Characterization of a moderately halo-acidophilic bacterium isolated from Lake Brown, Western Australia

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ABSTRACT

Compared to prevalent alkaline to neutral hypersaline environments, acidic hypersaline environments have been scarcely studied. However, they hold interest to many researchers in that these environments have similar geological and geochemical characteristics as those found in lithified strata on Mars. Fieldwork indicated that Lake Brown, located in Western Australia, possessed pH values of 3.1-4.5 and salinity between 13.0-23.0%. Water column, groundwater, and sediment samples were collected from the lake during the austral winter of 2005. These samples were analyzed with both traditional culture and molecular methods. Modified growth media and minimal media were designed to match the composition (Cl, Na, Mg, SO4, K, Ca, and Br) of Lake Brown surface and ground waters for the enrichment of microorganisms. One of the isolates obtained, Brown 1, can grow in media that possesses pH values of 3-7 with optimal growth at pH 4, salinity that ranged from 5% to saturation with optimal growth at 5% and could grow under temperatures that ranged from 20ºC to 65ºC with optimal grow occurring at 37ºC. The isolate's optimum growth conditions are similar to those found in Lake Brown. The isolate is a Gram-negative rod that forms yellow colonies on 17% Phytogel. Its 16S rRNA gene can be amplified with bacterial primers but not with archaeal primers. Its 16S rRNA gene sequence suggests that the isolate is a gamma proteobacterium. Studies on organisms isolated from environments such as Lake Brown, an acid hypersaline lake, can provide an opportunity to both expand our knowledge of terrestrial extremophiles and gain insight on the possible forms of life that might have existed on Mars.

Key Words: Moderate halo-acidophilic bacterium, Lake Brown, Western Australia, Mars-like conditions

1. INTRODUCTION

Prior to 2004, there has been evidence of previous acid saline environments on Mars [(1-4)]. Since then, the Mars Exploration Rover, Opportunity, has provided evidence *in situ* at the Meridiani Planum for the previous presence of these environments on the surface of Mars. In addition, the Rover, Spirit, as well as remotely sensed data from the various current orbitors, also have shown evidence of past acid brines in other locations besides Meridiani Planum on Mars [(5)]. This evidence includes sedimentary rocks composed of fine-grained siliciclastic materials, sulfate minerals-notably calcium sulfate, magnesium sulfate and jarosite, and hematite [(6-9)]. Thus, it is likely that there were acid saline groundwater and shallow surface water on Mars.

Acid saline lake systems have been recognized in Western Australia [(10-13)] and northwestern Victoria [(14-16)], as well as in Chile [(17)]. Naturally acid saline systems with pH values between 1.7 and 4 are common on the Yilgarn Craton of southern Western Australia [(18)]. Ergo, the acid saline systems in Western Australia are the best known terrestrial analogs to martian and sedimentary rocks because they are similar in (1) mineralogy (iron oxides, sulfates, jarosite, clays); (2) sedimentary structures that indicate deposition by winds and shallow surface waters; and (3) diagenetic features, including hematite concretions and displacive sulfate crystal molds.

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Lake Brown was one of 22 lakes in the Yilgarn Craton that was sampled during the austral winter of 2005. It possesses high salinity and at time of sampling, a pH of 4.5. It also possesses sedimentary formations that are similar to those found on Mars. This lake can serve as a modern terrestrial analog for the possible acid saline environments of Mars [19]. Due it its high salinity and acidic pH, it is likely to harbor novel microorganisms. The isolation and description of these novel isolates can increase our understanding of possible previous life on Mars. A bacterium that has been isolated from Lake Brown is described in this article. Studies of this organism and others that are isolated from salty acidic environments can help us understand the possible metabolic capabilities life would have had to possess to survive and possibly thrive on early Mars.

2. MATERIALS AND METHODS

2.1 Site description and sample collection Lake Brown is located about 278 km northeast of Perth (S 31° 07.815', W 118° 18.444') (Figure 1). The lake area is about 10.0 ac (4.0 ha) and lake circumference was measured to be 1.0 km. During June 2005, lake and ground water as well as sediment samples were taken from Lake Brown. During this period, the lake was at a flooding stage. The depth of the lake at its deepest was 30 cm, the lake water possessed a pH of 4.5 and temperature ranged from 12-22°C. The groundwater possessed pH values from pH 3.1-3.7 [18]. Water samples were collected in sterile 500 ml high density polyethylene bottles. The bottles were stored in coolers and flown back to UMR. Once at UMR, the samples were stored at 4°C until enrichments were prepared.

