RESEARCH ARTICLE

Different bacterial communities associated with the roots and bulk sediment of the seagrass *Zostera marina*

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Abstract

The bacterial community of *Zostera marina*-inhabited bulk sediment vs. root-associated bacteria was investigated by terminal restriction fragment length polymorphism and sequencing, and the spatial extension of the oxygen loss from roots was determined by oxygen microsensors. Extensive oxygen loss was found in the tip region of the youngest roots, and most of the rhizoplane of *Z. marina* roots was thus anoxic. A significant difference between the bacterial communities associated with the roots and bulk sediment was found. No significant differences were found between differently aged root-bundles. Terminal restriction fragments (TRFs) assigned to sulfate-reducing *Deltaproteobacteria* showed a relative mean distribution of 12% and 23% of the PCR-amplified bacterial community in the bulk-sediment at the two sites, but only contributed $< 2\%$ to the root-associated communities. TRFs assigned to *Epsilonproteobacteria* showed a relative mean distribution of between 5% and 11% in the root-associated communities of the youngest root bundle, in contrast to the bulk-sediment where this TRF only contributed $< 1.3\%$. TRFs assigned to *Actinobacteria* and *Gammaproteobacteria* also seemed important first root-colonizers, whereas TRFs assigned to *Deltaproteobacteria* became increasingly important in the root-associated community of the older root bundles. The presence of the roots thus apparently selects for a distinct bacterial community, stimulating the growth of potential symbiotic *Epsilon-* and *Gammaproteobacteria* and/or inhibiting the growth of sulfate-reducing *Deltaproteobacteria*.

Introduction

Seagrasses are important primary producers in coastal marine ecosystems. They present habitats and nursery grounds for many invertebrates and fish and serve as a major food source for many coastal birds. Seagrasses can affect microbial processes in the rhizoplane by excretion of amino acids (Wirsen *et al.*, 2002) and easily degradable sugars (McClung *et al.*, 1983; Welsh, 2000). They also oxidize the rhizosphere due to radial oxygen loss from the roots, although recent studies have shown that extensive oxygen leakage only happens from the very tip of younger root segments (Connell *et al.*, 1999; Jensen *et al.*, 2005; Frederiksen & Glud, 2006). Seagrasses can furthermore affect the microbial community in the bulk sediment by increasing the organic carbon load as detached leaves and decaying root and rhizome material.

Sulfate-reducing bacteria, which are the predominant bacteria involved in anaerobic degradation of organic matter in coastal marine sediments (Jørgensen, 1982), proliferate from the increased organic carbon load, and sulfate-reduction is increased in seagrass-inhabited sediments compared with nonvegetated sediments of the same origin (Holmer & Nielsen, 1997). Sulfide produced by sulfate-reducing bacteria is toxic to eukaryotic organisms (Bagarinao, 1992) and sulfide poisoning has been coupled to recent die-back events of seagrasses worldwide (Koch & Erskine, 2001; Pedersen *et al.*, 2004; Borum *et al.*, 2005; Holmer *et al.*, 2006). The plants can respond to this potential phytotoxin by development of a barrier to radial oxygen loss (Armstrong &

Armstrong, 2005), and such gas diffusion barriers can also be induced by organic acids (Armstrong & Armstrong, 2001).

Other types of bacteria can have beneficial effects on seagrasses by metabolizing toxic substances in the rhizoplane. Nitrate-reducing sulfide-oxidizing bacteria have recently been isolated from the roots of seagrasses (Küsel *et al*., 2006), and sulfide-oxidizing bacteria may be partly responsible for the oxidation of sulfide that has been observed during daytime in seagrass-inhabited sediments (Lee & Dunton, 2000) and *Spartina alterniflora* roots (Lee *et al*., 1999).

The bacterial community in the rhizosphere/bulk sediment of seagrasses has previously been studied by molecular analysis and cultivation approaches. Recent studies have shown that the bacterial community is different between vegetated and unvegetated sites and varies with season (James *et al*., 2006). Studies have also been concerned with specific groups of bacteria such as sulfate-reducing bacteria (Küsel *et al*., 1999; Nielsen *et al*., 1999; Finster *et al*., 2001), nitrogen-fixing bacteria (Bagwell *et al*., 2002) and acetogenic bacteria (Küsel *et al*., 1999), whereas the general bacterial diversity has only been described by molecular analyses of rhizosphere samples from a sediment horizon rich in rhizome and root material (Cifuentes *et al*., 2000). A description of the bacterial community and species diversity associated with the roots of seagrasses, and a comparison with the plant-inhabited bulk sediment, has, to our knowledge, not been reported.

In this study, the colonization patterns of bacteria associated with the roots of the seagrass *Zostera marina* were investigated in relation to different root ages, and the root-associated bacterial community was compared with the bacterial community in the surrounding bulk sediment. To obtain information about the diversity of root-associated and sediment bacteria, we made clone libraries from two different field sites. In addition, terminal restriction fragment length polymorphism (T-RFLP) analysis (Avaniss-Aghajani *et al*., 1994) of PCR-amplified 16S rRNA gene fragments was used to investigate differences in the root-associated bacterial community and the bacterial community in the plant-inhabited sediment, whereas oxygen microsensors were used to identify the spatial extension of the oxic microzone around *Z. marina* roots.

