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ORIGINAL ARTICLE

Roseobacter clade bacteria are abundant in coastal sediments and encode a novel combination of sulfur oxidation genes

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Roseobacter **clade bacteria (RCB) are abundant in marine bacterioplankton worldwide and central to pelagic sulfur cycling. Very little is known about their abundance and function in marine sediments. We investigated the abundance, diversity and sulfur oxidation potential of RCB in surface sediments of two tidal flats. Here, RCB accounted for up to 9.6% of all cells and exceeded abundances commonly known for pelagic RCB by 1000-fold as revealed by fluorescence *in situ* hybridization (FISH). Phylogenetic analysis of 16S rRNA and sulfate thiohydrolase (SoxB) genes indicated diverse, possibly sulfur-oxidizing RCB related to sequences known from bacterioplankton and marine biofilms. To investigate the sulfur oxidation potential of RCB in sediments in more detail, we analyzed a metagenomic fragment from a RCB. This fragment encoded the reverse dissimilatory sulfite reductase (rDSR) pathway, which was not yet found in RCB, a novel type of sulfite dehydrogenase (SoeABC) and the Sox multi-enzyme complex including the SoxCD subunits. This was unexpected as *soxCD* and *dsr* genes were presumed to be mutually exclusive in sulfur-oxidizing prokaryotes. This unique gene arrangement would allow a metabolic flexibility beyond known sulfur-oxidizing pathways. We confirmed the presence of *dsrA* by geneFISH in closely related RCB from an enrichment culture. Our results show that RCB are an integral part of the microbial community in marine sediments, where they possibly oxidize inorganic and organic sulfur compounds in oxic and suboxic sediment layers.**

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Introduction

Marine *Roseobacter* clade bacteria (RCB) are one of the most abundant bakterioplanktonic groups in oceans worldwide and are particularly important in organic sulfur cycling (Gonzalez and Moran, 1997; Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006). RCB are free-living, but they are also often found in epibiotic biofilms on macro-algae and various invertebrates (Wagner-Döbler and Biebl, 2006). Moreover, RCB are the dominant primary colonizers of submerged surfaces (Dang and Lovell, 2002). Accordingly, their 16S rRNA gene sequences and isolates have been frequently recovered from biofilms of deep-sea and coastal sediments (Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006; Brinkhoff *et al.*, 2008; Sass *et al.*, 2010). However, little is known about RCB in marine sediments. In 16S rRNA gene libraries from marine sediments, RCB accounted for 2–15% of all clones (Buchan *et al.*, 2005). In the only quantitative study, RCB accounted for 3–11% of all 16S rRNA genes in bulk DNA from coastal sediments (Gonzalez *et al.*, 1999), but their cell abundance in sediments is unknown.

RCB are metabolically heterogeneous and are capable of, for example, anoxygenic phototrophy, aromatic and organosulfur degradation. In water columns, RCB and SAR11 are the main groups involved in the demethylation of dimethylsulfoniopropionate and in routing it to the food web or to other compounds such as the climatically active

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DMS (Gonzalez *et al.*, 1999; Howard *et al.*, 2006; Curson *et al.*, 2011). In addition, a few cultivated strains oxidize inorganic sulfur compounds such as sulfite, sulfide and thiosulfate (Sorokin, 1995; Gonzalez *et al.*, 1999; Sass *et al.*, 2010).

The detection and phylogeny of the *soxB* gene in the Sox multi-enzyme (SOX) pathway for thiosulfate oxidation in environmental polymerase chain reaction (PCR)-based libraries and genome sequences confirmed the involvement of RCB in sulfur oxidation (Meyer *et al.*, 2007; Moran *et al.*, 2007; Newton *et al.*, 2010). *Sox* genes occur in various phylogenetic clades, including many *Proteobacteria* and *Chlorobi* (Petri *et al.*, 2001; Meyer *et al.*, 2007). The majority of these organisms encode a complete SOX pathway, whereas only a few groups such as some phototrophic and chemotrophic bacteria lack the subunits sulfur dehydrogenase SoxCD (Meyer *et al.*, 2007; Frigaard and Dahl, 2009; Gregersen *et al.*, 2011). These SoxCD-lacking organisms oxidize thiosulfate or sulfide and transiently form zero-valence sulfur, which is further oxidized to sulfite via the rDSR pathway (Hensen *et al.*, 2006; Grimm *et al.*, 2008). In RCB such as *Roseobacter denitrificans*, the complete SOX pathway including SoxCD is present; however, the rDSR pathway has not been found yet (Moran *et al.*, 2007, www.roseobase.org).

