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Characterization by 16S rRNA gene analysis and *in situ* hybridization of bacteria living in the hindgut of a deposit-feeding echinoid (Echinodermata)

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The hindgut caecum of the deposit-feeding echinoid *Echinocardium cordatum* harbours a symbiotic bacterial microflora, organized into layered mats around detrital particles owing to the proliferation of filamentous bacteria. The bacterial community was analysed using 16S rRNA gene analysis and fluorescence *in situ* hybridization. The purpose was to characterize its biodiversity and to identify its predominant members. The majority of the 16S sequences belong to the δ-Proteobacteria (61.5%), the Bacteroidetes and the Firmicutes constitute the two other main bacterial groups (respectively 23.1% and 15.4%). A total of 41% of the δ-Proteobacteria clones isolated belong to a bacterium of the genus *Desulfonema* for which a specific oligonucleotide probe was designed, enabling identification of its distribution in the nodules.

INTRODUCTION

The deposit-feeding echinoid *Echinocardium cordatum* has developed both a foregut and a hindgut caecum, hosting different microbial communities. The foregut caecum contains fermenters that seemingly allow the echinoid to metabolize refractory carbohydrates (Thorsen, 1998). The intestinal caecum harbours an abundant microflora (De Ridder et al., 1985a) performing sulphur reduction and fermentation; but also producing short-chained fatty acids and essential polyunsaturated fatty acids such as linolenic acid (Temara et al., 1991). The bacteria of this intestinal caecum are grouped into spherical nodules consisting of a peripheral bacterial mat and of a central detrital particle (plant fragment, shell, organic aggregates) (De Ridder, 1994). Filamentous bacteria predominate in the mat and seemingly have a prominent role in the nodule formation. On the basis of morpho-physiological and immunological approaches they were identified as sulphur-oxidizing *Thiothrix*-like bacteria (Brigmon & De Ridder, 1998). However, observations based on fluorescence *in situ* hybridization (FISH) analyses with general probes indicated that they could rather belong to the sulphate-reducing genus *Desulfonema* (Thorsen et al., 2003).

This study investigates, through 16S rRNA sequencing and a specifically designed FISH probe, the biodiversity of the bacterial consortium forming the nodules and more particularly the filamentous bacteria occurring in the mat. The aim of this work is to bring further identification of the predominant filamentous bacteria present in these nodules, but also to identify the other bacterial groups present, in order to propose functional aspects of the symbiosis.

MATERIALS AND METHODS

Collection of material

Thirty individuals of *Echinocardium cordatum* (Pennant, 1777) were collected in Wimereux (France). Diameter of the nodules ranged between 2 and 8 mm.

Microscopy

Nodules were fixed in 4% paraformaldehyde, dehydrated in alcohol series and xylene, and embedded in paraffin. Sections of 7 μm were mounted on aminosilane-coated slides. The slides were kept at 4°C in the dark until processing. Slides were deparaffinized in xylene (three times 5 min each), 96% ethanol (two times 5 min each), and 70% ethanol (once 5 min each).

DNA extraction and PCR

Nodules from three echinoids were placed in 10 μl of polymerase chain reaction (PCR) water, freeze–thawed five times and centrifuged at 10,000 rpm for 5 min; DNA was diluted ten times. One microlitre of this extract was used for amplification with 49 μl of Red'y'Star Mix (Eurogentec). The 16S rRNA gene was amplified with primers 8F and 1492R (Buchholz-Cleven et al., 1997). Amplification was performed as described in Gillan et al., 2005).

Cloning and sequencing

The PCR products were cloned with the TOPO TA kit using TOP10 *Escherichia coli* cells (Invitrogen, San Diego). Three libraries were constructed from the three hosts (24 clones from individual 'a', 10 from 'b' and 18 from 'c'). Plasmids were purified using the QIA prep spin miniprep (Qiagen) and sequenced partially with the primer 518F (Buchholz-Cleven et al., 1997) on ABI Prism 3100 (Applied Biosystems). Clones identified as *Desulfonema* genus were sequenced entirely with 518F, the vector based primers M13F and M13R (Buchholz-Cleven et al., 1997) and 1099F (Lane, 1991).

