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Phylogenetic Characterization of the Epibiotic Bacteria Associated with the Hydrothermal Vent Polychaete *Alvinella pompejana*

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***Alvinella pompejana* is a polychaetous annelid that inhabits active deep-sea hydrothermal vent sites along the East Pacific Rise, where it colonizes the walls of actively venting high-temperature chimneys. An abundant, morphologically diverse epibiotic microflora is associated with the worm's dorsal integument, with a highly integrated filamentous morphotype clearly dominating the microbial biomass. It has been suggested that this bacterial population participates in either the nutrition of the worm or in detoxification of the worm's immediate environment. The primary goal of this study was to phylogenetically characterize selected epibionts through the analysis of 16S rRNA gene sequences. Nucleic acids were extracted from bacteria collected from the dorsal surface of *A. pompejana*. 16S rRNA genes were amplified with universal bacterial primers by the PCR. These genes were subsequently cloned, and the resulting clone library was screened by restriction fragment length polymorphism analysis to identify distinct clone types. The restriction fragment length polymorphism analysis identified 32 different clone families in the library. Four of these families were clearly dominant, representing more than 65% of the library. Representatives from the four most abundant clone families were chosen for complete 16S rRNA gene sequencing and phylogenetic analysis. These gene sequences were analyzed by a variety of phylogenetic inference methods and found to be related to the newly established epsilon subdivision of the division *Proteobacteria*. Secondary structural model comparisons and comparisons of established signature base positions in the 16S rRNA confirmed the placement of the *Alvinella* clones in the epsilon subdivision of the *Proteobacteria*.**

Integrated bacterial associations with animals are widely distributed in both terrestrial and marine systems. Although many of these relationships appear obligatory, very little is known about the functional role of the bacteria. Epibiotic associations of bacteria with metazoans are common in the marine environment. Many of the affiliations are monospecific and invariant in nature, often involving a single bacterial species with a specific host group. Bioluminescent bacteria colonizing the light organ of the sepiolid squid *Euprymna scolopes* constitute a single species of *Vibrio fischeri* (32). Similarly, a sulfur-oxidizing bacterium found associated with the nematode *Laxus* sp. appears to be monospecific and closely related to a group of sulfur-oxidizing endosymbiotic bacteria grouped within the gamma subdivision of the division *Proteobacteria* (35). However, more frequently, these epibiotic assemblages are composed of a phenotypically diverse group of microorganisms, which further complicates resolving the contribution of each individual member with its host. One of the more dramatic examples of invertebrate-bacterium associations are those found in certain sponges. In the majority of the large marine sponges, dense populations of bacteria reside in the intercellular spaces constituting up to one-third of the volume of the sponge. These bacterial populations are composed typically of between four and seven different morphological types (44). Although there have been several attempts to isolate these bacteria free from their hosts, few of these isolates have been confirmed to be the symbionts. In these and other similar associations, the task of distinguishing and characterizing the important members of these epibiotic associations from those opportunistic bacteria has remained, until recently, intractable.

One of the more remarkable members of deep-sea hydrothermal vent communities is the colonial, tube-dwelling, polychaetous annelid *Alvinella pompejana* found inhabiting the walls of actively venting chimneys (9). Now thought to be one of the most thermotolerant metazoans known, *A. pompejana* appears restricted to the high-temperature vent sites from 17°S to 21°N on the East Pacific Rise (4, 10, 48). The harsh chimney environment is characterized by extreme temperature gradients (2 to 350°C) and high concentrations of sulfide and heavy metals in the vicinity of the worm colonies. A highly diverse and dense assemblage of microorganisms, including a filamentous morphotype, inhabits the dorsal surface of this worm (8, 17). This filamentous morphotype appears to dominate the population and is often found originating from small expansions of the worm's epidermis within the intersegmentary spaces (17). To date, this filamentous morphotype has eluded all attempts at culturing (11, 23, 36). Several studies have suggested that the bacteria participate in the nutrition of the host or in the detoxification of the host's immediate environment (1, 16). However, these hypotheses have remained merely suggestive because of the inherent difficulty in working with this deep-sea polychaete; *A. pompejana* rarely survives trips to the surface, and the dominant epibiont morphotypes have not yet been cultured successfully.

The analysis of 16S rRNA sequences has recently revolutionized the study of the ecology and diversity of natural populations of microorganisms over that which was possible with more traditional microbiological culturing techniques. By linking a sequence analysis approach with current cloning technol-

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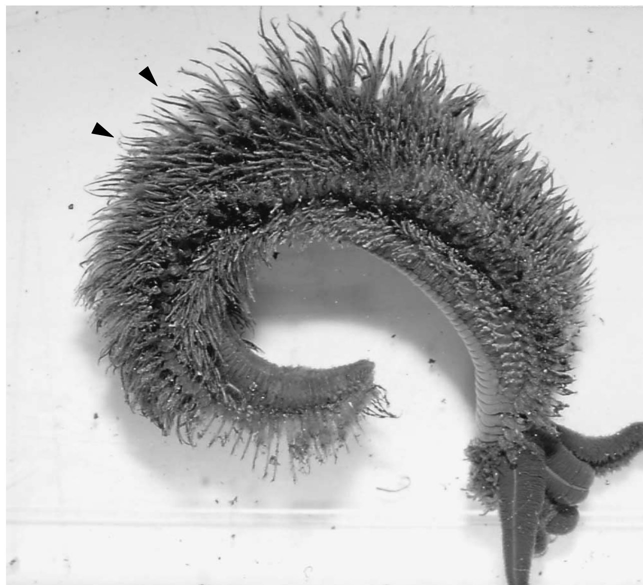

