

Genetic Diversity of Attached Bacteria in the Hindgut of the Deposit-Feeding Shrimp *Neotrypaea* (formerly *Callinassa*) *californiensis* (Decapoda: Thalassinidae)

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A B S T R A C T

Microbial colonization of marine invertebrate guts is widespread, but in general the roles that these bacteria play in the nutrition of their hosts are unknown. To examine the diversity and potential nutritional roles of hindgut microbiota in a deposit feeder, PCR-amplified 16S rRNA genes were cloned from the bacterial community attached to the hindguts of the thalassinid shrimp *Neotrypaea californiensis* exposed to different feeding treatments. Partial 16S rDNA sequences were analyzed for 30 clones for three shrimp per treatment for a total of 270 clones. No effects of host starvation or high-protein diets were apparent on hindgut bacterial community composition. Diversity analyses indicated high variability between bacterial communities in individual shrimp hindguts, but partial 16S rDNA sequences revealed remarkable species-level similarity (>98%) within clusters of sequences from the different shrimp hindguts, and many sequences from different shrimp hindguts were identical. Sequences belonged to three main groups of bacteria: *Cytophaga-Flavobacteria-Bacteroides* (CFB), proteobacteria, and gram-positives. Of the 270 sequences, 40% belonged to the α -proteobacteria, $\geq 5\%$ each to the γ - and ϵ -proteobacteria, and $\geq 20\%$ each to the gram-positive and CFB groups. All except one sequence are novel with $\leq 95\%$ sequence similarity to known genes. Despite weak similarity to known taxa, about 75% of the sequences were most closely related to known symbiotic and sedimentary bacteria. The bacteria in shrimp hindguts represent new species that have not yet been encountered in other environments, and gut environments may be a rich source of the difficult-to-culture and novel components of marine bacterial diversity.

Introduction

Microbial colonization of animal guts is ubiquitous. These symbioses range from pathogenic to mutualistic

and from facultative to obligate, and they can affect biogeochemical cycles more strongly than the simple sum of the partners alone. Nutritional mutualisms have long been recognized as important and obligate for both symbiotic partners in terrestrial systems such as ruminants and termites. The significance of such relationships

for the marine environment was not broadly recognized until the discovery of deep-sea hydrothermal vent systems. There is now accumulating evidence that microbial colonization of guts of marine invertebrates is widespread and that these interactions are mutually beneficial in nutrition [17].

Of gut regions (fore-, mid-, and hindgut), the hindgut is most amenable to harboring an indigenous microbiota in healthy animals [17, 39]. In this region, the main function is storage of fecal matter and the host's defenses against microbes (e.g. enzymes, surfactants, sloughing) are weakest. Furthermore, in contrast to the midgut where the main function is absorption, bacteria in the hindgut have access to leftover digesta and are not competing directly with their hosts for uptake of digested compounds. Studies on several invertebrate groups—insects, millipedes, and crustaceans—found that hindgut microbial communities were more dense and diverse than those in the respective foreguts, which reflected what was ingested [17]. The hindgut, hence, is a prime location for investigating nutritional mutualisms.

Detritivores, such as thalassinid shrimp, eat nutritionally poor food and are likely to benefit from associated gut microbes. Thalassinid shrimp, across genera, feeding mechanisms and substrata, recently have been identified as exhibiting extensive colonization of the hindgut cuticle [18, 37]. Thalassinids can significantly affect geochemical processes in marine sediments on a broad scale because of their wide distribution and considerable burrowing and feeding activities. Because of the great potential for these gut symbionts to contribute to diagenesis in marine sediments, it is important to know the identities and understand the nutritional and geochemical roles of bacteria living in the hindguts of these marine invertebrates.

We focused our study on the local thalassinid shrimp *Neotrypaea* (formerly *Callianassa*) *californiensis* because epimural rod bacteria were known to cover as much as 50% of its hindgut surface [18]. We conducted feeding experiments on *N. californiensis* collected from the field to investigate whether the hindgut bacterial community changes in response to the host's diet as has been observed with other invertebrates [e.g., 12, 17, 26]. As clues to nutritional function of the hindgut community, we specifically sought shifts in species composition by investigating phylogenetic associations and genetic diversity of partial 16S rDNA sequences obtained from attached hindgut bacteria. There was high variation in the overall community composition among individual hindguts both within a

treatment and between treatments. Nonetheless, many bacterial sequences from multiple shrimp hindguts grouped into clusters that, remarkably, had greater than species-level similarity. The data suggest that this hindgut–bacteria system might represent an obligate relationship rather than simply an opportunistic microbial response to the waning intensity of digestion.

Methods

Feeding Experiments and Hindgut Samples

Shrimp were collected on August 11, 1999, from False Bay, San Juan Island, Washington, and returned to the lab either for immediate dissection (field treatment) or for use in one of two feeding treatments: protein-based diet and starvation. Animals were kept in individual aquaria with 1- μ m filtered, running seawater and placed for 5 d in outdoor sea tables covered with black plastic sheets to simulate the darkness of shrimp burrows.

The protein treatment consisted of a 1:1 mixture of Sil-Co-Sil ground silica foundry sand (LM #125, Lane Mountain Comp.) and silt (#106 Ottawa, IL Mine, U.S. Silica Comp.) and 5% by weight pure soy protein powder (Challenge Protein 95, General Nutrition Corp.). The protein powder–sediment mixture in the protein treatment was changed daily to prevent bacterial fouling and to keep the food supply relatively constant under *ad libitum* feeding. During this change, which lasted about 1 h, animals were placed into separate aquaria with only filtered, running seawater. The starvation treatment had no sediment or food substrate, but the animals were otherwise handled in the same way as the protein-treatment animals throughout the experiments. At the end of each day of the 5-d feeding experiments, the presence of fecal rods in the aquarium was recorded. Shrimp hindguts were also examined for content without dissection. Because of the translucence of the posterior, ventral portion of the shrimp tail and the distinctive beige color of the foundry sand–silt mixture, the artificial food–sediment mixture could readily be distinguished from dark, native sediment.

To avoid sex-related differences in feeding rates and food preferences [e.g., 2, 6, 52, 53], only male shrimp were analyzed. Hindguts were dissected from three shrimp per treatment with alcohol-sterilized dissecting tools and immediately placed into sterile microfuge tubes with 100% EtOH. To prevent contamination by bacteria attached to the external body surface, different sets of tools were used for cutting open the outer cuticle of the body and for dissecting the inner body cavity.

A longitudinal incision was made along the hindguts. They were then pinned open on a previously UV-irradiated dissecting dish to remove fecal material, loose sand grains, and unattached bacteria. After the inner hindgut surface was rinsed with 70 or 100% EtOH and scraped with the tips of forceps to dislodge sediment grains, hindguts were placed into fresh tubes of 100% EtOH. All dissecting tools were alcohol-flame sterilized for each individual shrimp.

