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Detection of prokaryotic microbial communities of Çamaltı Saltern, Turkey, by fluorescein in situ hybridization and real-time PCR

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Abstract: Knowledge of the functions, interactions, and diversity of extremely halophilic microorganisms mostly comes from the results of studies performed in different salterns throughout the world. As model habitats, salterns allow the comparison of different techniques used for qualitative and quantitative analysis of halophilic communities in these hypersaline environments. Çamaltı Saltern is the biggest coastal solar saltern located on the Aegean coast of Turkey, and it produces most of the salt consumed in the country. In the present study, detection of prokaryotic communities of the Çamaltı Saltern was performed using 2 culture-independent methods. Real-time polymerase chain reaction (RT-PCR) and fluorescein in situ hybridization (FISH) techniques were evaluated to analyze the microbial populations of Çamaltı Saltern. Of the Çamaltı samples, 48% to 67% were hybridized with the EUB338 probe and 33% to 57% were hybridized with the ARC915 probe. Repeatability of the RT-PCR experiments with environmental DNA was considered insufficient. However, FISH analysis may be combined with RT-PCR and these 2 techniques may be used in tandem to rapidly reveal quantitative aspects of the microbial population of hypersaline environments.

Key words: Fluorescein in situ hybridization (FISH), real-time PCR, hypersaline environments, Çamaltı Saltern

Çamaltı Tuzlası/Türkiye prokaryotik mikrobiyal kommünitelerinin fluoresan in situ hibridizasyon (FISH) ve eş zamanlı PZR teknikleri ile tespiti

Özet: Ekstrem halofilik mikroorganizmaların fonksiyonları, etkileşimleri ve çeşitlilikleri üzerine bilinenler çoğunlukla dünyanın farklı yerlerindeki tuzlalarda gerçekleştirilmiş çalışmalardan sağlanmaktadır. Model bir habitat olarak tuzlalar, bu aşırı tuzlu ortamlardaki farklı halofilik komünitelerin kalitatif ve kantitatif tekniklerle kıyaslanmasına izin vermektedir. Çamaltı Tuzlası Ege Denizi kıyısında Türkiye'nin en büyük tuzlasıdır ve ülkede tüketilen tuzun çoğu burada üretilir. Bu çalışmada, Çamaltı Tuzlası'ndaki prokaryotik komünitelerin tespiti iki kültür bağımsız teknikle gerçekleştirilmiştir. Eş zamanlı PZR ve fluoresan in situ hibridizasyon tekniği (FISH) Çamaltı Tuzlası'ndaki mikrobiyal populasyonların analizi için kullanılmıştır. DAPI ile boyanan hücrelerin % 48 ila % 67'si EUB338 probu ile % 33 ila % 57'si ise ARC915 probu ile hibridizasyon göstermiştir. Çevresel DNA ile yapılan eş zamanlı PZR deneylerinin tekrarlanabilirliği yetersiz olarak değerlendirilmiştir. Bununla beraber, FISH analizleri eş zamanlı PZR kombine edilmelidir ve bu iki teknik birlikte hipersalin çevrelerin hızlı ve kantitatif özelliklerinin eldesinde kullanılabilir.

Anahtar sözcükler: Fluoresan in situ hibridizasyon (FISH), eş zamanlı PZR, aşırı tuzlu ortamlar, Çamaltı Tuzlası

Introduction

Extremely halophilic microorganisms require at least 2 M NaCl or an equivalent ionic strength for growth. Most grow in saturated or near-saturated brines. They are the principal inhabitants of hypersaline environments such as salt lakes and salterns (1,2).

Çamaltı Saltern is the biggest coastal solar saltern in Turkey and produces 500,000 t/year of sodium chloride. It is a multipond system that consists of 182 ponds covering 58 km^2. Seawater from the Aegean Sea flows into these shallow ponds. As the wind and sun evaporate the brine, it flows through a sequence of ponds of increasing salinity. In this discontinuous salinity gradient, the salt concentration in each pond is approximately constant over time. When saturation is reached, at approximately 9 times the salt content of the original sea water, crystallization begins. Therefore, multipond solar salterns have a range of salinities, from seawater to saturation, that are model habitats for studying microbial communities in hypersaline environments (1,2).

