First insights into the gut microflora associated with an echinoid from wood falls environments

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Abstract: Wood falls are organic substrates sunken on the ocean floor that house a diversified fauna of marine invertebrates. Among them, the echinoid *Asterechinus elegans* is found in various localities from the West-Pacific region and observations of its gut content indicate that it is a wood-feeding species. The diversity of the microflora associated with its gut content was investigated by 16S rRNA gene cloning analysis and identified *Proteobacteria*, *Planctomycete*, *Firmicutes*, *Cytophaga-Flexibacter-Bacteroides* and *Actinobacteria*. Clones were related to bacteria from the gut of phytophageous (including wood-feeding) animals but also from sulphide-rich environments such as whale carcasses, mangrove soils and marine sediments of notably hydrothermal vents and cold seeps. Furthermore, the analysis of the adenosine 5'-phosphosulphate (APS) reductase gene put in evidence the presence of several sulphide-oxidizing bacteria (SOB) belonging to the *Alpha-* and *Gamma-proteobacteria* while some sulphate-reducing bacteria (SRB) affiliated to the *Delta-proteobacteria* were also detected. APS reductase clones were related to thioautotrophic symbionts of various marine invertebrates including tube worms and gutless oligochaetes. These results suggest that a part of the bacterial community associated with the gut content of *A. elegans* would be able to participate in the wood digestion and therefore to the echinoid nourishment. Further analyses are however needed to clarify several aspects of this association.

Résumé : *Premières données sur la microflore intestinale associée à un oursin inféodé aux bois coulés.* Les bois coulés sont des substrats organiques d'origine terrestre situés sur les fonds océaniques et abritant une faune diversifiée d'invertébrés marins. Parmi eux, l'échinide *Asterechinus elegans* se retrouve à plusieurs endroits de la région Ouest-Pacifique et l'observation de son contenu digestif indique qu'il s'agit d'une espèce se nourrissant de bois. La diversité de la microflore associée à ce contenu digestif a été analysée par clonage du gène codant pour l'ARNr 16S qui a permis d'identifier des *Proteobacteria*, des *Planctomycete*, des *Firmicutes*, des *Cytophaga-Flexibacter-Bacteroides* et des *Actinobacteria*. Les clones étaient proches de bactéries originaires du tube digestif d'animaux phytophages (dont des xylophages) mais aussi d'environnements riches en sulfure d'hydrogène tels des carcasses de baleines, des sols de mangroves et des sédiments marins provenant notamment de sources hydrothermales et de suintements froids. De plus,

l'analyse du gène codant pour l'adénosine 5'-phosphosulphate (APS) réductase a mis en évidence la présence de plusieurs bactéries sulfo-oxydantes appartenant aux *Alpha*- et aux *Gamma-proteobacteria* tandis que quelques bactéries sulfato-réductrices affiliées aux *Delta-proteobacteria* ont aussi été détectées. Ces clones étaient proches de symbiontes associés à divers invertébrés marins dont des vers vestimentifères tubicoles et des oligochètes dépourvus de tube digestif. Ces résultats suggèrent qu'au moins une partie de la communauté bactérienne associée au contenu digestif d'*A. elegans* serait capable de participer à la digestion du bois et donc à l'alimentation de l'oursin. D'autres analyses sont cependant nécessaires afin de clarifier certains aspects de cette association.

Introduction

Wood falls, also called sunken woods, are large terrestrial organic substrates (trunks, branches, etc.) lying on the bottom of the ocean basin. They are drifted by rivers and marine currents and are particularly abundant on slopes of islands from the inter-tropical zone. A diversified fauna that includes mainly molluscs is specialized in the utilization of this organic matter that offers a source of nutriments below the euphotic zone, notably through chemosynthesis. Among these molluscs, Mytilidae mussels were the first organisms associated with wood falls for which the presence of chemoautolithotrophic bacterial symbionts was established. Indeed, the gills of these mussels house bacteria that use hydrogen sulphide produced by the microbial degradation of the wood and supply their host with carbon and energy (Gros & Gaill &, 2007; Gros et al., 2007; Duperron et al., 2008). Moreover, it has been shown that these mussels took sunken woods as a step in their colonization of hydrothermal vents and cold seeps (Distel et al., 2000; Samadi et al., 2007). Other invertebrates such as crustaceans, echinoderms, polychaetes and sipuncula are also found on wood falls where they represent a significant part of the fauna (Pailleret et al., 2007). However, although several species are known to feed on wood, their diet and the role of their gut microflora remain poorly understood.

