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Cleber C. Ouverney
*University of Southern California*, cleber.ouverney@sjsu.edu

Jed A. Fuhrman
*University of Southern California*

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Marine Planktonic Archaea Take Up Amino Acids

CLEBER C. OUVERNEY* AND JED A. FUHRMAN

Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-0571

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Archaea are traditionally thought of as “extremophiles,” but recent studies have shown that marine planktonic Archaea make up a surprisingly large percentage of ocean midwater microbial communities, up to 60% of the total prokaryotes. However, the basic physiology and contribution of Archaea to community microbial activity remain unknown. We have studied Archaea from 200-m depths of the northwest Mediterranean Sea and the Pacific Ocean near California, measuring the archaeal activity under simulated natural conditions (8 to 17°C, dark and anaerobic) by means of a method called substrate tracking autoradiography fluorescence in situ hybridization (STARFISH) that simultaneously detects specific cell types by 16S rRNA probe binding and activity by microautoradiography. In the 200-m-deep Mediterranean and Pacific samples, cells binding the archaean probes made up about 43 and 14% of the total countable cells, respectively. Our results showed that the Archaea are active in the uptake of dissolved amino acids from natural concentrations (nanomolar) with about 60% of the individuals in the archaeal communities showing measurable uptake. Bacteria showed a similar proportion of active cells. We concluded that a portion of these Archaea is heterotrophic and also appears to coexist successfully with Bacteria in the same water.

Archaea are normally regarded as organisms that thrive in high-temperature, high-salt, or extreme anaerobic environments, and they have generally been thought to be unable to compete against other microorganisms for limiting resources under “nonextreme” conditions (24, 30). However, ribosomal RNA genes and rRNA found in plankton of temperate (5 to 18°C) (7, 13, 14, 19, 28) and polar (−1.5°C) (22) aerobic seawater, usually from depths below the euphotic zone (>100 m deep), have revealed the widespread presence of Archaea, mostly close relatives of the Crenarchaeota, outside environments frequently associated with extreme conditions. Direct counts through fluorescent in situ hybridization (FISH) have provided the first look at these unique microorganisms, showing that they can constitute up to 60% of the cells in the planktonic habitat (15) but typically more like a few percent in the surface mixed layer and up to 30% at depths of a few hundred meters or more (8, 15, 22). Curiously, Crenarchaeota is a group originally thought to be made up only of thermophilic Archaea (7, 14, 30), and to date, no marine Crenarchaeota have been isolated in pure culture, and almost nothing is known about their activity and metabolic properties in situ (16, 24), including whether they are heterotrophs or autotrophs (e.g., ammonium or nitrite oxidizers). Their apparent widespread occurrence and persistence in the plankton alongside bacteria in deep water remains a mystery.

We can postulate three possible explanations for this persistence: (i) the Archaea grow as plankton on dissolved substances (such as dissolved organic matter or inorganic nutrients), similar to the way most planktonic bacteria are thought to grow (2, 10), (ii) the Archaea grow in some specialized microzones within the planktonic habitat, such as sinking particles or anaerobic zones in animal guts, and are released into the plankton where they grow slowly or not at all, or (iii) the Archaea originated at some remote source and were dispersed into the plankton but are dormant (17, 33), neither growing nor being removed at appreciable rates, but with sufficient cellular rRNA content to be detected through various molecular methods.

To choose among these possibilities and generally examine archaeal activity, we used a triple labeling technique, which we have termed substrate tracking autoradiography fluorescence in situ hybridization (STARFISH) (26), that allows us to characterize the picoplankton community diversity by phylogenetic groups and simultaneously determine metabolic activity of each group in situ (25, 26). The technique applies a general fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) for total cell counts, fluorescently labeled oligonucleotide probes for whole-cell in situ hybridization of specific target groups, and a tritiated nutrient for detection of cell function by autoradiography. For this study, we chose dissolved amino acids as substrates, for two reasons: (i) they are ubiquitous and known to be an important source of C, N, and energy for marine microheterotrophs (11, 31), and (ii) their availability as a mixture of 15 highly specific activity-tritiated compounds permits sensitive measurement of single-cell uptake autoradiographically, even by small, slowly growing cells (26).

Here, we present evidence, based on the STARFISH method, suggesting that free-living planktonic marine Archaea from 200-m depths in the Mediterranean and off the California coast are actively involved in the heterotrophic uptake of dissolved amino acids from aerobic seawater, with activities apparently comparable to those of the Bacteria (based on the extent of clustering of silver grains around cells in microautoradiography). Hence, Archaea appear to be successfully vying with other heterotrophic microorganisms for dissolved amino acids.

