BIOPAN-SURVIVAL I: EXPOSURE OF THE OSMOPHILES *SYNECHOCOCCUS SP.* (NAGELI) AND *HALOARCULA SP.* TO THE SPACE ENVIRONMENT

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ABSTRACT

The objective of this study was to determine the survivability of osmophilic microorganisms in space, as well as examine the DNA breakage in osmophilic cells exposed to solar UV-radiation plus vacuum and to vacuum only. The organisms used were an unidentified species of *Synechococcus* (Nägeli) that inhabits the evaporitic gypsum-halite crusts that form along the marine intertidal, and an unidentified species of the extremely halophilic genus *Haloarcula* (designated as isolate G) isolated from a evaporitic NaCl crystal. Because these organisms are desiccation resistant and gypsum-halite as well as NaCl attenuate UV-radiation, we hypothesized that these organisms would survive in the space environment, better than most others. The organisms were exposed to the space environment for 2 weeks while in earth orbit aboard the Biopan facility. Ground controls were tested in a space simulation facility. All samples were compared to unexposed samples. Survivability was determined by plate counts and the most probable number technique. DNA breakage was determined by labeling breaks in the DNA with ^{32}P followed by translation. Results indicate that the osmophilic microbes survived the 2 week exposure. The major cause of cell death was DNA damage. The number of strand breaks in the DNA from vacuum UV exposed cells was greater than the vacuum only exposed cells.
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INTRODUCTION

Questions regarding the origin and distribution of life throughout the universe abound. With the ability to expose microbes to the space environment in a controlled manner we can now address some of these questions. The goal of research in exobiology includes not only searching for extraterrestrial life, but also identifying the pathways through which life arose, evolved, and may have distributed itself throughout the universe. Interplanetary transfer is one mechanism by which life may distribute itself throughout the universe. Possible mechanisms of interplanetary transfer include spacecraft, and natural mechanisms such as meteorites, comets and interplanetary dust particles (Horneck and Brack, 1992). The interplanetary transfer of life is particularly relevant with regard to Mars and Earth because it is thought that the Shergotty, Nakhla and Chassigny (SNC) type meteorites traveled from Mars to Earth (Vickery and Melosh, 1987), and possibly meteorites from Earth traveled to Mars, providing a possible pathway for the transport of biological material between these two planets. Although no life or organics were found on Mars at either of the two Viking lander sites (reviewed by Mazur et al., 1978; Klein, 1979) next to Earth, Mars holds the best chance for the origin and early evolution of life in our solar system (for reviews see Banin and Mancinelli, 1995; Mancinelli and Banin, 1995). Recent evidence from Martian meteorite ALH84001 suggests that microbial life may indeed have evolved on Mars (McKay et al., 1996). It is unclear if this

pathway can transport life from one planet to the another, primarily because it is unclear if any organisms can withstand the rigors of the space environment for such a journey. A comprehensive theoretical study of the probability of microbes surviving ultraviolet radiation and ionizing radiation during ejection from the vicinity of red giant stars concluded that microbes could survive if they were shielded by a thin film of carbonaceous material (Secker et al., 1994). This study provides data leading to a better understanding of the potential for life to withstand the rigors of the space environment and interplanetary transfer.

Microbes Tested in the Space Environment

Microbes tested in the space environment to date include *Bacillus subtilis* spores, bacteriophage T-1, Tobacco Mosaic Virus, (Horneck and Brack, 1992, Horneck, 1993) and most recently osmophilic microbes. The results of these studies revealed that *Bacillus subtilis* spores will survive for years in space if protected against high solar UV-radiation flux (Horneck, 1993; Horneck et al., 1994), whereas viruses lose viability on the order of weeks to months (reviewed in Horneck and Brack, 1992). The lethal and mutagenic effects of heavy ions on microbes and the implications for microbes exposed to the space environment is reviewed by Horneck et al. (1994). The data suggest that most damage suffered by microbes exposed to the space environment is due to UV radiation, especially during the short term, but heavy ionizing radiation has a greater probability of being lethal (Horneck, et al., 1994). These studies reflect the effects of the space environment on representatives of a small number of microbial species (reviewed in Horneck and Brack, 1992). It has recently been shown that microorganisms inhabiting gypsum-halite crusts and NaCl crystals are extremely osmophilic and can withstand desiccation (Rothschild et al., 1994).