The microbial diversity of salterns around the world has been examined using culture-independent and culture-dependent techniques. The latter allow for the purification of single colonies and have been used to analyze the diversity of salterns in Eilat, Israel (3); Santa Pola, Spain (4,5); coastal Australia (6); Peru (7); and Tuz Lake in Turkey (8). A key culture-independent technique for microbial ecology studies is the fluorescein in situ hybridization technique (FISH). This method allows for rapid identification and enumeration of bacterial cells in an environmental sample and has been used to quantify microbial groups in marine sediments (9,10), freshwater habitats (11), and extremely acidic environments (12).

The principal concept of the technique is hybridization of the fluorescently labeled oligonucleotide probes with 16S rRNA molecules (13,14). FISH has been used for quantitative analysis of microbes in different saline environments. For *Bacteria* and *Archaea*, the probes EUB312 and ARC915, respectively, were used with considerable success. In addition, probe EHB412 was designed for the specific detection of *Salinibacter*-related organisms (15).

Clone library analysis, FISH, and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) have been used as culture-independent techniques in Israel (16), Tunisia (17), Spain (4), Australia (18), and Turkey (8). Real-time PCR (RT-PCR) and quantitative PCR (Q-PCR) are powerful tools for measuring bacterial populations in environments such as soils (19-22), activated sludge (23), and groundwater and sediment (24). RT-PCR is widely applied in microbial ecology to quantify the abundance and expression of taxonomic and functional gene markers within the environment. Q-PCR-based analyses combine 'traditional' end-point detection PCR with fluorescence detection technologies to record the accumulation of amplicons in 'real time' during each cycle of amplification. The detection of amplicons during the early exponential phase of the PCR enables the quantification of gene (or transcript) numbers when these are proportional to the starting template concentration. SYBR green fluorescent dye, 5' exonucleases, and labeled primers are the most commonly used RT-PCR compounds that report amplicon quantity (22,25). Previously, 16S rDNA was often used as the target gene in detection analyses by quantitative RT-PCR (19,26,27).

The aim of this study was to analyze the community structure of prokaryotic *Archaea* and *Bacteria* populations in Çamaltı Saltern, Turkey, using culture-independent methods. Therefore, the FISH technique was used to obtain qualitative and quantitative results from the microbial communities and RT-PCR was evaluated to obtain rapid and accurate results. This is the first application of the RT-PCR method based on the 16S rDNA primer and SYBR green fluorescent dye for detection of halophilic prokaryotic communities.

Materials and methods

Sample collection

Brine samples were acquired from 10 different ponds of the Çamaltı Saltern in July 2007. The total salt concentration of each sample was determined in situ with a hand refractometer (Eclipse), and the pH was measured at the sampling point (Table 1).

Nucleic acid extraction

For nucleic acid extraction, a combination of the methods of Cifuentes et al. (28) and Nogales et al. (29) was used. Microorganisms were filtered from 50 mL of sample on a 0.22-µm GV filter (Durapore, Millipore). The filter was cut into small pieces with sterile scissors and placed in RNAse- and DNAse-free 2-mL cryotubes containing 600 µL of extraction buffer (100 mM Tris-HCl, 100 mM EDTA, pH 8.0). First, 6 µL of lysozyme (3 mg mL^{-1}) was added and the tubes were incubated at 37 °C for 15 min. Next, 9 µL of proteinase K (150 mg mL^{-1}) and 60 µL of 10% sodium dodecyl sulfate (SDS) were added and the tubes were incubated at 37 °C for 30 min. After the addition of 120 µL of 5 M NaCl and 90 µL of CTAB solution (10% CTAB, 0.7 M NaCl), the tubes were incubated at 65 °C for 10 min, immersed in liquid nitrogen for 2 min, and incubated again for 2 min at 65 °C. The freeze-and-thaw steps were repeated 3 times. After that, 900 µL of a combination of phenol, chloroform, and isoamyl alcohol (25:24:1) (PCI) was mixed in, and the tubes were centrifuged at 16,000 × *g* for 5 min at 4 °C. The aqueous phase was removed and 1 volume of PCI was added. The tubes were vortexed and centrifuged again (2-3 times) until a clear interphase between the aqueous and the organic phases was observed. Finally, the nucleic acids were precipitated with ethanol and resuspended in 50 µL of sterile deionized water. The quality of the nucleic acids was assessed by electrophoresis in 1% agarose gel (LE, FMC Products, Rockland, ME, USA) and visualized under UV light after ethidium bromide staining. Extracts were stored at –70 °C.

