Symbiotic relationships of bacteria with higher organisms are commonly observed in nature; however, the functional role of these relationships is only rarely understood. This is particularly evident in epibiotic bacterial associations in the marine environment where the bacteria are often a diverse ensemble of microorganisms, thus complicating the identification of the functionally important members. Classical microbiological techniques, relying primarily on culturing these organisms, have provided an incomplete picture of these relationships. Molecular genetic techniques, focusing on the analyses of bacterial 16S rRNA sequences cloned directly from natural microbial populations, are now available which allow a more thorough examination of these associated bacterial populations. This study sought to characterize the epibiotic bacterial population associated with a very unique organism, *Alvinella pompejana*, using such a molecular approach.
Alvinella pompejana is a polychaetous annelid that inhabits active deep-sea hydrothermal vent sites along the East Pacific Rise. This worm colonizes the walls of actively venting high temperature chimneys and is thought to be one of the most thermotolerant metazoans known. The chimney environment is characterized by high concentrations of sulfide and heavy metals in the vicinity of the worm colonies. A 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; however, previous studies have been unable to confirm such a role. The primary goal of this study is to phylogenetically characterize the dominant epibionts through the analysis of 16S rRNA gene sequences.

Nucleic acids were extracted from bacteria collected from the dorsal surface of Alvinella pompejana. 16S rRNA genes were amplified with universal bacterial primers by the polymerase chain reaction (PCR). These genes were subsequently cloned and the resulting clone library was screened by restriction fragment length polymorphism (RFLP) analysis to identify unique clone types. Thirty-two distinct clone families were found in the library. Four of these families were clearly dominant, representing over 65% of the library. The main assumption in this study is that the numerical dominance of the phylotypes in the starting population will be reflected in the clone library. Thus, representative clones from the four most abundant clone families were chosen for complete gene sequencing and phylogenetic analysis. These gene sequences were analyzed using a variety of phylogenetic inference methods and were found to be related to the newly established
epsilon subdivision of the Proteobacteria. In future studies, these gene sequences will be used to construct specific oligodeoxynucleotide probes which can be used to confirm the morphology of the clone types in the epibiont population.
Phylogenetic Characterization of the Epibiotic Bacteria Associated with the Hydrothermal Vent Polychaete *Alvinella pompejana*

by

Michael A. Haddad

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Major Professor, representing Microbiology

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Chair of Department of Microbiology

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Dean of Graduate School

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Michael A. Haddad, Author
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# TABLE OF CONTENTS

**INTRODUCTION** .................................................................................................................................................. 1  
Ecology of *Alvinella pompejana* ......................................................................................................................... 3  
The Epibiotic Microflora of *Alvinella pompejana* .................................................................................................. 6  
Possible Role of the Epibionts .............................................................................................................................. 11  
Conclusions of Previous Research ....................................................................................................................... 13  
Use of the 16S rRNA Molecule .......................................................................................................................... 14  
Experimental Rationale ...................................................................................................................................... 15  

**METHODS** ........................................................................................................................................................ 18  
Sample Collection ............................................................................................................................................... 18  
Bacterial Removal and DNA Purification ............................................................................................................ 18  
Amplification of 16S rRNA Genes ....................................................................................................................... 19  
Construction and Screening of Clone Library ..................................................................................................... 20  
RFLP Analysis ...................................................................................................................................................... 21  
Gene Sequencing .................................................................................................................................................. 22  
Phylogenetic Analysis ........................................................................................................................................ 23  

**RESULTS** ......................................................................................................................................................... 25  
Clone Library Construction ................................................................................................................................. 25  
Restriction Fragment Length Polymorphism Analysis ......................................................................................... 25  
16S rRNA Gene Sequences ................................................................................................................................. 26  
Phylogenetic Analysis .......................................................................................................................................... 29  

**DISCUSSION** ................................................................................................................................................... 39  

**BIBLIOGRAPHY** ................................................................................................................................................. 45  

**APPENDICES** .................................................................................................................................................... 52
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Map showing active hydrothermal vent sites along the East Pacific Rise (EPR)</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Dorsal view of the holotype of (a) <em>Alvinella pompejana</em> and (b) <em>Alvinella caudata</em></td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Schematic outline of the experimental strategy used for the phylogenetic analyses of the epibiotic microbial community associated with the hydrothermal vent polychaete <em>Alvinella pompejana</em></td>
<td>17</td>
</tr>
<tr>
<td>4.</td>
<td>Scaled Illustration of the restriction patterns of 16S rDNAs representing 11 clone families identified in the alvinellid clone library.</td>
<td>27</td>
</tr>
<tr>
<td>5.</td>
<td>Phylogenetic tree showing the relationship of the alvinellid clones to the other members of the Proteobacteria.</td>
<td>30</td>
</tr>
<tr>
<td>6.</td>
<td>Proposed secondary structural model of clone APG 13B 16S rDNA</td>
<td>33, 34</td>
</tr>
<tr>
<td>7.</td>
<td>Proposed secondary structural model of clone APG 56B 16S rDNA.</td>
<td>35, 36</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Chemical composition of vent end-member fluid @ 21° N on the East Pacific Rise vs. ambient sea water</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>RFLP analysis results showing the numerical distribution of the alvinellid clone families.</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Signature base positions distinguishing the delta and epsilon subclasses of Proteobacteria and the alvinellid clones.</td>
<td>38</td>
</tr>
</tbody>
</table>
**LIST OF APPENDICES**

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Locus APG</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5A</td>
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</tr>
<tr>
<td></td>
<td>1496 bp DNA</td>
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<td>13b</td>
<td>54</td>
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<td></td>
<td>1502 bp rRNA</td>
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<td>3</td>
<td>44b</td>
<td>56</td>
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<tr>
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<tr>
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<td>1504 bp DNA</td>
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</table>
Phylogenetic Characterization of the Epibiotic Bacteria Associated with the Hydrothermal Vent Polychaete *Alvinella pompejana*

INTRODUCTION

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. As a result, whether these bacteria reside intracellularly (endosymbiotic) or extracellularly (episymbiotic or epibiotic), many of these affiliations can only roughly be defined as symbiotic. It has been impossible to determine whether they are mutualistic, where both members are benefited by the relationship, or merely commensal, where only a single member profits.

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* comprise a single strain of *Vibrio fischeri* (McFall-Ngai et al., 1991) 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. Several distinct morphological types of bacteria are found colonizing the gills of the rock crab *Cancer irroratus* and are believed to play an important role in the pollution of these organisms (Bodammer et al., 1981). Similarly, periphytic bacteria are associated with another crab, *Helice crassa*, found at tannery effluent sites, and may contribute
to the concentration of chromium in these environments (Johnson et al., 1981). An unnamed limpet species inhabiting deep-sea hydrothermal vent sites is also found with dense colonies of filamentous bacteria colonizing the gill epithelial surface (de Burgh et al., 1984).

Probably one of the more dramatic examples of invertebrate/bacteria associations are those found in certain sponges. In the majority of the large marine sponges, dense populations of bacteria reside in the intercellular spaces, comprising up to one-third of the volume of the sponge. These bacterial populations are typically composed of between 4-7 different morphological types (Smith et al, 1987). Although there have been several attempts to isolate these bacteria free from their hosts, few have been confirmed to be the symbionts. In these and other similar associations the task of distinguishing and characterizing the important members in 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 polychaetous annelid, *Alvinella pompejana*, found colonizing the walls of actively venting chimneys (Desbruyères et al., 1980). A highly diverse and dense assemblage of microorganisms, including an integrated filamentous morphotype, occupies the dorsal surface of this worm (Desbruyères et al., 1985; Gaill et al., 1987). To date, this filamentous morphotype has eluded all attempts at culturing (Durand et al., 1990; Jeanthon et al., 1992; Prieur, et al. 1990). Several studies have suggested that the bacteria participate in the nutrition of the host or in the detoxification of the host's immediate environment (Alayse-Danet et al., 1987; Gaill et al., 1988). However, due to the inherent difficulty in working with *A. pompejana*, which rarely survives trips to the surface, the apparent failure to
culture the dominant morphotypes, these hypotheses have remained merely suggestive.

