

Extremely Thermophilic Acidophilic Bacteria Convergent with *Sulfolobus acidocaldarius*

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SUMMARY

A series of extremely thermophilic acidophilic bacteria has been characterized as closely resembling the species *Sulfolobus acidocaldarius* except for a totally different guanosine–cytosine content in the DNA; some conceptual consequences of this situation are discussed. Both organisms also share special features, including a very characteristic type of ether lipid, with other extreme acidophilic thermophiles.

INTRODUCTION

There has recently been increasing interest in the characterization of extremely thermophilic bacteria, particularly those which are also adapted to strongly acidic conditions such as hot springs in certain volcanic areas. Brock, Brock, Belly & Weiss (1972) have described from such sources a small pleiomorphic bacterium, *Sulfolobus acidocaldarius*, strains of which grow at temperatures as high as 85 °C and pH values as low as 1.0. This facultative sulphur autotroph is at least partly responsible for the formation of sulphuric acid from volcanic sulphur in such locations (Eliermans & Brock, 1972). Earlier, Darland, Brock, Samsonoff & Conti (1970) described a mycoplasma-like organism from burning coal-tips, *Thermoplasma acidophila*, growing at up to 65 °C at pH values down to 0.5; more recently Brierley & Brierley (1973) described a pleiomorphic bacterium rather similar to *S. acidocaldarius* but obligately autotrophic on sulphur or ferrous iron, growing at up to 70 °C at pH 1 or above.

Though these three organisms seem to be distinct in several ways, they also share many characteristic features, presumably adaptive for their extreme habitats. We describe a series of isolates from a natural thermal acid source which appear indistinguishable from *S. acidocaldarius* except in the guanosine–cytosine (GC) content of their DNA, such that on this evidence alone they could be assigned to a different genus. We offer this account as evidence of the almost complete evolutionary convergence that is likely to be encountered in organisms from such extreme habitats. In an accompanying paper (Millonig, de Rosa, Gambacorta & Bu'Lock, 1975) the morphology, ultrastructure and mode of division of these organisms are described; the present paper gives their physiological and chemical characteristics with some discussion of their relationship to other extremely acidophilic thermophiles.

METHODS

Sources. In the Pisciarelli solfatara (Agnano, near Naples, Italy) are a series of small hot springs whose acidity is due to sulphuric acid, with an abundance of sulphur in the surrounding soil and on the water surfaces. The analyses (Table 1) were carried out by conventional methods, calcium and magnesium being assayed complexometrically, iron colorimetrically with *o*-phenanthroline, sulphate turbidimetrically as BaSO₄, chloride by Mohr titration, and sulphide plus sulphite by iodometric titration.

Isolation. Samples of thermal water from the temperature range 74 to 89 °C and the pH range 1.4 to 2.6, were collected in sterile bottles and enriched as soon as possible with 0.1 % yeast extract (Difco), adjusting the pH to 2.0 with H₂SO₄ or NaOH. Cultures usually became markedly turbid after 3 days' incubation at 75 °C; pure strains were obtained by two serial transfers from these cultures in fresh medium (see below).

Culture conditions. The standard culture medium comprised (g/l): yeast extract (Difco), 1.0; Casamino acids (Difco), 1.0; KH₂PO₄, 3.1; (NH₄)₂SO₄, 2.5; MgSO₄·7H₂O, 0.20; MgCl₂·2H₂O, 0.25; tap water to 1 l, pH adjusted with H₂SO₄. All pH values (high-temperature glass electrode) are corrected for the effect of temperature; for both natural sources and culture media the pH increases by 0.004 unit/°C.

In experiments with other carbon sources these were added at 2.0 g/l in place of the yeast extract and Casamino acids; in experiments on amino acid utilization, again at 2.0 g/l, the ammonium sulphate was also omitted from the medium.

For autotrophic growth, sublimed sulphur (S₈) was added at 10 g/l to the standard medium, and from a growth thus obtained an inoculum was transferred to basal salts medium with 10 g S₈/l; the culture vessel was gassed with CO₂ for 30 s, then closed and incubated for 7 to 10 days; growth was followed visually and by pH decrease.

