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Phylogenetic characterization of the bacterial assemblage associated with mucous secretions of the hydrothermal vent polychaete Paralvinella palmiformis

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Abstract: As part of an ongoing examination of microbial diversity associated with hydrothermal vent polychaetes of the family Alvinellidae, we undertook a culture-independent molecular analysis of the bacterial assemblage associated with mucous secretions of the Northeastern Pacific vent polychaete Paralvinella palmiformis. Using a molecular 16S rDNA-based phylogenetic approach, clone libraries were constructed from two samples collected from active sulfide edifices in two hydrothermal vent fields. In both cases, clone libraries were largely dominated by small epsilon, Greek-Proteobacteria. Phylotypes belonging to the Cytophaga–Flavobacteria and to the Verrucomicrobia were also largely represented within the libraries. The remaining sequences were related to the taxonomic groups Fusobacteria, Green non-sulfur bacteria, Firmicutes, γ- and δ-Proteobacteria. To our knowledge, this is the first report of the presence of Verrucomicrobia, Fusobacteria and green non-sulfur bacteria on hydrothermal edifices. The potential functions of the detected bacteria are discussed in terms of productivity, recycling of organic matter and detoxification within the P. palmiformis microhabitat.

Keywords: Paralvinella palmiformis; Hydrothermal vent; Phylogeny; Microbial diversity; 16S rRNA; Proteobacteria

1. **INTRODUCTION**

Over the past decade, our knowledge of the diversity and role of hydrothermal vents micro-organisms and fauna has considerably expanded. Representative species of both the *Bacteria* and *Archaea* domains, including chemolithoautotrophs, chemoorganoheterotrophs and mixotrophs, have been isolated [1-2]. The emergence of molecular methods, independent of traditional microbiological culturing techniques, has improved and accelerated our comprehension of the structure and composition of deep-sea hydrothermal vent microbial communities [e.g. 3-8]. Concurrent with microbiological investigations, more than 500 macro-faunal species from deep-sea vents have been described and their biology, systematic, physiology and ecology investigated [9-14]. Associations between vent metazoans, including vesicomyid clams, mytilid mussels, provannid gastropods, vestimentiferan tube worms, and their sulphide-oxidising bacterial endosymbionts have been the subject of many studies [e.g. 15-18]. However, despite of extensive work on the physiology of these invertebrate/sulphide-oxidising bacteria symbiosis, relatively little is known about other microbe/vent animal associations or interactions between micro-organisms, animals and mineralisation processes.

The work presented here is part of an ongoing project aiming at describing microbial assemblages associated with polychaete worms of the family *Alvinellidae*. The *Alvinellidae*, comprising the two genera *Alvinella* and *Paralvinella*, is a group of terebellomorph polychaetes endemic to deep-sea hydrothermal vents in the Pacific Ocean [19]. *Alvinellidae* species found at modern Eastern and Northeastern Pacific vents differ, but probably evolved from common ancestors that colonised Pacific spreading ridges more than 30 millions years ago, before the separation of the two Eastern Pacific ridge systems [20-21]. Among the alvinellids, *Alvinella* spp. inhabiting walls of actively venting high-temperature chimneys along the East-Pacific rise have been the most studied [22]. They differ from the confamilial genus *Paralvinella* by their obligate association with an epibiotic bacterial community thriving on their integument [9, 11]. The functional role of this obligate association remains unclear, mainly because the dominant filamentous epibiotic bacteria of these worms, which belong to the ε-*Proteobacteria* [3, 23] have yet to be cultured, and because *in vivo* experimentation is difficult with these polychaetes [11]. On the other hand, studies of other *Alvinellidae* species suggest that there are obviously novel microbial associations and that polychaetes-microbes combinations could affect the mineralogy of sulphide edifice structures [24].

This study focused on the microbial assemblage associated with the organic–rich mucous secretions of an other *Alvinellidae, Paralvinella palmiformis* [19]. This alvinellid species, the so-called “palm-worm”, is found only at hydrothermal vent sites along the Gorda, Juan de Fuca and Explorer Ridges in the Northeast Pacific ocean. One of the characteristic feature of this worm is its continual secretion of mucus, presumably as a way of clearing its bodywall of particulate debris. The presence of metallothioneines and high quantities of elemental sulphur in the mucus have led to the suggestion that mucous secretion may also allow the worm to eliminate accumulated toxins [25]. Secreted mucus is colonised by numerous bacteria, unidentified up to now, and by a diversified meiofauna [25]. Mucous secretions often form a loose sheath around the body of individual *P. palmiformis*, and shed mucus accumulates in the vent environment. These organic-rich secretions, in direct contact with hydrothermal fluid, represent *a priori* a high-quality substratum for microbial colonisation. We used molecular characterisation to evaluate the diversity and phylogenetic relationships of the organisms comprising the mucous microbial assemblage, and to consider their potential physiological nature.

2. **MATERIALS AND METHODS**

2.1 *Sample collection and processing*

Material was collected from two deep-sea hydrothermal vent fields located at Axial Volcano on the Juan de Fuca Ridge, during the NeMO (New Millennium Observatory) cruise. Collection of samples was performed on sulphide chimneys of CASM vent field (on T&S chimney, 130°01’W 45°59’N; depth, 1546 m) and ASHES vent field (on Hell chimney, 130°01’W 45°56’N; depth, 1580 m) on dives R626 and R632 (24 and 31 July 2001) of the ROV *Ropos*. These two vent fields are 6.2 kilometres apart. Mucus secretions of the annelid polychaete *Paralvinella palmiformis* were collected in areas containing patches of palm worms (*P. palmiformis*) on a substratum of marcasite (FeS_2) irregularly colonised by sulphide worms *Paralvinella sulfincola*.

Once aboard the ship, mucus secretions still attached to individual *P. palmiformis* were aseptically placed into sterile tubes containing sterile water, and frozen at –80 °C. Samples were transported frozen to the laboratory and thawed just before DNA extraction.

2.2 *DNA extraction and purification*

For each sample, DNA was extracted from approximately 4 g of thawed mucus (wet weight, corresponding to approximately 1g dry weight) following a modified version of the extraction protocol of Juniper *et al.* [26]. Both detergent-based and enzymatic lysis extraction procedure were combined. These relatively gentle methods without physical disruption steps avoid excessive shearing of DNA and reduce the risk of chimera formation during PCR [27].

For DNA extraction, samples were aseptically transferred into sterile centrifuge tubes, suspended in 10 ml TE-Na-1x lysis buffer (100 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0) and shaked during 30 seconds to remove cells from the mucus. This was followed by successive additions of 1 ml Sarkosyl (10%), 1 ml SDS (10%) and 200 µl proteinase K (20 mg/ml; Eurobio). Preparations were briefly vortexed, then incubated at 45 °C. After 1.5 h incubation, samples were centrifuged for 30 min at 6000 *g* and the supernatants were collected. The pellets were then resuspended in lysis buffer and the same volumes detergents and enzymes used previously were added. The samples were re-incubated at 45 °C and centrifuged after 2.5 h longer incubation (after a total of 4 h lysis, intact cells could not be observed by microscopy). Supernatants from the 1.5 h and 4 h lysis treatments were pooled and extracted with equal volumes of buffered (pH 8.0) PCI (Phenol/Chloroform/Isoamylic Alcohol: 25/24/1). After centrifugation 20 min at 14000 *g*, the upper aqueous phase containing nucleic acids was removed and extracted again with equal volumes of 100% chloroform (centrifugation 20 min at 14000 *g*). After centrifugation for 20 min at 14000 *g*, the final aqueous phase containing DNA was collected. To assure the precipitation of extracted DNA and its visualisation after centrifugation, samples were supplemented with 45 µl of eukaryotic DNA (herring sperm DNA solution at 6 mg/ml). DNA was then precipitated by addition of a 0.7 volume of 100% isopropanol and incubation overnight at –20 °C. After centrifugation at 14000 *g* for 40 min, the supernatants were poured off and the DNA pellets were air dried before being redissolved in 500 µl TE-1X buffer (10mM Tris/HCl, 2 mM EDTA, pH 7.5). Absorbance was determined at 230, 260, 280 and 320 nm, on a GenQuantII spectrophotometer (Pharmacia Biotech Inc., Uppsala, Sweden) to check the purity of the nucleic acids obtained and to determine their concentration.

