# --FINAL REPORT--
# WORKSHOP ON VIABILITY OF
# HALOPHILIC BACTERIA IN SALT DEPOSITS

## BACKGROUND

The isolation of viable organisms from a matrix that suggests their age is of the order of 200 million of years has the potential significance in three areas of interest to the National Aeronautics and Space Administration's program in Exobiology: it first relates to plans to revisit Mars. The physical characteristics of the planet preclude the occurrence of liquid water at present. However, there are surface features that suggest Mars had a considerable aqueous history at one time, and what appear to be paleolake basins (ca.3.5 Byr old) are being considered as prime landing sites for future missions. One such site is the so-called white-rock feature that may be the remnants of a lacustrine deposit. While the chemical composition of this deposit is unknown, its high albedo is suggestive of evaporites consisting of magnesium and sodium chloride. Given that Mars may have had an aqueous history, and if conditions were propitious for the origin and evolution of life, one can imagine an evolution that terminated once conditions on the planet precluded the existence of liquid water. If so, conditions must have been such that were suitable for the growth of organisms in an environment that gave rise to halite crystals. On earth such environments are commonly found in salterns in which extreme halophiles are found growing in brines highly enriched with respect to sodium and magnesium chloride. This implies that evidence for a putative chemical and/or biological evolution on Mars might be sequestered within halite crystals and suggests a rational search strategy for any program that purports to search for life. Secondly, the Agency

supports an active research program in areas concerned with Origin and Early Evolution of Life. The presence of organism (or their remnants) in ancient halites represents an untapped pool of evolutionary information. In a sense this material represents a snapshot into evolutionary history. Finally, evidence that putative martian biota or materials indicative of an earlier chemical and/or biological evolution were contained within halites would affect how standards related to planetary quarantine as well as issues related to "sample return" are viewed.

## INTRODUCTION

The workshop was held from August 19 to August 20 to consider the significance of finding viable extreme halophiles in halites associated with Permian-aged sedimentary deposits. The format consisted of tutorials that addressed issues related to the microbiology and geochemistry of the halite environment and concluded with recommendations that related the significance of this phenomenon to NASA's interest in planetary exploration and the early evolution of life. The attendees represented diverse scientific interests and a list of participants, their disciplines, and affiliations appears as Appendix A.

The occurrence of viable organisms from fossil material, including salt deposits, has been known for a considerable period of time. The age of these organisms is uncertain since the possibility that the organisms were of exogenous origin was not adequately resolved. Organic material indicative of a biological origin can be isolated from various so-called fossil sources. Studies on the stability of amino acids in fossil shells indicate that racemization occurs over time thus masking a convenient criterion of biological origin. There are a class of

amino acids, such as serine, threonine, cysteine, and arginine, that are rapidly degraded whereas others, such as glutamic acid, are relatively stable. Interestingly, while no amino acids were detected in dinosaur eggs found in Montana, eggs from Patagonia were reported to contain amino acids. No data exists at this time for the stability of amino acids in brines such as those found within the fluid inclusions of halite crystals. Such an environment is of particular interest in that the fluid phase consists of a saturated variety of salts and it is not clear whether the material within such inclusions occurs in the liquid bulk phase or is absorbed along the inner faces of crystal surfaces. Proteins and DNA have also been isolated from a variety of fossil materials. Radioimmunoassays can identify albumin and collagen extracted from frozen mammoths whose age is estimated to be 40 Kyr old. A DNA fragment (820 base-pairs) encoding the large subunit of ribulose 1,5-diphosphate carboxylase from Miocene magnolia species (17-20 Myr old) was amplified by the polymerase chain reaction (PCR) using primers based on ribulose 1,5-diphosphate carboxylase sequence from corn. Amber is a prolific source of fossil DNA. DNA from termites and bees encased in 30 Myr old amber has been amplified by PCR and bacterial DNA isolated from amber whose age is approximately 120 Myr old was amplified by PCR. These observations suggest that there are depositional situations that allow for the recovery of what are usually thought to be labile macromolecules and that such molecules retain sufficient information to lead to comparisons with contemporary cells.

Two of the attendees reported on isolating extreme halophiles from halite deposits while taking special precautions to exclude exgonous contamination. Both reported that organisms could be recovered from salt crystals, but that the ability to recover organisms were

markedly different. In one case, viable organisms were successfully isolated from only a few samples and led to the extrapolation that the sample size for successfully isolating bacteria could be about one Kg. On the other hand, samples from another site appeared to be a more prolific source of bacteria and suggested that gram size samples were sufficient for isolation. However, even in the latter case, there were regions that were "sterile" (i.e., from which no organisms could not be isolated). At the present time it is not clear why these investigators had such different experiences with respect to isolating organisms. It is clear that this anomaly needs to be resolved if halite crystals are to be used as source material.

