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Microbial diversity and complexity in hypersaline environments: A preliminary assessment

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The microbial communities in solar salterns and a soda lake have been characterized using two techniques: BIOLOG, to estimate the metabolic potential, and amplicon length heterogeneity analysis, to estimate the molecular diversity of these communities. Both techniques demonstrated that the halophilic Bacteria and halophilic Archaea populations in the Eilat, Israel saltern are dynamic communities with extensive metabolic potentials and changing community structures. Halophilic Bacteria were detected in Mono Lake and the lower salinity ponds at the Shark Bay saltern in Western Australia, except when the crystallizer samples were stressed by exposure to Acid Green Dye #9899. At Shark Bay, halophilic Archaea were found only in the crystallizer samples. These data confirm both the metabolic diversity and the phylogenetic complexity of the microbial communities and assert the need to develop more versatile media for the cultivation of the diversity of bacteria in hypersaline environments.
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Introduction

Hypersaline environments can be found on all continents and in most countries. They consist of two primary types: those that arose from seawater and hence contain sodium chloride as the predominant salt, and those that arose from nonseawater sources and contain different ion ratios. This situation is frequently found in soda lakes where the dominant anion is carbonate. The former are referred to as thalassohaline, whereas the latter are called athalassohaline. These athalassohaline systems are dominated by potassium, magnesium, or sodium and are frequently the sources of potash, magnesium metal, soda, and even borax if the waters were high in boron. Some examples of these are the Dead Sea, the alkaline soda lakes of Egypt (e.g., Wadi Natrun), the soda lakes of Antarctica, and Big Soda Lake and Mono Lake in California.

Sodium chloride-dominated environments can occur as natural inland salt lakes. The Great Salt Lake, Utah, is an example of such, but other examples are salt mine drainage waters, playas, natural coastal splash zones and tide pools, brine springs from underground salt deposits, and solar salterns.

Solar salterns are found worldwide. They may be fed by brine springs containing from 5% to 20% sodium chloride where solar and wind power evaporate the water and thus produce salt. More commonly, solar salterns are located on or near the seashore, and seawater is directed into the initial or inlet pan. From there, the water flows either through gravity or by pumping to new condensers as the brine becomes more and more concentrated with respect to total salts and sodium chloride. At total salt concentrations around 8–10%, the calcium salts begin to precipitate, forming gypsum layers in the pans until the majority of calcium has been removed. Finally, as the percentage of total salts approaches 40%, the brine is passed into the crystallizers. Here the sodium chloride precipitates, leaving an overlying layer of water high in potassium and magnesium and still containing some sodium and calcium salts. The major anion throughout the saltern is chloride.

Because of the increasing salinity of the environment, solar salterns are considered extreme environments with very restricted biology. Indeed, fish are not observed much beyond 4–5% salt. However, there is an extensive microbial community [13,18,19,26] in these salterns including the alga *Dunaliella* and frequently heavy populations of the brine shrimp *Artemia*. There are also frequently large populations of birds in a solar saltern and in many places, such as Bonaire, Netherlands Antilles, salterns have become protected nature preserves and bird breeding grounds.

Although it is known that both halophilic Bacteria and Archaea exist in the crystallizers (Ref. [27] and references cited above), there have been few studies examining the diversity of the microbial community and its variability over a prolonged time period. In addition, it is generally believed that the halophilic microbial community has limited metabolic capabilities, no doubt the result of early investigations into the organisms in brine [20]. The studies reported in this paper were designed to evaluate both the population diversity and the metabolic diversity in two solar salterns and an alkaline soda lake.

Materials and methods

Samples

All samples were collected aseptically and processed within less than 4 h of collection (Cargill Solar Salt Plant, Newark, California and Eilat Salt, Eilat, Israel) or on return to the laboratory for Shark Bay, Western Australia and Mono Lake, California. A list of the samples, their collection location, and dates is described in Table 1.