In coastal sediments, large amounts of hydrogen sulfide are released during degradation of organic matter and re-oxidized to sulfate at the sediment surface (Jansen *et al.*, 2009; Kamyshny and Ferdelman, 2010). Generally, *Gamma-* and *Epsilonproteobacteria* are considered as dominant sulfur-oxidizing organisms at marine sediment surfaces (Sievert *et al.*, 2008; Lenk *et al.*, 2011). In previous studies, we recovered alphaproteobacterial DsrAB sequences, which indicated that also yet unknown *Alphaproteobacteria* may be important to sulfur oxidation in coastal water columns and sediments (Lavik *et al.*, 2009; Lenk *et al.*, 2011). In this study, we investigated whether alphaproteobacterial RCB, some of which are known to oxidize sulfur compounds in the water column, could also play a role in sulfur oxidation in coastal sediments. We first studied the environmental abundance and diversity of RCB by comparative analysis of 16S rRNA and *soxB* genes in tidal sediments from the German Wadden Sea. Using fluorescence *in situ* hybridization (FISH), we quantified RCB in different types of marine sediments. Furthermore, we linked the 16S rRNA genes of uncultured RCB with sulfur oxidation genes using metagenomics, single geneFISH (Moraru *et al.*, 2010) and enrichment cultures of sulfur-oxidizing RCB.

Materials and methods

Sampling

The sediment was sampled in two tidal sites located in the East and in the North Frisian German Wadden Sea. Sediment cores from the Janssand intertidal sand flat (53°44′07″N, 007°41′57″E) were taken during repeated sampling campaigns in April 2005, August 2007 and May 2009 for catalyzed reporter deposition-FISH (CARD-FISH) and 16S rRNA gene libraries (Lenk *et al.*, 2011), and in June 2009 for cell sorting and 16S rRNA gene cloning (this study). Sediment from a silty tidal site of Koenigshafen at the island of Sylt (55.02544°N, 8.4317°E) was sampled in October 2008 for the enrichment culture and in October 2010 for the RCB-specific 16S rRNA gene library and CARD-FISH analysis. For a biogeographic survey of RCB, we also analyzed sediment samples of diverse origin (see Supplementary Table 1). Sediment processing is detailed in Supplementary Methods.

Enrichment of RCB

RCB were enriched from sediment sampled in October 2008 (site Koenigshafen, Island of Sylt, Germany). Initially, 2.5 ml of sediment from the anoxic layer of 3–4 cm depth were inoculated into 50 ml sulfate-free, sodium bicarbonate-buffered, artificial seawater medium (Widdel and Bak, 1992) containing 1 mM sulfide, 5 mM acetate and 5 mM nitrate. The headspace contained a N_2/CO_2 (80/20 ($v\,v^{-1}$)) atmosphere. After 1 month of incubation, the presence of nitrate reducers was indicated by gas formation. Subsequently, RCB were further enriched by repeated Media Processing Node series. FISH revealed high relative abundance of cells targeted by probe ROS537 (>50%) in the highest positive dilution (10^{-6}). This culture was used for substrate tests (details are given in Supplementary Figure 5).

CARD-FISH on sediment samples and enrichment cultures

The *in situ* abundance of RCB was assessed by CARD-FISH using probe ROS537 (Gonzalez and Moran, 1997; Eilers *et al.*, 2001) following an established protocol (Ishii *et al.*, 2004). Probe match analysis against the SILVA SSU Ref. database release 102 revealed 92% coverage for sequences of the marine *Roseobacter* clade. Only 3% of the sequences targeted by ROS537 were non-target organisms. RCB were counted along three vertical sediment profiles sampled in April 2005, August 2007 and May 2009. Probe ALF968 (Glöckner *et al.*, 1999) specific for *Alphaproteobacteria* was applied for double hybridizations. CARD-FISH on sediments of Sylt and enrichment cultures are detailed in Supplementary Methods.

