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Molecular Identification and Localization of Filamentous Symbiotic Bacteria Associated with the Hydrothermal Vent Annelid *Alvinella pompejana*

S. CRAIG CARY,[1]* MATTHEW T. COTTRELL,[1] JEFFREY L. STEIN,[2] FRANCISCO CAMACHO,[3] AND DANIEL DESBRUYÈRES[4]

College of Marine Studies, University of Delaware, Lewes, Delaware 19958[1]; Recombinant Biocatalysis, Inc., La Jolla, California 92037[2]; Department of Botany, Oregon State University, Corvallis, Oregon 97331[3]; and IFREMER, Centre de Brest, F-29273 Brest Cedex, France[4]

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Alvinella pompejana **is a polychaetous annelid that inhabits high-temperature environments associated with active deep-sea hydrothermal vents along the East Pacific Rise. A unique and diverse epibiotic microflora with a prominent filamentous morphotype is found associated with the worm's dorsal integument. A previous study established the taxonomic positions of two epsilon proteobacterial phylotypes, 13B and 5A, which dominated a clone library of 16S rRNA genes amplified by PCR from the epibiotic microbial community of an *A. pompejana* specimen. In the present study deoxyoligonucleotide PCR primers specific for phylotypes 13B and 5A were used to demonstrate that these phylotypes are regular features of the bacterial community associated with *A. pompejana*. Assaying of other surfaces around colonies of *A. pompejana* revealed that phylotypes 13B and 5A are not restricted to *A. pompejana*. Phylotype 13B occurs on the exterior surfaces of other invertebrate genera and rock surfaces, and phylotype 5A occurs on a congener, *Alvinella caudata*. The 13B and 5A phylotypes were identified and localized on *A. pompejana* by in situ hybridization, demonstrating that these two phylotypes are, in fact, the prominent filamentous bacteria on the dorsal integument of *A. pompejana*. These findings indicate that the filamentous bacterial symbionts of *A. pompejana* are epsilon *Proteobacteria* which do not have an obligate requirement for *A. pompejana*.**

The polychaetous annelid *Alvinella pompejana* inhabits the deep-sea hydrothermal vent environment, which is considered to be one of the most formidable biomes on earth. This environment is characterized not only by its high temperature but also by high concentrations of hydrogen sulfide (>1 mM) and heavy metals (0.3 to 200 μM), such as silver, copper, zinc, and cadmium (9). The papery dwelling tubes of *A. pompejana* encrust the walls of active chimneys at five of the known vent sites along the East Pacific Rise. The temperature of the worm's immediate environment ranges from 20°C to at least 40°C (7) and can be as high as 85°C (4), making *A. pompejana* one of the most thermotolerant metazoans yet described.

A distinguishing feature of *A. pompejana* and a congener, *Alvinella caudata*, which inhabits cooler locations in the same environments, is a unique epibiotic bacterial community associated with the worm's integument (11, 12). The most prominent members of the bacterial community are filamentous bacteria closely associated with hair-like structures protruding from the worm's integument. In addition to these conspicuous filaments, three other morphological types are present, namely, single cells distributed on the integument, clump-like associations located in the intersegmentary spaces, and filaments inserted into the worm's posterior parapodia. This community is also metabolically diverse, consisting of both aerobes and facultative anaerobes (20). The epibiotic bacteria include sulfur oxidizers, sulfate reducers, nitrifiers, nitrate respirers, denitrifiers, and nitrogen fixers. Furthermore, many of these strains are resistant to cadmium, zinc, arsenate, and silver and tolerate high concentrations of copper (15).

The association between *A. pompejana* and this bacterial community is certainly symbiotic in the most general sense; that is, the symbiosis consists of a cohabitation of dissimilar organisms. However, the manner in, and degree to which, either player benefits from the association are unclear and are the focus of our research. Almost nothing is known about the interaction between these symbionts and their invertebrate host, although it has been suggested that these epibiotic bacteria may provide nutrition to the worm or perhaps detoxify the worm's immediate environment (1). None of the bacteria cultivated from *A. pompejana* during several previous studies possess the filamentous morphology of the dominant bacteria in the community, so we performed our study of these filamentous bacteria using tools that do not require their cultivation.

In a previous study, a clone library was constructed of 16S rDNA genes amplified by PCR from the genomic DNA of the microbial community associated with *A. pompejana* (14). Thirty-two clone families were identified by their unique restriction fragment patterns, but four clone families (5A, 13B, 44B, and 56B) were especially abundant, accounting for over 65% of the clones in the library. Phylogenetic analysis of the 16S rRNA genes of the four dominant clone families placed them in two distinct clades within the epsilon subdivision of the *Proteobacteria.*

The hypothesis tested in the present study asserted that the most abundant clone families in the clone library of 16S rRNA genes correspond to the most abundant bacteria in the epibiotic community, that is, those with the filamentous morphotype. We tested the specificities and sensitivities of PCR primers designed to detect and distinguish the two most abundant clone families, 13B and 5A. Using these primers in a diagnostic PCR, we confirmed that these phylotypes are a ubiquitous feature of *A. pompejana* by testing several specimens of *A. pompejana* collected from locations along the East Pacific Rise

* Corresponding author. E-mail: caryc@udel.edu.

separated by as much as 450 km. The highly specific primers were also used to assess the occurrence of these phylotypes within the immediate vicinity of the worm colonies. Finally, we used the phylotype-specific oligonucleotides as in situ hybridization probes to confirm that the most abundant clone families in the library correspond to prominent filamentous bacteria on *A. pompejana.*

MATERIALS AND METHODS

Animal collection. Specimens of *A. pompejana* and *A. caudata* were sampled from active vent sites commonly known as 13°N (12°48′N, 103°56′W) and 9°N (9°50′N, 104°17′W) on the East Pacific Rise at a depth of approximately 2,620 m in November of 1994 and 1995. Animals were collected by the DSV *Alvin* and held in an insulated container which maintained the water at 5°C until surfacing. Once on board, individual specimens were held at 2°C until they were either frozen intact at −80°C or sampled for bacteria and nucleic acids as described below.

