Chapter 3

Linking microbial community structure and function in marine intertidal sediment by Mag-SIP

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Abstract

Substrate utilization by major microbial groups was investigated by using stable isotope probing combined with magnetic bead capture hybridization (Mag-SIP) in marine intertidal sediment. Sediment cores were incubated with different $^{13}$C-labeled substrates, and sectioned into surface (0-2 cm) and deeper (2-5 cm) layers. The surface layer contained the aerobic zone and the top of the anaerobic zone, whereas the deeper layer represented the sulfate-reduction zone. Specific 16S rRNA was isolated with a nested set of five probes targeting about 80% of the active community, and analyzed for $^{13}$C-labeling. We also determined total and active microbial community structure by constructing clone libraries for the 16S rRNA genes and the 16S rRNA. The sequences of 16S rRNA genes and rRNA were partially overlapped, and were dominated by Deltaproteobacteria (only in the deeper layer), Gammaproteobacteria, Cyanobacteria, and chloroplast sequences. In the deeper layer, Gammaproteobacteria dominated glucose consumption, and sulfate-reducing Deltaproteobacteria, specifically Desulfobacteraceae, were relatively important in acetate, propionate, and amino acid degradation. Surprisingly, Cyanobacteria and chloroplast sequences explained a substantial part of the rRNA library in the deeper layer, and our results indicate that these phototrophs may survive by glucose utilization under dark, anaerobic conditions. In the surface layer, all substrates were more evenly utilized by all major groups, indicating limited specialization at the phylogenetic level of this study. There were therefore strong contrasts in community characteristics and substrate utilization patterns between the two depth horizons, which are discussed in terms of general community functioning and the known characteristics of the microbial groups.
Coastal sediments are generally characterized by high inputs of organic matter, and play a prominent role in the marine carbon cycle (Middelburg and Soetaert, 2005). Most of the organic matter is rapidly mineralized by a diverse microbial community along a vertical sequence of electron acceptors (Braker et al., 2001; Edlund et al., 2008). Oxygen is generally found only in the top few millimeters of the sediment. Here, aerobic respiration is the dominant pathway and is primarily carried out by a diverse heterotrophic microbial community. Sulfate reduction is the main anaerobic pathway for organic carbon mineralization in coastal sediments (Canfield et al., 1993; Jørgensen, 1982). Under anaerobic conditions, a consortium of microbes is thought to be involved in organic matter degradation, with fermenting bacteria producing intermediates such as propionate and acetate which are degraded to carbon dioxide by sulfate-reducing bacteria. Sulfate reducers are specialized with respect to the utilization of these fermentation products (Widdel and Hansen, 1992). In addition, several sulfate reducers can only carry out an incomplete oxidation of these substrates, whereas others such as many Desulfobacteraceae are capable of complete oxidation to carbon dioxide (Canfield et al., 2005).

Microbial communities and their activities have been widely studied in marine sediments. The development of cultivation-independent molecular methods since the late 1980s has led to an explosion of papers on microbial diversity and community structure (Bowman and McCuaig, 2003; Kemp and Aller, 2004; Ravenschlag et al., 2001). One of the more recent developments in the study of microbial community structure and function is a combination of $^{13}$C-labeling and tracing isotope levels in biomarkers generally referred to as stable isotope probing (SIP) (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2000). Traditional SIP methods have been used in marine sediments (Boschker et al., 1998; Webster et al., 2006). Other developments are in novel single-cell methods, such as fluorescence in situ hybridization (FISH) coupled with micro-autoradiography (Sintes and Herndl, 2006) or with Raman microscopy (Huang et al., 2007) and nano-scale secondary-ion mass spectrometry (nanoSIMS) (Musat et al., 2008). However, each of these SIP methods has limitations, such as low phylogenetic resolution or a requirement for high levels of isotope incorporation. FISH-based methods also require relatively high cellular rRNA content and accessibility of probe target sites (Fuchs et al., 1998). Recently, we further developed a method that is based on stable isotope probing combined with magnetic bead capture hybridization of rRNA (Mag-SIP) by lowering the amount of rRNA needed and thereby making it applicable to major phylogenetic groups within environmental samples (MacGregor et al., 2002; Miyatake et al., 2009). Advantages of Mag-SIP are a combination of high sensitivity for $^{13}$C-incorporation in target rRNA and high phylogenetic resolution.

