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Characterization of halophiles isolated from solar salterns in Baja California, Mexico

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Abstract Solar salterns are extreme hypersaline environments that are five to ten times saltier than seawater (150–300 g L^{-1} salt concentration) and typically contain high numbers of halophiles adapted to tolerate such extreme hypersalinity. Thirty-five halophile cultures of both Bacteria and Archaea were isolated from the Exportadora de Sal saltworks in Guerrero Negro, Baja California, Mexico. 16S rRNA sequence analysis showed that these cultured isolates included members belonging to the *Halorubrum*, *Haloarcula*, *Halomonas*, *Halovibrio*, *Salicola*, and *Salinibacter* genera and what may represent a new archaeal genus. For the first time, metabolic substrate usage of halophile isolates was evaluated using the non-colorimetric BIOLOG Phenotype MicroArray™ plates. Unique carbon substrate usage profiles were observed, even for closely related *Halorubrum* species, with bacterial isolates using more substrates than archaeal cultures. Characterization of these isolates also included morphology and pigmentation analyses, as well as salinity tolerance over a range of 50–300 g L^{-1} salt concentration. Salinity optima varied between 50 and 250 g L^{-1} and doubling times varied between 1 and 12 h.

Keywords Halophile · Extreme hypersaline · BIOLOG · Solar saltern · Salinity tolerance · Substrate usage

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Abbreviations

PM	Phenotype MicroArray™
ESSA	Exportadora de Sal

Introduction

Solar salterns, found around the world, are extreme hypersaline habitats that contain thriving microbial ecosystems. There have been many investigations of the archaeal, bacterial, and eucaryal inhabitants in these environments using both culture and culture-independent techniques (Javor et al. 1982; Javor 1984; Diez et al. 2000; Benlloch et al. 2001; Casamayor et al. 2002; Litchfield and Gillevet 2002; Ovreas et al. 2003; Spear et al. 2003; Burns et al. 2004a, b; Ley et al. 2006; Maturrano et al. 2006a, b; Feazel et al. 2008; Rossello-Mora et al. 2008). Halophilic microbes are of interest for their potential industrial applications as well as their potential as candidates for finding life elsewhere in the solar system (Grant et al. 1998; Litchfield 1998; Landis 2001; Oren 2002a, b; Tehei et al. 2002). Furthermore, halophilic Bacteria and Archaea have been used to isolate halophages to better understand the ecology of haloviruses (Torsvik and Dundas 1974; Wais et al. 1975; Pauling 1982; Rohrmann et al. 1983; Wais and Daniels 1985; Daniels and Wais 1990, 1998; Nuttall and Dyall-Smith 1993; Bath and Dyall-Smith 1998; Dyall-Smith et al. 2003; Porter et al. 2005; Bath et al. 2006).

The solar saltern at the Exportadora de Sal (ESSA) saltworks located in Guerrero Negro, Baja California Sur, Mexico, has been studied for almost 30 years; however, many studies have focused on the microbial mats found in evaporation ponds at relatively moderate salinities (~100 g L^{-1}) (Garcia-Pichel et al. 1994; Hoehler et al.

2001; Des Marais 2003; Ley et al. 2006). Only a few studies of the more extreme saline habitats at ESSA have been performed, including early characterizations of cultured isolates from brine samples (Javor 1984) and more recent 16S rRNA gene sequence analysis to determine the community diversity within endoevaporite crystals (Spear et al. 2003). As part of a greater aim to elucidate host-virus dynamics in an extreme hypersaline environment, we sought to purify halophilic hosts from ESSA. In this report, our objective was to use dilution cultures to grow halophilic Bacteria and Archaea from saltern ponds and channels of differing salinities to capture a diversity of halophiles and to provide insights into their abundance and interaction with their environment. We report here the basic characterization of these cultures, including the first known 16S genetic sequencing analysis of ESSA water sample isolates and the first known report of the use of non-colorimetric BIOLOG PM plates in conjunction with cultured isolates from an extreme hypersaline habitat. The phylogenetic analysis of these cultured isolates shows the presence of several genera within these salterns, including *Salinibacter* and a novel genus from the Bitterns pond. A comparison of our metabolic data with previously published work both confirm and contradict the substrate usage profile of similar halophilic strains. This report offers a more comprehensive survey of ESSA water sample isolates than has been previously conducted.

Materials and methods

Water sample collection

In February 2006, water samples were collected from various salinity ponds at the ESSA facility located in Guerrero Negro, Baja California Sur, Mexico. The ESSA saltworks is the world's largest evaporative saltworks (>300 km^2) and is composed of a series of shallow (~1 m) evaporation ponds through which increasingly saline water is pumped, ranging from the salinity of the lagoon source water (~40 g L^{-1}) to saturation level (~350 g L^{-1}). When sodium chloride reaches supersaturation and precipitates, the brine is pumped out of the final crystallizing pond and the salt is harvested.

To sample a range of water sources at the upper end of the saltern salinity gradient, water was collected from evaporative ponds (ponds 9 and 11), a crystallizer pond (pond 12), a waste pond (the Bitterns pond), as well as from the inlet and outlet crystallizer channels (crystallizer channels 1 and 2). Ponds 9 and 11 contain gypsum precipitate, while pond 12 and the Bitterns pond contain halite. Ponds 11, 12, and the crystallizer channels have a pink coloration, and the Bitterns pond, containing magnesium salts, has a light green coloration. Salinity of each water source was measured by a VWR VistaVision handheld refractometer (VWR International LLC, Brisbane, CA, USA) and ranged between ~150 g L^{-1} (pond 9) to over 280 g L^{-1} (ponds 11 and 12). Water temperature was measured for each pond using a portable probe (Russell RL060P, Thermo Electron Corp., Beverly, MA, USA) and ranged between 16 and 19°C.

From the ponds and channels, 500 ml of water was collected for culturing purposes and kept cool during transport back to the lab at California State University, Long Beach by storing the bottles among frozen ice packs in a standard cooler. Two extinction dilution series were initially prepared in the field using freshly collected water from crystallizer channels 1 and 2. Two different media were used in these dilution series: 23% MGM and 23% DBCM (Burns and Dyall-Smith 2006; Dyall-Smith 2006) (see below). In order to maximize our chances of recovering numerically abundant halophiles, serial 100-fold dilutions ranging from 10^{-2} to 10^{-10} were carried out. The tubes were then transported back to the lab at ambient temperature and subsequently incubated at 42°C. The earliest that turbidity was observed in several of these tubes was 9 days after initial inoculation.

