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Citation

Published Version
doi:10.1073/pnas.92.16.7232

Accessed
July 8, 2017 2:50:33 PM EDT

Citable Link
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Domination of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site

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Communicated by Andrew H. Knoll, Harvard University, Cambridge, MA, May 1, 1995

ABSTRACT Microbial community structure in natural environments has remained largely unexplored yet is generally considered to be complex. It is shown here that in a Mid-Atlantic Ridge hydrothermal vent habitat, where food webs depend on prokaryotic primary production, the surface microbial community consists largely of only one bacterial phylogenetic type (phylotype) as indicated by the dominance of a single 16S rRNA sequence. The main part of its population occurs as an ectosymbiont on the dominant animals, the shrimp *Rimicaris exocolutata*, where it grows as a monocolonucleus within the carapace and on the extremities. However, the same bacteria are also the major microbial component of the free-living substrate community. Phylogenetically, this type forms a distinct branch within the δ-Proteobacteria. This is different from all previously studied chemosymbiotic endo- and ectosymbioses from hydrothermal vents and other sulfide habitats in which all the bacterial members cluster within the γ-Proteobacteria.

Hydrothermal vents in the deep sea are among the most productive ecosystems on earth. They are known for their unusual animal communities and extremely high biomass depending on carbon fixed via chemosynthesis rather than photosynthesis (1). At Pacific vents, this high productivity is concluded to be largely based on the activity of a number of different endosymbiotic bacteria of tubeworms and bivalves (2). In contrast, nothing is known about the identity of the major producers at Mid-Atlantic Ridge (MAR) vents. Some of these habitats are strikingly different from the Pacific vents in that the dominant animals are the highly mobile shrimp *Rimicaris exocolutata*, which carry dense populations of epibiotic bacteria (3).

These shrimp cluster on solid sulfide surfaces around warm vent water emissions, where they usually form a layer several specimens thick with an estimated density of about 25,000–50,000 individuals per m² (3, 4). They constantly ingest small sulfide particles and their guts are often densely packed with these solids (4, 5). One of the most unusual features of these shrimp is the extremely dense bacterial population growing inside their carapace and on their extremities (Fig. 1A and B). Several studies have claimed the presence of two or three distinct ectobacterial species on the basis of morphology (4–6). Indeed, using scanning electron microscopy (SEM), morphologically different rods and filaments of various thicknesses can be detected (Fig. 1A and B). They are inferred to be chemosymbiotic sulfur-oxidizers (2, 6) and may benefit from the animals’ movement in the gradients formed in the mixing zone of reduced and oxidized waters, as do ectosymbiotic bacteria on some marine nematode species (7).

Pacific and MAR vents harbor strikingly different animal communities, yet they are both characterized by the mass occurrence of invertebrate–bacteria associations. We investigated the phylogenetic identity and relative abundance of the epibions growing on the MAR vent shrimp to estimate their overall importance in the surface-associated microbial community. This community is the primary food source for higher trophic levels at the MAR, as inferred on the basis of observations indicating that the shrimp, the dominating macrofaunal element, graze bacteria either from their own bodies or from the sulfide surfaces (4, 5) and show no indication of appendages modified for filter feeding (ref. 5; C. L. Van Dover, personal communication). Furthermore, no significant population of actively filter-feeding animals has been described at this vent site.

Since the shrimp bacteria could not be cultured (E. V. Odintsova and H. W. Jannasch, personal communication), the 16S rRNA phylogenetic framework was used to determine the relationship of the epibions to known vent endosymbions, which cluster with all other analyzed sulfur-oxidizing symbionts (8, 9), and to quantify their abundance both on the shrimp body and in the free-living surface community.

MATERIALS AND METHODS

Specimens. Shrimp and solid sulfide blocks were collected by using the DSV (deep submergence vehicle) *Alvin* in 1993 during dives 2610 to 2623 at the Snakepit site (3). Animals were frozen immediately on board ship for nucleic acid extraction or fixed for SEM (10) or in situ hybridizations (11). Surfaces of solid sulfide blocks were scraped to a depth of 1–2 mm and that material was frozen for nucleic acid extraction or fixed for SEM. In total, eight patches each with an approximate radius of 10 cm were scraped on sulfide blocks collected during three different dives. The density of the sulfides was determined to average 1.9 g·cm⁻³. One sample from each dive was examined by SEM. *Escherichia coli* strain INVaF° was grown overnight in LB broth, centrifuged, washed, and stored frozen. Cells of *Methanobacterium thermooautotrophicum* were a generous gift of Lee Krumholz (Massachusetts Institute of Technology).