In the present study, we applied the Mag-SIP protocol to study substrate utilization patterns by major groups in the microbial community of an intertidal sediment. Sediment
cores were incubated with different $^{13}$C-labeled substrates, and were sectioned into surface (0-2 cm) and deeper (2-5 cm) layers. The aerobic/anaerobic boundary is mostly situated a few mm from the sediment surface in coastal sediments (Brune et al., 2000), so the surface layer is expected to contain both the aerobic and the top of the anaerobic zone, whereas the deeper layer represents the sulfate reduction zone (Oenema, 1990). 16S rRNA of major groups was captured with specific oligonucleotide probes and $^{13}$C-labeling levels were determined by micro elemental analyzer-isotope ratio mass spectrometry (µEA-IRMS) (Miyatake et al., 2009). We also constructed clone libraries for the 16S rRNA genes and for the reversed-transcribed 16S rRNA as an alternative method to identify major active populations in both layers (Martinez et al., 2006; Mills et al., 2005). The results show different community characteristics and substrate utilization patterns between the surface and deeper layers, and are discussed in terms of general community functioning and the known characteristics of the detected microbial groups.

**Materials and methods**

**Sampling and $^{13}$C labeling**

Sediment samples were collected at an intertidal flat in the Rattekaai area (51°26.341′N, 4°10.040′E) of the Oosterschelde bay (The Netherlands) in May 2008. The sampling location was characterized by extensive intertidal sand flats. Undisturbed sediment cores (internal diameter 5.2 cm) were sampled at low tide and immediately transported back to the laboratory. Approximately equimolar $^{13}$C amounts of uniformly labeled [D-$^{13}$C]glucose, [D-$^{13}$C]sodium propionate or [D-$^{13}$C]sodium acetate (50, 100 and 150 mM, respectively; 99% $^{13}$C; Cambridge Isotope Laboratories, Andover, MA, USA) were injected with the line injection method (19 injections of 17 µl each) into the top 5 cm of the sediment (Jørgensen, 1978). A [D-$^{13}$C]-labeled algal-derived amino acid mixture (13 mM; 98% $^{13}$C; Cambridge Isotope Laboratories) was also injected with the same method. Four cores for each substrate were incubated for 24 h in the dark at in-situ temperature (14°C), followed by sectioning in 0-2 cm (surface layer) and 2-5 cm (deeper layer) from the surface. From each core, twenty to twenty-five grams wet weight of each section were stored at -80°C. Unlabeled control cores (no labeled substrates added) were also processed.

**Nucleic acid extraction from the sediment**

Total community RNA was extracted using the phenol-chloroform method (pH 5.1) as described in Miyatake et al. (2009). For every treatment, RNA extracted from frozen sediment samples of two cores were combined as a total community RNA sample for subsequent magnetic bead capture hybridization. All treatments were processed in duplicate. Total community RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In order to estimate the amount of 16S rRNA
among total community RNA, RNA extracts were ran on a 5% polyacrylamide gel electrophoresis and were visualized by staining with ethidium bromide. DNA was extracted using the same method except that the extraction buffer and phenol had a pH of 7.0.

**Phylogenetic analysis of total community RNA, captured 16S rRNA, and total community DNA**

In order to identify major microbial groups in the community, we constructed both 16S rRNA genes and rRNA clone libraries from the two sediment horizons. In addition, clone libraries were constructed from 16S rRNA captured with each probe in order to check the specificity of the capture protocol (see below). Aliquots of total community (approximately 100 ng) and captured RNA (25 to 50 ng) were reverse transcribed with reverse primer DXR518 (5′-CGTATTACCGGGCTGCTGG-3′) (Nogales et al., 1999) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reverse transcripts were amplified with 10 cycles of PCR using primers 27F-DXR518 (Martinez et al., 2006; Mills et al., 2005). DNA extracted from the sediment was amplified directly using the same primers. PCR products were ligated into pGEM T-easy vector, and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). Positive clones were re-amplified with M13 primers, and sequenced with the 27F primer on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms were manually checked using the Chromas Lite software (http://www.technelysium.com.au/chromas_lite.html). Phylogenetic analysis was performed by using the fast aligner and treeing tools implemented in the ARB program package (Ludwig et al., 2004). Phylogenetic relationships were determined by inserting sequences from this study into an ARB tree composed of the Greengenes database (http://greengenes.lbl.gov/) to which additional *Deltaproteobacteria* clone sequences from marine environments had been added. Phylootypes (≥ 97% similarity in sequence) were defined and the final bootstrapped neighbor-joining tree with 1000 samplings was created in MEGA4 (Tamura et al., 2007) using the Jukes-Cantor model.