Phylogenetic analysis

Sequences were submitted to BLAST (Madden et al., 1996), aligned in Bioedit (Hall, 1999) and analysed using SeqPup 0.6f (Gilbert, 1996). Chimeras were checked at

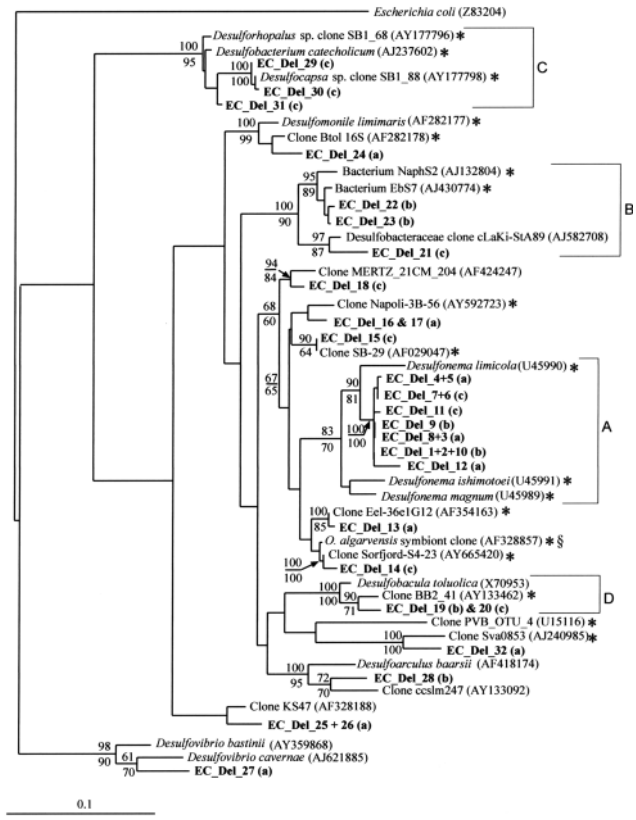

Figure 1. Maximum likelihood tree showing the relationships between the 16S rRNA gene clones of *Echinocardium cordatum* belonging to the δ-Proteobacteria. *Escherichia coli* served as the outgroup. A matrix of 556 nucleotides was used (RNA position 565–1121, *E. coli* numbering). Bootstrap values above 50% (100 resamplings) are shown, with upper and lower values representing distance and parsimony respectively. The bar represents the observed numbers of substitution per 10 nucleotides. *, marine clone or bacterium; §, symbiotic clone or bacterium. (a) host no. 1; (b) no. 2; and (c) no. 3. Identical clones from one host are grouped together.

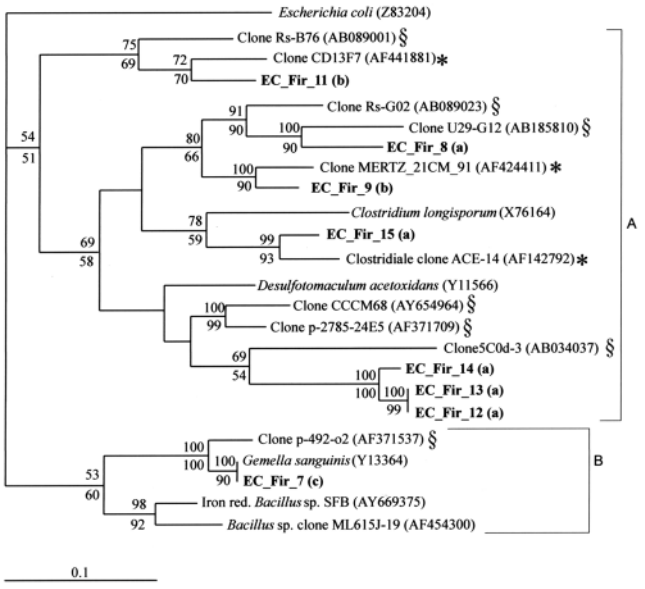

Figure 2. Maximum likelihood tree showing the relationships between the 16S rRNA clones of *Echinocardium cordatum* (EC_Fir, in bold) that belong to the Firmicutes. A matrix of 556 nucleotides was used (RNA position 565–1121, *Escherichia coli* numbering). See Figure 1 for symbol explanations.

