

MICROBIAL DIVERSITY IN HYPERSALINE ENVIRONMENTS

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ABSTRACT

It is generally believed that high salinity environments are sterile. After all, no fish can be seen swimming in them. Therefore, they must not support any life. Unfortunately, many people believe this to be true. However, there is an extremely important but usually neglected component of salinas and hypersaline systems - the microbial community. Not only are microbes able to withstand salt stress, but they serve several functions in the hypersaline environment. Both bacteria and the alga *Dunaliella* serve as food sources for the brine shrimp. Additionally, they both provide coloration to the ponds thereby increasing the temperature in the ponds by as much as 5-10°C. This increased temperature increases the rate of evaporation and hence causes faster precipitation of the halite crystals. Bacteria and fungi are further responsible for the degradation of organic matter and the conversion of organic nitrogen to ammonia which can be used by the algae and perhaps brine shrimp and brine flies. Thus, microbes are an integral part of the ecosystem.

There are several questions that need to be answered regarding the bacteria in hypersaline environments. Who is there, how stable is the community, how diverse is that community, what role(s) are the bacteria playing in the environment, how many organisms are present, and do any of these bacteria have a useful function for man?

This paper will answer some of these question by citing specific examples from the authors' own studies on microbial diversity in hypersaline systems as well as from the literature and describe some of the existing usefulness of some of the isolates to human problems and diseases as well as some of the interesting recent isolates. From the studies reported here, it is very obvious that hypersaline systems such as salt lakes and solar salterns are reservoirs of significant bacterial communities whose potential has barely been examined.

KEYWORDS: Bacteria, Archaea, salinas, diversity, molecular studies, cultivation

1. INTRODUCTION

Biodiversity generally refers to plants and animals ranging from algae to trees for the former and from insects to polar bears for the latter. In fact, there is an effort to describe all known plants and animals, under the Biodiversity Inventories Program (1). While this seems like a daunting task in itself, if one considers the neglected microbial component it becomes an impossible task. It simply is not known or even well estimated how many species/genera there are of the microscopic algae, fungi, bacteria, and viruses. Thus the study of the microbial diversity and community characteristics of extreme environments such as hypersaline lakes and solar salt works is vital to beginning to estimate this major compartment of biodiversity.

An extremely important but usually neglected component of salinas and hypersaline systems is the microbial community. Not only are microbes able to withstand salt stress, but they serve several functions in the hypersaline environment; both bacteria and the alga *Dunaliella* serve as food sources for the brine shrimp. Additionally, they both provide coloration to the ponds thereby increasing the temperature in the ponds by as much as 5-10°C. This increased temperature increases the rate of evaporation and hence causes faster precipitation of the halite crystals. The existence of fungi in these systems has only recently been noted (2), and their role in the total microbial community dynamics is completely unknown.

Bacteria, and probably the fungi, are furthermore responsible for the degradation of organic matter and the conversion of organic nitrogen to ammonia which can be used by the algae and perhaps brine shrimp and brine flies so common to hypersaline environments.

There are two groups of bacteria involved in the microbial diversity in solar salterns. The first group is from the domain *Bacteria*. This group contains the bacteria most people are familiar with; those microbes that are responsible for the production of wine and beer, the production of yogurt and some cheeses and fermented meats, the production of antibiotics, waste water treatment, etc. as well as the being the principle agents of many human and animal diseases. In this domain are some unusual *Bacteria* which can grow over very wide salinity ranges (*Halomonas*) or which seem to be a bridge (*Salinibacter*) between the *Bacteria* and the other domain of bacteria the *Archaea*.

The domain *Archaea* includes many unusual bacteria that can grow at very high temperatures (over 120°C), require very low temperatures for growth (less than 1° C), require very acid environments (less than pH 1), or very alkaline environments (pH over 12), very high pressures (1200 atm), and very high salt concentrations (greater than 15% salt). These latter are the bacteria that are dominant in the crystallizers.

Thus, there are several questions that need to be answered regarding the bacteria in hypersaline environments. Who is there, how stable is the community, how diverse is that community, what role(s) are the bacteria playing in the environment, how many organisms are present, and do any of these bacteria have a useful function for man?

This paper will answer some of these questions by citing specific examples from the authors' own studies on microbial diversity in hypersaline systems as well as from the literature and describe the usefulness of some of the isolates to human problems and diseases. From the studies to be reported, it becomes very obvious that hypersaline systems are reservoirs of significant bacterial communities whose potential has barely been examined.