FIG. 1. Dorsal view of a specimen of *A. pompejana*. Filamentous bacteria are associated with cuticular secretions forming dense aggregates extending from the worm's integument. Arrows indicate approximate positions of bacterial sampling for this study.

ogies, a more complete view of the structure and composition of microbial communities from many systems is beginning to emerge. Novel 16S rRNA genes have been identified from bacterioplankton samples collected from the Sargasso Sea which do not resemble any previously known bacterial species (2, 19). In addition, unique 16S rRNA genes belonging to the domain *Archaea* have recently been detected in the Pacific Ocean (6, 15). In these and other studies, 16S rRNA sequence analysis has provided a powerful means to examine the diversity of natural microbial populations in marine and freshwater systems, avoiding reliance on cultivatability. This study represents the first attempt to dissect and characterize the evolutionary relationships of a complex epibiotic microbial community without the necessity of cultivation. Characterizing the dominant members of the microflora of *A. pompejana* provides essential information necessary to resolve the role of these bacteria.

MATERIALS AND METHODS

Sample collection. Specimens of *A. pompejana* were collected in October 1991 during the joint French-U.S. Hydrothermal Environment Research Observatory expeditions at the Elsa vent site 13°N (2,620 m) on the East Pacific Rise (12°48′N, 103°56′W). Individual worms were collected from worm colonies along the walls of active chimneys with the deep-submergence vehicle *Alvin*. Specimens were kept cold ($<$4°C) in a thermally insulated container until surfacing. Once on board, the animals were immediately frozen in liquid nitrogen and kept at −80°C until needed.

Nucleic acid purification. A small patch of bacteria, including a portion of worm integument, was removed asceptically from the dorsal surface of frozen specimens of *A. pompejana* (Fig. 1). The sample was completely homogenized in 5 M guanidinium isothiocyanate–50 mM Tris (pH 7.4)–25 mM EDTA–0.8% 2-mercaptoethanol with a Dounce homogenizer. The homogenate volume was doubled with 50 mM Tris-Cl (pH 8.0)–25 mM EDTA and centrifuged at 14,350 relative centrifugal force for 15 min at room temperature to remove cellular debris. Nucleic acids were precipitated with isopropanol at room temperature for 1 h. The precipitate was collected by centrifugation, washed with ice-cold 70% ethanol, and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]). The extracted bulk nucleic acids were treated with proteinase K (a final concentration of 500 μg/ml at 50°C for 1 h) and extracted twice with an equal volume of phenol-chloroform (4:1) and once with an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acids were ethanol precipitated overnight at −20°C. The precipitate was collected following centrifugation as described above, washed with 70% ethanol, and resuspended in TE buffer.

To remove contaminating RNAs, the sample was treated with RNase A (33 ng/μl) for 10 min at 65°C and phenol purified. The integrity of the DNA sample was shown by agarose gel electrophoresis, and the final DNA concentration was determined spectrophotometrically.

Amplification and cloning of 16S rRNA genes. Bacterial 16S rRNA genes were amplified from the *Alvinella* microflora sample by the PCR with two general bacterial 16S rRNA primers: 27F (AGA GTT TGA TCM TGG CTC AG) and 1518R (ACR CCN ACC TAG TGG AGG AA) (18). PCR amplifications were performed on a thermal cycler (Coy Corporation, Ann Arbor, Mich.). The reaction conditions were as follows: 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$; 0.2 mM each dATP, dCTP, dGTP, and dTTP, 0.2 μM each amplification primer, 10 ng of purified template DNA, 2.5 U of *Taq* polymerase (Promega Corp., Madison, Wis.), in a total volume of 100 μl. Amplification profile conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 25 to 30 cycles. The resulting PCR products were ethanol precipitated and analyzed by agarose gel electrophoresis.

The amplified genes were cloned with the plasmid vector pCRII (Invitrogen, San Diego, Calif.) as described in the manufacturer's instructions. The ligation was performed with PCR products obtained from a reaction after only 25 cycles. The PCR product was ethanol precipitated prior to ligation. Plasmid DNA was prepared from each of the confirmed transformants by an alkaline lysis preparation protocol (42). The purified plasmids were resolved on a 1% agarose gel in 1× TAE (40 mM Tris-acetate [pH 7.6], 1 mM EDTA) and visualized with ethidium bromide staining. Transformant plasmid DNAs which comigrated with the 1.5-kb insert control were scored preliminarily as having a full-length 16S rRNA gene. Stab cultures of positive clones were made in minimal media for long-term storage.

RFLP analysis. To screen the clones into similar clone types, clones containing full-length inserts were subjected to restriction fragment length polymorphism (RFLP) analysis. The cloned insert was reamplified by use of the PCR conditions and primers described above. One microliter of a 1:10 dilution of each of the alkaline lysis plasmid DNA preparations was used as the starting template.