http://rdp.cme.msu.edu/. Distance, parsimony and maximum-likelihood trees were generated with the Phylip package (v. 3.6, Felsenstein, 2002). Distance trees were generated with 'Dnadist' using Jukes–Cantor distances, neighbour-joining, parsimony trees were generated with 'Dnapars' using unweighted ordinary parsimony, and maximum-likelihood trees were generated with 'Dnaml' (Ti/Tv=2.0; empirical base frequencies, one category of sites with a constant rate of variation). Statistical significance of the phylogenetic groups was tested by bootstrap analysis with 'Seqboot' and 'Consense' (100 bootstrap). Trees were created with Treeview (v. 1.6.6).

Fluorescence in situ *hybridization*

Nodules of 20 specimens were fixed in 4% paraformaldehyde and hybridized with the Cy3-labelled probes (Interactiva, Ulm) EUB338 and GAM42a (with comp. BET42a) (Gillan et al., 2005). A specific probe (DNMA-EC439: 5′-GCGGTTTCTTCCCGCCTG -3′, position 439–457 *E. coli* numbering) was designed to target the identified *Desulfonema* (PROBE-DESIGN of the ARB package; Ludwig et al., 2004). It contains at least one central mismatch to all known 16S rRNA sequences, including *Desulfonema* sequences as determined by Check Probe 2.1r from the RDP (http://rdp.cme.msu.edu/). Specificity was tested at formamide percentages between 10 and 60%. Formamide concentration for observation was 40%.

Nucleotide sequence accession numbers

The sequences obtained in this study have been assigned in the GenBank database under accession number sets AY222304 to AY222319 and AY845639 to AY845689.

RESULTS

δ-Proteobacteria group

Sixty-one and a half per cent of the clones belong to the δ-Proteobacteria. The Desulfobacterales are the major group represented by the retrieved clones (Figure 1), most of the smaller clusters are supported by high bootstrap values. The largest cluster formed contains 12 clones (Cluster A). These clones group with the filamentous sulphate-reducing genus *Desulfonema*, and were isolated from the three independent DNA libraries constructed. The specific oligonucleotide probe used in this study (DNMA-EC_439) was designed using three clones of that cluster (EC_Del_1, EC_Del_3 and EC_Del_7). These three clones, similar at 99.3%, originate from three different clone libraries, and show average sequence identities of 90.7%, 89.6%, and 89.3% with the species *D. magnum*, *D. ishimotoei* and *D. limicola* respectively. Cluster B holds three *Echinocardium cordatum* clones grouping with sulphate-reducing organisms such as bacteria NaphS2 and EbS7 isolated from marine sediments. Cluster C groups three clones respectively affiliated with the species