Halophile isolation and purification

Throughout this paper, salinity refers to the concentration of total salts used to prepare media or saline solution (i.e., NaCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, and KCl) (Dyall-Smith 2006). Two different media were used for initial enrichment culturing from the ESSA water samples: nutrient-rich MGM medium at 23% salinity (23% MGM); and DBCM medium at 23% salinity, which is limited in carbon source but enriched for vitamins and minerals (23% DBCM) (Burns and Dyall-Smith 2006; Dyall-Smith 2006). One milliliter of each water sample was diluted into 5 ml of each medium. This initial tube was designated as "undiluted (U)" and a serial dilution from this tube was carried out further for each type of medium (10^{-3}, 10^{-6}, and 10^{-8}). Tubes were incubated in a non-shaking incubator at 42°C. A *Halobacterium salinarum* strain was also used as a positive control and cultured in the same media and under the same conditions as the ESSA water samples. Growth in several of the tubes, including for *H. salinarum*, was observed in as little as 5 days post-inoculation.

Agar (Fisher Scientific, Pittsburgh, PA, USA) was initially washed three times to remove impurities before use (Dyall-Smith 2006) and liquid cultures were then streaked onto solid media plates made with the same liquid media as above. Plates were incubated at 42°C in a sealed plastic container along with a small beaker of nanopure water as a humidifier to prevent evaporation and salt precipitation on

plates. Colony growth was first observed after 11 days and continued for more than a month after initial plating. Furthermore, growth was observed on all plates representing each of the water samples collected. To maximize the culturable diversity recovered from the saltern, isolated colonies were picked from all of these plates and then restreaked at least four times before they were considered pure strains. Pure cultures were frozen in a sterile-autoclaved solution of 80% glycerol, 6% salt water, and 1 mM $CaCl_2$ (Dyall-Smith 2006) and stored at −80°C.

Carotenoid pigment extraction and gram staining

Carotenoid pigment analysis was carried out (Oren et al. 1995). Briefly, 3–5 ml of cells was filtered onto a 0.2 μm nylon membrane (Nylaflo 47 mm, Gelman Sciences, Ann Arbor, MI, USA) and the pigment extracted in glass scintillation vials with 3 ml of a 1:1 (v/v) methanol/acetone mixture. Vials were incubated in the dark at room temperature for 1 h and read spectrophotometrically between 400 and 700 nm (GENESYS 10UV Scanning, Thermo Electron Corp.). The methanol/acetone mixture was used as the blank. Gram staining was carried out using a protocol modified for halophiles (Dussault 1955).

PCR and phylogenetic analysis

All isolates were subjected to 16S rRNA gene PCR analysis using bacterial- and archaeal-specific primers to determine their initial classification. DNA was extracted from cultured isolates as previously reported (Dyall-Smith 2006). Cultured cells were pelleted and the supernatant discarded. The pellet was then resuspended in 200 μl of pure water and vortexed to lyse the cells, and an equal volume of Tris-buffered phenol (pH 8) was added to extract the proteins. The sample was vortexed vigorously and left at room temperature for 15 min before centrifuging at 16,000×*g* for 5 min. The aqueous phase was transferred to a clean microfuge tube and subjected to a second phenol extraction. The aqueous phase was once again transferred to a clean tube and DNA was precipitated by addition of two volumes of 95% ethanol and inversion by hand approximately 50 times. The tube was centrifuged at 16,000×*g* for 20 min and the ethanol decanted. The DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 16,000×*g* for 2 min. The ethanol was decanted and the DNA pellet was dried before resuspending in 1× Tris EDTA buffer and either incubated at 65°C for 1 h or left at room temperature overnight before being stored at −20°C. The bacterial primers used were GM3: AGAGTTTGATCMTGGC and GM4: TACCTTGTTACGACTT (Muyzer et al. 1995); and the archaeal primers used were A21f: TTCCGGTTGATC CYGCCGGA and 958r: YCCGGCGTTGAMTCCAATT (Radax et al. 2001). PCR reaction mixtures (20 μl) consisted of 1× PCR buffer (New England Biolabs, Ipswich, MA, USA), 10 nmol each dNTP (Brinkmann Eppendorf, Westbury, NY, USA), 10 pmol each primer (Operon, Huntsville, AL, USA), 1.5 U *Taq* polymerase (New England Biolabs), and 50–100 ng of extracted nucleic acids. Reaction conditions were as follows: initial denaturation (94°C for 5 min) followed by 30 cycles of denaturation (94°C for 30 s), annealing (53°C for 30 s), and extension (72°C for 90 s) and a final extension (72°C for 10 min), carried out in a PTC-100® Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Triplicate reactions for samples that showed positive amplification results were purified using commercial spin kits (Epoch Biolabs, Sugar Land, TX, USA). DNA samples and primers were sent to a commercial facility for sequencing (Macrogen Inc., Seoul, South Korea). Sequences were imported into the ARB software (Ludwig et al. 2004) and aligned with reference taxa from the database. Custom lane masks of aligned sequences were created encompassing unambiguous nucleotide positions common to all sequences resulting in the export of 1307 nt (bacterial tree) and 797 nt (archaeal tree). Phylogenetic analysis was performed using the PAUP* v. 4.0b software package (Rogers and Swofford 1998). Trees were constructed using the neighbor-joining and parsimony method with 1,000 bootstrap pseudoreplicates. Additional partial sequences for three strains (RBC-B-10^{-2}, RBC-B-10^{-3}, and CH-A-U) that were close relatives of full-length sequences were added manually into the tree using the ARB parsimony addition tool.

Salinity tolerance and carbon substrate usage

Growth curve baselines were established by growing the isolates in standard 23% MGM medium. Representative isolates from each phylotype were then grown to mid-log phase in 23% MGM medium before transferring 500 μl of cells to 125-ml culture bottles (Wheaton, Millville, NJ, USA) containing 20 ml of medium at varying salinities. The salinities used were 50, 100, 150, 200, 250, and 300 g L^{-1} (~0.7–4.5 M) at pH 7.5, which were prepared by varying the concentrations of total salts (Dyall-Smith 2006). Triplicate cultures of isolates at each salinity were incubated aerobically, along with negative controls, at 42°C with shaking at 100 rpm (C25 Classic Series, New Brunswick Scientific, Edison, NJ, USA). Cell density was measured spectrophotometrically (GENESYS 10UV Scanning, Thermo Electron Corp.) at 600 nm every 2, 8, or 12 h (depending on the growth rate of the isolate) between 3 and 7 days. Growth rate calculations were determined by the equation: $\mu = ((\log_{10} N_2 - \log_{10} N_1)\ 2.303)/(t_2 - t_1)$. The μMax value was calculated as the highest rate along the exponential portion of the growth curve.