Clone Libraries and DNA Sequencing

DNA was extracted from hindguts using a phenol–chloroform protocol (modified from [22]). Each hindgut was placed into a microfuge tube with 150 μ L of digestion buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 20 mM EDTA; 2% sodium dodecyl sulfate) and 2 μ L of proteinase K (10 mg/ml). Mixtures were incubated at 65°C for 2 h on a rotating carousel and transferred to pre-pelleted Phase Lock Gel I tubes (5 Prime \rightarrow 3 Prime, Inc.). DNA was extracted twice with an equal volume of phenol and twice with an equal volume of chloroform:isoamyl alcohol (24:1). RNase (0.2 mg/ml final concentration) was added to the aqueous phase and incubated for 1 h at 37°C. DNA was precipitated by adding 2 volumes of 100% EtOH, mixing gently, and storing at 4°C overnight. DNA was collected by centrifugation at 14,000 rpm for 15 min and washed with 1 ml of 70% EtOH, then vacuum dried, resuspended in 100 μ L of 10 mM Tris (pH 7.6)–1 mM EDTA (pH 8.0), quantified with a GeneQuant (Pharmacia) spectrophotometer, and stored at -20°C until use.

DNA (100 ng) extracted from the shrimp hindguts was PCR amplified with 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') universal bacterial 16S rRNA gene primers [9]. PCR amplification bias toward certain bacterial taxa was restricted by using a cycle number at the early phase of exponential increase in product concentration [9, 41]. PCR (0.8 mM deoxynucleoside triphosphates, 1 μ M of each primer, 1 mM MgCl_2 , 0.1 U/ μ L *Taq* DNA polymerase [Promega]) amplification consisted of a 70-s denaturation step at 94°C, followed by 21 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 2 min, followed by a final extension of 72°C for 10 min. PCR products were purified with the QIAquick PCR purification kit (Qiagen), cloned into the pCR 2.1-TOPO vector, and transformed into TOP10 *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen Corp.).

Positive clones with an insert of the correct size (\sim 1.6 kb) were shaken in Luria–Bertani broth with ampicillin (50 μ g/ml) at 37°C for 3–5 h (modified from [9]). Media containing whole cells were used as templates and amplified with vector-specific M13 forward and reverse primers (10% by volume of whole cells, 0.8 mM deoxynucleoside triphosphates, 1 μ M of each primer, 1 mM MgCl_2 , 0.1 U/ μ L *Taq* DNA polymerase [Promega] or display TAQ FL [Display Systems Biotech]). PCR amplification began with 3 cycles of 94°C for 70 s, 56°C for 45 s, 72°C for 90s, followed by 23 cycles of 90°C for 15 s, 56°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and concentrated at a 5:3 ratio at elution.

Thirty nonchimeric clones (see section on Phylogenetic Analyses) were chosen randomly from each shrimp hindgut. DNA was sequenced with the 8f (see above) and internal 519r (5'-GWA TTA CCG CGG CKG CTG-3') primers [9]. Cycle sequencing was done with the Thermo Sequenase II kit (Amersham Pharmacia Biotech, Inc.) and the products analyzed on a 373A DNA sequencer (Applied Biosystems, Inc.) or with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Inc.) and the products analyzed on a MegaBACE 1000 (Molecular

Dynamics). A consensus sequence for each clone was constructed from the forward and reverse strands using the software programs Sequence Navigator (Applied Biosystems Inc.) or Sequencher (Gene Codes Corp.). All consensus sequences corresponded to bases 32–512 of the *E. coli* numbering system [54].

Phylogenetic Analyses

Sequences were first checked for chimeras using the on-line program CHECK_CHIMERA at the Ribosomal Database Project II (<http://www.cme.msu.edu/RDP/html/analyses.html>). No chimeras were identified with this analysis. Possible chimeras arising from similar sequences within the samples were also checked manually [30]. Nine chimeras were found and excluded from analysis. Additional clones were sequenced and analyzed to bring the total number of clones per shrimp to 30.

Hindgut bacterial sequences were submitted to the BLAST program at the National Center for Biotechnology Information (<http://www.ncbi.nlm.gov/BLAST/>) to search for closely related sequences. The 10 to 20 most closely related sequences that had sequences at least as long as the hindgut bacterial sequences were used in phylogenetic analyses. All sequences were aligned preliminarily with the on-line Sequence Aligner software at the Ribosomal Database Project II Web site (<http://rdp.cme.msu.edu/html/analyses.html>). Final alignments were done manually based on the secondary structure of the *E. coli* 16S rRNA molecule [16]. Sequences were grouped by phyla according to the BLAST searches, and respective phylogenetic trees were constructed with the aligned sequences using PAUPSearch (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI). Consensus trees (50% majority rule) were constructed with uncorrected neighbor-joining distances [44] and bootstrapping of 1000 replicates. Negative branching was prohibited, and groupings from the consensus trees were excluded if they occurred in fewer than 50% of the bootstrap replicates. Otherwise, all other values were at default setting. Percent similarity was calculated from aligned sequences using the uncorrected distance matrix method in the Distances program (Wisconsin Package Version 10.0, Genetics Computer Group [GCG], Madison, WI). Phylogenetic trees were viewed in TreeView (version 1.5) [35]. Closely related sequences that did not affect the phylogenetic groupings of hindgut bacterial sequences were excluded to simplify the phylogenetic trees.

Diversity Analyses

Sanders–Hurlbert rarefaction was used to compare species diversities and to determine how well a sample reflected the total diversity among hindgut samples. A plot of the expected number of species versus the potential number of individuals in a sample addresses both the species richness (number of species present) and the evenness with which individuals are represented in a sample. Expected number of species, $E(S_n)$, was calculated as

$$E(s_n) = \sum_i^s \left[1 - \frac{\binom{N - N_i}{n}}{\binom{N}{n}} \right] \quad (1)$$

where N is the number of individuals in a collected sample, n is the number of individuals in a hypothetical, randomly selected smaller sample, s is the total number of species in the actual sample, and N_i is the number of individuals belonging to the i th species in that sample [24, 45]. It was developed for and has been used frequently in benthic studies [e.g., 27, 28] and occasionally in microbial studies [5, 33]. Rarefaction curves were generated with the software program Matlab (version 5.0, Math Works Inc.) using routines generously provided by Dr. Eugene D. Gallagher of the University of Massachusetts at Boston.

To examine the effect of feeding treatment on the hindgut bacterial diversity, percentage remoteness was calculated. Percentage remoteness (PR) is calculated as

$$PR = 100 - 100 \times \left[\frac{\sum_{i=1}^s \min(x_{i1}, x_{i2})}{\sum_{i=1}^s \max(x_{i1}, x_{i2})} \right] \quad (2)$$

where x_{i1} and x_{i2} are the amounts of the i th species in samples 1 and 2, respectively, and s is the total number of species found in the two samples [36]. Min and max refer respectively to the minimum and maximum number of species i found in either sample. PR indicates how dissimilar two samples are from each other. A value of 100% indicates that the samples have no species in common, and 0% indicates that they are identical. A PR value was calculated for the hindgut communities of each pair of shrimp.