Diamidino-2-phenylindole counts and FISH

Sample fixation was carried out by the protocol described previously (4), optimized for extreme halophiles. Procedures for hybridization, diamidino-2-phenylindole (DAPI) staining, and microscopy were applied according to the methods of Snaidr et al. (30). At least 2 filters were analyzed and cells were counted in 30 different microscopic fields. The probes used for in situ hybridization were ARC915 for *Archaea*, EUB338 for *Bacteria*, EHB412 for *Salinibacter* sp., and NON338 for nonspecific hybridization (5,31). Oligonucleotides labeled with the dye CY3 were purchased from Thermo (US). The formamide concentration in the hybridization buffer was 35%. Epifluorescence microscopy (Leica 6600 equipped with a Y3 filter for CY3 or a D filter for DAPI) was used to measure total cell numbers.

Real-time PCR

A standard curve of each cycle threshold (Ct) value against the log of the cell numbers of each test microorganism (*S. ruber* for *Bacteria* and *H. mediterranei* for *Archaea*) was determined by counting colony-forming units (CFUs) mL^{-1}. Templates of genomic DNA were extracted from 1 mL of freshly prepared *S. ruber* and *H. mediterranei* cell suspensions and then serially diluted in 10-fold stages. Aliquots (0.1 mL) of from 10^{-2} to 10^{-8} water-based serial dilutions were plated in triplicate onto 23% modified growth medium. The plates were incubated at 37 °C for 3-5 days and the CFUs were counted.

Total DNA from 10 different samples from 10 different ponds was extracted as indicated above. The DNA samples were then diluted in a series of 10-fold dilutions and used as a template in RT-PCR reactions. RT-PCR was performed using a Rotor-Gene Cycler (Corbett Research) real-time PCR system. PCR mixtures (25 µL) contained 12.5 µL of 1× SYBR green master mix (QIAGEN), 0.9 µM primer, 1 µL of DNA template, and ultrapure water for the balance. Initial denaturing was done for 3 min at 96 °C, followed by 35 cycles of 45 s of denaturing at 96 °C, 50 s of primer annealing at 50 °C, and 1 min of extension at 72 °C. Melting curve analysis and gel electrophoresis (2% agarose) of Q-PCR products revealed single amplicons of the predicted sizes for all primer pairs. The sequences of the forward primers were 21f 5'-TTCCGGTTGATCCTGCCGGA-3' (32) for *Archaea* and 27f 5'-AGAGTTTGATCATGGCTCAG-3' (33) for *Bacteria*. The reverse primer for *Archaea* was 342r 5'-GGTTACCTTGTTACGACTT-3' (33), and it was 517r 5'-ATTACCGCGGCTGCTGG-3' for *Bacteria*. Negative controls contained no template DNA. After completion of the PCR amplification cycles, a melting curve was generated for the resulting amplicon by measuring the loss of fluorescence from 45 to 92 °C.

Results

Salinity, pH, and coordinates of the 10 different samples are given in Table 1. Çamaltı samples fell in the range of 6%-30% salinity and 6.5-7.5 pH, indicating the heterogeneity of the samples.

FISH analysis

DAPI counts of the Çamaltı samples fell in the range of (1.21-3.2) × 10^7 cells mL^{-1}. Different types of cells (flat rods, curved rods, and cocci) were observed in all of the samples. The community was dominated by *Archaea* (Table 1), although *Bacteria* was also present. The visualizations of microorganisms hybridizing with EUB338, ARC915, and EHB412 are shown in Figures 1-3.

The sample with 6% salinity (sample 1) showed the highest *Bacteria* population according to FISH results with probe EUB338. Samples 2 and 3, with salinities of 30% and 28%, respectively, showed the highest *Archaea* population with probe ARC915.