Molecular genetic strategies now exist for the characterization of microorganisms which have previously not been observed in culture. The analysis of small subunit ribosomal RNA sequences (16S rRNA) cloned directly from natural populations has expanded the limits of microbial ecology and diversity over that which was possible with more traditional microbiological techniques. In other studies, 16S rRNA sequence analysis has provided a powerful means to examine the diversity of natural microbial populations in marine and fresh water systems avoiding the reliance on cultavatibility. These same techniques can be used to phylogenetically characterize the microflora associated with *Alvinella pompejana*. Characterizing the dominant members of the microflora would provide essential information necessary to determine the role of these bacteria. These same techniques can also be applied to other epibiotic microbial associations, which will no doubt add to our limited knowledge about these other associations.

**Ecology of *Alvinella pompejana***

*Alvinella pompejana* is a tube-dwelling, polychaetous annelid that colonizes actively venting chimneys at deep-sea hydrothermal vent sites (Desbruyères & Laubier 1980). *A. pompejana*, as well as its congener *A. caudata*, are placed within their own family, the Alvinellidae (Desbruyères et al., 1986), along with another genus *Paralvinella* (Desbruyères et al., 1985). All members of the family appear to be restricted solely to deep-sea hydrothermal vent sites (Desbruyères et al., 1991). The Alvinellidae range in distribution
from 17°S to 50°N along the East Pacific Rise (EPR) in the eastern Pacific Ocean (Figure 1), with *Alvinella* spp. being restricted to the high temperature vent sites from 17°S-21°N (Desbruyères & Laubier, 1991; Tunnicliffe et al., 1993). *Alvinella* spp. form dense colonies along the walls of actively venting chimney-like mineral structures. These worms build very stable tubes composed of both inorganic and organic material which are cemented to the chimney walls (Gaill et al., 1986; Vovelle et al., 1986). It has been suggested that these tubes assist in the stabilization of venting chimney structures by providing additional structural substrate for the further deposition of mineral precipitates (Tunnicliffe et al., 1990).

The immediate environment of *Alvinella pompejana* is one of the most extreme environments on the planet. *A. pompejana* is one of the few invertebrate species present at vent sites to live in close proximity to the hot hydrothermal vent effluent. These worms are found colonizing basal mounds, zinc-sulfide and black smoker chimneys where the core effluent temperature ranges from 80°C-350°C, depending on the chimney type (Desbruyères & Laubier, 1991). The mixing zone of the hot hydrothermal fluid with the cold ambient seawater produces a sharp temperature gradient, ranging from 350°C - 1.8°C within a few centimeters (Desbruyères et al., 1985). In one study, temperature readings within an alvinellid colony were 20-35°C at the tube opening, 100°C 10 cm inside the tube mass, and 249°C 20 cm inside the tube mass (Desbruyères et al., 1982). Recently, an actively swimming specimen of *A. pompejana* was observed resting on the submersible's temperature probe, which simultaneously registered a temperature of 105°C. This observation lead the authors to suggest that *A. pompejana* may hold the record for the upper
Figure 1. Map showing active hydrothermal vent sites along the East Pacific Rise (EPR). (a) The Alvinellidae range in distribution from 17°S to 50°N along the EPR, with *Alvinella* spp. being restricted to the high temperature vent sites from 17°S-21°N. (b) *Alvinella pompejana* specimens used in this study were collected from the Elsa vent field at the 13°N site. This figure was adapted from: Jollivet, D. 1993. Distribution et évolution de la faune associée aux sources hydrothermales profondes à 13°N sur la dorsale du pacifique oriental: le cas particulier des polychètes alvinellidae. These de doctorat de L'Université de Bretagne Occidentale. IFREMER, Centre de Brest.
temperature limit of any eukaryote (Chevaldonné et al., 1992). Although additional data are required to confirm this claim, it is believed that these organisms exist at environmental temperatures between 20°C and 60°C (Desbruyères & Laubier, 1991).

Another consequence of colonizing active chimneys at deep-sea hydrothermal vent sites is the direct exposure to high concentrations of heavy metal compounds and hydrogen sulfide contained in the vent effluent. Elevated concentrations of these compounds are well known to inhibit the growth and essential biochemical activities of microorganisms and eukaryotes (Collins et al., 1989; N.R.C., 1979). Table 1 shows concentrations of some of these compounds at a site at 21°N on the East Pacific Rise in comparison to average seawater. As is evident from this table, the vent water is acidic and enriched in hydrogen sulfide and biologically toxic heavy metals, such as arsenic, lead, and silver. Due to the proximity of the worm's tubes to the vent effluent, A. pompejana and its associated microflora are constantly exposed to these toxic compounds (Desbruyères et al., 1985; Desbruyères & Laubier, 1991).

The Epibiotic Microflora of Alvinella pompejana

A fascinating feature of both A. pompejana and A. caudata is the presence of an epibiotic population of bacteria associated with the worms' dorsal surface (Figure 2). These alvinellid species differ somewhat in the structural features that link the epibionts to their dorsal surface. A. pompejana posses modified dorsal digiform expansions heavily colonized by filamentous bacteria. Similar filamentous bacterial morphotypes are linked to modified posterior parapodia in A. caudata (Desbruyères & Laubier, 1991).
Table 1a. Chemical composition of vent end-member fluid at 21°N on the East Pacific Rise vs. ambient sea water.

<table>
<thead>
<tr>
<th></th>
<th>21°N</th>
<th>Ambient sea water</th>
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<tbody>
<tr>
<td>Li (μM)</td>
<td>891</td>
<td>26</td>
</tr>
<tr>
<td>Na (mM)</td>
<td>432</td>
<td>464</td>
</tr>
<tr>
<td>K (mM)</td>
<td>23.2</td>
<td>9.79</td>
</tr>
<tr>
<td>Rb (μM)</td>
<td>28</td>
<td>1.3</td>
</tr>
<tr>
<td>Be (nM)</td>
<td>15</td>
<td>0.02</td>
</tr>
<tr>
<td>Mg (mM)</td>
<td>0</td>
<td>52.7</td>
</tr>
<tr>
<td>Ca (μM)</td>
<td>15.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Sr (μM)</td>
<td>81</td>
<td>87</td>
</tr>
<tr>
<td>Ba (μM)</td>
<td>&gt;7</td>
<td>0.14</td>
</tr>
<tr>
<td>pH</td>
<td>3.4</td>
<td>7.8</td>
</tr>
<tr>
<td>Cl (mM)</td>
<td>489</td>
<td>541</td>
</tr>
<tr>
<td>Si (mM)</td>
<td>17.6</td>
<td>0.16</td>
</tr>
<tr>
<td>Al (μM)</td>
<td>5.2</td>
<td>0.01</td>
</tr>
<tr>
<td>SO4 (mM)</td>
<td>0</td>
<td>27.9</td>
</tr>
<tr>
<td>H2S (mM)</td>
<td>7.3</td>
<td>0</td>
</tr>
<tr>
<td>NH3 (mM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mn (μM)</td>
<td>960</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe (μM)</td>
<td>1664</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Co (nM)</td>
<td>213</td>
<td>0.03</td>
</tr>
<tr>
<td>Cu (μM)</td>
<td>35</td>
<td>0.007</td>
</tr>
<tr>
<td>Zn (μM)</td>
<td>106</td>
<td>0.01</td>
</tr>
<tr>
<td>Ag (nM)</td>
<td>38</td>
<td>0.02</td>
</tr>
<tr>
<td>Cd (nM)</td>
<td>155</td>
<td>1.0</td>
</tr>
<tr>
<td>Pb (nM)</td>
<td>308</td>
<td>0.01</td>
</tr>
<tr>
<td>As (nM)</td>
<td>247</td>
<td>27</td>
</tr>
<tr>
<td>Se (nM)</td>
<td>72</td>
<td>2.5</td>
</tr>
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Figure 2. Dorsal view of the holotype of (a) *Alvinella pompejana* and (b) *Alvinella caudata*. Arrows indicate approximate positions of bacterial sampling for this study. This figure was adapted from: Desbruyères, D. and L. Laubier. 1980. *Alvinella pompejana* gen. sp. nov., ampharetidae aberrant des sources hydrothermales de la ride Est-Pacifique. Oceanol. Acta 3: 267-274.
In both species the bacteria appear as a gray mat covering the majority of the worms' surfaces and are also found lining the interior of the worms' tube (Desbruyères et al., 1985; Gaill & Hunt, 1986). The density of the bacterial population seems to increase posteriorly, presumably responding to some environmental gradient (Desbruyères et al., 1980; Gaill et al., 1987). For the purposes of this discussion, only the details of the epibiotic microbial population associated with *A. pompejana* will be further emphasized.