For autotrophic growth on ferrous iron, 100 ml of the basal salts solution was supplied with 2 ml of sterile solution containing 25 g of FeSO₄·7H₂O in 95 ml distilled water and 5 ml 0.5 M-H₂SO₄. The flasks were inoculated with exponential phase cells centrifuged quickly from an optimal culture; after 5 days at 75 °C, pH 3.0, the Fe³⁺ ion was assayed with 0.01 N-KMnO₄ after acidification with 1 M-H₂SO₄.

For normal (heterotrophic) growth, small cultures were incubated either statically in ovens or in a gyrorotatory water bath. Larger cultures (25 l) were grown in a Terzano fermenter with slow agitation (20 rev./min or less) and aeration (2.5 to 3.0 l/min).

Growth was quantified turbidimetrically at 540 nm; a turbidity of 0.4 (10 mm cell) corresponds to 0.54 g dry cells/l. Cells were normally recovered by centrifuging at 20 000 g.

Stock cultures were maintained by cooling cultures grown on the standard medium to 4 °C and correcting the pH to 6.0, and were then renewed at monthly intervals.

Lysis. Cells were not lysed by lipase (Fluka) or lysozyme (Boehringer) added to the suspension at 2 mg/g cells in 0.05 M-tris buffer, pH 7.5, with or without 0.2 M-EDTA, but lysed rapidly with 0.5 % sodium lauryl sulphate.

Isolation and characterization of DNA. Growth-phase cells were lysed with 0.5 % sodium lauryl sulphate in 0.05 M-tris buffer with 0.15 M-NaCl, and the DNA isolated and purified by the method of Marmur (1961). Hydrolysis (Wyatt & Cohen, 1953) and separation of the bases by paper chromatography, followed by spectrophotometric assay (Bendich, 1957) was used to determine the guanosine and cytosine content. Alternatively, the DNA sample, following dialysis against 10 % (v/v) SSC (SSC = 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7), was examined by ultracentrifuging in a CsCl gradient using 1 extinction unit of DNA for each gradient, with an internal standard of *Xenopus laevis* DNA (% GC, 39.5), 0.004

extinction unit/gradient, labelled with ^{14}C (3×10^4 counts/min/mg). The gradient (4.1 ml, $n_D^{25} 1.4005$) was established with a Spinco L2/65B angular rotor 40 at 35000 rev./min for 72 h. Fractions of 0.01 ml were taken and diluted to 0.5 ml with 10% (v/v) SSC. Total DNA was measured spectrophotometrically. The marker DNA was localized by precipitation with cold 5% trichloroacetic acid and carrier bovine serum albumen for counting on glass-fibre filters. The % GC was calculated by the methods of Sueoka, Marmur & Doty (1959).

Antibiotic sensitivity. Sensitivity of the MT isolates and of a culture of *Bacillus acidocaldarius* (Agnano strains) to vancomycin (hydrochloride, Eli Lilly) and novobiocin (Ca salt, Boots Ltd) was established by the procedure of Brock *et al.* (1972).

Amino sugars. Lyophilized cells (50 mg) were treated with 4 M-HCl (5 ml) for 4 h at 100 °C, and the hydrolysate evaporated to dryness *in vacuo* (over P_2O_5 and NaOH) and taken up in water. After passage through a Dowex 50 (H^+) column, eluting first with water and then with M-aqueous ammonia, the eluate was chromatographed on Whatman 3MM paper (descending; 4:1:5, butanol-acetic acid-water). The Elson-Morgan-positive spots were eluted and assayed spectrophotometrically (Ashwell, 1957) as glucosamine.