Echinoderms associated with wood falls include notably the asteroids of the genus *Xyloplax* (Baker et al., 1986; Rowe et al., 1988; Janies & Mooi, 1999; Mah, 2006) and the ophiuroids *Ophiambix meteoris* Bartsch, 1983 and *O. aculeatus* Lyman, 1880 (Paterson & Baker, 1988). Echinoids occur either on the surface of the wood or in hollows and their relative abundance reaches more than 5% of the total fauna (Pailleret et al., 2007). Among them, *Asterechinus elegans* Mortensen 1942 is exclusively found on sunken woods and many specimens have been collected at various depths during recent scientific cruises in the Indo-Pacific region, including the Salomon Islands, the Vanuatu Islands and the Philippines. Up to then, *A. elegans* was only known from its description based on a single specimen sampled off the Admiralty Islands (Papua New Guinea) during the Challenger expedition in the 1870's (Mortensen, 1943). Interestingly, the intestine of this specimen was completely filled with wood fragments. The use of wood as a food source is unusual among echinoids (e.g., De Ridder & Lawrence, 1982). The diets of most regular sea urchins consist of plants (algae, sea-grasses) and to a lesser extend of epibiotic organisms attached to the plants. Paradoxically, echinoids seem to be unable to hydrolyze structural polysaccharides like native cellulose or lignin (Lawrence, 1982; Claereboudt & Jangoux, 1985). They consequently rely on their gut microflora to degrade their food like many other herbivores, from termites to mammals (e.g., Smith & Douglas, 1987). The capabilities of gut bacteria to digest macroalgae and phanerogams (sea-grasses) or some of their polysaccharide constituents have been demonstrated for different species of echinoids by several authors (review in De Ridder & Foret, 2001). These studies were based on cultivation methods testing metabolic abilities of isolated strains.

In that context, the access to *Asterechinus elegans* samples from deep wood falls provided a unique opportunity to investigate the gut bacteria of a wood-feeding echinoid. In this work, we mainly investigate the genetic diversity of the bacterial microflora found in the digestive tube, using 16S rRNA gene sequence analyses. The ability of the bacterial microflora to oxidize sulphide or to reduce sulphate was also tested by analyzing the gene coding for the APS reductase, an enzyme involved in both process. A morphological approach using scanning electron microscopy (SEM) was also tentatively performed although the samples were not adequately preserved to get

precise and thorough observations of bacterial morpho-types.

Materials and Methods

Sampling and microscopic analyses

Sunken woods were collected by trawling at depths ranging from 220 to 780 metres in the Big Bay of the Espiritu Santo Island (Vanuatu Archipelago) during the BOA1 and SAN-TOBOA cruises in 2005 and 2006, respectively. The area covered was between 14°59'44.8''S and 15°09'06.3''S in latitude and between 166°51'38''E and 166°55'28.2''E in longitude. Echinoids found attached to these woods were fixed in absolute ethanol and their gut content was examined with a Leica MZ75 binocular. For SEM, pieces of the digestive tract from ten different individuals were dissected, critical-point dried and mounted on stubs. They were then carefully opened, coated with gold and observed with a Jeol JSM-6100 scanning electron microscope. This procedure enabled the observation of the inside of the digestive tube (i.e., gut content and inner wall). Noteworthy, wood samples from the immediate surrounding were unfortunately not available for this study.

Sequence analyses of the 16S rRNA and APS reductase genes

Gut content from three specimens of *Asterechinus elegans* were removed with sterile tools and placed in sterile micro-centrifuge tubes. The internal wall of the digestive tract was scrapped to ensure a complete removal. Total DNA was then extracted from the samples with an Invisorb® Spin Tissue Mini Kit (Invitek) following the manufacturer's instructions. The gene coding for the bacterial 16S rRNA gene (ca. 1500 bp) was amplified with the bacterial primers 8F and 1492R (Buchholz-Cleven et al., 1997) using 5 µl of DNA template. PCR consisted of 30 cycles with denatura-tion at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 1 min. A ca. 400 bp-long fragment of the gene coding for the APS reductase alpha subunit was amplified with primers aps1F and aps4R (Blazejak et al., 2006). PCR conditions were 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min 30 sec. A final 10 min elongation step at 72°C was added at the end of both PCR protocols.

The PCR products were then purified with QIAquick columns (Qiagen) and cloned into TOP10 chemically competent *Escherichia coli* cells using the TOPO TA Cloning kit (Invitrogen). Clones containing the complete 16S rRNA gene or full-length fragment of the APS reductase gene, as revealed by PCR with the vector primers M13F and M13R, were selected for plasmid isolation with the QIAprep miniprep kit (Qiagen). Clones were sequenced on an ABI Prism 3100 genetic analyser with primer M13F. Only partial sequences were obtained for the 16S rRNA gene. The sequences were compared with those in the GenBank database using the basic local alignment search tool (BLAST) in order to find related species (Altschul et al., 1990). Each sequence was also checked for chimera formation using Chimera Check v2.7 program (Cole et al., 2003). Coverage values of the clones libraries were calculated according to Good (1953) with 97% of sequence similarity used as the criterion for sequence uniqueness. Representative sequences and their closest relatives were then aligned using Clustal X (Thompson et al., 1994) and neighbour-joining trees were generated with Paup (Swofford, 1998) using the Jukes and Cantor distance (Jukes & Cantor, 1969). Reliability of various inferred phylogenetic nodes was estimated by bootstrapping (1000 replicates) (Felsenstein, 1985). The sequences obtained in this study have been deposited in the EMBL database under Accession Numbers FM896882 to FM897005 for the 16S rRNA gene and FM878950 to FM879023 for the APS reductase gene.