Flow cytometry sorting of RCB and amplification of 16S rRNA gene

Surface sediment (0–3 cm) of Janssand site was sampled in June 2009 and preserved for FISH. Subsamples were sonicated (Lenk *et al.*, 2011) and

cells were purified via density gradient centrifugation (Fazi *et al.*, 2005). Purified cell fractions were hybridized via CARD-FISH with probe ROS537 and then sorted using a MoFlo flow cytometer (Cytomation Inc., Fort Collins, CO, USA) according to Sekar *et al.* (2004). Hybridized (ROS537-positive) cells were selected based on green fluorescent signals and side angle light scatter (SSC) by plotting SSC versus green fluorescence in a bivariant dot plot diagram. A total of 133 000 target cells were sorted and concentrated on a 0.2 μm polycarbonate membrane filter. To check the purity of the sorted cell fraction, a subsample was counterstained with 4′,6-diamidino-2-phenylindole (1 μg ml^{-1}) and the relative abundance of non-hybridized cells was determined. Amplification of the 16S rRNA gene was performed from filter pieces (Sekar *et al.*, 2004). PCR, cloning and sequencing of the amplification product was performed as described previously (Lenk *et al.*, 2011).

Fosmid library screening and sequence analysis
For metagenomic analysis, we screened two fosmid libraries that were established from Janssand sediment of 5–12 cm depth (Mussmann *et al.*, 2005) and from 490 cm sediment depth (K Bischof, unpublished) for the presence of *dsrAB* and *soxB*. We used primer pairs DSR1F/DSR4R (Loy *et al.*, 2009), rDSR240F/rDSR808R (Lenk *et al.*, 2011) and soxB432F/1446B (Petri *et al.*, 2001) according to PCR conditions previously published. After screening, selected clones were chosen for full-length sequencing of the insert (~40 kb). The fosmid insert sequences were determined by a shotgun approach. Sequencing approach, assembly and annotation are detailed in Supplementary Methods.

Simultaneous in situ hybridization of dsrA gene (geneFISH) and 16S rRNA
A *dsrA* targeting polynucleotide probe ds285 RCB (359 bp, GC content 65%) was designed based on the *dsrAB* sequence in fosmid ws101A12 using the PolyPro software (Moraru *et al.*, 2011). The target region of the *dsrA* probe showed 92.3% sequence identity to the alphaproteobacterial *dsrA* phylotype that was derived from the sulfidic enrichment. It displayed 66.3% sequence identity to the gammaproteobacterial *dsrA* phylotype detected in the enrichment. Fosmid copies were extracted from *Escherichia coli* clone cultures using the Spin Miniprep Kit (Qiagen, Hilden, Germany) and served as template for the synthesis of the double-stranded DNA polynucleotide (dsDNA probe). Dig-dUTP-labeled polynucleotide probes were synthesized using the PCR Dig Probe Synthesis Kit (Roche, Diagnostics, Mannheim, Germany). Primers *dsr*1-F, 5′-GAAGTATCCCGAGTCGAAGG-3′ and *dsr*1-R, 5′-GC GCCGGGCGGTGCATCTC-3′ targeting *dsrA* of fosmid ws101A12 were used for synthesis at 56 °C annealing temperature. The template and the primers applied for synthesis of probe NonPolyPro350 are described elsewhere (Moraru *et al.*, 2010). GeneFISH was performed according to Moraru *et al.* (2010). Details with regard to the protocol are given in Supplementary Methods.

Nucleotide sequences
The sequence data from this study have been submitted to the GenBank databases under accession No. JQ256774–JQ256779 (enrichment culture *dsrA*, *dsrB*, *soxC*, *soxD*), JQ256780–JQ256790 (fosmid clones), JQ256791–JQ256838 (16S rRNA gene) and JQ256839–JQ256904 (*soxB*).

Results

Abundance of RCB in marine sediments
We quantified RCB in surface sediments of the Janssand site in different seasons using probe ROS537 for CARD-FISH. Relative abundances in the upper 2 cm varied substantially between the summer and spring samples (Figure 1). Maximum relative and total abundances were reached in August 2007, where RCB accounted for up to 9.6% of all cells (2.5×10^8 cells ml^{-1}). In deeper, permanently anoxic, sulfidic layers down to 9 cm RCB made up 0.8% to 3.1% of all cells. Double hybridizations supported the specific detection of RCB, as all ROS537-hybridized cells also showed signals with probe ALF968 targeting *Alphaproteobacteria* (Figure 2). We also quantified RCB in other coastal sediments including Sylt sediments (Supplementary Table 1). Here, RCB constituted approximately up to 8% of all cells in the upper 1 cm of the sediment. In contrast, they made up <0.5% in 7–8 cm depth. RCB also occurred in other coastal habitats such as North and Baltic Sea sediments, where they accounted for approximately 2% of all cells, but RCB were not detected in significant numbers in hydrothermal (Guaymas Basin), arctic (Svalbard) or subtropical (Bermuda, Elba) sediments (Supplementary Table 1).