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Table 1 Description of the samples used in this study

Sample code	Saltern location	Sample location	Description and percent total salts (w/w)
ML-1-99	Mono Lake, California	Shore	Alkaline soda lake, pH 9, salinity 8%
SB-1-99	Shark Bay, Australia	Inlet from the Indian Ocean	ND
SB-0-99	Shark Bay, Australia	End of the first pond	ND
SB-F-99	Shark Bay, Australia	End of the flume pond	ND
SB-P-99	Shark Bay, Australia	Pump house	ND
SB-O3-99	Shark Bay, Australia	Crystallizer	ND
E-3-Inlet	Eilat, Israel	Inlet to saltern	3.9%, pH 6.9
E-3-200	Eilat, Israel	Concentrator	13.6%, pH 7.5
E-3-202	Eilat, Israel	Concentrator	17.4%, pH 7.6
E-3-203	Eilat, Israel	Concentrator	21.8%, pH 7.7
E-3-304	Eilat, Israel	Crystallizer	29.5%, pH 7.0
E-4-Inlet	Eilat, Israel	Inlet to saltern	3.9%, pH 8.18
E-4-200	Eilat, Israel	Concentrator	14.9%, pH 7.85
E-4-202	Eilat, Israel	Concentrator	20.8%, pH 7.79
E-4-304	Eilat, Israel	Crystallizer	27.9%, pH 7.39

ND=no data taken.

Enumeration methods

Two different procedures were used to determine the numbers of microorganisms in the sample: a surface-spread plating method was used for all samples, and direct epifluorescence counting of selected samples followed the procedure of Zweifel and Hägstrom [29]. For direct microscopy, aliquots of the samples were preserved in formalin in the field and refrigerated until examined after staining with 4′, 6-diamidino-2-phenylindole (DAPI).

All samples were plated in duplicate onto at least eight different media: modified R2A made with tap water (MR2A; Difco) and a modified casamino acid medium (MCAT) [16] prepared with 3–4%, 10%, 20%, and 25% solar salt (w/v). Samples were incubated at either room temperature or 37°C for up to 4 weeks. The sample from Mono Lake was cultured on 8% and 15% MR2A as well as MCAT, modified for the alkaline pH of the lake, containing per 1 L: 900 ml tap water, 30 or 100 g solar salt, 15 g casamino acids, 3 g sodium citrate, 2.5 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl, 25 g agar, and 5 g Na_2CO_3 in 100 ml, which was autoclaved separately; final pH 9 [23]. The Mono Lake sample was diluted in 8% solar salt (w/v) adjusted to pH 9 and incubated at 37°C. Because of the low nutrient levels in the Shark Bay waters (Mackle, personal communication), Bushnell–Haas media supplemented only with 3.3 g/l potassium acetate and 1 g/l pyruvic acid, 25 g Noble Agar (Difco), and the above four salt concentrations were also used (BHAP). All growths were scored according to pigment and approximate colony size. Plates containing fungi were considered contaminated and discarded because of the overgrowth by the fungi.

The effects of Acid Green Dye #9889/2AR reuse S_1 on the growth of culturable bacteria and archaea were also tested. The dye, which is water soluble and commonly used to increase the color of salterns, was added to enrichment flasks at the recommended final concentrations of 1.0% or 0.1%. After incubation, aliquots were removed and plated onto the above media, and samples were also taken and frozen for later fingerprint analysis.

Metabolic diversity

Metabolic diversity was determined for the Mono Lake sample using BIOLOG GN plates to which 145 μl of untreated brine was

Table 2 Total CFU per milliliter on MR2A and MCAT for all samples described in this paper

