Cah. Biol. Mar. (1998) 39 : 275-278

Spanning the thermal limits: an extreme eurythermal symbiosis

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Integrated bacterial associations with animals are widely distributed in both terrestrial and marine ecosystems. Many marine bacterial symbioses appear to be obligatory, thereby apparently defining the distribution of the host animal through the symbiont's requirements for energy. Whether the bacteria reside externally or endosymbiotically, in most cases we know little about their role in the association. Many of these symbioses are loosely classified as mutualistic, however in most cases this view has yet to be adequately tested.

Epibiotic associations appear the most widespread in marine systems. Often epibiotic symbionts exist as a phenotypically mixed population making it impossible to decipher the role of independent members. Even with significant technological advances our ability to understand these associations has been severely limited because in most cases the symbionts cannot be cultured free of the host to enable metabolic characterization. A powerful means to describe this type of association is to use a molecular genetic approach.

A conspicuous component of certain high temperature (40 - 105°C) vent habitats is the polychaetous annelid *Alvinella pompejana* Desbruyères & Laubier, 1980. *A. pompejana* is restricted to the walls of high temperature vent chimneys at the 9°, 13° and 21°N vent sites on the East Pacific Rise (Desbruyères et al., 1985). The immediate environment surrounding this habitat is characterized not only by its high temperature but also by high levels of hydrogen sulphide (>1 mM) and high concentrations of heavy metals (0.3 - 200 µM) such as silver, copper, zinc, and cadmium (Von Damm, 1995). *A. pompejana* is characterized by a dense, specific epibiotic microflora associated with the worms' dorsal integument (Desbruyères et al., 1985). Although electron microscopy studies of the *A. pompejana* epibionts suggest that they are highly diverse, two bacterial morphotypes, a filamentous sheathed and a rod-shaped form, appear to predominate. The filamentous form is integrated with specialized expansions of the intersegmentary parts while the rod-shaped forms appear less abundant but evenly distributed (Gaill et al., 1984).

In previous studies the alvinellid symbiont population has been implicated in both the nutrition of the host and in the detoxification of sulphide and heavy metals (Alayse-Danet et al., 1987). The evidence for this, however, is inconclusive. Stable isotope analysis and activities of key metabolic enzymes provide only a measurement of the epibiont population average and not an understanding of the specific roles of the symbionts. The focus of this study has been to use recently developed molecular genetic technologies to characterize the predominant symbionts within the mixed population and to determine their function in the symbiosis. Our specific objectives have been to: 1) phylogenetically characterize the dominant symbionts in the population, 2) determine the distribution of these symbionts both on the worm and within the vent environment, and 3) determine the metabolic capabilities within the structure of the symbiont population.

I. Phylogenetic characterization of dominant members of the epibiotic community

Nucleic acids were extracted from bacteria taken from the dorsal surface of *Alvinella pompejana* collected from the 13°N and 9°N vent sites on the East Pacific Rise. Small subunit ribosomal rRNA (16S rRNA) genes were amplified with universal bacterial primers by the polymerase chain reaction (PCR) and subsequently cloned into a plasmid vector (Haddad et al., 1995). The resulting clone library consisted of 139 transformants with correctly sized inserts. The library was then screened by restriction fragment length polymorphism (RFLP) analysis to identify distinct clone types (families). The RFLP analysis of the entire library identified thirty-two unique clone families. The four dominant families (5A, 13B, 44B, 56B) represented over 65% of the library. Four representatives of each of the dominant clone families were chosen for complete 16S rRNA sequencing and analysed using a variety of