The epibiotic microbial population associated with *Alvinella pompejana* is morphologically diverse. Electron microscopic observations have documented many morphotypes, including rod, coccoid, spiral, and filamentous bacteria (Gaill et al., 1987). Although it is impossible to accurately identify bacterial species based solely on morphology, these studies have served to document several interesting patterns of the distribution of these morphotypes in the population. These morphological forms have been found in several different distributions on the host, ranging from rod-shaped bacteria found randomly dispersed, to filamentous bacteria found colonizing specific locations on the worm (Desbruyères et al., 1985; Gaill et al., 1987). These filamentous morphotypes dominate the population representing over 60% of the microbial biomass (Gaill et al., 1987). Filamentous bacteria are found joined to small expansions of the worm's epidermis within the intersegmentary spaces (Gaill et al., 1987). These expansions are ≥ 10 μm in length and appear composed of glandular cells (Desbruyères et al., 1985). The filamentous bacteria are embedded in a polysaccharide secretion from the worm (Gaill et al., 1988), and have been shown to be in an active state of growth (Alayse-Danet et al., 1986). The appearance of this specific association and abundance of the morphological form in the population has lead others
to suggest a specific relationship between these filamentous bacteria and *A. pompejana* (Gaill et al., 1988).

The high diversity of the epibiotic microbial community associated with *Alvinella pompejana* is apparent not only from the morphology, but also in the wide range of metabolic capabilities detected in the community. Several attempts have been made at culturing the epibiotic bacteria free from *A. pompejana*. A preliminary study of the heterotrophic bacteria present in this community yielded 62 total isolates. All of the isolates were Gram-negative and rod-shaped in their morphology (Prieur et al., 1987). In later studies additional heterotrophs and several autotrophic bacteria were successfully isolated. The majority of these new isolates had resistance to one or more selected heavy metals, such as silver, copper, cadmium, zinc, and arsenic (Jeanthon et al., 1990; Prieur et al., 1990). Numerous sulfur-oxidizing and sulfur-reducing isolates were also obtained, as well as several isolates involved in the nitrogen cycle, and in the oxidation of manganese (Durand et al., 1990). Growth of these isolates was observed at temperatures ranging from 20°C to 80°C, at both atmospheric and *in situ* pressure (250 atm). Collectively, these studies complement early microscopy findings by demonstrating the microflora of *A. pompejana* is both morphologically and metabolically diverse. In all of these studies the dominant filamentous morphotype was never confirmed to be in culture, thus illustrating the limitations of these classical approaches.
Possible Role of the Epibionts

Several studies have speculated on the role of the epibiotic microbial community in the survival of *A. pompejana*. The primary hypothesis has been that the microflora in some way participates in the nutrition of the alvinellid, either by the worm directly feeding on the bacteria, or by a translocation of specific metabolites from the bacteria to the worm (Gaill et al., 1988). *A. pompejana* has a complete digestive system similar to other ampharetids (Saulinier-Michel et al., 1990). Analysis of both the mouth structures and lumenal contents reveal the presence of bacterial cells (Desbruyères et al., 1983). This suggests that direct feeding on bacteria does occur, but its significance to the overall nutrition of the host remains unknown.

Comparison of $\delta^{13}$C values of *A. pompejana* and *Riftia pachyptila*, a gutless, vent invertebrate that harbors endosymbiotic chemosynthetic bacteria, suggests that these two organisms derive their organic carbon from a similar source (Desbruyères et al., 1983). The nutrition of *Riftia pachyptila* is provided entirely by the resident chemosynthetic endosymbionts (Cavanaugh et al., 1981; Felbeck, 1981). At this time it is unknown whether the endosymbionts provide nutriment via some translocation product or are merely selectively digested by the host. Examinations of the worm's anatomy reveals disorganized collagenous fibers underlying the worm's cuticle at the intersegmentary spaces (Gaill et al., 1987) as well as highly vascularized underlying epithelium (Desbruyères et al., 1985). Some of the rod-shaped bacteria present in the population have thin filamentous structures which attach to the worm's cuticle (Gaill et al., 1987). These observations have led
the authors to suggest the potential for the passage of metabolites from the bacteria to the worm (Alayse-Danet et al., 1987; Gaill et al., 1988).

A second hypothesis for the role of the epibionts is in the detoxification of the immediate environment of *Alvinella pompejana* (Alayse-Danet et al., 1987; Gaill et al., 1988). As stated previously, this alvinellid lives in an environment with high concentrations of biologically toxic heavy metals and hydrogen sulfide. Many bacteria are known to possess strategies for toxic heavy metal resistance, such as sequestering metals in the cell wall or in intracellular compartments, enzymatic modification of these metals to less toxic forms, or by rapidly expelling these metals from the cytoplasm (Silver et al., 1989). It is thought the epibiotic microflora of *A. pompejana* could participate in the detoxification the worms' environment through the concentration of toxic heavy metals. A similar function for the epibiotic bacteria of the crab *Helice crassa* has also been proposed (Johnson et al., 1981). These bacteria colonize the carapace and gills of the crab and participate in the concentration of chromium.

Hydrogen sulfide, at concentrations less that one-thousandth that found in vent habitats, is highly toxic to almost all living systems. Sulfide poisons certain respiratory proteins, such as cytochrome c oxidase (N.R.C., 1979), involved in the use of molecular oxygen in aerobic respiration. Several novel mechanisms exist in certain invertebrates to cope with the elevated sulfide levels in the vent systems. *Riftia pachyptila* possesses a sulfide binding protein in the blood which tightly binds free sulfide thus preventing access to sulfide sensitive cellular constituents (Arp et al., 1983; Powell et al., 1983). Marine organisms that are routinely exposed to toxic levels of locally produced sulfide often possess high activities of sulfide oxidizing systems (Somero et al., 1983). Although the blood of *A. pompejana* possesses
extremely high binding efficiencies for oxygen in order to deal with the elevated temperatures and hypoxic conditions (Toulmond et al., 1990), no specific sulfide binding capability has been reported. It is conceivable that the microbial community associated with *A. pompejana* oxidizes available sulfide within the tube, thereby detoxifying the immediate environment of the worm.