Results

At the time of dissection, all field treatment hindguts contained dark, native sediment. During each day of the 5-d feeding experiments, fecal pellets and (or) contents were observed in the hindguts of shrimp from the protein treatment but not in shrimp from the starvation treatment.

Analysis of partial 16S rDNA sequences indicated that bacteria in shrimp hindguts belonged to three phyla: *Cytophaga-Flavobacteria-Bacteroides* (CFB), gram-positive bacteria, and proteobacteria (Fig. 1). The 270 sequences from all shrimp grouped into twelve different clusters each showing >98% similarity within clusters (Table 1). Species in bacteria are defined by 97% identity in 16S rRNA sequence [46]; therefore, we assumed each cluster to represent a separate species. The total number of species per hindgut sample thus ranged from 4 to 9 (Fig. 1). In addition, >50% of the clones in each of 10 clusters were composed of clones from multiple shrimp and treatments and displayed identical sequences. This suggests that bacterial diversity within hindguts may be restricted.

Proteobacteria made up 53% of all the clones, with α -proteobacteria representing >77% of all proteobacteria (Table 1). Thirty percent of all the clones analyzed were in the Alpha-III cluster. This cluster contained the most common clones found overall and made up 74% of all α -proteobacterial clones. Twenty percent of the rest of the α -proteobacterial clones were in cluster Alpha-II, 4% in Alpha-I, and 3% in Alpha-IV. Clones from clusters Alpha-II, -III, and -IV grouped strongly together (100% bootstrap value) but grouped weakly (51%) with an α -proteobacterium from the accessory nidamental gland of the squid *Loligo pealei*, the nearest known neighbor at <88% similarity (Fig. 1A, Table 1). Within each of these three clusters sequence similarity was >98%, but the sequence similarity among the clusters was only 91–94%. Thus these three clusters appear to be closely related species that do not have known, close relatives in the database. Cluster Alpha-I grouped with an unidentified rumen bacterium, but they shared only 85% sequence similarity (Table 1).

Only 5 and 7% of the proteobacteria clones belonged to the ε -subdivision and the γ -subdivision, respectively (Table 1). Cluster Epsilon-I was most closely related to a Japan Trench sediment clone with 93% sequence similarity, and cluster Epsilon-II shared 94% sequence similarity with its closest neighbor, a deep-sea sediment clone (Table 1, Fig. 1A). The clone in Gamma-I was the only identifiable one, showing 99% sequence similarity to *Pseudoalteromonas denitrificans*. Cluster Gamma-II grouped (85% bootstrap value) with sulfur-oxidizing symbionts and *Methylomonas* species (about 90% similarity).

The next most common phylum was the CFB group constituting 25% of all the clones and forming two clusters (Table 1). Cluster CFB-I grouped with *Ornithobacterium rhinotracheale*, an isolate from an avian respiratory tract, and *Blattabacterium* sp., an endosymbiont of a cockroach (Fig. 1B), but was most similar to several *Cytophaga* species (Table 1). Cluster CFB-II was most closely related to the bacteroid *Marinilabilia* (also *Cytophaga salmonicolor*).

Gram-positive bacteria made up the remaining 22% of clones (Table 1). They formed two clusters more closely related to each other than to other known bacteria, but differed from one another by 10% (Fig. 1C). The closest known relative to both clusters was an unidentified clone from Japan Trench sediment with about 80% sequence similarity (Table 1).

Neither phylogenetic nor diversity analyses indicated a relationship between hindgut bacterial community and