Each PCR product was digested with two restriction endonucleases, *Hae*III and *Mbo*I (Promega), each of which recognizes a 4-bp restriction site. Five microliters of each PCR reaction product was digested with 2.5 U of each enzyme in 0.5× restriction buffer (5 mM Tris-Cl, 5 mM $MgCl_2$, 25 mM NaCl, 0.5 mM dithiothreitol [pH 7.9]) at 37°C for 2 h. The final reaction volume was 10 μl. The entire reaction volume was electrophoresed through a 3% NuSieve agarose gel in 1× TAE (42).

Gene sequencing. A representative clone from each of the four numerically dominant clone families resolved by the RFLP analyses was sequenced bidirectionally with a combination of both automated and manual plasmid sequencing. In addition, for intraclone family comparisons, five clones from each of the four dominant families were partially sequenced from the 5′ and 3′ regions of the 16S rRNA gene. Template plasmid DNA for both sequencing techniques was prepared with Magic Mini plasmid purification kits (Promega). Automated sequencing was performed on an ABI model 373A automated gene sequencer. Manual sequencing was performed by standard dideoxynucleotide-terminated sequencing protocols with a Sequenase v2.0 DNA sequencing kit (U.S. Biochemical Corp.) and α-^{35}S-dATP.

Phylogenetic analysis. The alvinellid clone 16S rDNA sequences were aligned manually with a subset of other aligned bacterial 16S rRNA sequences obtained from the Ribosomal Database Project (RDP) (29). In addition, the 16S rRNA sequence for *Desulfurella acetivorans* (EMBL accession number X72768) was aligned and included in the analysis. The conserved regions and established secondary structural models of the 16S rRNA sequence were used as guides to ensure a correct alignment of the homologous regions of the sequences. Sequence data were manipulated with the Genetic Data Environment v2.0 sequence analysis software package (45). Phylogenetic trees were edited with the Treetools program (provided by Mike Maciukenas from the University of Illinois at Urbana-Champaign for the RDP). Programs used to infer phylogenetic trees are contained in the Phylip v3.5c software package (13). DNADIST was used to calculate evolutionary distances with the Kimura two-parameter model for nucleotide change and a transition/transversion ratio of 2.0 (26). Phylogenetic trees were reconstructed from evolutionary distance data by the neighbor-joining method (41), implemented through the program NEIGHBOR. Parsimony trees were reconstructed with DNAPARS. A total of 100 bootstrapped replicate resampling data sets for both DNADIST and DNAPARS were generated with SEQBOOT with random sequence addition and global rearrangment. Secondary structural models for the cloned 16S rDNA sequences were obtained with the gRNAID v1.4 program (provided by Shannon Whitmore) and optimized by comparison with other established 16S rRNA secondary structures (20).

RESULTS

Clone library construction. DNA was isolated from a sample of the epibiotic microflora obtained from an *A. pompejana* worm. The bacterial 16S rRNA genes were amplified from this preparation and cloned directly into a plasmid vector. From

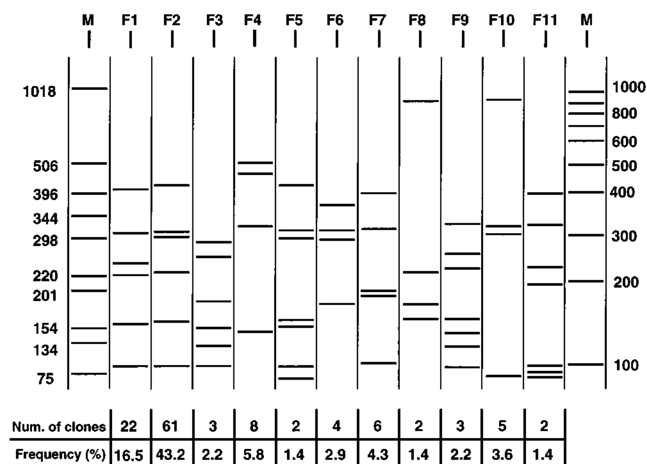

FIG. 2. Scaled illustration of the restriction patterns of 16S rDNAs representing 11 clone families identified in the alvinellid clone library. Each of these families contain two or more representative clones. Molecular weight standards (M) are included for comparison. The fragment sizes are given in base pairs. The table at the bottom of the figure shows the numerical distribution of the alvinellid clones within each specific family containing more than one representative.