Fixation of bacteria. Frozen *A. pompejana* were thawed on ice, and their hair-like structures with associated bacteria were removed from the dorsal integument and fixed for 2 h in 4% formaldehyde freshly prepared in phosphate-buffered saline (PBS). The samples were centrifuged for 60 s at 12,250 × *g*, and the fixative was replaced with 500 μl of PBS before the samples were stored at 4°C.

DNA purification. The hair-like structures with associated bacteria were aseptically removed from freshly collected *A. pompejana* for DNA purification. Clumps of these structures ranging in volume from 50 to 100 μl with their associated bacteria were homogenized in 1 ml of 5 M guanidine thiocyanate–50 mM Tris-HCl (pH 7.4)–25 mM EDTA–0.8% 2-mercaptoethanol. A brief centrifugation was performed to remove the bulk of the mineral grains, and the homogenates were stored at −80°C until DNA extraction was performed. Aliquots (100 μl) of the thawed homogenates were incubated for 1 h with 25 μl of 20% Chelex 100 (23) while being mixed on a rotating wheel. Following a brief centrifugation to remove the Chelex 100, total nucleic acids were extracted with an IsoQuick nucleic acid extraction kit (MicroProbe Corporation, Bothell, Wash.). In accordance with the manufacturer's instructions, the first extraction was performed at 65°C for 10 min and the second extraction of the aqueous phase was done at room temperature. A final concentration of 0.3 M sodium acetate and an equal volume of isopropanol were added to the final aqueous phase to precipitate the nucleic acids that were collected by ultracentrifugation at 24,000 × *g* for 30 min. The pellet was rinsed with 70% ethanol, dried, and dissolved in 50 μl of water. The nucleic acids were quantified spectrophotometrically.

RNA purification. Bacteria were removed and homogenized in guanidine thiocyanate by the method described above for DNA purification. The homogenate volume was doubled with TE (50 mM Tris-HCl [pH 7.6], 25 mM EDTA), and the nucleic acids were precipitated with isopropanol at room temperature. The precipitate was collected by centrifugation, washed with 70% ethanol, resuspended in TE, and treated with proteinase K (500 μg/ml, 55°C, 50 min). The samples were then extracted with phenol-chloroform (4:1), ethanol precipitated, and again suspended in TE. DNA and RNA were separated and purified by isopycnic centrifugation as described by Cary et al. (5) and quantified spectrophotometrically. A final concentration of 1 μg of RNasin (Promega, Madison, Wis.)/ml was added to the RNA samples prior to storage at −80°C.

Probe design. Computer-assisted analyses of the 5A and 13B 16S rRNA gene sequences (14) revealed two variable regions that were ideally suited for the construction of oligodeoxynucleotide probes. The region from nucleotides 1240 to 1268 (*Escherichia coli* numbering system) was chosen as the probe site, because it has proven to be very useful for differentiating symbiotic bacteria in past studies. Five additional representatives of the 5A and 13B clone families (14) were sequenced through this region to verify sequence identity. Potential target sites for probes 19 to 20 nucleotides long were selected and compared to sequences available through the Ribosomal Database Project (17) and GenBank. Oligodeoxynucleotides 19 bases long designated 13B1242R and 5A1243R were synthesized with sequences complementary to a portion of this variable region in clones 13B and 5A, respectively.

Probe specificity. The specificity of each of the clone-specific probes was tested by dot blot hybridization. Total RNA (50 ng) from the bacterial community of *A. pompejana* and a range of representative prokaryotic and eukaryotic organisms was vacuum blotted onto Zeta Probe nylon support membranes (Bio-Rad Laboratories, Richmond, Calif.). 16S rDNA PCR products amplified from clones 5A and 13B were denatured with 0.1 N NaOH and included in the dot blot analysis as controls. The clone-specific oligonucleotide probes were labeled at their 5′ termini with [^{32}P]dATP by following the protocol of Sgaramella and Khorana (22). Labeled probes were purified on C_{18} reverse-phase Sep-Pak columns (Millipore, Milford, Mass.) as described by Lane et al. (16). The hybridization procedures were as reported by Cary et al. (5), and the final wash temperatures used were 5°C below that required to fully remove the probe from the nylon membranes. In addition to the clone-specific probes, a universal probe (1406R), ACGGGCGGTGTGTRC (16), and a eubacterium-specific probe (338R), GCTGCCTCCCGTAGGAGT (3), were used as positive control probes to assess the presence and accessibility of target on the nylon membranes. The complement to the universal probe (1406F) served as a control for nonspecific binding.