**Conclusions of Previous Research**

The majority of epibiotic associations of bacteria with metazoans are poorly understood. Deciphering the functional role of the bacteria in these relationships is often confounded by the complexity of the associated bacterial communities. Nowhere is this problem more evident than with the epibiotic bacterial community associated with the deep-sea hydrothermal vent polychaete *A. pompejana*. This epibiotic microflora has been shown to be both morphologically and metabolically diverse; however, the exact relationships between the bacteria and the polychaete have yet to be definitively established. It has been suggested that the bacteria participate in the nutrition of the worm, and/or in the detoxification of the worms' environment. Previous experiments have been unable to prove either of these hypotheses. One of the main difficulties in researching such a relationship is the viability of the microorganisms in culture. The filamentous bacterial form, which has been suggested to play an important symbiotic role, has not been observed in previous culture attempts. It is conceivable that these bacteria are pleiomorphic and might already be in previously established culture collections, however the reliance on morphology for the characterization of this population is a major weakness
in these studies. Even if the pleiomorph of the filamentous bacteria did appear in culture, it would be difficult to identify its spatial distribution in the worm's environment. In order to circumvent these difficulties and begin to understand the phylogenetic diversity of the alvinellid epibiont community, an experimental strategy developed for the analysis of mixed populations of free-living microorganisms was chosen. This approach is based the philosophy that, like organelles, microorganisms can be studied without cultivation. The approached used in this study is based on the sequence and phylogenetic analysis of bacterial 16S rRNA genes cloned directly from natural microbial communities.

Use of the 16S rRNA Molecule

Nucleic acid sequence comparisons have provided an invaluable perspective on the evolutionary relationships of microorganisms. Microbial systematics had previously relied on certain phenotypic characters of microorganisms, which were later found to be poor phylogenetic indicators. The most utilized molecule for nucleic acid sequence comparisons among microorganisms is the small subunit ribosomal RNA (16S rRNA). The 16S rRNA molecule is functionally and evolutionarily homologous among all organisms, due to its essential role in protein synthesis, and is not laterally transferred between microorganisms (Olsen et al., 1986). Thus, the phylogenetic relationships between the 16S rRNAs are reflective of the evolutionary relationships of the organisms. The sequence of the 16S rRNA molecule, or its gene, are mosaics of both highly conserved and variable regions, which extends the utility of this molecule to a wide phylogenetic range (Woese, 1987).
The utility of 16S rRNA sequence analysis has extended into the study of the ecology and diversity of natural populations of microorganisms. By linking the sequence analysis approach with current cloning technologies, a more complete view of the structure and composition of microbial communities is beginning to emerge. Novel 16S rRNA genes from bacterioplankton samples collected from the Sargasso Sea do not resemble genes from known bacterial species (Britschgi et al., 1991; Giovannoni et al., 1990). In addition, unique 16S rRNA genes belonging to the domain Archaea have recently been detected in the Pacific Ocean (Fuhrman et al., 1992). These analyses have also been extended to the analysis of microorganisms associated with marine snow (DeLong et al., 1993) and to the examination of the endosymbiotic bacteria associated with the gutless bivalve Solemya reidi (Cary, 1994). These molecular techniques are ideally suited to analyze the microbial community associated with Alvinella pompejana.

Experimental Rationale

The primary goal of this study is to characterize the evolutionary relationships of the predominant bacteria present in the epibiotic population of microorganisms associated with Alvinella pompejana. The dominant member(s) of this population are filamentous bacteria. Due to the abundance of these bacteria in the population and the intimate association with A. pompejana (see previous section), this bacterial form is believed to play an important symbiotic role. Therefore, its further characterization may be important in defining the nature of this symbiosis.

In this study, cloning and sequencing methodologies were matched to genetically dissect the bacterial community associated with Alvinella
pompejana (Figure 3). A specimen of *A. pompejana* was collected from an active hydrothermal vent site at 13°N on the EPR and was preserved frozen to maintain the integrity of the nucleic acids. A small patch of bacteria was removed from the dorsal integument of the animal. DNA was purified from this sample. The 16S rRNA genes were amplified using the polymerase chain reaction (PCR) with general bacterial primers. These primers are known to hybridize to the 5' and 3' ends of the 16S rRNA genes of all known bacteria. Thus, this provides a representation of the complete bacterial 16S rRNA genes present in an environmental DNA sample. These primers have proven successful in amplifying diverse assemblages of bacterial 16S rRNA genes from natural populations. The amplified 16S rRNA genes were subsequently cloned into a plasmid vector to separate and propagate the amplified genes. In order to identify unique phylotypes and determine their numerical distribution in the library, each cloned 16S rRNA gene was screened using restriction fragment length polymorphism (RFLP) analysis. Assuming the numerical dominance of the phylotypes in the library is reflective of the actual distribution in the natural community, representatives from the four most dominant clone types were chosen for complete gene sequencing and phylogenetic analysis.
EXPERIMENTAL APPROACH

Alvinella pompejana

Isolate bacteria

Extract and purify DNA

Amplify 16S rRNA genes

Construct clone library

Group clones using RFLP analysis

Sequence and phylogenetically analyze representatives of the numerically dominant clone types

Figure 3. Schematic outline of the experimental strategy used for the phylogenetic analysis of the epibiotic microbial community associated with the hydrothermal vent polychaete Alvinella pompejana.
METHODS

Sample Collection.

Specimens of *Alvinella pompejana* were collected in October 1991 during the joint French-US HERO (Hydrothermal Environment Research Observatory) expeditions at the Elsa vent site 13°N (2620m) on the East Pacific Rise (12°48'N, 103°56'W). Individuals were collected from worm colonies along the walls of active chimneys using the mechanical arm of the Deep Sea Research Vessel Alvin. The 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.

Bacterial Removal and DNA Purification.

A large patch of bacteria, including a portion of worm integument, was removed from a frozen *A. pompejana* individual using sterile forceps. The bacterial sample was completely homogenized in 5M guanidinium isothiocyanate, 50mM Tris (pH 7.4), 25mM EDTA, 0.8% 2-mercaptoethanol, using a Dounce homogenizer. The homogenate volume was doubled with 50mM Tris-Cl (pH 8.0), 25mM EDTA, and centrifuged at 10,000 rpm in a tabletop centrifuge for 15 minutes at room temperature to remove cellular debris. Nucleic acids were precipitated with 0.8 volumes of 100% isopropanol at room temperature for 1 hour. The precipitate was collected by centrifugation at maximum speed in a tabletop centrifuge for 30 minutes, washed with ice cold 70% ethanol, resuspended in TE buffer (10mM Tris-Cl, 1mM EDTA pH 8.0). The extracted bulk nucleic acids were digested with proteinase K (a final concentration of 500µg/ml at 50°C for 1 hour). The
sample was then extracted twice with an equal volume of phenol/chloroform (4:1) and once with an equal volume of chloroform/isoamyl alcohol (24:1). For each extraction the phases were allowed to completely separate and the aqueous layer was retained. Nucleic acids were precipitated overnight with 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of cold 100% ethanol at -20°C. The precipitate was collected following centrifugation as above, washed with 70% ethanol, and resuspended in TE.

To remove contaminating RNA's the sample was treated with RNase (33 ng/µl) for 10 minutes at 65°C, and phenol purified as above. The integrity of the DNA sample was determined by agarose gel electrophoresis through a 0.8% agarose gel in 1X TAE (40mM Tris-acetate, 1mM EDTA). The final DNA concentration was determined spectrophotometrically.

Amplification of 16S rRNA Genes.

Bacterial 16S rRNA genes were amplified from the environmental sample using the polymerase chain reaction (PCR) using two general bacterial 16S rRNA primers: EubB (5'-AGA GUU UGA UCM UGG CUC AG-3') and EubA (5'-ACR CCN ACC TAG TGG AGG AA-3'); (Giovannoni, 1991). PCR amplifications were performed on a Coy thermal cycler (Coy Corporation, Ann Arbor, MI). The reaction conditions were: 50mM KCl, 10mM Tris-HCl (pH 8.4); 2.5mM MgCl2; 0.2mM each dATP, dCTP, dGTP, dTTP; 0.2µM of each amplification primer; 10ng of purified template DNA; 2.5U Taq polymerase (Promega), in a total volume of 100µl. Amplification profile conditions were 95°C for 1 min., 55°C for 1 min., and 72°C for 3 min. for 30 cycles. The resulting PCR products were ethanol precipitated and analyzed by gel electrophoresis as described above.
Construction and Screening of Clone Library.