2. MATERIALS AND METHODS

2.1 Samples

The sources of saline (approximately the salinity of seawater) and hypersaline (greater than seawater and up to saturation) waters for this paper were salterns in Puglia, Italy and San Francisco, CA. All samples were collected aseptically in sterile 1 liter bottles and processed as soon as possible. One to four liters of each sample were centrifuged to obtain a pellet of the microorganisms for the molecular analyses of the whole microbial community, while dilutions were made directly from the original samples for the cultivation work.

2.2 Cultivation Methods

The samples were diluted and spread plated onto varying salt concentrations using media previously described (3). The inoculated plates were incubated at temperatures near the in situ temperatures of the samples, and the resulting colonies were counted for up to six weeks.

2.3 Noncultivation Methods

Molecular techniques have greatly increased our ability to analyze microbial community composition by allowing us to search for microbes which cannot grow or grow extremely slowly and hence are missed with normal cultivation techniques. The method used in these studies is the amplicon length heterogeneity procedure (LH) (4). The DNA was extracted from the pure cultures or the cell pellets of the whole community, and the 16S rRNA genes were amplified using universal primers with a fluorescent tag on the 5' end. The polymerase chain reaction (PCR) products were separated on a SpectruMedix SCE 9610 (SpectruMedix LLC.) sequencer which separated the PCR products on the basis of base pairs. For molecular identification, nonfluorescently tagged PCR products were obtained using the same primers, cloned, and sequenced as described previously. (5)

Both traditional cultivation and the molecular methods have their limitations, so it is essential that all available techniques be used.

3. RESULTS

3.1 Puglia, Italy

The plan of the first salina to be described is shown in Figure 1. This salina is located on the eastern side of Italy in the province of Bari. It is located next to a residential and business area that borders the ocean. Portions of the data reported here are from a thesis (6).

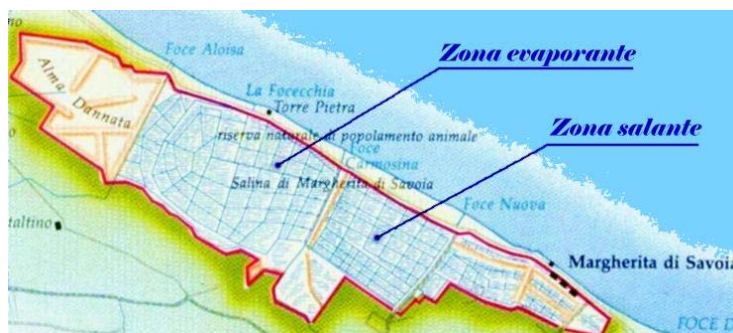


Figure 1. Plan of the Salina de Margherita di Savoia.

Samples were taken from four different salinities within the salt works and shipped back to the US for processing. Both cultivation and molecular techniques were then used to estimate the microbial diversity within the salina and across the different salinities. For the molecular techniques, the first step in evaluating the whole uncultivated community composition is to use a PCR-based fingerprinting method.

Table 1 shows the fingerprint results for the *Archaea* which were found only in the ponds having the higher salt concentrations. The plate washes columns refer to overgrown plates that contained too many bacterial colonies to count. This growth was simply washed off of the agar with the appropriate salt concentration. These plate washes were then subjected to the same molecular analyses as the centrifuged pellets. The pure cultures were from purified and isolated colonies from the initial cultivation techniques. The major operational Taxonomic Units (OTUs) were found in the base pair lengths separated at 508.7 for all three ponds and especially in the plate washes and the whole community samples. This implies that there is a common set of genera residing in these three ponds. On the other hand, the data also indicate that there is not a consistent

pattern for the other OTUs implying a microbial diversity not anticipated for the high salt concentrations.

Table 1. Relative abundances of the halophilic Archaea based on LH fingerprints. WC equals whole community analyses, PW is the too numerous to count plate washes, and PC stands for the pure cultures that were isolated and identified.

Base Pair Length (OTUs)	15.7% Pond			20.2% Pond			28% Pond		
	WC	PW	PC	WC	PW	PC	WC	PW	PC
500.7						0.12			
501.4						0.2			
501.9			0.2	0.2		0.1			
502.7						0.2			
503.2			0.1	0.1					
506.1	0.3			0.1			0.2		
506.7		0.1							
508.7	0.2	3.6		0.4	2.0		0.2	2.0	
509.2		0.5							
509.9		0.8					0.2	1.5	
510.6	0.1			0.1				0.4	
511.9	0.5			0.4			0.5		

The data in Figure 2 clearly demonstrate that the plate washes produced more OTUs than did the whole community analyses or the selection of pure cultures. One explanation for this is the long incubation times used for the plates, up to 6 weeks, which allowed the slow growers to appear. It is also possible that cross feeding allowed more organisms to appear while there were limitations due to inherent problems with the PCR (7). The greatest diversity was noted for the 15 % salinity pond which is not surprising as this salinity supports the growth of most halophilic Archaea as well as most halophilic *Bacteria*.