PCR sensitivity. The sensitivities with which the 13B and 5A clone-specific primers could detect their targets in PCR amplifications were tested with plasmids mixed with genomic DNAs. Probes 13B1242R and 5A1243R were matched with a universal bacterial forward primer, EubB (13), to amplify a 1.2-kb portion of the 16S rRNA gene. Plasmids from clones 13B and 5A were serially diluted from concentrations approximating 10 ng to 1 fg in 10-fold increments. The total DNA concentration in the reaction mixture was maintained at 10 ng by the addition of an appropriate amount of a mixed genomic DNA extracted from free-living marine bacteria. The amplifications were performed on a PTC 100 thermal cycler (MJ Research, Watertown, Mass.). The reaction mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide, 0.2 μM each primer, and 2.5 U of *Taq* DNA polymerase (Promega) in a total volume of 100 μl. The thermocycling conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. This cycle was repeated 35 times.

Screening of *A. pompejana*, other invertebrates, and rock surfaces for the 13B and 5A phylotypes. The bacterial primer EubB was paired with primers 13B1242R and 5A1243R to assay for the presence of phylotypes 13B and 5A, respectively, on specimens of *Alvinella* spp. collected from both the 9°N and 13°N sites by the methods described above. Other invertebrate genera and rock surfaces found in the vicinity of *A. pompejana* colonies were included in the analysis. The PCR volumes were 20 μl, and the concentrations of the reagents were the same as those used to test the probe sensitivities, except for the concentration of the template DNA, which was 2 to 4 ng/μl. The thermocycling was performed with a RoboCycler Gradient 96 thermocycler (Stratagene, La Jolla, Calif.), with thermocycling conditions including a 3-min denaturation at 94°C, a 3-min primer annealing by a touchdown regimen (8), and a 3-min extension at 72°C. The temperature of primer annealing was 66°C during the first six cycles and decreased 1°C every sixth cycle to 60°C, at which point 10 cycles were performed. A hot start (6) was performed by adding the primers to the reaction mixtures, which were warmed to 85°C before the initial denaturation at 94°C.

The amplification products were cut with the tetrameric restriction enzymes *Hae*III and *Mbo*I, and the resulting fragments were resolved by agarose gel electrophoresis with 3% NuSieve (FMC, Rockland, Maine) agarose. The restriction patterns were compared to those produced by control amplifications with plasmid DNAs from clones 13B and 5A as templates.

Labeling oligonucleotides with fluorochromes for in situ hybridization. The phylotype-specific oligonucleotides used for the in situ hybridization experiments were modified versions of those used in the PCR assays. The modifications included shifting the 3′ ends 1 or 2 bases in the 5′ direction and adding 4 or 5 bases to the 5′ ends to incorporate two additional mismatches. The 22-mer oligonucleotides 13B1244R (AGTTTTGCTTCTCTTTGTCCTT; *E. coli* nucleotide positions 1244 to 1265) and 5A1244R (GATTTCGCTTCTCATTGTCC TC; *E. coli* nucleotide positions 1244 to 1265) differed from each other by five 1-base mismatches. These oligonucleotides with 5′ amino linkers (Genset, La Jolla, Calif.) added were labeled with either fluorescein isothiocyanate (FITC) or Texas Red (Molecular Probes, Eugene, Oreg.) by incubating 100 μg of oligonucleotide with 40 μg of the fluorochrome dissolved in dimethylformamide. The reaction mixture was incubated in 0.1 M sodium bicarbonate buffer (pH 9.2) overnight at room temperature. The labeled oligonucleotide was purified from the reaction mixture by Sephadex G-25 gel filtration and 20% polyacrylamide gel electrophoresis and with a Nensorb 20 nucleic acid purification cartridge (DuPont, NEN, Boston, Mass.).

In situ hybridization. The PBS in which fixed samples of hair-like structures were stored was replaced with 27 μl of hybridization solution composed of 2× SSPE (1× SSPE is 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, and 1 mM EDTA [pH 7.4]), 5 mg of autoclaved and sheared herring sperm DNA/ml, 5× Denhardt's solution (50× Denhardt's solution contains 5 g of Ficoll, 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin, and H_2O to 500 ml), and 7.5% sodium dodecyl sulfate.

The in situ hybridization experiment included seven treatments. The first was a control incubated in hybridization solution without probe for 18 h at 55°C to assess the sample's autofluorescence. The second, third, and fourth treatments were controls used to assess the nonspecific binding of probes 338R, 13B1244R, and 5A1244R, respectively. These three controls were incubated for 12 h at 55°C with 20 ng of unlabeled oligonucleotides 338R, 13B1244R, and 5A1244R per μl and then for 6 h at 55°C with 2 ng of FITC-labeled 338R, 13B1244R, and 5A1244R per μl, respectively. The fifth, sixth, and seventh treatments were used to assess the specific binding of probes 338R, 13B1244R, and 5A1244R, respectively. Specific binding was assessed by using incubations containing heterologous probes as follows. Preincubation of these three treatments for 12 h at 55°C with 20 ng of unlabeled oligonucleotides 13B1244R, 338R, and 338R per μl, respectively, was followed by a 6-h incubation at 55°C with 2 ng of FITC-labeled 338R, 13B1244R, and 5A1244R per μl, respectively. After the 18 h of incubation, all seven treatments were centrifuged for 60 s at 12,250 × *g* and the hybridization solutions were replaced with 0.5 ml of 0.2× SSPE containing 1 μg of 4′,6-diamidino-2-phenylindole (DAPI)/ml for a 2-h wash at 55°C. The hair-like structures were then transferred to glass slides and covered with 10 μl of Citifluor (Ted Pella, Redding, Calif.) and a coverslip. The simultaneous hybridization with FITC-labeled 13B1244R and Texas Red-labeled 5A1244R was performed with-