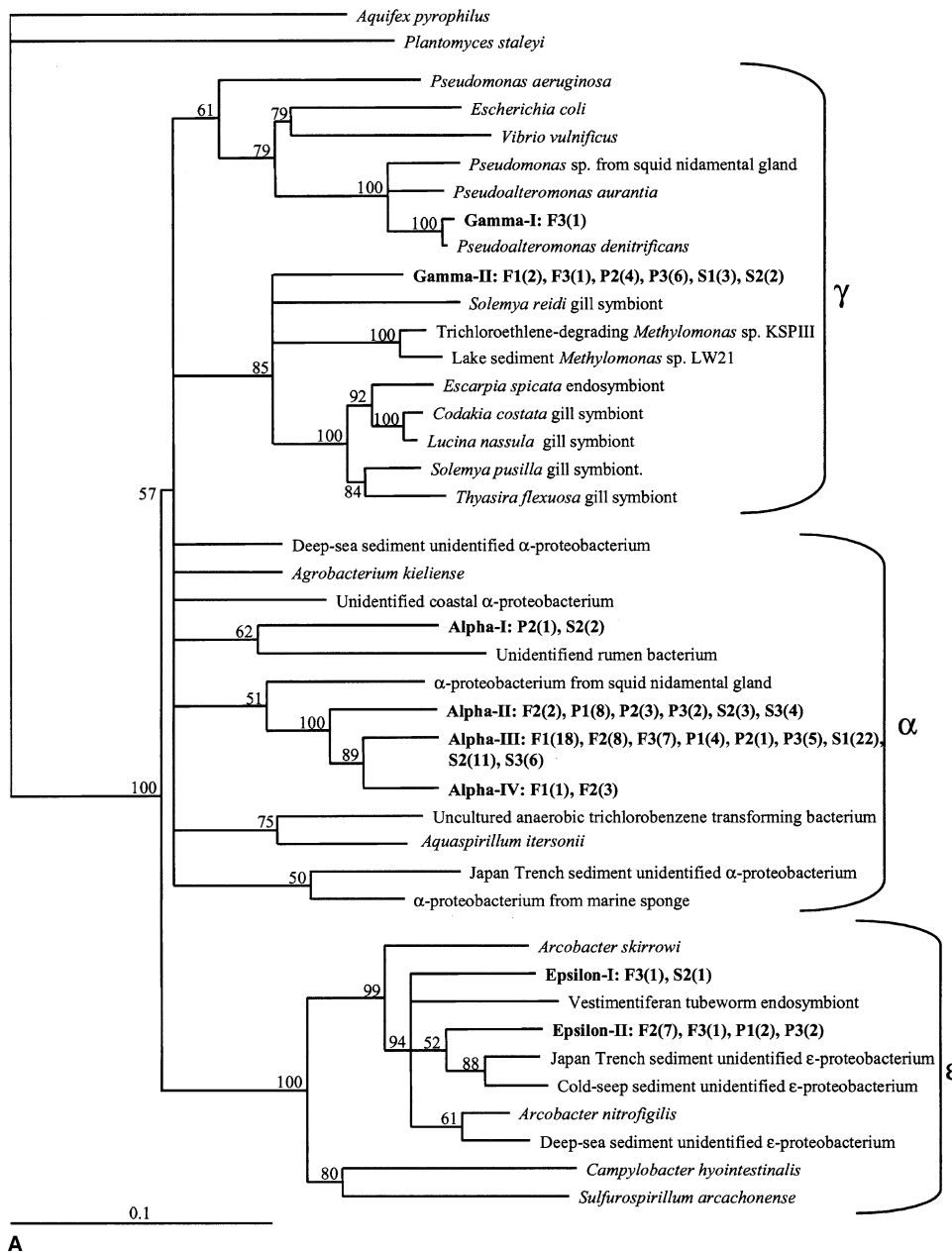


Fig. 1. Neighbor-joining trees of 16S ribosomal genes of attached (a) proteobacteria, (b) CFB group, and (c) gram-positive bacteria cloned from nine shrimp hindguts. F1–3, field treatment shrimp; P1–3, protein treatment shrimp; and S1–3, starvation treatment shrimp. Numbers in parentheses after individual labels are the number of clones from a given shrimp that belong to the same phylogenetic grouping. Numbers at or near nodes are bootstrap values from 1000 replicates. The bar represents 10% sequence divergence; γ , α , and ϵ in Fig. 1A refer to the respective subdivisions of proteobacteria. Boldfaced groupings are sequences from this study. Accession numbers of sequences from top to bottom used in these trees are as follows: Proteobacteria: M83548, M34126, AF237678, AE000474, X56582, AF022407, X82135, X82138, L25709, AB015603, AF150800, AF165909, L25712, X95229, U62130, L01575, AB015520, D88524, U70678, AF001764, AF034931, AJ009467, AB000477, AB015246, AF218241, L14625, D83061, AB013832, AB015256, L14627, AB015529, AF097689, and Y11561. CFB group: AE000474, X75622, U87105, AJ005972, U85888, AF182021, U63938, AB017047, AB001333, and M62422. Gram-positive bacteria: AE000474, X75272, AF001770, AB013835, AF061007, Y11621, AF014818, AF202263, AF201898, and AB023576. See Table 1 for the accession numbers of sequences from this study.

host diet. We found no trend in species composition with respect to feeding treatment, nor could we identify particular species as being specific to a particular host diet (Fig. 1, Table 1). In fact, the largest source of variation was between individuals rather than between treatments. Percentage remoteness calculations showed high dissimilarities within (57% to 80%) and between treatments (37.84% to 86.79%). Great overlap in the two ranges of PR indicates that the differences seen in the hindgut communities can be attributed to individual variation rather than to treatment effect. Rarefaction analyses also failed to reveal any trend in overall species diversity with respect to feeding

treatment. Species are evenly distributed in a sample when the rarefaction curve has a steep initial slope and quickly reaches a plateau. If two rarefaction curves cross at a particular value of $E(S_n)$, the one with the higher initial slope shows the greater evenness in abundance among species. Diversity in the hindgut communities as shown by both species richness and evenness was highly variable (Fig. 2), suggesting that hindgut bacterial composition did not vary strongly with host diet over the limited duration of our experiment.

The shapes of the rarefaction curves also showed that analysis of more than 30 clones would be necessary to

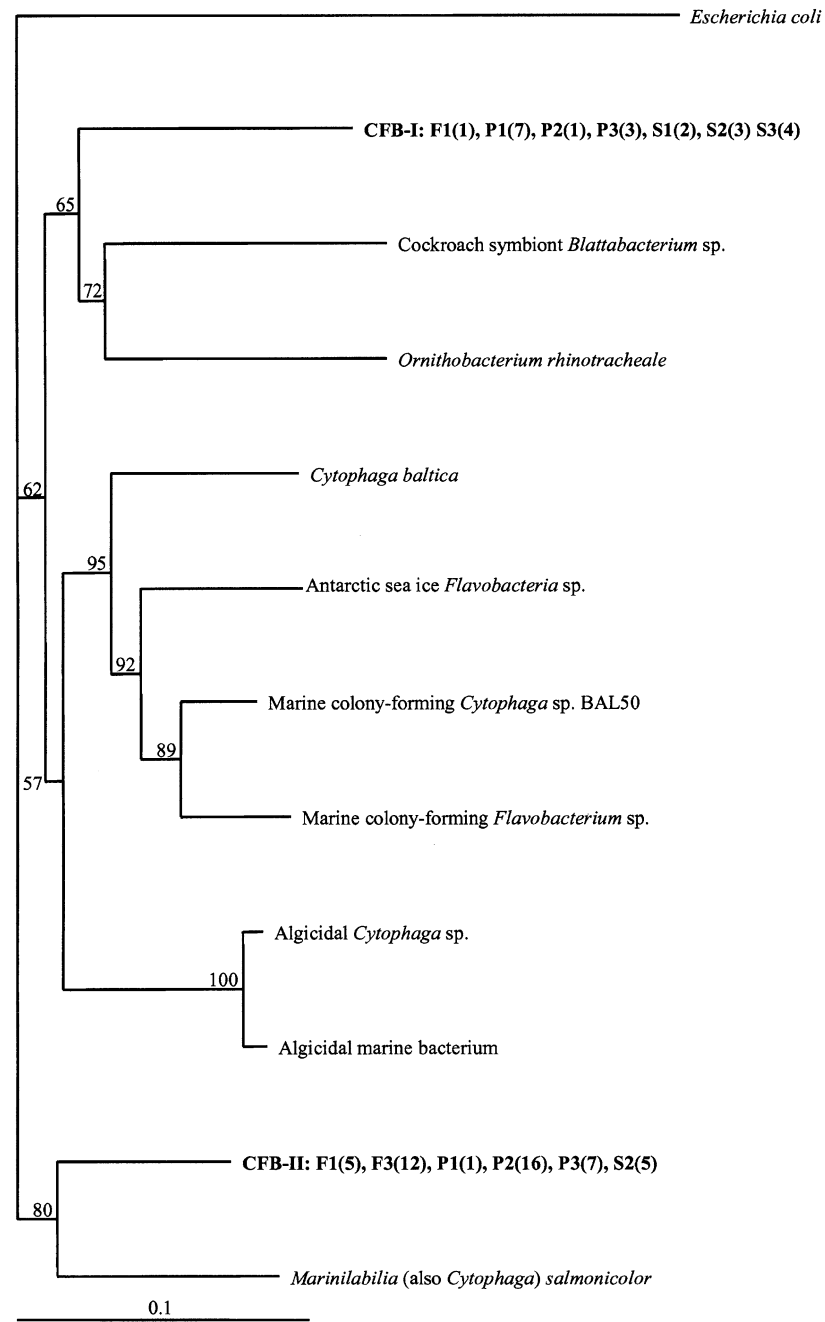
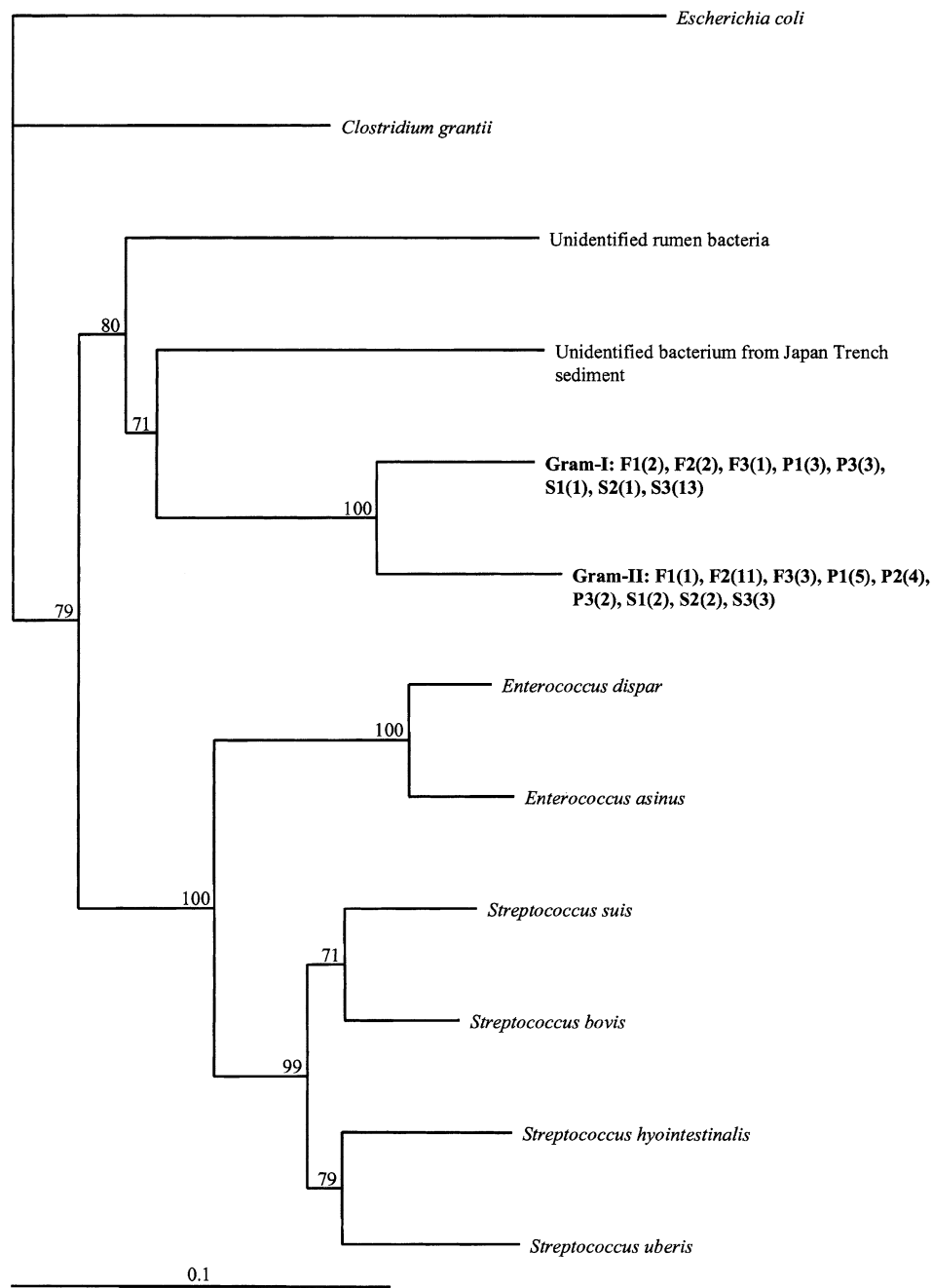