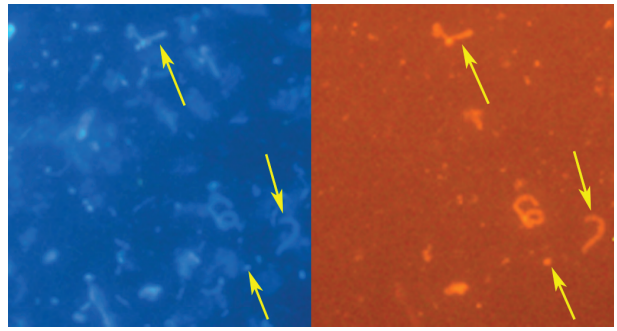

Figure 1. Visualization of microorganisms hybridizing with EUB338.

Real-time PCR (quantification of 16S rRNA genes)

Ct values of cultured cells were obtained within a linear reaction range of the standard curves (Table 2). A standard curve was constructed using different cell concentrations of pure cultures of *Salinibacter ruber* and *Haloferax mediterranei*. Quantification showed a

Table 1. Quantification of *Bacteria* and *Archaea* in samples from Çamaltı Saltern, by FISH and RT-PCR.

Sample	GPS coordinates	NaCl	pH	Total DAPI counts mL^{-1} ± SD[a]	EUB338	ARC915	Ct value for *Archaea*	Ct value for *Bacteria*
1	38′28″47N 26′56″11E	6%	7	1.26 × 10^7 ± 0.43	67%	33%	15	20
2	38′29″25N 26′54″16E	30%	7	1.59 × 10^7 ± 0.09	45%	55%	18	-
3	38′29″56N 26′53″37E	28%	6.9	1.21 × 10^7 ± 0.12	43%	57%	22	27
4	38′30″29N 26′54″16E	24%	7	2.9 × 10^7 ± 0.21	52%	48%	20	20
5	38′30″10N 26′53″52E	28%	6.5	3.2 × 10^7 ± 0.42	48%	52%	17	25
6	38′29″57N 26′53″37E	19%	7.2	2.42 × 10^7 ± 0.32	55%	45%	18	23
7	38′30″21N 26′54″06E	28%	7	1.28 × 10^7 ± 0.62	49%	51%	15	20
8	38′29″24N 26′54″35E	30%	7.2	1.56 × 10^7 ± 0.28	57%	43%	15	25
9	38′28″48N 26′55″45E	22%	7.5	1.71 × 10^7 ± 0.65	53%	47%	-	25
10	38′28″41N 26′55″01E	18%	7.4	1.68 × 10^7 ± 0.45	58%	42%	15	20

[a]: Standard deviation
-: not determined

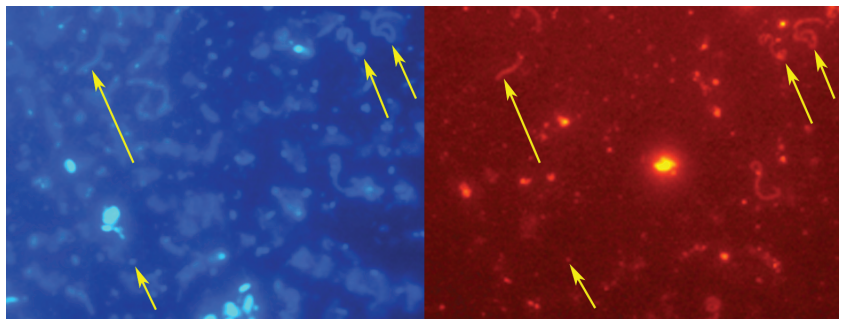

Figure 2. Visualization of microorganisms hybridizing with ARC915.