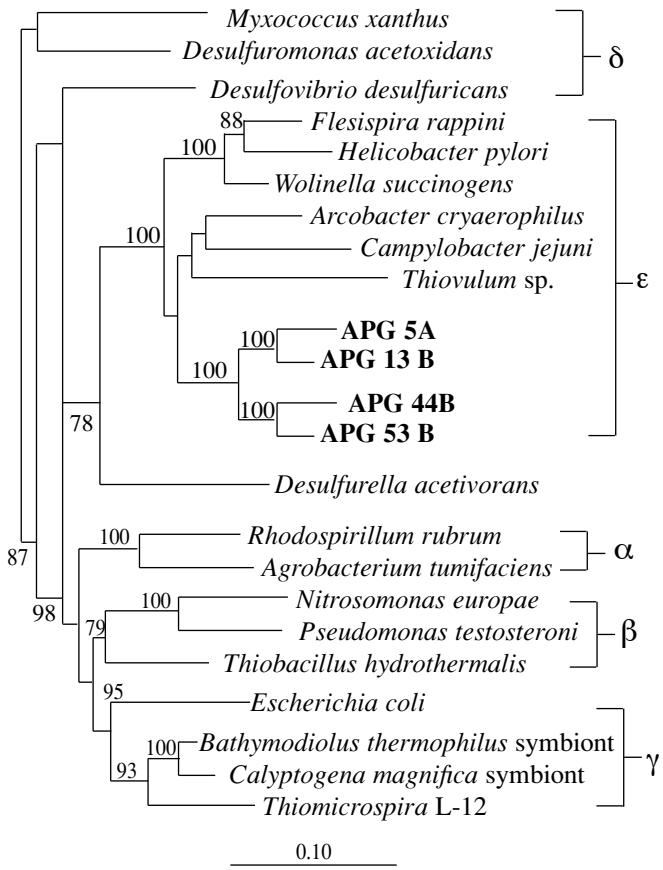

Figure 1. Phylogenetic tree showing the relationship of the alvinellid clones to the other members of the *Proteobacteria*. This tree was inferred from 16S rRNA sequence data using the neighbour-joining method. Molecular sequences for all reference strains (except *Desulfurella acetivorans*) were obtained from the RDP. APG indicates 16S rRNA sequences cloned from the epibiotic microbial population of an *Alvinella pompejana* individual. A total of 1084 nucleotide positions were included in the analysis. The tree was rooted with the sequence of *Bacillus subtilis*. Scale bar indicates 0.1 fixed mutations 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). Taken from Haddad et al., (1995). Reprinted with permission from *Applied Environmental Microbiology,* 61: 1679-1687 copyright 1995.

phylogenetic inference methods. All of the gene sequences were found to be related to the newly established epsilon subdivision of the Proteobacteria (Fig. 1). 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. These were the first representatives of epsilon proteobacteria to be characterized from hydrothermal vents communities. Subsequent studies of the epibionts of the MAR shrimp *Rimicaris exoculata* Williams & Rona, 1986 (see Polz and Cavanaugh, 1995) and members of the free-living microbial community from the Loihi Seamount (Moyer et al., 1995) have identified an abundance of epsilon representatives.

II. Molecular identification and localization of the dominant phylotypes

Our next objective was to determine if the dominant families in the clone library represented the filamentous morphotype that dominated the epibiotic community. Nucleic acid probe technology based on 16S rDNA signature sequences provided the resolution necessary to match the dominant clone types to specific members of the microbial community on *Alvinella pompejana* (Cary et al., 1997). Two oligodeoxynucleotide probes targeting the 16s rRNA were designed for the PCR and in situ hybridization that would distinguish the 2 dominant clone families (5A and 13B) from all known bacterial 16s rRNAs. The probes were each labelled with either fluoroscein isothiocyanate (FITC) or Texas Red. The hair-like dorsal expansions were removed intact from worm specimens and subjected to a well controlled series of both PCR and in situ hybridization experiments.

The PCR based experiments (Table 1) revealed that the two phylotypes 5A and 13B are regular features of the bacterial community associated with *Alvinella pompejana* taken from 2 geographically isolated vent sites (13°N, 9°N EPR). Assaying the surfaces around colonies revealed that these phylotypes are not entirely restricted to *A. pompejana*

Table 1. Occurrence of clone families 13B and 5A on biotic and abiotic surfaces assayed by restriction digestion of PCR products. Reprinted with permission from *Applied Environmental Microbiology (Applied Environmental Microbioloty,* 63: 1124-1130) copyright 1997.

Sample	Primers 13B1242R & EubB			Primers 5A1243R & EubB		
	Amplified	Restriction pattern = 13B	n	Amplified	Restriction pattern = 5A	n
Alvinella pompejana integument, 13°N	3	3	3	3	3	3
	3	3	3	3	3	3
" gut	2	2	2	2	2	3
" tube	2	1	3	3	3	3
Alvinella caudata integument	3	0	3	3	3	3
" gut	3	0	3	3	0	3
Paralvinella sp. mucous[a]	5	4	8	8	0	8
" gut	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 *Paralvinella* sp., *Riftia pachyptila* and basalts were collected on dives when no *Alvinella pompejana* were collected.

but may have a significant free-living component. Only one of these phylotypes, 13B, was found to be a primary component of the epibiotic community associated with the congener *A. caudata* Desbruyères & Laubier, 1986.

These same hair-like structures were subject to a series of in situ hybridization experiments with the phylotype specific probes for 13B and 5A. Both phylotypes were identified and localized on *Alvinella pompejana* as the prominent filamentous bacteria. Interestingly, in many of the specimens examined, the distribution of bacteria appears to exist in a gradient dominated in the proximal end by 13B and at the dorsal end by 5A. Studies are now underway to examine the distribution of the phylotypes on worms collected from colonies that are clearly experiencing different thermal and chemical conditions.