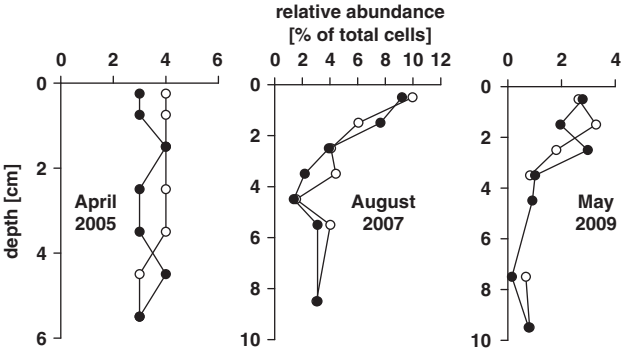

Figure 1 Relative abundance of marine RCB determined by CARD-FISH (probe ROS537) in vertical sediment profiles in April 2005, August 2007 and May 2009. Sediment core A, white circles; sediment core B, black circles.

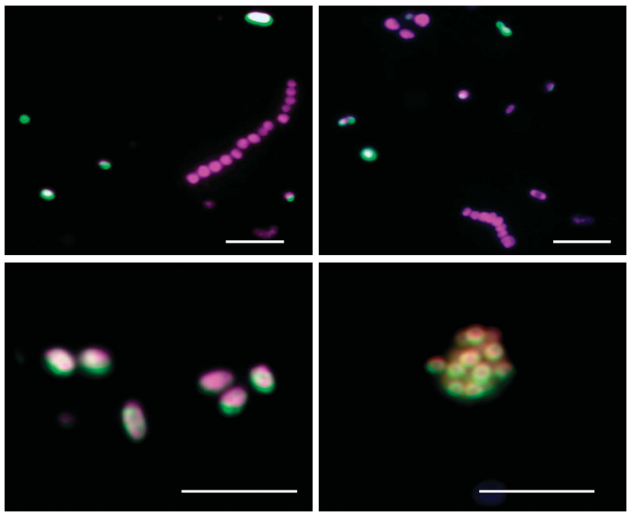

Figure 2 Epifluorescence microscopy images of marine RCB in Janssand sediments. For all images, green (Alexa 488): cells that were identified by RCB-specific probe ROS537; red (Alexa 594): *Alphaproteobacteria* that hybridized with the general probe ALF968. Only RCB are simultaneously stained in green and red. The scale bar corresponds to 5 μm.

16S rRNA and soxB gene diversity of sedimentary RCB

To study the diversity of RCB, we constructed and sequenced 16S rRNA gene libraries from both Janssand and Sylt sediments and a *soxB* library from Janssand sediment. In previously generated, *Bacteria*-targeted 16S rRNA gene libraries from Janssand sediments (Lenk *et al.*, 2011), out of 458 analyzed clones, 10 sequences related to RCB were recovered. To study the RCB diversity in more detail, we generated a 16S rRNA gene library from probe ROS537-hybridized cells that we mechanically enriched from sediment samples by fluorescence-activated cell sorting. The sorted cells served as template for PCR and subsequent cloning and sequencing. Out of 30 clones, 14 sequences grouped with RCB. Despite a sorting purity of 97%, the non-RCB sequences were diverse but displayed several mismatches to probe ROS537 and likely originated from contaminating cells in the sorted cell fraction. Biased cell lysis and PCR probably favored amplification of non-target cells over hybridized RCB cells. To recover specifically RCB sequences from the Sylt sediments, we used probe ROS537 as a reverse primer and combined it with a general forward primer for PCR. Here, we recovered 34 partial, RCB-related sequences. In total, we obtained 58 RCB-related 16S rRNA sequences from the two tidal sediments. These sequences were highly diverse (79–99% sequence identity (SI)) and affiliated with sequences of cultured and uncultured organisms from biofilms, pelagic and benthic habitats (Figure 3). The closest cultured relatives were among the genera *Sulfitobacter*, *Thalassobacter*, *Roseobacter* and others. Clones JSS_4432 and KH_5078 grouped with *Tateyamaria pelophila* (97% SI), a facultatively anaerobic, sulfite and