Results

Observations

Asterechinus elegans is a regular echinoid of small size, the test diameter of the collected specimens never exceeding 25 mm (Fig. 1A). The wall of the digestive tube was very thin and translucent, the gut content being visible through the stomach and the intestine (Fig. 1B). Observations on the gut content of all individuals (n = 20) revealed that they were mainly composed of numerous wood fragments of different size, shape and colour (Fig. 1B). For instance, some were small light cubes while others were large dark twigs of up to 7 mm long. Sediments were also occasionally present in various proportions from virtually absent to about a third of the gut content, depending on the individuals.

Despite the poor quality of the samples fixation, it was possible to observe gut bacteria inside the digestive tube using SEM. Several morphotypes were detected but are not described here because of their bad preservation. However, filamentous bacteria of about 10 µm in length and 0.2 µm in width were found in the intestine, over the digestive wall and in contact with wood fragments (Fig. 1C & D). They were particularly abundant in all echinoid individuals and appeared as a dominant morphotype, reaching a density from 3.5×10^3 to 1.5×10^4 individuals per square millimeter of intestinal tissue; in contrast, this morphotype was scarce in the stomach.
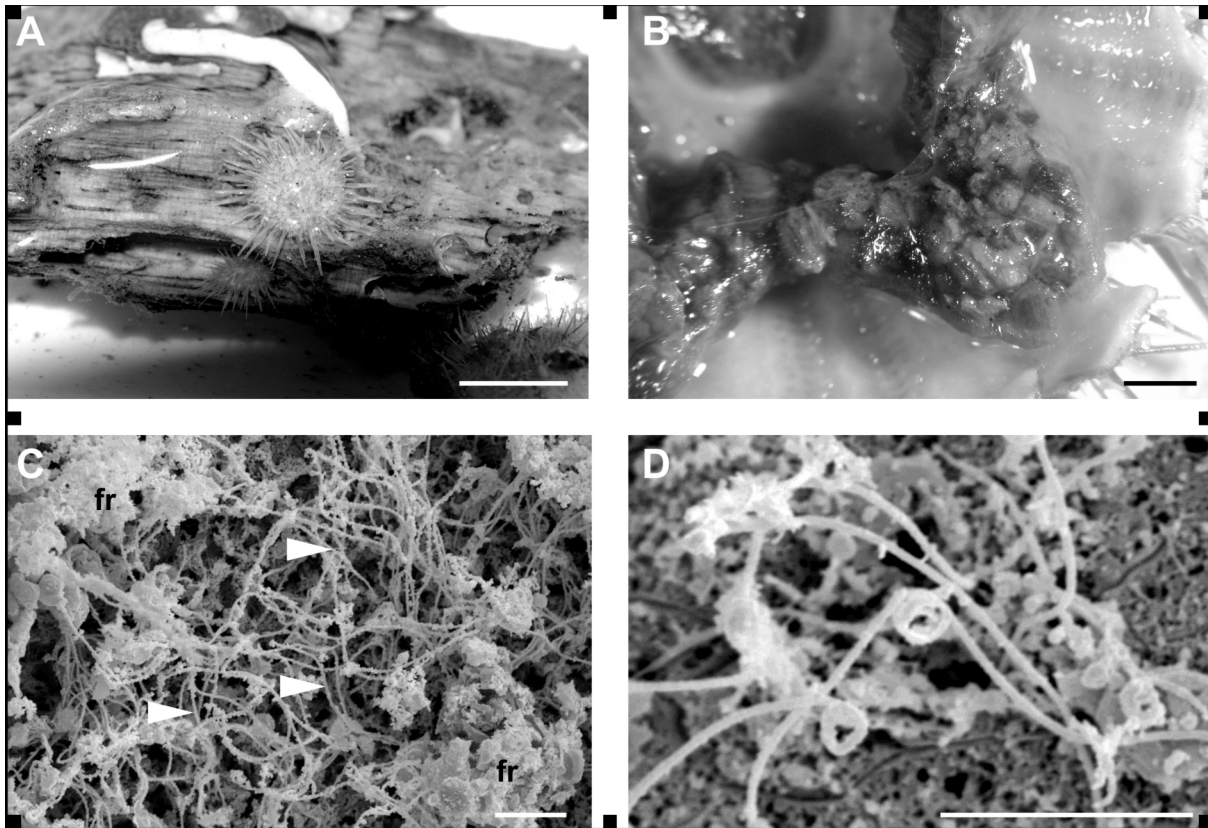
Figure 1. A. *Asterechinus elegans* on a piece of sunken wood. **B.** Intestinal festoon filled with wood fragments. **C & D.** Filamentous bacteria (arrows) inside the intestine, in contact with food remnants (fr). Scale bars = 2 cm for A, 1 mm for B and 5 μm for C and D.