Nucleic Acid Manipulation. Nucleic acids were extracted for two purposes: (i) PCR, cloning, and sequencing for phylogenetic analysis of the shrimp epibions and (ii) quantitative slot-blot hybridization to determine the relative abundance of these bacteria in the total community.

DNA for PCR was extracted from bacterial scrapings from the shrimp carapace by using enzymatic (lysozyme and proteinase K) and detergent-based (SDS) lysis (12). This relatively gentle method avoids excessive shearing of DNA and reduces the risk of chimera formation during PCR (13). PCR of the 16S rRNA gene using the amplification primers 27F and 1492R (14), cloning using the TA cloning kit (Vector Laboratories), and magnetic bead sequencing were essentially as described (15). Direct sequencing of PCR products obtained from DNA of the scraped bacteria was done to determine if one sequence predominated to such an extent that each sequence position would be unambiguously identifiable. In addition, a partial sequence for each of 26 randomly selected clones was deter-

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Abbreviations: MAR, Mid-Atlantic Ridge; SEM, scanning electron microscopy.
Bartlesville, OK) was used as published (16). Positive control nucleic acids from organisms classified in the three domains [sensu Woese et al. (17)] were included for Eukarya (R. exceatus-wood tissue), Archaea (M. thermogranolithicum), and Bacteria (E. coli). These were treated the same way as the shrimp and sulfide samples, except that tail tissue was ground in liquid nitrogen prior to bead beating.

Phylogenetic Analysis. The secondary structure of the shrimp-epibiont 16S rRNA was reconstructed manually by using templates published in the Ribosomal Database Project (RDP) (18) to ease the identification of homologous sequence positions. Sequence alignment was done manually in the Genetic Data Environment (GDE) (19). All reference sequences and the basic alignment to which the epibiont sequence was added were drawn from the RDP. The final data set included 989 nucleotide positions. Phylogenetic distance and bootstrap analyses were performed with the programs DNADIST with Jukes and Cantor correction, SEQBOOT, and FITCH, all contained in the PHYLIP 3.4 package (20) implemented through the GDE. Parsimony analysis, including bootstrapping, was done in PAUP 3.0 (21).

Probe Construction and Synthesis. To confirm the source of the dominant sequence and to determine the distribution of the corresponding organisms in the environment, an epibiont-specific probe and a negative control probe complementary to the RNA-like strand were constructed. A search against GenBank by using BLAST (22) and against the Ribosomal Database Project by using CHECK-PROBE identified a sequence stretch (5'-ATCTTCTCCCCAGACTCT-3', E. coli position 653–672) with which no sequence in all the data bases had less than three mismatches, two of which were always in the middle region. A negative control probe containing two mismatches (5'-ATCTTCTCTTAACAGACTCT-3') was constructed to be used as a test for unspecific labeling. Synthesis and purification of the probes were as described (15) except that enzymatic biotinylation was used (see below).

In Situ Hybridizations. Whole fixed anterior bodies of shrimp were embedded in paraffin, sectioned, mounted on glass slides, and deparaffinized (23). The oligonucleotide probes were labeled with a biotin-ddUTP conjugate (Boehringer Mannheim) according to the supplied instructions. Hybridizations were performed at 42°C followed by three 15-min washes at 46°C and subsequent incubation in avidin-conjugated fluorescein isothiocyanate (FITC) (Vector Laboratories) (15). Documentation was done on a Zeiss Axioscope microscope.

Slot-Blot Hybridizations. To determine the relative abundance of the shrimp epibions compared with total Bacteria, Archaea, and Eukarya, quantitative slot-blot analysis was performed. Nucleic acids were blotted on nylon membranes as described (16) except that Zeta-Probe membranes (Bio-Rad) were used. For the final quantification of total shrimp-epibiont nucleic acids, extracts of all bacteria-containing body parts of 10 individuals were pooled. Five replicates of the pooled nucleic acids were blotted to account for between-set variability. For estimation of the sulfide surface-associated microbial community, nucleic acids were extracted from the eight different patches of sulfide surface scrapings collected from the shrimp habitat on three different days. These were pooled after nucleic acid extraction and blotted in three replicates. Positive control nucleic acids from organisms representing the three domains [sensu Woese et al. (17)] were blotted in duplicate. All standard nucleic acids (see below) were blotted on the same membrane as the samples for hybridization.