DNA was then purified by cesium chloride (CsCl) density gradient centrifugation as described by Juniper *et al.* [26].

2.3 *Amplification and cloning of 16S rDNA genes*

Community 16S rDNAs were selectively amplified by PCR with universal oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of the 16S rRNA genes. The bacterial forward primer was SAdir (5'-AGA GTT TGA TCA TGG CTCA GA-3') corresponding to positions 8-28 in the *Escherichia coli* 16S rRNA and the bacterial reverse primer was S17rev (5'-GTT ACC TTG TTA CGA CTT-3'), corresponding to positions 1493-1509. 16S rDNAs, intergenic sequences and part of the 23S rDNA of the *Archaea* were amplified with the specific forward primer 21Fa (5'-TTC CGG TTG ATC CTG CCG GA-3') and a specific reverse primer 23SA-REV (5'-CTT TCG GTC GCC CCT ACT-3') (position 257-234 on *Thermococcus celer* 23S rDNA sequence). Libraries were produced from community rDNA by amplifying approximately 100 ng of bulk DNA in 50 µl reaction mixtures containing (final concentrations) 1X PCR buffer (QBIOgene, France), 200 µM of each deoxyribonucleoside triphosphate, 100 µM of each forward and reverse primer and 2.5 U of *Taq* polymerase (QBIOgene). Reaction mixtures were incubated in a Robocycler Gradient 96 (Stratagene, La Jolla, CA.). The cycling program was as follows: 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 48 °C for 1.5 min, 72 °C for 2 min and a final extension period of 6 min 72 °C.

After verification of the quality of the amplifications on a 0.8% (w/v) agarose gel, the PCR products were cloned directly to separate them from each other, by the TA cloning method, using a TOPO TA Cloning® kit (pCR2.1 vector), according to the manufacturer's instructions (Invitrogen). Clone libraries were constructed by transforming *E. coli* TOP10F' cells. For each *Paralvinella palmiformis* mucous sample, three bacterial clones libraries were constructed from three independent PCR amplifications and cloning experiments.

2.4 *Screening of 16S rDNA clones by Amplified Ribosomal DNA Restriction Analysis (ARDRA).*

Each recombinant colony was suspended in 100 µl of sterile deionized water. Ribosomal DNA inserts from recombinant clones were amplified by PCR from 5 µl of colony suspension, using the same reaction mixtures as described above. The PCR program was the same as that for the initial amplification of the rDNA (see above), except that the initial step lasted 10 minutes (for cell lysis). Insert-containing clones of the six libraries were then screened by Amplified Ribosomal DNA restriction Analysis (ARDRA) using 5 U of the restriction endonucleases *Hha*I and *Rsa*I according to the manufacturer's instructions (New England Biolabs). The DNA fragments were separated by gel electrophoresis on a 2% agarose (agarose type XI,

Sigma) gel run in TAE-1x (Tris-Acetate-EDTA) buffer. The clones were separated into different phylotypes based on the ARDRA banding patterns.

2.5 *16S rDNA sequencing and phylogenetic analysis.*

At least one gene representative of each unique phylotype was completely sequenced using fluorescent dideoxy terminators (Sanger method [28]). For dominant patterns (more than 20 clones per pattern), five clones were partially sequenced to check for the homogeneity of the group. Sequencing procedures were as described previously [29] and the work was carried out by Genome Express S.A. (Grenoble, France) with automatic DNA sequencers (Perkin Elmer). Sequences were assembled with the SeqMan program (DNASTAR software; Madison, Wis., USA). These sequences were checked for the presence of chimeras using the Ribosomal Database Project's (RDP) CHIMERA_CHECK program [30] and by using the BLAST (Basic Local Alignment Search Tool) [31] program to compare 16S rDNA partial sequences.

Sequences were compared to those in available databases using the BLAST [31] network service to determine approximate phylogenetic affiliations. Partial sequences were compiled and aligned with the rDNA sequences from the Ribosomal Database Project, using the GCG CLUSTALW program [32]. Alignments were refined manually, using the SEAVIEW program [33]. All the trees presented were constructed by the PHYLO-WIN program [33], on the basis of evolutionary distance [34] and maximum likelihood [35] methods. The robustness of inferred topologies was tested by the bootstrap resampling [36] of trees calculated on the basis of evolutionary distance (neighbor-joining algorithm with Kimura two-parameters correction [37]) and maximum likelihood. If ARDRA patterns were not identical but related sequences displayed over 97% sequence similarity, only one of the sequences was retained for phylogenetic analysis.

The EMBL accession numbers of the 51 sequences used in this study are AJ441198 to AJ441248.

2.6 *Accumulation curves and comparison of diversity in the two environments*

To determine if the clones libraries were representative of the microbial diversity of the samples, accumulation curves were constructed for the different ARDRA banding patterns of 16S rDNA clones of each sample of *P. palmiformis* mucus secretions. Numbers of different ARDRA patterns in clone libraries were determined after digestion with the two restriction endonucleases (the accumulation curve of each

sample comprised cumulative ARDRA patterns of the three bacterial clones libraries constructed for each sample). The 16S rDNA clonal libraries of the *P. palmiformis* mucous samples from the two vent fields (CASM and ASHES), were compared statistically by determining differences between homologous coverage curves and heterologous coverage curves [38] as in Singleton [39]. The homologous coverage (Cy) of the library of 16S rRNA genes called Y, by a sample from Y, is calculated by the following formula:

$Cy = 1 - (Ny / n)$

where *Ny* is the number of unique sequences in the sample and *n* is the total number of sequences. The heterologous coverage (Cyx) of Y, by a sample from X from another collection of sequences, is defined by the formula:

$Cyx = 1 - (Nyx / n)$

where *Nyx* is the number of sequences in a sample of Y that are not found in a sample of X and *n* is the number of sequences in the sample of Y. *Ny* and *Nyx* were calculated for different levels of phylogenetic distance (D) to generate coverage curves Cy(D) and Cyx(D). Differences between the homo- and heterologous coverage curves were determined by the Cramér-von Mises test [40] and compared by a Monte-Carlo test procedure [41]. The compared sample clones libraries were constructed following the same procedure: each was generated by pooling of libraries obtained from three independent amplifications. The two libraries comprised nearly the same number of clones (141 clones in one case against 143 in the other case). The CLUSTALW program [32] was used to create an alignment containing all of the sequences in the two libraries, and to calculate their phylogenetic distances (the sequences of all phylotypes were copied the same number of times that they occured in the sample). Then, phylogenetic distances data were transferred to a program, written in Fortran, that calculated homo- and heterologous coverage curves [40] and compared these values [41] using the formula of Singleton [39] (for detail on the statistical method, please refer to this article). Because of the large variability of p-values with the seed of the random number generator used, the number of Monte-Carlo simulations was increased tenfold as per Singleton [39]. This assured stability of the p-values whatever the random number generator seed used, even though the coverage curves quickly approach 1 when phylogenetic distance increases.

3. **RESULTS**

3.1. General microbial rDNA composition of the clone libraries from *Paralvinella palmiformis* mucus.

In this study, bacterial clone libraries were constructed from mucus secretions of *Paralvinella palmiformis* from CASM (T&S chimney) and ASHES (Hell chimney) vent fields, collected during the NeMO oceanographic cruise. Mucous cocoons were collected in areas of medium to high temperature where patches of *P. palmiformis* mixed to patches of *P. sulfincola.* The studied samples represent composition of bacterial assemblages associated with mucous cocoons of *P. palmiformis* from Juan de Fuca faunal assemblages of type II [14, 42].