MATERIALS AND METHODS

Sample preparation. Two samples were collected outside the Villefranche-sur-Mer Bay, an oligotrophic environment in the French Mediterranean Sea (43°40.79′N, 7°17.88′W) on 21 July 1998, one sample at the surface (22.4°C) and one sample at 200 m (17.0°C) deep (Table 1), where we had previously detected nearly 60% of the total cells as Archaea (15). Similarly, two samples were collected in Monterey Bay, Northern California, at 36°45.49′N and 122°01.04′W on 14 July 1999, one sample at the surface (14.0°C) and one sample at 200 m deep (8.7°C) (Table 1). The chlorophyll maximum was detected at a 28-m depth in the Villefranche Bay and at 20 m deep in Monterey Bay. All samples were
used unfiltered and divided into two live 100-ml subsamples in sterile culture flasks wrapped in aluminum foil and incubated with a mixture of 15 tritiated amino acids (premixed by Amersham, catalog no. TRK440, with an average specific activity of about 50 Ci/mmol) at trace levels of 5 nM at in situ temperatures. Surface samples from both sites were incubated for 13 h, while the 200-m-deep samples from Villefranche were incubated for 19 h, and the samples from Monterey Bay were incubated for 13 h. Controls consisted of two 100-ml subsamples from each depth killed in 10% formalin for 1 h prior to addition of 3H-labeled amino acids at 5 nM concentrations. Nutrient uptake was monitored over time for all of the live and killed subsamples by withdrawing 2-ml aliquots with replicates from each subsample, filtering each aliquot onto a 0.2-μm-pore-size Nucleopore polycarbonate filter, rinsing the filter four times with about 2 ml of 1× phosphate-buffered saline (PBS) and measuring the radioactivity with a scintillation counter by using EcosintA scintillation cocktail. In addition, total counts (cells per milliliter) were determined with DAPI, and the radioactivity level in units of disintegrations per minute (DPM) was normalized to the cell concentration (DPM/cell = Table 1). Once cells reached saturation (no further increase with time) levels, 3H-labeled-amino-acid uptake was halted by addition of a petri dish (to prevent the filters from drying out), cut into eight equal pieces with a new, clean razor blade, and carried to the darkroom.

In the darkroom, cells were transferred from the filters onto Teflon slides by placing each one-eighth piece of the Nucleopore filter only (peeled off the Millipore filter) cell face down over a single emulsion-coated well, again with the aid of the night vision scope and a safe light positioned 2 m away.

Emulsion exposure and development. The slides were left to dry in total darkness for 30 min before they were placed in a light-proof box and stored at 4°C for 3 days for emulsion exposure. The emulsion was developed to Kodak specifications as follows: 2 min in Dektol developer, a 10-s stop in deionized water, and 5 min in fixer. The slides were washed in deionized water for 2 min, the pieces of the Nucleopore filters were peeled off, and slides were air dried and prepared for whole-cell hybridization. At this point, the slides could be stored at −80°C for several weeks without noticeable changes in results.

In situ hybridization. In situ hybridization took place in 10 μl of hybridization solution per well, containing 5× SET (1× SET is 150 mM NaCl–20 mM Tris–Cl [pH 7.8])–1 mM EDTA), 0.2% bovine serum albumin (acyetylated; Sigma), 10% deer transferrin (molecular weight, 500,000; Pharmacia), 0.01% polyadenyllic acid (Sigma), and 0.1% sodium dodecyl sulfate (SDS) as described by DeLong et al. (9) and Braun-Howland et al. (3), and 50 ng of a 5-ng/μl concentration of Cy3-labeled oligonucleotide probe (Table 2). The slides were incubated at 43°C for 3 h, briefly washed with ~5 ml of 0.2× SET at 43°C, and finally immersed three times in 0.2× SET at 43°C for 10 min each time. After air drying, the slides were mounted in glycerol–10× SET (50:50 [vol/vol]) solution under a 24×-mm cover glass and kept at −20°C for at least 1 h before they were observed by fluorescence and transmitted light microscopy.