Carbon substrate usage by isolates was measured using the BIOLOG Phenotype MicroArray™ plates, PM1 and PM2a (Biolog, Inc., Hayward, California, USA), for at least one member of each genus except the *Halovibrio* strain (9-C-U), which did not achieve high enough cell density to allow for its usage in the BIOLOG method. Each 96-well microtiter plate has 95 wells that contain a single substrate per well with one water negative control well, for a total of 190 substrates. Substrate usage was evaluated non-colorimetrically based on cellular growth measured spectrophotometrically. We initially conducted the BIOLOG experiments by using 23% salt water for all the isolates tested. We then repeated the experiments for two of the bacterial isolates, *Halomonas* sp. (9-10^{-3}) and *Salicola* sp. (9-A-U), at their optimal salinity, 5 and 15%, respectively. Cells were grown to mid-log phase using optimal salinity for each respective isolate (i.e., 5, 15, or 23% MGM medium) then pelleted for 10 min at 10,000 rpm (Sorvall RC-5B; DuPont Instruments) at 4°C. The media supernatant was discarded and the pellets were washed a total of three times by resuspending in 5, 15, or 23% Salt Water (total salts) devoid of nutrient medium, vortexing vigorously, and then pelleting the cells at 10,000 rpm for 10 min in between washes. After the final wash, cells were diluted with the respective salt water solution to 0.1 OD_{600nm} and 135 µl was then aliquoted into each well of the PM plates. Triplicate plates were used for each isolate and were incubated at 42°C in sealed plastic bags as described above. Cell density was measured in a Multiskan MCC plate reader (Thermo Electron Corp.) at 600 nm once per day for 7 days. Positive carbon source usage for each isolate was determined as wells whose mean absorbance was significantly higher than the water control at any point during the 7 days as determined by using the "*t*" test ($n = 3$, $df = 2$).

Cluster analysis, which was used to compare patterns of substrate usage across halophile strains, was performed using PC-ORD v. 4 (MjM Software, Glenedon Beach, OR, USA). Clustering was based on binary data (usage/non-usage) for each of the 190 substrates analyzed using the Sørensen (Bray–Curtis) distance measure with UPGMA linkage.

Results

Morphology and genetic diversity of halophile cultures from ESSA

Our lab purified a total of 35 halophile strains isolated from six different extreme hypersaline water sources from the ESSA salterns. Cultures were acquired from both the 23% MGM nutrient-rich and 23% DBCM vitamin-enriched media. A few of the isolates were obtained in dilutions of up to 10^{-6} (e.g., CH-10^{-6}), suggesting that they were found in relatively high abundance in the salterns, while the majority of isolates were obtained from undiluted enrichments (e.g., 9-A-U).

Phase contrast microscopy showed that most of the isolates were bacilli while some exhibited a triangular shape. Gram staining was conducted on three of the bacterial isolates (9-A-U, 9-10^{-3}, and CH-10^{-6}) and seven of the archaeal isolates (CH-B-U, Bitterns-U, 11GM-10^{-3}, 12-10^{-3}, 11-10^{-6}, Bitterns-10^{-3}, and RBC-A-10^{-3}) and all were found to be gram negative. Pigment extractions of these same ten isolates revealed that all of the archaeal isolates expressed three absorbance peaks around 466, 496, and 529 nm, signifying the presence of the carotenoid bacterioruberin. The *Salinibacter* isolate (CH-10^{-6}) was red pigmented showing weakly extractable carotenoid peaks at 481 nm and a shoulder at 508 nm, consistent with pigmentation of *Salinibacter ruber* strains (Anton et al. 2002).

16S rRNA gene sequence analysis was conducted to identify our halophile cultures. We were able to successfully sequence 26 of the 35 cultured isolates and our results show that our collection groups into at least four bacterial and three archaeal genera (Fig. 1). The bacterial isolates included members of the *Salicola*, *Halovibrio*, *Halomonas*, and *Salinibacter* species (Fig. 1a). Our collection contains only one member from each of the *Halovibrio*, *Halomonas*, and *Salinibacter* clades, but several members of the *Salicola* clade were recovered. The *Halovibrio*-like culture was most closely affiliated with the sequence of the originally named "*Pseudomonas halophila*" str. DSM 3050 (Fendrich 1988), which has been proposed for renaming (Sorokin and Tindall 2006).

The archaeal cultures were represented by several members from the *Halorubrum* and *Haloarcula* genera (Fig. 1b). In fact, the majority of our ESSA isolates (12 of 26 sequenced) belong to two subclades within the *Halorubrum* genus (Fig. 1b). Interestingly, the third archaeal clade in our collection contains only one member, Bitterns-U, which shows high similarity (97–98%) to uncultured environmental samples and to a poorly characterized cultured isolate (Fig. 1b).

Salinity tolerance and optima

As a matter of course, the ESSA isolates were tested for their salinity tolerance. The bacterial isolates tended to show a broader salinity tolerance range compared to the archaeal isolates (Fig. 2a–c). Figure 2d shows the optimal growth salinities for all isolates tested. The *Halomonas* (9-10^{-3}) and *Salicola* (9-A-U) bacterial strains had optimal growth at 5 and 15% salinity, respectively, while the