All attempts to amplify archaeal 16S rDNA from these samples failed, whatever the approach used, while the same procedure applied to hydrothermal samples from hotter areas allowed a good amplification of archaeal 16S rDNAs. For this study, only bacterial 16S rDNA products were obtained. To represent for the best the bacterial diversity of the samples, the CASM and ASHES libraries were both constructed by the compilation of three clone libraries stemmed from three independent PCR amplifications and cloning experiments. A total of 141 insert-containing clones for the CASM library and of 143 for the ASHES one were screened by ARDRA using two restriction enzymes with tetra-nucleotide recognition sequences. As demonstrated by a precedent study [43], RFLP (and consequently ARDRA) patterns created by the use of tandem restriction endonucleases, with tetra-nucleotide recognition sequences, allow the detection of the wide majority of the taxa present in a library. The use of two restriction enzymes significantly decreases the probability to retrieve different sequences types within a given phylotype. For the CASM and the ASHES libraries, sixty-four and fifty-nine phylotypes (unique RFLP profiles, called OTUs) were respectively detected, thirty of which were shared by both libraries. The 16S rDNA of at least one representative of each phylotype was partially sequenced. It has been observed that micro-organisms displaying DNA-DNA hybridisation above 70%, and belonging therefore to the same species, share usually more than 97% 16S rRNA sequence similarity [44]. Consequently, sequences differing less than 3% were considered as related groups for phylogenetic analysis and only fifty-one 16S rDNA genes were completely sequenced. Chimeric sequences accounted for only 0.7% (n=1) of the CASM clone library and 1.4% (n=2) of the ASHES clone library. They were excluded from the phylogenetic analysis. Chimeras recombination sites (breakpoints) were detected. In other respects, the phylogenetic position of two sequences (*P. palm.* C 70 and *P. palm*. C

72) of the CASM library, unlikely to be chimeras, was unresolved. BLAST analysis did not provide any evidence that these sequences were related to any other sequence. Highly-conserved regions characteristic of bacterial sequences were retrieved within these sequences. Almost the same phyla were represented in the populations from both vent fields. Sequences belonging to the ε-*Proteobacteria*, the δ-*Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, the *Verrucomicrobia*, the Green non-sulfur bacteria, the *Fusobacteria* and the Firmicutes were detected in both libraries and few sequences belonging to the γ-*Proteobacteria* were moreover detected in the sample from ASHES.

3.2. Accumulation curves and statistical analysis

It is highly probable that the diversity in the samples might not be fully covered. However, the decrease in the rate of phylotype detection of accumulation curves (Fig. 1), indicated that the major part of the diversity in the libraries was detected.

The two clonal libraries were statistically analysed to determine if differences between the samples could have been detected. For statistical calculations, the CASM and ASHES libraries were respectively called X and Y, the homologous coverage of Y by a sample from Y was defined as Cy and the heterologous coverage of Y by a sample of X was defined as Cyx. To compare and determine if the ASHES and CASM libraries were significantly different, the heterologous coverage of the ASHES library by the CASM library (Cyx) has been calculated. The ASHES clones were found to be significantly different from the CASM clones (Pyx=0.003). More information on the nature of this difference was obtained by examination of the distribution of $(Cy-Cyx)^2$ as a function of the evolutionary distance D (Fig. 2). At low phylogenetic distance ($D < 0.22$), the libraries differed. On the other hand, they shared many groups of deep relatedness. The typical 16S rRNA sequence difference between microbial divisions or phyla is 20 to 25% [45]. Based on this principle, we can conclude that the populations of CASM and ASHES differed at the species and genera levels but were comparable at high taxonomic level (phylum or division). Comparison of the p-values calculated for both libraries has demonstrated that the ASHES population was more diverse than the CASM one (Pxy=0.090 and Pyx=0.003).

3.3. Phylogenetic analysis of the bacterial assemblages.

Phylogenetic analysis of the Juan de Fuca samples clustered the phylotypes into twenty-six groups: six within the ε-*Proteobacteria*, one within the γ-*Proteobacteria*, three within the δ-*Proteobacteria*, five within the *Cytophaga-Flavobacterium-Bacteroides* group, five within the *Verrucomicrobia*, two within the Green non-sulfur bacteria, two within the *Fusobacteria* and two within the Firmicutes (Fig. 3 and 4). None of the phylotypes corresponded to published 16S rRNA sequences. Within both clone libraries, sequences distribution was approximately the same in terms of phyla and divisions. No sequence was affiliated with the deep-rooted branches *Thermotogales* and *Aquificales* (Fig. 4) of which all members are, to date, thermophiles or hyperthermophiles.

- **ε-*Proteobacteria***. The majority of the sequences were assigned to the ε-*Proteobacteria*, accounting for 55.3% of the CASM library and for 41.2% of the ASHES one (Fig. 3). Phylogenetic analysis clustered the sequences of ε-*Proteobacteria* into six groups within which four (groups I, III, V and VI) have already been described for Atlantic and/or Southern-East Pacific hydrothermal vent sequences [4, 8]. 16S rRNA secondary structural features [8] characteristics of these four groups were retrieved in our clone sequences. The majority of the ε-*Proteobacteria* were part of a new clade, the group IV, accounting for 37.6% of the CASM library and 36.4% of the ASHES one. These sequences differed from those of the other groups of ε-*Proteobacteria* by the nucleotide signature GCGCGC at the beginning of the domain II of the 16S rRNA secondary structure (positions 575 to 480 of *Escherichia coli* 16S rRNA sequence numbering). Among this group, some sequences were closely related to the Mid-Atlantic Ridge sequence VC2.1-Bac1 identified from an *in situ* growth chamber deployed on an hydrothermal chimney (91–92% sequence similarity) (Table 1) [7]. Some other sequences were closely related to sequence NKB9 detected in cold seeps of the Nankai Trough (94 to 96% similarity). No sequences belonging to the Atlantic clone groups C or D (Atlantic clone group D corresponds to the South Pacific clone group I [4]) described by Corre *et al.* [8] were detected in our samples. Group II and III were related to 16S rRNA sequences associated with hydrothermal vent animals. In that way, group III sequences were related to the sequence of the ectosymbiont of *Rimicaris exoculata* (90-94% similarity) [6], a shrimp endemic to Atlantic and Indian oceans. Group II was distantly related to the sequence of one of the episymbiont of the East-Pacific polychaete *Alvinella pompejana* [3, 23].