STARFISH cell count. For each microscopic field observed, the following four types of counts were recorded: (i) total DAPI cell counts with UV excitation (Olympus type UM-MUW UV filter; excitation wavelength, 330 to 385 nm; emission wavelength, >420 nm), (ii) probe fluorescence cell counts, which included autofluorescent and probe-labeled cell counts with a green-light excitation filter (Chroma Cy3, #U-M4007), (iii) microautoradiography counts (cells with associated silver grains) under transmitted light, and (iv) counts for cells labeled with a fluorescent probe and simultaneously labeled autoradiographically (overlap between green excitation and transmitted light). Based on these four counts, we quantified the abundance of cells labeled with the fluorescent probes as a percentage of the total DAPI-stained cells (the number of labeled cells with the probe divided by the number of DAPI-stained cells [count iii divided by i above]). Nearly 600 of the DAPI-stained cells were included in the computation of the percentages. Likewise, the abundance of all cells labeled autoradiographically with the tritiated amino acids (count iii divided by i above) and that of cells

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**TABLE 1. Oligonucleotide probe sequences, Excherichia coli positions, length, and references**

<table>
<thead>
<tr>
<th>Name/target</th>
<th>Sequence (5′-3′)^*</th>
<th>Position/length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control^a</td>
<td>CTAGTGTAGCCCGCTGCAG</td>
<td>N/A±18</td>
<td>26</td>
</tr>
<tr>
<td>Bacteria</td>
<td>ACCGTGTGTGCGGGCC</td>
<td>927±17</td>
<td>3</td>
</tr>
<tr>
<td>Archaea</td>
<td>GCCGTTCGTGGCTGCCCTGGG</td>
<td>338±24</td>
<td>15</td>
</tr>
<tr>
<td>Marine GI Crenarchaeota-1</td>
<td>CTGTTGACCACTTTAGGTT</td>
<td>541±18</td>
<td>15</td>
</tr>
<tr>
<td>Marine GI Crenarchaeota-2</td>
<td>TTAGGGCCATAATCTMTCTCT</td>
<td>554±20</td>
<td>19</td>
</tr>
<tr>
<td>Marine GI Euryarchaeota</td>
<td>TTAGGGCCATAAAAKCGAC</td>
<td>554±20</td>
<td>19</td>
</tr>
</tbody>
</table>

^a Not expected to bind to any organism.  
^b K = G or T, M = A or C, S = C or G.  
^c N/A, not applicable.
TABLE 3. Percentage of total DAPI cells that are either autofluorescent (NP = no probe), labeled with fluorescent probes (CON = control probe; ARC = Archaea probe mix; BAC = Bacteria probe), labeled by microautoradiography (MAR) with 3H-labeled amino acids, or simultaneously labeled with both (probe and 3H-labeled amino acids) for surface and 200-m-deep Mediterranean Sea samplesa

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>NP</th>
<th>MAR</th>
<th>NP and MAR</th>
<th>CON</th>
<th>MAR</th>
<th>CON and MAR</th>
<th>ARC</th>
<th>MAR</th>
<th>ARC and MAR</th>
<th>BAC</th>
<th>MAR</th>
<th>BAC and MAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.0 ± 1.2</td>
<td>30.5 ± 3.7</td>
<td>0.7 ± 0.4</td>
<td>5.0 ± 0.7</td>
<td>29.0 ± 1.7</td>
<td>0.7 ± 0.3</td>
<td>4.2 ± 1.1</td>
<td>29.3 ± 1.8</td>
<td>1.0 ± 0.6</td>
<td>56.2 ± 1.9</td>
<td>32.2 ± 1.8</td>
<td>27.3 ± 1.9</td>
</tr>
<tr>
<td>200</td>
<td>2.3 ± 0.7</td>
<td>33.3 ± 1.7</td>
<td>0.0 ± 0.0</td>
<td>0.83 ± 0.4</td>
<td>31.0 ± 3.8</td>
<td>0.0 ± 0.0</td>
<td>42.8 ± 5.5</td>
<td>39.5 ± 2.6</td>
<td>24.2 ± 4.0</td>
<td>46.3 ± 2.1</td>
<td>36.0 ± 2.5</td>
<td>30.8 ± 1.9</td>
</tr>
</tbody>
</table>

a Each set of three columns is from a pair of duplicates from each depth.

labeled both with the probe and the tritiated amino acids (count iv divided by i above) are presented.

Counts were acquired on an Olympus BMax epifluorescence microscope equipped with a UPlanApo objective lens (100×), a type HBO 100-Hg vapor lamp, and the filters previously described. The images were captured and intensified by using a microchannel plate image intensifier (COHU Intensified CCD camera model 5515-2001/0000), and the background was reduced by image averaging with a model DSP-2000 image processor (Dage-MTI, Inc., Michigan City, Ind.). The images were visualized with a Sony Trinitron color video monitor (model PVM-1353MD). This video system could display cells in real time with fluorescence considerably less than the fluorescence directly detectable by eye. Photomicrographs and microautoradiographs were captured with a Scion LG3 capture card and NIH Image software version 1.61.