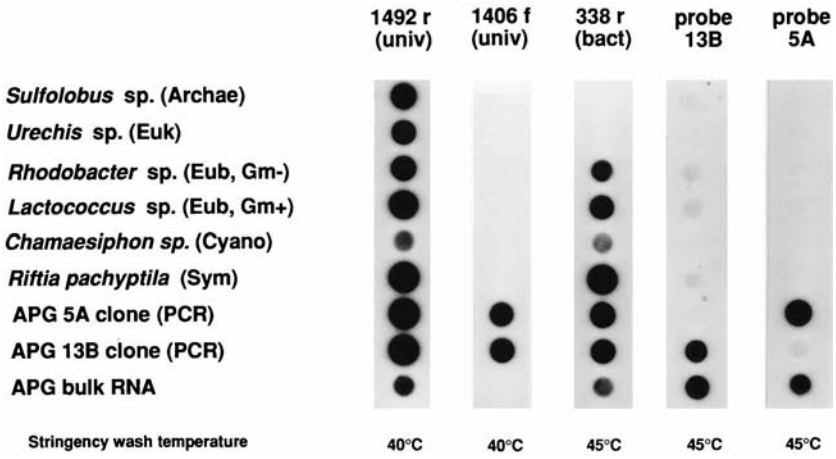

FIG. 1. Dot blot hybridization demonstrating the specificities of probes 13B1242R and 5A1243R. Fifty nanograms per dot of archaebacterial (Archae), eukaryotic (Euk), eubacterial (Eub), and host and symbiont (Sym) rRNAs was immobilized on nylon support membranes along with denatured 16S rRNA PCR products generated from clones 13B and 5A and bulk RNA extracted directly from the *A. pompejana* microflora. These samples were hybridized with the specified ^{32}P-labeled probes, including a universal (univ) probe (1406R), a complement of the universal probe (1406F), which served as a negative control, a eubacterial probe (338R), and the probes specific for phylotypes 13B and 5A. The high specificity of each probe demonstrates the efficacy of this technique. Stringency conditions are shown for each probe. Cyano, cyanobacterium; APG, *A. pompejana* generated; bact, bacterial.

out competing oligonucleotide added. The other hybridization conditions were the same as those for the probes used separately.

Slides were examined with a Nikon Microphot-FXA epifluorescence microscope fitted with filter set XF23 (485-nm excitation, 535-nm band pass emission; Omega Optical, Brattleboro, Vt.) for visualization of FITC, filter set XF67 (365-, 490-, and 575-nm excitation; 460-, 540-, and 630-nm emission) for simultaneous visualization of FITC and Texas Red, and filter set XF05 (365-nm excitation, 400-nm-long pass emission) for visualization of DAPI. The widths of filaments were measured with an ocular micrometer at a magnification of ×1,200. Black and white photomicrographs were taken with Kodak Tmax 400 ASA film, and color photomicrographs were taken with Fuji Color 400 ASA film. The FITC- and Texas Red-labeled and DAPI-stained samples were photographed with exposure times of 30 and 17 s, respectively.

RESULTS AND DISCUSSION

Probe specificity in dot blot hybridization and sensitivity in PCR. Comparative sequence analysis of the two dominant phylotypes, 5A and 13B (14), with more than 1,000 known bacterial 16S rRNAs revealed two unique target sites ideal for clone-specific oligonucleotide probes. The larger and more variable one chosen for these experiments was in the region of nucleotides 1240 to 1280 (*E. coli* numbering system) and provided the highest degree of within-clade differentiation. To maximize the performance of these oligonucleotides both as primers in PCR and as probes for in situ hybridization, it was critical that they be relatively short, to minimize problems with cellular permeability, yet long enough to be highly specific. The 19-mer probes designed for the 13B and 5A phylotypes differed from each other by 3 bases, conferring on them a high degree of intersymbiont specificity.

Dot blot hybridizations performed on rRNAs from a wide range of archaeal, bacterial, and eukaryotic taxa, as well as 13B and 5A rDNAs amplified by PCR, demonstrated that the oligonucleotides were specific for their targets (Fig. 1). Probes 13B1242R and 5A1243R clearly hybridized exclusively to the rDNAs of their respective phylotypes, demonstrating their specificities at the indicated stringency (0.2× SSPE, 45°C). No cross-reactivity was observed with any of the nonhomologous target nucleic acids, whose accessibility to probes was demonstrated by the hybridization of universal probe 1492R. Furthermore, in a turbo blot analysis of the entire 16S rRNA clone library constructed by Haddad et al. (14), the oligonucleotides 13B1242R and 5A1243R did not hybridize to any of the cloned rDNAs representing the 30 additional clone families (4).

