Extremophiles (2012) 16:585–595
DOI 10.1007/s00792-012-0458-8

ORIGINAL PAPER

Amino acid-assimilating phototrophic heliobacteria from soda lake environments: *Heliorestis acidaminivorans* sp. nov. and '*Candidatus* Heliomonas lunata'

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Received: 5 January 2012 / Accepted: 19 April 2012 / Published online: 16 May 2012
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Abstract Two novel taxa of heliobacteria, *Heliorestis acidaminivorans* sp. nov. strain HR10B^T and '*Candidatus* Heliomonas lunata' strain SLH, were cultured from shoreline sediments/soil of Lake El Hamra (Egypt) and lake water/benthic sediments of Soap Lake (USA), respectively; both are highly alkaline soda lakes. Cells of strain HR10B were straight rods, while cells of strain SLH were curved rods. Both organisms were obligate anaerobes, produced bacteriochlorophyll *g*, and lacked intracytoplasmic photosynthetic membrane systems. Although the absorption spectrum of strain HR10B was typical of other heliobacteria, that of strain SLH showed unusually strong absorbance of the OH-chlorophyll *a* component. Major carotenoids of both organisms were OH-diaponeurosporene glucosyl esters, as in other alkaliphilic heliobacteria, and both displayed an alkaliphilic and mesophilic phenotype. Strain HR10B was remarkable among heliobacteria in its capacity to photoassimilate a number of carbon sources, including several amino acids. Nitrogenase activity was observed in strain HR10B, but not in strain SLH. The 16S ribosomal RNA gene tree placed strain HR10B within the genus *Heliorestis*, but distinct from other described species. By contrast, strain SLH was phylogenetically more closely related to neutrophilic heliobacteria and is the first alkaliphilic heliobacterium known outside of the genus *Heliorestis*.

Keywords Anoxygenic phototrophic bacteria · Heliobacteria · Bacteriochlorophyll *g* · Alkaliphiles · Soda lakes · Extreme environments

Communicated by F. Robb.

This paper is dedicated to the memory of Howard Gest, co-discoverer of the heliobacteria.

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Introduction

Heliobacteria are anoxygenic phototrophic bacteria that contain a unique photosynthetic pigment, bacteriochlorophyll (Bchl) *g*, a pigment absent in all other anoxygenic phototrophs (Madigan 2001; Madigan and Ormerod 1995). In heliobacteria, photosynthetic pigments and associated proteins reside in the cytoplasmic membrane rather than in highly differentiated intracytoplasmic membranes typical of purple bacteria or the chlorosomes of green bacteria (Miller et al. 1986; Madigan and Ormerod 1995).

In nature, heliobacteria have been isolated from either soils or hot springs, and to date, none have been obtained from lake water or seawater or their sediments (Stevenson et al. 1997; Asao and Madigan 2010). This is in contrast to purple and green phototrophic bacteria, which are ubiquitous in aquatic environments such as freshwater and saline lakes, estuaries, and marine ecosystems (Imhoff 2001; Madigan 1988; Pfennig 1967, 1978). Phylogenetically, heliobacteria form a monophyletic clade within the phylum *Firmicutes*, the *Bacillus*/*Clostridium* subdivision of Gram-positive bacteria (Madigan and Ormerod 1995). Heliobacteria can be divided into two phylogenetically distinct

clades, one consisting of the genera *Heliobacterium*, *Heliobacillus*, and *Heliophilum*, and the other solely of the genus *Heliorestis*. Notably, pH profiles of heliobacteria track phylogeny; species in the former clade are neutrophilic, whereas species of *Heliorestis* are alkaliphilic (Asao and Madigan 2010).

Species of *Heliorestis* include *Heliorestis daurensis* (Bryantseva et al. 1999), *Heliorestis baculata* (Bryantseva et al. 2000b), and *Heliorestis convoluta* (Asao et al. 2006). All of these organisms were isolated from shoreline sediments/soils of soda lakes. Soda lakes are major alkaline environments characterized by large amounts of carbonates and, in some cases, high NaCl concentrations as well (Jones et al. 1998). Besides the alkaliphilic *Heliorestis* species and various cyanobacteria, several alkaliphilic purple bacteria have also been isolated from soda lake environments (Asao et al. 2007, 2011; Imhoff et al. 1978, 1979; Madigan 2003). Interestingly, while soda lakes are known for their high primary productivity driven primarily by cyanobacteria and purple sulfur bacteria (Grant et al. 1990; Jones et al. 1998), no heliobacteria, including alkaliphilic species, have shown the capacity for autotrophic growth (Sattley et al. 2008; Asao and Madigan 2010). Moreover, although some heliobacteria can grow in darkness by pyruvate fermentation (Kimble et al. 1994; Pickett et al. 1994), alkaliphilic heliobacteria apparently cannot; photoheterotrophy appears to be their sole form of metabolism (Asao et al. 2006; Bryantseva et al. 1999, 2000a, b).

In this paper, we describe two new alkaliphilic heliobacteria from Egyptian and USA soda lake environments, strain HR10B and strain SLH, respectively. Together, these organisms show a variety of unusual physiological, ecological, photosynthetic, and phylogenetic properties not previously observed in heliobacteria. Collectively, these properties form the basis for our description of these heliobacteria as new taxa, *Heliorestis acidaminivorans* sp. nov. and '*Candidatus* Heliomonas lunata'.

Materials and methods

Inoculum, enrichment, and isolation

Strain HR10B was isolated from shoreline sediment/soil samples taken near Lake El Hamra (Wadi El Natrun), Egypt; samples were collected by MTM as described previously (Asao et al. 2006) and stored at 4 °C. Enrichment cultures were established in 10-ml screw-capped tubes completely filled with the alkaline medium 1/2 S (Asao et al. 2006) and incubated at 34 °C under high-intensity incandescent light (70 μmol quanta m^{-2} s^{-1}). Isolations were carried out in repeated agar tube dilutions (Imhoff 2006) in medium 1/2 S.