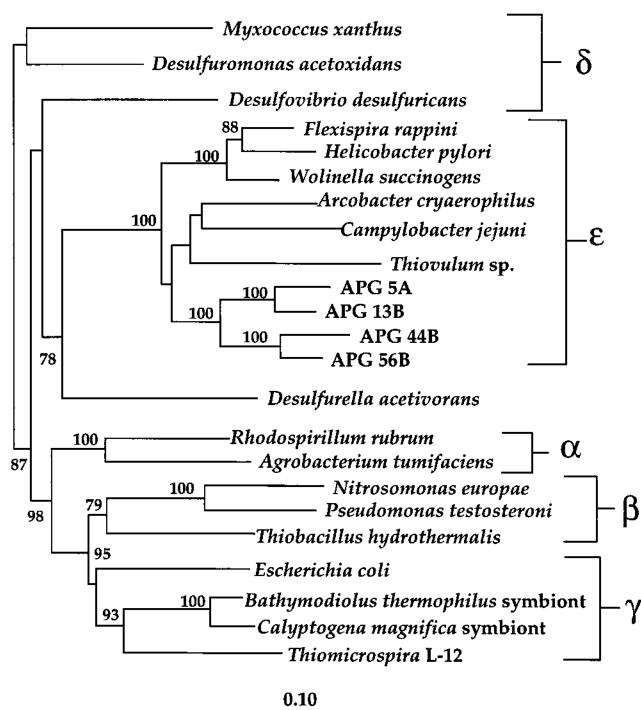

FIG. 3. Phylogenetic tree showing the relationship of the alvinellid clones to the other members of the *Proteobacteria*. This tree was inferred from 16S rRNA sequence data by the neighbor-joining method. Molecular sequences for all reference strains (except *D. acetivorans*) were obtained from the RDP. Boldface type indicates 16S rRNA sequences cloned from the epibiotic microbial population of a single *A. pompejana* specimen. A total of 1,084 nucleotide positions were included in the analysis. The tree was rooted with the sequence of *Bacillus subtilis*. The scale bar indicates 0.1 fixed mutation per nucleotide position. Numbers refer to bootstrap values for each node out of a total of 100 replicate resamplings (values below 75 are not shown).

two separate cloning attempts, a total of 162 putative transformants were screened and 139 of these contained inserts of the correct size. The first cloning experiment resulted in poor transformation efficiency due to the competency of the host cells. Comparison of the two cloning attempts provides no information as to the robustness and randomness of the libraries. Clones containing full-length inserts from both attempts were grouped into one library, with the clones from the first attempt being designated APG #A, while those from the second library were designated APG #B.

To identify unique clone types, clones confirmed to contain a full-length insert were subjected to RFLP analysis. The clones were then grouped into families on the basis of their restriction pattern; that is, clones with identical restriction patterns were placed into the same family. The restriction analysis of the 139 full-length clones identified 32 distinct clone families. Figure 2 is a schematic representation of the restriction patterns of 11 representative clone families. This figure illustrates some of the diversity of restriction patterns seen in the library.

Figure 2 also shows the numerical distribution of each of the clone families with more than one representative. Four of these families, designated F1, F2, F9, and F13, dominated the library, representing 16.5% (23 clones), 43.2% (60 clones), 5.8% (8 clones), and 4.3% (6 clones) of the library, respectively. The remaining families were represented by fewer members, with the majority of the families containing only one member. Verification of the RFLP clone family groupings was obtained by comparing partial 16S rRNA sequences of five representative clones from each of the four dominant families. In each case, all representatives from each family showed complete identity over the 500 bases sequenced. On the basis of the assumption that the numerical dominance of the bacterial species will be reflected in the clone library, representative clones from the four most dominant families (F1, F2, F9, and F13) were chosen for complete sequencing of the cloned insert. Because there is no evidence at this time to support this assumption, it has merely served as a starting point in the analysis of the microbial population associated with *A. pompejana*. The clones representing these four families are as follows: APG 5A (F1), APG 13B (F2), APG 56B (F9), and APG 44B (F13).

The 16S rRNA gene sequences for the four alvinellid clones have been deposited in GenBank under the following accession numbers: clone APG 5A, L35523; clone APG 13B, L35520; clone APG 44B, L35521; and clone APG 56B, L35522.

Phylogenetic analysis. Three different analyses were performed to determine the correct phylogenetic placement of the four alvinellid clones: (i) phylogenetic tree reconstruction inferred from both evolutionary distance and character-based calculations, (ii) comparison of secondary structural models of the clone 16S rRNA with other established 16S rRNA secondary structures, and (iii) comparison of established signature base positions in the 16S rRNA molecule.

Figure 3 is a phylogenetic tree inferred from the neighbor-joining method of phylogenetic tree reconstruction. This analysis includes the four alvinellid clone 16S rRNA sequences, a subset of reference sequences from the RDP, and the 16S rRNA sequence from a newly characterized bacterium, *D. acetivorans* (38). Regions of ambiguous alignment, i.e., positions 1 to 11, 69 to 102, 128 to 132, 176 to 226, 265 to 269, 451 to 480, 840 to 869, 999 to 1045, 1124 to 1185, 1262 to 1298, and 1357 to 3′ terminus (by the *Escherichia coli* numbering system) were excluded from the analysis. A total of 1,061 nucleotide positions was used in the analysis. Bootstrap values corresponding to 100 replicate resamplings are shown above the lines leading to each branch, unless designated otherwise.

The four alvinellid clone 16S rRNA sequences form their own cluster within the epsilon subdivision of the division *Proteobacteria* (Fig. 3). The alvinellid clones cluster together to the