**Oligonucleotide probes**

Based on the rRNA clone library, biotin-labeled oligonucleotide probes targeting major groups and unlabeled helper and competitor probes (Table 3.1) were selected and purchased from Ocimum Biosolutions (IJsselstein, The Netherlands). The nested set of probes targeted approximately 80% of the different rRNA sequences recovered from the two sediment layers. The testing of the probes for total bacterial rRNA (EUB338), *Deltaproteobacteria* (DELTA495a), and *Desulfobacteraceae* (Dbact653) is described in Miyatake et al. (2009). The DELTA495a probe also targets most *Gemmatisimonadetes* (Lücker et al., 2007). It was used in combination with a competitor probe (cDELTA495a) to avoid capture of *Gammaproteobacteria*, which have only a single mismatch in the probe target region. In order to increase the yield, unlabeled 21-mer helper probes (Fuchs et al., 2000; MacGregor
et al., 2002) complementary to the consensus sequences upstream and downstream of the Dbact653 probe target sites were also used. Two probes targeting Cyanobacteria/chloroplast (CYA361 and CYA762) (Schönhuber et al., 1999) were tested with the Mag-SIP protocol. CYA361 was selected as it gave higher yields with sufficient coverage of clones in the rRNA library. Gammaproteobacteria are a generally abundant in marine sediments (Bowman and McCuaig, 2003; Ravenschlag et al., 2001). However, the commonly used probe for Gammaproteobacteria (GAM42a) (Manz et al., 1992) could not be used directly without further adaptation and testing of the protocol as it targets the 23S rRNA. Using the ARB software, we therefore designed another specific probe (BG553) and matching helper probes that target the 16S rRNA of most Beta/Gammaproteobacteria (Table 3.1). In this study, this probe is basically Gammaproteobacteria specific as we did not detect any Betaproteobacteria-related sequences in any of the clone libraries, including the rRNA library derived from the rRNA captured with this probe. Betaproteobacteria are typically a minor component of the microbial community in marine sediments (Bowman and McCuaig, 2003; Hunter et al., 2006). Optimal formamide concentrations (Table 3.1) in terms of capture efficiency versus specificity were determined as previously described (Miyatake et al., 2009) and gave more than 90% of target specificity as checked with clone libraries from captured 16S rRNA.
Table 3.1. 16S rRNA-targeted probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>% FA&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>25</td>
<td>Most Bacteria</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>DELTA495a</td>
<td>AGT TAG CCG GTG CTT CCT</td>
<td>45</td>
<td>Most Deltaproteobacteria, Most Gemmatimonadetes</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td>cDELTA495a</td>
<td>AGT TAG CCG GTG CTT TT</td>
<td>45</td>
<td>Competitor of DELTA495a</td>
<td>(Macalady et al., 2006)</td>
</tr>
<tr>
<td>Dbact653</td>
<td>TTC CCT CTC CCA TAC TCA</td>
<td>25</td>
<td>Most Desulfobacteraceae</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_up_help</td>
<td>CCC CGG AAG TGC AYT TGA WAC</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>Dbact653_down_help</td>
<td>GTG GAA TTC CTG GTG TAG AGG</td>
<td>25</td>
<td>Helper probe for Dbact653</td>
<td>(Miyatake et al., 2009)</td>
</tr>
<tr>
<td>CYA361</td>
<td>CCC ATT GCG GAA AAT TCC</td>
<td>20</td>
<td>Most Cyanobacteria and many chloroplast</td>
<td>(Schönhuber et al., 1999)</td>
</tr>
<tr>
<td>BG553</td>
<td>CGC CCA GTA ATT CCG ATT</td>
<td>60</td>
<td>Most Betaproteobacteria, Most Gammaproteobacteria</td>
<td>This study</td>
</tr>
<tr>
<td>BG553_up_help</td>
<td>AAC CGC CTR CGN RCG CTT TA</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>This study</td>
</tr>
<tr>
<td>BG553_down_help</td>
<td>AAC GCT YGC ACC CTM CTG ATT</td>
<td>60</td>
<td>Helper probe for BG553</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>1</sup>Percent formamide (FA) in hybridization buffer for hybridizations at 20°C