- **C*ytophaga–Flavobacterium–Bacteroides* (CFB) group.** A significant part of the sequences accounting for 17.2% of the CASM library and for 27.3% of the ASHES library was assigned to the CFB (*Cytophaga–Flavobacterium–Bacteroides*) division (Fig. 4). All the sequences detected in this study could be assigned to five phylogenetic clades, the groups XI to XV. Within this CFB division, clone sequences were related to the *Cytophaga-Flavobacteria* cluster. CFB have already been detected from an Atlantic hydrothermal vent [7]. Clones of the group XIII, accounting respectively for 2.8% and for 7.7% of the CASM and ASHES libraries, were closely related (93% similarity) to the Atlantic clone VC2.1-Bac22. Most notably, the majority of the CFB sequences belonged to the group XIV and were distantly related (percent similarity below the approximate genus threshold value of 93%) to the marine species *Marinilabilia salmonicolor* [46] or to the Guaymas basin sediment clone SB-5 [47].
- ***Verrucomicrobia*.** Numerous sequences were assigned to five groups (groups XVI to XX) within the recently described division *Verrucomicrobia* [48] (Fig. 4). These sequences represented, respectively, 13.5% and 9.8% of the libraries from CASM and ASHES. All the sequences from this study were very distantly related to sequences of published strains and published environmental sequences (79 to 89% similarity).
- **Minor groups within the clone libraries: Firmicutes, *Fusobacteria*, Green non-sulfur bacteria (GNS), δ-*Proteobacteria* and γ-*Proteobacteria*.** Sequences belonging to other taxonomic groups and accounting for 0.7 to 7% of the libraries, were retrieved in the samples. They belonged to the Firmicutes, the *Fusobacteria*, the Green non-sulfur bacteria, the δ-*Proteobacteria* and the γ-*Proteobacteria* (for detail of their respective proportions, see Fig. 3-5). Several Firmicutes, δ- and γ-*Proteobacteria* have been isolated from hydrothermal chimney samples [29, 49-50] and from the microflora associated with *Alvinella pompejana* [51-52]. One phylotype of group VII of γ-*Proteobacteria* was affiliated (91% sequence similarity) with the endosymbiont [18] of the Northeast Pacific vent vestimentifer *Ridgeia piscesae* inhabiting the same chimneys as *P. palmiformis.* In other respects, one sequence of group VIII of δ-*Proteobacteria* could be assigned to the genus *Desulfuromusa* (96% 16S rDNA similarity with *Desulfuromusa succinoxidans*). Members of this genus are obligate anaerobic, mesophilic, heterotrophic sulphur-reducing bacteria inhabiting Denmark Fjord mud and Guaymas basin sediments [53]. Clones of the Green non-sulfur bacterial group have distant

relatives in Guaymas sediments [47]. Finally, several sequences from the mucous cocoons of *P. palmiformis* were affiliated to *Fusobacteria.*

4. DISCUSSION

The mucous secretions of *Paralvinella palmiformis* were colonised by a highly diverse microflora. In the following sections we consider results from both samples in order to identify trends and highlight possible ecological significance.

4.1. Methodological considerations.

rDNA sequences are efficient molecular markers for phylogeny at genus and species level. Using molecular data to infer physiology and biogeochemical activity of microbes in the ecosystem is considerably more challenging. Physiological predictions based only on the phylogenetic position of a sequence must be made with caution. However, if a phylogenetic analysis places, with high bootstrap value, a sequence within a monophyletic group where all known organisms share a particular trait, then it is reasonable to propose that this trait occurs in the organism detected by its sequence.

Since only two *P. palmiformis* mucous samples were analysed, it is important to consider the representativeness of the data. The molecular phylogenetic approach followed in this study has inherent limitations. The extraction of nucleic acids from environmental samples can introduce biases through selective cell lysis efficiency and nucleic acid extraction quality. The extraction step can can be sensitive to the presence of abundant polysaccharides and minerals in samples such as those extracted in this study. Measures employed here to reduce extraction bias included the use of both detergent-based and enzymatic cellular lysis, combining the products of repeated cell lyses treatments, the checking of lysis efficiency through microscopic examination and the purification step using cesium chloride density gradient centrifugation. Potential bias introduced during PCR amplification is more difficult to assess and control. Clone abundance in the library does not necessarily reflect rDNA sequence abundance in the nucleic acid extracts because saturation during the later cycles of amplifications can modify sequences ratios [54]. Biases can also be caused by primer selectivity and PCR-mediated chimeric gene amplification. Finally, the cloning step is also subject to bias.

In addition to methodological artifacts, the genomic properties of the natural microbial communities themselves constitute a source of bias [55]. It has been demonstrated that the quantities of PCR-generated

16S rDNA fragments are correlated to the genome size and the number of 16S rRNA genes (the number of 16S rDNA genes can vary for a strain from 1 to 14 copies) [55]. This level of genomic detail has not been documented in natural microbial communities. Finally, 16S rRNA genes operon microheterogeneity, and even macroheterogeinity, as reported for *Haloarcula marismortui* (5% difference between two expressed 16S rRNA genes) [56], can lead to a misinterpretation and an overestimation of community diversity. These genomic properties need to be taken into account when drawing conclusions from molecular ecological studies.

The sequence richness of the libraries developed in this study suggests that they were not subject to major methodological selectivity. Statistical analysis demonstrated that the CASM and ASHES sample libraries were significantly different. It seems probable that the most important ecological roles played by the micro-organisms are conserved in both environments even if these roles are not played by exactly the same populations. If the two libraries had had a quantitatively similar molecular composition, this would have been evidence that the relationship between *P. palmiformis* and the bacterial lineages associated with its mucus secretions was obligatory, implying a symbiotic or a consortium relationship. Hypercomplex consortia composed of many taxonomic groups have already been described [47]. However, even if the two sampled bacterial populations had yielded similar clone libraries, it could not be concluded that their similarity was stable. In the present case, it seems probable that the bacterial community is not exclusively structured by biotic factors and notably by the relationship with the polychaete, but also by abiotic factors. Even if the two studied microbial communities were sampled from the same type of faunal assemblage, they both came from areas where physico-chemical conditions were variable. In this type of habitat, it is likely there are populations successions. The molecular approach used here theoretically allows the detection of slow growing or dormant micro-organisms, in addition to those whose growth and abundance were favoured by physico-chemical and biotic conditions that predominated at the time of sampling. The resulting clone libraries would therefore tend to reflect the absolute genetic diversity of the sampled populations, and not just quantitative dominance by particular organisms.

4.2. Phylogenetic analysis of the bacterial assemblages and metabolic hypotheses.

The libraries from the CASM and ASHES samples contained a very broad bacterial diversity both at high and low taxonomic levels (from phylum to infraspecific diversity). Most sequences from both libraries were

affiliated with the ε-*Proteobacteria* subclass. The second and third most represented taxonomic groups were the *Cytophaga-Flavobacteria* and the *Verrucomicrobia* while remaining sequences were distributed among the Firmicutes, the *Fusobacteria*, the Green non-sulfur bacteria (GNS), the δ- and the γ-*Proteobacteria*. In most cases, sequences were closely related to environmental sequences that were detected only by molecular methods. It seems reasonable to think that the three taxonomic groups represented by numerous sequences, i. e. the ε-*Proteobacteria,* the *Cytophaga-Flavobacterium-Bacteroides* and the *Verrucomicrobia*, play a significant role within this ecosystem.

- **ε-*Proteobacteria***. The subclass ε-*Proteobacteria* comprises a complex group of Gram-negative, microaerophilic and/or anaerobic, chemoorganoheterotrophic or chemolithotrophic bacteria that are found in a variety of habitats [57]. Many members of this subclass are eukaryotic pathogens. Although this group is very heterogeneous, microaerophily and sulphur metabolism are very common features. In all the deep-sea vent microbial communities studied to date, ε-*Proteobacteria* phylotypes have been found to be dominant, accounting for 40 to 98% of the bacterial clone libraries [4-8]. This study expands the geographic distribution of the ε-*Proteobacteria* to the deep-sea hydrothermal vents of the Juan de Fuca Ridge and increases the number of representative sequences. While the functional ecology of this dominant group has not been studied, there have been some measurements of RuBPcase (Ribulose Biphosphate Carboxylase) activity and labelled substrate uptake in samples of the episymbionts of *Alvinella pompejana*, where ε-*Proteobacteria* predominate [58-59]. Contradictory results, some of which have never be reproduced, argue for overall heterotrophic or autotrophic metabolism (or perhaps mixotrophy) among the *A. pompejana* ε-*Proteobacteria* [11, 58-59]. Several strains belonging to the Atlantic clone group D have been recently isolated from Guaymas hydrothermal chimney samples and from *Alvinella pompejana* samples (from tubes of polychaetes or episymbiont biomass) [57, 60]. These strains, referenced as *Caminibacter hydrogeniphilus* and strain Ex-18.2 (and strain Am-H not placed on the phylogenetic tree) grew chemolithoautotrophically using H_2, formate or pyruvate as electron donor and sulphur or nitrate as terminal electron acceptor. The absence of representative of this group in our samples could be explained by the moderate thermophilic to thermophilic nature (Topt. 45-60 °C) characterising all the strains isolated to date. Physiological and metabolic inferences based only on the phylogenetic position must be made with caution, especially in a heterogeneous division such as the ε-*Proteobacteria*. Nonetheless, considering the ubiquity and

prevalence of this group at deep-sea vents, it is reasonable to propose that at least some of the populations sampled here were chemolithoautotrophs that contributed new organic matter to the vent ecosystem.