Strain SLH was cultured from a Soap Lake water/benthic sediment sample obtained from a depth of 23 m. Samples were collected as described previously (Asao et al. 2007) and stored at 4 °C. Within 2 weeks of sampling, enrichments were established in 17-ml screw-capped tubes completely filled with medium 1/2 S modified to contain 0.05 % (w/v) $Na_2S{\cdot}9H_2O$ and incubated at 25 °C under low-intensity incandescent light (35 μmol quanta/m^2/s). Isolations were pursued in repeated agar tube dilutions using medium 1/2 S modified to contain 2–10 mM sulfide and 1.5 % NaCl and incubated anaerobically under incandescent light (35–70 μmol quanta/m^2/s) at 32 °C.

The pure culture of strain HR10B and the highly enriched culture of strain SLH were stored frozen in growth medium containing 10 % DMSO at −74 °C.

Media and growth conditions

Cultures of strain HR10B were routinely grown in 10-ml screw-capped tubes completely filled with medium SHC. Medium SHC contained per liter of deionized water: EDTA, 5 mg; KH_2PO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 200 mg; $CaCl_2{\cdot}2H_2O$, 75 mg; NH_4Cl, 0.5 g; sodium acetate, 1 g; sodium pyruvate, 1 g; yeast extract, 0.1 g; vitamin B_{12}, 20 μg; trace elements (Wahlund et al. 1991), 1 ml; BICINE buffer (Sigma, St. Louis), 1.63 g; $NaHCO_3$, 2.5 g; Na_2CO_3, 2.5 g; $Na_2S{\cdot}9H_2O$, 0.25 g. The pH of the medium was adjusted to 9–9.2 after autoclaving by the addition of sterile 5 N HCl or NaOH and immediately dispensed into sterile tubes. Cultures of strain SLH were grown routinely in 10-ml screw-capped tubes completely filled with medium SOP. The latter is medium SHC lacking sodium acetate and supplemented with NaCl (15 g/l) and biotin (1 mg/l). Cultures of both organisms were incubated in incandescent light (70 μmol quanta/m^2/s) at 30–32 °C.

The following physiological tests were performed on phototrophic cultures of the axenic strain HR10B and the highly enriched strain SLH. In testing for growth as a function of pH, medium SHC or SOP was supplemented with one of the following buffers (final concentrations 10 mM): MOPS (for pH 6.5–7.5), BICINE (for pH 8–9), CAPSO (for pH 9.5–10); CAPS (for pH 10.5–11) (all buffers, Sigma, St. Louis, MO, USA). In testing for photoassimilation of organic carbon sources, single carbon substrates were added to tubes of medium SHC or SOP lacking organic compounds. Tests for pyruvate fermentation were done in medium SOP–S (medium SOP lacking sulfide) reduced with 0.05 % (w/v) ascorbic acid and incubated in darkness.

In testing for growth factor requirements, cultures of both strains were grown in media lacking yeast extract. In

testing for nitrogen source utilization, single fixed nitrogen sources were added to N-free media. Nitrogenase activity was tested by acetylene reduction assays on cells grown photoheterotrophically in medium SHC or SOP modified to contain only 1 mM NH_4Cl or glutamine as sole nitrogen source (Kimble and Madigan 1992). In testing for sulfide tolerance, cultures were grown in media containing 0–20 mM sulfide; media lacking sulfide contained 0.05 % (w/v) sterile sodium ascorbate plus 0.05 % (w/v) sodium thiosulfate as a reduced biosynthetic sulfur source. Because heliobacteria form extracellular elemental sulfur globules from sulfide that interfere with turbidity measurements, growth in sulfide tolerance experiments was assessed by microscopic cell counts using a Petroff–Hausser Cell Counter. For other physiological tests, cultures were prepared in triplicate, and growth was measured as optical densities (OD) at 700 nm.

Microscopy and pigment analyses

Electron microscopy was performed as described previously (Kimble et al. 1995). Phase-contrast photomicrographs were taken on agar-coated slides using an Olympus B-MAX 60 photomicroscope. Absorption spectra of intact cells were obtained in 30 % bovine serum albumin previously reduced with sodium ascorbate (1 mg/ml) in an anoxic glove bag for 4–16 h. For in vitro spectra, pigments were extracted in 100 % methanol under aerobic conditions in darkness. All spectra were obtained using a Hitachi U-2000 double-beam spectrophotometer. Carotenoids were identified on the basis of absorption spectra, retention times in HPLC, and analysis of relative molecular weights after purification as previously described (Takaichi et al. 2003).

Genetic properties

Genomic DNA of strains HR10B and SLH was extracted using the Puregene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA), and 16S rRNA genes were amplified as described previously (Asao et al. 2007). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD, USA) and sequenced at the Genome Sequencing Center, Washington University in St. Louis.

Reference 16S rRNA gene sequences were downloaded from GenBank and alignments performed using the ClustalW program implemented within MacVector 7.2.3 (Oxford Molecular, Beaverton, OR, USA). The resulting sequence alignments were visually examined and edited manually. The program CHIMERA_CHECK (Cole et al. 2003) was employed to confirm that the entire sequence of the 16S rRNA gene amplified from the *Can.* H. lunata culture was most similar to that of other species of heliobacteria. A 16S rRNA gene tree was generated using PHYLIP version 3.68 (Felsenstein 1989). A neighbor-joining tree was constructed from a distance matrix based on the F84 algorithm (transition/transversion ratio, 2.0; empirical base frequencies) using the program DNADIST, which was then imported into the program NEIGHBOR. Bootstrap analysis was conducted on 1,000 replications. The 16S rRNA gene sequences of strains HR10B and SLH were deposited in GenBank under accession numbers EU908049 and EU910943, respectively.

Results

Enrichment and isolation

Enrichment cultures for anoxygenic phototrophic bacteria using shoreline sediment/soil of Lake El Hamra (Egypt) as inoculum in an illuminated alkaline, sulfide-reduced medium turned green within 3 days. Microscopic examination revealed motile long rods along with corkscrew-shaped cells resembling *Heliorestis convoluta* (Asao et al. 2006). In subsequent transfers, the long rod dominated, and absorption spectra of intact cells signaled the presence of Bchl *g*. Although phase-bright endospore-like structures were observed in the first transfer from the primary enrichment, they were not observed in later cultures or in single colonies picked from shake tubes. The pure culture of this rod-shaped heliobacterium was designated as strain HR10B.