Hybridization and magnetic bead capturing

Ribosomal RNA from specific groups of microorganisms was isolated with the Mag-SIP protocol as described in Miyatake et al. (2009). In brief, 20–40 µg total RNA was hybridized with a specific biotin-labeled probe and the 16S rRNA-probe hybrids were captured with streptavidin coated hydrophobic paramagnetic beads (Dynabeads MyOne Streptavidin T1; Invitrogen). Captured 16S rRNA was released from the beads, and approximately 500 ng of the captured material used for stable isotope analysis. Small aliquots of captured material were used for clone library construction.
\textit{\textsuperscript{13}C analysis of captured 16S rRNA}

Isotope ratio analysis of captured 16S rRNA was performed by \textmu EA-IRMS consisting of a wet oxidation interface (LC IsoLink; Thermo Fisher Scientific, Bremen, Germany) coupled on-line to an isotope ratio mass spectrometer (DELTA V Advantage; Thermo Fisher Scientific) (Krummen et al., 2004) as described in Miyatake et al. (2009). Samples (50 \(\mu\)l) were directly injected into this \textmu EA-IRMS operating in bulk injection mode. Standard curves ranging from 0-1000 ng of carbon were made with phthalic acid. Stable carbon isotope ratios were expressed as \(\delta^{13}\text{C}\) values calibrated against the international standard Vienna Pee Dee Belemnite (VPDB). The delta-notation is defined as:

\[
\delta^{13}\text{C}_{\text{sample}} (\text{‰}) = \left[ \left( \frac{R_s}{R_{st}} \right) - 1 \right] \times 1000
\]

(1)

, where \(R_s\) is the ratio of \textsuperscript{13}\text{C}/\textsuperscript{12}\text{C} in the sample and \(R_{st}\) is the ratio of the international standard VPDB (0.0111797). The measured \(\delta^{13}\text{C}\) values were corrected for the protocol blank:

\[
\delta^{13}\text{C}_{\text{RNA}} (\text{‰}) = \left[ \left( \frac{\delta^{13}\text{C}_{\text{sample}} \times C_{\text{sample}}}{C_{\text{sample}} - C_{\text{blank}}} \right) \left( \frac{\delta^{13}\text{C}_{\text{blank}} \times C_{\text{blank}}}{C_{\text{blank}}} \right) \right]
\]

(2)

, where \(\delta^{13}\text{C}_{\text{sample}}\) is the \(\delta^{13}\text{C}\) value of the sample, \(C_{\text{sample}}\) is the amount of carbon in the sample, \(\delta^{13}\text{C}_{\text{blank}}\) is the \(\delta^{13}\text{C}\) value of the blank, and \(C_{\text{blank}}\) is the amount of carbon in the blank (Boschker, 2004).

\textit{Nucleotide sequence accession numbers}

Nucleotide sequences obtained from total community RNA, DNA, and probe-captured 16S rRNA have been deposited in the Genbank/DDBJ/EMBL database under accession numbers GQ449821 to GQ450274.
Results

Comparison of 16S rRNA gene and rRNA clone libraries

Clone libraries were constructed from both 16S rRNA genes and rRNA for the sediment surface (0-2 cm) and deeper (2-5 cm) layers. Between 80 and 100 clones were obtained for each of the four clone libraries. Figure 3.1 shows phylogenetic relationships of 16S rRNA genes and rRNA clone sequences from both layers. Clones with 97% sequence similarity were considered to represent similar phylotypes, so only representative clones are shown. Approximately 35 µg of DNA and 15 µg of RNA per 1 g (dry weight) sediment were extracted from the surface layer, and somewhat lower amounts from the deeper layer (approximately 15 µg g⁻¹ of DNA and 10 µg g⁻¹ of RNA).

