Eucaryotic Diversity in a Hypersaline Microbial Mat[▽][†]

Leah M. Feazel,[1][‡] John R. Spear,[2][‡] Alicia B. Berger,[3][‡] J. Kirk Harris,[1] Daniel N. Frank,[1] Ruth E. Ley,[4] and Norman R. Pace[1]*

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347[1]; Division of Environmental Science and Engineering, Colorado School of Mines, Golden, Colorado 80401[2]; Cancer Biology Program and Pathology Department, School of Medicine, Stanford University, Palo Alto, California 94305[3]; and School of Medicine, Washington University, St. Louis, Missouri 63108[4]

To determine the eucaryotic diversity of the hypersaline Guerrero Negro microbial mat, we amplified 18S rRNA genes from DNA extracted from this mat and constructed and analyzed clone libraries. The extent of eucaryotic diversity detected was remarkably low, only 15 species among 890 clones analyzed. Six eucaryotic kingdoms were represented, as well as a novel cluster of sequences. Nematode sequences dominated the clone libraries.

Biologically rich, laminated, photosynthetic microbial mats flourish on the floors of evaporating ponds at the Exportadora de Sal saltworks in Guerrero Negro, Mexico. This industrial operation pumps seawater out of the Laguna Ojo de Liebre, on the west coast of Baja California; the water is then gravity fed through ~185 km^2 of evaporative basins of increasing salinity. Microbial mats, 6 to 8 cm thick with a tofu-like consistency, have developed on the bottom of these ~1-m-deep ponds. The mats at Guerrero Negro have received considerable attention with regard to biogeochemistry and microbiology (1, 10, 13, 17). Photosynthetic microbiota dominate the top few millimeters of the mat complex and produce oxygen during the day (10). The mat is permanently severely anoxic in the deeper layers, and throughout the mat at night, with high sulfide levels from metabolic sulfate reduction (15). Recent, small subunit rRNA gene sequence surveys have identified in the mat a spectacular diversity of bacterial species (10). Although a few eucaryotic rRNA gene sequences were detected, they were rare, amounting to ~0.5% of the total rRNA genes detected using universal PCR primers.

Most eucaryotic phylogenetic diversity is microbial in nature. Yet, the environmental diversity and distribution of microbial eucaryotes remain poorly understood and difficult to observe with the classical methods of microscopy and culture. Recent culture-independent molecular studies have expanded the known extent of eucaryotic phylogenetic diversity, including unique microbes from environments considered "extreme," such as anoxic deep sea trenches (19), the McMurdo Dry Valleys of Antarctica (7), the endolithic environments of Yellowstone (20), anoxic sediments (2, 6), and others (11, 16). In considering that the chemical and bacterial complexity of the Guerrero Negro mat might also indicate high eucaryotic diver-

sity, we undertook to determine the eucaryotic complexity of mat samples. Analyses were conducted through sequence-based identification of microbial eucaryotes based on PCR amplification and sequence analysis of small subunit rRNA genes.

In brief (detailed methods can be found in the supplemental material online and as described previously in references 10 and 20), DNA was purified from 10 vertical sections of ~6-cm cores (the top 6 mm was in millimeter-sized sections; below that were four sections of 1 cm each) from the well-studied site Pond Four (near Pond Five) at the saltworks site and used as the template for eucaryote-specific PCR amplification of the small-subunit rRNA gene. PCR products were cloned, and >2,000 randomly selected clones were screened by restriction fragment length polymorphisms (RFLP). Approximately 300 representative RFLP types were sequenced, and the results were compared to database sequences. About 50% of the sequences were representative of eucaryotes (many archaea were detected). Overall, this survey of eucaryotic diversity in the Guerrero Negro mat is based on 890 RFLP-type clones. This is a relatively limited sampling, but it is sufficient, from a population statistical point of view, to gain some insight into the local eucaryotic diversity in the context of high bacterial diversity (10).

Phylogenetic analyses of the sequences were conducted to identify the resident eucaryotes. The overall census is summarized in Fig. 1, and the distribution of sequences is given in Table 1. In light of the high bacterial diversity, the eucaryotic diversity detected was surprisingly low, only ~15 species-level groups representing six eucaryotic kingdom-level phyla were identified. Diversity estimates were utilized and they predicted 16 eucaryotic "species" in this sample (see methods described in the supplemental material). All sequences, with the exception of one cluster, were closely related to those of organisms previously described or to environmental sequences previously encountered. The novel sequences that had no kingdom-level affiliation with known sequences we termed Guerrero Negro Eucaryal Group 1 (GNEG1). GNEG1 sequences are all closely related, ~99% identity in rRNA sequence, and possibly allelic. This indicates that the same or highly similar organisms occur