Liquid enrichment cultures using Soap Lake (Washington State, USA) 23-m water/benthic sediment as inoculum turned pale green after 8 months of phototrophic incubation. Other enrichment cultures inoculated with Soap Lake chemocline water, mixolimnion water, or shoreline sediment did not turn green, even after 3 years of incubation. Microscopic examination of the 23-m enrichment revealed motile curved rods and non-motile filamentous cells, along with a number of spheroplasts. Endospores were not observed, but absorption spectra of the enrichment culture indicated the presence of Bchl *g*. After repeated agar tube dilutions, the putative heliobacterium was obtained as a highly enriched culture containing mostly the curved rod along with a few cells of a small, rod-shaped contaminant. The curved rod-shaped heliobacterium was designated strain SLH. Despite the repeated attempts using agar dilution tubes, isolation of strain SLH in axenic culture was unsuccessful. Although no physical association between phototroph and chemotroph was apparent from microscopic observations, the two organisms could not be separated under our growth conditions.

Morphology and pigments

Cells of strain SLH were curved rods, about 0.6 μm in diameter by 2–7 μm long (Fig. 1a–d), and often formed into pairs or chains. Dividing cells typically formed a bent structure with an axis at the septum between the cells (Fig. 1c). In scanning electron micrographs, cells of strain SLH also showed "connecting structures" between cells,

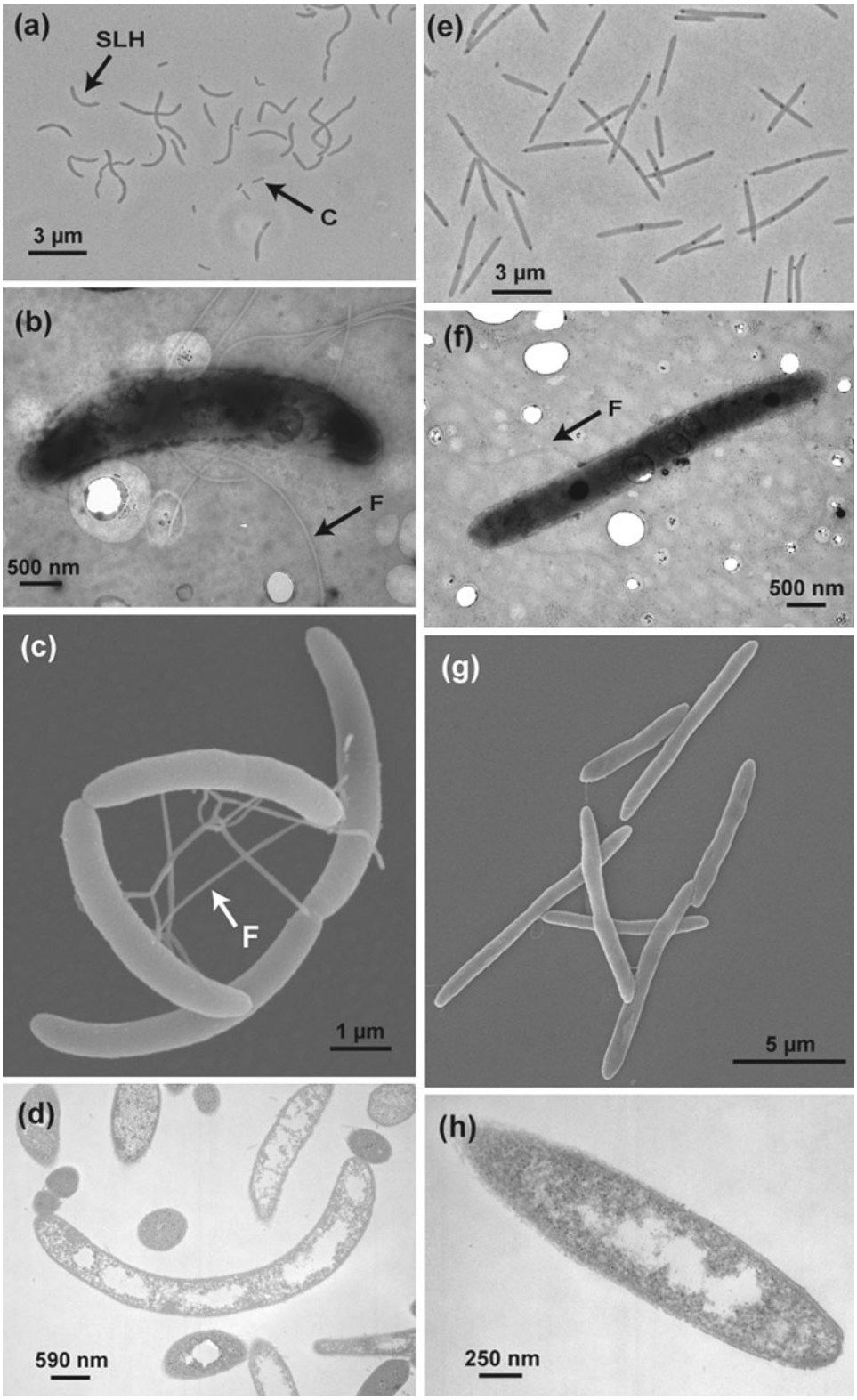

Fig. 1 Morphology and structures of cells of '*Candidatus* Heliomonas lunata' strain SLH (**a**–**d**), and *Heliorestis acidaminivorans* strain HR10B (**e**–**h**). **a, e** Phase-contrast photomicrographs. *SLH* strain SLH, *C* chemotrophic bacterium. **b, f** Negatively stained electron micrographs. *F* flagellum. **c, g** Scanning electron micrographs. **d, h** Transmission electron micrographs of thin sections of cells

reminiscent of similar structures observed in *Heliorestis convoluta* (Asao et al. 2006) (Fig. 1c). Cells of strain SLH were motile by means of flagella (Fig. 1b). Cells of strain HR10B were long and straight motile rods, 0.6–0.9 μm in diameter and 3–12 μm long (Fig. 1e–h); motility was by flagella, which were inserted laterally (Fig. 1f). In the stationary phase, cells occasionally formed chains. Electron micrographs of thin sections of cells of strains HR10B and SLH revealed smooth cell walls, indicating the absence of an outer membrane. Moreover, as is true of all known heliobacteria, neither intracytoplasmic photosynthetic membranes nor chlorosomes were observed in thin sections of cells of strain HR10B or SLH (Fig. 1d, h).