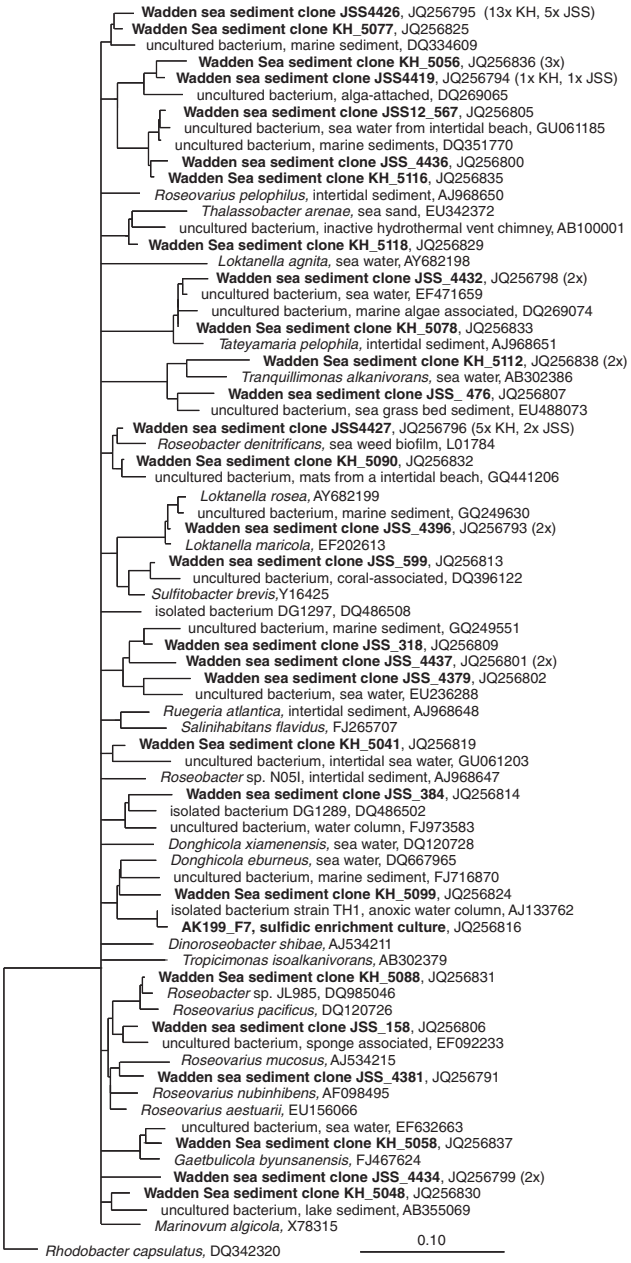

Figure 3 Consensus tree of alphaproteobacterial 16S rRNA gene sequences from intertidal sediments of Janssand (JSS) and Sylt (KH). Bar indicates 10% sequence divergence.

thiosulfate oxidizing strain that was previously isolated from the study site (Sass *et al.*, 2010).

To test whether the detected RCB at Janssand have the potential to oxidize inorganic sulfur compounds, we screened a *soxB* gene library for RCB-related sequences. In total, 66 *soxB* sequences were recovered, which grouped into 35 operational taxonomic units (Supplementary Figure 1). The majority of sequences (41 sequences, 17 operational taxonomic units) affiliated with *Alphaproteobacteria*. Among these, 39 sequences (15 operational taxonomic units) consistently clustered with SoxB