* Corresponding author. Mailing address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 90309-0347. Phone: (303) 735-1864. Fax: (303) 492-7744. E-mail: nrpace@colorado.edu.
‡ These authors contributed equally to this work.
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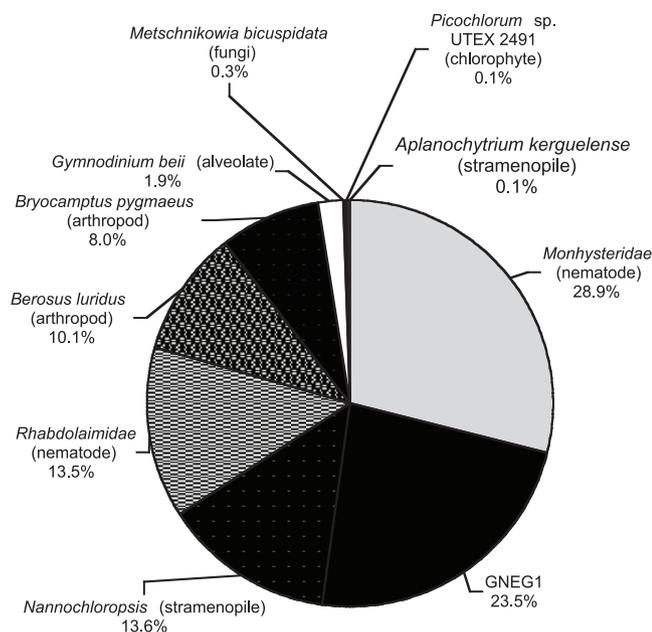▽ Published ahead of print on 9 November 2007.

FIG. 1. Guerrero Negro eucaryotic diversity. This pie chart shows the relative diversity of each of the eucaryotic species detected in the mat, based on the 890 clones screened.

throughout the mat. Phylogenetic analyses of these sequences indicate a deeply branching eucaryotic lineage that is not "fast clock" and has no specific affiliation with any other clusters within the eucaryotic domain. Attempts to visualize the corresponding organism by fluorescence in situ hybridization were unsuccessful due to the overwhelming autofluorescence observed in these mats.

The most abundant eucaryal sequences detected, approxi-

mately half, were those of nematodes. In order to resolve the relationships of the sequences, we conducted an all-nematode phylogenetic analysis, which was largely congruent with that of Meldal and colleagues (11; see Fig. S1 in the supplemental material). Figure 2 shows the local grouping of the *Monhysteridae* and *Rhabdolaimidae* families, with which the Guerrero Negro sequences are affiliated. The limited variation in both groups of sequences (Table 1) is potentially allelic and in any case indicates that only two kinds of nematodes dominate the eucaryotic diversity of the mat at the site sampled.

The unusual properties of these nematodes, including their anaerobic lifestyle, their resistance to sulfide poisoning, and their persistence in the hypersaline state, encouraged efforts to cultivate them. A representative of the *Monhysteridae* family, a *Diplolaimelloides* sp. (Fig. 2), was cultured on 1.5% agar containing 1% NaCl and spread lightly with an autoclaved emulsion of the mat (see materials and methods described in the supplemental material). Microscopic examination (see Fig. S2 in the supplemental material) showed a pharynx typical of bacterivorous, free-living nematodes, sexual dimorphism of the tails, and anterodorsal ocelli that are red pigmented with a small white spot at the anterior tip (3, 4, 9).

Other conspicuous sequences in the mat include those of an insect and a crustacean, both of which are closely related to the rRNA sequences of named organisms (Table 1). The sequences are found mainly in the upper portion of the mat and presumably represent grazers of the mat. The only conspicuous photosynthetic organism detected by sequence (99% identity), the stramenopile *Nannochloropsis gaditana*, was located mainly at mid-depth in the mat. Presuming that the organism represented by the sequence conducts oxygenic photosynthesis, this may indicate that oxygen production occurs more deeply in the mat than generally considered.