Sample code	MCAT	Pigment	R2A	Pigment	BHAP	Soda
E-3-Inlet	2×10^4	1×10^2	3.5×10^5	1×10^2	ND	ND
E-3-200	2×10^4	2×10^3	8×10^4	5×10^3	ND	ND
E-3-202	1.5×10^7	5.0×10^6	4.0×10^7	5.0×10^6	ND	ND
E-3-203	6.1×10^4	6.3×10^3	2.7×10^5	2.5×10^5	ND	ND
E-3-304	8.6×10^4	8.6×10^4	1.4×10^5	1.4×10^5	ND	ND
E-4-Inlet	6.0×10^2	1.0×10^2	3.1×10^3	2.5×10^2	ND	ND
E-4-200	2.5×10^5	1.0×10^5	1.5×10^5	1.3×10^5	ND	ND
E-4-202	6.0×10^6	5.0×10^5	6.0×10^6	5.0×10^5	ND	ND
E-4-304	8.5×10^3	8.5×10^3	2.1×10^5	2.1×10^5	ND	ND
SB-1-99	1.2×10^3	1.0×10^2	4.5×10^3	1.0×10^2	2.8×10^3	ND
SB-0-99	8.5×10^3	1.0×10^2	4.5×10^3	1.0×10^2	1.5×10^4	ND
SB-F-99	1.3×10^5	<100	1.0×10^5	5.0×10^3	7.0×10^4	ND
SB-P-99	6.5×10^5	1.0×10^4	1.2×10^5	3.0×10^4	9.0×10^4	ND
SB-O3-99	$>3.0\times10^5$	$>3.0\times10^5$	$>3.0\times10^5$	$>3.0\times10^5$	$>3.0\times10^5$	ND
ML-1-99	ND	ND	5.4×10^4	3.0×10^4	ND	1×10^2

ND=not determined.

Table 3 Positive BIOLOG GN results with Eilat and Mono Lake samples

Substrate	Eilat			Mono Lake
	E-3-Inlet	E-4-Inlet	E-4-200	
α-Cyclodextrin		+		
Dextrin	+	+		
Glycogen	+	+		
Tween 40	+	+	+	+
Tween 80				+
N-Acetylglucosamine		+		+
Adonitol				+
L-Arabinose		+		+
D-Arabitol				+
Cellobiose		+		
D-Fructose	+	+		
D-Galactose	+	+		+
Gentiobiose		+	+	+
α-D-Glucose		+		
m-Inositol			+	
α-D-Lactose		+		
Lactulose			+	+
Maltose	+	+		+
D-Mannitol	+	+		
D-Mannose		+		+
D-Mellibiose		+		
β-Methyl-D-glucoside				+
D-Psicose		+		
D-Sorbitol		+		
Sucrose	+	+		+
D-Trehalose	+	+		+
Turanose				+
Xylitol		+		
Methyl pyruvate	+	+		+
Monomethyl succinate	+			
Acetic acid	+			
cis-Aconitic acid	+	+		
Citric acid	+	+		
Formic acid			+	
D-Galacturonic acid lactone				+
D-Gluconic acid	+	+		
D-Glucuronic acid		+		
β-Hydroxybutyric acid	+			
γ-Hydroxybutyric acid	+			
β-Hydroxy phenylacetic acid			+	
α-Keto butyric acid		+		
α-Keto glutaric acid	+	+		+
D,L-Lactic acid	+	+		+
Propionic acid	+	+		
Quinic acid	+	+		
D-Saccharic acid				+
Succinic acid	+	+		
Bromo succinic acid	+	+		
Succinamic acid	+			
Alaninamide				+
D-Alanine	+	+		
L-Alanine	+	+		+
L-Alanylglycine	+	+		
L-Asparagine	+	+		
L-Aspartic acid	+	+		
L-Glutamic acid	+	+		
Glycyl-L-aspartic acid		+		
Glycyl-L-glutamic acid	+	+		
L-Histidine	+	+		+
Hydroxy-L-proline	+	+		
L-Leucine		+		
L-Ornithine	+	+		+
L-Phenylalanine	+			
L-Proline	+	+		
L-Pyroglutamic acid	+			
D-Serine		+		
L-Serine	+	+		
L-Threonine	+	+		
Urocanic acid		+		
Inosine		+	+	
Uridine		+		
Thymidine		+		
2-Amino ethanol			+	
2,3-Butanediol			+	
Glycerol		+		
D,L-α-Glycerol phosphate		+		
Glucose-1-phosphate		+		
Glucose-6-phosphate		+		
Total number of substrates used	39	57	9	23