- ***Cytophaga–Flavobacterium–Bacteroides* (CFB) group.** The label CFB was originally used for *Cytophaga-Flexibacter-Bacteroides* [61]. *Cytophaga-Flavobacteria* are mainly aerobic, although some microaerophilic, capnophilic or anaerobic strains are also classified within this group [62]. Known *Cytophaga-Flavobacteria* are all chemoorganoheterotrophs and especially proficient at degrading macromolecules such as protein, chitin, pectin, agar, starch and cellulose. These bacteria are ubiquitous, abundant in organic-rich habitats and probably play a major role in the turn-over of organic matter in nature [62].
- ***Verrucomicrobia*.** This is the first report of *Verrucomicrobia* from deep-sea hydrothermal vents. This division has few cultivated members and is represented by an ever-increasing number of environmental sequences from diverse habitats including forest and agricultural soils, marine deep-sea sediments, freshwater environments and the pelagic marine environment [45]. Some members of this division are prosthecate bacteria [63]. A remarkable feature is that all of the isolates from this division preferentially use sugars as substrates for growth [48].
- **Minor groups within the clone libraries: Firmicutes, *Fusobacteria*, Green non-sulfur bacteria (GNS), δ-*Proteobacteria* and γ-*Proteobacteria*.** To our knowledge, this study is the first to attest the presence of representatives of the *Fusobacteria* and the Green non-sulphur taxonomic groups on hydrothermal edifices. Although heterotrophy is a very common feature within the Firmicutes phylum, the diversity of metabolic types among isolated strains makes it difficult to hypothesise about the metabolic properties of the sequences present in our samples. Similarly, while the δ-*Proteobacteria* includes the sulphur-oxidising endosymbionts of vent and seep-associated metazoans [18], there are also many dissimilatory sulfate-reducing δ-*Proteobacteria* and function cannot be infered from phylogenetic data alone when environmental sequences have no close cultured relatives. The GNS bacteria, recognised in 1987 as a division-level group [64], are still poorly represented by sequences from cultured organisms even though the number of environmental sequences continues to increase [45]. The cultured GNS representatives isolated to date display a wide range of phenotypes making difficult any metabolic inference from phylogeny. Finally, several sequences from the mucous

secretions of *P. palmiformis* were affiliated with the *Fusobacteria.* Representative of this taxonomic group are obligately anaerobic mesophilic bacteria fermenting proteinaceous substrates.

Many of the detected sequences grouped robustly within assemblages of micro-organisms that all displayed heterotrophic properties. The second and the third most represented groups within the clone libraries, affiliated with the *Cytophaga-Flavobacteria* and the *Verrucomicrobia* groups, comprise exclusively organoheterotrophs capable of degrading proteinaceous or saccharidic substrates. As discussed above, the *Cytophaga-Flavobacteria* are known for their proficiency in degrading biomacromolecules. Members of these two groups, and perhaps some members of the minor lineages detected in the samples, are most probably decomposers involved in the degradation of mucus secretions and other organic debris that accumulates within the *P. palmiformis* microenvironment. The biochemical composition of the mucus of *P. palmiformis* has not been analysed. The organic portion (10% of total dry weight) of the mucus of a related species from Eastern Pacific vents, *P. grasslei*, was found to consist of 60% sugars and 40% proteins [65].

4.3. Possible implication of bacteria in the detoxification of the ecological niche of *P. palmiformis.*

The ecological niche of *P. palmiformis* is submitted to a constant rain of mineral particles and to toxic gases, calling for special adaptations. The mucus secreted continually by this worm is one of these adaptive responses. Mucus secretions play several roles: (i) they protect the epidermis of the worm from mineral particles and predation; (ii) they detoxify the direct environment of the animal by trapping some mineral particles like iron sulphur, copper sulphur, zinc sulphur and by concentrating trace elements such as uranium; (iii) they would perhaps act also as a transport system for toxic particles absorbed by the worm [25]. The mucus secreted by the animals is colonised by bacteria. As demonstrated by this study, this colonising microflora is genetically widely diverse. There is evidence that some bacteria detected in the mucus samples detoxify the environment by using some mineral particles as electron donors or acceptors for their metabolism. Thermodynamic models indicate that mixing environments, like the ecological niche of *P. palmiformis*, are favourable for chemosynthetic reactions of sulphide oxidation below 38 °C [66]. Moreover, the oxidation of sulphide and elemental sulphur are widely accepted as the principal chemosynthetic reactions leading to production of primary biomass in ridge ecosystems [66-67]. Consequently, it is highly probable that sulphide-oxidisers occur within the microbial communities

associated to the worm. Among the three major taxonomic groups detected are the ε-*Proteobacteria* the unique taxonomic group, to date, with representatives involved in sulphur cycling. Among the cultivated ε-*Proteobacteria* from diverse habitats occur sulphur-reducers as well as sulphide-oxidisers. Hydrogen sulphide is notably oxidised by the marine species of the genera *Arcobacter* and *Thiovulum* [68]. Our environmental sequences are distantly related to these species and it is presently impossible to infer that an organism with a given sequence belongs without doubt to a sulphide-oxidising group. However, there is evidence that some ε-*Proteobacteria* from deep-sea vents are involved in sulphur cycling. This hypothesis is supported by the isolation from this ecosystem of strains which are all sulphur-reducers [57, 60] and by the white sulphur-like coloration of some uncultured ε-*Proteobacteria* [4]. Further investigations will be necessary to identify the metabolic capabilities of the micro-organisms detected by their sole sequence and to determine whether or not some are sulphide-oxidisers. On the other hand, some sequences could be assigned to bacterial groups in which detoxifying activities bacterial are common features. It is quite possible that sequences of group XXIII related to the *Fusobacteria* species *Propionigenium maris* (93% similarity) would be involved in detoxification phenomenon. It has been demonstrated that two strains of this species, isolated from marine hemichordate and polychaete burrows, were able to detoxify the environment of these animals by debrominating the bromoaromatic compounds produced by these animals [69].

4. 4. Conclusion.

The bacterial assemblages associated with the mucous secretions of *Paralvinella palmiformis* were mainly composed of ε-*Proteobacteria*, *Cytophaga-Flavobacteria* and *Verrucomicrobia* and to a lesser extent of *Fusobacteria,* Firmicutes, δ-*Proteobacteria*, γ-*Proteobacteria* and Green non-sulfur bacteria. The presence of *Verrucomicrobia, Fusobacteria* and Green non-sulfur bacteria is a first-time report for the hydrothermal vent ecosystem. Although metabolic characteristics cannot be assumed from phylogeny, when a sequence groups robustly within a group of organisms sharing identical properties, physiology can be cautiously inferred. A specialised heterotrophic microflora, including bacteria of the groups *Cytophaga-Flavobacteria* and *Verrucomicrobia,* may be decomposing the mucus secretions and other organic debris in the *Paralvinella palmiformis* habitat. As well, we hypothesise that some of the ε-*Proteobacteria* sequences identified in the two samples represent sulphide oxidising primary producers.

Note. While this paper was under review, the presence of ε-, δ-, γ-, α-*Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* and *Verrucomicrobia* was reported from a molecular survey of the bacterial diversity of the East-Pacific Rise giant vent tubeworm *Riftia pachyptila* [70].