Materials and methods

Sampling sites

Samples were collected from a densely colonized *Z. marina* bed in Ellinge, Kulhuse, Roskilde Fjord, Denmark, in July 2005 and from a sparsely vegetated *Z. marina* bed (< 10 shoots m^{-2} at the place and time of sampling) near Rungsted Harbour, Denmark, in August 2005. Ellinge is located in the northern part of Roskilde Fjord, a narrow and shallow estuary (mean depth 3 m, surface area 123 km^2) and Rungsted is located in Øresund, which is a narrow strait connecting the Baltic sea with the North Sea. Both sites are characterized by a water depth of *c*. 1 m, a low tidal range (< 10 cm) and sandy, nutrient-rich sediments. The salinity was 12‰ and 13‰ and the water temperature 24 °C and 20 °C at the time of sampling at Ellinge and Rungsted, respectively.

Plant and sediment sampling

Plants with surrounding sediment were randomly collected with a spade at a water depth of *c*. 1 m and immediately transported on shore, where the sediment was carefully separated from the plant roots by washing the roots with autoclaved seawater. Roots from four (Ellinge) and six (Rungsted) different plants were divided according to differently aged root bundles (first three root bundles only), put into separate plastic bags and placed on ice until return to the laboratory, where they were stored at -80 °C until further analysis. Two additional plants were randomly collected and returned in water from the sampling site to the laboratory for further analysis with oxygen microsensors. Three sediment cores of > 10 cm length were collected within clusters of plants using perspex core tubes (20×5 cm). In the laboratory, the sediment cores were sectioned into oxidized (sed-ox) (light gray, 1–2 cm upper horizon, Rungsted only) and reduced (sed-red) (black, 5 cm below the oxidized horizon, both sites) sediment samples. Plant roots were removed from the sediment and the sediment was homogenized with a spoon and frozen at -80 °C until DNA extraction was performed 7–14 days later.

Microsensor measurements

Microscale oxygen measurements were performed on plants within 1 h from collection. All oxygen measurements were made with Clark-type oxygen microsensors with tip diameters of *c*. 10 μm (Revsbech, 1989). Root surface oxygen concentrations at different distances from the root tip were measured by replanting each plant in a plastic box containing sieved (mesh size 1 mm) sediment and placing the plant in saturating light (*c*. 470 μmol photons m^{-2} s^{-1}) in a 5 L aquarium. Before each measuring point, the microsensor tip was positioned at the root surface while observed under a dissecting microscope and gently covered with sediment. Steady-state oxygen concentrations were obtained within 15–30 min after reburial of the roots [more details in Jensen *et al*. (2005)].

DNA extraction

Homogenized sediment (0.5 g) was used for DNA extraction of the sediment cores. All root material from each root

bundle was crushed with a sterilized pestle for < 1 min in a sterilized mortar and the entire root bundle material was transferred to the bead beating tube using the first buffer in the protocol to ease transfer of all material. DNA from both sediment and root samples were then extracted by a commercialized bead beating method (Yu & Mohn, 1999). The mortar was used in order to improve the extraction of DNA from potential endophytes, as initial studies had shown, that the roots remained partly intact using the bead beating method only. The DNA was cleaned up using the Genomic Mini spin kit (A&A Biotechnology, Gdynia, Poland) following the manufacturer's instructions.

PCR amplification

16S rRNA gene fragments were amplified with the primers 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) (Weinbauer *et al.*, 2002) and 1492r (5′-ACG GYT ACC TTG TTA CGA CTT-3′) (Lane, 1991). For T-RFLP analysis, the forward primer was 5′-end labeled with TET. Reaction mixtures (25 μL) contained 2 μL DNA extract, 15 pmol of each primer (TAG, Copenhagen, Denmark) and a Ready-To-Go PCR Bead (GE Healthcare, Uppsala, Sweden). PCR amplification was carried out in a Peltier Thermocycler (DNA Engine DYAD). After a denaturation step of 3 min at 94 °C, amplification reactions were carried out with 35 cycles of: denaturation (25 s, 94 °C), primer annealing (40 s, 54 °C), a primer extension step (1 min 45 s, 72 °C), followed by a final extension step of 7 min at 72 °C. PCR products of the expected size were gel purified using the Gel-Out kit (A&A Biotechnology, Gdynia, Poland) following the manufacturer's instructions.

T-RFLP

Seventeen microliters gel purified PCR product, 10 U restriction enzyme and 1/10 of manufacturer's recommended buffer (New England Biolabs) were gently mixed before digestion. Digestion of all samples was performed in a Perkin-Elmer thermocycler (PE 9600) for 12 h at 65 °C (*Tsp*509I). Samples were transferred to a microtiter plate, 0.2 μL MegaBACE ET 900 RT-Size standard was added and the samples were desalted with sephadex G-50 (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. After denaturation of the DNA at 94 °C for 2 min, the samples were kept on ice before T-RFLP analysis was performed in an automated DNA sequencer (MegaBace 1000 DNA Sequencing System, GE Healthcare, Uppsala, Sweden). Samples were injected at 3 kV for 3 min and electrophoresis was carried out at 6 kV for 4 h. Electropherograms were analyzed by comparison with the internal standard using the GENETIC PROFILER, version 2.2 software (GE Healthcare, Uppsala, Sweden).