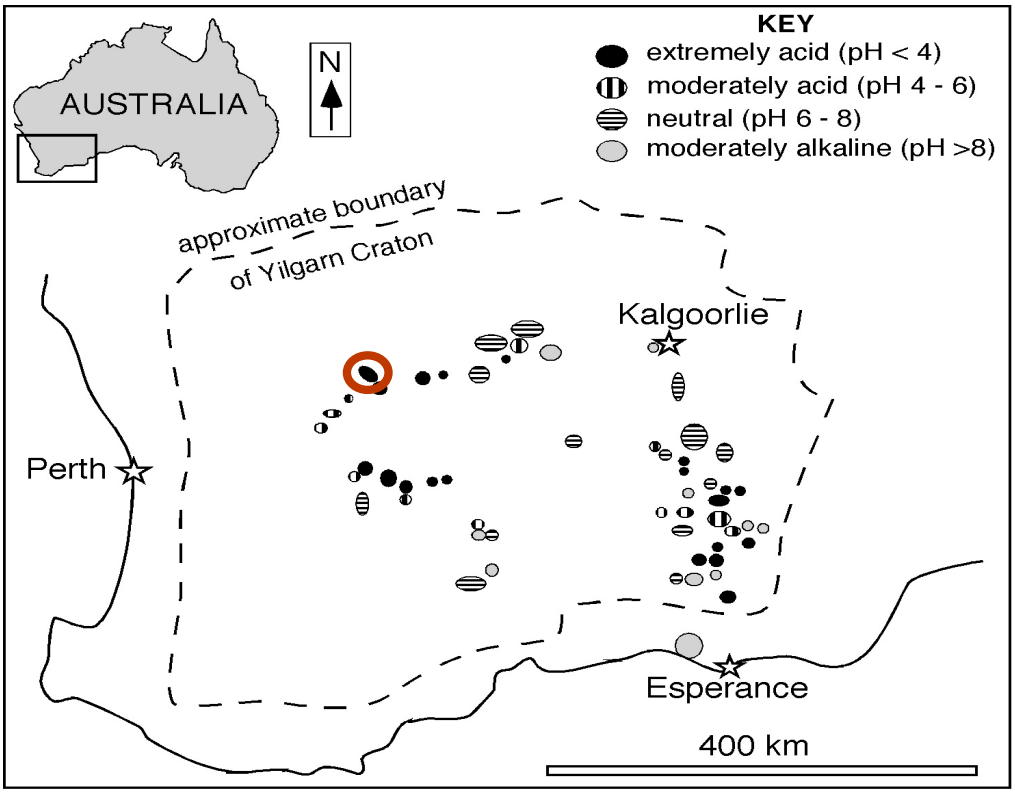

Figure 1 Map of South Western Australia showing the pH ranges of the lakes that are present in this region. Lake Brown is designated with a red circle.

2.2 Growth media, isolation and enrichment culture condition Modified growth medium (MGM) was prepared with a 30% stock solution mixed with deionized water. The 30% Stock Solution contained the following (g/L deionized water): NaCl 240g, $MgCl_2 \cdot 6H_2O$ 30g, $MgSO_4 \cdot 7H_2O$ 35g, KCl 7g, NaBr 0.8g, $NaHCO_3$ 0.2g. $CaCl_2$ $2H_2O$ was added slowly from a 1M sterile stock to give a final concentration of 0.5g/L. The pH was adjusted to 4.0 with 1M HCl. Oxoid peptone, 0.75% and 0.15% of yeast extract were added to MGM as carbon sources. Citric acid was used for the pH

buffer. Solid medium, 1.5% MGM phytogel plates, were prepared for the isolation of colonies. Phytogel was autoclaved separately from MGM to prevent hydrolysis of the gel. The incubation temperature was 37°C.