Fig. 1. Continued

B

represent the overall diversity in some of these hindguts (Fig. 2). A rising curve implies that there are likely additional species that have not been sampled, whereas a curve that has reached a plateau implies that most of the species in the community have been sampled. For four hindguts, rarefaction curves had approached an asymptote (P3, P1, S3, and M2, Fig. 2), suggesting that the clonal diversity in these hindguts was well represented, whereas the curves for the other five hindguts (S2, M3, P2, M1, SI, Fig. 2) were still in the phase of rapid increase, suggesting that addi-

tional bacterial diversity was present in the hindgut. A combined rarefaction curve of data from all nine shrimp hindguts further shows the high variability between individuals, but the shape of the curve indicates that the diversity of hindgut bacteria found in this population of shrimp, regardless of the nutritional state of the shrimp, was well represented by this study (Fig. 2). The fact that most points representing individual shrimp fall below the combined curve indicates non-random and non-uniform distribution of clones among individuals [25].



C

Fig. 1. Continued

Discussion

The results of this study suggest that shrimp hindguts harbor a small, core group of unique bacteria as symbionts. Only one of 270 sequences cloned from shrimp hindguts could be identified to the species level. Most of the sequences (>94%) had no close relatives in the database and shared <90% similarity with their nearest neighbor (Table 1). There was remarkable repetition of clonal sequences, however, with species-level sequence

similarity among all nine shrimp hindguts. Ten of the 12 clusters identified contained identical sequences from multiple hindguts. Eleven of these clusters could be identified as novel species. Some clusters were more closely related to each other than to known groups, suggesting that shrimp hindguts may harbor new species as well as entirely new groups of bacteria restricted to this environment.

The sequences that we found overlapped taxonomically with hindgut isolates from other thalassinids identified by

Table 1. Characteristics of 12 bacterial clusters identified from 270 partial 16S rDNA sequences cloned from 9 different shrimp hindguts^a

Cluster	Length (bp)	# of clones	# of shrimp represented (# of treatments)	Within-group % similarity	% of identical sequences (# of shrimp)	Nearest neighbor(s)	% similarity with nearest neighbor(s)	Accession # of unique sequence(s)
Alpha-I	436	3	2 (2)	>99.0	0	Unidentified rumen bacterium	<85	AF434073 to AF434075
Alpha-II	435	22	6 (3)	>98.3	50 (5)	Squid nidamental gland α -proteobacterium	<87	AF434060 to AF434072
Alpha-III	437	82	9 (3)	>98.1	70 (9)	Squid nidamental gland α -proteobacterium	<88	AF434076 to AF434102
Alpha-IV	437	4	2 (1)	>99.7	75 (2)	Squid nidamental gland α -proteobacterium	<87	AF434103 to AF434104
Epsilon-I	459	2	2 (2)	100	100 (2)	Deep-sea sediment ϵ -proteobacterium	<95	AF434108
Epsilon-II	459	12	4 (2)	>99.3	83 (4)	Japan Trench sediment ϵ -proteobacterium	<93	AF434105 to AF434107
Gamma-I	480	1	1 (1)	—	—	<i>Pseudoalteromonas denitrificans</i>	99.1	AF434117
Gamma-II	479	18	6 (3)	>99.1	61 (6)	Gill symbionts, <i>Methylomonas</i> spp.	<90	AF434109 to AF434116
CFB-I	475	21	7 (3)	>98.7	71 (6)	<i>Cytophaga</i> spp. ^b	<83	AF434154 to AF434160
CFB-II	475	46	6 (3)	>99.1	74 (6)	<i>Marinilabilia salmonicolor</i>	<86	AF434141 to AF434153
Gram-I	511	26	8 (3)	>99.2	62 (8)	Japan Trench sediment unidentified bacterium	<81	AF434118 to AF434128
Gram-II	511	33	9 (3)	>99.2	73 (9)	Japan Trench sediment unidentified bacterium	<79	AF434129 to AF434140

^a Phylogenetic affiliations of the groupings are as follows: alpha I–IV: α -proteobacteria; epsilon I–II: ϵ -proteobacteria; gamma I–II: γ -proteobacteria; CFB I–II: *Cytophaga–Flavobacteria–Bacteroides* group; I–II: gram-positive bacteria. % similarity is from uncorrected distance matrix. (‘—’) indicates not applicable.