Cloning

A total of five different cloning reactions were performed. DNA extracts from the roots and the sediments at the respective sites were pooled together into five different groups; roots and sed-red (Ellinge) and roots, sed-red and sed-ox (Rungsted). Fresh PCR products of three parallel PCRs were gel purified as described above and grouped together before ligation into the pCR 2.1-Topo vector (Invitrogen Life Technologies) and transformation into Top 10 *Escherichia coli* competent cells (Invitrogen Life Technologies) following the manufacturer's instructions.

Sequencing

Plasmids were purified using the Qiaprep Spin Miniprep Kit (Qiagen) before sequencing. The directions of the inserts were analyzed by PCR amplification using one of the M13 internal primers and 1492r. Sequence reactions were performed using the internal M13 primer that ensured sequencing of the 5′-end of the gene fragments. Primers used in addition to the internal M13 primers for nearly full-length 16S rRNA gene sequences were 519r (5′-GWA TTA CCG CGG CKG CTG-3′), 532f (5′-CAG CMG CCG CGG TAA TSC-3′), 907r (5′-CCGTCAATTCMTTRAGTTT-3′) and 926f (5′-AAA CTY AAA KGA ATT GAC GG-3′) (Lane, 1991).

Sequence analysis

16S rRNA gene sequences were assembled using ASSEMBLY LIGN software (Oxford Molecular, Cambridge, UK) and closest related sequence defined bacteria as well as the closest cultured bacterium were found using the BLAST function at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Potential chimeric sequences were identified using the CHECK_CHIMERA program and the CLASSIFIER at the Ribosomal Database Project (http://rdp.cme.msu.edu), as well as by blasting partial sequences. Eighteen potential chimeric sequences were checked for their restriction site. Two terminal restriction sites that occurred after a potential chimeric event were removed from the T-RFLP analysis and the chimeric sequences were then given no further consideration. Similarity percentages were calculated using the pairwise alignment function in the software package BIOEDIT (Hall, 1999). Nucleotide sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). Evolutionary distances were calculated based on the Jukes and Cantor correction (Jukes & Cantor, 1969) and phylogenetic trees were reconstructed by the neighbor joining method (Saitou & Nei, 1987) as implemented in the TREECON version 1.3b software (Van de Peer & De Wachter, 1994).

Analysis of T-RFLP

The sequences from the clone libraries were analyzed with different tetrametric restriction enzymes using WEBCUTTER 2.0 (http://rna.lundberg.gu.se/cutter2/). *Tsp*509I was found to be the best enzyme to separate the sequences from the clone libraries and was therefore selected as the restriction enzyme for T-RFLP analysis.

Terminal restriction fragments (TRFs) < 50 bp and larger than 900 bp, as well as TRFs < 200 U above the baseline were excluded from the analysis. TRFs corresponding to TRFs from plant organelles and *Cyanobacteria*, as well as TRFs after a presumed chimeric event, were also removed before further analysis. The relative abundance of each TRF was determined using the maximal signal intensity of each individual TRF in relation to the total maximal signal intensity of all included TRFs. In this regard, it should be remembered that T-RFLP is not a quantitative description of the structure of the original microbial community. This is primarily due to the potential artifacts and biases from the extraction of DNA and the PCR method in general (further information in a review by Wintzingerode *et al.*, 1997). Different T-RFLP profiles can, however, still describe trends in differences between sites, but one cannot conclude whether the differences observed are caused by an increased presence of microorganisms with a specific TRF or whether the differences are caused by an increase or absence of other bacteria.

Statistical and community analysis

The standardized TRFs were analyzed with nonmetric multidimensional scaling (MDS) (Clarke, 1993) through the use of the program package PRIMER (Clarke & Warwick, 2001). Similarities were calculated using the Bray-Curtis similarity coefficient (Bray & Curtis, 1957) on the relative abundance data and statistical analysis was performed using the ANOSIM function in the software package. We also tried to normalize the data according to the procedure of Dunbar *et al.* (2000), but this yielded no change in the statistical differences between the samples. The normalization caused a number of TRFs to be eliminated, and as some of these were actually present in the clone library, the data were chosen to be presented without normalization. The statistical method chosen does not emphasize rare species and does not take the absence of a species as an indicator of difference. Therefore, it is seen that avoiding normalization of the data is the best approach in this case. In this regard it should also be mentioned that the highest and lowest total fluorescence was from root samples even after elimination of TRFs from plastids.

The different sequences in the clone libraries were assigned ± 1–2 bp to their respective *Tsp*509I restriction site and their relative mean contribution to the root-associated bacterial community of the first, second and third root bundle as well as their mean contribution to the bacterial community in the different sediment horizons were calculated for each of the two sites.