RESULTS

Levels of prokaryotic cell-specific activity (DPM per cell) at the end of the incubation with the 3H-labeled-amino-acid mixture in the Villefranche Bay and the Monterey Bay samples were within the same order of magnitude (Table 1). Killed controls were ±1% as radioactive as their live sample counterparts. Live surface prokaryotic-cell-specific activity levels between the two sites were <10% apart, but the Villefranche Bay 200-m-deep prokaryotic cells had a specific activity 2.4-fold greater than 200-m-deep prokaryotes in Monterey Bay (Table 1). Autoradiography for both samples confirmed that cells in the killed control did not take up the 3H-labeled amino acids at detectable levels (Table 1).

For the in situ hybridization counts, the negative-control counts were not subtracted from the probe counts unless specified. In all samples, the background level determined by the “no-probe” and the “control probe” counts consisted mostly of autofluorescent cells. These autofluorescent cells constituted <5% of the total counts on average, and ≤10% of those cells were detected taking up dissolved amino acids.

Villefranche-sur-Mer. In the surface water, Archaea-labeled cell counts were low and statistically indistinguishable from the control probe counts (Table 3). Cells in the “no probe” counts were primarily autofluorescent cells such as cyanobacteria, whose pigment fluorescence is normally indistinguishable from the Cy3-probe fluorescence, and these did not generally take up the 3H-labeled amino acids (Table 3).

In the 200-m-deep water, however, cells labeled with the archaeal probe comprised about 43% (7.83 × 104 cells/ml) of the prokaryotic community (Fig. 1 and Table 3). These presumed Archaea were mostly curved rods (0.6 to 1.8 μm in length) or spheres (~0.6 μm in diameter), and 57% of all such Archaea were also detected taking up 3H-labeled amino acids (Fig. 1 and Table 3). Control counts for both “no probe” and “control probe” were less than 3% of the total DAPI-detected cells at this deep-water site (Table 3).

In addition to the archaeal probes, a Cy3-labeled probe targeting the Bacteria domain (5) was used in the same experiment. In the surface sample, 56% (2.09 × 104 cells/ml) of the DAPI cells were labeled with the bacterial probe, and half of those Bacteria-probe-labeled cells were simultaneously detected in the autoradiography taking up 3H-labeled amino acids (Table 3). At 200 m deep, almost half of the cells were labeled with the bacterial probe and two-thirds of those cells were also detected taking up 3H-labeled amino acids (Table 3). When we add the Archaea probe cell count to the Bacteria probe cell count, the total in surface waters is 56% (all bacteria), and at 200 m, the total is statistically indistinguishable from 100% of the DAPI-countable cells. The reason for the lower percentage of the total in the bacterium-dominated surface is unknown but may reflect an underestimate from the...
single bacterial probe compared to the multiple archaeal probes.

Monterey Bay. The overall average percentage of autoradiography-positive counts in the Monterey Bay samples was nearly twice as high as that of the Villefranche Bay samples, with 60% of the total cells at the surface and 62% at 200 m deep being detected autoradiographically (Table 4). Archaea-probe counts (5%) were indistinguishable from the control counts at the surface. In the 200-m-deep sample from the Monterey Bay, the percentage of probe-labeled cells for all probes used was lower than those respective counts at the Monterey surface sample. Bacteria probe counts made up 44% (1.36 × 10⁸ cells/ml) of the cells while Archaea counts increased to 14% (4.34 × 10⁴ cells/ml) compared to the surface. About 64% of the Bacteria probe-labeled cells and 60% of the Archaea probe-labeled cells (number of probe-labeled cells and cells simultaneously recorded in autoradiography divided by the number of probe-labeled cells alone) were detected in autoradiography at this 200-m-deep site (Table 4).

**DISCUSSION**

This investigation used FISH in combination with autoradiography to assess both the abundance and activity of Archaea in a mixed natural marine prokaryotic community.