added to each of the 96 wells. These microtiter plates were incubated at the same temperatures as the petri plates. Further confirmation of the whole community's ability to utilize some of the BIOLOG substrates was achieved by using biometer flasks [2] that contained Bushnell–Haas broth (Difco) supplemented with 3% solar salt and Na_2CO_3 (pH 9.8). Substrates were added at 0.09 g/flask. The flasks were inoculated with 10 ml of the untreated sample. The respired CO_2 was trapped in 10 ml of standardized 0.1 N NaOH (Fisher), removed periodically, and back titrated to the phenolphthalein endpoint with 0.1 N standardized HCl (Fisher). The volume of HCl used for the titration was converted to milligrams CO_2 respired and plotted as cumulative CO_2 [24]. All samples were tested in triplicate with the controls containing no added substrate.

Whole community fingerprinting

Fingerprints were determined based on the amplicon length heterogeneity (ALH) procedure of Rappé *et al* [21] and Suzuki *et al* [25]. For the Bacteria, primers 6-FAM-5′ 27F (5′-6-FAM-AGAGTTTGATCMTGGCTCAG-3′) and unlabeled 355R (5′-GCTGCCTCCCGTAGGAGT-3′) [12] were used. For the Archaea, the primers were designed specifically for the halophilic archaea based on 16S rRNA sequences in GeneBank: 6-FAM-5′ 1HF (5′-6-FAM-ATTCCGGTTGATCCTGCCGG-3′) and H30-5R (5′-GTTACCCCACCGTCTACCT-3′) were used.

For the Eilat samples, the 16S rDNA was harvested from the cell pellet obtained after centrifugation of 4–5 l of sample and the 16S rDNA obtained using the Fast DNA Spin Kit for Soil (BIO 101; Vista, CA). For other samples, 100–500 ml of the samples was concentrated by centrifugation or the complete growth on MCAT medium [15] was harvested with the appropriate salt solution and then extracted using the BIO 101 system. Other modifications are as described in Mills *et al* [17]. The final concentrations in the PCR reaction mixture were: PCR buffer, 0.25 mM $MgSO_4$, 0.25 U *Tff* DNA polymerase (Promega, Madison, WI), 250 mM dNTPs (Boehringer Mannheim, GmbH, Germany), 0.25 mM forward and reverse primers (Invitrogen Life Technology, Gaithersburg, MD), 6-FAM labelled primer synthesized by Perkin-Elmer Biosystems (PE-Biosystems, Foster City, CA), and 5–40 ng template DNA with DEPC-treated water to final volume. The PCR was performed

in a PTC-100 Programmable Thermal cycler (MJ Research, Watertown, MA) under the following conditions: 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, repeat of 28 cycles with a final extension time of 10 min at 72°C.

After PCR of the 16S rDNA with the above primers, the resulting products were separated on a 4.25% polyacrylamide denaturing gel using an ABI 377 sequencer (PE Biosystems, Foster City, CA), which separated the products on the basis of their electrophoretic mobility, and each peak was quantified based on its fluorescence intensity according to ABI GeneScan Software. With this procedure, no PCR-product cleanup is required before electrophoresis and the system is able to separate the products that have a single base pair difference. Replicate extractions were analyzed to demonstrate reproducibility of the method, and peaks appearing in two of the replicates and accounting for greater than 1% of the operational taxonomic units (OTUs) were accepted as part of the analysis.

Results

Bacterial enumerations

The maximum numbers of colony-forming units (CFU) per milliliter on various media are listed in Table 2. In all cases, the maximum numbers were found on media containing solar salt concentrations approximating those of the sample (specific salt concentrations are not shown). In general, the maximum numbers of CFU were recovered on R2A medium, and for the Eilat samples, the counts were often two orders of magnitude higher on this medium. This implies that the bacterial community should have the ability to metabolize pyruvate, glucose, and starch, and prefer to grow on reduced concentrations of casamino acids and proteose peptone than are found in the MCAT medium. DAPI epifluorescence counts were 1.0×10^6, 1.5×10^7, and 1.3×10^8 for E-3-202, E-3-203, and E-3-304, respectively. For the latter two samples, these were two to three orders of magnitude higher than the plate counts on any of the eight media, and this was the typical range of the direct counts when using the Zweifel and Hägstrom [29] method. Without the toluene step, counts were invariably greater than 10^{11} (data not shown) indicating the presence of a large number of ghost cells.