Anhydrobiosis and the Physiology of Osmophily

Desiccation tolerance has been reported for a variety of organisms including, bacteria, yeast, fungi, plants, insects and crustacea (e.g., Clegg, 1986; Crowe et al., 1992; Csonka and Hanson, 1991; see Potts 1994 for a review). The role of water in the structure function relationships of desiccated cells has been inferred from biophysical studies of purified proteins (e.g., Carpenter, 1993). Mechanisms of death due to anhydrobiosis (extreme desiccation) include lipid, protein and nucleic acid irreversible phase changes such as denaturation and structural breakage (Cox, 1993; Dose et al., 1992). For example, upon desiccation carbohydrates, lipids and nucleic acids undergo Maillard reactions that lead to cross linking and eventual irreversible polymerization of the molecule (Cox, 1993). One of the specific purported mechanisms of death due to anhydrobiosis in prokaryotes is the dehydration of DNA leading to breakage (Dose et al., 1992; 1991 Dose et al., 1995; Dose and Gill, 1994). Desiccation-induced damage in nucleic acids (as occurs upon exposure to the space environment) represents the accumulation of all the damage that has occurred during desiccation because there is no cell growth. Because it is unlikely that repair mechanisms operate in air-dried cells, this damage will become manifest only upon rehydration (Setlow, 1992).

Radiation and Life

The effects of solar UV-radiation on microbial life and evolution have been reviewed extensively (e.g., Cadet and Vigny, 1990; Horneck and Brack, 1993; Rothschild, 1991). In summary, nucleic acid bases absorb UV-radiation strongly, with maximum absorbance at 260 nm. Proteins also absorb UV-radiation with a peak at 280 nm. It has been well established that killing of cells by UV-radiation is due primarily to its action on DNA, so that UV-radiation near 260 nm is an effective lethal agent. The several defects in DNA caused by radiation include, the formation of pyrimidine dimers, pyrimidine(6-4)pyrimidone photoadducts, DNA protein crosslinks and strand breaks (e.g., Cadet and Vigney, 1990). More recently it has been reported that damage and plasmid inactivation increase with increasing photon energy. This also occurs with increases in dehydration brought about by increasing levels of desiccation from vacuum

(Wehner and Horneck, 1994; Wehner and Horneck, 1995). Because the organisms in this study are not metabolically active during exposure to the space environment, repair mechanisms are not functioning. Under the conditions of anhydrobiosis, protective mechanisms such as pigments, salts (NaCl and gypsum) may act as sunscreens for the organism are of primary importance (Mancinelli and Shulls, 1978; Garcia-Pichel and Castenholz, 1991; Olson and Pierson, 1986; Pierson and Olson, 1989).

Biopan is a pan shaped biological facility developed by the European Space Agency (ESA) to fly in earth orbit on Russian recoverable satellites (DeLonge, 1993). The overall objective of the Biopan series of experiments is to understand the responses of microbes to space vacuum (desiccation) and solar UV-radiation. This study focused on the osmophilic microbes *Synechococcus* (Nägeli) inhabiting evaporitic gypsum-halite crusts that formed along the marine intertidal, and on *Haloarcula*-G isolated from an evaporitic NaCl crystal obtained from a salt evaporation pond. Because they are osmophiles and because gypsum-halite and NaCl attenuate UV-radiation, we hypothesized that these organisms would survive exposure to the space environment during a flight on Biopan.