Nucleotide sequence accession numbers

All nucleotide sequences obtained in this study has been deposited in GenBank under accession no. EF028917–EF029033 and EF036251–EF036313.

Results

Microsensor analysis

The oxygen concentration at the root surface of the first root bundle varied between 0% and 80% of air saturation, with the highest oxygen concentrations within the root tip region (Fig. 1). No oxygen was detected at the root surface of the second root bundle, although several attempts were made.

Molecular analysis

DNA was retrieved from all samples except for one of the third root bundles from the Rungsted locality. Because one of the collected plants did not have an intact third root bundle, we ended up with four replicate DNA samples from the third root bundles at the Rungsted site compared with six from the first and second root bundles. Several TRFs corresponding to TRFs from plastids and potential chimeric sequences were removed before further T-RFLP analysis.

Phylogenetic analysis

The results of the affiliation of the sequences are summarized in Table 1. Further information about the clones (Clone library from which the sequence was obtained, % similarity to closest uncultured and cultured organism, length of the sequence and *Tsp*509I *in silico* restriction site) is given in supplementary material.

The most striking difference between the clone libraries from the bulk sediment vs. the clone libraries from the roots was the abundance of clones affiliating with either *Epsilon-* or *Deltaproteobacteria*. *Deltaproteobacteria* were the most abundant group in the clone libraries from the sediment (17–28% of bacterial sequences) whereas presumed *Epsilonproteobacteria* were the most abundant group in the clone libraries from the roots (17–35% of bacterial sequences) (Table 1). The majority of the sequences presumed to represent *Epsilonproteobacteria* grouped together with either *Arcobacter* sp. or *Sulfurimonas* sp./*Thiomicrospira* sp. (Fig. 2).

The majority of the presumed *Deltaproteobacteria* derived from the bulk sediment clone libraries and their closest affiliates among cultured organisms are found within the order *Desulfobacterales*. Three sequences deriving from the

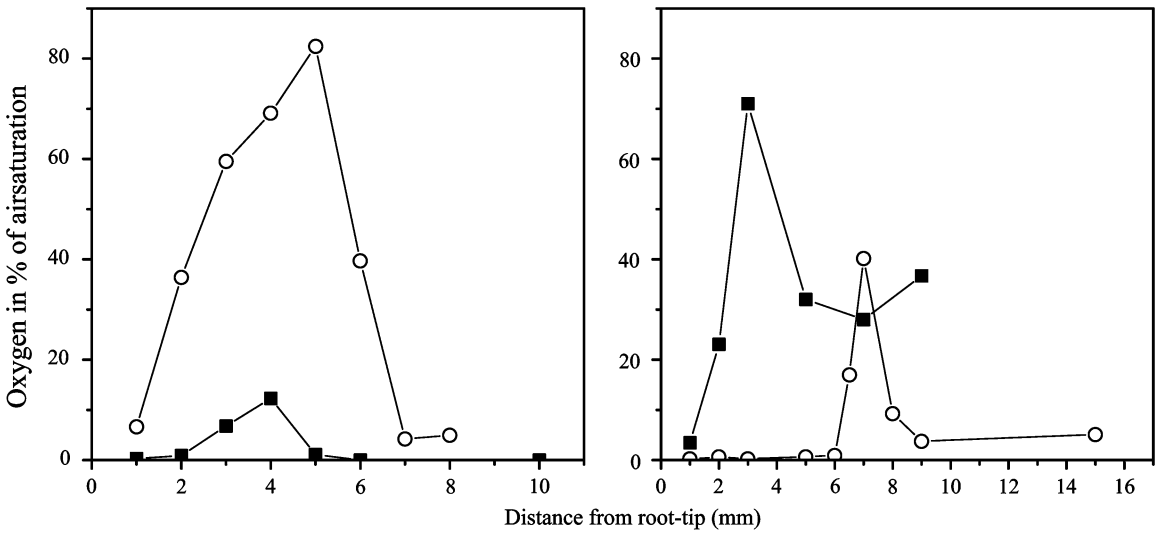

Fig. 1. Oxygen concentration at the root surface of *Zostera marina* roots from two different plants in Rungsted (left) and Ellinge (right), respectively. The oxygen concentration was measured at various distances from the root apex of roots from the first root-bundle. No oxygen was detected on the root surface of roots from the second root-bundle (data not shown).