The clones obtained were often most closely related to environmental clones derived from other marine sediment studies (Fig. 3.1). Diversity, in terms of the number of clones detected at the ≥ 97% level, appeared to be approximately similar between the surface layer (53 phylotypes, both 16S rRNA genes and rRNA clones) and the deeper layer (46 phylotypes), within the limitations of the study. The rRNA libraries were somewhat less diverse than the 16S rRNA gene libraries, with 35 and 37 16S rRNA gene phylotypes, and 27 and 23 rRNA phylotypes for the surface and the deeper layer, respectively. The 16S rRNA gene libraries were rather different between the surface and the deeper layers, suggesting a major difference in community structure (Fig. 3.1), although some 16S rRNA gene phylotypes were found in both layers. The rRNA phylotypes were dominated by several abundant phylotypes in the Deltaproteobacteria (only in the deeper layer), Gammaproteobacteria, Planctomycetes, Cyanobacteria and chloroplast. Many but not all 16S rRNA gene phylotypes were matched by rRNA phylotypes recovered from the same layer. Most clones affiliated with Cyanobacteria and chloroplast were detected in both layers, and were mainly found in a limited number of highly abundant phylotypes. The chloroplast clones were mostly related to diatoms. For the rRNA libraries, there was a clear difference in Deltaproteobacteria phylotypes between the two layers. Desulfobacteraceae clones were recovered much more frequently from the deeper layer, and there was a shift from Desulfuromusa- to Desulfocapsa-related clones between the surface and the deeper layer. The lower number of rRNA clones from Desulfobacteraceae in the surface layer was mainly due to the absence of Desulfococcus- and Desulfosarcina-related clones, while sequences related to clone OS02-TRNA-76 were more abundant both in the rRNA library (Fig. 3.1) and in probe-captured rRNA, where they accounted for about 80% of the clones in the surface layer (data not shown). The rRNA phylotypes belonging to the Gammaproteobacteria were rather diverse in comparison with other groups, and many of the dominant phylotypes were found in both layers. They were most closely related to environmental clones, although some were distantly related to Thiomicrospira.
Figure 3.1. (Continue to the next page) Neighbor-joining tree showing the affiliation of clones obtained from 16S rRNA genes and 16S rRNA to closely related sequences. Clones designated OS2 are derived from DNA and RNA in the surface layer, and clones designated OS25 are from the deeper layer. Clones with designations containing DNA and TRNA are derived from DNA and total community RNA, respectively. Numbers and letters in parenthesis indicate number of clones in a phylotype (≥ 97% similarity in sequence) and type of sequences (D for DNA-derived, R for RNA-derived). The RNA clones from the 2-5 cm layer are derived from Miyatake et al. (2009). Bootstrap values represent 1000 replicates and only values greater than 50% are reported. The scale bar indicates 10% estimated phylogenetic divergence.
Figure 3.1. Cont.
In order to get a comprehensive overview, the distribution of clones belonging to major microbial groups in the 16S rRNA gene and rRNA libraries in both layers was compared (Fig. 3.2). There were obvious differences in community structure between the surface and the deeper layer. *Desulfobacteraceae* clones were found in similar proportions (6%) in 16S rRNA gene libraries of both layers, but were more abundant in the rRNA library of the deeper layer (19%) than of the surface layer (2%). *Deltaproteobacteria* not belonging to the *Desulfobacteraceae* were more abundant in the deeper layer (7%) than in the surface layer (4%) in the 16S rRNA gene libraries, whereas the opposite was true for the rRNA libraries (deeper layer 3%, surface layer 7%). *Gammaproteobacteria* clones were found in almost the same numbers in both 16S rRNA gene and rRNA library of both layers. *Cyanobacteria* and chloroplast had more or less the same proportion in the 16S rRNA gene library of both layers (5-9%), but were generally much more abundant in the rRNA libraries of both layers (19 and 30% for *Cyanobacteria*, 14% for chloroplast of the deeper layer). Clones belonging to the *Bacteroidetes*, *Alphaproteobacteria* and others were hardly detected in the rRNA libraries even though they were sometimes abundant in the 16S rRNA gene libraries. *Planctomycetes* were not covered by our Mag-SIP probes, and were more abundant in the rRNA than 16S rRNA gene libraries of both layers. Finally, the proportion of 16S rRNA gene phylotypes that were also found in the rRNA library is also indicated in Figure 3.2, and was in total lower in the surface layer than in the deeper layer (29 and 39%, respectively). Interestingly, all *Cyanobacteria* and chloroplast 16S rRNA gene phylotypes in both layers and all 16S rRNA gene phylotypes from the *Desulfobacteraceae* in the deeper layer were found in rRNA library, suggesting that the majority of the members belonging to these groups were viable and growing.