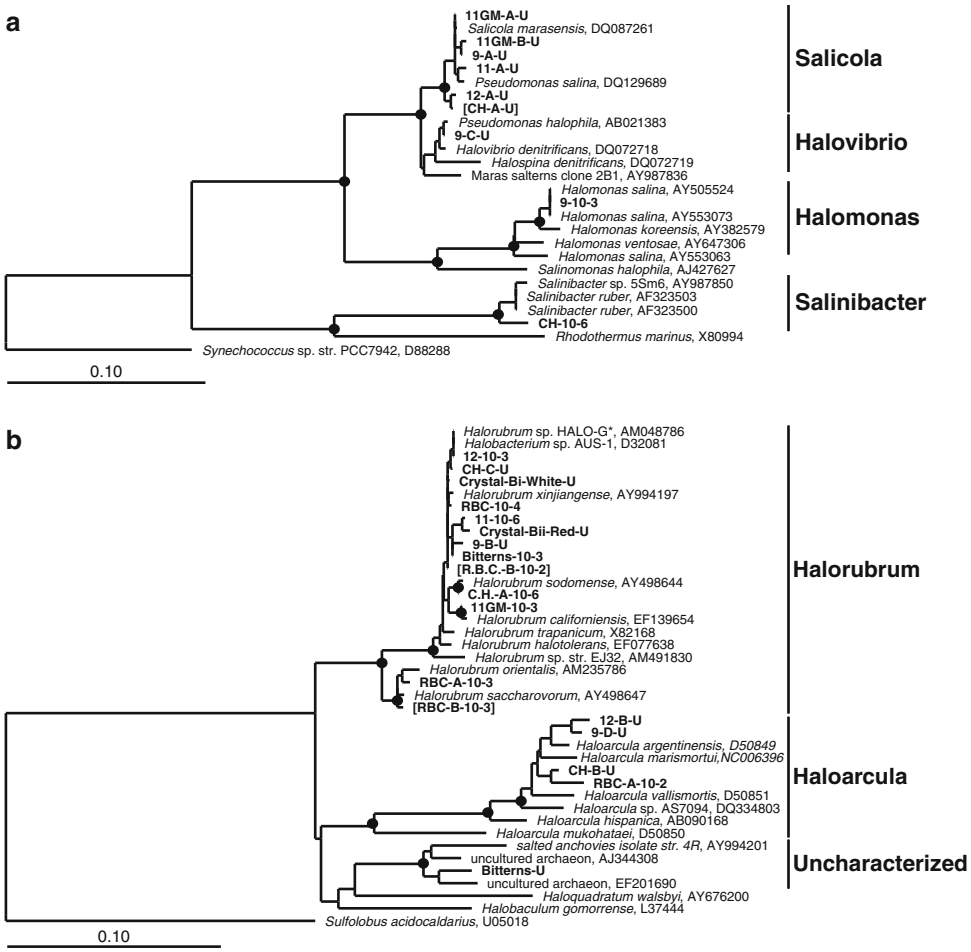

Fig. 1 Unrooted dendrogram depicting relationships among 16S rRNA genes of cultured **a** Bacterial and **b** Archaeal ESSA halophiles (*bolded*) and closely related cultures (*italics*) and environmental clone library (*plain*) sequences. The trees were constructed using neighbor-joining analysis of an alignment of 1307 and 797 nucleotide positions for the Bacteria and Archaea, respectively (gaps and ambiguous residues were excluded using a custom filter in ARB). *Closed circles* represent nodes where bootstrap confidence values of ≥90% were observed for neighbor-joining and parsimony trees expressed as percentages of 1,000 replicates for both. Lineages in brackets were partial 16S sequences added to the tree using the parsimony addition tool in ARB. *Synechococcus* and *Sulfolobus* used as outgroups for the Bacteria and Archaea, respectively

Halovibrio (9-C-U) bacterial strain grew equally well at 15 and 20% salinities. The optimal growth salinities for all the archaeal cultures were between 20 and 25%. As expected, the *Salinibacter* isolate was an exception that expressed an optimal salinity at and did not grow below 20%, similar to its archaeal counterparts (Fig. 2d), confirming that this isolate is truly halophilic and not merely halotolerant, as are the other Bacteria in our collection.

The *Halomonas* isolate displayed a very high μMax of 0.68 h^{-1} at optimal salinity (Fig. 2b), compared to 0.05–0.17 h^{-1} for the other isolates tested (Fig. 2), and had a generation time of 1 h, while doubling times for the other isolates varied between 4 and 15 h, with the *Salinibacter* strain exhibiting the slowest growth rate of all the isolates tested at 15 h (Fig. 2d).

Substrate usage analysis

Before now, substrate usage data has conventionally been presented in tabular format. However, we wanted to provide the reader with a more easily interpretable dataset, therefore, we opted to present the results in a graphical format. The substrate usage patterns for all of the purified strains tested at optimal salinity are summarized in Fig. 3 and they reveal a broad variability in substrate usage, even among closely related strains. The *Haloarcula* strain used the fewest substrates, 10/190, about 5% of the total number of substrates tested (Fig. 3a); while the *Halomonas* strain used the most, 119/190, about 63% of the substrates (Fig. 3e). Generally, the bacterial cultures (*Halomonas*, *Salicola*, and *Salinibacter* strains) were able to utilize more of the substrates than the archaeal samples. This is reflected in the UPGMA dendrogram based on substrate usage as all three representative bacterial cultures, especially the two halotolerant isolates, clustered closely (Fig. 4). Surprisingly, although all five *Halorubrum* strains tested are very closely related (Fig. 1), and showed similar salinity tolerances and growth rates (Fig. 2), they showed highly diverse metabolic profiles in both the number and type of substrates utilized (compare Fig. 3f–j). The 12-10^{-3} and 11-10^{-6} *Halorubrum* isolates used 15 and 16 substrates, respectively, while the Bitterns-10^{-3}, 11GM-10^{-3}, and RBC-A-10^{-3} isolates used 34, 41, and 49 substrates, respectively. This diverse pattern of utilization among *Halorubrum* isolates was reflected in the clustering results (Fig. 4).

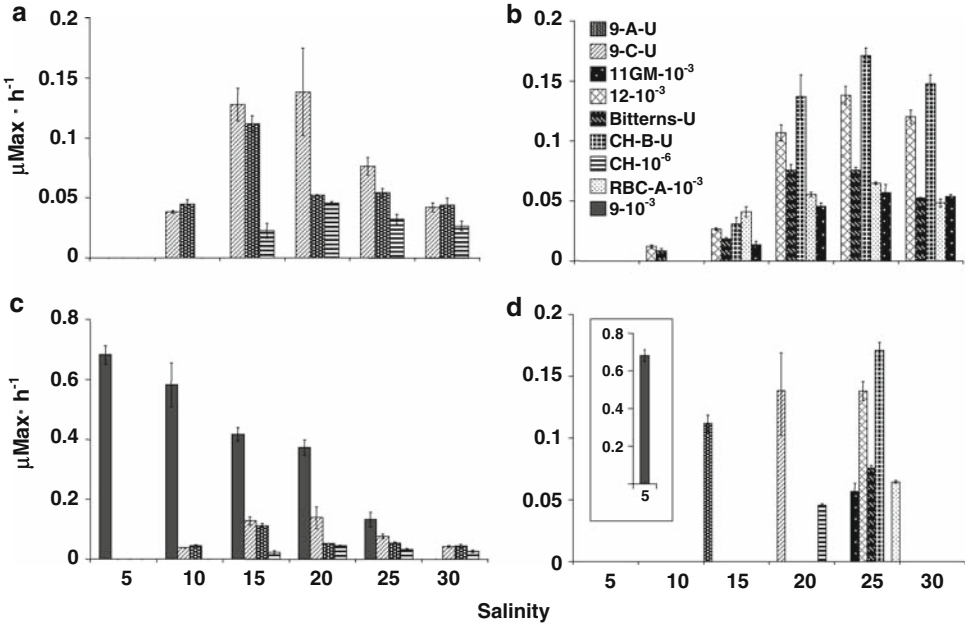

Fig. 2 Maximal growth rates (μMax) at varying salinities for **a** Bacterial and **b** Archaeal cultures. **c** Includes the very fast-growing *Halomonas* (9-10^{-3}) strain with other Bacterial strains for comparison (note scale difference). **d** Growth rate (μMax) at optimal growth salinities for each culture; *inset* shows *Halomonas* (9-10^{-3}) strain (note scale difference). *Bars* represent mean ± 1 SD of calculations from triplicate flasks

Individual substrate usage data from the PM1 and PM2 plates are presented in Tables S1 and S2, respectively, in the Electronic Supplementary Materials section. Only one carbon source, acetic acid, was used universally by all the halophiles assayed (Tables 1, S1, S2). There was also exclusive usage of certain substrates by the bacterial cultures isolated from Pond 9 as well as by samples isolated from the crystallizer inlet channel (Table 1). The profiles of the bacterial isolates tended to overlap, reflecting shared carbon sources (Table 1). For example, all three of the bacterial samples shared exclusive usage of γ-amino butyric acid, putrescine, and L-lysine. In contrast, there was very little overlap in substrate usage among the Archaea as a group, as the only substrate common to all of them was acetic acid (Tables 1, S1, S2). The *Haloarcula* archaeal and *Salinibacter* bacterial samples, which were both purified from the crystallizer inlet channel (CH), were the only isolates to use α-cyclodextrin (Table 1). Twenty-one substrates, including several amino acids and both tween 20 and tween 40, were used only by the bacterial cultures (9-A-U and 9-10^{-3}) that were isolated from Pond 9, the lowest salinity pond that was sampled in this study (Table 1). Despite its central role in many metabolic pathways, pyruvic acid was used by only four of the ten isolates (Tables 1, S1, S2). Only 16 of 190 total substrates were not utilized by any isolate when isolates were tested at their optimal salinity (Tables 1, S1, S2).