It was critical that the clone-specific probes perform with maximum specificity and sensitivity when used as primers in PCR. In principle, only one copy of the template is required for a successful PCR amplification, but past studies of 16S rRNA genes have shown that even under optimal conditions, at least 75 copies are needed to visualize a positive amplification (4). In addition, this lower end of detection can be dramatically reduced in the presence of nonhomologous template, and for this reason we added genomic DNA from a community of free-living bacteria to the dilutions of plasmid DNA used in the sensitivity analyses. When matched with the forward primer EubB, the clone-specific primers 13B1242R and 5A1243R amplified their target templates at concentrations of as low as 1 and 10 fg, respectively, in the presence of nonhomologous template (Fig. 2). This sensitivity is equivalent to approximately 200 copies of the gene, since the 1.2-kb target gene was

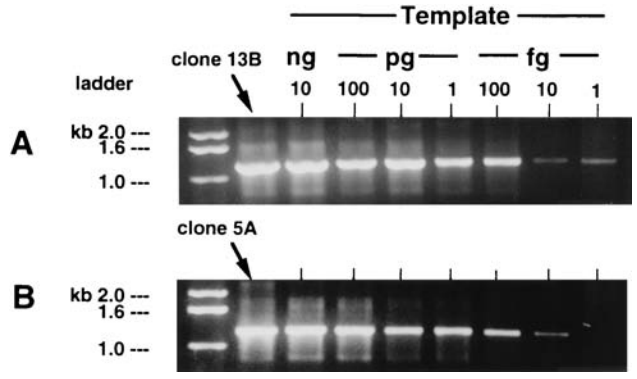

FIG. 2. Ethidium bromide-stained agarose gels of PCR products generated with primers 13B1242R (A) and 5A1243R (B) paired with primer EubB. The templates were plasmids containing clones 13B and 5A added at the concentrations indicated above each lane. The total concentration of DNA added to each reaction mixture, including the plasmid DNA, was 10 ng, with the balance being genomic DNA extracted from a natural community of free-living bacteria. The sizes of the molecular size markers are indicated on the left.

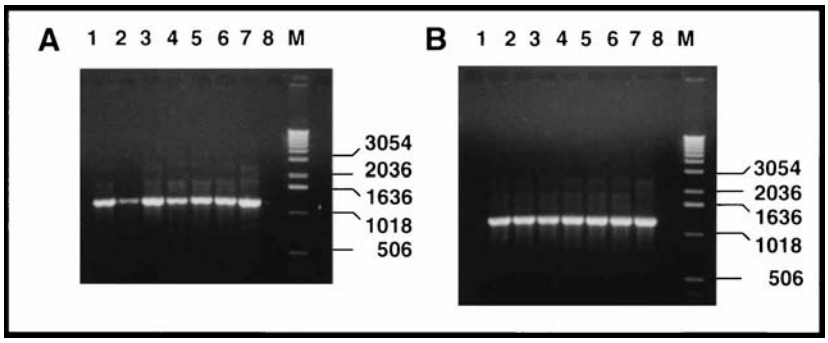

FIG. 3. Ethidium bromide-stained agarose gels of PCR products generated with primer EubB paired with primers 13B1242R (A) and 5A1243R (B). The template DNAs were purified from *A. pompejana* collected from 9°N (lanes 1 to 3) and 13°N (lanes 4 to 6) on the East Pacific Rise. Plasmid DNAs from clones 13B (panel A, lane 7) and 5A (panel B, lane 7) were the positive controls. Negative controls (lane 8) were reaction mixtures without template DNA added. The sizes of molecular size markers (lanes M) are indicated on the right of the gels in base pairs.

carried within a 3.9-kb plasmid vector. Similar experiments carried out on a dilution series of genomic DNA from the microbial community associated with *A. pompejana* gave similar results (4).

Occurrence of 13B and 5A on *A. pompejana*. One of our primary objectives in this study was to determine if phylotypes 13B and 5A are recurring features of the microbial community associated with *A. pompejana*. It was important to test this idea, because the library of 16S rRNA genes with which the community was initially characterized came from a single specimen of *A. pompejana* collected from the 13°N site (14). It was not possible to know if these phylotypes are ubiquitous features of *A. pompejana* after examining only a single specimen. Primers 13B1242R and 5A1243R paired with primer EubB produced a 1.2-kb amplification product from all six of the *A. pompejana* specimens collected at the 13°N and 9°N sites (Fig. 3 and Table 1). These PCR products produced the same *Hae*III-*Mbo*I restriction fragments as those produced in the products of control amplifications of 13B and 5A clone family plasmids (4). This confirmed that phylotypes 13B and 5A occur on *A. pompejana* organisms inhabiting both the 9°N and 13°N sites on the East Pacific Rise. From the presence of these phylotypes on several animals collected from two sites separated by 450 km, we infer that the 13B and 5A phylotypes are constant features of *A. pompejana.*