The specific probes used in this study targeted *Deltaproteobacteria*, *Desulfobacteraceae*, *Gammaproteobacteria*, *Cyanobacteria* and chloroplast, and the clones from these groups accounted for approximately 80% of the rRNA sequences in both sediment layers. We also produced rRNA libraries from the rRNA isolated with the different probes in the Mag-SIP protocol. Specificity of all the probes was better than 90%. There was in general good agreement between the phylogenies of the captured rRNA and total rRNA clone libraries. Sufficient rRNA for isotope analysis could be isolated with all probes from both layers. *Desulfobacteraceae* explained only 1-2% of the rRNA library from the surface layer (Fig. 3.2), showing that also relatively minor populations can be targeted by the Mag-SIP protocol.
Figure 3.2. Proportion of clones affiliated with major groups among each library derived from 16S rRNA genes or 16S rRNA in either the surface layer (0-2 cm) or the deeper layer (2-5 cm). Total numbers of clones sequenced are indicated as n. Shaded part of the bars in 16S rRNA gene libraries indicates proportion of clones which were also found in the rRNA library (at the level of ≥ 97% sequence similarity) in each layer.

Substrate incorporation by major groups within the microbial community

There were clear contrasts in $^{13}$C-label incorporation of rRNA captured by the different probes between the substrates and sediment layers (Fig 3.3). For the surface layer of the sediment, differences in labeling levels among the major microbial groups were relatively small (within a factor of 2) and often rather similar to total bacterial rRNA labeling. Labeling levels detected in Gammaproteobacteria were higher than total bacterial rRNA with both $^{13}$C-glucose and -propionate, and the same was true for Deltaproteobacteria and Desulfobacteraceae for $^{13}$C-propionate. Label incorporation in Cyanobacteria/chloroplast was detected with all substrates in the surface layer, but to a somewhat lower degree than the other groups. Labeling levels for other probe-substrate combinations were similar to each other.

Differences in label incorporation between substrates and microbial groups were much more pronounced in the deeper layer representing the anaerobic sulfate-reducing zone of the sediment. For glucose-labeled sediment, Gammaproteobacteria clearly showed higher incorporation than the other groups, but they were relatively less important for the other substrates. Desulfobacteraceae were in general more highly labeled with propionate, acetate, and amino acid than the other groups. Labeling in Cyanobacteria/chloroplast was
detected only with glucose and to a much lesser extent with propionate, suggesting that these phototrophic organisms were able to utilize glucose under anaerobic conditions in the dark. Overall, although some similarities in label distribution were therefore found between the surface and the deeper layer, the differences in labeling between phylogenetic groups and substrates were much more pronounced in the deeper layer.

Figure 3.3. The increase in δ13C ratios between labeled sediments and unlabeled control sediment (Δδ13C) for the different captured-16S rRNA fractions. The sediment were incubated with [13C]glucose, [13C]propionate, [13C]acetate, or [13C]amino acid. Data for 13C-amino acid labeled sediment were normalized to the amount of 13C as added with 13C-acetate. Some of the results from the deeper layer are derived from Miyatake et al. (2009); the results with probes BG553 and CYA361 are new as are all results from the 13C-amino acid labeling experiment. Unlabeled controls had δ13C values between -15 and -20 ‰, within the typical range for marine phytobenthos and Bacteria (Boschker and Middelburg, 2002; Coffin et al., 1990) and differences in δ13C value between duplicate analysis of unlabeled controls was less than 2 ‰.
Discussion

We used two complementary approaches to study active microbial communities in intertidal marine sediment. First, we compared 16S rRNA gene- and rRNA-derived clone libraries to indicate potential active groups. Second, we used Mag-SIP to study $^{13}$C labeling of major phylogenetic groups within the microbial community in order to determine substrate utilization patterns. Despite some prominent exceptions (Fegatella et al., 1998; Flär dh et al., 1992), rRNA content is generally related to growth rate and is highest in growing cells (Kerkhof and Kemp, 1999; Moeseneder et al., 2005). Clone libraries made from rRNA are therefore generally considered to emphasize the metabolically active populations, whereas 16S rRNA gene libraries are more representative of the numerically abundant population (Duineveld et al., 2001; Gentile et al., 2006; Mills et al., 2005). In our study, the distribution of clones derived from DNA and RNA only partially overlapped, and this is in agreement with previous studies (Mengoni et al., 2005; Nogales et al., 2001).