Table 1. Number of partial 16S rRNA gene sequences assigned to different phylogenetic groups from five different clone libraries derived from *Zostera marina* inhabited rhizoplane and sediment (Sed-ox, Sed-red) at two different sites

	Rhizoplane	Rungsted Sed-ox	Sed-red	Ellinge rhizoplane	Sed-red
Alphaproteobacteria	3	4	2	3	2
Deltaproteobacteria	2	7	4	0	9
Gammaproteobacteria	9	4	4	0	4
Epsilonproteobacteria	12	0	1	5	3
Clostridia	1	0	0	4	0
Planctomycetes	0	3	3	1	0
Bacteriodetes	6	3	3	14	7
Actinobacteria	1	2	3	0	1
Fusobacteria	0	0	0	0	1
Unidentified	1	4	1	2	4
Plastids/Cyanobacteria	24	0	3	37	1
Total	59	27	24	66	32

Sed-ox, oxidized sediment horizon; Sed-red, reduced sediment horizon.

upper sediment horizon and one deriving from the reduced sediment horizon in Rungsted had closest cultured organisms affiliating within the order *Myxococcales*, whereas two sequences displayed 86% similarity to the unclassified sulfur and iron-reducing *Deltaproteobacterium* 103 as closest cultured organisms (see supplementary material).

T-RFLP analysis of the bacterial communities

T-RFLP analysis of 16S rRNA gene fragments was used together with sequencing to investigate the root-associated bacterial community regarding root age and to compare the root-associated bacterial community with the bacterial community in the surrounding sediment. Analysis using the ANOSIM function in the PRIMER software package showed significant differences between the root-associated bacterial community in all of the three different root bundles and the surrounding sediment ($P < 0.5\%$, $R = 0.74$–1). A significant difference was also found between the bacterial community in the upper oxidized sediment horizon and the lower reduced sediment horizon at the Rungsted locality ($P = 0.2\%$, $R = 0.72$). No significant differences were found between the root-associated bacterial communities of the different root bundles; however, MDS analysis indicated that although not significantly different, there was a trend toward a different bacterial community associated with the roots of the first and the third root bundle at both sites (Fig. 3).

TRFs with a mean total contribution between 66% and 86% of the T-RFLP-derived bacterial community in the five