To optimize the relation between signal strength and specificity of the probes, midpoint dissociation temperatures (T_m) for all oligonucleotides were determined experimentally. The procedure of Raskin et al. (24) was employed except that the hybridization and wash buffer recommended for the Zeta-Probe membranes and 10-min washes at the specific temperatures were used. Probes were labeled with polynucleotide

*The sequence discussed in this paper has been deposited in the GenBank data base (accession no. U29081).
kinase to an approximate specific activity of $5 \times 10^5$ cpm$\mu$g$^{-1}$. For determination of the $T_d$ values of the domain-specific probes, the respective positive control nucleic acids served as templates. For the shrimp epibiont-specific probe, nucleic acids from scrapings had to be used, since no culture of these bacteria exists. Care was taken to avoid contamination by rinsing bacteria-containing body parts with sterile water several times before gently scraping off epigrowth. Values read from the $T_d$ curve and from the standard curve (see below) may lead to an overestimation of the relative abundance of the shrimp epibiont when related to other samples caused by possible additive contribution of nonspecific hybridization. However, the scrapings were extremely pure as judged by repeated comparisons of the signal obtained by the universal probe measuring total Bacteria (25) and the epibiont-specific probe (see Table 1).

Hybridizations were done overnight at 40°C (except Eukarya at 30°C). Based on the experimentally determined $T_d$ values, the wash temperatures were 42°, 50°, 60°, and 56°C for the Eukarya (26), Archaea (25), Bacteria (25), and the epibiont-specific probes, respectively. For qualitative analysis, blots were exposed on Reflection NEF-496 (DuPont) film. Quantification was done using a Fujix Bio Imaging Analyzer BAS2000 phosphor imager and BAS2000 Image File Manager 2.1 analysis software. Standard nucleic acids from shrimp epibiont scrapings and positive controls were spotted in different concentrations. Least-squares linear regression analysis on the standard curves yielded $r^2 > 0.98$ in all cases. To back-calculate from total amount of nucleic acids to amount per volume of sulfide substrate, the density value of 1.9 g ml$^{-1}$ was used.

### RESULTS AND DISCUSSION

Remarkably, only one bacterial sequence or “phyotype” was recovered from nucleic acids extracted from the shrimp epibionts. A single 16S rRNA gene was evident by direct sequencing. Furthermore, of the 26 clones analyzed by partial sequencing, 23 contained the same sequence in all nucleotide positions, whereas the remaining 3 were unreadable. To test if this dominance was reflected in the makeup of the shrimp-epibiont population in situ, an epibiont-specific 16S rRNA-targeted oligonucleotide hybridization probe and a negative control probe with two introduced mismatches were constructed. These probes were used in in situ hybridizations (Fig. 1). A probe complementary to all known Bacteria 16S rRNAs (25) served as a positive control.

*In situ* hybridization and epifluorescence microscopy confirmed the genetic identity of all three morphotypes of shrimp symbionts on the 16S RNA level (Fig. 1). The specific probe gave signal patterns identical to those of the universal bacterial probe (Fig. 1 D and F) with no background staining from the negative control probe (Fig. 1 I and J). Both thin and thick filaments were stained (Fig. 1 C and D). Regions of active growth within these filaments are discernible because the strength of the signal is correlated to cellular RNA content, which is a measure of its physiological state (27) (Fig. 1 C and D and G and H). The small rods are less visible, yet staining in a region where only these cells grow showed an intense fluorescence with the specific probe (Fig. 1 E and F). These results are supported by the quantitative hybridizations, which gave essentially identical signals between the universal bacterial probe and the epibiont-specific probe when applied to nucleic acids extracted from shrimp bodies (Table 1).