Two separate clone libraries were constructed from a single *A. pompejana* specimen. The amplified genes were cloned using a plasmid vector (pCRII, Invitrogen) specifically designed to accommodate PCR products that contain single non-template dependent additions of deoxyadenosines at the 3' ends of the DNA duplexes. The only significant difference between the two libraries was that in the first attempt the host cells were less competent and resulted in a low transformation efficiency.

The clone libraries were constructed using the TA Cloning System version 1.3 (Invitrogen) following the manufacturer's instructions, with the following modifications. The ligation reaction was performed with PCR products obtained from a reaction with a reduced number of cycles (30) and that had been ethanol precipitated prior to ligation. The transformants were plated onto LB agar plates (10g tryptone, 10g NaCl, 5g yeast extract, 15g bacto-agar/L, pH 7.0) containing the antibiotic kanamycin (50µg/ml) with 25μl of X-Gal (40mg/ml) spread evenly across the agar plates. White colonies were scored and picked from all available plates and confirmed by restreaking. Stab cultures of positive clones were made in LB agar for long-term storage.

Plasmid DNA was prepared from each of the confirmed transformants using an alkaline lysis preparation protocol (Sambrook et al., 1989). Confirmed white colonies were grown in 5ml of LB media containing kanamycin (50µg/ml) overnight at 37°C with vigorous shaking. Two milliliters of this culture was used in the plasmid preparation. The purified plasmids were resolved on a 1% agarose gel in 1X TAE and visualized with ethidium bromide staining. A plasmid known to contain the correct size insert and one lacking an insert (blue) were used as size standards.
Transformant plasmid DNAs which co-migrated with the 1.5 kb-insert control were scored as having a full-length 16S rRNA gene.

RFLP Analysis.

In order to sort the clones into similar clone types, clones containing full-length inserts were subjected to RFLP analysis. The cloned insert was re-amplified using the same PCR conditions and the primers used previously to establish the library. This amplification step was required to generate a sufficient amount of isolated product for the restriction analysis. 1μl of a 1:10 dilution of the each of the alkaline lysis plasmid DNA preparations was used as the starting template.

Each PCR product was digested using two restriction endonucleases, *Hae* III and *Mbo* I (Promega), each of which recognize a four-base pair restriction site. 5μl of each PCR reaction was digested with 2.5U of each enzyme in 0.5X restriction buffer (5mM Tris-Cl, 5mM MgCl₂, 25mM NaCl, 0.5mM DTT pH 7.9) at 37°C for 2 hours. The final reaction volume was 10 μl. The reaction was stopped by the addition of 0.5μl of 500mM EDTA (pH 8.0). The entire reaction volume was electrophoresed through a 3% NuSieve agarose gel (FMC) in 1X TAE using a standard 6X loading buffer (Sambrook et al., 1989). NuSieve agarose was used in these analyses because it has been specifically developed for resolving DNA products less than 1000 bp. A third tracking dye, orange G, was added to the mixture (0.35% w/v final concentration) to aid in the running consistency between gels.
Gene Sequencing

Representative clones from each of the four numerically dominant clone families resolved by the RFLP analyses were bidirectionally sequenced using a combination of both automated and manual plasmid sequencing. Automated sequencing was performed on an ABI model 373A automated gene sequencer at the Center for Gene Research and Biotechnology (Oregon State University).

Manual sequencing was performed by standard dideoxynucleotide-terminated sequencing protocols using a Sequenase v2.0 DNA sequencing kit (U.S. Biochemical Corp.) Sequencing reactions were performed following the supplier's instructions. Template plasmid DNA was prepared using Magic Mini plasmid purification kits (Promega). Two μg of purified DNA were denatured for each sequencing reaction. Template was denatured as follows: 2 μg of plasmid DNA were brought to a final concentration of 0.18M NaOH/0.18mM EDTA and incubated for 5 min. at room temperature. The DNA was precipitated with 0.1 volumes of 3M sodium acetate and 2 volumes of ice cold 100% ethanol for 30 min. at -70°C. The precipitate was collected by centrifugation at 20,000 rpm at 4°C for 30 min. in a Beckman TL-100 refrigerated tabletop ultracentrifuge. The pellet was washed in ice cold 70% ethanol and centrifuged. The pellet was allowed to air dry and was stored at -70°C until used for sequencing. Sequencing reactions were resolved by electrophoresis through a 7% polyacrylamide gel using Long-Ranger acrylamide (AT Biochem).
Phylogenetic Analysis

The alvinellid clone 16S rDNA sequences were manually aligned to a subset of other aligned bacterial 16S rRNA sequences obtained from the Ribosomal Database Project (RDP) (Larsen et al., 1993). 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 insure a correct alignment of the homologous regions of the sequences. Sequence data were manipulated using the Genetic Data Environment (GDE) v2.0 sequence analysis software package (Smith et al., 1992). Regions of ambiguous alignment i.e. positions 1-11, 69-102, 128-132, 176-226, 265-269, 451-480, 840-869, 999-1045, 1262-1298, 1357-3' terminus (using the E. coli numbering system) were excluded from the analysis. A total of 1061 nucleotide positions were used in the analysis. Phylogenetic trees were edited using 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 (Felsenstein, 1993). DNADIST was used to calculate evolutionary distances with the Kimura 2-parameter model for nucleotide change and a transition/transversion ratio of 2.0 (Kimura, 1980). Phylogenetic trees were reconstructed from evolutionary distance data using the neighbor-joining method (Saitou et al., 1987), implemented through the program NEIGHBOR. Parsimony trees were reconstructed using DNAPARS. A total of 100 bootstrapped replicate resampling data sets for both DNADIST and DNAPARS were generated using SEQBOOT, with random sequence addition and global rearrangement. 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 (Gutell, 1993).
RESULTS

Clone Library Construction

DNA was isolated from a sample of the epibiotic microflora obtained from an *A. pompejana* individual and the bacterial 16S rRNA genes were amplified from this preparation. Two separate attempts were made at cloning these amplified genes. Both attempts used the TA Cloning System v1.3 (Invitrogen); however the first attempt utilized a version in which the competency of the cells was significantly lower. In the earlier attempt a total of 40 white transformants were obtained and all were screened for correct sized (1.5kb) inserts. Only 4 transformants was found to contain full-length inserts. The second attempt using more competent host cells resulted in 249 total white transformants. A total of 162 white transformants was screened and 135 of these contained the correct size inserts. Clones containing full-length inserts from both attempts were grouped into one library resulting in a final total of 139 clones. Clones from the first attempt are designated "APG #A", while those from the second library are designated "APG #B".

Restriction Fragment Length Polymorphism Analysis

In order to identify unique clone types, clones confirmed to contain a full-length insert were subjected to RFLP analysis. The insert was first amplified from purified plasmid DNA and the resulting amplification product was digested with two restriction endonucleases, *Hae* III and *Mbo* I. The clones were then grouped into "families" based on 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 4 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.

Table 2 shows the numerical distribution of each of the clone families. Four of these families, designated F1, F2, F9, and F13, dominated the library, representing 16.4% (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. Based on 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).

16S rRNA Gene Sequences

The 16S rRNA gene sequences for the four alvinellid clones have been deposited in GenBank under the following accession numbers: APG 5A, L35523; APG 13B, L35520; APG 44B, L35521; and APG 56B, L35522.
Figure 4. Scaled illustration of the restriction patterns of 16S rDNAs representing 11 clone families identified in the alvinellid clone library. Each of these families contain 2 or more representative clones. Molecular weight standards (M) are included for comparison. The fragment sizes are given in base pairs.
Table 2. RFLP analysis results showing the numerical distribution of the alvinellid clone families.