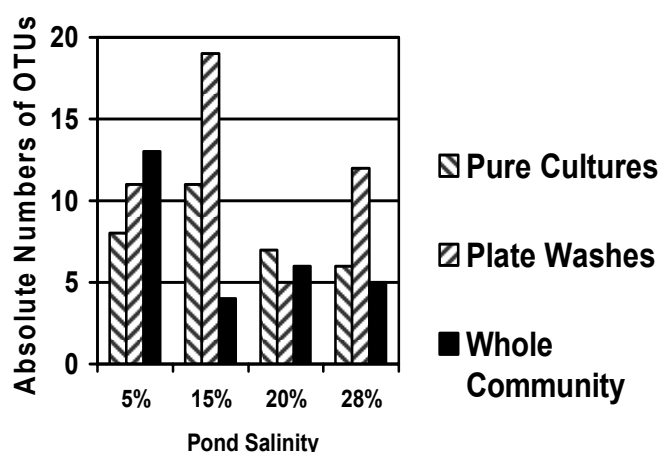


Figure 2. Comparison of the Operational Taxonomic Units (OTUs) for the domain *Bacteria*.

Over sixty isolates were tested by standard microbiological methods and identified. The members of the domains *Bacteria* and *Archaea* are listed in Table 2 along with the medium and salt concentration they were originally isolated from. Both the 15% and 28%

salt ponds contributed eight different genera while the other two ponds contributed six genera each. The genus *Halomonas* was found throughout the salt works. The haloarchaea *Halorubrum* and *Haloferax* were isolated only from ponds containing 15% or more salt.

Table 2. Summary of identification of members of the domains *Bacteria* and *Archaea*.

5% Pond	15% Pond	20% Pond	28% Pond
<i>Halobacillus</i> (MCAT 8%) <i>Halomonas</i> (R2A 4% & BHAP 8%) <i>Halovibrio</i> (R2A 15%) <i>Marinobacter</i> (R2A 8%) <i>Pseudomonas</i> (R2A 15%) <i>Salicola</i> (R2A 22%)	<i>Brevibacterium</i> (MCAT 4%) <i>Halobacillus</i> / <i>Bacillus</i> (R2A 4% & 15%) <i>Halomonas</i> (MCAT 8%) <i>Halorubrum</i> (BHAP 15%) <i>Idiomarina</i> (R2A 15%) <i>Rhodovibrio</i> (BHAP 15%) <i>Salinivibrio</i> MCAT 4% & 8%	<i>Haloferax</i> (R2A 8%) <i>Halomonas</i> (BHAP 8% & 15% & R2A 8%) <i>Halorubrum</i> (R2A 22%) <i>Idiomarina</i> (MCAT 8%) <i>Marinobacter</i> (MCAT 15%) <i>Salinivibrio</i> (R2A 4%)	<i>Halobacillus</i> (R2A 15% & BHAP 8%) <i>Halomonas</i> (BHAP 4% & 15% & MCAT 4%) <i>Idiomarina</i> (R2A 15%) <i>Marinococcus</i> (R2A 22%) <i>Salicola</i> (R2A 22%) <i>Salinicoccus</i> (0.1MCAT 8%) <i>Salinivibrio</i> (R2A 15%)

3.2 Cargill Solar Salt Plant

Similar studies were conducted at the Cargill Solar Salt Plant in Newark, California. However, these studies lasted over several years and seasons so it was possible to examine the microbial diversity over both time and space. Figure 3 summarizes the data for the domain *Bacteria* and demonstrates the wide diversity of bacteria in this system. The largest quadrant is for unidentified microbes, followed by the cytophaga/flavobacterium group (most likely *Salinibacter*), and the gammaproteobacteria group which includes the genus *Halomonas* which was noted to be widespread in the Puglia saltern.

A spatial and seasonal comparison of the archaeal clones which were sequenced is shown in Figure 4. The SF4 and SF5 refer to different sampling times while the 8 refers to the 8% salinity pond, the 10 refers to the approximately 20% pond, and the 26 refers to the pond with 26% salt. Spatially there are significant differences in the dominant haloarchaea and there are significant changes in the community structures for 10A and 26 between the sampling periods of SF4 and SF5, December and June the following year. The genus *Halobacterium* was not found in pond 10A in December, but was almost 5% of the community in June. Other changes were decreases in STR B1T and *Halorubrum lacusprofundi* in June with a concomitant increase in unidentified clones. However, pond 26 had the greatest changes with a complete shift in the dominant microbes from 9 genera/species to only three with miscellaneous isolates (i.e. present at less than 1%) characterizing the archaeal community.