The results of the hybridizations indicate a high degree of specificity in the shrimp-epibiont association with a single bacterial phyotype, suggesting that a single species or multiple species with identical 16S rRNA are growing on the shrimp body. The probing region for the specific oligonucleotide and the negative control were chosen in a highly variable area of the 16S rRNA molecule, so it is expected that only very closely related phyotypes would possess a similar or identical sequence stretch. For example, among the closest relatives within the e-Proteobacteria, *Thiovulum* has 5 and all other species have 4 mismatches in the specific region. If the epibionts are a single species, their distinct morphotypes appear unusual. Although pleomorphy can be found frequently in a variety of bacterial groups, it is usually a function of changing growth conditions. On the shrimp body, the different cell types occur side by side. If this is caused by physicochemical variation on a microscale or if three different developmental stages of a single species are present cannot be determined. However, given the extremely limited diversity of epibionts and specificity, the association may be defined as a symbiosis (28)—i.e., a stable association between a specific bacterial group and the shrimp host.

The obtained 16S rRNA sequence was used to reconstruct phylogenetic relationships of this symbiont phyotype to other known bacteria. It forms a deep branch in the e subdivision of the Proteobacteria (Fig. 2). This is distinctly different from previously analyzed ecto- and endosymbionts of animals from sulfidic habitats, including hydrothermal vents, which all cluster within the γ-Proteobacteria (Fig. 2) (8, 9). However, association with animals is the best-known mode of living in the e-Proteobacteria, although all examples are parasitic. Sulfur metabolism and microaerophilic are also common in this group and there exists a moderate relationship between the *R. extrabaculata* symbiont and the sulfide-gradient organism *Thiovulum* sp. (29).

To address the question of numerical importance of the ectosymbionts in the surface microbial community, oligonucleotide slot-blot hybridization on nucleic acids from different sources using probes specific for the shrimp epibiont and for the three domains Bacteria, Archaea, and Eukarya was performed (Fig. 3, Table 1). Most surprisingly, the quantification indicates that the shrimp epibiont is also the dominant phyotype on the sulfide surface, contributing half of the detectable bacterial rRNA (Table 1). This is supported by SEM observation of the characteristic filaments on the sulfide surfaces (Fig. 4). Qualitative slot-blot analysis showed a complete absence of signals from the Archaea and Eukarya probes (Fig. 3), which parallels results obtained by using lipid biomarker analysis of surface sulfides from a Pacific hydrothermal vent site (30).

The main biomass of bacteria at these vents appears to be associated with the shrimp bodies. Assuming that the 16S rRNA content in an average bacterial cell is about 23% of the total nucleic acids and 15 fg (31), we can calculate from the quantitative probing (Table 1) a rough estimate of cell numbers. Even though rRNA content may reflect activity of cells
rather than their actual numbers, the variation in ribosome content for actively growing cells in the environment is thought to vary only 5- to 10-fold (32, 33). Thus, an average shrimp would carry $8.5 \times 10^6$ bacteria, which would lead to $2.1 \times 10^{11}$ to $4.2 \times 10^{11}$ bacteria per m$^2$ with a density of 25,000 to 50,000 host organisms per m$^2$. This is considerably higher than the $4.9 \times 10^8$ bacteria similarly estimated for 1 m$^2 \times 1$ mm$^2$ slice of the sulfides. Likewise, the planktonic bacterial community abundance appears less important than the epibiont population. Assuming an average of $5 \times 10^6$ bacterial cells per ml of seawater, as reported for a Pacific vent (34), it would take 17 ml of seawater at the MAR vents to equal the number of bacteria on a single shrimp, or 425 to 850 liters to equal that of a 1-m$^2$ surface covered with shrimp. Considering that chemical gradients over or close to surfaces supply energy for most of the primary productivity at vent ecosystems, the planktonic community under direct influence of the vents may be comparatively small. On the other hand, the shrimps' dwelling close to the surfaces may enable their epibionts to reach high growth rates. This view is supported by the fluorescent signal obtained in the in situ hybridizations (Fig. 1), which was unusually strong for naturally occurring bacteria and is indicative of high rRNA content. Thus, the epibiotic phylogeny appears to be not only the dominating bacterium in the surface community but also the essential producer at the base of the food chain leading up to the macroinvertebrates at the MAR vents.

While the shrimps' behavior may keep the symbionts under favorable growth conditions in the fluctuating and unstable environment of oxygen and sulfide gradients (35), the exact role of the epibiotic symbionts for their host is not clear yet. They probably serve directly as a nutritional source, since in a preliminary analysis, stable carbon isotope ratios of shrimp tissues resembled those of ectosymbiotic scrapings rather than those of sulfides (C. L. Van Dover, personal communication). However, the ratio of symbiont-specific to total bacterial nucleic acids in gut samples is closer to that of the sulfides.