Occurrence of 13B and 5A in the vicinity of *A. pompejana* colonies. Assaying of a diverse collection of microbial communities collected from various surfaces in the vicinity of *A. pompejana* colonies revealed that phylotypes 13B and 5A are not restricted to *A. pompejana* (Table 1). Phylotype 13B was associated with *A. pompejana* integument, gut, and dwelling tube, as well as with *Paralvinella* sp. mucus, *Riftia pachyptila* tube, and basaltic rock. Similarly, phylotype 5A was not associated with only *A. pompejana* integument, gut, and dwelling tube; it was also detected on *A. caudata* integument. The amplification products which did not produce the same *Hae*III-*Mbo*I restriction fragments as those of the cloned 13B and 5A templates probably represent phylotypes related to 13B and 5A. How closely related to 13B and 5A these phylotypes might be is difficult to assess from the restriction analysis alone, but they are likely to be at least as different from 13B and 5A as 13B and 5A are from each other. Digestion with *Hae*III and *Mbo*I is able to resolve 13B and 5A with rRNA genes that are 96% similar. The occurrence of phylotype 13B on rock surfaces and invertebrate taxa other than *Alvinella* indicates that this epsilon proteobacterial phylotype does not have an obligate requirement for *A. pompejana*. In addition, phylotype 5A occurred on both *A. pompejana* and *A. caudata*, indicating that it is not an obligate symbiont of *A. pompejana.*

TABLE 1. Occurrence of clone families 13B and 5A on biotic and abiotic surfaces assayed by restriction digestion of PCR products

Sample	Primers 13B1242R and EubB			Primers 5A1243R and EubB		
	No. of samples that amplified	No. of amplifications with restriction pattern of 13B	No. of samples assayed	No. of samples that amplified	No. of amplifications with restriction pattern of 5A	No. of samples assayed
Alvinella pompejana integument, 13°N	3	3	3	3	3	3
Alvinella pompejana integument, 9°N	3	3	3	3	3	3
Alvinella pompejana gut	2	2	3	2	2	3
Alvinella pompejana tube	2	1	3	3	3	3
Alvinella caudata integument	3	0	3	3	3	3
Alvinella caudata gut	3	0	3	3	0	3
Paralvinella sp. mucus[a]	5	4	8	8	0	8
Paralvinella sp. gut[a]	0	0	1	1	0	1
Riftia pachyptila tube[a]	2	2	4	4	0	4
Tevnia sp. tube	0	0	3	2	0	3
Basaltic rock[a]	2	2	4	4	0	4
Sulfide rock	0	0	3	2	0	3

[a] The *Paralvilella* sp., *Riftia pachyptila*, and basalts were collected on dives when no *A. pompejana* specimens were collected.