^b Several sequences, *Cytophaga* sp. BAL50, Algicidal *Cytophaga* sp., Algicidal marine bacterium, had the same % similarity to this cluster.

morphophysiological methods. Bacteria belonging to γ -proteobacteria and the CFB group were present in the hindguts of other thalassinid shrimp species [19, 38]. These prior studies did not identify any gram-positive bacteria, α -proteobacteria, or ϵ -proteobacteria, but they did note some unidentified taxa that might have belonged to these groups. Thus, the differences in isolation and characterization methods between our and previous studies might account for some of the differences seen in hindgut communities. The general similarities of our results to prior work, however, indicate that shrimp hindguts might be a habitat restricted to particular groups of bacteria.

By design of the extraction procedure, attached hindgut bacteria examined in this study were considered to be

resident and not sediment bacteria that had passed through the gut undigested. The lack of a trend between hindgut bacterial community composition and feeding treatment supports this hypothesis. During the 5-d experiments, shrimp in protein and starvation treatments were not exposed to sediment and seawater native to the habitat from which the shrimp were collected. If the attached hindgut community were transient and replenished frequently from the environment, a difference in this community would be expected among treatments. However, shrimp in the protein and starvation treatments had a similar attached hindgut community to shrimp from the field treatment.

Although the sediment community at this site has been not studied, results from the few studies that have com-

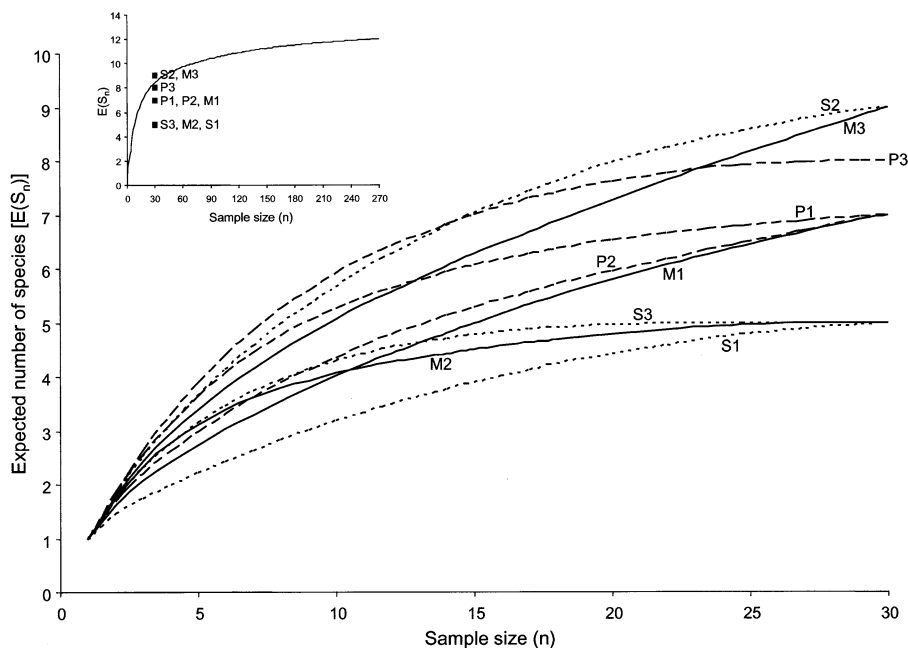


Fig. 2. Rarefaction curves for 30 partial 16S rDNA sequences from nine different shrimp hindguts. Conspecificity is defined by >97% sequence similarity (see Results section). Solid lines are for the field treatment; dashed lines, for the protein treatment; and dotted lines, for the starvation treatment. Labels for the individual shrimp hindguts are near their respective lines. Inset: Rarefaction curve plotted with data pooled from all hindguts. Labeled black squares indicate the endpoints of the rarefaction curves from each of the individual shrimp hindguts.

pared the deposit-feeder gut bacteria community with the sediment community indicate that the two communities are different [19, 48, 51]. Only one other study has used molecular techniques in investigating the hindgut microbiota of a marine deposit feeder, a sea cucumber, and it also examined the surrounding sediment community. Different isolates and different cloned bacterial sequences were obtained from the two communities [51]. In a study on other thalassinid shrimp, a significant difference was found in the digestive capabilities between the resident gut bacteria and bacteria living in burrow linings [19]. Differences in bacterial isolates were also found between attached (resident) communities and whole-gut (resident plus transient) communities [19, 38]. The *N. californiensis* hindgut bacterial community also appears to differ from the sediment community. Bacteria in the δ - and β -subdivisions of the proteobacteria, and *Plantomyces* which are generally found in many different sediments [e.g., 7, 15, 29, 34, 42, 47, 49] and have been cloned and sequenced from nearby sediments of Puget Sound [15, 34], were conspicuously missing from shrimp hindguts.

The lack of a trend with host feeding conditions also suggests that the bacterial community found in the hindguts of *N. californiensis* is invariant on a short, dietary term with the ambient environment in the hindgut (i.e., with leftover digesta of host diet). The high individual variability among shrimp hindguts indicates that this bacterial community may be directly controlled by its host or tightly coupled to the host's physiology.

A specific physiological state of the host that may have a direct influence on the bacterial community in the hindgut is the molt stage of the host; during molting of the exoskeleton the chitinous hindgut lining is replaced as well. Using culturing and morphological and physiological identification methods, Dempsey et al. [11] found high individual variability in the colony types and the numbers of colony-forming units that could be isolated from individual penaeid shrimp guts. He suggested that the high individual variability might be attributed to the molt stage of the shrimp. A study of the effect of molting on hindgut bacterial flora in a desert millipede showed that the new hindgut lining was devoid of microbes [8]. It is not known how hindguts in general become repopulated after molting.