Continued examination of the microflora has revealed a high diversity of genes encoding dissimilatory sulphite reductase - an enzyme essential for dissimilatory sulphate reduction. This would suggest that a significant component of the community have the capacity to respire anaerobically, a trait consistent with high temperature anoxic environments.

III. Habitat characterization and evolution of an *Alvinella* colony

Recent studies have now focused on characterizing the tube habitat of *Alvinella pompejana* (Cary et al., 1998). A special temperature logger (the "Mosquito") was designed to incorporate a 20 cm long titanium tube (0.75 cm dia.) which allowed placement of the temperature sensor inside the small *Alvinella* tube opening (Fig. 2A). Temperature probe measurements taken 6 cm inside the venting tube averaged 68°C (± 6.33°C) with frequent spikes exceeding 81°C (Fig. 2B). Measurements made at the tube opening averaged 22°C (± 2.5°C) while those taken only a few centimetres away were generally lower than 17°C. Much of the micro-variation seen in the surveys is probably generated by the turbulent mixing occurring throughout the colony or actual changes in plumbing of the porous chimney system. No indication of tidal cycle or strong harmonic periodicity was reflected in any of the series.

The data presented here demonstrate that the Pompeii worm inhabits an environment where an unprecedented temperature gradient exists within the space of only a few centimetres. The posterior end of the worm experiences temperatures near 70°C while the anterior encounters 20°C or lower - an apparent gradient of almost 50°C. This would suggest *A. pompejana* is not only the most thermotolerant metazoan described, but the most eurythermal organism on the planet. These observations are consistent with the thermal stability of collagen fibrils taken from connective tissue of *A. pompejana* (Gaill, 1993). We are continuing

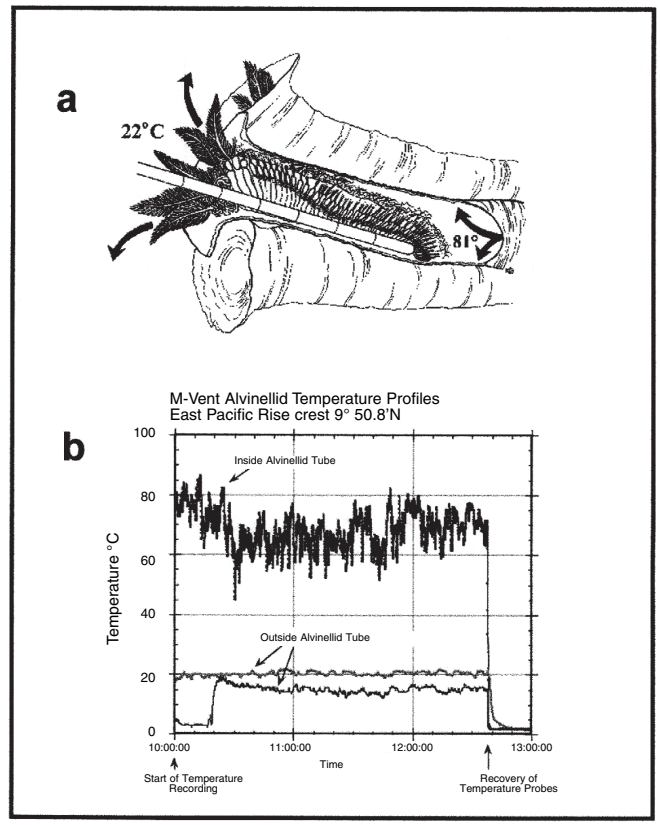

Figure 2. **(a)** A cut away representation of the temperature probe placement in a Pompeii worm tube. Noted temperatures are maximal values obtain during one deployment. (**b)** Inside and two representative outside temperature profiles over a 3 hour period for a single deployment. The temperature recorders were set to measure at 2 second intervals over the duration of each deployment. The micro-variation observed in the profiles was probably generated by the turbulent mixing of fluids throughout the colony. Spectral analysis of the temperature records provided no evidence of tidally-influenced cycles or strong harmonic periodicities. Reprinted with permission from *Nature (Nature* 391: 545-546) Copyright (1998), Macmillan Magazines Ltd.

these studies by chemically characterizing the redox conditions in these tubes using micro-water sampling techniques and voltametric microelectrode technologies.

Biotechnological research efforts are now targeted at characterizing the eurythermal properties of enzymes from the Pompeii worm and its bacterial symbionts. Our hopes are to discover industrially relevant biocatalysts that maintain high activity over extended temperature ranges. Over 400 genes isolated from the epibiotic community have currently been sequenced. Additionally, probing of a symbiont Fosmid genomic library with probes specific to the 2 dominant phylotypes corroborated the in situ probing results and will allow the characterization of protein coding domains specifically linked to the ribosomal operon.

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