Lipids. Total lipid was extracted from lyophilized cells by Soxhlet extraction (48 h) in 1:1 chloroform solution. The resolution of the lipid classes was carried out on a silica gel column (Merck Kieselgel 60, 230 mesh), eluting with chloroform (neutral lipids), acetone (glycolipids) and methanol (phospholipids). For experiments with labelled precursors sodium [^{14}C]acetate (610 $\mu\text{Ci}/\text{mg}$) was purchased from New England Nuclear Corp., Boston, Massachusetts, U.S.A.; DL-[^{14}C]mevalonolactone (99 $\mu\text{Ci}/\text{mg}$) was purchased from The Radiochemical Centre, Amersham, Buckinghamshire. In this experiment, the organism was grown in 25 l batch cultures and the labelled substrate, sodium [^{14}C]acetate (0.25 mCi) or DL-[^{14}C]mevalonolactone (0.25 mCi), added to the cultures at the beginning of the exponential phase over a 3 h period by a peristaltic pump. Cells were harvested in the stationary phase by continuous-flow centrifugation, then washed with 0.1 M-NaCl, freeze-dried and extracted continuously (Soxhlet). The lipid extract was evaporated and treated with 37% HCl in methanol (6%, v/v) under reflux; after 6 h the mixture was diluted with water (1:1) and extracted three times with *n*-hexane. This extract was chromatographed on a silica gel column (Merck Kieselgel 60, 230 mesh) in light petroleum (b.p. 40 to 70 °C) and increasing amounts of ether; the cyclic glycerol diether was recovered in the 50% ether fractions as a viscous oil. The radioactivity was measured by a Beckman LS-250 liquid scintillation system [efficiency 88 to 91% by internal standardization in 10 ml of the following solution: toluene, 1 l; 2,5-diphenyloxazole (PPO), 10 g; 1,4-di(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP), 0.5 g].

RESULTS

Isolation

Samples of thermal water were collected in the Pisciarelli solfatara, from small pools with a temperature range of 74 to 89 °C and a pH range of 1.4 to 2.6; these waters generally contain about 5 g dissolved salts/l, and a proximate analysis of a typical sample is given in Table 1. By enrichment and serial transfer (see Methods) six isolates were obtained, of which four showed maximum growth temperatures of 85 °C, one 80 °C, and one 89 °C. Two strains, designated MT3 and MT4, were retained for further study; unless specified, data refer primarily to the MT3 strain.

Table 1. Proximate analysis of thermal water sample from Pisciarelli

Temperature (°C)	74–89
pH	1.4–2.6
Residue at 180 °C (g/litre)	4.9
Radioactivity (¹⁴ C) (pCi/ml)	15
Cations (mM)	
Ca ²⁺	1.0
Mg ²⁺	Not detected
Fe ²⁺	Trace
Anions (mM)	
SO ₄ ²⁻	18
Cl ⁻	15
S ²⁻ , HSO ₃ ⁻	0.1

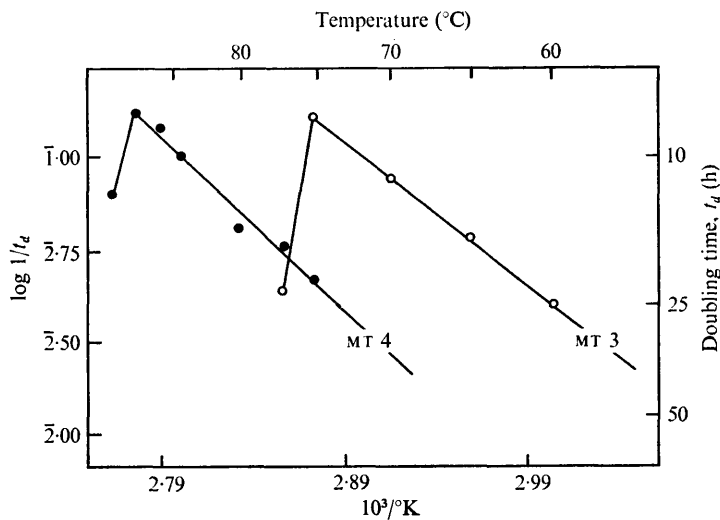


Fig. 1. Arrhenius plots of growth of MT3 and MT4 at pH 3.0.

Maintenance

We were unable reliably to maintain cultures on agar media, or by freezing ($-20\text{ }^{\circ}\text{C}$) or lyophilization. Cultures were therefore maintained on liquid media (see Methods). It was observed that cultures which were cooled from the optimum growth temperature rapidly lost viability if left at the 'optimum' pH (2.0 to 3.0) but remained viable at the lower temperature if the pH of the medium was raised, e.g. to 6.0. Cultures of MT3 are deposited at the Torry Research Station, Aberdeen.