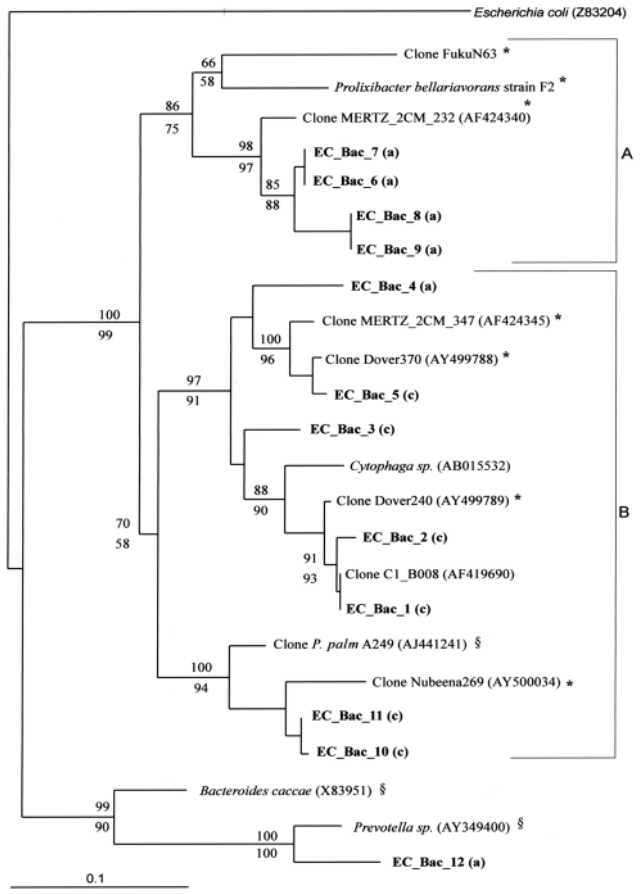

Figure 3. Maximum likelihood tree showing the relationships between the 16S rRNA clones of *Echinocardium cordatum* (EC_Bac, in bold) that belong to the Bacteroidetes. A matrix of 557 nucleotides was used (RNA position 565–1122, *Escherichia coli* numbering). See Figure 1 for symbol explanations.

Desulfobacterium catecholicum, *Desulfocapsa* sp. and *Desulforhopalus* sp. Two clones group with *Desulfobacula toluolica* in Cluster D. One clone groups with good bootstrap values with the species *Desulfovibrio cavernae* and *D. bastinii*. Remaining clones group with uncultured clones occurring in marine sediments.

Bacteroidetes group

23.1% of the clones belong to the Bacteroidetes. The clones are distributed in two main clusters (Figure 2). Both are composed of clones isolated from marine sediments. Cluster A is composed of four clones grouping with Bacteroidetes clones affiliated with the species *Prolixibacter bellariavorans* (AY918928). The Cluster B groups seven clones with marine clones as well as a *Cytophaga* sp. (EC_Bac_2 and _1) and *P. palm* A249, a clone originating from the microbial community in the mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis*. One clone, EC_Bac_12, groups with good bootstrap values with *Bacteroides caccae* and *Prevotella* sp. occurring in the human gut and oral cavity.

Firmicutes group

15.4% of the clones belong to the Firmicutes. The clostridiales (Cluster A) are the most represented group (Figure 3). Five isolated clones group with species and uncultured clones found in other gut environments such as *Clostridium lactatifermentans* in the caeca of chicken, clones Rs-B76 and Rs-G02 in the gut of the termite *Reticulitermes speratus*, pig gut clones p-4247-4Wa3, p-2785-24E5, and clones U29-G12 and 5C0d-3 in the rumen microbiota of cattle. The Bacillales (Cluster B) are represented by one clone, grouping with high bootstrap values