This survey of the mat eucaryotes clearly is not comprehen-

TABLE 1. Overall eucaryotic diversity[a]

Affiliation	No. of clones identified (total $n = 890$)[b]	No. of clones identified per layer			% Identity to the nearest organism by BLAST comparison	Intragroup sequence divergence
		Top (1–2 mm) ($n = 335$)	Middle (3–6 mm) ($n = 250$)	Bottom (0.7–3.7 cm) ($n = 185$)		
Monhysteridae (nematode)	257	142	60	55	95% to *Diplolaimelloides meyli*	0.07[d]
GNEG1	209	52	58	99	Unaffiliated[c]	0.02
Stramenopila spp.	121	1	101	19	99% to *Nannochloropsis gaditana*	0.06[e]
Rhabdolaimidae (nematode)	120	34	76	10	96% to *Rhabdolaimus* cf. *terrestris* 1Z	0.05[f]
Insecta (arthropod)	90	87	3	0	98% to *Berosus luridus*	0.04
Crustacea (arthropod)	71	52	19	0	97% to *Bryocamptus pygmaeus*	0.01
Alveolata	17	0	5	17	98% to *Gymnodinium beii*	0.03
Ascomycota (fungi)	3	1	2	0	99% to *Metschnikowia bicuspidata*	N/A[g]
Chlorophyta	1	0	1	0	97% to *Picochlorum* sp. strain UTEX 2491	N/A
Stramenopila	1	0	1	0	95% to *Aplanochytrium kerguelense*	N/A

[a] The frequency of each eucaryotic species is shown both overall and in three chemically distinct layers of the mat. Also shown are the nearest relatives, found by BLAST, and the intragroup sequence divergence.
[b] *n*, number of clones analyzed.
[c] Unaffiliated, ≤87% sequence identity to any previously sequenced rRNA gene.
[d] Based on 16 clones with sequences from nucleotide positions 3253 to 7823.
[e] Based on 22 clones with sequences from nucleotide positions 3259 to 7817.
[f] Based on 10 clones with sequences from nucleotide positions 3253 to 7823.
[g] N/A, not applicable. Only one clone was sequenced, so no divergence can be calculated.
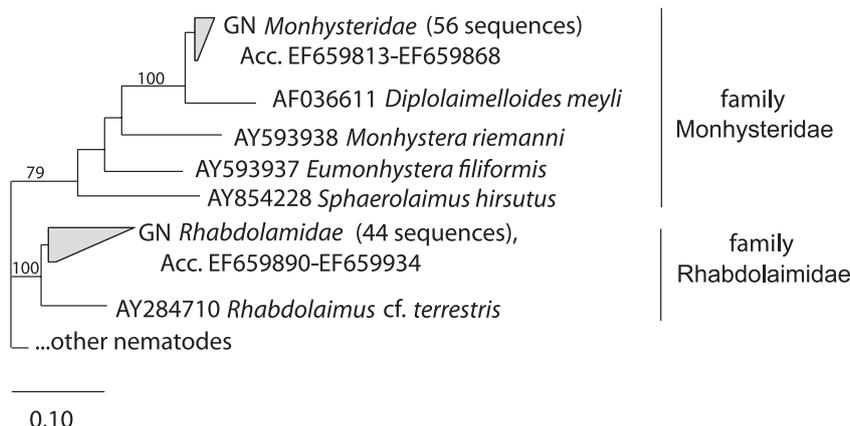
FIG. 2. Nematode phylogeny. This phylogenetic tree shows the phylogenetic relationships between the nematodes sequenced in this study and previously studied nematode species. Scanning electron microscopy images of a nematode from the mat, a fully expanded nematode phylogeny, and more images are available in the supplemental material online.

sive; most of the sequences derive from a limited diversity of organisms, and sequences representative of some groups were detected only once (Table 1). Nonetheless, and with acknowledgment of the caveats of molecular technology (e.g., PCR bias, variability in rRNA gene dosage [14], etc.), based on our study of multiple environments (6, 10, 17, 20), we believe the frequency with which rRNA genes are detected probably is a rough measure of the corresponding biomass.

The eucaryotic diversity of the Guerrero Negro mat is surprisingly sparse considering the vast bacterial diversity in the same setting. What might account for this disparity in species richness of bacteria and eucaryotes? The answer may lie in the differences in intrinsic metabolic capabilities of bacteria and eucaryotes. Bacteria collectively exhibit broad metabolic capabilities and can occupy the many chemical niches that are generated during the physiological function of the mat community. In contrast, the metabolic versatility of eucaryotes seems more limited, basically fermentative in the absence of bacterial symbionts, though capable of survival under high sulfide, fermentative, anaerobic conditions. The dominant organisms in the mat, the nematodes, evidently are bacterivorous. The *Monhysteridae* nematodes are commonly found in marine sediments and are prevalent at the oxic-anoxic boundary (18). Although such organisms comprise only a small fraction of the total Guerrero Negro mat biomass, they are conspicuous and possibly play an important role in the community, for instance by imparting churning action and microbial/chemical transport within the mat (8). The diverse organisms detected provide good subjects for study of the ways in which eucaryotes adapt to environmental stresses such as extreme anoxia and drastic diel sulfide fluctuations.

Nucleotide sequence accession numbers. Eucaryotic sequences were submitted to GenBank with accession numbers EF659781 to EF659934.

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