Morphology

The morphology and ultrastructure of MT isolates are fully described in the accompanying paper (Millonig *et al.* 1975). Under phase contrast the organism is spherical, about $1.0\text{ }\mu\text{m}$ in diameter, revealing some irregularity ('tumbling') when in Brownian motion, somewhat less marked than in *S. acidocaldarius* (Brock *et al.* 1972, and our own observations). No viable cells passed through a membrane filter with $0.45\text{ }\mu\text{m}$ pores. Cells are deformed or destroyed in smear preparations, indicating the lack of a rigid cell envelope; on exposure to pH 3.0 at room temperature cells become vacuolated and the contents coagulate.

Table 2. *Effect of pH on growth rate of MT3 at 75 °C*

pH	1.5	2.0	2.5	3.0-4.5	5.0
Doubling time (h)	20.0	17.0	12.0	7.9	10.5

Table 3. *Growth and Fe²⁺ oxidation in cultures of MT3*

Culture	Final Fe ³⁺ (mg/ml)	Growth
Basal salts		
Inoculated	1.05	+++
Not inoculated	0.64	—
Basal salts + 0.1 % yeast extract		
Inoculated	0.91	++
Not inoculated	0.61	—

Cultures were incubated for 5 days; total Fe was 1 mg/ml.

Temperature and pH effects

The temperature range of cultures of MT3 and MT4 grown at pH 3.0 is shown by the Arrhenius plots, Fig. 1. Strain MT3 grows in the range 50 to 80 °C, optimally at 75 °C and not detectably at 45 or at 83 °C. Strain MT4 grows in the range 63 to 89 °C, optimally at 87 °C and not detectably at 60 or at 92 °C. At the optimal temperatures, pH 3.0, the doubling times for MT3 and MT4 were 7.9 and 7.5 h respectively. Above the normal growth range, strain MT3 remained viable after 30 to 60 min at 90 °C but not after 120 min; strain MT4 survived 100 °C for 5 and 15 min but was killed after 30 min.

At optimal temperatures, broad pH optima were found from pH 3.0 to pH 4.5, but there was no growth at pH 1.0 or 5.5 (Table 2).

Measured by turbidity, growth in optimal conditions reached typical maxima at an E_{540} of 0.3 in static cultures and 0.45 in fermenter cultures, corresponding to about 0.37 and 0.54 g dry cells/l respectively.

Heterotrophic growth

No MT isolates grew heterotrophically under nitrogen. Good growth was obtained on media with yeast extract, tryptone, or Casamino acids, provided that their concentration was not greater than 0.2 %. Growth is markedly inhibited at higher nutrient concentrations, as in *Sulfolobus* and *Thermoplasma*. On defined media (containing NH₄ salts), both MT3 and MT4 utilized glucose, xylose, sucrose, lactose, maltose or rhamnose as sole carbon source, but not cellobiose, sorbitol, or mannitol; supplied as sole carbon and nitrogen sources, amino acids (glycine, valine, leucine, arginine, lysine, alanine, glutamate, aspartate, serine, methionine) did not support growth. The organisms are less nutritionally demanding than *Thermoplasma* and their range of heterotrophic nutrition is within that described for *S. acidocaldarius* (Brock *et al.* 1972). The organisms are not even moderately halophilic, since no growth is observed when 1 % (w/v) NaCl is added to the standard medium.

Autotrophic growth

After passage from heterotrophic growth on yeast extract through yeast extract-sulphur medium to a medium supplied only with sulphur, CO₂ and inorganic salts, moderate growth occurred during 7 days, confirmed by direct phase-contrast microscopy, and the pH was decreased from 3.5 initially to 2.2. Autotrophic growth also occurred with ferrous iron as the oxidizable substrate, but iron oxidation was not more marked in the presence of yeast extract (Table 3). *Sulfolobus* likewise shows facultative autotrophic growth on sulphur but

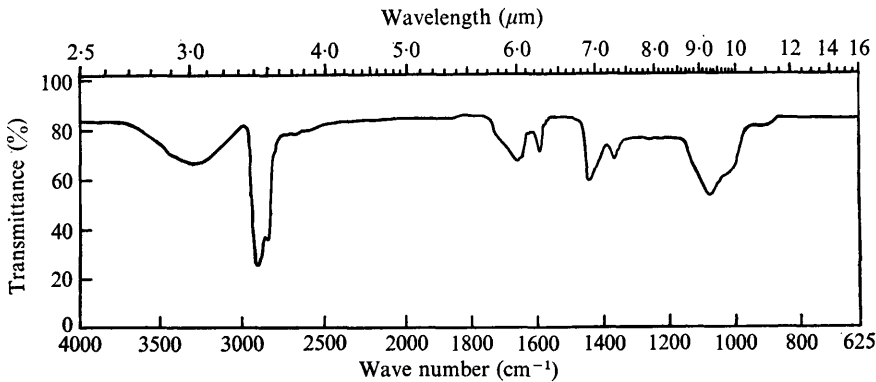


Fig. 2. Infrared spectrum, in chloroform solution, of total lipids of strain MT3.