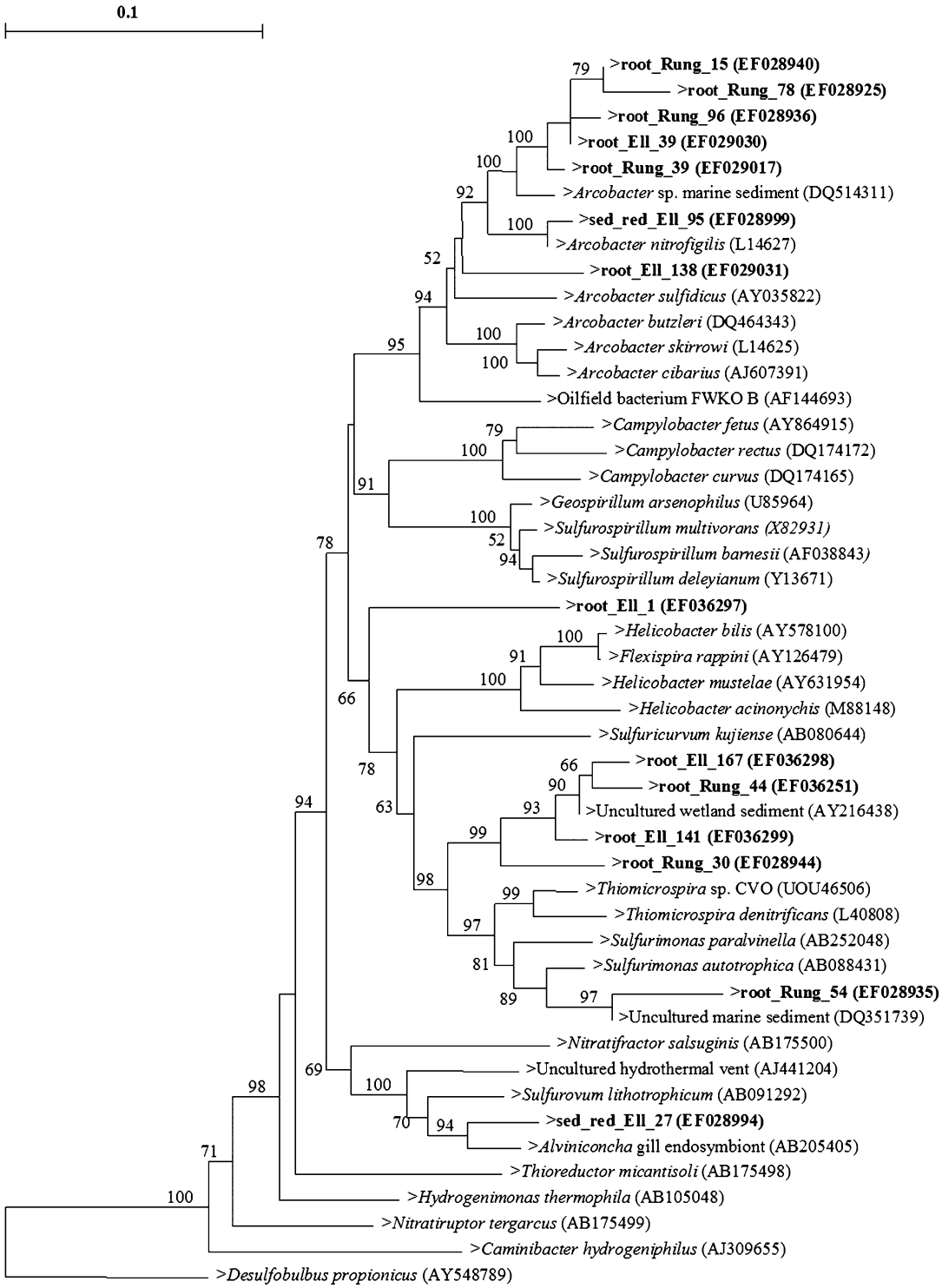

Fig. 2. Relationships between partial 16S rRNA genes of sequences related to *Epsilonproteobacteria*. The tree was constructed by the neighbor-joining method (39) using the Jukes and Cantor correction (24). *Desulfobulbus propionicus* was used as outgroup. Values indicate the percentage of 100 replicate trees supporting the branching order; values below 50 are omitted. Sequence root_Rung_15 represents a total of three sequences > 99% identical, whereas sequence root_Rung_39 and sed_Elli_95 represents a total of two sequences > 99% identical.

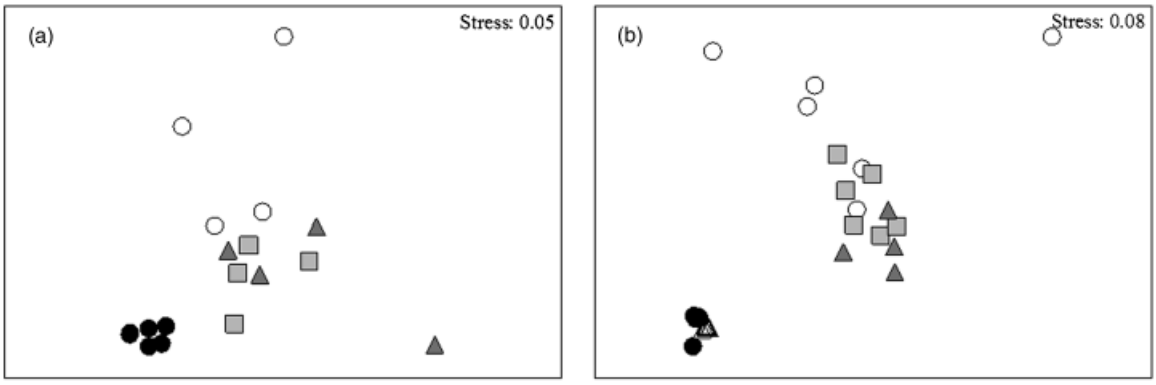

Fig. 3. MDS plots of T-RFLP profiles of 16S rRNA gene fragments digested with *Tsp*509I, from (a) Ellinge (b) Rungsted. ○ (r1), ■ (r2), ▲ (r3), △(Sed-ox) and ● (Sed-red). r1–r3 indicate the different root-bundles (r1. being the youngest), whereas Sed-ox and Sed-red indicate the oxidized sediment horizon (1–2 cm surface sediment) and reduced sediment horizon (5 cm below the oxidized horizon), respectively.

different groupings (root bundle 1–3, sed-ox and sed-red) at the two sites could be assigned to retrieved clones, and TRFs displaying a minimum mean distribution of $> 5\%$ are shown in Fig. 4.

TRF 130 (presumed sulfate-reducing *Deltaproteobacteria*) showed the highest mean contribution (Rung: $23 \pm 4\%$, Ell: $12 \pm 4\%$) in both of the anaerobic sediment horizons followed by TRF 148 (presumed *Bacteriodetes*) (Rung: $10 \pm 2\%$, Ell: $10 \pm 3\%$). Both of these TRFs also contributed the most in the oxidized sediment horizon investigated in Rungsted (TRF 130: $18 \pm 1\%$, TRF 148: $19 \pm 2\%$). This was in contrast to the root bundles where TRF 130 only contributed 0.1–2% and TRF 148 contributed 0.3–4.0% to the bacterial communities different root bundles. Another difference was that TRF 82 (presumed *Epsilonproteobacteria*) contributed most with $11 \pm 4\%$ to the bacterial community associated with the roots of the first root bundle in Ellinge compared with 0.4% in the sediment. TRF 650 (presumed *Actinobacteria*) contributed most to the bacterial community of the first root bundle in Rungsted ($18 \pm 26\%$), followed by TRF 555 (presumed *Gammaproteobacteria*, *deltaproteobacteria*); $10 \pm 7\%$, TRF 153 (presumed *Gammaroteobacteria*); $6 \pm 7\%$, TRF 450 (presumed *Gammaproteobacteria*); $6 \pm 10\%$, TRF 616 (presumed *Bacteriodetes*); $6 \pm 5\%$ and TRF 82 (presumed *Epsilonproteobacteria*); $5 \pm 2\%$.

A shift toward a different root-associated bacterial community was seen in the second and the third root bundle. TRF 650 (presumed *Actinobacteria*) was still the highest contributor in the root-associated bacterial community of the second root bundle in Rungsted ($10 \pm 10\%$), whereas TRF 64 (presumed other types of *Deltaproteobacteria*) contributed with $11 \pm 4\%$, $19 \pm 14\%$ and $18 \pm 4\%$ to the bacterial community associated with the second root bundle in Ellinge, third root bundle in Ellinge and Rungsted, respectively.