MATERIALS AND METHODS

Two types of organisms were used in this study: An unidentified species of the cyanobacterium *Synechococcus* (Nägeli) inhabiting gypsum-halite crystals collected from the marine intertidal area along the coast of Baja California, Mexico (described by Rothschild et al., 1994). The other was a pure culture of an unidentified species of the extremely halophilic genus *Haloarcula*, designated as isolate G, (isolated from a NaCl crystal) in mid-log phase growth that had been washed 5 times in 25% NaCl by centrifugation and re-suspended in 25% NaCl. A sufficient amount of each sample was placed onto a 7 mm diameter quartz disc and allowed to dry so that a thin film containing the microbes dried onto the disc, such that microbes were not shading each other. For the flight experiment these discs were placed into Biopan sample holders that were then placed into the Biopan facility (DeLonge, 1993). Three samples were exposed to the sun's solar radiation and space vacuum and 3 samples were exposed to vacuum only.

Biopan was launched 14 June 1994. The organisms were exposed to the space environment for 15 days. The temperature aboard the satellite was measured with thermocouples and the radiation monitored by a dosimeter. After landing, the samples were removed from the facility and shipped back to the laboratory for analysis.

The number of survivors of *Haloarcula*-G in each sample were determined using plate counts and the most probable number method (MPN) (Koch, 1994). Samples were placed in a 25% NaCl solution and 10-fold serial dilutions prepared. Using the spread plate technique, 0.1 mL aliquots of the dilutions were plated onto casein medium, as described by Rothschild et al. (1994), solidified with 1.5% agar. From the serial dilutions, 10 μL aliquots were placed into microtiter plate wells containing 100 μl of liquid medium for MPN determinations. The *Haloarcula* agar and microtiter plates were incubated for three weeks at 30°C (their growth temperature optimum) and growth monitored.

Survival of the *Synechococcus sp.* (Nägeli) samples was based on rates of carbon and nitrogen fixation that were compared to control samples stored in the laboratory during the flight. This particular species produces extracellular material making MPN determinations unreliable. Rates of carbon fixation were performed by determining the production of acid-stable C from $NaH^{14}CO_3$ (Rothschild et al., 1994). Briefly, samples were placed in clear glass vials, and incubated in the presence of an aqueous solution of $NaH^{14}CO_3$ (~1.0 μCi mL^{-1}) under lights. The reactions were stopped by the addition of formalin and placed in the freezer for 1 hr. Aliquots were removed to 7 mL scintillation vials, acidified (to pH < 3) with 50% acetic acid and air dried. The dried samples were resuspended in warm water, the scintillation fluid Ecolume (ICN Biomedicals, Irvine, CA) added, and the counts accumulated with a Packard scintillation

counter. Dark controls were performed similarly except that for 1 h before and during the incubation in the glass vials, the outsides of the vials were wrapped in aluminum foil. Killed controls (autoclaved) were run similarly to eliminate the possibility of abiotic incorporation. Nitrogen fixation rates were performed using the acetylene reduction assay (Postgate, 1972). Samples was placed into 10 mL clear glass serum vials. Acetylene, generated from calcium carbide, was injected into the headspace of each chamber to yield an atmosphere containing not less than 20% acetylene. The vials were incubated in light (900-1200 μmol · m^{-2} · s^{-1}) or dark, at temperatures inside the vials of ~25°-26° C for 5 h. As a control to determine the amount, if any, of ethylene produced endogenously, a sample was incubated similarly, but without acetylene. Additionally, autoclaved (1 h. at 121 °C), or formalin treated (submerged 2 hrs. in 37% formaldehyde followed by drying overnight) killed controls were also tested. Periodically gas samples were collected and analyzed for ethylene production using a Perkin-Elmer model Sigma 3 gas chromatograph equipped with a flame ionization detector.

DNA isolation and breakage analyses were performed by lysing the cells using the GENE-TRAK® Systems (Framingham, MA, USA) sample processing instrument (SPI), and the DNA extracted with phenol/chloroform/isoamyl alcohol (25:24:1 by volume) and precipitated with ethanol according to standard procedures (Sambrook et al., 1989). The amount, if any, of nicking (breakage) of the purified DNA was determined by labeling the nicked sites with [α-^{32}P]dATP using a modification of a standard nick translation protocol (i.e., in the absence of DNase I). Three types of controls were run to assess nicking during extraction: DNA prepared from unexposed cells, DNA purified commercially (i.e., lambda DNA), and commercially purified lambda DNA exposed to the same purification protocol as the other samples.