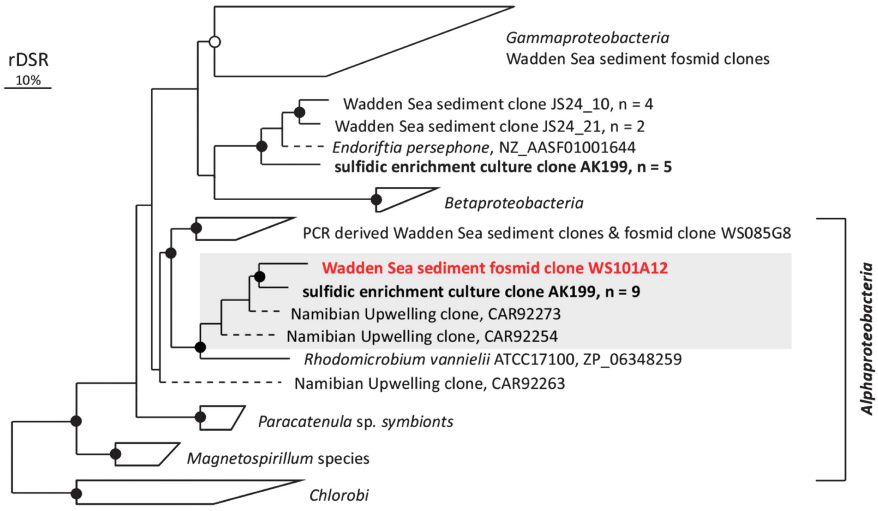

Figure 4 Phylogenetic reconstruction (RaxML) of DsrAB protein sequences encoded on Wadden Sea sediment fosmids from Janssand and DsrAB sequences retrieved from the sulfidic enrichment culture AK199 from Koenigshafen (in bold). Gray shading indicates sequences likely originating from members of the marine *Roseobacter* clade. Bootstrapping values are indicated with >70% (closed circles) and >50% (open circles) RAxML bootstrap support. The bar indicates 10% sequence divergence.

sequences of cultured RCB. Similar to the results obtained with the 16S rRNA genes, the RCB-associated SoxB sequences were highly diverse (46–96% amino-acid SI). They were only distantly related to sequences of known cultured representatives such as *Oceanibulbus indolifex* (JSS043, 86% SI) and sulfite-oxidizing *Silicibacter pomeroyi* (JSS037, 82% SI).

Metagenomic analysis of sulfur-oxidizing bacteria in a tidal sediment

To reveal genomic and metabolic features of sulfur-oxidizing microbes, in particular of RCB, we screened fosmid clones for genome fragments encoding *dsrAB* or *soxB*. Out of ~24 000 fosmids, we identified 11 fosmids of interest with insert sizes ranging from 30 to 43 kb. Eight fosmids carried the *dsrAB* gene and two fosmids carried the *soxB* gene, while one fosmid (ws101A12) carried both genes (Figure 5 and Supplementary Table 2). The deduced DsrAB or SoxB amino-acid sequences of eight fosmids grouped with *Gammaproteobacteria* (Supplementary Table 3). The derived SoxB sequences of two fosmids including fosmid ws101A12 affiliated with *Alphaproteobacteria* (Figure 5 and Supplementary Figure 1). The DsrAB sequence derived from fosmid clone ws101A12 was most closely related to sequences of uncultured, alphaproteobacterial sulfur-oxidizing prokaryotes (SOP) detected in sulfidic waters off the coast of Namibia (84% sequence identity SI) (Figure 4). The closest cultured relative was *Rhodomicrobium vannielli* (78% SI), a photoautotrophic alphaproteobacterium. The SoxB sequence of fosmid ws101A12 affiliated with the marine *Roseobacter* clade (Supplementary Figure 1). It had the highest sequence identity to the SoxB sequence of *Sagittula stellata* (86% SI). Furthermore, the comparison of intrinsic nucleotide signatures of this genomic fragment using the TaxSOM software (Weber *et al.*, 2011) provided additional evidence for a *Roseobacter* clade-related affiliation of ws101A12 and supported *Oceanicola batsensis* as closest known relative (data not shown).

Gene content on fosmid clone ws101A12

The 35 kb insert of fosmid ws101A12 contained 37 open reading frames (ORFs) (Figure 5 and Supplementary Table 2). In 18 out of 37 predicted proteins, the closest homologs were found in genome sequences of known RCB. This fosmid clone harbored a gene set of 14 genes in the Sox multi-enzyme pathway (*soxTRSVWXYZABCDEF*, ORFs 1–14) including the sulfur dehydrogenase SoxCD encoding genes (ORF 11, ORF 12). All deduced protein sequences displayed highest sequence identity to *sox* genes of marine RCB (Supplementary Table 2). In addition, the same metagenomic fragment encoded a nearly complete rDSR operon (*dsrABEFHCMKLJOPN*, ORFs 24–35, ORF 37). Of these, seven deduced protein sequences displayed significant sequence similarity to homologs in *Alphaproteobacteria* (ORFs 24–26, 29, 32–34). Between the rDSR and SOX operons, three subunits of a putative sulfite dehydrogenase were encoded (*soeCBA*, ORFs 17–19). The deduced proteins showed highest sequence similarity to homologs among RCB (62–82% SI; Supplementary Table 2) including the SoeABC subunits of *Ruegeria pomeroyi* DSS-3 (YP_168752-54).