2.3 Morphological, physiological and biochemical characterization Once isolated, Brown 1's cell morphology was examined by light microscopy, motility was determined by wet mounts, and Gram reaction was determined by using a Gram-stain kit (Difco, MI). The isolate was also tested for oxygen tolerance/requirement and its range of tolerance and optimum conditions of temperature, pH and salinity. All tolerance tests were performed in triplicate. The presence of cytochrome oxidase and catalase was determined after growth on MGM agar poised at pH 4 and containing 5% salt [20]. To determine carbon utilization, assays were also performed with MGM media that was amended with filter-sterilized carbon sources. Cultures that achieved twice the OD_{660nm} of the uninoculated controls were considered as positive growth.

2.4 DNA extraction, PCR amplification, RFLP and sequencing of 16S rRNA gene fragments DNA was extracted from the isolate by using an UltraClean soil DNA isolation kit (MO Bio Laboratories, Solana Beach, CA, USA) per the manufacturer's instructions. Primers targeting the nucleotide positions 27-1392, based on the 16S rRNA gene sequence of *E. coli*, were used to amplify the 16S rRNA sequence of Brown 1. The sequence of the 27 forward primer is 5'AGAGTTTGATCMTGGCTCAG-3' and 1392 reverse primer sequence is 5'ACGGGTGTGTRC-3'. The PCR conditions for amplifying DNA from Brown 1 were 95°C for 5 minutes, 34 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute and 30 seconds, extention for 10 minutes at 72°C. The PCR reaction mix was 50 µL PCR master mix containing 2.5 U taq DNA polymerase, 20 pmol of each primers, 5 µL 10X PCR buffer, 5.0 mM MgCl2, 500 µL dNTP and 2 µL purified DNA extract.

2.5 Sequencing and phylogenetic analysis Amplified DNA was purified by using a PCR product purification kit (Qiagen, Valencia, CA) and was sequenced on a 3130 Applied Biosystem Sequencer (Foster City, CA) by using Big Dye 3.1 chemistry at the UMR cDNA Resource Center (Rolla, MO). Since this sequencer can provide about 700 base pair reads, two readings were necessary, one from the forward direction using forward primer (27F) and the other from the reverse direction using reverse primer (1392R). The assembled sequence was analyzed by conducting BLAST searches of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rDNA sequence of Brown 1 and each sequence of its closely related neighbors were aligned by using CLUSTAL X 1.83 [21]. Maximum likelihood trees were constructed by using PAUP (4.0 beta) [22].

3. RESULTS AND DISCUSSION

3.1 Phenotypic characterization Brown 1 was isolated from Lake Brown surface water. Its colony morphology was yellow, smooth, circular and <1 mm in diameter on phytogel (Figure 2A). The Gram reaction indicated that it is Gram negative and microscopic examination indicated they are rod-shaped with round shaped structures in the middle of the body of their cells (Figure 2B). It could grow in NaCl concentrations ranging from 5% to saturation with optimum growth occurring at 5% (Figure 3A). The isolate could grow under pH conditions from 4.0 to 6.0 with optimum growth at 4.0 (Figure 3B). Its optimum temperature for grow was 37°C and it tolerated a range from 20-60°C (Figure 3C).

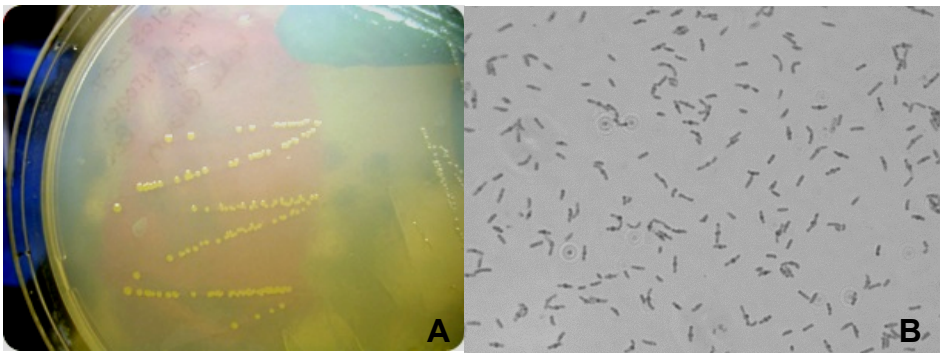

Figure 2 Images of Brown 1. Image A shows the yellow colonies formed by Brown 1. Image B is a visible light microphotograph of Brown 1.