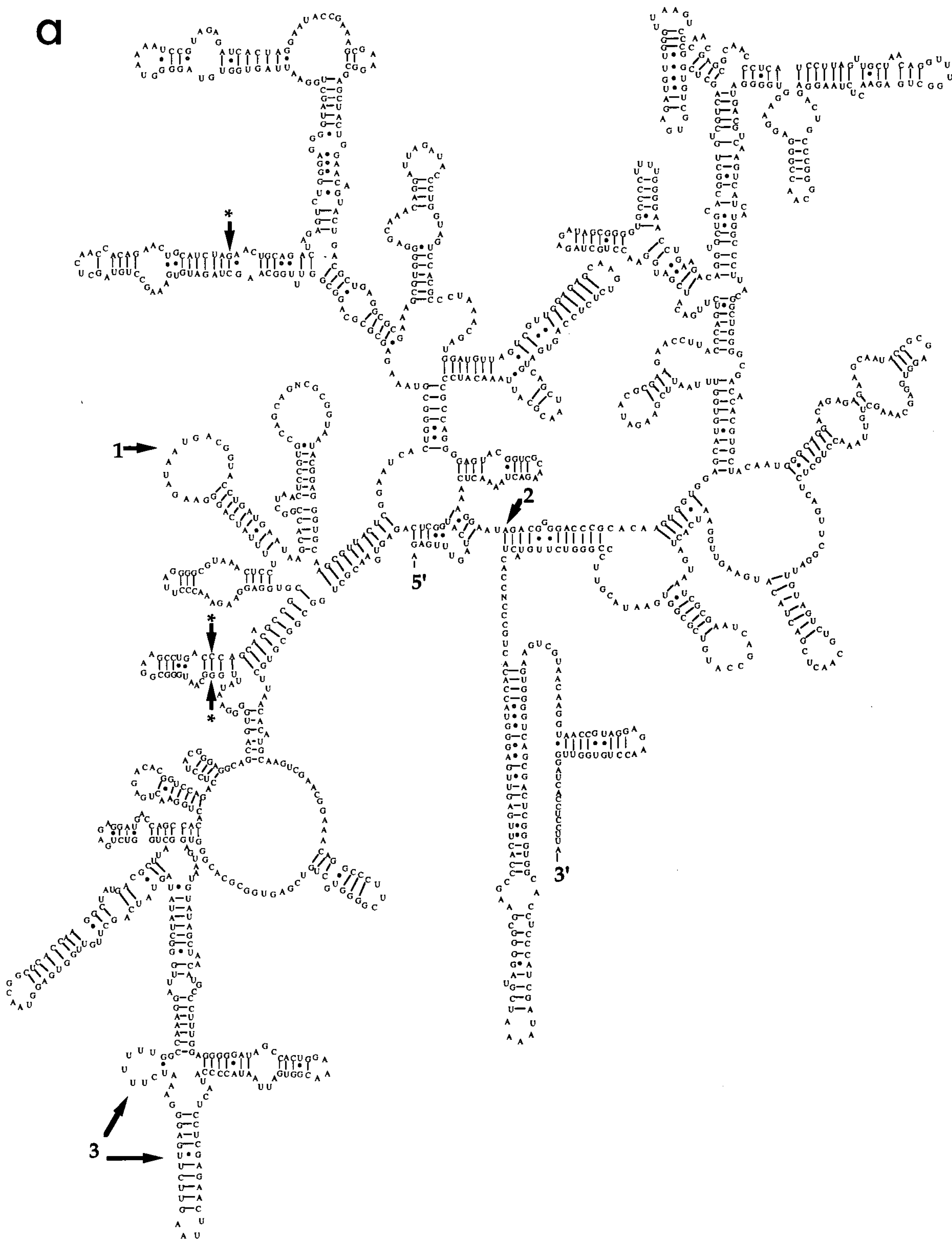

FIG. 4. Proposed secondary structural model of clones APG 13B (a) and APG 56B (b) 16S rRNA. Numbered positions denote secondary structural features characteristic of the epsilon subdivision of the *Proteobacteria* which is discussed in text. Positions with asterisks denote signature nucleotide discrepancies which were further examined for compensatory base pair changes. This structural model was obtained with the gRNAID v 1.4 program and comparative analysis with other bacterial 16S rRNA structures (20).