iron autotrophy was not noted (Brock *et al.* 1972); *Thermoplasma* is an obligate heterotroph (Darland *et al.* 1970) and the organism of Brierley & Brierley (1973) is obligatorily autotrophic for sulphur or iron.

Coat substance, antibiotic sensitivity, and lysis

As judged by electron microscopy (Millonig *et al.* 1975) the structured extra-membrane material of MT cells, which does not confer rigidity to the cells, is not peptidoglycan; analytically, the cells contain a small proportion of glucosamine (9 mg/g dry wt in MT4, 11 mg/g dry wt in MT3). *Sulfolobus* contains similar amounts of glucosamine, which is absent in *Thermoplasma* (Brock *et al.* 1972). All MT strains were resistant to vancomycin, which blocks muramic acid transfer, at concentrations ≤ 5.0 mg/ml, but susceptible to novobiocin at or above 0.01 mg/ml; as a control, *Bacillus acidocaldarius* was inhibited by 0.005 mg or more novobiocin/ml and 0.020 mg or more vancomycin/ml. The antibiotic sensitivity of the MT strains parallels that of *Sulfolobus* (Brock *et al.* 1972) and *Thermoplasma* (Darland *et al.* 1970).

At neutral pH and room temperature, cells were not lysed by lysozyme and/or lipase, with or without added EDTA, but were rapidly and completely lysed by 0.5 % sodium lauryl sulphate. Similar properties are more fully described for *T. acidophila* (Belly & Brock, 1972). On lysis of centrifuged cells the pH rose from 3.5 to 6.3, suggesting that the intracellular pH is close to neutrality.

Lipids

Lyophilized MT cells contain about 10 % (w/w) total lipids, comprising 70 % polar lipids, 10 % less polar, and 20 % neutral lipids, approximately. The infrared spectrum of the total lipids (Fig. 2) is very characteristic, showing the total absence of ester linkages (no peak at 1740 cm⁻¹) and a high proportion of ether links (signal at 1100 cm⁻¹). The predominantly isoprenoid nature of the alkyl component of these lipids is apparent from the selectivity with which [2-¹⁴C]mevalonate is incorporated into the total lipids (Table 4). We have made parallel observations on the lipids of *S. acidocaldarius*, with essentially similar results, and chemical details are given elsewhere (de Rosa, Gambacorta, Minale & Bu'Lock, 1974a).

Table 4. *Incorporation of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonolactone into MT3 lipids*

Precursor	Percentage incorporation into	
	Total lipids	Cyclic glycerol diether*
[2- ¹⁴ C]acetate†	2.6	1.74
DL-[2- ¹⁴ C]mevalonolactone‡	10.7	3.21

* Isolated from the total lipids after hydrolysis with HCl-MeOH.

† 5.11×10^8 c.p.m. (0.41 mg) to 25 l culture, affording 453 mg lipids.

‡ 5.11×10^8 c.p.m. (2.53 mg) to 25 l culture, affording 415 mg lipids.

DNA base composition

High molecular weight DNA, homogeneous in the ultracentrifuge and apparently free from satellite DNA species, was prepared from both MT3 and MT4 cultures. By quantitative chromatographic separation of the bases, after hydrolysis, the % GC for MT3 was determined as 42 % and that for MT4 as 39 %. By preparative CsCl-gradient ultracentrifugation in a mixture with ¹⁴C-labelled *Xenopus laevis* DNA (% GC, 39.5) values of 45 and 39 % were obtained for these samples.