Comparison of the *dsr* locus of fosmid ws101A12 with the gammaproteobacterial fosmid clones and available SOP genomes pointed at the presence of previously uncharacterized ORFs (ORFs 21–23) that are consistently located upstream of *dsrA* (Figure 5). ORFs 21–22 are conserved hypothetical proteins

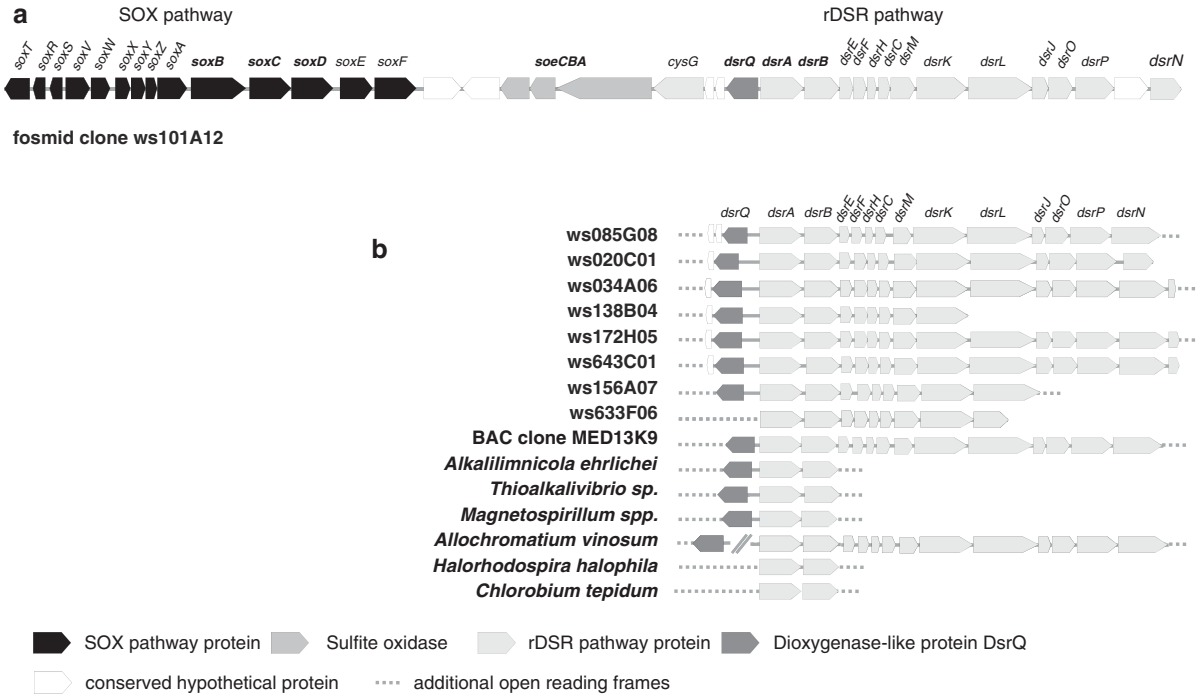

Figure 5 (**a**) Genomic organization of *sox*, *soe* and *dsr* loci in fosmid clone ws101A12. (**b**) Gene neighborhood of the *dsrAB* locus encoded on Wadden Sea sediment fosmid clones and in genomes of selected SOP.

of which homologs were found in a few SOP. The deduced protein sequences of ORF 23 weakly matched a taurine catabolism dioxygenase (Supplementary Table 2), but displayed sequence motifs typical for dioxygenases (Supplementary Figure 2). Comparative genome analysis of the *dsr* loci revealed that the ORF23 homologs are located upstream of *dsrAB* in nearly all our fosmid clones and in most (facultatively) aerobic chemotrophic SOP (Figure 5). In contrast, homologs are not associated with the *dsr* locus in the genomes of the sulfur-oxidizing endosymbionts *Ruthia magnifica* and *Vesicomyosocius oktuanii* and of the anaerobic phototrophs *A. vinosum* and *Halorhodospira halophila*. It is moreover absent in the genomes of the strictly anaerobic, phototrophic *Chlorobi*.