Discussion

Bacterial communities in plant-inhabited aquatic sediments are considered to be greatly influenced by the presence of the roots, due to release of easily degradable sugars, small organic acids and amino acids as well as an oxygenation of the otherwise anaerobic sediment. It has often been assumed in the literature that oxic conditions prevail in seagrass-inhabited sediments due to the radial oxygen loss from the roots. However, it was recently shown that the zone of extensive radial oxygen loss is limited to small areas at the tip of new root segments while other parts of the root system do not leak oxygen (Connell *et al.*, 1999; Jensen *et al.*, 2005; Frederiksen & Glud, 2006), and this was also true for the plants in this study. Some differences to the extent of the oxygenated zone, especially at the densely inhabited site, were, however, apparent. The differences may be due to the potential effects of the radial oxygen loss from the roots of other plants. Sulfide and organic acids have been shown to induce a barrier to radial oxygen loss in other plants (Armstrong & Armstrong, 2001, 2005), and an extensive oxidation of the rhizosphere could diminish the effects of such products. Further studies are needed to confirm these observations, but in general it can be concluded that anaerobic rather than oxic conditions prevail in *Z. marina*-inhabited sediments, which may have profound influence on rhizosphere bacterial community structure and activity.

The most dominant TRF (TRF 130) found in the bulk sediment affiliated with sulfate-reducing *Deltaproteobacteria*, which is consistent with the results of Cifuentes *et al.* (2000), who found a dominance of sulfate-reducing *Deltaproteobacteria* in their clone library from *Zostera noltii*-inhabited sediments. Mutualistic or symbiotic associations between seagrasses and sulfate-reducing bacteria have previously been suggested (e.g. Welsh, 2000; Smith *et al.*, 2004), yet our results based on 16S rRNA gene (this study) and amplification of *dsrA* (S.I. Jensen *et al.*, unpublished data)