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Figures and tables

Table 1. Distribution and phylogenetic affiliations of bacterial 16S rDNA sequences associated with mucus secretions of *Paralvinella palmiformis* from Axial Volcano (CASM and ASHES), Juan de Fuca.

Fig. 1. Accumulation curves of the diversity detected in the two bacterial clone libraries. Accumulation curves (CASM (σ) and ASHES (λ) vent fields) represent the sequential detection of cumulative phylotypes following ARDRA patterns (determined after digestion with two restriction endonucleases) after grouping of phylotypes with sequences sharing more than 97% similarity (sequences differing less than 3% were considered as related groups for phylogenetic analysis, and this criterion was used for the accumulation curves. Thus grouped, the number of phylotypes decreased from 59 to 43 for CASM and from 59 to 46 for ASHES).

Fig. 2. Statistical comparisons of the CASM and ASHES libraries. X and Y are the 16S rRNA gene sequence libraries from CASM and ASHES, respectively. Homologous Cy and heterologous Cyx coverage curves were calculated. Coverage curves versus phylogenetic distance (D) are plotted with the following symbols : squares (ν) for Cy(D) and triangles (σ) for Cyx(D). The grey line (λ) indicates the value of $(Cy-Cyx)^2$ at each value of phylogenetic distance. The phylogenetic distance D was calculated using the clustalW program of GCG [32].

Fig. 3. Phylogenetic position and relative representation of *Proteobacteria* 16S rDNA sequences from the CASM and ASHES bacterial clone libraries. The tree topology shown was developed by the neighbor-joining method, using *Bacillus subtilis* as the outgroup. 16S rRNA reference sequences were obtained from GenBank (accession numbers are indicated in the figure). The letters C (CASM) and A (ASHES) within clone names refer to sample origin. For the analysis, 984 sites were used, with 500 bootstrap replicates. Scale bar indicates the expected number of changes per sequence position. The figure on the right represents the relative proportion of each group of clones as a percentage of the total clones in each library.

Fig. 4. Phylogenetic position and relative representation of bacterial 16S rDNA sequences (without the *Proteobacteria*) from the CASM and ASHES bacterial clone libraries. The tree topology shown was obtained by the neighbor-joining method, using *Methanococcus vulcanus* as outgroup. 16S rRNA reference sequences were obtained from GenBank (accession numbers are indicated in the figure). The letters C (CASM) and A (ASHES) within clone names refer to sample origin. For the analysis, 1049 sites were used, with 500 bootstrap replicates. Scale bar indicates the expected number of changes per sequence position. The figure on the right represents the relative proportion of each group of clones as a percentage of the total clones in each library.

Table 1

Phylogenetic affiliation	Type sequence	Accession number	Clone number in CASM library	Clone number in ASHES library	Closest relative in the phylogenetic tree	Sequence similarity*
ε-*Proteobacteria*						
Group I	*P. palm.* C 43	AJ441198	2	0	Atlantic clone VC1.2-Cl26	94 %
	P. palm. C/A 78	AJ441199	2	2	Pele's vent clone PVB OTU6	93 %
	P. palm. *C*	AJ441200	2	0	Atlantic clone VC1.2-Cl26	90 %
		AJ4412	3	0	Atlantic clone VC1.2-Cl26	89 %
Group II			3	0	*Alvinella pompejana* APG44B	88 %
Group III	*92*		3	2	*Rimicaris* ectosymbiont	94 %
		01AJ44	1	0	*Rimicaris* ectosymbiont	90 %
Group IV	*P. palm.* C 73		14	12	Deep-sea sediment clone NKB9	95 %
	P. palm. C 140	1202	3	4	Deep-sea sediment clone NKB9	94 %
	P. palm. C/A 13		13	7	Deep-sea sediment clone NKB9	96 %
	P. palm. C 84		17	19	Deep-sea sediment clone BD2-1	96 %
	P. palm. C/A 114	AJ4412	1	5	Deep-sea sediment clone NKB9	94 %
	P. palm. C/A 113		2	4	Atlantic clone VC2.1-Bac1	91 %
	P. palm. C/A 18		3	1	Atlantic clone VC2.1-Bac1	92 %
Group V	*P. palm.* C/A 55	03	2	1	Atlantic clone VC1.2-Cl10	95 %
	P. palm. C/A 64		1	1	Atlantic clone VC1.2-Cl10	91 %
Group VI	*P. palm.* C/A 220	AJ4412	6	1	Guaymas strain Ex-18.1	90 %
γ-*Proteobacteria*	*P. palm.* C/A 89					
Group VII	*P. palm.* C/A 138		0	1	*Ridgeia piscesae* endosymbiont	91 %
δ-*Proteobacteria*	*P. palm.* C/A 26	04AJ44				
Group VIII	*P. palm.* C/A 39		1	1	*Desulfuromusa succinoxidans*	96 %
Group IX			0	5	*Desulfonema magnum*	85 %
Group X	*P. palm.* A 79	1205	0	1	*Geobacter bremensis*	83 %
CFB						
	P. palm. C/A 79	AJ4412	0	2	Flexibacter flexilis	82 %
Group XI	*P. palm.* A 205		1	1		82 %
Group XII	*P. palm.* A 229		4	11		93 %
Group XIII		06	1	7	**Cellulophaga lytica**	86 %
Group XIV	*P. palm.* A 21		4	3		86 %
	P. palm. C/A 100		1	1	Atlantic clone VC2.1-Bac22	86 %
	P. palm. C/A 20	AJ4412	1	0	Marinilabilia	86 %
	P. palm. C/A 221		12	9		85 %
	P. palm. C/A 42	07	0	1		87 %
	P. palm A/C 22		0	2	salmonicolor	87 %
	P. palm. C 67		0	1		90 %
	P. palm. C/A 33	AJ4412	0	1		87 %
Group XV	*P. palm.* A 53				*Marinilabilia salmonicolor*	
Verrucomicro	*P. palm.* A 10	08	1	0	Guaymas sediment clone SB-5	86 %
	P. palm. A 249		0	3	Guaymas sediment clone SB-5	82 %
bia	*P. palm.* A 12		8	5	Guaymas sediment clone SB-5	79 %
		AJ4412	0	1	Guaymas sediment clone SB-5	86 %
Group XVI	*P. palm.* C 85		1	0	Guaymas sediment clone SB-5	80 %
	P. palm. A 54		2	0	Guaymas sediment clone SB-5	81 %
Group XVII	*P. palm.* C/A 139	09	2	2	Yellowstone clone OPB56	81 %
Group XVIII	*P. palm.* A 228		5	1		89 %
	P. palm. C 136	AJ4412	0	2	**Verrucomicrobium spinosum**	86 %
Group XIX	*P. palm.* C 41					
Group XX	*P. palm.* A/C 285		1	0	**Verrucomicrobium spinosum**	88 %
	P. palm. C/A 24	10	1	5		79 %
	P. palm. A 24		0	1	**Verrucomicrobium spinosum**	82 %
Green non-		AJ4412				
	P. palm. C 37		2	2	Deep-sea sediment clone	93 %
sulfur	*P. palm.* C/A 25		0	6		82 %
	P. palm. A 17	11AJ44				
Group XXI			5	4	BD2-18	83 %
Group XXII	*P. palm.* C/A 63		5	3		86 %
	P. palm A 11	1212				
Fusobacteria			2	0	Deep-sea sediment clone	
	P. palm. C/A 51		2	0		