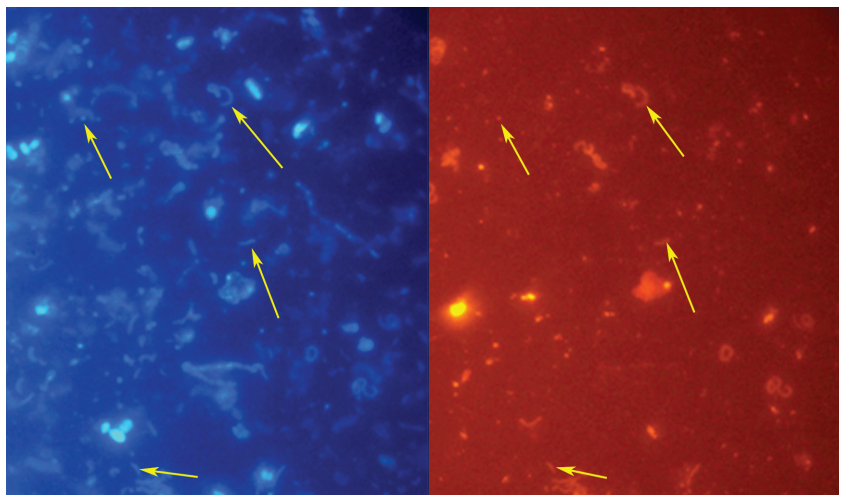

Figure 3. Visualization of microorganisms hybridizing with EHB412.

Table 2. Ct values of cultured cells.

Dilution factor	**Colony forming units (cells mL^{-1})**	**Ct values (*Archaea*)**
10^{-2}	2.22×10^{7}	15
10^{-3}	1.75×10^{6}	18
10^{-4}	1.67×10^{5}	22
10^{-5}	2.26×10^{4}	26
10^{-6}	3.46×10^{3}	30
Dilution factor	**Colony forming units (cells mL^{-1})**	**Ct values (*Bacteria*)**
10^{-2}	1.03×10^{7}	25
10^{-3}	1.65×10^{6}	28
10^{-4}	1.67×10^{5}	32
10^{-5}	1.58×10^{4}	36
10^{-6}	1.76×10^{3}	40

linear relation between cell concentrations and RT-PCR threshold cycles. Conventional PCR was able to detect extracted DNA samples at the minimum concentration according to cell density, which was diluted 1000 times (10^{-3}) (Table 2).

Ct values obtained from total DNA extracts of the pond samples are shown in Table 1. The Ct value was 15 for the samples dominated by *Archaea* populations, especially the samples with saturated NaCl. The Ct value for *Bacteria* was 20-25. There is a direct correlation between the Ct value and dilutions from archaeal and bacterial genomic DNA. According to this correlation, as much as a 10^{-6} dilution of the genomic DNA (*S. ruber* and *H. argentinensis*) could be detected after 30 cycles. Archaeal 16S rRNA genes were dominant in all samples, and bacterial rRNA genes were also detected in all samples. However, the repeatability of the Ct values was not high when compared with the repeatability of the FISH results.

Melting curve results in RT-PCR studies showed that 2 specific peaks, at 91 °C and at 85 °C, belonged to archaeal and bacterial amplicons, respectively (Figure 4).

Discussion

Hypersaline environments can be classified (1,7) as thalassohaline (concentrated seawaters with NaCl as the major salt) or athalassohaline (saline waters that are rich in anions other than chloride and/or cations other than sodium). The Çamaltı Saltern is a thalassohaline hypersaline environment.

Salterns in Eilat, Israel (3); Santa Pola, Spain (4,5); and coastal Australia (6) contained 1.3×10^7, 1×10^7 to 5×10^7, and 1.2×10^7 cells mL^{-1}, respectively, while hypersaline environments in Delta del Ebro (Spain) harbored up to 10^8 cells mL^{-1} (34) and solar salterns in La Palma (Canary Islands, Spain) contained more than 10^8 cells mL^{-1}. Similarly, Çamaltı Saltern contains 10^7 cells mL^{-1}.

Our results from FISH with fluorescently monolabeled (5'-Cy3) oligonucleotide probes indicated that the Çamaltı samples ranged from 48% to 67% of all DAPI-stained cells hybridized with the EUB338 probe, and from 33% to 57% of all DAPI-stained cells hybridized with the ARC915 probe.

A direct correlation was observed between the salinity of the samples and archaeal populations. High salinity samples contained higher numbers of *Archaea*, while low salinity samples (e.g., sample 1) were dominated by bacterial populations.