The Indian Ocean waters entering the Shark Bay solar saltern (SB) are considered oligotrophic (Mackel, personal communication). Thus, it is not surprising that higher numbers of SB-CFU were frequently enumerated on the BHAP medium than on the MCAT medium. Also, the numbers of bacteria in the SB samples with less than 12% salt were generally lower than those found in the Eilat samples. This again indicates that these organisms have fewer nutrients for growth than are found in more organically enriched waters.

The only countable plates with the Mono Lake samples were those containing 8% solar salt. The inclusion of the soda medium at an elevated pH closer to that of Mono Lake did not result in improved recovery of CFU.

Metabolic diversity

Samples from Eilat and Mono Lake were placed into BIOLOG GN plates, incubated at 37°C, and read for up to 4 weeks. The concern here was not the time to color development or rate of metabolism, but the determination of the general metabolic potential and how consistent this would be in various saline environments over time. As noted previously [15], only samples containing less than 15% salts gave reproducible results because the salts interfere with the indicator dye. Thus, the results for only four samples are shown in Table 3. Only one compound, Tween 40, was used by all four samples. The data, however, indicate a very extensive metabolic potential especially in the inlet samples with 41% and 60% of the 95 potential substrates used by E-3-Inlet and E-4-Inlet, respectively. As the salt concentrations increased (E-4-200), the percentage of substrates metabolized dropped to 9.5%. The Mono

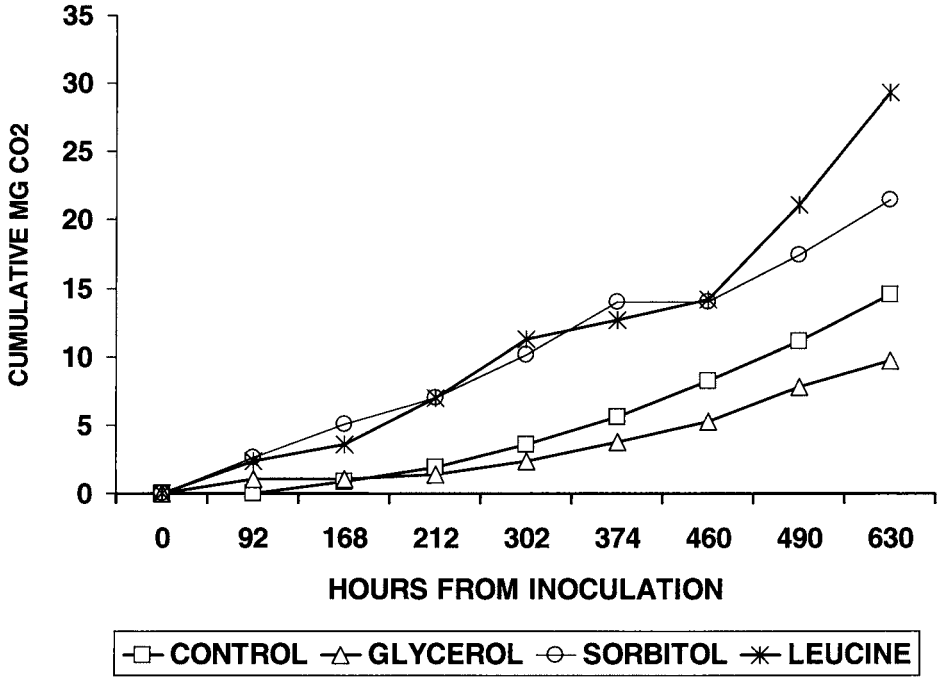

Figure 1 Carbon dioxide respiration by the whole community from Mono lake.