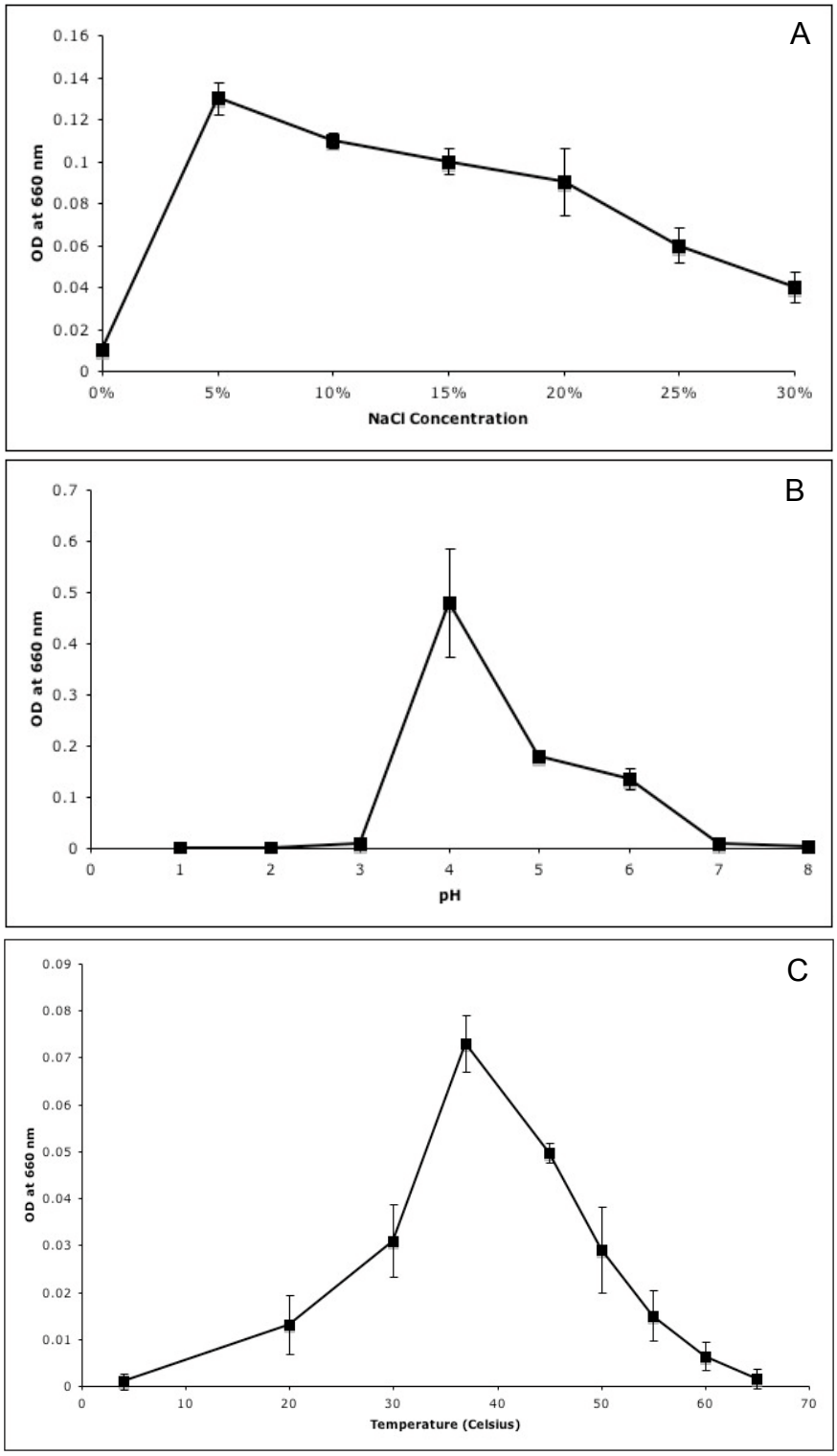

Figure 3 Growth tolerances and optima of isolate Brown 1 for NaCl concentrations (A), pH (B) and temperature (C). Standard deviations are given.

3.2 Phylogenetic analysis A total of 1313 base pairs were read of the 16S rRNA gene sequence of isolate Brown 1. When its sequence was compared to other known sequences, the closest relatives (95% matches) were *Salinisphaere* sp. ARD M17, *Salinisphaera shabanense* isolate E1L3A, and an unidentified gamma proteobacterium designated strain KT0813. A phylogenetic tree showing Brown 1 and related organisms is given in Figure 4.