RESULTS

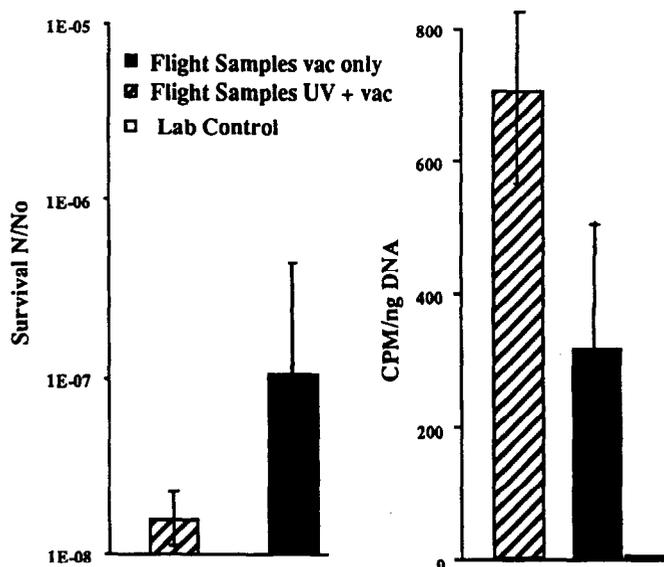During the flight the temperature averaged –10 °C and ranged from –18 °C to + 15 °C, and the total solar

Fig. 1. Data from *Haloarcula*-G samples after exposure to solar UV-radiation and vacuum, or vacuum only during the Biopan flight. The bars indicate the standard error. Survival is depicted in the left panel (N = the number of survivors as determined by MPN; No = the number of surviving cells in the laboratory controls). The right panel depicts the incorporation of [α-^{32}P]dATP] into DNA of flight samples and lab controls.
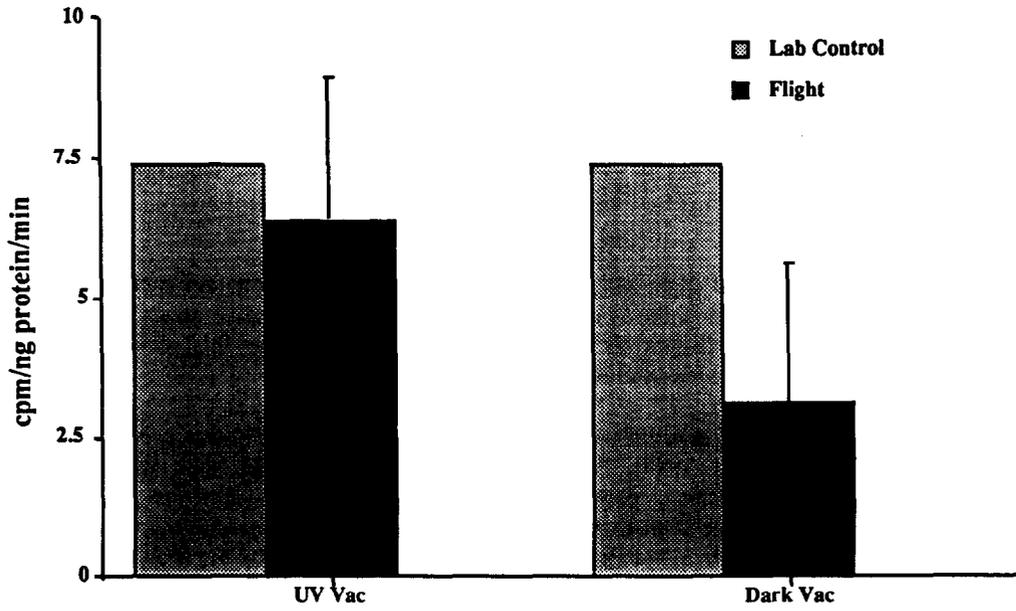
Fig. 2. Carbon fixation data from *Synechococcus* samples after exposure to solar UV-radiation and vacuum, or vacuum only during the Biopan flight. The bars indicate the standard deviation.
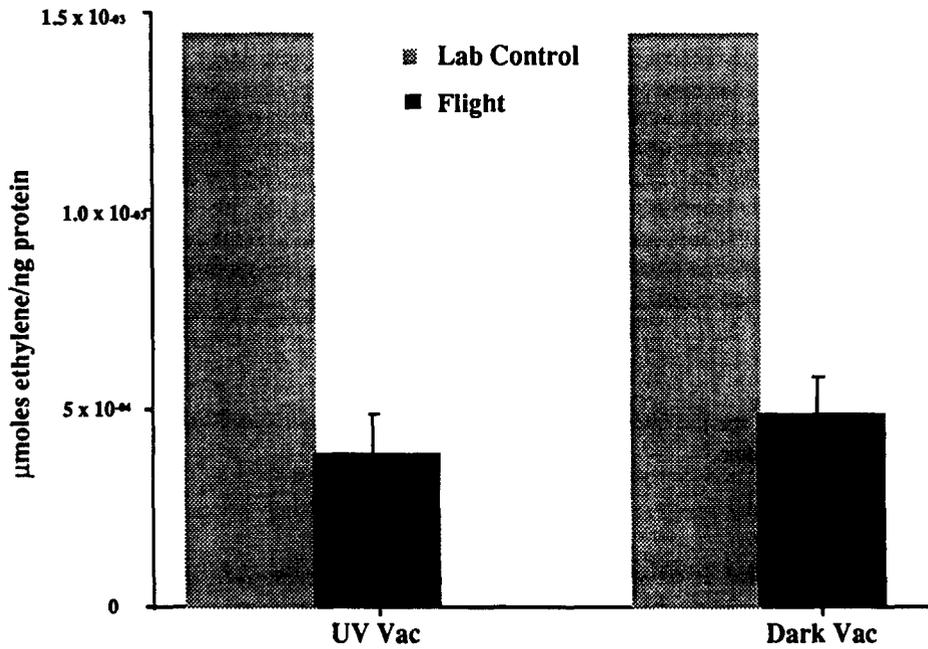