Table 4 Minimal numbers of OTUs from the Eilat salt works samples using halophilic Archaea primers

Sample locations (date sampled)	Base pair area percentage[a]					
	241	244	245	246	247	249
E-1-202 (August 1996)			64	36		
E-4-202 (February 1998)			45	40	15	
E-1-304 (August 1996)	7	5	11	52	18	8
E-2-304 (January 1997)	3	12	6	52	15	11
E-3-304 (August 1997)	8	6	11	48	18	10
E-4-304 (February 1998)			12	66	21	

[a]Values shown are the area percentage of each OTU.

Lake sample, which is at about 8% salt, fell in between these two salt concentrations at 32% of the substrates utilized.

Based on the BIOLOG results, three substrates were selected to determine if indeed they were metabolized by the whole community in Mono Lake. L-leucine, sorbitol, and glycerol were added to replicate biometer flasks. The data (Figure 1) show that both L-leucine and sorbitol were respired by the microbial community, but that glycerol could not serve as a significant source of carbon when the community was incubated in Bushnell–Haas broth with ammonium nitrate as the nitrogen source.

Whole community fingerprints

The Eilat crystallizer, Mono Lake, and several of the Shark Bay samples have been subjected to ALH analysis (ALHA) following PCR with universal bacterial or archaeal primers. The results are listed in Tables 4 and 5.

The Eilat samples were analyzed using archaeal primers that had been modified for the halophilic Archaea based on the known sequences in GeneBank. The community displayed a limited range of Archaea with the greatest variety appearing in the crystallizers (Table 4). During all four sampling periods, the crystallizers had a common OTU, which contained 246 bp. In addition, during the 1996–1997 sampling events in the crystallizer (E-1-304 and E-2-304), there was a total of six OTUs. During the last sampling in February 1998, however, the number of OTUs decreased to three but the major component still had 246 bp. As might be expected, the OTUs found in the lower salinity pan, E-1-202 and E-4-202, had only two or three halophilic Archaea OTUs with the predominant one at 245 bp in both 1996 and 1998.

Subsequently, the bacterial primers 6-FAM-5′ 27F and 355R were also used. At the same time, analyses were also performed on the Shark Bay samples. These results (Table 5A) show a much broader distribution of OTUs of bacteria in these systems. For Mono Lake, the major OTUs found from the culture plates were at 319, 342, 349, and 351 bp. This contrasts with the major OTUs for the whole community (uncultivated) sample at 318, 348, 349, 350, and 352. The only overlap is with the 349-bp group.

When the Shark Bay pump (SB-P) and brine (SB-B) samples were analyzed, the predominant OTU for SB-P was at 325 bp (45%) with 349 and 354 providing for 23% and 17%, respectively, of the total community population. These results are similar to what was found with the Mono Lake sample. The brine sample, however, contained a major peak at 325 bp (45%) with 318, 334, and 351 providing the remaining community composition.

Attempts to obtain PCR products with the bacterial primers with the Shark Bay crystallizer plate-cultured community were unsuccessful, implying that there were nondetectable levels of halophilic bacteria in that sample. However, when the SB-03 (crystallizer) samples were treated with different concentrations of dye to determine the effects of the dye on microbial growth, OUTs representing bacteria were detected. The results in Table 5A show that at a final concentration of 0.1% dye, the community is

Table 5 Minimal numbers of OTUs observed with the samples from Mono Lake and Shark Bay

Sample locations	Bacterial primers Base pair area percentage																				
	318	319	323	325	334	338	339	342	343	345	348	349	350	351	352	353	354	355	363	366	372
Whole community — Mono Lake	16		1	1				11			14	21	14		14		7			1	
Cultured community — Mono Lake		25					2	18	10		3	21		18				3			
Shark Bay — brine	17			45	23									15							
Shark Bay — pump house	9	12			6					13		23				10	17		10		
Shark Bay — crystallizer+0.1% dye									91	9											
Shark Bay — crystallizer+1.0% dye						67		22	11												