Phototrophic cultures of both strains HR10B and SLH were green. However, cultures of strain SLH were consistently a lighter shade of green than those of strain HR10B; cultures of the latter showed a color similar to that of other heliobacteria (Asao and Madigan 2010). In performing absorption spectra of intact cells, cell suspensions were maintained under anoxic condition to minimize the spontaneous oxidation of Bchl *g* to Chl *a* (Gest 1994).

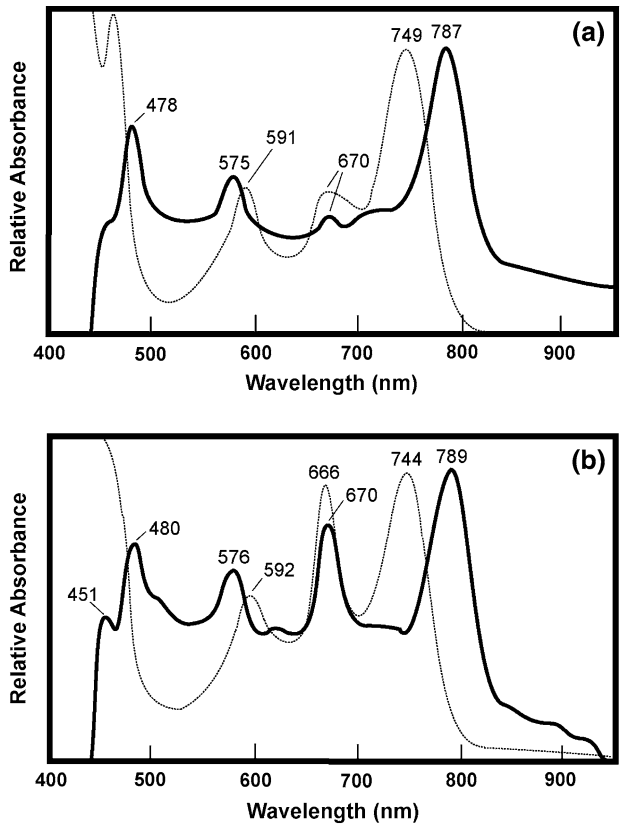

Fig. 2 Pigments of *Heliorestis acidaminivorans* strain HR10B and '*Candidatus* Heliomonas lunata' strain SLH. **a** Absorption spectra of intact cells (*solid line*) and methanol extract (*dotted line*) of strain HR10B. **b** Absorption spectra of intact cells (*solid line*) and methanol extract (*dotted line*) of strain SLH. Spectra of intact cells were performed under anoxic conditions

Absorption spectra of intact cells of strains HR10B and SLH showed maxima at 787 and 789 nm, respectively, indicative of Bchl *g* (Fig. 2a, b). In both organisms, maxima at 670 nm were present due to absorption by 8^1-hydroxy-chlorophyll (OH-Chl) *a*, the primary electron acceptor in the heliobacterial reaction center (Neerken and Amesz 2001). Notably, however, the ratio of OH-Chl *a* to Bchl *g* absorption in cells of strain SLH was significantly higher than in cells of strain HR10B (Fig. 2) or in that reported from all other heliobacteria (Asao et al. 2006; Asao and Madigan 2010).

Spectra of methanol extracts of cells of strains HR10B and SLH confirmed the presence of Bchl *g*, with absorption maxima at 749 and 744 nm, respectively (Fig. 2) (Brockmann and Lipinski 1983; Kobayashi et al. 1991; Asao et al. 2006). Consistent with the spectrum of intact cells, OH-Chl *a* absorbance (666 nm) in methanol extracts of cells of strain SLH was significantly higher than that of strain HR10B (Fig. 2).

HPLC elution profiles of carotenoids extracted from both strains SLH and HR10B (data not shown) were consistent with those obtained from *Heliorestis daurensis* (Takaichi et al. 2003) and indicated that both strains contained OH-diaponeurosporene glucosyl 16:0 ester as the major carotenoid. In strain HR10B, this pigment comprised 70 % of all carotenoids. Other carotenoids in strain HR10B included diaponeurosporene (10 %), OH-diaponeurosporene glucosyl 16:1 ester (10 %), and OH-diaponeurosporene glucosyl 18:1 ester (10 %). In strain SLH, OH-diaponeurosporene glucosyl 16:0 ester comprised 80 % of all carotenoids, with small amounts of diaponeurosporene (10 %) and OH-diaponeurosporene glucosyl 16:1 ester (10 %) present as well; OH-diaponeurosporene glucosyl 18:1 ester was not detected in extracts of cells of strain SLH.

Physiology: salinity, pH, and temperature

The pH response of strain HR10B clearly showed an alkaliphilic phenotype; optimal growth occurred between pH 8–9 (range 8–10.5) (Fig. 3a). Strain HR10B did not grow below pH 7.5. The mixed culture of strain SLH also grew best at alkaline pH, with optimum growth at pH 9 and no growth below pH 7.5 or above pH 10 (Fig. 3a).

Growth of strain HR10B did not require NaCl, although low levels were growth stimulatory; optimal growth occurred at NaCl concentrations of 0.5–4 % (Fig. 3b). Likewise, growth of strain SLH did not require NaCl, but the addition of 1–3 % NaCl enhanced phototrophic growth significantly (Fig. 3b). The optimum growth temperature for strain HR10B was 30–37 °C (range 18–45 °C). By contrast, strain SLH grew best at 25–30 °C (range 18–37 °C) (data not shown).