<table>
<thead>
<tr>
<th>Clone Family</th>
<th>Number of Clones</th>
<th>% of Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 1(^a)</td>
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<td>16.5</td>
</tr>
<tr>
<td>F 2</td>
<td>60</td>
<td>43.2</td>
</tr>
<tr>
<td>F 3</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 4</td>
<td>3</td>
<td>2.2</td>
</tr>
<tr>
<td>F 5</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 6</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 7</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 8</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 9</td>
<td>8</td>
<td>5.8</td>
</tr>
<tr>
<td>F 10</td>
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<td>F 11</td>
<td>4</td>
<td>2.9</td>
</tr>
<tr>
<td>F 12</td>
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</tr>
<tr>
<td>F 13</td>
<td>6</td>
<td>4.3</td>
</tr>
<tr>
<td>F 14</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 15</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>F 16</td>
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<tr>
<td>F 17</td>
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<tr>
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<td>F 32</td>
<td>1</td>
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</tr>
</tbody>
</table>

\(^a\) Highlighted rows indicate the four numerically dominant clone families chosen for phylogenetic characterization.
Phylogenetic Analysis

Three different types of analyses were performed to determine the correct phylogenetic placement of the four alvinellid clones: 1) phylogenetic tree reconstruction inferred from both evolutionary distance and character based calculations, 2) comparison of secondary structural models of the clone 16S rRNA to other established 16S rRNA secondary structures, and 3) comparison of established signature base positions in the 16S rRNA molecule.

Figure 5 is a phylogenetic tree inferred by 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, Desulfurella acetivorans (Rainey et al., 1993). Bootstrap values corresponding to 100 replicate re-samplings are shown above the lines leading to each branch, unless otherwise designated. This analysis included a total of 1061 nucleotide positions.

The four alvinellid clone 16S rRNA sequences form their own cluster within the epsilon subdivision of the Proteobacteria. The alvinellid clones cluster together to the exclusion of other species, supported by a bootstrap value of 100%. This cluster clearly groups within the epsilon Proteobacteria, which is 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%, respectively, 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.
Figure 5. 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 neighbor-joining method. Molecular sequences for all reference strains (except Desulfurella acetivorans) was obtained from the RDP. Boldface type 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).
Despite the high confidence placed in the branch associating the alvinellid cluster with the epsilon Proteobacteria, the position of the alvinellid cluster within this subdivision is not supported as strongly. In the tree shown in Fig. 5, the alvinellid cluster appears as a sister group to the *Campylobacter/Thiovulum* group. This branch is supported by bootstrap values of only 57% by the neighbor-joining method, and 66% by the parsimony method. Examination of the alternative branching orders obtained with these analyses showed the alvinellid cluster varied in its position within the epsilon subdivision. The alvinellid cluster also appeared as a deeper group to the epsilon Proteobacteria (data not show). This variation was dependent on the nucleotide positions included in the analyses; however, the affiliation of the alvinellid cluster with the epsilon Proteobacteria was consistently recovered.

Higher-order structural features of the 16S rRNA molecule are useful in the phylogenetic characterization of microbial groups (Woese, 1987b). Comparison of the 16S rRNA secondary structural models between distantly related groups, i.e. at the phyla and subdivision levels, can reveal structural features which support their phylogenetic relatedness. Comparison of these structural features between closely related groups is usually not as informative. Because 16S rRNA sequences for the alvinellid clones APG 5A/APG 13B and APG 44B/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.
Figures 6 and 7 show 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 were observed which support the placement of the alvinellid clones within the epsilon Proteobacteria. Three secondary structural features characteristic of the epsilon Proteobacteria (then referred to as the *Campylobacter Thiovulum* subdivision) had been previously reported (Lane et al., 1992). All three of these structural features have been observed in our alvinellid clones (position numbers are those of the 16S rRNA sequence of *E. coli*): 1) a conserved deletion of a helix corresponding to positions 455-477, 2) a unique compensatory base pair change at position 921 (U → A) and 1396 (A → U), and 3) a "long-short" stem arrangement involving stem structures at positions 184-193 and 198-219 respectively (Figure 6 and 7). In addition to these, another feature was observed which has been reported among the epsilon Proteobacteria (Rainey et al., 1993): an insertion of a (C) in a conserved loop structure corresponding to positions 1357-1365. This was observed independently of the discovery by Rainey and colleagues (1993).

In order to confirm the results from the previous two phylogenetic analyses, an examination of the signature base positions distinguishing the subdivisions of the Proteobacteria was undertaken. Signature base positions are specific positions within the 16S rRNA molecule which have been used to distinguish various members of the bacterial, archace, and eukaryal domains (Woese, 1987). Because the epsilons are a newly recognized subdivision of the Proteobacteria, the signature base positions for these members have only recently been compiled (Rainey et al., 1993). In order to confirm the composition of these nucleotide positions, complete 16S rRNA
Figure 6. Proposed secondary structural model of clone APG 13B 16S rRNA. Numbers indicate regions which are discussed in text. This structural model was obtained using the gRNAID v 1.4 program and comparative analysis with other bacterial 16S rRNA structures (Gutell 1993).
Figure 7. Proposed secondary structural model of clone APG 56B 16S rRNA. Numbers indicate regions which are discussed in text. Positions with asterisks denote signature nucleotide discrepancies which were further examined for compensatory base pair changes. This structural model was obtained using the gRNAID v 1.4 program and comparative analysis with other bacterial 16S rRNA structures (Gutell 1993).
sequences representing 51 available epsilon Proteobacteria obtained from the RDP were examined. The signature base positions for the four alvinellid clones were compiled separately for comparison.

Table 3 shows the signature base positions compiled for the epsilon and delta Proteobacteria. A total of 72 signature positions were examined. Out of these 72 positions, 48 were found to differ between the delta and epsilon subdivisions. These signature base positions were also examined in the alvinellid clones. Out of the 48 positions distinguishing the deltas from the epsilons, only four were found to differ between the epsilons and the alvinellid clones (see highlighted positions in Table 3). In order to rule out the possibility that these differences were due to experimental artifacts, these same positions were re-examined 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 (Figure 7). Compensatory base pair changes are changes in variable nucleotides which preserve the conserved secondary structure of the 16S rRNA molecule (Gutell et al., 1990). This type of comparison is not possible with the difference at position 108 because it occurs in a non-stem structure.
<table>
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<th>Position</th>
<th>Delta</th>
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a The information for the delta subclass was taken from Table 4 of Woese (1987) and Table 1 of Rainey et al (1993). The information for the epsilon subclass was compiled from an alignment of 51 16S rRNA sequences of epsilon Proteobacteria. Y, pyrimidine; R, purine; N, any nucleotide; N. D., not determined.

b Upper case, major base; if no other specified, it accounts for >90% of assayable cases. Lower case, minor occurrence base; found in <15% of assayable cases (or in only one sequence).

c Shaded positions indicate nucleotide differences between the epsilon Proteobacteria and the alvinellid clones.
DISCUSSION

Bacterial/invertebrate symbioses are well recognized in marine systems. However, defining the fidelity of these relationships is frequently problematic. Differentiating relationships that are commensal, where only one partner gains benefit from the relationship, from those that are truly mutualistic, where both partners profit, is difficult. The partners are usually so intricately tied that resolving the level of interaction has been impossible. Although considerably more is known about monospecific endosymbiotic associations in certain marine invertebrates (Cavanaugh, 1985; Fisher, 1990), there are numerous cases of diverse microbial assemblages, found obligately associated with the external surfaces of certain invertebrates, which have eluded sufficient explanation (de Burgh & Singla, 1984; Johnson et al., 1981; Richards et al., 1982). Molecular genetic techniques now provide an avenue by which to study these tightly associated symbioses.