Figure 1. A. *Asterechinus elegans* sur un morceau de bois coulé. **B.** Portion de l'intestin rempli de fragments de bois. **C & D.** Bactéries filamenteuses (flèches) à l'intérieur de l'intestin, en contact avec des restes alimentaires (fr). Echelle= 2 cm pour A, 1 mm pour B et 5 μm pour C et D.

Sequences analysis of the 16S rRNA gene

Partial bacterial 16S rRNA gene sequences (401-563 bp) were recovered from the gut content of three sea urchin specimens (Figs 2 & 3). A library of 124 clones (48% of coverage value) was obtained and sequences with at least 97% of similarity were gathered, giving 65 operational taxonomic units (OTU). Nineteen OTUs included more than one clone and 9 of them were shared by at least two echinoids. A BLAST search indicated that the majority of the clones (79 clones) were identified as *Proteobacteria*, mainly from the *Alpha*-subclass, followed by *Gamma*-, *Delta*-, and *Beta-proteobacteria* with respectively 51, 22, 3 and 3 clones. Other clones were identified as *Firmicutes* (19 clones), *Planctomycete* (16 clones), *Cytophaga-Flexibacter-Bacteroides* (7 clones), *Actinobacteria* (1 clone) or were not identified (2 clones). Most of the clones were related to marine bacteria occurring in the water column, in sediments or on invertebrates (mainly corals and sponges). Some clones were also affiliated to bacteria originating from the digestive tract of phytophageous animals including the vegetarian monkey *Colobus guereza* Rüppell 1835 (86-88% of sequence identity, ID hereafter), the larva of the emerald ash borer (96-100% ID) and the caterpillar of the cabbage white butterfly (99% ID). Other clones were similar (90-98% ID) to bacteria associated with sulphide-rich environments such as hydrothermal vents and cold seeps sediments, whale carcasses and mangrove soils. Finally, a few clones were close to bacterial symbionts of a hydrothermal vent mussel (94% ID) and a cold seep clam (95% ID), this latter symbiont being thioautotrophic.

Sequences analysis of the APS reductase gene

Full sequences (389-395 bp) of the APS reductase gene fragment were recovered from the same samples than those used for the 16S rRNA gene (Fig. 4). A library of 74 clones (46% of coverage value) was obtained and sequences with at least 97% of similarity were gathered, giving 40 OTUs. A BLAST analysis indicated the presence of both SOB and SRB. SOB, belonging to the *Alpha*- and *Gamma-proteobacteria*, formed the majority of the library with 35