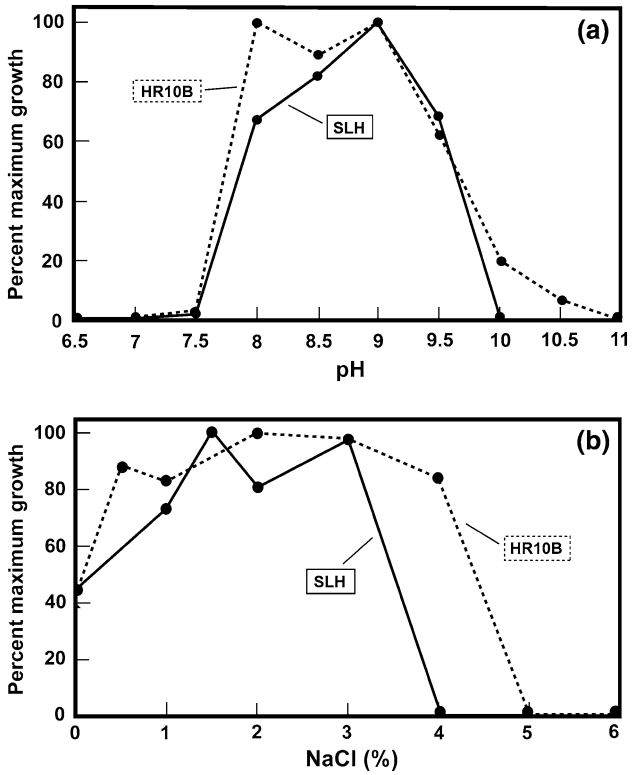

Fig. 3 Influence of pH (**a**) and NaCl concentrations (**b**) on photoheterotrophic growth of *Heliorestis acidaminivorans* strain HR10B (*dotted line*) and '*Candidatus* Heliomonas lunata' strain SLH (*solid line*). Growth of strains HR10B and SLH was scored on the first subculture after 11–15 days of phototrophic incubation. *Each data point* represents the mean OD_{700} of three replicate cultures

Physiology: carbon and nitrogen metabolism

Strain HR10B was unusual among all known heliobacteria in that it photoassimilated a wide variety of carbon substrates. Of special interest was the fact that photoheterotrophic growth of strain HR10B was luxurious on 10 mM concentrations of several amino acids, including arginine, glutamate, and lysine. As expected, strain HR10B also photoassimilated acetate or pyruvate (10 mM in each case), substrates widely used by other heliobacteria (Madigan and Ormerod 1995). Table 1 summarizes the carbon metabolism of strain HR10B.

Determining the substrate utilization profile of strain SLH was complicated by the fact that the culture was not axenic. Nevertheless, some nutritional conclusions could be reached since the curved rod phototroph was typically the dominant organism in the mixed culture (Fig. 1a). Dense phototrophic cultures of strain SLH were only obtained when pyruvate or malate (10 mM) were the carbon sources. Surprisingly, acetate (10 mM plus bicarbonate) did not support growth. Less dense cultures were observed with 10 mM concentrations of alanine, lysine, serine, threonine, or butyrate, or 0.1 % yeast extract. When sucrose (10 mM) was added as a carbon source in place of pyruvate in medium SOP, prolific growth of the chemotroph but not the heliobacterium occurred as confirmed microscopically; such cultures were milky white and unpigmented. Photoautotrophic growth with sulfide (1 mM) as electron donor, or dark fermentative growth at the expense of pyruvate (Kimble et al. 1994), was not observed in either strain HR10B or SLH.

Strain HR10B had no growth factor requirement. By contrast, growth of strain SLH required a trace amount of yeast extract (0.01 % w/v). In the absence of this small amount of yeast extract, growth ceased after the first subculture. Addition of a vitamin mixture containing *para*-aminobenzoic acid, folic acid, biotin, vitamin B_{12}, nicotinic acid, pantothenic acid, thiamine, and vitamin B_6 did not replace yeast extract for supporting growth of strain SLH. Riboflavin (1 mg/l) was also tested as a potentially required vitamin, but for unknown reasons its addition completely inhibited growth of both strains HR10B and SLH.

Table 1 Carbon substrates supporting photoheterotrophic growth of strain HR10B

Substrates	Carbon substrates	Growth[a]
C_2 or C_3 acids	Pyruvate (10 mM)	++
	Acetate (10 mM)	++
	Propionate (10 mM)	+
Amino acids	Alanine (10 mM)	++
	Arginine (10 mM)	++
	Glutamate (10 mM)	++
	Glutamine (10 mM)	++
	Histidine (10 mM)	++
	Lysine (10 mM)	+++
	Serine (10 mM)	++
Complex	Casamino acids (0.1 %)	+
	Yeast extract (0.1 %)	+
Negative controls	None (light incubation)	–
	None (dark incubation)	–

Photoheterotrophic growth was tested in medium SHC lacking pyruvate and acetate (final pH 9). A single carbon source was added to individual tubes. Growth was scored on the first subculture after 11–20 days of incubation. Carbon substrates tested but that did not support photoheterotrophic growth of strain HR10B included: alpha-ketoglutarate, asparagine, aspartate, butanol, ethanol, formate, fructose, fumarate, galactose, glucose, glycerol, glycine, lactate, malate, mannitol, methanol, methionine, proline, propanol, ribose, succinate, sucrose, threonine, valerate (each tested at 10 mM); butyrate, caproate, citrate (each tested at 5 mM), benzoate (2 mM), and ascorbic acid (0.1 %)

+++, > 0.4 OD_{700}; ++, 0.3–0.4 OD_{700}; +, 0.2 OD_{700}; –, 0–0.1 OD_{700}

[a] Growth was measured as optical density (OD) at 700 nm. Growth was scored based on the average OD_{700} of triplicate cultures

Strains HR10B and SLH utilized several fixed nitrogen sources, including ammonium, yeast extract, casamino acids, and any of a number of individual amino acids (added at 5 mM in each case), including alanine, asparagine, aspartate, arginine, glutamate, glutamine, histidine, lysine, methionine, or serine. By contrast, neither strain HR10B nor strain SLH grew in media containing $NaNO_3$ (10 mM), urea (2 mM), or proline (5 mM) as the nitrogen source.

Suspensions of cells of strain HR10B grown on limiting (1 mM) levels of NH_4Cl or glutamine produced ethylene ($H_2C{=}CH_2$) from acetylene ($HC{\equiv}CH$) in standard acetylene reduction assays (data not shown), demonstrating the presence and activity of nitrogenase (Kimble and Madigan 1992). By contrast, strain SLH grown under the same conditions did not reduce acetylene to ethylene, suggesting that either a nitrogenase system is absent from this organism or was not de-repressed under these N-starved conditions.