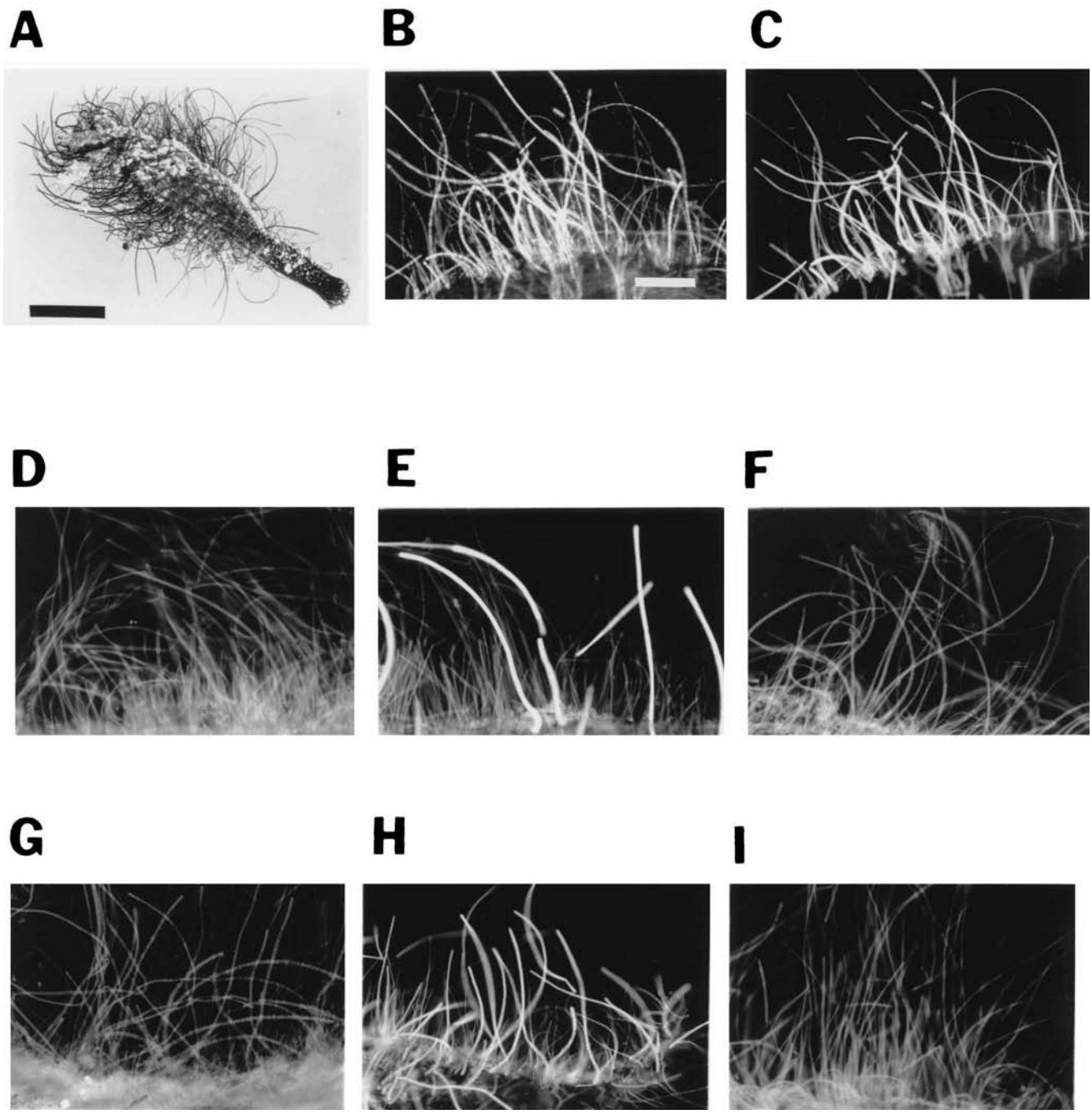

FIG. 4. Localization of phylotypes 13B and 5A. (A) Transmitted light micrograph of a single hair-like structure colonized by filamentous bacteria; (B) bacterial community stained with DAPI; (C) the same field of view shown in panel B viewed with the filter set for visualizing the FITC-labeled bacterial probe 338R; (D) hybridization of FITC-labeled 338R preceded by preincubation with unlabeled 338R; (E) hybridization with FITC-labeled 13B1244R; (F) hybridization of FITC-labeled 13B1244R preceded by preincubation with unlabeled 13B1244R; (G) control with no probe added; (H) hybridization with FITC-labeled 5A1244R; (I) hybridization of FITC-labeled 5A1244R preceded by preincubation with unlabeled 5A1244R. A constant exposure time of 30 s was used in panels C to I, allowing the direct comparison of intensities. Bars, 250 μm (A) and 100 μm (B to I).

Localizing phylotypes 13B and 5A on *A. pompejana*. The hair-like structures that are firmly attached to the integument of a freshly collected *A. pompejana* detached intact after a freeze-thaw cycle (Fig. 4A). Transmission electron and scanning electron micrographs (10, 12) revealed that the portion covered with filamentous bacteria projects above the surface of the integument while the portion lacking filamentous bacteria is anchored in the integument. Our hypothesis maintained that phylotypes 13B and 5A represent these prominent filamentous bacteria.

The accessibility of the ribosomes of the filamentous bacteria to oligonucleotide probes was assessed with bacterial probe 338R. All of the filamentous bacteria that stained with DAPI (Fig. 4B) also bound the FITC-labeled 338R (Fig. 4C), indi-

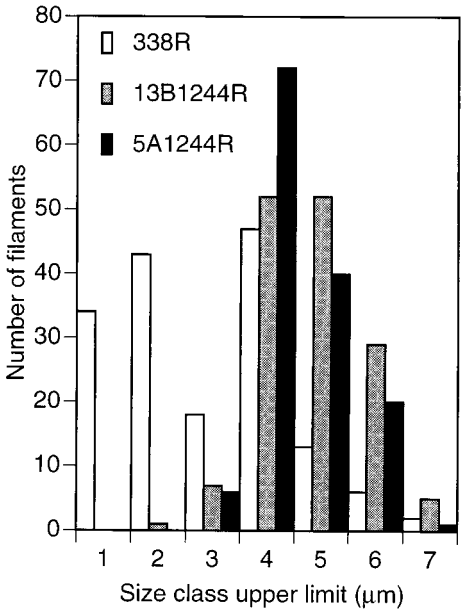

FIG. 5. Frequency distribution of the diameters of filamentous bacteria associated with *A. pompejana*. The filamentous bacteria were measured after hybridization with FITC-labeled probe 338R, 13B1244R, or 5A1244R.

cating that the rRNAs of these cells were readily accessed by the oligonucleotide. The specificity of the probe binding was confirmed by the ability of the unlabeled 338R to block the binding of the fluor-labeled 338R (Fig. 4D). This reduced the fluorescence intensity to that of cells with no probe added (Fig. 4G). We infer that the unlabeled 338R blocked the binding of the labeled probe by occupying the specific probe binding site, because the addition of 13B1244R did not block the binding of the fluor-labeled 338R (Fig. 4C).

FITC-labeled oligonucleotides 13B1244R and 5A1244R detected phylotypes 13B and 5A, respectively, in the bacterial community (Fig. 4E and H). Unlike probe 338R, these phylotype-specific probes bound to only a subset of the filamentous bacteria. The diameters of the filaments to which probe 338R bound ranged from 1.0 to 7.0 μm (Fig. 5), with two local maxima at 2 and 4 μm. Probe 13B1244R hybridized to filaments with diameters ranging from 2.0 to 7.0 μm (mean = 4.4 μm, standard deviation = 0.84, n = 146), and probe 5A1244R bound to filaments with diameters ranging from 3.0 to 6.5 μm (mean = 4.1, standard deviation = 0.74, n = 139) (Fig. 5). Simultaneous incubation with FITC-labeled 13B1244R and Texas Red-labeled 5A1244R demonstrated that these two probes hybridized to different filaments (Fig. 6), indicating that phylotypes 13B and 5A represent different populations.

The specificities of binding of the phylotype-specific probes were confirmed by competition experiments. Preincubation with unlabeled 13B1244R and 5A1244R blocked binding of FITC-labeled 13B1244R and 5A1244R, respectively (Fig. 4F and I), reducing fluorescence to the level observed when no probe was added (Fig. 4G). The possibility that phylotypes other than 13B and 5A could have been detected by the in situ hybridization must be considered in light of the fact that the diagnostic PCR produced some amplicons with *Hae*III-*Mbo*I restriction fragments different from those of phylotypes 13B and 5A. There are two reasons why our interpretation of the in situ hybridization results was not compromised in this way.