The results confirmed that Archaea can constitute a large fraction of the picoplankton. In addition, these results showing archaean abundance greater at the subsurface depth and declaring to nearly 40% of the total prokaryotic community at 200 m in the Mediterranean are similar to FISH results reported by Fuhrman and Ouverney (15). The lower percentages at 200 m deep off the California coast are in the range of Fuhrman and Ouverney’s (15) counts from San Pedro Channel as well as a report from DeLong et al. (8), who found approximately 20% of Archaea of the total cell count at 100 m deep, offshore from Moss Landing, Calif., using polyribonucleotide probes with whole-cell in situ hybridization. Similar trends in archaean abundance, but using dot blot hybridization methods, have been reported in California (19) and Antarctica (20, 21, 23). We did not find a significant number of archaeal cells near the surface. Previously, Massana et al. (19) and DeLong et al. (8) detected a relatively small amount of Archaea, mostly members of group II, near the surface off the coast of California by dot blot hybridization.

Results from microautoradiography applied in combination with FISH showed Archaea taking up dissolved organic nutrients at nanomolar concentrations under oxygenic and apparent nonextreme conditions. Measurement of in situ nutrient uptake by heterotrophic bacteria via autoradiography was first suggested in the mid 1960s by Brock and Brock (4) to better understand the nutritional requirements of microorganisms as well as estimate prokaryotic cell growth rate and production. Brock and Brock (4) also noticed that autoradiography alone was limited in assigning function to specific prokaryotic groups due to the lack of distinct morphological features among these microorganisms. STARFISH seems to help break the bacterioplankton “black box” not only in function (through autoradiography) but also in type (through FISH). Similar protocols have been proposed and applied in activated-sludge microbial communities (18) and other marine Bacteria (6).

Members of both the Archaea and the Bacteria domains were shown to coexist at the 200-m depth of the Mediterranean and California sites and apparently successfully compete for the same dissolved amino acid pool. Although this kind of microautoradiography is not yet an accurate way to measure the amount of uptake, there was not a qualitative difference between the labeling of the Archaea and Bacteria. These findings contradict the former conventional belief that Archaea are restricted to extreme environments due to lack of competitiveness against other microorganisms (24, 30). They also show that a significant portion of the archaean community is using dissolved organic matter. Thus, these organisms are living at least partly heterotrophically, and this gives us a useful physiological clue as to their role in the ecosystem.

The percentage of total cells detected taking up tritiated amino acids varied among the Mediterranean and the California sites and with water depth within each site. The results show a higher proportion of autoradiographically active cells near the surface of Monterey Bay (60% of total cells, 1.28 × 10⁹ cells/ml) than near the surface of the Villefranche Bay (30% of total cells, 1.1 × 10⁹ cells/ml). Although the causes of such variations are not currently known, a higher percent of active cells may be attributed in part to higher productivity levels normally associated with zones of upwelling, such as the Monterey Bay, compared to oligotrophic waters, such as the Mediterranean Sea.

In comparison to previous results from similar environments, we are not aware of previous autoradiography experiments in Monterey, although near-shore samples from Southern California showed a wide range of percentages active, generally 35 to 85% (12, 17). We are unaware of previous published autoradiography results for samples from the Mediterranean for comparison, although our own unpublished experiments with samples from within Villefranche Bay (July 1996, about 500 m closer to shore and considerably shallower than the samples from this study) showed that 73 to 86% of the cells were active autoradiographically with 5 mM added amino acids (method described by Tabor and Neihof [32]). We do not know enough about these systems at this point to explain these differences, and they may have methodological as well as ecological causes.

The limits of sensitivity of STARFISH have not yet been determined, but they are compounded by the previously described limitations of both FISH (27) and autoradiography (29). The portion of probe-labeled archaean cells that were not detected in the autoradiography, for example, probably represents a group of cells growing at a rate below our detection level and possibly not growing at all (26).
Previous work looking at the activity of whole-cell prokaryotic organisms by culture-independent methods required that an organism be recognized by its unique morphology through direct microscopic observations. Examples of such organisms include the aquatic filamentous Leucothrix mucor (4), as well as Microthrix parvicella and Nostocoida limicola found in activated sludge (1). However, measuring nutrient uptake by organisms with unique morphology in a mixed community is possible only once the classification system is determined, which normally requires pure cultures, and “morphospecies” may contain large genetic variations. Since the majority of microorganisms fall within a few morphotypes, alternative methods such as STAR-FISH (6, 18, 26) currently provide the only techniques for collecting information on in situ activity by specific microorganisms with nondistinct morphologies, which includes most of the bacterioplankton of natural systems.

Overall, the results reported here shed new light on microbial activity in ocean mid-depth waters. They suggest that Archaea, given their high abundance and activity, probably play a significant role in the organic and possibly inorganic biogeochemical cycles in the world’s oceans, especially at greater depths.

ACKNOWLEDGMENTS

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