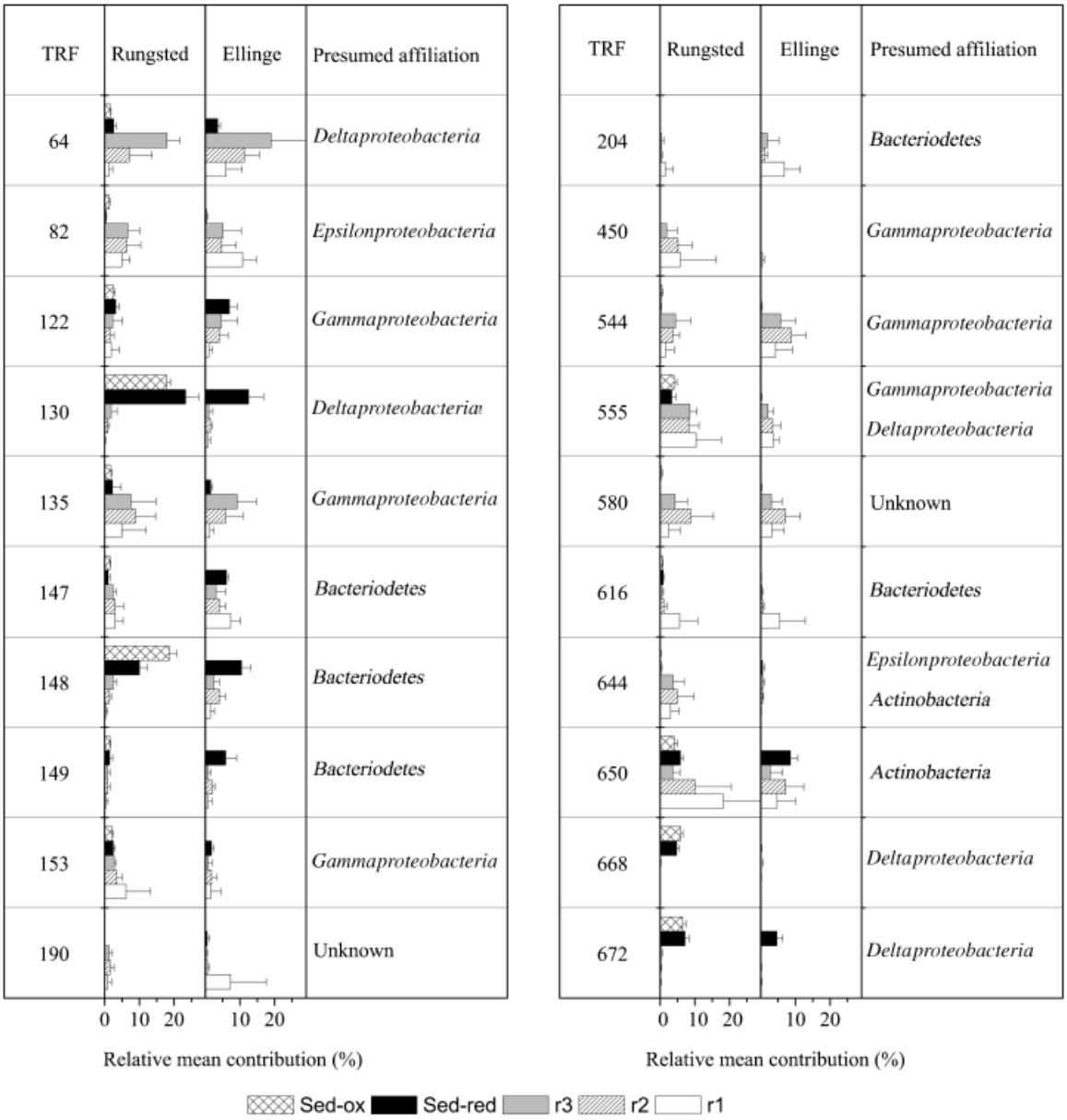

Fig. 4. The relative distribution of TRFs displaying a relative mean contribution of > 5% in one of the selected groupings in either of the two sites. r1–r3 indicates root-bundles 1–3 (r1 being the youngest), whereas Sed-ox and Sed-red indicates the oxidized sediment horizon (1–2 cm surface sediment) and reduced sediment horizon (5 cm below the oxidized horizon), respectively. Note that the oxidized sediment horizon was not investigated at Ellinge.

do not indicate such a relationship for *Z. marina*. We do not argue against the fact that sulfate-reducing bacteria are of major importance in the overall biogeochemistry of seagrass-inhabited sediments, but we speculate that their role regarding the plants may be indirect instead of mutualistic in terms of *Z. marina*.

TRF 64, assigned to *Deltaproteobacteria* with 86% similarity to the iron- and sulfur-reducing *Deltaproteobacterium* 103 (AY835391), did seem to be important root colonizers, but their late colonization indicates that they may be involved in the root decomposition rather than forming a mutualistic symbiotic relationship with the plant. In this regard, it should be noticed that most biogeochemical studies in seagrass-inhabited sediments have been conducted using sediment cores or roots separated from the plants. This study shows that there is a clear difference between the root-associated bacterial community and the bacterial community in the bulk sediment, and this complicates the interpretation of results from core analysis.

Epsilonproteobacteria, *Gammaproteobacteria* and *Actinobacteria* appeared to be important root colonizers. *Actinobacteria* have previously been associated with terrestrial

plants as endophytic diazotrophs, potential antagonists toward phytopathogens and as phytopathogens (e.g. Callaham *et al.*, 1978; Goodfellow & Williams, 1983; Loria *et al.*, 2006). However, *Actinobacteria* have only recently been considered important in the marine environment (Ward & Bora, 2006), and their potential role for *Z. marina* can thus only be speculated on.

A prevalence of sequences affiliating with *Epsilonproteobacteria* in the clone library was found from the roots, and based on the T-RFLP profiles they contributed significantly to the overall bacterial community associated with the roots; however, they appeared to be less-important bulk sediment colonizers. The closest cultured bacteria, to many of the *Epsilonproteobacterial* sequences, *Arcobacter nitrofrigilis,* was isolated from the salt marsh plant *S. alterniflora*, and is as Candidatus *Arcobacter sulfidicus* capable of nitrogen fixation (McClung *et al.*, 1983; Wirsen *et al.*, 2002). Many *Epsilonproteobacteria* as well as *Gammaproteobacteria* oxidize sulfide with oxygen and nitrate as electron acceptors (Campbell *et al.*, 2006) and a recent study has shown that nitrate-reducing sulfide-oxidizing bacteria are present in the rhizoplane of *Halodule wrightii* roots (Küsel *et al.*, 2006).

Sulfide intrusion into seagrass tissue has been coupled to recent die-back events (Koch & Erskine, 2001; Pedersen *et al.*, 2004; Borum *et al.*, 2005; Holmer *et al.*, 2006), and although chemical oxidation of sulfide occurs spontaneously in the presence of oxygen, microbially mediated sulfide oxidation is a much faster process (Jørgensen & Revsbech, 1983). It is therefore intriguing to suggest a potential symbiotic relationship between the obtained *Epsilonproteobacterial* and/or *Gammaproteobacterial* sequences and *Z. marina*. However, as these bacteria remain uncultured, their potential physiology at this stage can only be speculated on.

In conclusion, this study shows that the bacterial community associated with the roots of *Z. marina* differs from the bacterial community in the bulk sediment. Sulfate-reducing *Deltaproteobacteria* are predominant in the bulk sediment, whereas *Epsilonproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* appear to be successful root colonizers. This study points to potential physiological interactions between these bacteria and *Z. marina*, which need further investigation. Along with further detailed analysis of the rhizosphere microenvironment, the use of methods such as FISH, MAR-FISH and focused culturing approaches could help reveal the exact role of seagrass root colonizers and the importance of bacteria–seagrass interactions.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Sequence details.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574.6941.2007.00373.x (This link will take you to the article abstract.)

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