Optimum growth of strain HR10B was obtained in the absence of sulfide. Although near optimum growth was obtained at 1 mM sulfide, cultures containing >3 mM sulfide showed significantly lower cell yields. However, this suboptimum growth was still observed at sulfide levels as high as 20 mM, indicating that strain HR10B was highly sulfide tolerant. By contrast to strain HR10B, strain SLH showed an absolute requirement for sulfide. Maximum growth of the SLH culture was obtained at 1 mM sulfide. However, as for strain HR10B, cultures of strain SLH containing >3 mM sulfide showed lower cell yields, yet growth still occurred at 20 mM sulfide (data not shown).

Phylogeny

For strain HR10B, genomic DNA was isolated and the 16S rRNA gene amplified and sequenced. For the mixed culture of strain SLH, a 16S rRNA gene product was also amplified and the PCR product was adjudged to be that of a single DNA sequence as assessed from the sequence chromatogram. Using the program CHIMERA_CHECK (Cole et al. 2003), the entire length of this 16S rRNA gene sequence showed the highest similarity to the 16S rRNA genes of other species of heliobacteria. This indicates that the PCR product obtained from strain SLH was indeed the heliobacterial component of the mixture and also not a chimera containing sequences from both the heliobacterium and the chemotrophic organism.

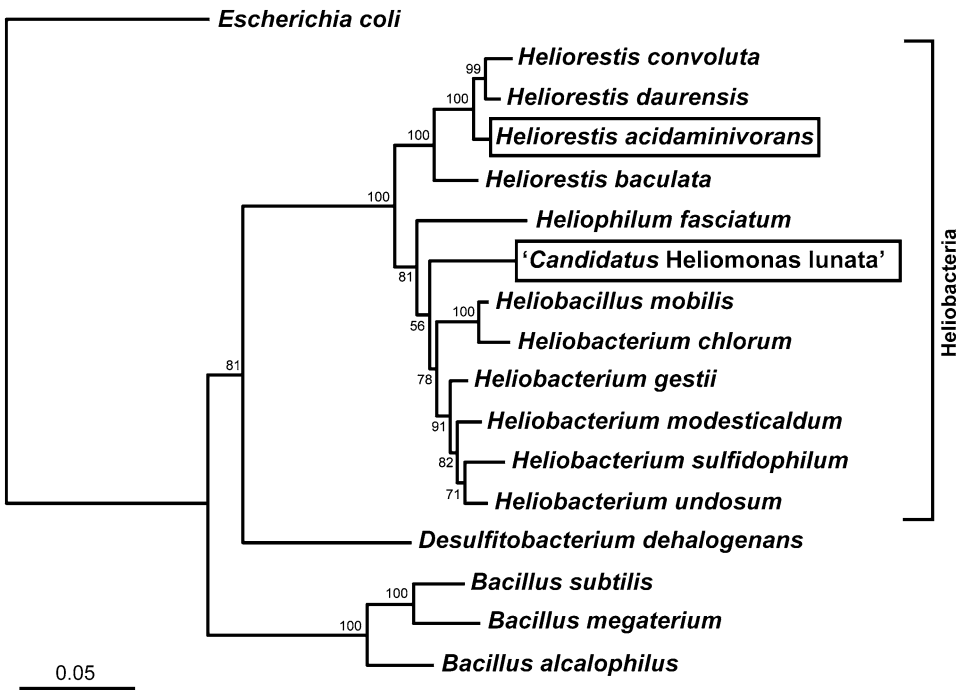

Fig. 4 A neighbor-joining 16S rRNA gene tree of heliobacteria; 1261 nucleotide positions were compared. The tree was constructed using PHYLIP version 3.68 (Felsenstein 1989). Bootstrap analysis was conducted on 1,000 replications and bootstrap confidence values >50 % are indicated at the nodes. The tree was rooted using *Escherichia coli* (*Gammaproteobacteria*) as an outgroup. All sequences have been deposited in GenBank as follows: *Escherichia coli* (J01859), *Bacillus megaterium* (X60629), *Bacillus subtilis* (AJ276351), *Bacillus alcalophilus* (X76436), *Desulfitobacterium dehalogenans* (L28946), *Heliorestis baculata* (AF249680), *Heliorestis convoluta* (DQ266255), *Heliorestis daurensis* (AF079102), *Heliophilum fasciatum* (L36197), *Heliobacterium sulfidophilum* (AF249678), *Heliobacterium undosum* (AF249679), *Heliobacterium modesticaldum* (U14559), *Heliobacterium gestii* (L36198), *Heliobacterium chlorum* (M11212), *Heliobacillus mobilis* (U14560), *Heliorestis acidaminivorans* strain HR10B (EU908049); '*Candidatus* Heliomonas lunata' strain SLH (EU910943)

In a 16S rRNA gene tree, strain HR10B grouped within the genus *Heliorestis* clade. The closest relatives of strain HR10B were *Heliorestis* (*Hrs*.) *daurensis* and *Hrs*. *convoluta*, with 16S rRNA gene sequence similarity of 97.9 and 97.0 %, respectively (Fig. 4). By contrast, strain SLH did not group within the genus *Heliorestis* clade, but instead was more closely related to neutrophilic heliobacteria, such as species of *Heliobacterium* and *Heliobacillus* (Fig. 4). In a pattern reminiscent of that observed with *Heliophilum fasciatum* (Ormerod et al. 1996), the 16S rRNA gene sequence of strain SLH was remarkably divergent from that of any cultured heliobacterium (Fig. 4). The closest relatives of strain SLH, *Heliobacillus mobilis* and *Heliobacterium gestii*, had rRNA gene sequences less than 94 % identical to that of strain SLH (Fig. 4), highlighting the unique phylogeny of strain SLH.

Discussion

Since the discovery of heliobacteria in the early 1980s (Gest 1994), the number of new species of these unusual anoxygenic phototrophs has steadily increased (Asao and Madigan 2010). Since the late 1990s, alkaliphilic heliobacteria were cultured from soda lakes in Siberia and Egypt (Bryantseva et al. 1999; Bryantseva et al. 2000a, b; Asao et al. 2006). Here, we have described two new alkaliphilic heliobacteria, and compared their major characteristics with those of other alkaliphilic heliobacteria in Table 2.