The bacterial isolates utilized more amino acids than the archaeal isolates with the *Halomonas* strain using all of the primary amino acids tested (Table 2). The *Haloarcula* strain used none of the primary amino acids tested, and two of the isolates, *Halorubrum* sp. (12-10^{-3}) and *Sp. novum* (Bitterns-U), used only one primary amino acid (Table 2). Tyrosine, cysteine, and tryptophan are not included in the BIOLOG PM plates and were not tested.

Interestingly, there also appeared to be an effect of salinity on substrate usage among the Bacteria. When we compared the BIOLOG substrate usage results for the two moderately halophilic bacterial strains at their optimal salinity (5 or 15%) with results at the culturing salinity (23%), we observed a dramatic increase in overall substrate usage at the lower salinity and some changes in the usage of particular substrates. For example, when the *Halomonas* (9-10^{-3}) isolate was tested at 23% salinity, it utilized only 19 substrates, whereas at 5%, it utilized 119 carbon sources, 15 of which overlapped (data not shown). Similarly, the *Salicola* (9-A-U) culture used 34 substrates when tested at 23% salinity, but grew on 106 substrates at 15%, with 26 substrates overlapping at both salinities (data not shown). Acetic acid was still utilized by all the isolates irrespective of the salinity at which they were tested.

Discussion

Halophilic Bacteria and Archaea from the ESSA salterns have been previously isolated from brine water and investigated, including the first description of *Haloarcula* (Javor et al. 1982), but genetic analysis was not performed on those cultures. In this study, the 26 sequenced isolates, representing four characterized bacterial and two characterized archaeal genera, were dominated by *Halorubrum*, *Salicola*, and *Haloarcula* strains. Several studies have retrieved *Salinibacter*-related species in hypersaline

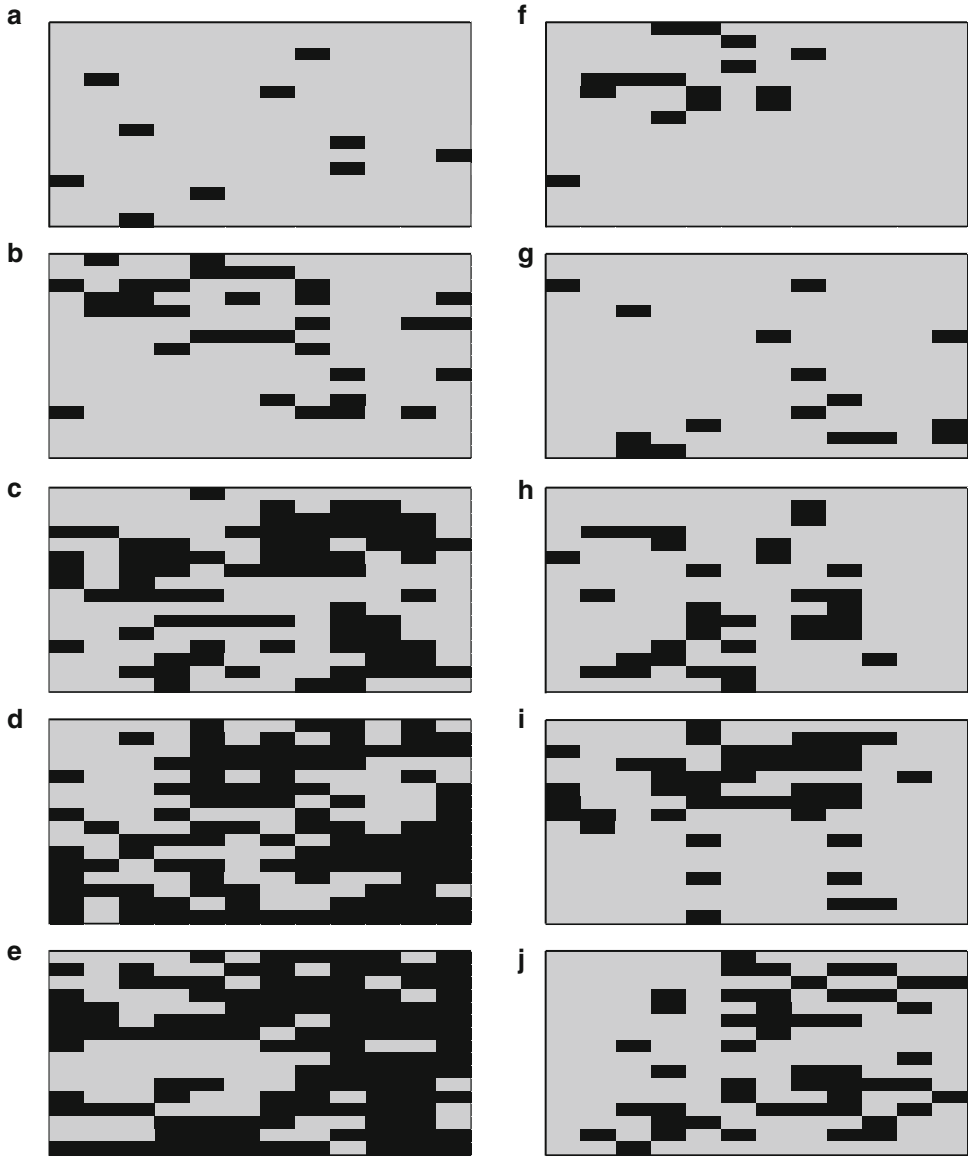

Fig. 3 Carbon source usage of representative isolates via BIOLOG PM assay plates. **a** *Haloarcula* sp./CH-B-U, **b** *Species novum*/Bitterns-U, **c** *Salinibacter* sp./CH-10^{-6}, **d** *Salicola* sp./9-A-U, **e** *Halomonas* sp./9-10^{-3}, **f** *Halorubrum* sp./12-10^{-3}, **g** *Halorubrum* sp./11-10^{-6}, **h** *Halorubrum* sp./Bitterns-10^{-3}, **i** *Halorubrum* sp./11GM-10^{-3}, **j** *Halorubrum* sp./RBC-A-10^{-3}. *Black boxes* represent statistically significant substrate usage at any point during the 7-day experiments. Experiments were conducted in triplicate. Individual substrate information presented in Supplementary Material Tables S1 and S2