Fig. 3. Nitrogen Fixation data from *Synechococcus* samples after exposure to solar UV-radiation and vacuum or vacuum only during the Biopan flight. The bars indicate the standard deviation.

samples of *Haloarcula*-G had fewer DNA breaks than the solar UV exposed samples (Fig. 1). The *Haloarcula*-G data from the plate counts (data not shown) and MPN method were in agreement and within the experimental error of the MPN data shown in the figure. The rates of N-fixation and C-fixation in the flight samples of *Synechococcus sp.* (Nägeli) revealed that the organisms were still able to metabolize, but the rate of fixation was reduced in the flight samples compared to the laboratory control samples (Fig. 2). UV radiation dose (200 nm - 400 nm) to the organisms was 10^4 KJ m^{-2}. *Haloarcula*- G and *Synechococcus* survived exposure to the space environment (Figs. 1, 2 and 3). The vacuum only exposed

DISCUSSION

Data obtained from the Biopan 1 flight and ground studies show that our original hypothesis was correct, that is, *Haloarcula*-G and the halophilic *Synechococcus* inhabiting gypsum-halite crusts survived exposure to both solar UV radiation and vacuum. In fact there survival rate was higher than all organisms tested to date except *Bacillus* spores (Horneck and Brack, 1992).

Damage to cellular DNA by UV radiation as well as DNA repair of the damage has been well documented (for a review see Friedberg et al., 1995). The anhydrobiotic conditions of the space environment preclude the involvement of repair mechanisms playing a significant role in the survival of the organisms tested in the present study. As a result, the DNA damage observed is cumulative and reflects the total damage to the DNA. However, damage other than breaks in the DNA will not be detected by the analytical methods used in this study. The anhydrobiotic conditions produced by space vacuum leads to cellular DNA damage through strand breaks (Dose and Gill 1995). The data from this study, however, suggests that solar UV radiation may be the primary factor in killing these cells through DNA damage and is in agreement with the data obtained by Horneck et al., (1994) and Linberg and Horneck (1991).