Strain HR10B shares the same habitat with another alkaliphilic heliobacterium, the morphologically unique *Heliorestis convoluta* (Asao et al. 2006). Both strain HR10B and *Hrs*. *convoluta* were obtained from shoreline sediment/soil. Such habitats had been the only soda lake environments where heliobacteria were cultured until strain SLH was cultured from the Soap Lake water/benthic sediment sample here. Discovery of strain SLH from an aquatic environment indicates that the habitats of heliobacteria are not strictly limited to soils (Stevenson et al. 1997) or hot spring microbial mats (Bryantseva et al. 2000a; Kimble et al. 1995), and thus it is possible that these phototrophs inhabit other aquatic environments as well but have thus far remained undetected.

The fact that both strains, HR10B and SLH, were heliobacteria was quickly apparent from the presence of Bchl *g*. Notably, however, the absorption spectrum of cells of strain SLH showed an unusually high OH-Chl *a* maximum, which has not been observed in any other heliobacteria, including strain HR10B. The high OH-Chl *a* maximum in intact cells of strain SLH assayed under anoxic conditions may be due to differences in the OH-Chl *a* to Bchl *g* ratio in the photosynthetic reaction center of this organism, and such a possibility warrants further investigation.

Like all alkaliphilic heliobacteria characterized to date, the major carotenoid in cells of both strains HR10B and SLH was OH-diaponeurosporene glucosyl ester (Takaichi et al. 2003). Nevertheless, the finding that cells of strain SLH had OH-diaponeurosporene glucosyl ester as their major carotenoid was surprising in light of the organism's phylogeny. Strain SLH is more closely related to neutrophilic heliobacteria—all of which contain 4,4′-diaponeurosporene as their major carotenoid (Takaichi et al. 1997)—than to species of *Heliorestis*. We thus hypothesize that strain SLH represents a "transitional species" of heliobacteria, one sharing properties with both known phylogenetic clades of heliobacteria.

In light of the rather restricted carbon nutrition of previously described heliobacteria (Asao and Madigan 2010), an unusual trait of strain HR10B was its capacity to photoassimilate a wide assortment of carbon sources, including several amino acids. It is perhaps this capacity that defines a special niche for strain HR10B among phototrophic bacteria in soda lake environments. Good photoheterotrophic growth of strain SLH occurred only with pyruvate or malate as carbon sources, but surprisingly, acetate, which is photoassimilated by all other heliobacteria (Asao and Madigan 2010), did not support growth. Certain amino acids also served as carbon sources for strain SLH, although these did not support growth yields comparable to those on pyruvate or malate. If, as suspected, malate is actually catabolized by the heliobacterial component of the SLH coculture, this heliobacterium would be the only species known to use malate, an excellent substrate for virtually all phototrophic purple non-sulfur bacteria (Madigan 1988; Madigan and Jung 2009). However, a pure culture would be required for unequivocal evidence that malate was photocatabolized by strain SLH.

Strain HR10B had no growth factor requirement, as was also true of *Hrs. convoluta* (Asao et al. 2006). Other species of heliobacteria, including neutrophilic species, require biotin as a growth factor (Asao and Madigan 2010), and thus the two Lake El Hamra heliobacteria are unique in this respect. By contrast, the culture of strain SLH required a small amount of yeast extract, which could not be replaced by a mixture of vitamins. It is therefore likely that strain SLH requires a growth factor(s) not tested here.

Strain HR10B was capable of N_2 fixation, and this capacity has been well established from physiological and genomic analyses of other heliobacteria (Kimble and Madigan 1992; Madigan 1995; Sattley et al. 2008). Surprisingly, however, cell suspensions of strain SLH did not exhibit nitrogenase activity. If true, the absence of a nitrogenase system in this organism would be unprecedented among heliobacteria, and thus the capacity for N_2 fixation in this organism should be addressed in future studies.

Table 2 Properties of alkaliphilic heliobacteria

Property	*Heliorestis acidaminivorans* strain HR10B	'*Candidatus* Heliomonas lunata' strain SLH[a]	*Heliorestis baculata*	*Heliorestis convoluta*	*Heliorestis daurensis*
Morphology	Straight rod	Curved rod	Rod/curved rod	Coil	Coil/bent filament
Cell dimensions	0.6–0.9 × 3–12 μm	0.6 × 2–7 μm	0.6–1 × 6–10 μm	0.6 μm wide and variable length	0.8–1.2 μm wide with a length up to 20 μm
Motility	Flagella	Flagella	Peritrichous flagella	Motility mechanism unknown	Peritrichous flagella
pH optimum	8–9	8–9.5	8.5–9	8.5	9
NaCl optimum (%)	0.5–4	1.5–3	0.5–1	0–1	0
Optimum temperature (°C)	30–37	25–30	30	30–35	25–30
Growth factor requirement	None	Yeast extract	Biotin	None	Biotin
Nitrogen fixation	+	−	nd	+	nd
Phylogeny (% 16S rRNA gene sequence similarity to strain HR10B)	100	91.7	95.8	97.0	97.9
Carbon source photoassimilated (+ bicarbonate)	P, A, casamino acids, PR, YE; amino acids (Ala, Arg, Glu, Gln, His, Lys; Ser)	P, BR, malate, YE; amino acids (Ala, Lys, Ser; Thr)[a]	P, A; L	P, A, BR; PR	P, A; PR
Habitat	Wadi El Natrun (Egypt) shoreline sediment	Soap Lake (Washington State, USA) water/benthic sediment	Soda lake (Siberia) shoreline soil	Wadi El Natrun (Egypt) shoreline soil	Soda lake (Siberia) shoreline soil

Data were obtained from this study and from Bryantseva et al. (1999, 2000b), Asao et al. (2006), and Asao and Madigan (2010). No alkaliphilic heliobacteria, including strains HR10B and SLH, can grow in darkness by pyruvate fermentation

P pyruvate, *A* acetate, *PR* propionate, *YE* yeast extract, *BR* butyrate, *L* lactate