**FIG. 2.** A 16S rRNA-based phylogenetic tree showing the relationship of the *R. exoculata* symbiont to known free-living and symbiotic bacteria within the five subdivisions (α, β, γ, δ, and ε) of the Proteobacteria. Representatives of the Gram-positive phylum and the Fusobacteria were included as outgroups to the Proteobacteria. The tree is based on a distance matrix calculated from aligned sequences. Bootstrap values higher than 50/100 are shown. An essentially identical tree was obtained when parsimony algorithms were used on the same data set (data not shown). Scale bar indicates percent nucleotide difference per sequence position.

**FIG. 3.** Qualitative community analysis by oligonucleotide slot-blot hybridization of total nucleic acids extracted from the shrimp carapace and the sulfide substrate. The Archaea (Arc) domain level probe gave negative results for both carapace and sulfides, indicating absence of Archaea. Results from the Eukarya (Euk) probe indicate absence of eukaryotic nucleic acids from the sulfide community and presence in low amounts in the carapace community, which probably stem from shrimp tissue. Only the Bacteria (Eub) and the epibiont-specific (Rex) probe gave strong hybridization on carapace and sulfides. No hybridization was observed with the negative-control (Neg) probe.

**FIG. 4.** Scanning electron micrograph of sulfide surface, showing bacterial filaments resembling the shrimp epibionts. Arrows point at cells embedded in the substrate of sulfide particles that precipitate from the vent waters. (Scale bar = 25 μm.)
unpublished results) and the observed ingestion of sulfides by the massive shrimp population (4, 5) is likely to remove significant numbers of attached bacteria. Assuming that all bacteria on the sulfides are affected indiscriminately, the free-living population of the epibionts may achieve high densities through its symbiotic life stage. While bacterial species growing exclusively on the sulfides can increase their numbers only through growth in situ, symbionts falling off the shrimp bodies likely serve as a continuous inoculum replenishing their substrate population. Thus, an indirect yet mutually favorable interaction between the two partners arises, further enabling them to reach high biomass under otherwise adverse conditions.

One of the most intriguing results of the phylogenetic approach to microbial ecology (36, 37) has been the discovery of the overwhelming diversity of microorganisms, which had been previously unrecognized. For example, it was inferred from DNA reassociation kinetics that the equivalent of 4000 bacterial species was present in 1 g of forest soil (38). In addition, cloning and sequencing of 16S rRNA genes from natural communities have produced extensive lists of uncultured microorganisms, many of which represent unknown phylogenetic lineages (for reviews see refs. 39 and 40). In the light of these findings, the extreme dominance of one bacterial phyotype in the microbial surface community at the MAR vents appears highly unusual. A similar phenomenon has so far been measured only once, in short-lived bloom situations in a pelagic environment (41). However, quantitative studies assessing the importance of single species in a total community are still rare, and more examples from diverse environments will have to be collected before unifying principles of microbial diversity, dominance, and community structure will emerge. Symbiotic association appears to be a fundamental characteristic of hydrothermal vent communities. In both Pacific and Atlantic vents the driving forces behind the close interaction between bacteria and invertebrates (35) may be similar, yet result in the respective dominance of two phenomenologically very different types of association, ecto- and endosymbiosis. Information about bacterial community structure in natural environments is still scarce. We have shown that limited diversity and dominance of one species on the macrofaunal level may be reflected in a microbial community. Given how common symbiotic interactions are in many environments, comparative measurement of the numerical representation of symbiotic species may shed light on the true significance of neutral and beneficial interactions among organisms in nature.

Note Added in Proof. Recently, 16S rDNA sequences derived from ε-Proteobacteria have been detected in clone libraries obtained from a bacterial mat at a hydrothermal vent (42) and from an epibiotic community of a vent polychaete (43).

We thank Chief Scientist C. L. Van Dover, the captain and crew of the R/V Atlantis II and DSV Alvin, J. Robinson for help on board, and L. Krumholz for generously providing cells of *M. thermooautotrophica*. In addition, we thank anonymous reviewers for helpful comments. This work was supported by a grant from the National Science Foundation.