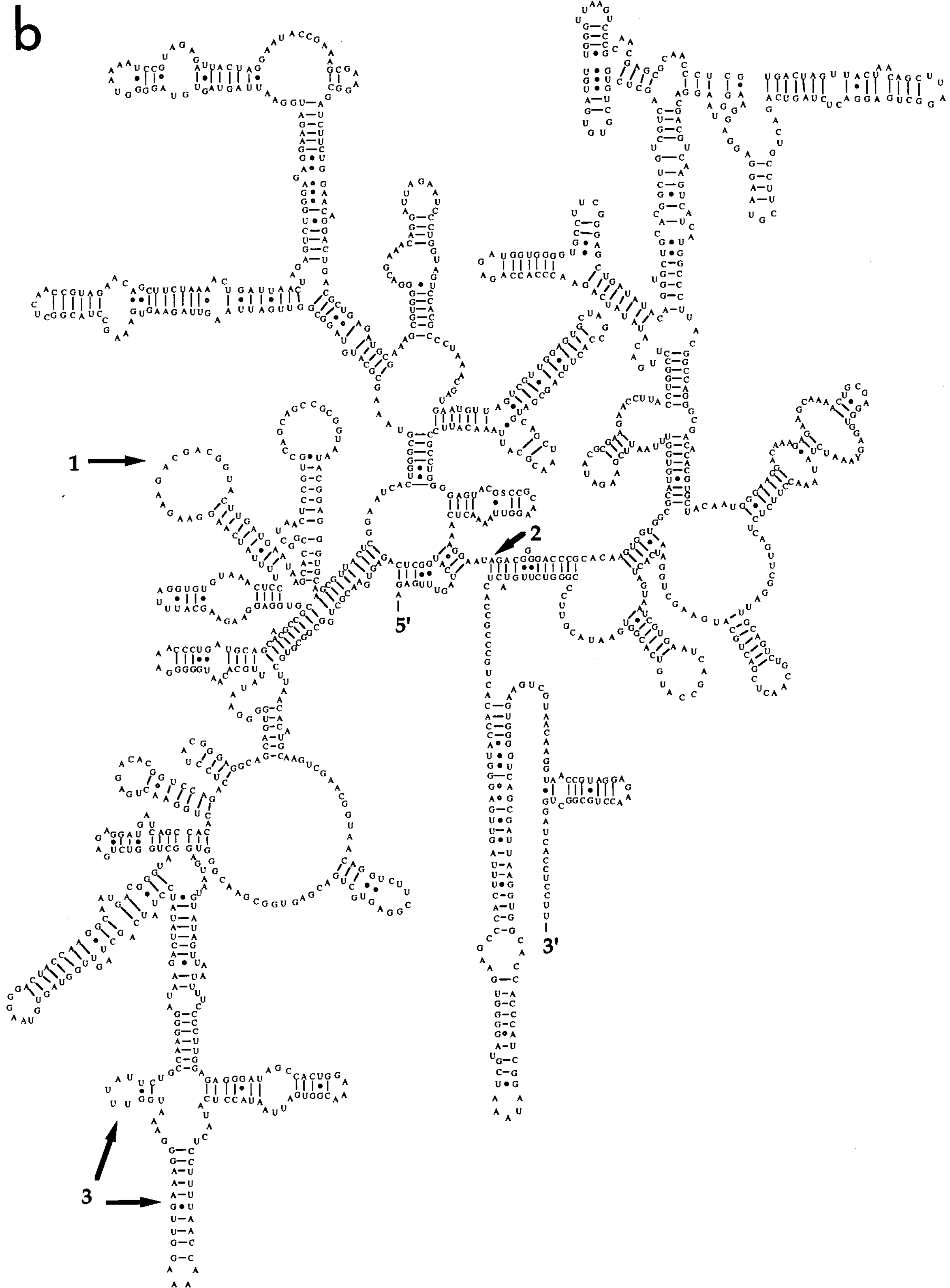

FIG. 4—*Continued.*

TABLE 1. Signature base positions distinguishing the delta and epsilon subdivisions of the *Proteobacteria* and the alvinellid clones[a]

Position	Signature base position[b]		
	Delta	Epsilon	APG clones
6	G	G	N.D.
7	G	G	N.D.
44	G	G	G
50	A	A	A
107	G	G	G
108	**C**	**C:u**	**A:c**
124	G	A	A
129	U	U	U
129:1	A	A	A
233	Y	C:R:u	G:a
236	G	A	A
237	C	U	U
242	C:g	U:c	U
284	G:c	G	G
370	**C**	**C**	**C:G**
371	G:a	U:g:a	R
390	C:u	A:c:u	Y
391	**G**	**G**	**G:C**
398	C	C	C
438	G:u	U	U
449	A	A	A
485	G	G	G
496	G:a	A	A
502	A:g	A:u	A
513	C	C	C
538	G	G	G
543	U:c	U	U
554	U:a	C	C
564	U:c	C	C
640	**A**	**A**	**R**
689	R:u	G	G
690	G:a	G	G
698	Y:a	C	C
722	G:a	A	A
760	G	U:g	G
812	G	C	C
822	R:u	A:U:g:c	R
823	R:u	A	A
825	A:g	R	G
871	U	U	U
875	U:c	Y	C
877	Y:a	U	U
878	Y:a	A:U:c:g	Y
916	G	G	G
929	G	A	A
947	G:u	G	G
948	Y	C	C
976	G	G:a	G
1015	A:g	G:a	G:a
1024	G:c	G	G
1026	G	G:u	G
1116	Y	C	C
1120	Y:G	Y:A	A:C
1153	N	R:U	U:G
1219	A	R	R
1233	R	G	G
1234	C:a	C	C
1246	G:u	R	G
1252	A	A:U	A:u
1260	G:Y	A:g	A:g
1291	C:g	Y	C
1297	Y	Y	U
1298	C:A	C	C:a
1325	C	C:g	C
1421	Y	U	U
1426	U:R	U	U
1431	Y:a	Y	C
1437	C	C:g:a	G
1441	G:u	A	A
1443	G	R	G

[a] The information for the delta subclass was taken from Table 4 of Woese (50) and Table 1 of Rainey et al. (38). The information for the epsilon subdivision was compiled from an alignment of 51 16S rRNA sequences of the epsilon subdivision of the *Proteobacteria*. Positions in boldface indicate nucleotide differences between the epsilon subdivision of the *Proteobacteria* and the alvinellid clones.

[b] Uppercase letter, major base (if no other specified, it accounts for >90% of assayable cases); lower case letter, minor-occurrence base (found in <15% of assayable cases or in only one sequence). Y, pyrimidine; R, purine; N, any nucleotide; N.D., not determined.

exclusion of other species, supported by a bootstrap value of 100%. This cluster clearly groups within the epsilon subdivision of the *Proteobacteria*, also supported by a bootstrap value of 100%. An identical tree topology was also obtained with the parsimony method, with bootstrap values of 99 and 100% showing the robustness of these two branches (data not shown). Clones APG 5A and APG 13B are more closely related within this clade, as are APG 44B and APG 56B.