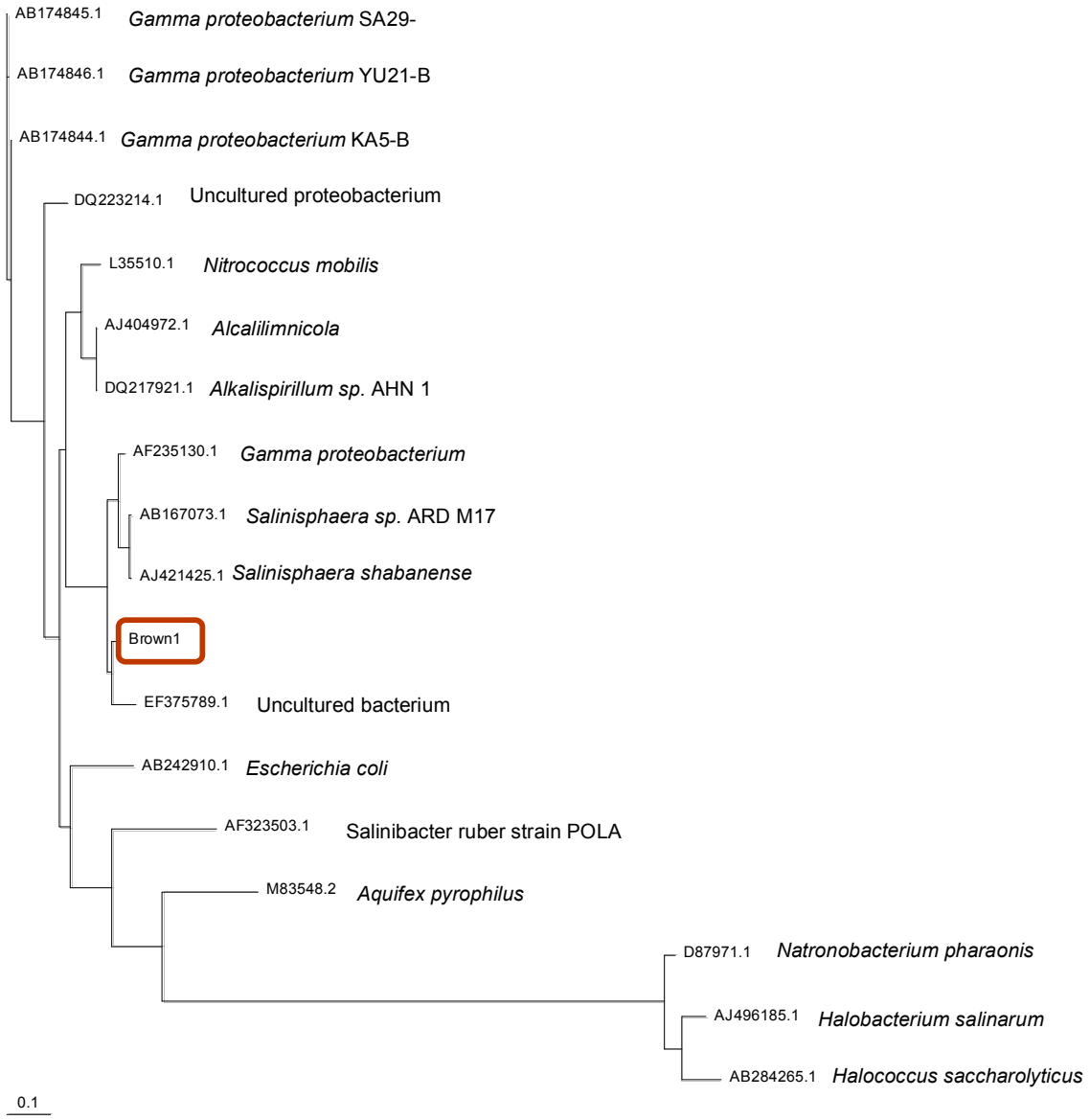

Figure 4 Phylogenetic tree demonstrating the relation of Brown 1 to its closest relatives.

3.3 Comparison of Brown 1 to *Salinisphaera shabanense* Very few organisms, that are closely related to isolate Brown 1, have been isolated and characterized. The most closely related (95% similarity of their 16S rRNA gene sequences) isolated and characterized organism is *Salinisphaera shabanensis*. This organism was isolated from the brine-seawater interface of the Shaban Deep of the northern Red Sea [23]. Table 1 provides a comparison between the two isolates.