The relationship between the hydrothermal vent polychaetes Alvinella spp. and their associated microflora has intrigued investigators since the discovery of these unique organisms (Desbruyères & Laubier, 1980b). Both A. pompejana and A. caudata possess morphologically and metabolically diverse assemblages of microorganisms associated with the worms' dorsal surfaces, some of which appear specifically attached (Gaill et al., 1987; Jeanthon & Prieur, 1990; Prieur et al., 1990b; Prieur et al., 1987a); Desbruyères, 1985 #1636. It has been suggested that the dominant integrated morphotype, a filament, plays a significant role in the either the nutrition of its host or in the detoxification of the worms' immediate environment (Gaill et al., 1988). The primary focus of this study was to examine the diversity of
this unique microflora and, through the approach presented here, phylogenetically characterize 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 over 70% of the total number of clones, with the remaining families consisting of only one or a few members. There is no evidence to suggest that 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 is in agreement with previous electron microscopic observations, which demonstrated that a filamentous morphotype dominated the microbial biomass (Gaill et al., 1987). The choice of clone types to phylogenetically characterize 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 Proteobacteria. Both methods gave a bootstrap value of 100%, illustrating the robustness of this placement. In the phylogenetic tree presented here (Figure 5) 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
Proteobacteria to the exclusion of other species; however this analysis is not able to resolve the precise placement of the cluster within the epsilon subdivision with a high degree of confidence.

Two additional comparative analyses of 16S rRNA sequences are commonly used to provide support for the phylogenetic affiliations of organisms: 1) the comparison of specific structural features in 16S rRNA secondary structural models and 2) the identification of specific signature base positions in the 16S rRNA sequence (Woese, 1987b). Both of these analyses were carried out with the sequences of the alvinellid clones, in order to lend additional support to the phylogenetic position obtained with the tree inference methods.

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 Proteobacteria. Lane and colleagues (1992), reported three important structural features which were conserved among several members of the epsilon subdivision (then referred to as the Thiovulum-Campylobacter 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 1357-1363 (E. coli numbering system) was found in all of the alvinellid clones. The significance of this insertion was first reported in Desulfurella acetivorans, a more distant relative of the epsilon Proteobacteria (Rainey et al., 1993). 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 proteobacterial member, Campylobacter sputorum subsp. sputorum (Gutell, 1993), and of a
general alignment of epsilon 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 out 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 Proteobacteria.

The presence of certain 16S rRNA signature nucleotides of the Proteobacteria (Woese, 1987a) provides the final criterion with which to evaluate the phylogenetic position of an organism within the phylum. Because the epsilons are a newly recognized member of the Proteobacteria, the signature base positions for this group have only recently been compiled (Rainey et al., 1993). A total of 72 positions were examined in 51 available epsilon 16S rRNA sequences from the RDP. Comparison of these signatures to the alvinellid clones was consistent with their placement in the epsilon Proteobacteria. Only four positions were found to differ significantly between the alvinellid clones and the epsilons, 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 (DeLong et al., 1993).

Recently a newly isolated bacterium, Desulfurella acetivorans, was phylogenetically characterized as a distant relative to the epsilon Proteobacteria, possibly representing a new subclass of this phylum (Rainey et al., 1993). 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 study confirms the phylogenetic position of \textit{D. acetivorans} proposed by Rainey and co-workers; however, it has identified several discrepancies between a number of the key signature nucleotides for the epsilon Proteobacteria used in their analysis (data not shown). 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 characters of a particular group of microorganisms based solely on 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 which form the two clusters comprising the epsilon subdivision of the Proteobacteria (Eaton et al., 1993; Lau et al., 1987; Lee et al., 1992; Paster et al., 1988; Paster et al., 1991; Romaniuk et al., 1987; Sly et al., 1993; Thompson et al., 1988; Vandamme et al., 1991). A survey of the conserved phenotypic characters among these epsilon genera reveals that the majority are adapted to environments low in oxygen; they range from microaerophiles to some which are able to grow anaerobically (Holt et al., 1994; Kreig et al., 1984; Vandamme et al., 1991). In addition, all of these genera, except the sulfur-oxidizing marine bacterium \textit{Thiovulum} sp. have been found associated with a eukaryotic organism as a host, occurring in digestive tracts, reproductive organs, or oral cavities of higher vertebrates. These conserved phenotypic characters are consistent with the environment of the alvinellid epibionts. As well as being associated with a metazoan host,
these bacteria would also be adapted to the hypoxic conditions within the
tubes of *A. pompejana* (Chevaldonné et al., 1991).

In conclusion, the novel molecular approach described here has
allowed the assessment of the microbial diversity of the epibiotic bacterial
population associated with *A. pompejana*. From an established 16S rRNA
clonal library containing 32 distinct clone variants, several dominant
phylotypes were characterized as members of the epsilon subdivision of the
Proteobacteria. Future studies are currently underway to design and test
oligodeoxynucleotide probes specifically targeting regions of the *Alvinella*
cloned 16S rRNA's. Previous studies have established the utility of such
probes in identifying the morphology of a cell type through *in situ*
hybridization techniques (Amann et al., 1991; Amann et al., 1990; Reysenbach
et al., 1994); Giovannoni, 1988 #533. These experiments will determine the
morphology of the clone types. The probes can then be used to screen large
insert DNA libraries established directly from epibiont nucleic acid samples.
This approach has been applied before to locate functional genes in free-living
marine archebacteria (Stein, 1994). The functional genes can then be related
to specific clone types, contributing additional information about the
metabolic potential of these epibionts. Through these molecular approaches
the function of the relationship between the *Alvinella* spp. and their
associated bacterial population can be determined without the need to culture
the organisms.


APPENDICES
APPENDIX 1

LOCUS APG5A 1496 bp DNA 11-AUG-1994

DEFINITION  Epibiont of Alvinella pompejana of 16S rRNA gene encoding 16S ribosomal RNA.

KEYWORDS  16S ribosomal RNA.

SOURCE  Epibiont of Alvinella pompejana, 5A from APG.

ORGANISM  Epibiont of Alvinella pompejana

Bacteria/Prokaryota; Proteobacteria; subgroup: epsilon.

REFERENCE  1 (bases 8 to 1542)

AUTHORS  Haddad,M.A., Camacho,F. and Cary,S.C.

TITLE  Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete, Alvinella pompejana

JOURNAL  in prep, 0-0 (1994)

STANDARD  full automatic

COMMENT  Data kindly submitted in computer readable form by:
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[1] Author requested hold until 01-OCT-1994

FEATURES  Location/Qualifiers
rRNA  1..1496

/gene="16S rRNA"
/product="16S ribosomal RNA"

BASE COUNT  415 a  321 c  446 g  311 t  3 others

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TTAACACATG CAAGTCGAAC
61 GGTAACAGAT CTTCGGAGTG CTGACGAGTG GCGAACGGGT
GAGTAAGGTA TAGCTAACTT
121 GCCTCTTGGA GAGGGATAGC CACTGGAAAG GGTGATTAAT
ACCTCATACT CCTCTTAACT
181 ATAAGGTTAA GAGGGAAATG GTTTATTCCG CCAAGGGATA
GGGCTATATG GTATCAGTTA
241 GTTGGTGAGGT AGTAGATGGAG GGTGATGAAG