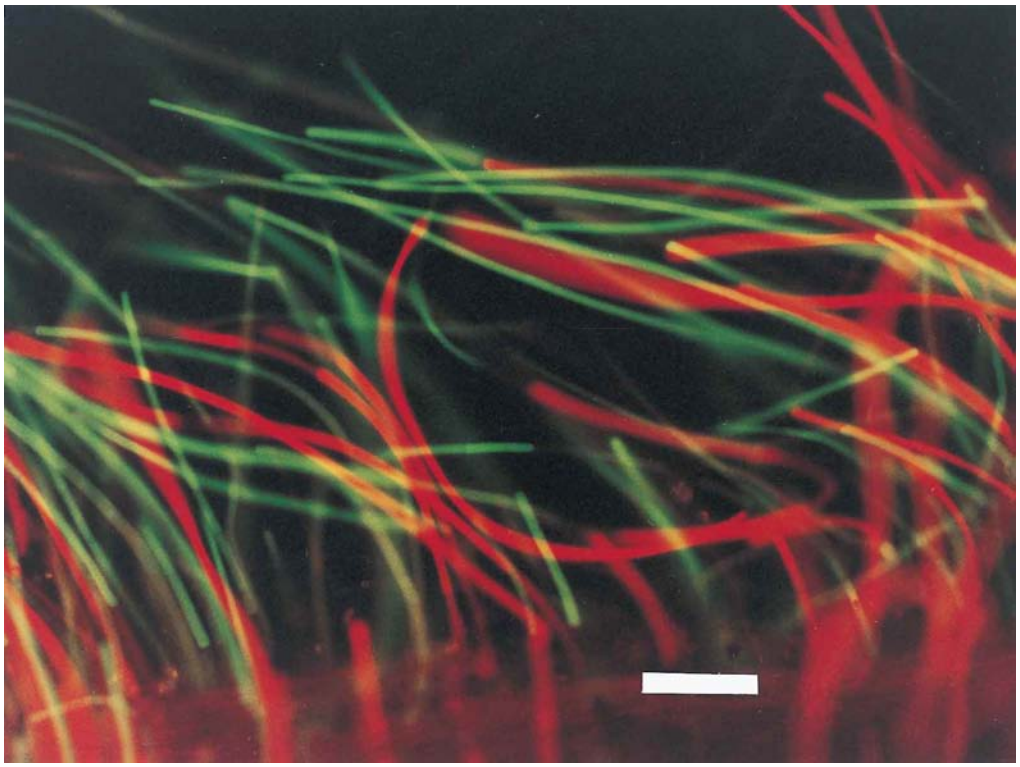

FIG. 6. Epifluorescence micrograph of filamentous bacteria on *A. pompejana* visualized by simultaneous hybridization with FITC-labeled 13B1244R and Texas Red-labeled 5A1244R. Bar, 50 μm.

First, the oligonucleotides employed for the in situ hybridizations were 22-mers with five 1-base mismatches between them. In principle, these should have been more specific than the 19-mers with three 1-base mismatches used in the diagnostic PCR. Second, and more importantly, the restriction digests of the diagnostic PCR assays of *A. pompejana* contained only fragments characteristic of phylotypes 13B and 5A, indicating the absence of closely related phylotypes on *A. pompejana* that could have potentially compromised the interpretation of the in situ hybridization results.

The results of these analyses conclusively demonstrated that phylotypes 13B and 5A are both filamentous bacteria which are prominent features of the hair-like structures on the dorsal integument of *A. pompejana*. The filamentous bacteria associated with *A. pompejana* are epsilon *Proteobacteria* (14). In addition, these findings support the notion that the dominance of clone families 13B and 5A in the library of 16S rRNA genes (14) reflects the dominance of these two phylotypes in the epibiotic assemblage.

Perspective and implications. In several recent reports, unique epsilon *Proteobacteria* have been found closely associated with invertebrate hosts and free living in hydrothermal vent environments. The dominant filamentous bacterium associated with the shrimp *Rimicaris exoculata*, found at Mid-Atlantic Ridge hydrothermal vents, is also a member of the epsilon *Proteobacteria* (19). Similarly, 60% of the clones in a library of 16S rRNA genes amplified by PCR from a microbial mat at Pele's Vent on Loihi Seamount, Hawaii, were epsilon *Proteobacteria* (18). These findings suggest that in addition to the better-known chemoautotrophic symbiotic gamma *Proteobacteria*, bacterial taxa aligned with the epsilon *Proteobacteria* likely play an important role in the ecology of hydrothermal vent ecosystems.

Earlier studies attempted to examine the bacterial population associated with *A. pompejana* by selective culturing techniques and gross enzyme analyses (1, 2, 21). While those studies provided many isolates of diverse metabolic capabilities, including several thermophiles, the numerical significance of these strains to the population is uncertain. Many of the isolates metabolize sulfide and are resistant to heavy metals, but none were confirmed to be the authentic epibionts. The dominant filamentous form was not isolated. The molecular genetic approach presented here combines the specificity of phylogenetic analysis with the efficacy of in situ hybridization. This allowed us to match phylotype with morphotype and to determine the significance of these dominant forms as persistent members of the *Alvinella* microflora and as free-living members of the local habitat.

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