It has been shown in insects that the chitinous hindgut lining is a suitable site for bacterial attachment [4], but apparently it is suitable for only a limited number of bacterial taxa in *N. californiensis*. What potential interactions might such a specific hindgut community have with its host shrimp? The hindgut bacteria could be parasites living on the host structures, such as the chitinous lining, or on nutrients that the host enzymes have digested. This scenario seems unlikely, however, as all the shrimp examined in this study appeared healthy. Moreover, hindgut bacteria in general do not compete with their hosts for nutrients since absorption of nutrients by hosts occurs in the midgut. Hindgut bacteria may merely be commensals benefiting from the relatively high food concentration due

to selective feeding by the host shrimp (as opposed to nonselective ingestion of bulk sediment) and from a low risk of predation without significantly affecting the host shrimp.

Another plausible, and perhaps more interesting, interaction appears to be a nutritional mutualism. Marine bacteria are known to secrete extracellular enzymes [50], and many bacteria associated with marine invertebrate guts can produce enzymes that their hosts cannot produce (reviewed in [17]). Any of the hydrolysates resulting from bacterial enzymes that are absorbed by the shrimp would represent a benefit to the shrimp host.

Shrimp can take up the hydrolysates via two possible mechanisms: absorption through the hindgut cuticle and reflux to the foregut through anal intake of water. Hindgut absorption of short-chain fatty acids from fermentation and of other small organic molecules has been demonstrated in various insects, including cockroaches [1, 20, 31]. In addition, bacteria secreting chitinase may help to enhance this flux by creating pores through which the shrimp can absorb organic compounds. Crustaceans have been observed to take up water from the anus [10, 13]. Most anal intake of water was observed just prior to defecation, and in some of the shrimps examined water taken in from the anus was observed to move forward all the way to the abdominal region [13]. Anal intake of water would serve as a mechanism to keep detached bacteria, released extracellular enzymes, and other bacterial enzyme-derived hydrolysates within the animal and possibly carry them all the way to the stomach, where digestion and subsequent absorption in the midgut could occur.

The attached hindgut bacteria of *N. californiensis* represent good candidates for nutritional mutualisms, although their nutritional roles cannot yet be established. Many of these bacteria grouped with endosymbionts, some of which, such as rumen bacteria and sulfur-oxidizing symbionts, are known to provide nutrition to their hosts (Fig. 1). The model from vertebrate hindgut mutualisms is that hindgut bacteria provide the host with short-chain fatty acids derived from digesta [3]. The gut bacteria most closely related to rumen bacteria might similarly be providing short-chain fatty acids to their shrimp host. Many of the enzymatic activities typically exhibited by the groups of bacteria found are those important for the breakdown of refractory compounds such chitin and cellulose. *Cytophaga* spp. are known to attach to surfaces and exhibit strong chitinase and cellulase activities. The one clone that could be identified, *Pseudoalteromonas* (for-

merly *Alteromonas* [14]) *denitrificans*, has amylase, alginate, and chitinase activities, as well as denitrification with gas formation [23]. In other studies, isolates from other thalassinid shrimp hindguts were found to exhibit significant agarase, amylase, cellulase, chitinase, lysozyme, and protease activities [19, 38]. Because the hindgut functions in storage of fecal material, hindgut bacteria would have relatively long exposure to the undigested compounds that hydrolyze slowly. Such a system would also alter the organic content and quality that is excreted as fecal matter.

Marine deposit feeders exert significant effects on diagenesis and organic-matter preservation. Most particles that reach the seabed pass through the guts of deposit feeders, with those not digested passing through several times before their eventual burial [43]. Because the gut environment is a geochemical “hotspot” where some conversions are accelerated both by the animal’s own digestive hydrolysis and by the actions of bacteria [40], it is important to understand how the metabolic capabilities of gut communities differ from those of sediment communities. Diets of deposit feeders may change twice, once in transition from the larva to the settled juvenile and again when gut volume becomes sufficient to allow deposit feeding [21]. Simultaneous monitoring of community composition of the gut flora may provide keys to dietary mutualisms. Because these shrimp have a planktonic stage, it is also important to consider whether the gut microbial community is established then, if the symbiosis is obligate, or whether this community changes after metamorphosis to the benthic lifestyle or only after change to a diet of sediment. A gut community acquired during the planktonic stages would be a source of metabolic capabilities that native sediment bacteria might not possess and could have profound implications for understanding nutrient cycling in sediments and between pelagic and benthic environments.

Seafloor and pelagic animals are notably diverse. Most known phyla are marine, and many are diverse in species, lifestyle, and diets. It appears likely—given the unrelatedness of the bacteria that we have characterized to known taxa and the unique chemical environments of animal guts [e.g., 32]—that propagules from gut floras of animals may account for a substantial fraction of the bacterial taxa in the sea that have so far evaded culture and that substantial, as-yet-unrecognized microbial diversity may reside in the guts of marine organisms. A corollary is that novel culture media and unsteady culture conditions may be required to elicit growth of these

microbes. A vast and ever-evolving biogeochemical potential exists in the interactions among gut microbes, marine invertebrates, and dietary substrates.

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References

- Bayon C, Mathelin J (1980) Carbohydrate fermentation and by-product absorption studied with labelled cellulose in *Oryctes nasicornis* larvae (Coleoptera: Scarabaeidae). *J Insect Physiol* 2:833–840
- Behmer ST, Joern A (1994) The influence of proline on diet selection: Sex-specific feeding preferences by the grasshoppers *Ageneotettix deorum* and *Phoetaliotes nebrascensis* (Orthoptera: Acrididae). *Oecologia* 98:76–82
- Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* 70:567–590
- Bignell DE (1984) The arthropod gut as an environment for microorganisms. In: JM Anderson, AMD Rayner, DWH Walton (eds) *Invertebrate–Microbial Interactions*. Cambridge University Press, Cambridge, UK, pp 205–227
- Boehm MJ, Madden LV, Hoitnik HA (1993) Effect of organic matter decomposition level on bacterial species diversity and composition in relationship to pythium damping-off severity. *Appl Environ Microbiol* 59:4171–4179
- Castro BG, Guerra A (1990) The diet of *Sepia officinalis* (Linnaeus, 1758) and *Sepia elegans* (D'Orbigny, 1835) (Cephalopoda, Sepioidea) from the Ría de Vigo (NW Spain). *Sci Mar* 54:375–388
- Cifuentes A, Antón J, Benlloch S, Donnelly A, Herbert RA, Rodríguez-Valera F (2000) Prokaryotic diversity in *Zostera noltii*-colonized marine sediments. *Appl Environ Microbiol* 66:1715–1719
- Crawford CS, Minion GP, Boyers MD (1983) Intima morphology, bacterial morphotypes, and effects of annual molt on microflora in the hindgut of the desert millipede, *Orthoporus ornatus* (Girard) (Diplopoda: Spirostreptidae). *Int J Insect Morphol Embryol* 12:301–312
- Crump BC, Armbrust EV, Baross JA (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol* 65:3192–3204
- Dall W (1967) The functional anatomy of the digestive tract of a shrimp *Metapenaeus bennettiae* Racek & Dall (Crustacea: Decapoda: Penaeidae). *Aust J Zool* 15:699–714
- Dempsey AC, Kitting CL, Rosson RA (1989) Bacterial variability among individual Penaeid shrimp digestive tracts. *Crustaceana* 56:267–278
- Feinberg L, Jorgensen J, Haselton A, Pitt A, Rudner R, Margulis L (1999) *Arthromitus* (*Bacillus cereus*) symbionts in the cockroach *Blaberus giganteus*: Dietary influences on bacterial development and population density. *Symbiosis* 27:109–123
- Fox HM (1952) Anal and oral intake of water by Crustacea. *J Exp Biol* 29:583–599
- Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int J Syst Bacteriol* 45:755–761
- Gray JP, Herwig RP (1996) Phylogenetic analysis of the bacterial communities in marine sediments. *Appl Environ Microbiol* 62:4049–4059
- Gutell R, Larsen N, Woese C (1994) Lessons from an evolutionary rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* 58:10–26
- Harris JM (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microb Ecol* 25:195–231
- Harris JM (1993) Widespread occurrence of extensive epimural rod bacteria in the hindguts of marine Thalassinidae and Brachyura (Crustacea: Decapoda). *Mar Biol* 116:615–629
- Harris JM, Seiderer LJ, Lucas MI (1991) Gut microflora of two saltmarsh detritivore thalassinid prawns, *Upogebia africana* and *Callinassa kraussi*. *Microb Ecol* 21:277–296
- Hassal M, Jennings JB (1975) Adaptive features of gut structure and digestive physiology in the terrestrial isopod *Philosicia muscorum* (Scopoli) 1763. *Biol Bull Woods Hole* 149:348–364
- Hentschel BT (1998) Intraspecific variations in $\delta^{13}\text{C}$ indicate ontogenetic diet changes in deposit-feeding polychaetes. *Ecology* 79:1357–1370
- Hoelzel AR, Green A (1992) Analysis of population-level variation by sequencing PCR-amplified DNA. In: AR Hoelzel (ed) *Practical Approach Series: Molecular Genetic Analysis of Populations*, vol xvii. IRL Press at Oxford University Press, New York, pp 159–187

23. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) *Bergey's Manual of Determinative Bacteriology*. Williams & Wilkins, Baltimore
24. Hurlbert SH (1971) The nonconcept of species diversity: A critique and alternative parameters. *Ecology* 52:577–586
25. Jumars PA (1976) Deep-sea diversity: Does it have a characteristic scale? *J Mar Res* 34:217–246
26. Kane MD, Breznak JA (1991) Effect of host diet on production of organic acids and methane by cockroach gut bacteria. *Appl Environ Microbiol* 57:2628–2634
27. Lamshead PJD (1993) Recent developments in marine benthic diversity research. *Océanis* 19:5–24
28. Lamshead PJD, Platt HM, Shaw KM (1983) The detection of differences among assemblages of marine benthic species based on an assessment of dominance and diversity. *J Nat Hist* 17:859–874
29. Li L, Kato C, Horikoshi K (1999) Bacterial diversity in deep-sea sediments from different depths. *Biodiversity Conservation* 8:659–677
30. Liesack W, Weyland H, Stackebrandt E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microb Ecol* 21:191–198
31. Maddrell SHP, Gardiner BOC (1980) The permeability of the cuticular lining of the insect alimentary canal. *J Exp Biol* 85:227–237
32. Mayer LM, Schick LL, Self RL, Jumars PA, Findlay RH, Chen Z, Sampson S (1997) Digestive environments of benthic macroinvertebrate guts: Enzymes, surfactants and dissolved organic matter. *J Mar Res* 55:785–812
33. Mills AL, Wassel RA (1980) Aspects of diversity measurement of microbial communities. *Appl Environ Microbiol* 40:578–586
34. Nold SC, Zhou J, Devol AH, Tiedje JM (2000) Pacific Northwest marine sediments contain ammonia-oxidizing bacteria in the β subdivision of the *Proteobacteria*. *Appl Environ Microbiol* 66:4532–4535
35. Page RDM (1996) Tree View: An application to display phylogenetic trees on personal computers. *Comput Applic Biosci* 12:357–358
36. Pielou EC (1984) *The Interpretation of Ecological Data*. John Wiley & Sons, New York
37. Finn EH, Nickell LA, Rogerson A, Atkinson RJA (1999) Comparison of gut morphology and gut microflora of seven species of mud shrimp (Crustacea: Decapoda: Thalassinidea). *Mar Biol* 133:103–114
38. Finn EH, Rogerson A, Atkinson RJA (1997) Microbial flora associated with the digestive system of *Upogebia stellata* (Crustacea: Decapoda: Thalassinidea). *J Mar Biol Assoc UK* 77:1083–1096
39. Plante CJ, Jumars PA, Baross JA (1990) Digestive associations between marine detritivores and bacteria. *Annu Rev Ecol Syst* 21:93–127
40. Plante CJ, Mayer LM, King GM (1996) The kinetics of bacteriolysis in the gut of the deposit feeder *Arenicola marina*. *Appl Environ Microbiol* 62:1051–1057
41. Polz MF, Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* 64:3724–3730
42. Ravenschlag K, Sahn K, Pernthaler J, Amann R (1999) High bacterial diversity in permanently cold marine sediments. *Appl Environ Microbiol* 65:3982–3989
43. Rhoads DC (1974) Organism–sediment relations on the muddy sea floor. *Oceanogr Mar Biol Annu Rev* 12:263–300
44. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molec Biol Evol* 4:406–425
45. Smith W, Grassle JF (1977) Sampling properties of a family of diversity measures. *Biometrics* 33:283–292
46. Stackebrandt E, Goebel BM (1994) Taxonomic note: A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44:846–849
47. Todorov JR, Chistoserdov AY, Aller JY (2000) Molecular analysis of microbial communities in mobile deltaic muds of Southeastern Papua New Guinea. *FEMS Microb Ecol* 33:147–155
48. Unkles SE (1977) Bacterial flora of the sea urchin *Echinus esculentus*. *Appl Environ Microbiol* 34:347–350
49. Urakawa H, Kita-Tsukamoto K, Ohwada K (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology* 145:3305–3315
50. Vetter YA, Deming JW (1999) Growth rates of marine bacterial isolates on particulate organic substrates solubilized by freely released extracellular enzymes. *Microb Ecol* 37:86–94
51. Ward-Rainey N, Rainey FA, Stackebrandt E (1996) A study of the bacterial flora associated with *Holothuria atra*. *J Exp Mar Biol Ecol* 203:11–26
52. Weissburg M (1992) Functional analysis of fiddler crab foraging: Sex-specific mechanics and constraints in *Uca pugnax*. *J Exp Mar Biol Ecol* 156:105–124
53. Weissburg M (1993) Sex and the single forager: Gender-specific energy maximization strategies in fiddler crabs. *Ecology* 74:279–291
54. Woese CR, Gutell R, Gupta R, Noller HF (1983) Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol Rev* 47:621–669