These Biopan experiments provide the initial data on the ability of osmophilic (halophilic) microbes to survive the space environment, and thus a glimpse into their potential for interplanetary transfer. The data from this experiment suggests that these types of organisms may survive interplanetary transfer aboard spacecraft on the order of months to years, especially if they are protected from solar UV radiation. In light of the recent finding from the Martian meteorite ALH84001 suggesting that microbes may have lived on early Mars (McKay et al., 1996) the results of the research reported here become more relevant to the question of the interplanetary transport of microbes during the early history of our solar system and the origin of life on Earth and Mars. Three important hypotheses may be generated from these observations: 1) Life arose independently on Earth and Mars; 2) Life arose on earth and was transported to Mars; or 3) Life arose on Mars and was transported to Earth.

ACKNOWLEDGMENTS

REFERENCES

Banin, A., and R. L. Mancinelli, Life on Mars? I. The chemical environment. *Adv. Space Res.*, 15(3), 163-170 (1995).

Cadet, J. and P. Vigny, The photochemistry of nucleic acids, in *Bioorganic Photochemistry*. Vol., 1 edited by H. Morrison, pp. 1-72, Wiley and Sons, New York, NY (1990).

Carpenter, J. F. Stabilization of proteins during freezing and dehydration: Application of lessons from nature, *Cryobiology* , 30, 220-221 (1993).

Clegg, J. S., The physical properties and metabolic status of Artemia cysts at low water contents: the "water replacement hypothesis", in *Membranes, metabolism and dry organisms, 1st ed.* edited by A. C. Leopold, pp. 169-187, Cornell University Press, Ithaca, NY (1986).

Cox, C. S., Roles of water molecules in bacteria and viruses, *Origins of Life.* 23,29-36 (1993).

Crowe, J. H., F. A. Hoekstra , and L. M. Crowe, Anhydrobiosis. *Annu. Rev. Physiol* 54, 579-599 (1992).

Csonka, L. N. and A. D. Hanson, Prokaryotic osmoregulation: genetics and physiology, *Annu. Rev. Microbiol.* 45, 569-606 (1991).

Delonge, R.. *Design of Experiment Hardware for the ESA Space Facility BIOPAN.* Forschungsbericht. DLR-FB 93-06. Deutsche Forschungsanstalt für Luft- und Raumfahrt, Köln, Germany (1993).

Dose, K., A. Bieger-Dose, M. Labusch, and M. Gill, Survival in extreme dryness and DNA-Single strand Breaks. *Adv. Space Res.* 12(4), 221-229 (1992).

Dose, K., A. Bieger-Dose, R. Dillman, M. Gill, O. Kerz, A. Klein, H. Meinert, T. Nawroth, S. Risi and C. Stridde, ERA-Experiment :Space Biochemistry. *Adv. Space Res.* 16(8), 119-129 (1995)

Dose, K., A. Bieger-Dose,O. Kerz, and M. Gill, DNA strand breaks limit survival in extreme dryness. *Origins of Life* , 21, 177-187 (1991).

Dose, K., and M. Gill, DNA stability and survival of Bacillus subtilis spores in extreme dryness. *Origins of Life,* 25, 277-293 (1994).

Friedberg, E. C., G. C. Walker, and S. Wolfram. DNA Repair and Mutagenesis. ASM Press Washington DC (1995).

Garcia-Pichel, F. and R. W. Castenholz, Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment, *J. Phyc.* 27, 395-409 (1991).

Horneck, G. and A. Brack, Study of the origin, evolution and distribution of life with emphasis on exobiology experiments in earth orbit, *Adv. Space Biol. Med.,* 2, 229-262 (1992).

Horneck, G. H. Bücker and G. Reitz, Long term survival of bacterial spores in space. *Adv. Space Res.* 14(10), 41-45 (1994).

Horneck, G., Responses of *Bacillus subtilis* spores to space environment: results from experiments in space, *Origins of Life,* 23,37-52 (1993).

