjus *et al.*, 1998; Melis, 1999). The same may be true of CO₂ stress, and we are currently investigating this possibility. Other causes of damage may be related to carboxylation. Some enzymes are regulated by the addition of CO₂—carboxylation or carbamylation. For example, Ru-BisCO, the primary enzyme of carbon fixation, requires carbamylation of lysine residue 201 for activation (reviewed in Bowyer and Leegood, 1997). Carboxylating and carbamylating enzymes are also used in amino acid, carbohydrate, and lipid metabolism. Normally, carboxylation is regulated by a system of protein carboxylases.

However, under high pCO₂, indiscriminant carboxylation events might occur, resulting in uncontrolled enzyme activation, inactivation, or both processes, which in turn results in growth inhibition or even cell death. We are currently planning experiments to test this hypothesis.

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ABBREVIATIONS

Ga, billion years ago; p, partial pressure.

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