Group XXIII Group XXIV **Firmicutes** Group XXV Group XXVI **Unresolved** **Chimeras**	*P. palm.* C/A 17 *P. palm.* C 70 *P. palm.* C 72	AJ4412 13AJ44 1214 AJ441234 AJ441215 AJ441235 AJ441236 AJ441237 AJ441216 AJ441217 AJ441218 AJ441219 AJ441238 AJ441220 AJ441221 AJ441239 AJ441240 AJ441241 AJ441242 AJ441222 AJ441243 AJ441223 AJ441244 AJ441224 AJ441225 AJ441245 AJ441226 AJ441246 AJ441227 AJ441228 AJ441247 AJ441229 AJ441248 AJ441230 AJ441231 AJ441232 AJ441233	1	2	BD2-18 Deep-sea sediment clone BD2-3 Deep-sea sediment clone BD2-3 Deep-sea sediment clone BD2-3 Deep-sea sediment clone BD2-3 Guaymas sediment clone SB-34 Guaymas sediment clone SB-34 Guaymas sediment clone SB-34 *Propionigenium maris* *Propionigenium maris* *Syntrophomonas wolfei* *Clostridium litorale*	

Figure 1

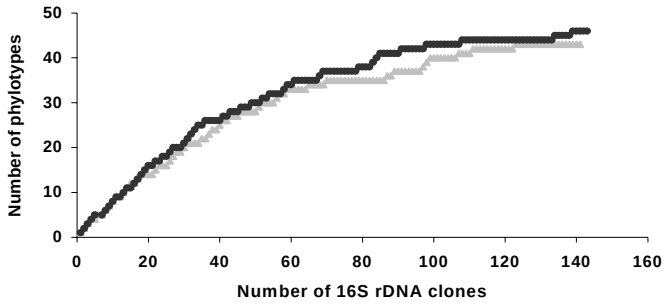
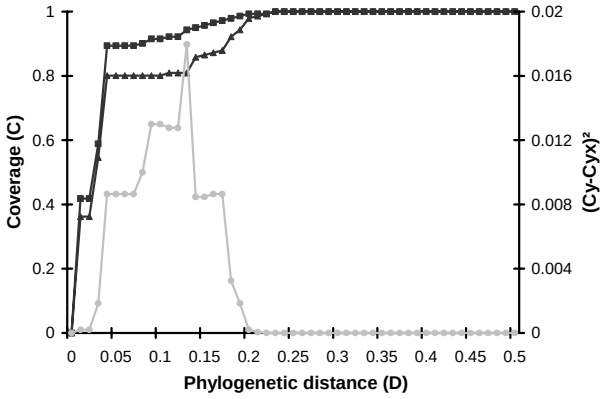
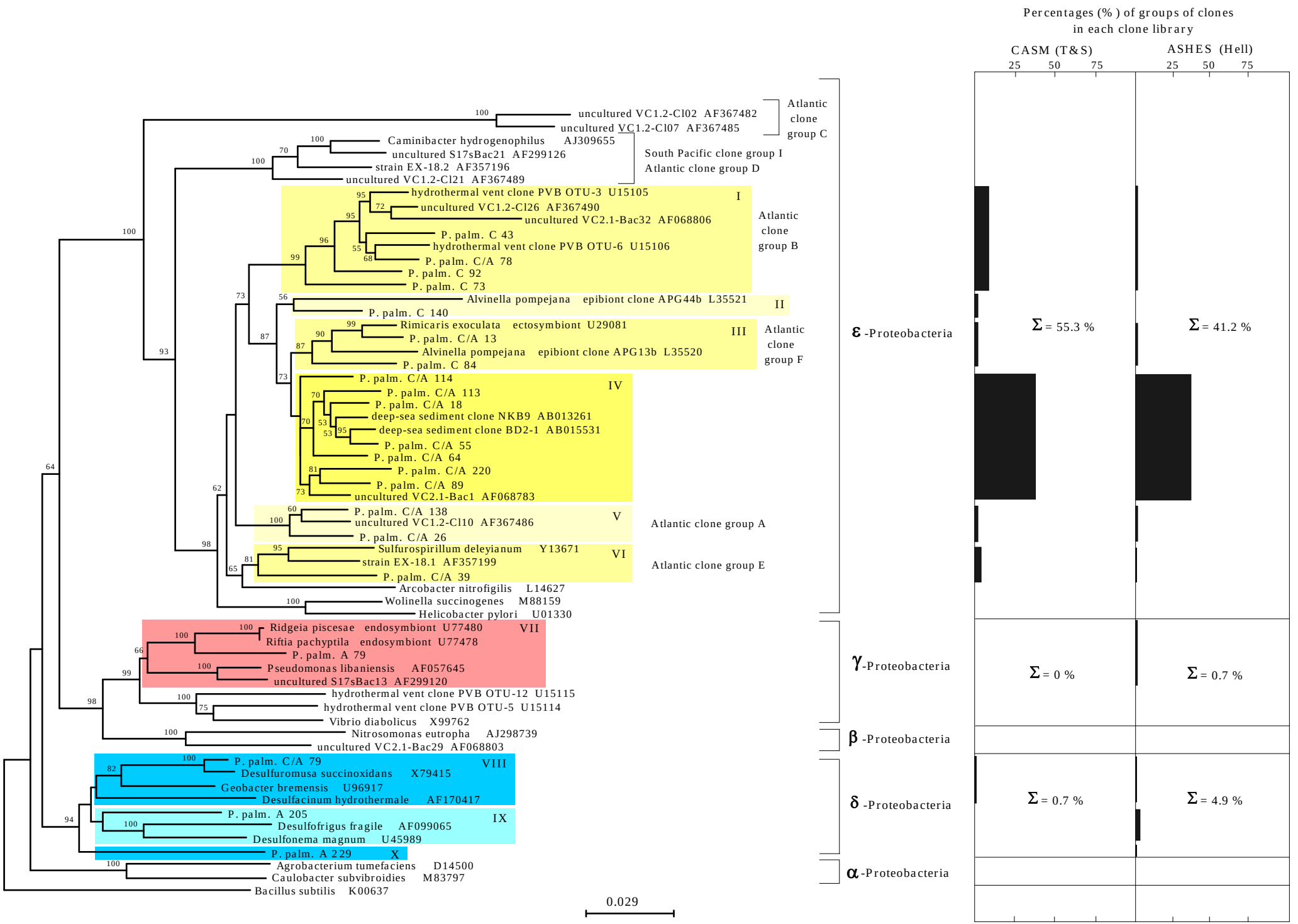
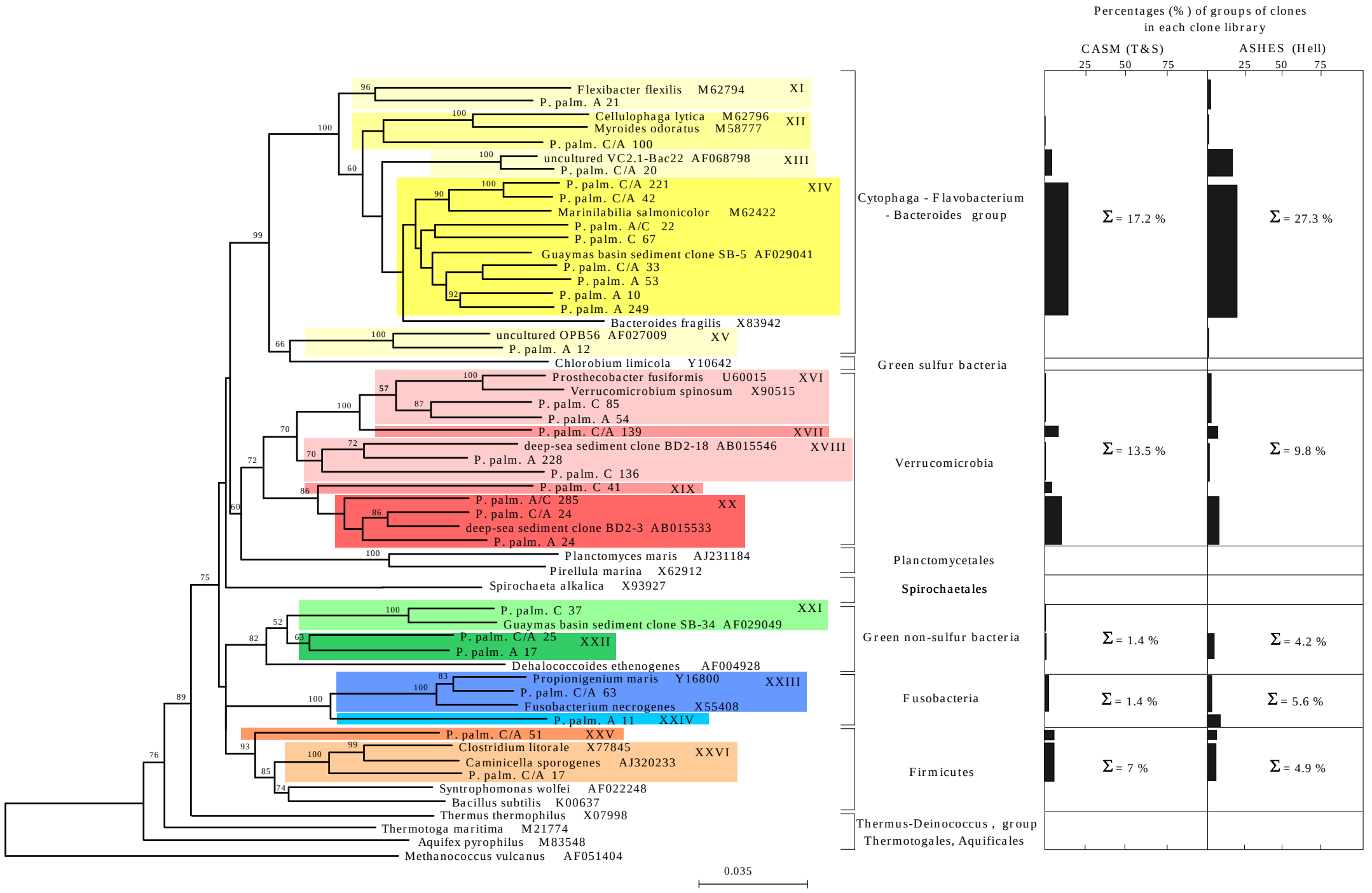