Sample Locations	Archaeal Primers Base pair area percentage					
	238	240	242	243	244	247
Shark Bay crystallizer-cultured community	1.2		2.7	93.7		
Shark Bay crystallizer+0.1% dye	3.9	1.8		96.2		
Shark Bay crystallizer+1.0% dye			2.4	96.9		

composed of a single OTU (91% of the community composition), whereas at a final concentration of 1% dye, the major OTU is at 338 bp (67% of the community composition is represented by this OUT) with the remainder at 342 bp (22%) and 343 bp (11%). Thus, while there is some selectivity in the community by the dye, there is still some overlap in the fingerprints, and significantly, the dye allowed the halophilic bacterial community to grow and be detected.

With the archaeal primers, only three samples from Shark Bay gave significant PCR products. The crystallizer community cultured on MCAT was washed from the plate and extracted and the two dye-exposed crystallizer samples were tested with 6-FAM-5′ 1HF and H305R. These results are shown in Table 5B. Both the dye-exposed samples and the cultured whole community samples gave essentially the same pattern. The major OTU is found with a base pair of 243 and various amounts of either 238-, 240- or 242-bp OTUs. It is interesting that regardless of treatment, the halophilic Archaea OTUs are more similar than those found with the bacterial primers. Attempts to obtain a fingerprint of an archaeal community in the Mono Lake sample were unsuccessful despite 8% salinity and pH 9.8 (Melack, personal communication); a salinity and pH at which we would have expected the presence of Archaea.

Discussion and conclusions

The data presented here emphasize the diversity both over time and in location of the microbial communities in hypersaline environments. It also indicates some of the potential similarities in these hypersaline communities. Since its introduction by Garland and Mills [8] in 1991, BIOLOG GN plates have been used to indicate the potential metabolic capabilities of communities in numerous environments. These range from rhizosphere communities [7] and composts [5] to hydrocarbon-polluted soils [28]. We had previously examined the metabolic potential of a solar saltern in San Francisco over time and had noted that major temporal differences were observed in the metabolic potential of samples taken from the same saltern pans [15]. Although the differences were not as great in the Eilat saltern, it was still not possible to predict from one time period to the next which substrates would be used by the community except for Tween 40 (Table 3). However, analysis by simple matching coefficient of the carbon utilization patterns showed that those saltern pans with densities less than 1.08 formed a tight cluster, and these samples also routinely used α-ketoglutaric acid and D,L-lactic acid [14].

Interest in soda lakes has centered primarily on the isolation and characterization of individual microorganisms [11]. Little attention has been paid to the community as a whole and/or its metabolic potential. This is the first report of the whole community metabolic potential from an alkaline soda lake and demonstrates that these environments contain communities capable of metabolizing a broader range of substrates than previously assumed. In general, for both the solar salterns and the soda lakes, media have traditionally been composed of casamino acids, and occasionally, a simple carbon source such as glucose [9]. Given the variety of carbon sources that can potentially be consumed, it is time to reevaluate our isolation techniques and develop media that will allow for isolation of a greater diversity of halophiles.

The importance of media constituents is clearly demonstrated by the increased numbers of CFUs found with the MR2A medium both at Shark Bay and at the Eilat saltern (Table 2). Media also need not be complex, as demonstrated by the large numbers of isolates obtained on BHAP medium, which is chemically defined and contained acetate and pyruvate as sole carbon sources. This medium also contained only inorganic nitrogen sources indicating that peptones and casamino acids are not required by all halophilic bacteria.

Because of the limitations of culturing microorganisms due to medium composition, redox conditions, and various temperature and pH requirements, there has been increasing interest in examining the 16S rDNA profiles of natural microbial assemblages. Benlloch *et al* [3] first reported the community diversity of the crystallizer ponds in a solar saltern in Santa Pola, Spain. They noted that the diversity of halophilic Bacteria was low and that of the halophilic Archaea was high, as expected, and that the gene sequences they purified and analyzed did not match those of isolates from the same ponds [3]. This latter finding again emphasizes the need for new media and approaches for culturing halophilic bacteria. In fact, the recent report of a new genus of red halophilic Bacteria that grow in the crystallizer ponds [1] confirms the limitations of our current techniques for discovering the diversity and biotechnological potential of halophilic bacteria.