Table 1 Comparison between Brown 1 and its closest characterized relative, *Salinisphaera shabanensis*

Characteristic	Brown 1	*Salinisphaera shabanensis* *
Gram reaction	-	-
Cell shape	rod	coccus
Motility	+	+
Colony color	yellow	brown
NaCl growth range	5% to saturation	1-28%
NaCl optimum concentration	5%	10%
pH growth range	4-6	4-8
pH optimum	4	6.5-7.5
Temperature growth range	20-60°C	5-42°C
Temperature optimum	37°C	30-37°C
Catalase	-	+
Cytochrome oxidase	-	+
Respiration	aerobic	microaerophilic and fermentative

*Data from reference 23

Isolate Brown 1 grew well on the following carbon sources: D-ribose, D-glucose, inositol, glucarate, ethanol, phenylalanine, and benzoic acid. It could also grow on serine, isoleucine, sorbitol, glutamine, glycine, fructose, lactose, asparagines, dulcitol, D-xylose, and ornithine. The following carbon sources did not support growth: cysteine, coumaric acid, aspartic acid, methanol, gallic acid, fumaric acid, glutamic acid,vanillic acid, adonitol, bromobenzoic acid or chlorobenzoic acid.

Isolate Brown 1 is novel species isolated from an acidic saline lake, Lake Brown, in Western Australia and possibly represents a new genus. There are numerous differences but most notable is the difference in cell shape and the absence of catalase and cytochrome oxidase activity of Brown 1 and thus providing support for it representing a new genus.

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REFERENCES

1. Burns, R.G., "Gossans on Mars", Lunar and Planetary Science Conference, 18th, Houston, TX, Mar. 16-20, Proceedings (A89-10851 01-91), 1987.

2. Burns, R.G., "Schwertmannite on Mars: Deposition of This Ferric Oxyhydroxysulfate Mineral in Acidic Saline Meltwaters", Lunar and Planetary Science Conference, 25th, Houston, TX, Mar. 14-18, Proceedings p. 203, 1994.

3. Clark, B.C., A.J. Castro, C.D. Rowe, A.K. Baird, P.H. Evans, H.J. Rose, P. Toulmin, K. Keil III, and W.C. Kelliher, "Inorganic analyses of Martian surface samples at the Viking Landing site", Science, **194**, 1283-1288, 1976.

4. Clark, B.C., "Chemical and physical microenvironments at the Viking landing sites", J. Mol. Evol., **14**, 13-31, 1979.

5. Morris, R.V., Klingelhöfer, G., Bernhardt, B., Schröder, C., Rodionov, D.S., de Souza, P.A., Yen, A., Gellert, R., Evlanov, E.N., Foh, J., Kankeleit, E., Gütlich, P., Ming, D.W., Renz, F., Wdowiak, T., Squyres, S.W., Arvidson, R.E. 2004. Mineralogy at Gusev Crater from the Mössbauer Spectrometer on the Spirit Rover. Science, **305**, 833-837.

6. Clark, B.C., R.V. Morris, S.M. McLennan, R. Gellert, B. Jolliff, A.H. Knoll, S.W. Squyres, T.K. Lowenstein, D.W. Ming, N.J. Tosca *et al.*, "Chemistry and mineralogy of outcrops at Meridiani Planum", EPSL, **240**, 73-94, 2005.

7. Klingelhöfer, G., R.V. Morris, B. Bernhardt, C. Schröder, D.S. Rodionov, P.A. Souza Jr., A. Yen, R. Gellert, E.N. Evlanov, B. Zubkov, J. Foh, U. Bonnes, E. Kankeleit, P. Gütlich, D.W. Ming, F. Renz, T. Wdowiak, S.W. Squyres, R.E. Arvidson, "Jarosite and hematite at Meridiani Planum from Opportunity's Mössbauer spectrometer", Science, **306**, 1740-1745, 2004.