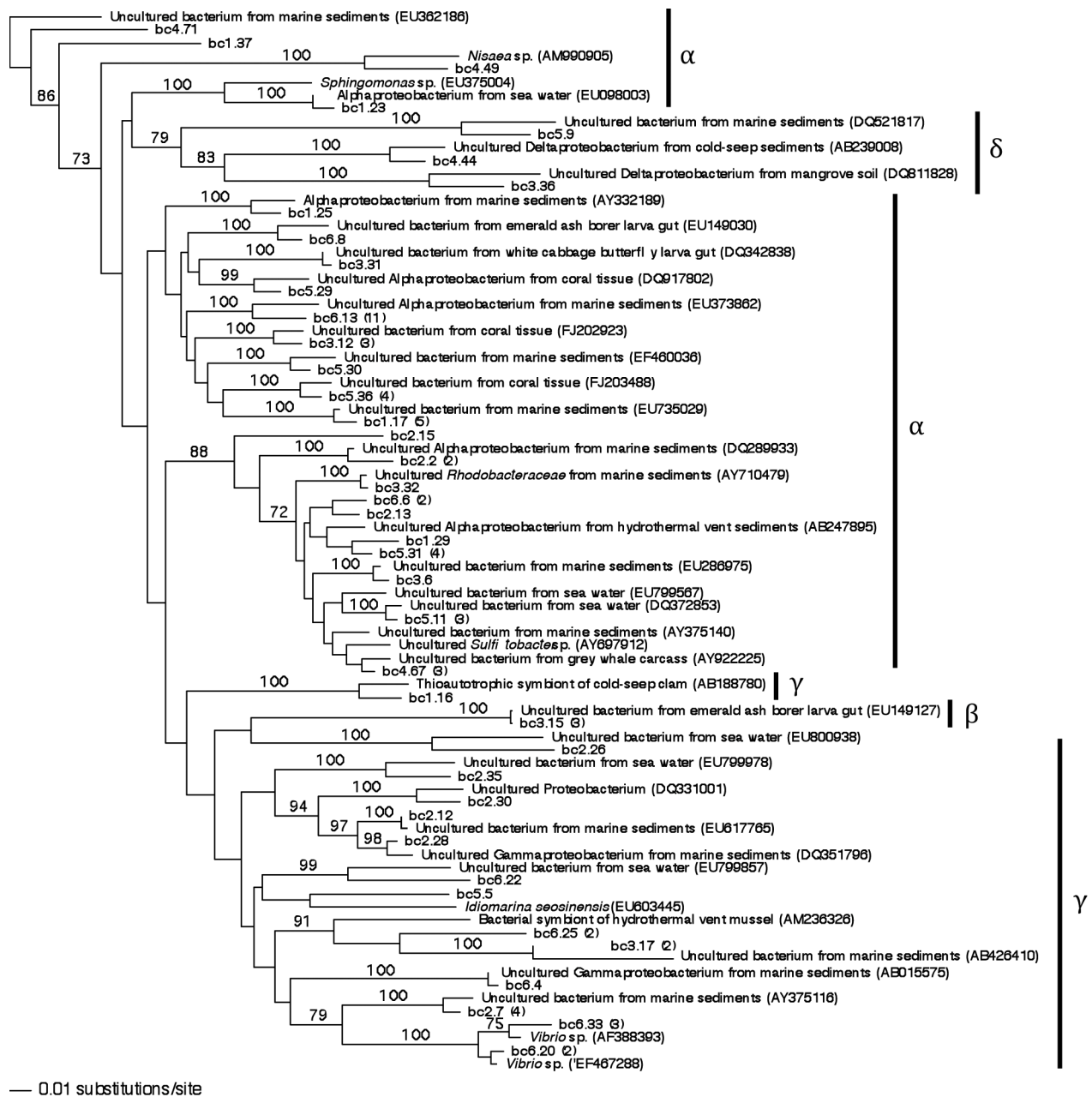**Figure 2.** Unrooted phylogram constructed using 16S rRNA gene sequences of *Proteobacteria* (sequences length ranging from 401 to 550 bp) from the gut content of *Asterechinus elegans*. The tree was built with the neighbour-joining method. Bootstrap values are indicated at nodes (only values > 70 are shown) while figures in brackets indicate the number of identical clones. Sequences obtained in the present study are note «bc». α, β, γ and δ stand for respectively *Alpha-*, *Beta-*, *Gamma-* and *Delta-proteobacteria*.