There were clear contrasts in microbial community structure (at the level of $\geq 97\%$ sequence similarity) and label distribution of rRNA captured by the different probes between the surface (0-2 cm) and the deeper (2-5 cm) layers. The surface layer contained the aerobic zone and the top of the anaerobic zone, whereas the deeper layer represented the dissimilatory sulfate reduction-dominated zone (Oenema, 1990). The Mag-SIP study revealed clear differences in substrate use between the different major phylogenetic groups. In the deeper layer, glucose was primarily incorporated by Gammaproteobacteria, whereas Deltaproteobacteria, more specifically Desulfobacteraceae, were involved in acetate and propionate consumption (Fig. 3.3). Under anaerobic sulfate-reducing conditions, a consortium of microbes is thought to be involved in organic matter degradation, with fermenting bacteria producing intermediates such as propionate and acetate that are degraded to carbon dioxide by sulfate-reducing bacteria (Canfield et al., 2005). Our results are in agreement with this model of anaerobic mineralization and suggest that, among the heterotrophic bacteria, the major active phylogenetic groups are also the main functional groups in sulfate-reducing marine sediment.

In contrast, differences in labeling between groups and substrates were much smaller in the surface layer of the sediment, indicating limited specialization in substrate range between groups. The surface layer also contained the top of the anaerobic sediment and it may well be that the small differences in labeling detected were actually due to incorporation by Bacteria in the anaerobic part of the surface layer. It could be that the variety of electron acceptors (e.g. oxygen, nitrate, metal oxides and sulfate) available to the microbial community in the surface layer allowed them to use a wider range of organic substrates. It is also possible that the dominant subclades within the main phyla, as detected in this study and generally found in environmental studies (Bowman and McCuaig, 2003; Edlund et al., 2008), would show differences in substrate range, but our results suggest that
the diverse microbial community found in the surface of marine sediments is functionally redundant in terms of carbon utilization. 

Gammaproteobacterial sequences were the most abundant in the clone libraries (Fig. 3.2) and were responsible for much of the glucose incorporation, especially in the deeper layer (Fig. 3.3). Their labeling with glucose in ∆δ¹³C was approximately four times higher in the deeper layer than in the surface layer (Fig. 3.3), which was unexpected as similar amounts of label were added. This is most likely due to less dilution of ¹³C label into a lower bacterial biomass of the deeper sediment layer, or decreased competition for glucose with other members of the microbial community. Although Gammaproteobacteria such as Escherichia coli and some Shewanella species are known for anaerobic growth by fermentation of glucose (Bowman et al., 1997; Ivanova et al., 2001; Lovley, 1991), their sequences are only distantly related to Gammaproteobacteria clones from this study, with approximately 90% sequence similarity. Approximately one-third of the Gammaproteobacteria clones in this study are most closely related to sulfur-oxidizing symbionts and their free-living relatives, which are thought to be chemoautotrophs involved in the oxidative part of the sulfur cycle (Distel et al., 1994; Nercessian et al., 2005). It is however difficult to draw conclusions on which of the diverse Gammaproteobacteria detected were the dominant glucose users.

Deltaproteobacteria were a major group, as expected for marine sediment where sulfate reduction is important, and a clear difference in the active subclades of the community was detected between the sediment layers studied (Fig. 3.1). In the deeper layer, Desulfobacteraceae clones related to Desulfosarcina and Desulfococcus were abundant. This Desulfosarcina-Desulfococcus group is ubiquitous and sometimes predominates microbial communities in anaerobic coastal sediments (Edgcomb et al., 1999; Ravenschlag et al., 2000; Sahm et al., 1999). The Desulfosarcina-Desulfococcus group are generally complete oxidizing members of the sulfate-reducing bacteria that are able to use a wide range of substrates, including acetate and propionate, but typically do not utilize carbohydrates (Canfield et al., 2005; Widdel and Hansen, 1992). Our labeling results for the deeper layer are generally in agreement with this physiology, as Desulfobacteraceae played major role in acetate and propionate (and amino acid) utilization. Webster et al. (2006) applied traditional DNA-SIP to study the use of ¹³C-glucose and acetate in anaerobic intertidal sediment and their results partially agree with our study, as Desulfobacteraceae were indicated as major consumers of acetate. However, they also found that glucose was used by the majority of the microbial community, whereas we found clear dominance of Gammaproteobacteria. This may be due to differences in methods applied such as lower substrate concentrations and shorter incubation time used in this study. For Desulfobacteraceae, the relatively minor labeling with glucose may suggest some direct incorporation but is also in agreement with the use of labeled fermentation products produced by for instance Gammaproteobacteria, which were dominant glucose utilizers (Fig. 3.3). In contrast, the Mag-SIP results from the surface sediment layer suggest that Deltaproteobacteria,
including *Desulfo bacteraceae*, were able to use glucose as well as all other substrates (Fig. 3.3). The rRNA clone library from the surface layer was dominated by a cluster related to *Desulfuromusa* in the other *Deltaproteobacteria* and a cluster only related environmental clones in the *Desulfo bacteraceae* (Fig. 3.1). The genus *Desulfuromusa* and its relatives includes sulfur- and metal oxide-reducing bacteria as well as fermentative organisms capable of utilizing glucose (Liesack and Finster, 1994). The dominant cluster in the *Desulfo bacteraceae* detected in the surface layer is commonly found in marine sediments, and is closely related to subclade IB as defined by Klepac-Ceraj *et al.* (Klepac-Ceraj *et al.*, 2004) and to the sulfate-reducing endosymbionts found in *Olavius* species (Dubilier *et al.*, 2001). They are generally treated as sulfate reducers, and our results suggest that they may have a wide substrate range and may even be able to use other electron acceptors beside sulfate as generally found in the top layer of marine sediments.