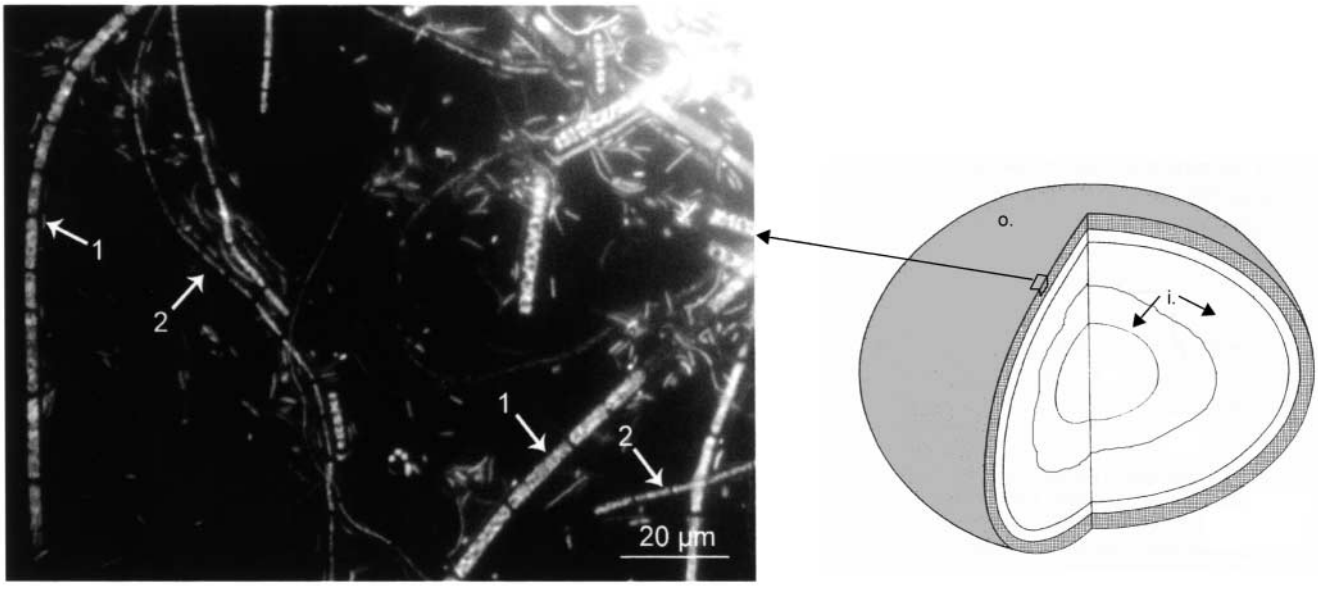

Figure 4. EUB338 stained preparation showing the two types of multicellular filamentous bacteria (Type1 and Type 2 filaments) and their predominance zone on a schematic view of the nodules. O, outer region of the nodule; I, inner region of the nodule.

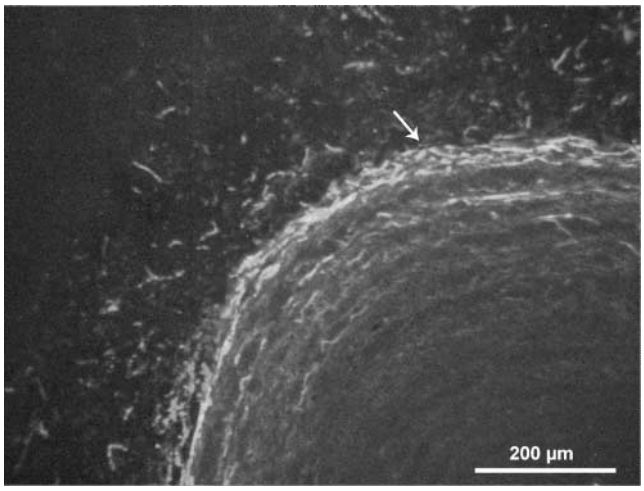

Figure 5. Cross section in a nodule showing the peripherally localized hybridization of the specific probe designed for the *Desulfonema* sequences (DNMA-EC439) with Type 1 filaments.

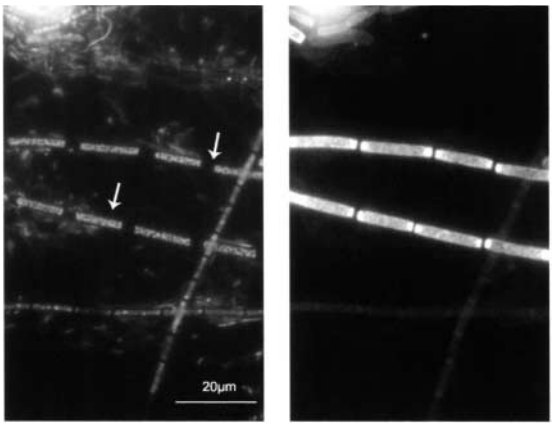

Figure 6. Type 1 filaments (arrows) shown with 4′-6-diamidino-2-phenylindole staining (left panel) and with the specific probe designed for the *Desulfonema* sequences (DNMA-EC439) identified in this study (right panel).

with the species *Gemella sanguinis*. The other clones isolated group with bacteria found in marine sediments.