8. McLennon, S.M., Bell, J.F., Calvin, W.M., Christensen, P.R., Clark, B.C., de Souza, P.A., Farmer, J., Farrand, W.H., Fike, D.A., Gellert, R., Ghosh, A., Glotch, T.D., Grotzinger, J.P., Hahn, B., Herkenhoff, K.E., Hurowitz, J.A., Johnson, J.R., Johnson, S.S., Jolliff, B., Klingelhofer, G., Knoll, A.H., Learner, Z., Malin, M.C., McSween, H.Y., Pocock, J., Ruff. S.W., Soderblom, L.A., Squyres, S.W., Tosca, N.J., Watters, W.A., Wyatt, M.B., and Yen, A., "Provenance and diagenesis of the evaporite-bearing Burns formation, Meridiani Planum, Mars", Earth and Planetary Science Letters, **240**, 95-121, 2005.

9. Squyres, S.W., J.P. Grotzinger, R.E. Arvidson, J.F. Bell III, W. Calvin, P.R. Christensen, B.C. Clark, J.A. Crisp, W.H. Farrand, K.E. Herkenhoff, J.R. Johnson, G. Klingelhöfer, A.H. Knoll, S.M. McLennan, H.Y. McSween Jr., R.V. Morris, J.W. Rice Jr., R. Rieder, and L.A. Soderblom, "*In situ* evidence for an ancient aqueous environment at Meridiani Planum, Mars", Science, **306**, 1709-1745, 2004.

10. Alpers, C.N., R.O. Rye, D.K. Nordstrom, L.D. White, and B.S. King, "Chemical, crystallographic and stable isotope properties of alunite and jarosite from acid-hypersaline Australian lakes", Chem. Geol., **96**, 203-206, 1992.

11. Mann, A. W, "Hydrogeochemistry and weathering on the Yilgarn Block, Western Australia- ferrolysis and heavy metals in continental brines", Geochimica et Cosmochimica Acta, **47**, 181-190, 1983.

12. McArthur, J.M., J. Turner, W.B. Lyons, and M.F. Thirlwall, "Salt sources and water-rock interaction on the Yilgarn Block, Australia: Isotopic and major element tracters", Appl. Geochem., **4**, 79-92, 1989.

13. McArthur, J.M., J. Turner, W.B. Lyons, A.O. Osborn, and M.F. Thirlwall, "Hydrochemistry on the Yilgarn Block, Australia: Ferrolysis and mineralization in acidic brines", Geochim. Cosmochim. Acta., **55**, 1273-1288, 1991.

14. D.T. Long, N.E. Fegan, W.B. Lyons, M.E. Hines, P.G. Macumber, and A. Giblin, "Geochemistry of acid brines: Lake Tyrrell, Victoria, Australia", Chem. Geol., **96**, 33-52, 1992.

15. D.T. Long, N.E. Fegan, J.D. McKee, W.B. Lyons, M.E. Hines, and P.G. Macumber, "Formation of alunite, jarosite, and iron oxides in a hypersaline system: Lake Tyrrell, Victoria, Australia", Chem. Geol., **96**, 183-202, 1992.

16. Macumber, P.G., “Hydrological processes in the Tyrrell Basin, southeastern Australia”, Chem. Geol., **96**, 1-18, 1992.

17. Risacher, F., Alonso, H., and Salazar, C., “Hydrochemistry of two adjacent acid saline lakes in the Andes of northern Chile”, Chem. Geol., **187**, 39-57, 2002.

18. Benison, K.C., B.B. Bowen, F.E. Ikuenobe, E.A. Jagniecki, D.A. LaClair, S.L. Story, M.R. Mormile, and B. Hong, “Sedimentary processes and products of ephemeral acid saline lakes in southern Western Australia”, J. Sed. Res., **77,** 366-388, 2007.

19. Benison, K.C. and B.B. Bowen,”Acid saline lake systems give clues about past environments and the search for life on Mars”, Icarus, **183**, 225-229, 2006.

20. R.M. Smibert and N.R. Krieg, “General Characterization”, *Manual of methods for general bacteriology*, edited by P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips, 409-443, American Society for Microbiology, Washington, D.C.

21. J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins, “The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools”, Nucleic Acids Res., **24**, 4876-4882, 1997.

22. D.L. Swofford, “PAUP*. Phylogenetic Analysis Using Parsimony (*and other 338 Methods). Version 4.0b 10”, Sinauer Associates, Sunderland, MA, 2003.

23. Antunes, A., W. Eder, P. Fareleira, H. Santos, and R. Huber, “Salinisphaere shabanensis gen. nov., sp. nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea”, Extremophiles, **7**, 29-34.