Figure 2




Percentages (%) of groups of clones
in each clone library
CASM (T&S)
ASHES (Hell)
25 50 75
25 50 75
uncultured VC1.2-Cl02 AF367482
uncultured VC1.2-Cl07 AF367485
Atlantic clone group C
Caminibacter hydrogenophilus AJ309655
uncultured S17sBac21 AF299126
strain EX-18.2 AF357196
uncultured VC1.2-Cl21 AF367489
South Pacific clone group I
Atlantic clone group D
hydrothermal vent clone PVB OTU-3 U15105
uncultured VC1.2-Cl26 AF367490
uncultured VC2.1-Bac32 AF068806
P. palm. C 43
hydrothermal vent clone PVB OTU-6 U15106
P. palm. C/A 78
P. palm. C 92
P. palm. C 73
I
Atlantic clone group B
Alvinella pompejana epibiont clone APG44b L35521
P. palm. C 140
II
Rimicaris exoculata ectosymbiont U29081
P. palm. C/A 13
Alvinella pompejana epibiont clone APG13b L35520
P. palm. C 84
III
Atlantic clone group F
P. palm. C/A 114
P. palm. C/A 113
P. palm. C/A 18
deep-sea sediment clone NKB9 AB013261
deep-sea sediment clone BD2-1 AB015531
P. palm. C/A 55
P. palm. C/A 64
P. palm. C/A 220
P. palm. C/A 89
uncultured VC2.1-Bac1 AF068783
IV
P. palm. C/A 138
uncultured VC1.2-Cl10 AF367486
P. palm. C/A 26
V
Atlantic clone group A
Sulfurospirillum deleyianum Y13671
strain EX-18.1 AF357199
P. palm. C/A 39
VI
Atlantic clone group E
Arcobacter nitrofigilis L14627
Wolinella succinogenes M88159
Helicobacter pylori U01330
ε -Proteobacteria
Σ = 55.3 %
Σ = 41.2 %
Ridgeia piscesae endosymbiont U77480
Riftia pachyptila endosymbiont U77478
P. palm. A 79
Pseudomonas libaniensis AF057645
uncultured S17sBac13 AF299120
VII
hydrothermal vent clone PVB OTU-12 U15115
hydrothermal vent clone PVB OTU-5 U15114
Vibrio diabolicus X99762
Nitrosomonas eutropha AJ298739
uncultured VC2.1-Bac29 AF068803
γ -Proteobacteria
Σ = 0 %
Σ = 0.7 %
β -Proteobacteria
P. palm. C/A 79
Desulfuromusa succinoxidans X79415
Geobacter bremensis U96917
Desulfacinum hydrothermale AF170417
VIII
P. palm. A 205
Desulfofrigus fragile AF099065
Desulfonema magnum U45989
IX
P. palm. A 229
X
δ -Proteobacteria
Σ = 0.7 %
Σ = 4.9 %
Agrobacterium tumefaciens D14500
Caulobacter subvibroidies M83797
α -Proteobacteria
Bacillus subtilis K00637
0.029


Percentages (%) of groups of clones
in each clone library
CASM (T&S)
ASHES (Hell)
25 50 75
25 50 75
Flexibacter flexilis M62794 XI
P. palm. A 21
Cellulophaga lytica M62796 XII
Myroides odoratus M58777
P. palm. C/A 100
uncultured VC2.1-Bac22 AF068798 XIII
P. palm. C/A 20
P. palm. C/A 221 XIV
P. palm. C/A 42
Marinilabilia salmonicolor M62422
P. palm. A/C 22
P. palm. C 67
Guaymas basin sediment clone SB-5 AF029041
P. palm. C/A 33
P. palm. A 53
P. palm. A 10
P. palm. A 249
Bacteroides fragilis X83942
uncultured OPB56 AF027009 XV
P. palm. A 12
Chlorobium limicola Y10642
Prosthecobacter fusiformis U60015 XVI
Verrucomicrobium spinosum X90515
P. palm. C 85
P. palm. A 54
P. palm. C/A 139 XVII
deep-sea sediment clone BD2-18 AB015546 XVIII
P. palm. A 228
P. palm. C 136
P. palm. C 41 XIX
P. palm. A/C 285 XX
P. palm. C/A 24
deep-sea sediment clone BD2-3 AB015533
P. palm. A 24
Planctomyces maris AJ231184
Pirellula marina X62912
Spirochaeta alkalica X93927
P. palm. C 37 XXI
Guaymas basin sediment clone SB-34 AF029049
P. palm. C/A 25 XXII
P. palm. A 17
Dehalococcoides ethenogenes AF004928
Propionigenium maris Y16800 XXIII
P. palm. C/A 63
Fusobacterium necrogenes X55408
P. palm. A 11 XXIV
P. palm. C/A 51 XXV
Clostridium litorale X77845 XXVI
Caminicella sporogenes AJ320233
P. palm. C/A 17
Syntrophomonas wolfei AF022248
Bacillus subtilis K00637
Thermus thermophilus X07998
Thermotoga maritima M21774
Aquifex pyrophilus M83548
Methanococcus vulcanus AF051404
0.035
Cytophaga - Flavobacterium
- Bacteroides group
Green sulfur bacteria
Verrucomicrobia
Planctomycetales
Spirochaetales
Green non-sulfur bacteria
Fusobacteria
Firmicutes
Thermus-Deinococcus , group
Thermotogales, Aquificales
Σ = 17.2 %
Σ = 27.3 %
Σ = 13.5 %
Σ = 9.8 %
Σ = 1.4 %
Σ = 4.2 %
Σ = 1.4 %
Σ = 5.6 %
Σ = 7 %
Σ = 4.9 %