The isolation of DNA from amber was described and suggested as a model for approaching similar problems in halite crystals. The conditions that affect the preservation of DNA are those related to the redox potential, low oxygen concentration, and low water activity that exists within the fluid inclusions. In the studies that were described, pgm quantities of bacterial DNA from a bacterial symbiont of bees, were isolated and compared with DNA from extant bacteria. Even though the DNA had undergone considerable degradation the information content was such as to permit describing the relationship of the organisms trapped in the amber to contemporary organisms.

In addition to the microbiology, geology, and geochemistry discussions, there was a series of presentations on physical chemistry and physical instrumentation directed at the question of determining the age of the organic material in evaporites and other geological specimens.

a. Richard Mathies of the Chemistry Department of the University of California at Berkeley discussed the bacteriorhodopsin of halobacteria and how it may be studied in its various states using laser Raman vibrational spectroscopy. He discussed the Raman microscope which allows the examination by Raman spectroscopy of very small volumes. In one case he presented data from a single frog rod (Volume 100). He proposed the following uses of the Raman microprobe:

1. To provide in situ characterizations of carotenoids and rhodopsins in halobionts,
2. To show that cultured populations are (or are not) derived from bacteria in the halites,
3. To discover new mutants and bacteriorhodopsins.

He also discussed the ability to fabricate micro-chemical analysis systems on a chip. This would allow biochemistry on much smaller samples (1 $\mu$ liter) allowing direct analysis of very small samples taken from halite.

b. Richard Keller of Los Alamos National Laboratories reviewed their work on sensitive fluorescent detection of single molecules. He proposed the following

possible uses of the techniques:

1.Sizing of DNA fragments in a single bacteria without PCR.

2.Determination that a specific DNA sequence is present or absent in a single bacterium by using a fluorescence tagged hybridization probe.

3.Determine the presence or absence of a particular protein using tagged antibody that fluoresces only when bonded to an antigen.

4.Rapid sequencing of single DNA fragments from a single bacterium.

c. Richard Leapman of NIH reviewed the potential of electron microscopy to:

1.Characterize individual cells of constituent macromolecular assemblies.

2.Provide structural and compositional information on cells.

He reviewed the classes of microscopy.

1.Transmission EM for internal structure.

2.Scanning EM for surface structure.

3.Scanning transmission EM for quantitative microanalysis.

He discussed electron energy loss spectroscopy and energy dispersive X-ray spectroscopy for elemental analysis.

All of the above methods used with appropriate preparative technique could provide information at the cellular and subcellular level on membrane structure, ion gradients, total nucleic acids, and total cell mass.

d. Edgar Hare of the Carnegie Institute of Washington discussed chemical methods of dating samples based primarily on racemization of amino acids. There is a large database

obtained from laboratory experiments on racemization at higher than ambient temperatures and analysis of dated fossil material. Since each amino acid has its own rate of racemization, this presents up to 19 parameters per sample. There is data on samples that have been exposed to varying water activities. Hydrated DNA undergoes breakdown largely by depurination. There appears to be little or no data on material in saturated brines or salt crystals.

e. Robert Finkel of Lawrence Livermore Laboratory introduced the subject of dating using isotope ratio as determined by an accelerator mass spectrometer. He drew the distinction between primordial nuclides and cosmo genic nuclides. The latter have lifetimes ranging from days to 3He which is stable. He considered the case of 14C with a halflife of 5730 years. In a best-case scenario, we estimated that a determination could be made on 20 micrograms of carbon (the order of 108 cells or about a colony growing on an agar surface) which would tell if those cells were older than 30,000 years. This could classify carbon in samples as being of some minimum age.

f. Lee Riciputi of Oak Ridge National Laboratory discussed the Rb-Sr dating of fluid inclusion. A: "Individual fluid inclusions are selected and then extracted using micropippettes ($<10$ micron diameter). The extracted fluid is then processed on cation exchange columns to separate the Rb and Sr, and these are run using thermal ionization mass spectrometry; isotope dilution is used to determine the Rb and Sr content of the samples at the same time the isotope ratios are measure.

Preliminary work was done using fluid inclusions $>200$ microns in diameter, as the contamination level was fairly high; with completion of a new class 100 clean room and using exchange columns designed for small samples, inclusions of 50 microns should be possible.

For very small samples, ORNL has special home-built, multi-stage mass spectrometers with extremely high sensitivity that can analyze orders of magnitude less material than conventional instruments. In addition, a new type of instrument, a multicollector ICP-MS designed for the analysis of isotope ratios offers the potential of direct analysis of fluid inclusion brines without the need for chemical separation. If the potential of this instrument proves out, it may be possible to analyze individual fluid inclusions in the 10 micron size range. The Rb-Sr dating technique currently offers the only method of directly dating the age of fluids trapped in various evaporite minerals. If different types of fluid inclusions can be related to different geological occurrences, this technique can date each of these events. This technique is probably appropriate for dating material 10's of millions of years old and older, with potential precision of a few percent."

g. T. C. Onstott of the Department of Geology of Princeton University discussed 40Ar/39Ar dating.