[a] All physiological studies were conducted in the coculture

Both strains, HR10B and SLH, were highly sulfide tolerant. Although sulfide levels in Lake El Hamra are low (Imhoff et al. 1978), those in Soap Lake can be amazingly high (several millimolar), with two studies reporting over 100 mM sulfide in monimolimnion waters (Rice et al. 1988; Sorokin et al. 2007). Thus, growth at 20 mM levels of sulfide by strain SLH was not surprising. Other alkaliphilic heliobacteria have also been reported to be quite sulfide tolerant (Asao et al. 2006; Bryantseva et al. 1999). In addition to having high concentrations of sulfide, the monimolimnion of Soap Lake is also extremely hypersaline, with up to 140 g/l total dissolved solutes (Rice et al. 1988; Oremland and Miller 1993; Sorokin et al. 2007; Taher 1999; Walker 1974). However, surprisingly, strain SLH had no obligatory salt requirement and grew best with only low levels of NaCl. In light of this, it would seem more likely that active growth of this organism could only occur in Soap Lake mixolimnion waters, where salt levels are much lower (Rice et al. 1988). It is thus possible that the culture of strain SLH originated from cells that became attached to particulate matter in the mixolimnion and sank to the sediments. However, countering this hypothesis is the fact that several enrichments using Soap Lake chemocline or mixolimnion water, or shoreline soil, were not positive for a strain SLH-like organism or any other heliobacterium. Thus, the true habitat of strain SLH in Soap Lake is open to question. Some purple bacteria isolated from the deep waters of Soap Lake have shown a similar low or no salinity requirement (Asao et al. 2011).

Taxonomic conclusions

Based on its phylogeny and alkaliphilic phenotype, strain HR10B is clearly a species of *Heliorestis*. However, the organism shows physiological traits not reported from other heliobacteria and is phylogenetically distinct from all other known species of *Heliorestis*. Therefore, we propose that strain HR10B be designated as a new species of the genus *Heliorestis*, as *Heliorestis acidaminivorans* sp. nov. Strain SLH, although alkaliphilic, shows a novel phylogeny that places it outside of the *Heliorestis* clade. Moreover, because its 16S rRNA gene sequence is quite divergent

from that of any described heliobacterium (less than 94 % identical to its nearest neighbor), strain SLH likely represents a new genus of heliobacteria (Rosselló-Mora and Amann 2001). Due to the non-axenic condition of the strain SLH culture, such a new taxon must have '*Candidatus*' status, and we thus propose the name '*Candidatus* Heliomonas lunata' to accommodate strain SLH into taxonomy. Descriptions of both *Heliorestis acidaminivorans* sp. nov. and '*Candidatus* Heliomonas lunata' follow.

Species description of *Heliorestis acidaminivorans* sp. nov.

Heliorestis acidaminivorans; a·cid·a·min·í·vor'·ans; N.L. fem. adj., feeding on amino acids; *Heliorestis acidaminivorans*, the amino acid-eating *Heliorestis*.

Cells are straight rods measuring 0.6–0.9 × 3–12 μm and motile by flagella. These are anaerobic anoxygenic phototrophs. Cell suspensions are green and cells contain Bchl *g* and OH-diaponeurosporene glucosyl esters as photosynthetic pigments. Absorption maxima of anoxic intact cell suspensions occur at 787 nm (major) and 670 nm (minor). Cells lack intracytoplasmic membranes or chlorosomes. It is alkaliphilic and mesophilic; optimum growth occurs at pH 8–9 (range 8–10.5) and 30–37 °C (range 18–45 °C). NaCl is not required, but growth optimum is at 0.5–4 % NaCl. There are no growth factor requirements. Cells show nutritionally diverse photoheterotrophic growth and grow photoheterotrophically (with bicarbonate present) on acetate, alanine, arginine, casamino acids, glutamate, glutamine, histidine, lysine, propionate, pyruvate, serine, or yeast extract as carbon sources. NH_4Cl, yeast extract, casamino acids, alanine, asparagine, aspartate, arginine, glutamate, glutamine, histidine, lysine, methionine, serine, and N_2 serve as nitrogen sources. Photoautotrophy or pyruvate fermentation is not observed. Type strain HR10B^T has been isolated from shoreline soil of Lake El Hamra, Wadi El Natrun (Egypt) and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as DSM 24790, in the American Type Culture Collection (ATCC) as ATCC BAA 2399 and in the National Collection of Marine Algae and Microbiota (NCMA) as NCMAB119.

Description of '*Candidatus* Heliomonas lunata' gen. nov. and sp. nov.

'*Candidatus* Heliomonas lunata;' L. *candidatus*, a candidate to denote a provisional taxonomic assignment (Murray and Stackebrandt 1995); *Heliomonas*; *Heliomonas*; G. n. *helios*, the sun; G. fem. n. *monas*, a unit; *Heliomonas*, the solar unit; *lu·na'ta*; L. fem. adj. *lunatus*, crescent-shaped; *Heliomonas lunata*, the solar unit of crescent-shaped cells.

Cells are curved rods measuring 0.6 × 2–17 μm and motile by flagella. These are anaerobic anoxygenic phototrophs. Cell suspensions are green and cells contain Bchl *g* and OH-diaponeurosporene glucosyl esters as photosynthetic pigments. Absorption maxima in anoxic intact cell suspensions occur at 789 nm (major) and 670 nm (major). Absorption at 670 nm in intact cells kept anoxic during assay is significantly higher than in other species of heliobacteria. Cells lack intracytoplasmic membranes or chlorosomes and are alkaliphilic and mesophilic. Optimum growth occurs at pH 8–9.5 (range 8–9.5) and 25–30 °C (range 18–37 °C). NaCl is not required, but 1–3 % NaCl is growth stimulatory. Growth is best photoheterotrophically on pyruvate or malate as carbon sources. Moderate growth is also observed with alanine, butyrate, lysine, serine, threonine, or yeast extract as carbon sources. Photoautotrophy or pyruvate fermentation is not observed. The coculture containing strain SLH was obtained from 23-m water/benthic sediment of Soap Lake, Washington (USA).

Acknowledgments This work was supported in part by the US National Science Foundation Grants 0237576 and 0950550. We thank Dr. Holly Pinkart, Central Washington University, and Deborah O. Jung, Southern Illinois University Carbondale, for help in sampling at Soap Lake. We thank Steven J. Schmitt, SIU Micro-Imaging and Analysis Center, for electron microscopy, and Prof. Aharon Oren, Hebrew University Jerusalem, for nomenclatural advice.

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