Because these values were so dramatically different from the range reported by Brock *et al.* (1972) for *S. acidocaldarius* (% GC, 60 to 68), which in other respects the MT isolates so closely resemble (see Discussion), we sought an independent determination. Samples of high molecular weight DNA were prepared from MT3 and from a culture of *S. acidocaldarius* strain 98/3 (stock culture provided by J. A. Mosser), and despatched under coded labels to Dr S. Ayad (Department of Biochemistry, University of Manchester) who by analytical CsCl ultracentrifugation obtained the following values: for MT3 (measured against DNA from *Micrococcus lysodeikticus*), $\rho^{25} = 1.707$ g/cm³, % GC = 47.9; for *S. acidocaldarius* (measured against DNA from *Bacillus subtilis*), $\rho^{25} = 1.729$ g/cm³, % GC = 70.4.

DISCUSSION

As already noted (de Rosa, Gambacorta, Millonig & Bu'Lock, 1974*b*), the MT series of isolates is extremely similar to the organisms designated as *S. acidocaldarius* by Brock *et al.* (1972), and the majority of the results presented here are to be taken as substantiating the closeness of this resemblance. However, the two are not strictly identical, the most fundamental difference being in the % GC content of the DNA. The magnitude of this difference would, in other circumstances, be taken to indicate not merely different species but also different genera. However, in the present case it accompanies the kind of close similarity in other characters which would be expected between different strains of the same species. These organisms are indigenous to particularly stringent habitats in which we would *a priori* expect strong convergence of characters, and in an extreme case these convergent adaptive characters may so totally dominate descriptions of the organisms that the normal indicators of phyletic affinity, other than the GC content, are no longer accessible. In our preliminary account (de Rosa *et al.* 1974*b*) we argued that until more satisfactory discriminatory criteria are available, both the MT organisms and *S. acidocaldarius* should be classed together, in a 'form/habitat' group, Caldariella, which would also include both the mycoplasma-like *T. acidophila* of Darland *et al.* (1970) and the un-named pleiotropic Fe, S autotrophic organism of Brierley & Brierley (1973). The latter has a GC ratio of 54 to 60 %; for

Thermoplasma, Belly & Brock (1972) report 24 to 28.5 % GC, but Freundt (1972) gives a revised figure of 46 % which is within our range for MT isolates. We would note recent implications of considerable heterogeneity amongst organisms morphologically classed as *S. acidocaldarius* (Mosser, Mosser & Brock, 1974; Bohlool & Brock, 1974).

Some aspects of our data for the MT organisms which either differ from or extend descriptions of *S. acidocaldarius*, additional to those in the accompanying paper (Millonig *et al.* 1975), require comment.

The need to devise conditions for the maintenance of liquid cultures as stock led to observations which clearly demonstrate that acid-tolerance in MT is an active process dependent upon continued metabolism. In particular, if cultures growing at the optimum temperature and pH are cooled to temperatures at which active growth no longer occurs, viability is lost and the cell contents are irreversibly altered. To maintain viability at sub-optimal temperatures it is necessary to provide a medium in which the hydrogen ion concentration is substantially closer to that which apparently exists inside the cells, a clear indication that acidophily depends upon the existence of metabolically driven mechanisms for maintaining the pH gradient across the cell membrane.

The very characteristic isoprenoid ether lipids of MT3 (unpublished) are also found, with quantitative differences only, in *S. acidocaldarius*. They are based on a cyclic diether of glycerol and a unique bifunctional saturated isoprenoid C₄₀ moiety (de Rosa *et al.* 1974a), and they are closely related to (or identical with) those partly characterized in *Thermoplasma acidophila* by Langworthy, Smith & Mayberry (1972). These lipids might well constitute a chemical marker for the 'Caldariella' group, just as the biogenetically related diphitanyl glycerol diether lipids of *Halobacterium cutirubrum* constitute a marker for the polyphyletic assembly of extreme halophiles (Kates, 1972). In both cases, the replacement of ester-linked by ether-linked lipids is presumably advantageous in stabilizing the membrane towards environmental stress, and indeed on naive chemical grounds it would seem more obviously advantageous in the extreme acidophiles. However, it is the branched and cyclic nature of the hydrophobic moiety which may be the more significant characteristic, since this will tend to form bilayers which are densely but irregularly packed, the effect being similar to that produced by the incorporation of cholesterol into normal acyl-lipid bilayers. This will both stabilize and fluidize the bilayer system and may prove to be a key factor in permitting these organisms to function in their extreme habitat.

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