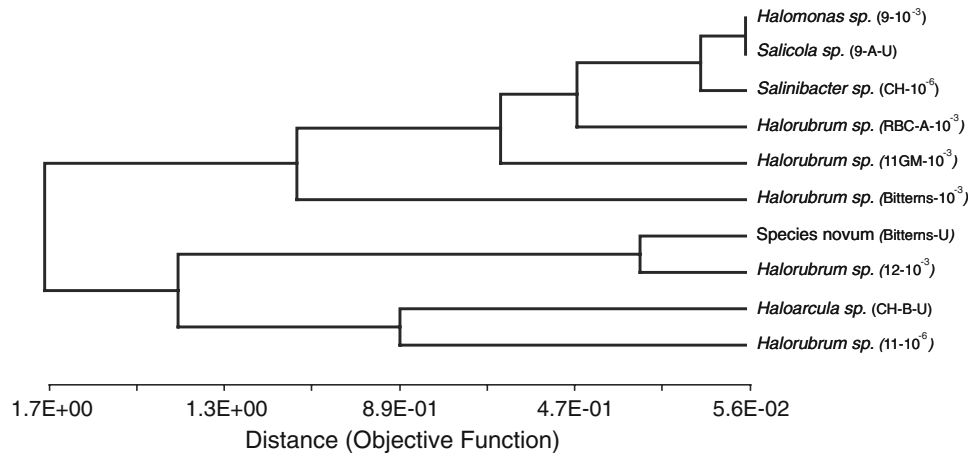

Fig. 4 Cluster analyses of BIOLOG substrate usage data by halophilic cultures. Dendrogram shows UPGMA clustering method of Sørensen (Bray–Curtis) distances calculated from use/non-use of 190 substrates

Table 1 Preferred usage of specific substrates

Carbon source	Preferred usage
N-Acetyl-D-glucosamine	None
D-Sorbitol	None
L-Fucose	None
D-Galactonic acid-γ-lactone	None
D-Psicose	None
D-Galacturonic acid	None
Phenylethylamine	None
Gelatin	None
N-Acetyl-D-galactosamine	None
N-Acetyl-neuraminic acid	None
D-Arabitol	None
L-Glucose	None
Sebacic acid	None
Sorbic acid	None
L-Homoserine	None
Hydroxy-L-proline	None
Pyruvic acid	Used only by four isolates (9-10^{-3}, 9-A-U, Bitterns-U, 11GM-10^{-3})
α-Cyclodextrin	Exclusive use by inlet channel isolates (CH-B-U, CH-10^{-6})
γ-Amino butyric acid	Exclusive use by all bacterial isolates tested (9-10^{-3}, 9-A-U, CH-10^{-6})
D-Tartaric acid	Exclusive use by all bacterial isolates tested (9-10^{-3}, 9-A-U, CH-10^{-6})
Putrescine	Exclusive use by all bacterial isolates tested (9-10^{-3}, 9-A-U, CH-10^{-6})
L-Lysine	Exclusive use by all bacterial isolates tested (9-10^{-3}, 9-A-U, CH-10^{-6})
L-Proline	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
D-Alanine	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Glycerol	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
D-Mannitol	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
L-Glutamic acid	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Tween 20	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Tween 40	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
L-Glutamine	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
2-Aminoethanol	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Pectin	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
I-Erythritol	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
D-Fucose	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
D-Raffinose	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
L-Sorbose	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
δ-Amino valeric acid	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Quinic acid	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
L-Ornithine	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
D,L-Octopamine	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
2,3-Butanediol	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
2,3-Butanone	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
3-Hydroxy 2-butanone	Exclusive use by Pond 9 isolates (9-10^{-3}, 9-A-U)
Maltose	Used only by two of three bacterial isolates
L-Asparagine	Used only by two of three bacterial isolates
β-Methyl-D-glucoside	Used only by two of three bacterial isolates
Maltotriose	Used only by two of three bacterial isolates
Adenosine	Used only by two of three bacterial isolates
L-Serine	Used only by two of three bacterial isolates
L-Threonine	Used only by two of three bacterial isolates
γ-Cyclodextrin	Used only by two of three bacterial isolates
D-Melezitose	Used only by two of three bacterial isolates
α-Keto valeric acid	Used only by two of three bacterial isolates
Dihydroxy acetone	Used only by two of three bacterial isolates
Acetic acid	Universally used by all ten isolates tested
2-Deoxy-D-ribose	Used by eight of ten isolates
Acetoacetic acid	Used by seven of ten isolates
α-Keto-glutaric acid	Used by seven of ten isolates
Propionic acid	Used by seven of ten isolates
N-Acetyl-D-glucoasaminitol	Used by seven of ten isolates

Table 2 Primary amino acid usage among the ESSA halophile isolates

Amino acid	*Halomonas* 9-10^{-3}	*Salicola* 9-A-U	*Salinibacter* CH-10^{-6}	*Sp. nov.* Bitterns-U	*Haloarcula* CH-B-U	*Halorubrum*				
						11GM-10^{-3}	12-10^{-3}	11-10^{-6}	Bitterns-10^{-3}	RBC-A-10^{-3}
L-Asparagine	X		X							
L-Aspartic acid	X									
L-Proline	X	X								
L-Alanine	X	X		X		X	X		X	
L-Serine	X		X							
L-Glutamic acid	X	X								
L-Glutamine	X	X								
L-Threonine	X		X							
L-Arginine	X	X	X							X
Glycine	X								X	
L-Histidine	X	X	X						X	X
L-Isoleucine	X	X	X			X		X		X
L-Leucine	X	X	X			X		X		X
L-Lysine	X	X	X							
L-Methionine	X	X	X					X		
L-Phenylalanine	X									
L-Valine	X	X	X					X		
Total	17	11	10	1	0	3	1	4	3	4

habitats from other geographical locations (Anton et al. 2000, 2002; Mutlu et al. 2008; Rossello-Mora et al. 2008), but not from the ESSA salterns (J. Anton, personal communication). Thus, we are the first group to successfully culture a *Salinibacter* species from ESSA. Additionally, past studies in the ESSA salterns (Javor 1984) have suggested that the Bitterns ponds did not support culturable halophiles. These greenish, magnesium-rich waste ponds did not show the red pigmentation indicating blooms of carotenoid-rich halophiles as do the nearby crystallizing ponds; however, our recovery of a *Halorubrum* sp. strain in a 10^{-3} dilution culture (Bitterns-10^{-3}) suggests that this microbe was present in moderate abundance.

Our other isolate from the Bitterns pond (Bitterns-U), may be a representative of a novel genus. The carotenoid pigmentation and salinity tolerance and optimum of the Bitterns-U isolate were characteristic of other extremely halophilic Archaea; however, phylogenetically the Bitterns-U sequence clustered with a unique group of archaeal sequences. The only other culture in this clade was isolated from salted anchovies (Moschetti et al. 2006) and was identified as not showing proteolytic capabilities, but has not been further characterized to date.