Interestingly, *Cyanobacteria* and chloroplast clones - expected in the surface layer - were also highly abundant in the deeper dark anaerobic layer. All *Cyanobacteria* clones were affiliated with *Oscillatoriales* and the chloroplast clones with diatoms. Moreover, all the *Cyanobacteria* 16S rRNA gene phylotypes and most chloroplast 16S rRNA gene phylotypes were also found in the rRNA library in both layers, implying that they were viable and growing even in the lower dark and anaerobic layer. An alternative explanation is that ribosomal content of these phototrophs decreases only slowly relative to protein synthesis rate even if they are starving or in stationary phase for prolonged periods of time, as has been found in some heterotrophic bacteria (Givskov *et al.*, 1994). However, *Cyanobacteria* and diatoms were capable of utilizing all the tested $^{13}$C-substrates in the surface layer and incorporated glucose in the deeper layer, which clearly shows that they were metabolically active in both layers. Active heterotrophic growth of *Cyanobacteria* and diatoms on glucose under aerobic conditions in the dark is well known (Lewin and Hellebust, 1976; Smith and Underwood, 2000). Our results suggest that they may also be able to survive for prolonged periods by utilizing glucose, probably via fermentation, under the anaerobic conditions found deep within marine sediments. Based on 16S rRNA libraries and on the incorporation of bromodeoxyuridine into DNA, viability and growth of *Cyanobacteria* in dark anaerobic conditions has also been observed in Baltic Sea sediment (Edlund *et al.*, 2008). Under anaerobic conditions, many *Cyanobacteria* are able to gain energy from fermentation of storage carbohydrates accumulated during photoautotrophic growth, but fermentation of external organic substrates in free-living *Cyanobacteria* is poorly documented (Stal and Moezelaar, 1997). Heterotrophic growth in these phototrophs is probably used to survive dark periods either during the night or during longer periods when they are mixed into the sediment by for instance bioturbation. Our results suggest that they may remain viable and active in the dark anaerobic conditions probably to be able to respond quickly when light becomes available. Many of the *Cyanobacteria* and diatoms in marine sediments may therefore be mixotrophs, suggesting that the functional distinction between phototrophic primary producers and heterotrophic bacteria is blurred.
Similar to other studies (Bowman and McCuaig, 2003; Edlund et al., 2008), several dominant 16S rRNA phylotypes were generally detected within the major phylogenetic groups targeted with the Mag-SIP protocol in this study. It would be interesting to further extend the Mag-SIP protocol to these dominant phylotypes. This would involve developing and testing new probe sets, for which the 23S rRNA may also offer interesting options (Fuchs et al., 2001). With a limited number of probe sets, it would be possible to cover the dominant rRNA clones. For instance, three probes would be necessary to cover most of the Desulfobacteraceae and two additional probes would cover most of the Deltaproteobacteria in the studied sediment (assuming that relevant probes can be designed). The Mag-SIP protocol is likely sensitive enough for this, as we showed that we were able to isolate 16S rRNA for $^{13}$C-analysis from groups accounting for 1-2% of the total 16S rRNA clones. Other possible developments lie in the combination of Mag-SIP with novel single-cell methods such as nanometer-scale secondary-ion mass spectrometry (NanoSIMS) (Behrens et al., 2008; Musat et al., 2008). The Mag-SIP approach therefore is a very valuable asset to study relationships between microbial community structure and functioning in a range of environments.

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