Figure 2. Phylogramme non enraciné construit à partir des séquences du gène ARNr 16S des *Proteobacteria* (longueur des séquences comprises entre 401 et 550 pb) provenant du contenu digestif *d'Asterechinus elegans*. L'arbre a été établi par la méthode du neighbour-joining. Les valeurs de bootstrap sont indiquées aux nœuds (seules les valeurs > 70 sont montrées) tandis que les nombres entre parenthèses indiquent le nombre de clones identiques. Les séquences obtenues au cours de cette étude sont notées «bc». α, β, γ and δ correspondent respectivement aux *Alpha-*, *Beta-*, *Gamma-* et *Delta-proteobacteria*.
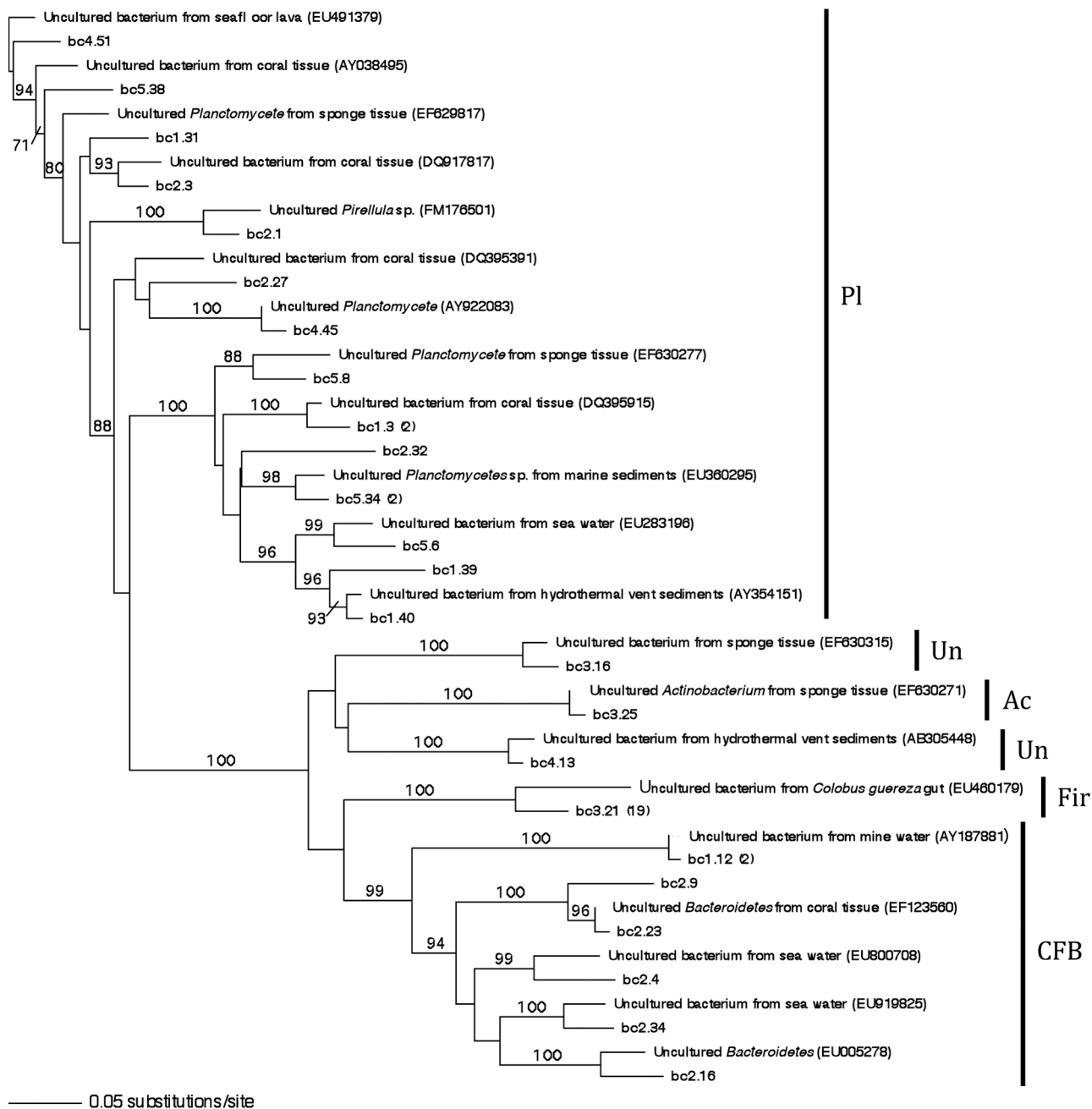
Figure 3. Unrooted phylogram constructed using 16S rRNA gene sequences of *Firmicutes*, *Planctomycete*, *Cytophaga-Flexibacter-Bacteroides* and *Actinobacteria* (sequences length ranging from 401 to 563 bp) from the gut content of *Asterechinus elegans*. The tree was built with the neighbour-joining method. Bootstrap values are indicated at nodes (only values > 70 are shown) while figures in brackets indicate the number of identical clones. Sequences obtained in the present study are noted «bc». Ac: *Actinobacteria*, CFB: *Cytophaga-Flexibacter-Bacteroides*, Fir: *Firmicutes*, Pl: *Planctomycete*, Un: Unidentified.

Figure 3. Phylogramme non enraciné construit à partir des séquences du gène ARNr 16S des *Firmicutes*, des *Planctomycete*, des *Cytophaga-Flexibacter-Bacteroides* et des *Actinobacteria* (longueur des séquences comprises entre 401 et 563 pb) provenant du contenu digestif *d'Asterechinus elegans*. L'arbre a été établi par la méthode du neighbour-joining. Les valeurs de bootstrap sont indiquées aux nœuds (seules les valeurs > 70 sont montrées) tandis que les nombres entre parenthèses indiquent le nombre de clones identiques. Les séquences obtenues au cours de cette étude sont notées «bc». Ac: *Actinobacteria*, CFB: *Cytophaga-Flexibacter-Bacteroides*, Fir: *Firmicutes*, Pl: *Planctomycete*, Un: Unidentified.
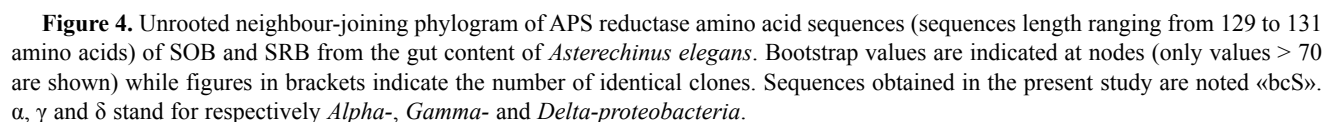
Figure 4. Unrooted neighbour-joining phylogram of APS reductase amino acid sequences (sequences length ranging from 129 to 131 amino acids) of SOB and SRB from the gut content of *Asterechinus elegans*. Bootstrap values are indicated at nodes (only values > 70 are shown) while figures in brackets indicate the number of identical clones. Sequences obtained in the present study are noted «bcS». α, γ and δ stand for respectively *Alpha-*, *Gamma-* and *Delta-proteobacteria*.