In terms of growth rates and salinity optima of isolates from previously characterized genera, our findings were in close agreement with past studies which have shown that bacterial isolates from saline habitats tend to have higher growth rates and lower salinity optima than halophilic archaea (Ventosa et al. 1998). This was particularly true of the *Halomonas* strain that grew best at the lowest salinity tested (5%), a finding similar to that found for *Halomonas salina* (Valderrama et al. 1991). The *Salicola* isolate showed optimal growth at 15%, which was the same as found for *Salicola marasensis* (Maturrano et al. 2006a, b), although unlike that strain, our 9-A-U strain did not grow above 25% salt. The growth of our *Halovibrio* isolate at 15 and 20% salinities implies that it is moderately halophilic, as similarly described for the *Halovibrio variabilis* isolate (Fendrich 1988). As expected, the major exception to these trends among bacterial cultures was the *Salinibacter* sp. isolate which showed optimal growth at, and not below, 20% total salts and had a doubling time of 15 h, the slowest in our study. These findings are in close accordance with those for the *S. ruber* type strain (Anton et al. 2002).

The major focus of this paper is the use of the BIOLOG Phenotype MicroAssay™ (PM) plates in conjunction with cultured isolates from solar salterns. The use of these convenient plates is very popular, as evidenced by a search of the scientific literature, but not without controversy. BIOLOG plates have been used to evaluate the substrate usage of cultured isolates and they have also been used to assess in situ community functional profiles, including hypersaline systems (Litchfield et al. 2001; Litchfield and Gillevet 2002). A number of critiques have pointed out several caveats regarding their use, especially with direct environmental samples (Glimm et al. 1997; Smalla et al.

1998; Preston-Mafham et al. 2002). Issues such as inoculum size, incubation times, and statistical analysis have been raised and discussed in order to determine the best way to generate accurate, reliable, and relevant data sets. Overall, we found studies involving the use of BIOLOG plates, both environmental and culture-based, to lack consistency, standardization, as well as proper statistical analysis to address plate-to-plate variability. We therefore set out to establish a protocol that we hope can serve as a standardized method for analyzing the metabolic activity of cultured halophile isolates resulting in accurate and reliable data.

One common theme we observed regularly in the literature was the lack of replication in BIOLOG experiments, whether whole environmental samples or cultured isolates were tested. When replication was employed, duplicate plates were used [as with the BIOLOG GN system (Mata et al. 2002)] or triplicate substrates on the same plate were used [as with the EcoPlate™ product (Weber et al. 2007, 2008)]; however, most of the papers, we reviewed did not actually *cite* the use of replicates, which is a basic standard for any experiment. Furthermore, we were unable to find reference to the use of a stringency test to address plate-to-plate variability. Instead, some groups have applied the method introduced by Garland and Mills (1991) to normalize their data.

When evaluating carbon substrate usage, we could not achieve reliable results using the commonly used EcoPlate™, which relies on the reduction of tetrazolium dye to assay for respiration. This may have been due either to the high salinity of our medium (Litchfield et al. 2001) or because at least some of our isolates were unable to reduce this dye. Therefore, we used the non-colorimetric BIOLOG Phenotype MicroAssay™ plates (PM1 and PM2a) to assay nutritional usage by measuring growth spectrophotometrically instead of measuring respiration. To date, no other group has published results of the carbon source usage of cultured halophile isolates via the BIOLOG PM plates (C. Litchfield, personal communication). By using cultured isolates and triplicate plates per isolate, by incubating the plates in a plastic bag with a beaker containing water to prevent evaporation, and by taking regular readings over a seven day period, we addressed several concerns that had been raised by other investigators in regards to the use of BIOLOG plates (Preston-Mafham et al. 2002). Furthermore, by applying statistical stringency to account for plate-to-plate variability (i.e., the "*t*" test), we were able to generate statistically reliable data in relation to carbon source usage to aid in a polyphasic description of our cultured halophiles.

The data sets acquired from our BIOLOG experiments place us in a position to compare the metabolic data of our current ESSA halophiles to previously published carbon source screenings of *Halomonas* species type strains (Mata et al. 2002), *Salicola* isolates (Maturrano et al. 2006a, b), as well as earlier cultured halophiles from Baja California, Mexico, and the Western Salt Company, San Diego Bay, Chula Vista, USA (Javor 1984). The *Halomonas* strain in our collection ($9\text{-}10^{-3}$) showed a very similar metabolic profile to most of the 21 *Halomonas* strains studied in the Mata et al. (2002) study. Just as 19 of their 21 *Halomonas* strains were able to grow on succinic acid, L-alanine, and L-serine as a sole carbon source, so, too, did $9\text{-}10^{-3}$. Similarly, $9\text{-}10^{-3}$ was unable to grow on *N*-acetyl-D-galactosamine and sebacic acid, just as 19 of 21 *Halomonas* type strains were unable to grow on these substrates. The only difference between our *Halomonas* strain and those tested earlier is that our strain is able to utilize D-serine, whereas only 2 of the 21 previously tested *Halomonas* strains were able to use this substrate (Mata et al. 2002).

Our *Salicola* culture (9-A-U) seems to be more similar—phylogenetically and metabolically—to the *S. marasensis* strains that were isolated from Peruvian salterns (Maturrano et al. 2006a, b), than to *Salicola salis*, which was isolated from Algeria (Kharroub et al. 2006). Like *S. marasensis*, the 9-A-U *Salicola* isolate was able to utilize several sugars, as well as the amino acid alanine; but similar to *S. salis*, 9-A-U was able to grow using acetic acid and Tween 80.

Because genetic analysis was not conducted on the cultured halophiles from Javor (1984), we are unable to know if some of those early isolates are phylogenetically similar to those in our current collection; however, Javor described the isolation of several pigmented colonies (red, orange, and pink), which are typical of archaeal species. Because of their distinct morphology, we can confirm that Javor and our group isolated *Haloarcula* (Javor 1984). Overall, a comparison of our data with Javor's shows similar carbon source usage results with more than half of the substrates that were tested having been utilized; however, usage of the rest of the substrates was dramatically different between the two culture sets (Table S3). For example, almost all of the earlier isolates (19 of 21) were able to metabolize glycerol, whereas in the current study, glycerol was used exclusively by the Pond 9 bacterial isolates, the *Salicola* and *Halomonas* spp.; 20 of 21 earlier isolates grew on pyruvate, while only four of ten of the current isolates were able to do so; and while 15 of 21 earlier isolates utilized sucrose, only one of the ten current isolates was able to metabolize this substrate. Interestingly, our *Haloarcula* strain (CH-B-U) showed a completely different carbon source usage profile compared to Javor's *Haloarcula* strain (GN-1). Both GN-1 and CH-B-U grew on acetic acid and neither metabolized lactic acid, but the results of the rest of the carbon sources tested by Javor (1984) are completely opposite from our own findings.