Fluorescence in situ *hybridization*

In all individuals, two types of multicellular filamentous bacteria were detected using the EUB338 probe. Figure 4 shows a diagram of the nodules and the zone in which these two types of filaments are observed. Type 1 filaments were approximately 2.5 to 3.0 μm in diameter and type 2 filaments never exceeded 1.5 μm in diameter. The specific probe DNMA-EC439 designed in this study, hybridized with the Type 1 filaments (Figures 5&6), these were revealed to be spatially concentrated in an upper 50 μm layer of the nodules. Type 2 multicellular filaments could not be positively identified with any of the general probes used in this study. Gamma Proteobacteria were not detected in the nodules using the GAM42a probe.

DISCUSSION

Most (75%) of the clones obtained in this study were affiliated with marine sediment bacteria. *Echinocardium cordatum* swallows sediment in bulk and probably supplies its caecum with symbionts (De Ridder et al., 1985b). Cloning and sequencing have shown that three main bacterial groups proliferate in the nodule, the δ-Proteobacteria, the Bacteroidetes and the Firmicutes. No γ-Proteobacterial sequences were found in the nodules (negative FISH results using the probe GAM42a), consequently *Thiothrix* bacteria do not occur in the nodule, contradicting previous observations by Brigmon & De Ridder (1998). These results could, however, be related to the occurrence of γ-Proteobacteria in the nodules at particular stages of the host's life span. This study supports the observations of Thorsen and co-workers (2003) and indicates that a *Desulfonema* bacterium is a recurrent member of the nodule microflora, as 16S sequences affiliated with the genus *Desulfonema* were detected in each clone library. *Desulfonema* were localized more precisely in the nodules with the use of probe DNMA-EC439, specific to the *Desulfonema*-related sequences. In all investigated individuals, it constantly hybridized to the predominant Type 1 filaments, i.e. to the bacteria that seemingly builds the peripheral mats and the frame of the nodule. Indeed, the characteristics of the *Desulfonema* group fit this role as these bacteria are gliding and produce extra cellular matrix (ECM) (Widdel, 1981). Moreover, when growing in co-cultures, the *Desulfonema* filaments can aggregate into nodules, in association with other bacterial morphotypes such as rods and spirochaetes (K. Harris, personal communication).

Desulfonema bacteria are sulphate-reducers; sources of sulphate are multiple in the gut of echinoids: secretions (sulphated mucopolysaccharides), food bolus (detritus and seawater) and the nodule itself (ECM). According to Thorsen (1998), the intestinal caecum of *E. cordatum* could act as a fermentation chamber; organic matter is present in the nodule, reducing conditions prevail in its inner layers and end products such as volatile fatty acids (butyrate, acetate, propionate) occur in the caecum lumen. Interestingly, 15.4% of the clones obtained in this study were affiliated to fermentative bacteria found in other gut environments. These mainly belong to the Firmicutes and are affiliated with organisms occurring in the digestive tube of arthropods (Egert et al., 2003), birds (Van der Wielen et al., 2002) and mammals (Sakamoto et al., 2004). Metabolite exchanges must occur between the bacteria and their echinoid host because the intestinal caecum is both a highly absorbing and an excretory organ (Warnau et al., 1998). Among the echinoids, the presence of an intestinal caecum is a unique feature solely observed in the order Spatangoida. The species developing this organ are rare and belong to phylogenetically dispersed families (Loveniidae, Schizasteridae and Asterostomatidae) (De Ridder, 1994; Stockley et al., 2005). In that context, further observations are in progress; they aim to answer the significance of this hindgut symbiosis, for example by examining the influence of environmental or morphofunctional parameters, and to identify possible pressure-factors common to all symbiotic spatangoids species.

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