Figure 4. Phylogramme non-enraciné construit à partir des séquences en acides aminés de l'APS reductase (longueur des séquences comprises entre 129 et 131 acides aminés) des bactéries sulfo-oxidantes et sulfato-réductrices provenant du contenu digestif *d'Asterechinus elegans*. L'arbre a été établi par la méthode du neighbour-joining en utilisant la distance de Jukes et Cantor. Les valeurs de bootstrap sont indiquées aux nœuds (seules les valeurs > 70 sont montrées) tandis que les nombres entre parenthèses indiquent le nombre de clones identiques. Les séquences obtenues au cours de cette étude sont notées «bcS». α, γ and δ correspondent respectivement aux *Alpha-*, *Gamma-* et *Delta-proteobacteria*.

of the 40 OTUs while SRB were all affiliated to the *Delta-proteobacteria* and included 5 OTUs. Sequences identities were however rather weak with percentages of similarity ranging from 79 to 93% for SOB and from 83 to 98% for SRB. Among SOB, one OTU, identified as a *Gamma-proteobacterium*, was particularly well-represented as it accounted for more than 40% (30 clones) of the total clones and was present in all samples analysed. Most of the other OTUs were represented by a single clone. SOB were related to bacteria from sulphide-rich environments such as peat soils (82-93% ID), ocean ridges (79-93% ID) and marine sediments (79-91% ID) but also to bacterial endosymbionts of various marine invertebrates (80-88% ID). The latter were the tube worms *Oligobrachia haakon-mosbiensis* Smirnov, 2000 and *Sclerolinum contortum* Smirnov, 2000, the nematode *Robbea* sp. and the gutless oligochaetes worms *Olavius ilvae* Giere & Erseus 2002 and *O. algarvensis* Giere, Erseus & Stuhlmacher 1998. SRB were related to various bacterial species including *Desulfotalea psychrophila*, *Desulfovibrio* sp., *Desulfo-monile tiedjei* and *Desulfocapsa sulfexigens* but also to an epibiotic symbiont of the hydrothermal shrimp *Rimicaris exoculata* Williams & Rona 1986.

Discussion

The food diet of the sea urchin *Asterechinus elegans* is composed almost exclusively of wood materials, but sediments can occasionally occur inside the gut. The echinoid should thus obtain nutriments from the digestion of the wood fragments. As herbivorous sea urchins generally lack specific enzymes, they depend greatly on their gut microflora to digest plant structural compounds such as native cellulose (De Ridder & Foret, 2001). Gut bacteria can indeed digest various species of algae and are able to hydrolyse several polysaccharides (De Ridder & Foret, 2001). Harris (1993) described the types of associations between gut bacteria and aquatic invertebrates. Briefly, ingested bacteria may be lysed and absorbed or they may survive in the gut and are then called transient bacteria. Transient bacteria are either directly eliminated in the faeces or they can proliferated somewhat before egestion. Another category of gut bacteria is the resident bacteria that are symbiotic and form stable populations inside the digestive tract. As for other invertebrates, *A. elegans* probably digests a part of the bacteria ingested with the wood but clones from genetic analyses should not correspond to lysed bacteria as we obtained undegraded DNA. Clones may thus correspond to transient bacteria occurring in the gut content and displaced with it during the digestive transfer and/or to resident bacteria staying in the digestive tube and not removed by the passage of food.

Cloning analyses of the 16S rRNA gene indicate that the bacterial microflora from the digestive tube of *A. elegans* is highly diversified. Although more than 120 sequences were obtained, the coverage value of the clone library reaches only 48%, indicating that the bacterial diversity is under-estimated. Despite this fact, about half of the OTU are detected more than once in at least two echinoids, showing a relative homogeneity in the bacterial community associated with the gut content. Interestingly, some clones are close to bacteria from the digestive tract of phytophagous and even wood-feeding animals. These are *Colobus guereza*, a strictly vegetarian monkey, the cabbage white butterfly caterpillar and the emerald ash borer larva, a pest that feeds exclusively on ash trees. This suggests that some bacteria, transient and/or resident, from the gut of *A. elegans* could be able to participate in the digestion of the wood fragments.