241 GTTGGTGAGGG TAAAAGCGCT CCAAGACC ATGACGCTAGC
TGGTCTGAGA GGATGATCAG
301 CCACACTGGA ACTGAGACAC GGTCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC
361 AACAATGCGGG AAACCTGTAT GCAGCGACGC CGCGTGGAAG GAAAGACCTCT TACGTAATCTG
421 ATAGTATGCT TGCCGTAATA CGGAGGTGTC AAGCGTTACT CGGAATCACT GGGCGTAAAG
481 GCAGCAGTGG GGAATATTGC
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AGCTAACTGT GGGCGTAAAG
601 ATCGAGATGCGA AAAAGTGGG GAGCAACACAG GATTAGATA
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661 ACGATGAATG TTATCGGTTG GAGTGCTAGC CACTTCATG
ATGCAGCTAA CGCATTTAAA
721 AACTGCGGCTT GGTAGTCCCG CCGCAAGGTT AAAACTCAAA
CTGGTGATGTC CAGCCCTAA
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GAACCTTTACC GGGCTCTGAC
841 ATGCGAGGGC ACCTACCGG AAGCGAAAT CGCGGGATGG
AGCAAATCTTA TAAACCTTCT
901 CAACTTTACC GGGCTCTGAC
961 ATATACCAGA ACCCACCAGA GATGGTGGGG TGCCTTCGGA
AGCTGCGCTA CAGCCGTTGC
1021 ACGGCTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG
TGCCGACCG CAGCCGCTAA
1081 TCGTGACTAG TTACTAACAG CATAGGCTGA GGACTCTAGT
CAGACTGCTT TCGTAAGGAG
1141 GAGGAGGTGG GGGACGACGT CAAGTCATCA TGGCCCTTAC
GGCCAGGGGC ACACACCG
1201 AGTACTATA TAAACCTTCT
1261 CTCAGTTCGG ATTGCAGTCT GCAACTCGAC TGCATGAAGC
TGGAATCACT AGTAATCCGT
1321 ATCGAGCCAT GTCACGCTGA ATACGTTCCC GGGTCTTGTA
CTCACGCCTT TCACACCATG
1381 GAGGTTGATT TCACCCGATG CGGGGATGCT AAATAGGCTA
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APPENDIX 2

LOCUS APG13b 1502 bp rRNA 11-AUG-1994

DEFINITION Epibiont of Alvinella pompejana of 16S rRNA gene encoding 16S ribosomal RNA.

KEYWORDS 16S ribosomal RNA.

SOURCE Epibiont of Alvinella pompejana, 13B from APG.

ORGANISM Epibiont of Alvinella pompejana
Bacteria/Prokaryota; Proteobacteria; subgroup: epsilon.

REFERENCE 1 (bases 8 to 1542)

AUTHORS Haddad,M.A., Camacho,F. and Cary,S.C.

TITLE Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete, Alvinella pompejana

JOURNAL in prep, 0-0 (1994)

STANDARD full automatic

COMMENT
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[1] Author requested hold until 01-OCT-1994

FEATURES Location/Qualifiers
rRNA 1..1502

BASE COUNT 424 a 319 c 437 g 320 t 2 others

ORIGIN

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GAGTAATGTA TAGTTAATTT
121 TCCCCTTGGGA GAGGGATAGC CACTGGAAAC GGTGATTAAT
ACCTCATACT CCTTTTAACC
181 AAAAGGTGGA AAGGGAAATG GTTTATTCTG CCAAGGGATA
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241 GTTGGTAGTG TAAGGGACTA CCAAGGCAAT GACGGGTAGC
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361 ACAATGGGGG AAGACGACGG TACTTGATGA ATACGGACCG GCAGGAATTT TAGGGTGTA
421 AACTCCTTTT ATCAAGGAAG AAGACGACGG TACTTGATGA ATACGGACCG GCAGGAATTT
481 TGCCAGCAGC CGCGGTAATA CGGAGGAGGC AAGCGTTACT ATGGAATTT TAGGGTGTA
541 CGCATGTAGG GGATTTGTAC AGTTAGAAGT GAAAGCCTAC GCCTTTTAGG TTTAACAG
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661 ATCCGTAGAG ATTACTAGGA ATACCGAAAAG CAGGGACGAT GCAGGCAGC GAAGGCGAT
721 GTGGAATTT TAGGGTGTA
781 ATCCGTAGAG ATTACTAGGA ATACCGAAAAG CAGGGACGAT GCAGGCAGC GAAGGCGAT
841 ATTCCGCTTG GGAGAGTGTTTTG AAGATGAAAA GTACGTGCTT
901 CAAGGATTTT TAGGGTGTA
961 CATATCTAGG GCAGTACGC ATGGAATTT TAGGGTGTA
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APPENDIX 3

LOCUS APG44b 1502 bp DNA 11-AUG-1994

DEFINITION Epibiont of Alvinella pompejana of 16S rRNA gene encoding 16S ribosomal RNA.

KEYWORDS 16S ribosomal RNA.

SOURCE Epibiont of Alvinella pompejana, 5A from APG.

ORGANISM Epibiont of Alvinella pompejana
   Bacteria/Prokaryota; Proteobacteria; subgroup: epsilon.

REFERENCE 1 (bases 8 to 1542)

AUTHORS Haddad,M.A., Camacho,F. and Cary,S.C.

TITLE Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete, Alvinella pompejana

JOURNAL in prep, 0-0 (1994)

STANDARD full automatic

COMMENT Data kindly submitted in computer readable form by:
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[1] Author requested hold until 01-OCT-1994

FEATURES Location/Qualifiers
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   /gene="16S rRNA"
   /product="16S ribosomal RNA"

BASE COUNT 399 a 344 c 449 g 310 t

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   GTGAGTAGTG TATAGCTAAT
   121 ATGCCCTTTG GAGGGGGATA GCCACTAGAA ATGGTGATTA
   ATACCCCATA CTACTTCTTC
   181 TCACAAGAGT TGATGGGAAA TCTTTTTTGG CCAAAGGATT
   GGGCTATACG GTATCAGCTT
   241 GTTGGTGAGG TAACTGCTCA CCAAGGCTAT GACGCCTAGC
   TGGTCTGAGA GGATGATCAG
APPENDIX 4

LOCUS APG56B  1504 bp DNA  11-AUG-1994

DEFINITION Epibiont of Alvinella pompejana of 16S rRNA gene encoding 16S ribosomal RNA.

KEYWORDS 16S ribosomal RNA.

SOURCE Epibiont of Alvinella pompejana, 5A from APG.

ORGANISM Epibiont of Alvinella pompejana
  Bacteria/Prokaryota; Proteobacteria; subgroup: epsilon.

REFERENCE 1 (bases 8 to 1542)

AUTHORS Haddad, M.A., Camacho, F. and Cary, S.C.

TITLE Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete, Alvinella pompejana

JOURNAL in prep, 0-0 (1994)

STANDARD full automatic

COMMENT Data kindly submitted in computer readable form by:
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[1] Author requested hold until 01-OCT-1994

FEATURES Location/Qualifiers
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  /product="16S ribosomal RNA"

BASE COUNT 397 a 341 c 459 g 305 t 2 others

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  121 TGCCCTTTGG AGGGGGATAG CCACTGGAAA CGGTGATTAA
TACCCCATAC TCCTCGAGAA
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661 CACGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
721 GAGGCGCGAA AGCGTGGGGA GCAAACAGGA TTAGATACCC TGTTAGTCCA CGGCTAAAC
781 GATGGATGTT AGTCGTTGGG GAGCAAGTCT CTCCAGTGAT GCCAGCTAACC GATTAACAT
841 CCCGCACAGG GAGTACCGGT GCAAGACTAA AACTCAAAGG AATAGACCGG CAGGCGCCACA
901 AGTGTTGGAG CATTGGTTTT AATTCAAGA TACGGCGAAGA ACCTTAACCC TGGAGATACG
961 CACGGCTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
1021 CTCATCCTTA GTTGCTAACA GGTTTGGCTG AGAACTCTAA AGAGACTGCC CGGGCAACCG
1081 GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGCCCTTA GGAGGAAGGT GGGGATGACG
1141 GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGCCCTTA GGAGGAAGGT GGGGATGACG
1201 CTACAATGGG CAGGACAGAG AGAAGCAATA CCGCGAGGTG GAGCAAAGCT GTAAACCTTG
1261 CTCTCAGTTC GGATTGTAGT CTGCAACTCG ACTACATGAA GGGGATGACG TCAAGTCATC
1321 CATAACGGGC ATGGCGCGG TCAAGTCATC ATGGCCCTTA GGAGGAAGGT GGGGATGACG
1381 ATGGGAGGTG AGTCCACCGG AAGCGGGGAT GCTAAAATAG CTCTCAGTTC GGATTGTAGT
1441 ATGGGAGGTG AGTCCACCGG AAGCGGGGAT GCTAAAATAG CTCTCAGTTC GGATTGTAGT
1501 CTCT