Klein, H. P., Simulation of the Viking biology experiments: An overview. *J. Molec. Evol.* 14, 161-165 (1979).

Koch, A. L., Growth Measurement, in *Methods for General and Molecular Bacteriology,* edited by P. Gerhardt, pp.248-277 (1994).

Lindberg, C., and G. Horneck, Action spectra for survival and spore photoproduct formation of Bacillus subtilis irradiated with short-wavelength (200-300 nm) UV at atmospheric pressure in vacuo. *J. Photochem. Photobiol. B: Biol,* 11, 69-80 (1991).

Mancinelli, R. L. and A. Banin, Life on Mars? II. Physical Restrictions. *Adv. Space. Res.,* 15, (3)171-178 (1995).

Mancinelli, R. L. and W. A. Shulls, Airborne bacteria in an urban environment, *Appl. Environ. Microbiol.* 35, 1095-1101 (1978).

Mazur, P., E.S. Barghoorn, H.O. Halvorson, T.H. Jukes, I.R. Kaplan and L. Margulis, Biological implications of the Viking mission to Mars, *Space Sci. Rev.,* 22, 3-34 (1978).

McKay, D. S., E. K. Gibson Jr., K. L. Thomas-Keperta, H. Vali, C S. Romanek, S. J. Clemett, X. D. F. Chiller, C. R. Maechling, and R. N. Zare, Search for past life on Mars: Possible relic biogenic activity in Martian meteorite ALH84001, *Science,* 273, 924-930 (1996).

Olson, J.M. and B. K. Pierson, Photosynthesis 3.5 thousand million years ago, *Photosynth. Res.* 9, 251-260 (1986).

Pierson, B. K. and J. M. Olson, Evolution of photosynthesis in procaryotes,. in *Microbial Mats. Physiological Ecology of Benthic Microbial Communities,* edited by Y. Cohen and E. Rosenberg, pp. 403-427, American Society for Microbiology, Washington, D.C. (1989).

Postgate, J. R., The acetylene reduction test for nitrogen fixation, in *Methods in Microbiology*, Vol. 6B, edited by J. R. Norris,and D. W. Ribbons, pp. 343-356, Academic Press, London, (1972).

Potts, M., Desiccation Tolerance of Prokaryotes, *Microbiol Rev.*, 58, 755-805 (1994).

Rothschild, L. J., Earth analogs for Martian life. Microbes in evaporites, a new model system for life on Mars, *Icarus,.* 88, 246-260 (1991).

Rothschild, L. J., L. J. Giver, M. R. White and R. L. Mancinelli, Metabolic activity of microorganisms in gypsum-halite crusts. *J. Phyc.*, 30, 431-438 (1994).

Sambrook, J., E. F. Fritsch, and T. Maniatis, Nick Translation of DNA. In *Molecular cloning a laboratory Manual*. Vol., 2. Section 10.6. Cold Spring Harbor, NY (1989).

Secker, J. J. Lepock, and P. Wesson, Damage due to ultraviolet and ionizing radiation during the ejection of shielded micro-organisms from the vicinity of M_o Main sequence and red giant stars, *Astrophys. Space Sci.* 219, 1-28 (1994).

Setlow, P., I will survive: protecting and repairing spore DNA, *J. Bacteriol.*, 174, 2737-2741 (1992).

Vickery, A. M., and H. J. Melosh, The large crater origin of SNC meteorites, *Science*, 237, 738-743 (1987).

Wehner, J. and G. Horneck, Effects of vacuum UV and UVC radiation on dry *E. coli* plasmid pUC19 I. Inactivation, *lacZ*⁻ mutation induction and strand breaks, *J. Photochem. Photobiol. B: Biol.*, 28, 77-851995

Wehner, J. and G. Horneck, Effects of vacuum UV and UVC radiation on dry *E. coli* plasmid pUC19 II. Mutational specificity at the *lacZ* gene, *J. Photochem. Photobiol. B: Biol.*, 30, 171-177 (1995).