Early Q-RT-PCR applications were mainly for the detection and quantification of pathogens, such as *Salmonella* spp. (35), *Listeria monocytogenes* (36), and *Vibrio cholerae* (37). More recently, applications of Q-RT-PCR were extended to environmental samples. Takai and Horikoshi (2000) used a Universal PCR primer set and a universal probe that were designed for Eubacteria and *Archaea* (38). In the literature, it was reported that the microbial quantity in an anaerobic sample measured by Q-RT-PCR using a universal probe was 40-fold greater than those measured by conventional culture methods (39).

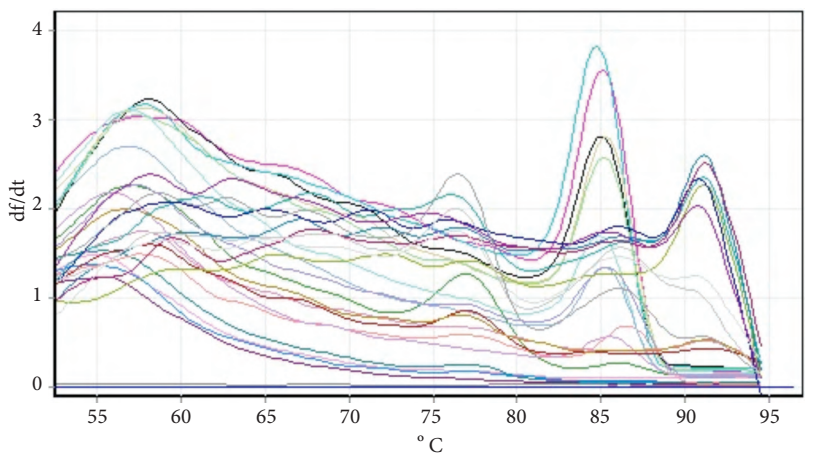

Figure 4. Melting curve results in a real-time PCR study.

Standard curves are often generated using purified DNA isolated from cultured cells and then applied to quantify environmental samples. Standard curves may also be based on the cell number determined from either a 16S rDNA copy number or CFUs (39). We have used direct correlation between CFUs and Ct values.

Most primer pairs may produce nonspecific products with complex environmental samples of high microbial diversity. This may invalidate applications of Q-RT-PCR using SYBR green I as the detection reagent (40). Therefore, we used melting curve analysis to ensure the quality of the amplified product in our study.

Ideally, amplicon length should be 50-150 bases for optimal PCR efficiency in RT-PCR (25). Primers used in this study generated very long amplicons (approximately 300 and 500 bp), and this may have resulted in poor amplification efficiency. Repeatability of the RT-PCR experiments with environmental DNA was considered insufficient with these primer sets. In cases in which longer amplicons are necessary, optimization of the thermal cycling protocol and reaction components may be necessary. Future studies may be carried out by using primer sets that generate shorter amplicons, as suggested.

As previously observed in other coastal salterns (4,5), the prokaryotic community of Çamaltı Saltern was found to be dominated by *Archaea* by using both FISH and RT-PCR methods. In this study, universal primers were used, but in some cases, RT-PCR could not detect the major groups in the sample. The purity of the DNA sample is an especially desirable feature for reliable results. Therefore, quantification of members of microbial communities based on rDNA structures should be further improved by using a combination of universal, bacterial, and archaeal primer and probe sets in future studies. However, attempts to get "clearer" DNA may lead us to underestimate the diversity of the sample being analyzed.

There is no single technique available that can capture the entire diversity of a microbial community. It was reported that using the fluorogenic PCR method combined with other culture-independent molecular techniques should facilitate elucidation of the structures, functions, and interactions of natural microbial communities (38). The rRNA operon copy number of the halophilic organisms could be illusory and runs the risk of resulting in false positive results. Overestimated microbial populations could be obtained after quantitative PCR, which directly reflects the rRNA gene numbers in the samples. Therefore, analyses of hypersaline communities should include an independent technique, such as FISH, which should ideally not be affected by rRNA copy numbers. In this study, FISH was used as a complementary technique.

Consequently, combining FISH and RT-PCR for the microbial population analysis of saline environments such as Çamaltı Saltern could give us more reliable and rapid results. However, further studies including different approaches of Q-RT-PCR that use specific probes and primers may reveal the population structures of halophilic prokaryotes not only at a domain level, but also at a genus as well as species level.

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