Cloning analysis of the 16S rRNA gene also revealed the presence of bacteria related to microorganisms from sulphide-rich environments such as whale carcasses, mangrove soils or marine sediments from notably hydrothermal vents and cold seeps. One clone is even close to a thioautotrophic bacterial symbiont associated with a cold seep clam. The presence of sulphide inside the digestive tract of the sea urchin is indeed emphasized by the analysis of the APS reductase gene that evidenced the co-occurrence of SRB and SOB. SRB are known to use H_2, originating from the anaerobic fermentation of the cellulose, in order to reduce sulphate into hydrogen sulphide (Leschine, 1995). The latter is thus produced during degradation of the wood lying on the seafloor (Dubilier et al., 2008; Laurent et al., 2009) and is a source of energy for chemoautolithotrophic SOB. In wood falls, some SOB presumably live in symbiosis with various marine invertebrates and provide their host with energy and carbon (Dubilier et al. 2008). This kind of association has been demonstrated for instance in the gills of Mytilidae mussels living on sunken woods (Gros & Gaill, 2007; Gros et al., 2007; Duperron et al., 2008). Our results suggest that symbiotic SOB (and SRB) would also be present in the digestive tract of *Asterechinus elegans*. These bacteria could participate in the nourishment of the sea urchins either by providing them with small organic molecules or by being digested. Furthermore, cloning analysis of the APS reductase gene put in evidence a particularly interesting OTU. Indeed, it accounts for more than 40% of the total clones, is found in all samples analysed and is related to various thioautotrophic symbionts from marine invertebrates. The corresponding bacterium could thus represent a key member of the microbial community associated with the gut content of the echinoid.

Interestingly, it should be noted that there is a discrepancy between the SOB library where a lot of clones are close to marine invertebrates endosymbionts and the

16S rRNA gene library where only a few sequences are related to such bacteria. This is due to a bias originating from the genetic databases as we used BLAST to identify the clones (Altschul et al., 1990). Indeed, APS reductase gene sequences are not numerous in GenBank (a few hundreds) and a significant part (about 10%) is related to endosymbionts. The probability to have an endosymbiont as best relative is therefore rather high even with a low sequence similarity. On the other hand, the proportion of 16S rRNA gene sequences from endosymbionts is trifling compared to the huge amount of sequences contained in databases for this gene (more than one million). Moreover, sulphur-oxidizing symbioses evolved independently on multiple occasions from many different bacterial lineages (Dubilier et al., 2008). Consequently, endosymbionts from various invertebrate hosts are not gathered in a same mono-phyletic group that would be representative of a symbiosis.

The presence of methanogenic and methanotrophic bacteria was also investigated by targeting genes coding for the alpha subunits of respectively the methyl-coenzyme M reductase (Lueders et al., 2001) and the particulate methane monooxygenase (Duperron et al., 2007) (data not shown). Indeed, hydrogen from the wood fermentation could be an electron donor for methanogens that would produce methane as an energy source for methanotrophs. However it was not possible to amplify these genes during PCR experiments. Although several technical reasons including primers mismatches can explain this result, another possi-bility is that such bacteria are not present in the gut of *Asterechinus elegans*. If this is the case, it would be in accordance with observations of wood degradation in the marine environment where SRB out-compete methanogens for H_2 due to the abundance of sulphate (Leschine, 1995). This process also occurs in Mytilidae mussels from wood falls where only thiotrophic symbionts are found in the gills while methanotrophic bacteria are absent, although the lat-ter are found in related mussels from hydrothermal vents and cold seeps (Duperron et al., 2005; Duperron et al., 2008). *Archaea* were also targeted using specific primers (DeLong, 1992) but in this case also, PCR amplifications failed despite the numerous conditions tested, suggesting that *Archaea* are not present.

The present work indicates that the bacterial community occurring in the digestive tube of *Asterechinus elegans* is highly diversified and could participate in the digestion of wood fragments. Further analyses are however needed to demonstrate direct assimilation, by the echinoid, of organic molecules derived from the wood bacterial degradation. Stable isotopes analyses, for instance, could be used to determine if the carbon source for the sea urchin comes from the wood or from the bacteria. Moreover, a morpho-logical approach completed with FISH experiments (ade-quately fixed samples were unfortunately not available for FISH analyses) still needs to be realized. This approach could indeed allow to (1) to quantify the different bacterial groups or important clones obtained, and (2) to describe and identify the bacterial morphotypes observed inside the gut, especially the dominant and recurrent filamentous bacteria. Finally, the bacteria found in the digestive tube of *A. elegans* should be compared to those occurring in the surrounding environment and more particularly on the sunken woods to determine if the sea urchin harbours transient or resident (i.e., symbiotic) bacteria.

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