It is clear that these two studies, separated by 24 years, revealed some very different results. The time difference between the two may suggest that the makeup of the culturable halophile community has changed during that duration. Alternatively, the same species could have merely adapted over time by gaining or losing-specific enzymatic activity, which may be a possible explanation for the differences seen between GN-1 and CH-B-U.; however, it is likely that both culture studies are only scratching the surface of the true diversity in a saltern system. It should also be noted that the methodology to test carbon source usage differed between our current study and the earlier one. In Javor's study, substrates were not tested as sole carbon sources, but were instead added to buffered complex medium (CM-B).

The primary advantage of using the high-throughput BIOLOG PM system is the ability to compare large numbers of substrates in different categories across test strains, for example numerous carbohydrates, including both D- and L-isomers. Our ESSA isolates showed some carbohydrate usage including sugars that serve as key metabolic intermediates, with the majority being able to grow on 2-deoxy-D-ribose and fructose-6-phosphate. The *Salinibacter* strain used several sugars, including sucrose, maltose, D-fructose, and α-D-glucose. None of the cultured isolates utilized L-glucose or D-sorbitol, and only the Pond 9 isolates, representing the *Salicola* and *Halomonas* bacterial genera, were able to take up glycerol. Our data contrasts with the results by Sher et al. (2004), Hochuli et al. (1999), and Javor (1984), which showed that glycerol was used by both *S. ruber* and *Haloarcula hispanica*. In fact, Hochuli et al., used glycerol to track the biosynthesis pathways for the primary amino acids in *H. hispanica*.

Additionally, the BIOLOG PM1 and PM2a plates contained 17 of the 20 primary amino acids, allowing us to evaluate their usage as a sole carbon source. The difference in primary amino acid usage between the bacterial and archaeal isolates from this study was very striking. The bacterial species we evaluated utilized between 10 and 17 of the amino acids tested, compared to their archaeal counterparts, which used fewer than four amino acids, with the fast-growing *Halomonas* sp. using the most and the *Haloarcula* sp. using none.

All but two isolates tested, including all the *Halorubrum* strains, showed a unique amino acid usage profile. The 12-10^{-3} *Halorubrum* and the unknown Bitterns-U cultures showed identical primary amino acid usage because they both grew only on L-alanine. The most utilized amino acids were L-alanine, L-isoleucine, and L-leucine, which were each used by six of the ten halophiles evaluated—three archaeal and all three bacterial. The *Haloarcula* isolate we tested (CH-B-U) was the only cultured halophile in our study that did not utilize any of the 17 primary amino acids tested as a sole carbon source. This seems to support the assertion made that *H. hispanica* does not require any uptake of extracellular amino acids for growth (Falb et al. 2008). Our data conflict with those from an early characterization of *H. hispanica*, which was shown to grow using arginine, glutamic acid, lysine, and glutamine as sole carbon sources (Torreblanca et al. 1986). However, we cannot claim that our *Haloarcula* isolate represents the same species or strain as those used in Torreblanca et al. (1986).

Relating these BIOLOG experimental findings to the in situ ecology of halophile growth is challenging, but because some of the substrate usage was exclusive to isolates cultured from a specific collection site, one could hypothesize that metabolic activity is a reflection not just of the species, but is also a reflection of the specific environmental conditions. For example, it may be that the exclusive use of 21 substrates by the two bacterial pond 9 isolates, *Halomonas* and *Salicola*, and the exclusive use of α-cyclodextrin by the archaeal *Haloarcula* and bacterial *Salinibacter* isolates from the crystallizer inlet channel reflects adaptation to available substrates in their specific hypersaline environment. If so, this could transcend a species-specific metabolic profile and instead provide insight into the possible metabolic requirements needed for surviving in a specific part, or micro-environment, of an extreme hypersaline system. Past studies have shown adaptation to hypersalinity, such as differential RNA and protein expression (Bidle 2003; Bidle et al. 2007, 2008), and that convergent evolution in hypersaline environments was a likely explanation for the similar genomic features (e.g., GC content) and amino acid bias found in halophiles, both Archaea and Bacteria (Mongodin et al. 2005; Paul et al. 2008). While salinity may play a central role in selecting for specific halophiles in these ponds, our finding, of exclusive substrate usage by halophiles of different genera and domains isolated from the same collection site, suggests possible 'micro-environment' adaptation based on substrate availability in the environment.

In regards to the amino acid usage differences observed between the bacterial and archaeal isolates, one could hypothesize that the Archaea must synthesize most or all of the primary amino acids due to the absence or low abundance of amino acids in the higher salinity ponds. However, the *Salinibacter* strain (CH-10^{-6}) was one of the cultures that used a majority of amino acids tested (10 of 17), and it thrives in upper salinity ponds alongside its archaeal counterparts (Anton et al. 2000, 2002; Rossello-Mora et al. 2003, 2008; Mutlu et al. 2008). Thus, these differences may instead reflect more general physiological differences in the usage of amino acids between halophilic Bacteria and Archaea. Interestingly, investigations with

brine samples demonstrated that amino acids were not taken up in situ by *S. ruber*, despite the fact that in situ the halophilic 'square Archaea' and in culture the *S. ruber* strain M31 did indeed take up amino acids (Rossello-Mora et al. 2003).

One of the most striking findings was that all of the bacterial isolates, including the *Salinibacter* strain, used more substrates than their halophilic archaeal counterparts. This strategy of diversified carbon usage may provide a way for Bacteria to compete in hypersaline habitats where they are living with halophilic Archaea that often form abundant blooms. This may be particularly important for groups that are living at supraoptimal salinity, such as the *Halomonas* sp. in this study. We also found potential evidence of ecological niche differentiation among the halophilic Archaea. The finding that our *Halorubrum* isolates showed widely diverging patterns of substrate usage suggests that differences in substrate usage may have contributed to their diversification.

Based on phylogenetic and physiological data, we can conclude that the culturable halophile community isolated from ESSA is diverse and shows both similarities and differences to halophile strains isolated previously from ESSA and elsewhere. On one hand, the ESSA halophile community shows a stable community structure, with the presence of organisms that are very similar to other *Halomonas*, *Salicola*, and *Salinibacter* spp. strains; while on the other hand the ESSA community shows variability over time as witnessed by the metabolic differences between earlier ESSA halophile cultures and current isolates, especially with the *Haloarcula* sp. isolates. Furthermore, the ESSA isolates show a very unique substrate usage profile, even among closely related species, but also what could be adaptive micro-environmental signatures based on the exclusive use of particular substrates by diverse isolates which share a specific habitat within the salterns. Coupled with the discovery of a potentially new genus recovered from the Bitterns waste pond, further investigation of this extreme environment's microbial population is warranted to provide a more complete ecological picture of these fascinating microorganisms.

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