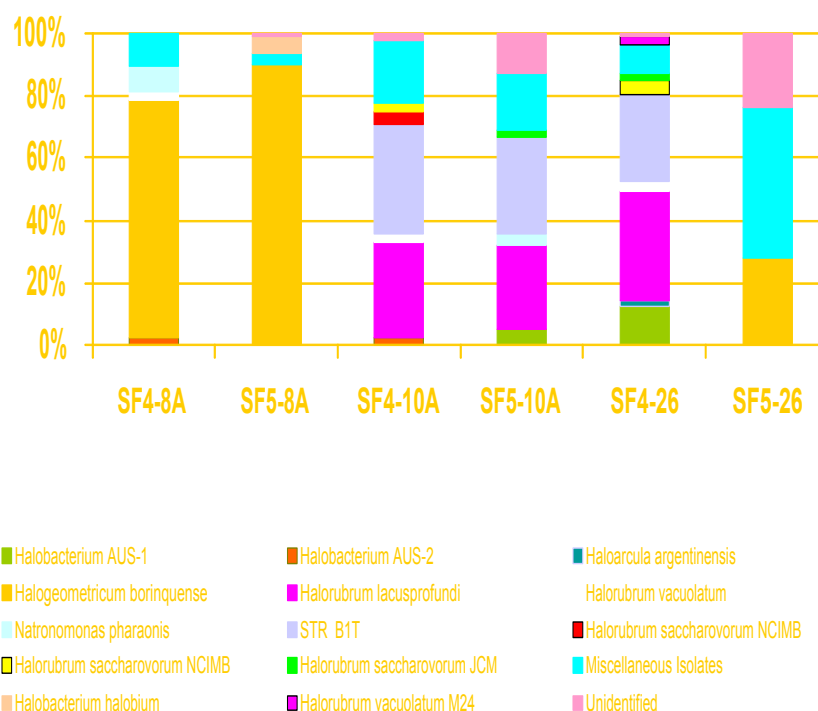


Figure 3. Relative abundances of the *Bacteria* clones and pure cultures found in the Cargill Solar Salt Plant ponds.

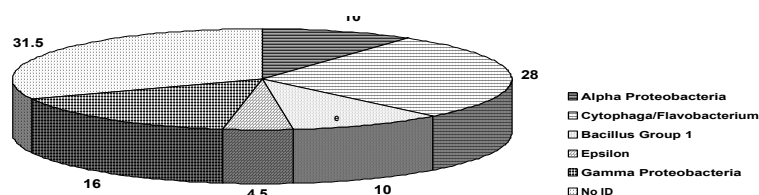


Figure 4. Archaeal clone identifications using the RDP, version 8 data base.

4. DISCUSSION

The data presented above clearly show that the bacterial and archaeal communities of solar salterns are dynamic both spatially and temporally. Other work has shown the spatial variations (8-10) as did a study of the solar saltern at, Shark Bay, WA, Australia where spatial variability was also noted via molecular techniques along with traditional cultivation methods (11).

However, temporal studies have been few. Litchfield et al. summarized some of their work on the salterns in Bonaire, Netherlands Antilles (2) where again there were major differences in the total numbers of bacteria and numbers of pigmented bacteria over time. When those studies were completed, the molecular techniques had not been developed, much less applied to the environment. Thus it is important to look at these salterns over time to see what microbes might be there at different time periods and to determine if there are metabolic changes related to different seasonal communities.

The biodiversity noted in this paper begs the question as to what useful microorganisms might be among these communities. We do know that many of these bacteria contain potentially useful enzymes such as amylases, proteases, etc. (12,13). Also, since the isolation and identification of the genus *Halomonas* (14), this organism has proven to be

a source for many useful products, especially its internal compatible solute ectoine (15). Other potential uses for the haloarchaea were recently reviewed (16), and it was noted that the membranes of the haloarchaea are particularly useful in drug delivery systems as well as in photographic applications.

New bacteria are being isolated and identified from solar salterns. Most recently the genus *Salinibacter* was cultured (17) after the use of molecular techniques had shown its existence (10) for several years. More surprisingly, in 2004, two groups (18,19) reported the first successful cultivation of the Walsby's square bacterium which had first been observed microscopically and reported in 1980 (20). Both of these bacteria have great potential to help us understand the physiological ecology of salterns and the growth characteristics of a square bacterium. How many more ecologically and evolutionarily important microbes exist in salinas we do not know, but we do know that if the solar salterns are destroyed, we will lose a significant source of microbial biodiversity.

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