Further studies along a salinity gradient by Martínez-Murcia *et al* [16] showed decreasing numbers of amplicons as the salinity increased for the halophilic Bacteria with the opposite for the halophilic Archaea. In this study, the authors used restriction digests of the PCR-amplified 16S rDNA. Since then, others have examined the community structure throughout two salterns at different times of the year using the 5S rRNA fingerprinting technique [6]. The authors found the same fingerprints regardless of location or time of sampling. This procedure did not involve PCR, and Casamayor *et al* [6] also noted that none of the sequences from the crystallizer ponds matched those of known halophilic Archaea. Similarly, they found that in the lower salinity ponds, the 5S rNA sequence represented members of the Proteobacteria and Gram-positive bacteria and none of the generally reported [4] flavobacteria, α-Proteobacteria or halophilic Archaea. Because of the limitations of the method, however, discrimination of overlapping electrophoretic bands may have masked sequences of other members of the community [6].

We used the ALH procedure, which has been developed and used in other environments [17,21,22,25], to examine the diversity along the salinity gradient in the Eilat saltern. The ALH procedure is based on the direct analysis of fluorescently labeled PCR products, and separations of one base pair are highly reproducible. With this procedure, we indeed found an overall increase in the numbers of halophilic archaeal amplicons with increasing salinity in the Eilat saltern (Table 4). The one exception to this occurred in February 1998 where the same amplicons were found in both the 15% and 28% salinity ponds. Studies on the Eilat samples with universal Bacteria primers are currently under way.

Whole community and whole cultured community samples from Mono Lake were extracted and subjected to PCR using the universal primers for Bacteria. There were 10 amplicons from the bulk whole community sample and eight amplicons with the cultured community; three of these overlapped with the samples taken directly from the lake surface waters, and in both, a major

amplicon that had 349 bp occurred. In a different approach, using density gradient gel electrophoresis, Hollibaugh *et al* [10] investigated changes in the water column whole community fingerprint during a mixing event in Mono Lake. Partial sequences showed similarities, but no direct matches, with known organisms in the α- and γ-Proteobacteria and Cytophagales. Because of the differences in techniques, it is not possible to compare the results of these two studies directly. However, it is encouraging to note that their sequence analyses showed similarities to known organisms just as our results show some convergence of OTUs between the whole community sample and that obtained from a petri plate culture. They were also unable to detect Archaea in these Mono Lake waters (Hollibaugh, personal communication).

Samples from the brine and pump house ponds at Shark Bay similarly failed to indicate the presence of halophilic Archaea, and we were unable to detect halophilic Bacteria in the cultured crystallizer community. However, when samples from the crystallizer pond were treated with varying concentrations of dye, a distinct halophilic Bacteria fingerprint was obtained, depending on the dye concentration (Table 5A). The differences in the fingerprints disappeared when halophilic Archaea primers were used (Table 5B).

Furthermore, the fingerprints from the whole community Mono Lake and Shark Bay-P-99 samples (Tables 2 and 5A) showed similar major amplicons at 349 bp. This is unexpected based on the geographic and environmental differences between the two locations. Shark Bay is in Australia and has very oligotrophic waters, whereas Mono Lake is an alkaline soda lake in California, but is also oligotrophic. At both locations, the total salinity was greater than 8%. Thus, total salt concentration and total nutrients would seem to be more important determinants of community structure rather than geographic location.

The general picture that emerges from these studies of metabolic potential and ALHA is one of great metabolic versatility and significant variability in the microbial community structure in solar salterns and soda lakes. Thus, estimation of the biodiversity and its potential biotechnological applications requires more extensive investigations of these dynamic saline environments than has occurred so far.

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