Higher-order structural features of the 16S rRNA molecule are useful in the phylogenetic characterization of microbial groups (50). Because 16S rRNA sequences for the alvinellid clones APG 5A and APG 13B and for APG 44B and APG 56B are very similar, it would be unlikely that reconstruction of a 16S rRNA secondary structural model for all four clone sequences would reveal significant defining characters. For this reason, secondary structural models for only two of the alvinellid clones were generated. The alvinellid clones APG 13B and APG 56B were chosen to represent their respective groups.

Figure 4 shows the proposed secondary structures of two alvinellid clone sequences, APG 13B and APG 56B. Both of these structures are consistent with the general topological features of other established bacterial 16S rRNA secondary structures. Several structural features which support the placement of the alvinellid clones within the epsilon subdivision of the *Proteobacteria* were observed. Three secondary structural features characteristic of the epsilon subdivision of the *Proteobacteria* (then referred to as the *Campylobacter-Thiovulum* subdivision) have been reported previously (28). All three of these structural features were observed in our alvinellid clones (position numbers are those of the 16S rRNA sequence of *E. coli*): (i) a conserved deletion of a helix corresponding to positions 455 to 477, (ii) a unique compensatory base pair change at positions 921 (U to A) and 1396 (A to U), and (iii) a long-short stem arrangement involving stem structures at positions 184 to 193 and 198 to 219 (Fig. 4a and b). In addition to these, another feature was observed which has been reported among the epsilon subdivision of the *Proteobacteria* (38), an insertion of a cytosine in a conserved loop structure corresponding to positions 1357 to 1365.

To confirm the results from the previous two phylogenetic analyses, we compared specific base positions of the cloned sequences with signature base positions distinguishing the subdivisions of the *Proteobacteria*. Table 1 shows the signature base positions compiled for the epsilon and delta subdivisions of the *Proteobacteria*. A total of 72 signature positions were examined. Of these 72 positions, 48 were found to differ be-

tween the delta and epsilon subdivisions. These signature base positions were also examined in the alvinellid clones. Of the 48 positions distinguishing the deltas from the epsilons, only 4 were found to differ between the epsilons and the alvinellid clones (see highlighted positions in Table 1). To rule out the possibility that these differences were due to experimental artifacts, these same positions were reexamined in the clone 16S rRNA secondary structural models. The discrepancies at positions 370, 391, and 640 were seen in two of the alvinellid clones, APG 44B and APG 56B. Secondary structure analysis for clone APG 56B supports these three discrepancies, revealing compensatory base pair changes at the adjoining positions in the stem structures of the molecule (Fig. 4b). Compensatory base pair changes are changes in variable nucleotides which preserve the conserved secondary structure of the 16S rRNA molecule (21). This type of comparison is not possible with the difference at position 108 because it occurs in a nonstem structure.

DISCUSSION

Bacterium-invertebrate symbioses are well recognized in marine systems. However, defining the fidelity of these relationships is frequently problematic. The partners are usually so intricately interrelated, both morphologically and metabolically, that resolving the level of interaction has been impossible. Although considerably more is known about monospecific endosymbiotic associations in certain marine invertebrates (3, 14), there are numerous cases of diverse microbial assemblages, found obligately associated with the external surfaces of certain invertebrates, which have eluded sufficient explanation (5, 25, 39). Molecular genetic techniques now provide a tool with which to dissect and study these highly integrated symbioses.

The hydrothermal vent polychaetous annelid *A. pompejana* possesses a morphologically and metabolically diverse assemblage of microorganisms associated with the worm's dorsal surfaces, some of which appear specifically attached (8, 17, 24, 36, 37). It has been suggested that the dominant integrated morphotype, a filament, plays a significant role in either the nutrition of its host or in the detoxification of the worm's immediate environment (16). The primary focus of this study was to examine the diversity of this unique microflora and, through the approach presented here, to characterize phylogenetically several of the dominant phylotypes.

The initial RFLP analysis of the alvinellid clone library grouped the 139 clones into similar clone types or families. The analysis identified 32 distinct clone families. Four of these clone families collectively represented more than 69% of the total number of clones, with the remaining families consisting of only one or a few members. There is no evidence to suggest the assumption that the distribution of clone types in the library represents the actual distribution in the natural population. However, the numerical distribution of the dominant clone families agrees with previous electron microscopic observations, which demonstrated that a filamentous morphotype dominated the microbial biomass (17). The choice of clone types to characterize phylogenetically was based on their numerical dominance in the library.

The 16S rRNA sequences of individual alvinellid clones representing the four dominant clone families and a subset of other representative bacterial 16S rRNA sequences were used to infer their evolutionary relationships. Both parsimony and distance phylogenetic inference methods placed the four alvinellid clones as a unique cluster within the epsilon subdivision of the division *Proteobacteria*. Both methods gave a bootstrap value of 100%, illustrating the robustness of this placement. The alvinellid cluster is shown as a sister lineage to the *Campylobacter-Thiovulum* clade. However, this association is not strongly supported; the corresponding bootstrap values are 57 and 66%, obtained with the neighbor-joining and parsimony methods, respectively. The next most strongly supported placement of the alvinellid cluster is as a deeper-branching lineage within the epsilon subdivision. It is clear that the alvinellid clones form their own cluster within the epsilon subdivision of the *Proteobacteria* to the exclusion of other species; however, this analysis was not able to resolve the precise placement of the cluster within the epsilon subdivision with a high degree of confidence.