"Viable halobacteria recovered from "rock" salt may have been sequestered within the intergranular pores or within fluid inclusions. The latter may be either primary, i.e., formed at the time of deposition, or secondary, i.e., formed during some later recrystallization event. The time at which fluid and presumably halobacteria last moved through the intergranular pores can be determined by dating pore filling K-rich sulfate phases, such as polyhalite. This was recently accomplished by utilizing the 40Ar/39Ar laser microprobe. Thin sections of salt form the WIPP site were irradiated by neutrons, placed within an ultra-high vacuum system beneath a microscope, and polyhalite crystals approximately 20-50 u in size were selectively ablated with a

focussed laser beam and the Ar released analyzed in a mass spectrometer. Over 20 analyses fall on an isochron yielding an age of 215 $\pm$ 2 Ma. Portions of halite grains rich in fluid inclusions were also selectively decrepitated with the laser beam. The Ar released from the fluid inclusions lie within error of the polyhalite isochron and establishes their age as being quite ancient and not due to recent meteoric interaction.

h. Harold Morowitz of George Mason University discussed thermal inactivation of bacteria in terms of absolute reaction rate theory which yield enthalpies and entropies of activation for the inactivation process. From experiments on bacteria in salt solution and dried in salt crystals at elevated temperatures, extrapolation is possible to the temperature history of the evaporites to determine if long-term survival is theoretically reasonable and to explore possible methods of protection in a crystal.

Appendix A

## PARTICIPANTS


RAUL CANO
Biological Sciences Department
California Polytechnic State University
San Luis Obispo CA 93407
Phone: (805) 756-2440
FAX: (805) 756-1419

SHERWOOD CHANG
Exobiology Branch
Ames Research Center M/S 239-4
Moffett Field CA 94035-1000

DAVID DesMARAIS
Ames Research Center M/S 239-4
Moffett Field CA 94035-1000

JACK FARMER
Planetary Biology Branch
Ames Research Center
239-4
Moffett Field CA 93035-1000

ROBERT FINKEL
Lawrence Livermore Laboratory
Box 808
M/S 232
Livermore CA 94550
Phone: (510) 422-2044

JAMES FREDERICKSON
P.O.Box 999
Richland WA 99352
Phone: (509) 375-3908
FAX: (509) 375-6666

J. PETER GOGARTEN
Department of Molecular and Cell Biology
University of Connecticut
Storrs CT 06269-3044
Phone: (203) 486-4061
FAX: (203) 486-1784

WILLIAM GRANT
University of Leicester
P.O.Box 138
Department of Microbiology
Medical Sciences Building
University Road
Leicester LEI 9HN
ENGLAND
Phone: 44-533-522-948
FAX: 44-533-523-013

EDWARD HARE
Carnegie Institute of Washington
5251 Broad Branch Road, N.W.
Washington D.C. 20015-1305
Phone: (202) 686-2410

LAWRENCE HOCHSTEIN
Planetary Biology Branch
Ames Research Center
239-4
Moffett Field CA 93035-1000

THOMAS HOERING
Carnegie Institute of Washington
5251 Broad Branch Road, N.W.
Washington D.C. 20015-1305
Phone: (202) 686-2410

RICHARD A. KELLER
CST-2
Mail Stop M 888
Los Alamos National Laboratory
Los Alamos NM 87544
Phone: (505) 667-3028

MAX KENNEDY
Industrial Research, Ltd.
Gracefield Research Centre
Gracefield Road - P.O. Box 31-310
Lower Hutt NEW ZEALAND
Phone: 64-4-569-0000
FAX: 64-4-569-0132

L. PAUL KNAUTH
Department of Geology
Arizona State University
Tempe AZ 85287-1404

RICHARD LEAPMAN
Biomedical Engineering and Instrumentation Branch
National Center for Research Resources
National Institutes of Health
Bethesda MD
Phone: (303) 496-2566

RICHARD MATHIES
Chemistry Department
University of California
Berkeley CA 94720
Phone: (510) 642-4192

MICHAEL MEYER
NASA Headquarters, Mail Code SL
300 E. St., S.W.
Washington D.C. 20546-0001
Phone: (202) 358-0307

HAROLD MOROWITZ
Mail Stop 1D6
George Mason University
4400 University Drive
Fairfax VA 22030-4444
Phone: (703) 993-2173
FAX: (703) 993-2175

T.C. ONSTOTT
Department of Geology
Princeton University
Princeton NJ 08544
Phone: (609) 258-1234

DENNIS W. POWERS
Star Route Box 87
Anthony TX 79821
FAX: (915) 581-8636

LEE RICIPUTI
Chemical Analytical Sciences Division
Oak Ridge National Laboratory
P.O. Box 2008
Oak Ridge TN 37831-6365

NELSON R. SHAFFER
Mineral Resources Section
Indiana Geological Survey
611 N. Walnut Grove
Bloomington IN
Phone: (812) 855-2687
FAX: (812) 855-2862

RUSSELL VREELAND
Department of Biology
West Chester University
West Chester PA 19383
Phone: (215) 436-2479