The comparison of completed secondary structural models for two of the alvinellid clone 16S rRNAs with other established bacterial 16S rRNA secondary structures revealed several features that supported the inclusion of the alvinellid clones within the epsilon subdivision of the *Proteobacteria*. Lane and colleagues (28) reported three important structural features which were conserved among several members of the epsilon subdivision (then referred to as the *Campylobacter-Thiovulum* subdivision). All three of these structural features were present in the alvinellid clones. In addition, an insertion of a cytosine residue in the conserved loop structure corresponding to nucleotide positions 1357 to 1363 (*E. coli* numbering system) was found in all of the alvinellid clones. The significance of this insertion was first reported in a 16S rRNA structural analysis of *D. acetivorans*, a more distant relative of the epsilon subdivision of the *Proteobacteria* (38). A thorough analysis of other members of the epsilon subdivision supports this insertion as being highly conserved. Examination of both the 16S rRNA secondary structure of another epsilon subdivision proteobacterial member, *Campylobacter sputorum* subsp. *sputorum* (20), and of a general alignment of epsilon subdivision 16S rRNA sequences obtained from the RDP suggests an equivalent insertion at this position. Examination of other proteobacterial 16S rRNA sequences showed that an insertion at this position occurs in only 10 of 925 available 16S rRNA sequences from the four other established proteobacterial subdivisions. Thus, this insertion has the potential to become a defining character of the epsilon subdivision of the *Proteobacteria*.

The presence of certain 16S rRNA signature nucleotides of the division *Proteobacteria* (50) provides the final criterion with which to evaluate the phylogenetic position of an organism within the division. Because the epsilon subdivision is a newly recognized member of the *Proteobacteria*, the signature base positions for this group have been compiled only recently (38). A total of 72 positions were examined in 51 available epsilon subdivision 16S rRNA sequences from the RDP. Comparison of these signatures with those of the alvinellid clones was consistent with their placement in the epsilon subdivision of the *Proteobacteria*. Only four positions were found to differ significantly between the alvinellid clone sequences and those of the epsilon subdivision, and at least three of these differences appear to be real characteristics of the clone sequences. In each of these cases, the nucleotide differences were supported by compensatory base pair changes in adjoining nucleotide positions of the clone secondary structure in which they occur. This type of analysis has been used previously to rule out nucleotide differences which might be due to sequencing errors or PCR artifacts (7).

Recently, a newly isolated bacterium, *D. acetivorans*, was characterized phylogenetically as a distant relative to the epsilon subdivision of the *Proteobacteria*, possibly representing a new subdivision of the *Proteobacteria* (38). These investigators

used phylogenetic tree reconstruction and comparison of signature nucleotides of 16S rRNA sequences to infer the evolutionary relationship of this thermophilic sulfur-reducing bacterium. The analysis presented in the current report confirms the phylogenetic position of *D. acetivorans* proposed by Rainey and coworkers; however, it has identified several discrepancies between a number of the key signature nucleotides for the epsilon subdivision of the *Proteobacteria* used in their analysis. Although it is not possible to resolve these inconsistencies at this time, further examination of additional epsilon representatives will more firmly establish the epsilon nucleotide signatures for use in future studies.

It is often difficult to infer the conserved phenotypic characteristics of a particular group of microorganisms on the sole basis of their phylogenetic relatedness. However, phenotypic qualities that appear in ancestral branches and within more closely related species may provide some insight into the metabolic potential and ecology of unknown but related organisms. Currently, there are seven known genera that form the two clusters comprising the epsilon subdivision of the *Proteobacteria* (12, 30, 31, 33, 34, 40, 43, 47, 49). A survey of the conserved phenotypic characteristics among these epsilon subdivision genera reveals that the majority are adapted to environments low in oxygen; they range from microaerophiles to some which are able to grow anaerobically, reducing available seawater sulfate (22, 27, 49). In addition, all of these genera, except the sulfur-oxidizing marine bacterium *Thiovulum* sp., have been found associated with eukaryotic organisms. These conserved phenotypic characteristics are consistent with the environment of the alvinellid epibionts. As well as being associated with a metazoan host, these bacteria must also be adapted to the hypoxic conditions within the tubes of *A. pompejana* produced by elevated temperatures and the abundance of reduced compounds in the chimney end-member fluids (4).

In conclusion, the molecular approach described here has allowed, for the first time, the assessment of the microbial diversity of a complex epibiotic bacterial population associated with a marine invertebrate. From an established 16S rRNA clone library, several dominant phylotypes were characterized as members of the epsilon subdivision of the division *Proteobacteria*. These are the first marine epsilon subdivision members characterized from hydrothermal vent systems. Studies are currently under way to design and test oligodeoxynucleotide probes specifically targeting regions of the *Alvinella* epibiont cloned 16S rRNAs. These clone-specific probes are being used currently for in situ hybridization experiments to verify the morphology of the clone types. The same probes will then be used to screen high-molecular-weight DNA libraries established directly from epibiont nucleic acid samples. This approach has been applied before to identify recombinants containing functional genes from an unculturable free-living marine archaeoplankton (46). Through these molecular approaches, the metabolic potential of the specific members of the epibiotic community and possible function of their relationship with *A. pompejana* may be determined without the need to culture the organisms.

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