Enrichment and molecular characterization of rDSR-encoding RCB

To confirm the hypothesis that the *dsr* locus in fosmid ws101A12 indeed belonged to RCB, we screened for RCB-related 16S rRNA and *dsrAB* genes in a sulfidic enrichment culture from anoxic sediments. After repeated transfers, RCB dominated the enrichment and accounted for >50% of all cells, whereas *Gamma-* and *Epsilonproteobacteria* were less abundant (Supplementary Figure 3). From this enrichment culture, bacterial 16S rRNA and *dsrAB* genes were amplified, cloned and sequenced. Consistent with our FISH results, 16S rRNA gene sequences (55 clones) affiliated with RCB, *Gamma-* and *Epsilonproteobacteria*. Only a single RCB phylotype was identified that most closely affiliated with the nitrate-reducing strain TH1 (98% SI), and with *Donghicola eburneus*, an acetate-oxidizing aerobe (96% SI). A sequence obtained from a denaturing gradient gel electrophoresis analysis with RCB specific primers matched the clone library-derived sequence, which strongly supported the presence of only one RCB phylotype in the enrichment (Supplementary Figure 4).

Two different DsrAB phylotypes were identified in the enrichment culture. One phylotype was most closely related to the DsrAB encoded on the RCB-affiliated fosmid clone ws101A12 (93% SI). The second phylotype affiliated with gammaproteobacterial DsrAB sequences previously retrieved from Janssand sediments (85% SI). To test whether the RCB in the enrichment also possess *soxCD* genes, we amplified a fragment using novel primers that target *soxC* at the 5′ end and *soxD* at the 3′ end. We recovered a fragment of approximately 1130 bp in size that was directly sequenced and had the highest nucleotide sequence identity to *soxC* of fosmid ws101A12 (83% SI). The corresponding *soxD* sequence displayed 73% nucleic acid sequence identity to *soxD* of fosmid ws101A12 and 76% to *soxD* of *R. pomeroyi*. As no pure culture of the *dsr*-encoding RCB organism could be recovered, we monitored their growth in the enrichment upon supply with organic and inorganic sulfur compounds and organic acids. Under nitrate-respiring conditions, the addition of inorganic sulfur compounds

stimulated growth of RCB. Strongest growth of RCB was observed with dimethylsulfoxide and taurine under aerobic conditions (Supplementary Figure 5). As other sulfur-oxidizing bacteria were present in the enrichment, we cannot completely rule out that the observed RCB relied on metabolites released by these organisms.

In situ localization of dsrA in RCB cells in the enrichment using geneFISH

As a 16S rRNA gene was absent on the metagenomic fragment and as we could not obtain a pure RCB culture from the enrichment, we combined 16S rRNA CARD-FISH with gene-targeted FISH (Moraru *et al.*, 2010). To link directly the RCB identity and the *dsrA* gene at the single-cell level, we designed a polynucleotide probe dsr285_RCB (Supplementary Figure 6) that targeted the alphaproteobacterial *dsrA* phylotype of fosmid ws101A12 (Supplementary Table 4) and hybridized it to the sulfidic enrichment culture. The sequence identity between the probe and the target *dsrA* sequence was 91.9%, which is sufficient to ensure specific binding (Moraru *et al.*, 2011). GeneFISH of *dsrA* clearly localized the gene in those cells that were simultaneously hybridized with the 16S rRNA targeting probe ROS537 (Figure 6). Because of known methodological issues (Moraru *et al.*, 2010), only approximately 50% of all RCB were targeted by the polynucleotide probe. Cells not hybridized to probe ROS537 also gave no *dsrA* geneFISH signals. The second DsrAB phylotype in the enrichment belonged to *Gammaproteobacteria* and displayed 66.3% sequence identity to the geneFISH probe. Therefore, it was not targeted by probe dsr285_RCB (Supplementary Table 4). Hybridization with the negative control probe NonPolyPr350 (Supplementary Figure 6) resulted in background level unspecific binding and showed that the probe did not bind unspecifically to cellular components (Figure 6). We also attempted to quantify the abundance of *dsrA*-encoding RCB in surface sediment. However, the low detection efficiency of geneFISH in sediment samples due to high particle background hampered any further *in situ* quantification.

Discussion

Abundance and diversity of RCB in marine sediments

The marine *Roseobacter* clade often accounts for a substantial fraction of coastal bacterioplankton, but so far it was unknown, how abundant they are in coastal sediments. Here, we provide evidence that RCB also reach high total abundances in marine sediments, which outnumbered RCB in bacterioplankton by up to three orders of magnitude (Eilers *et al.*, 2000; Alderkamp *et al.*, 2006). Our results

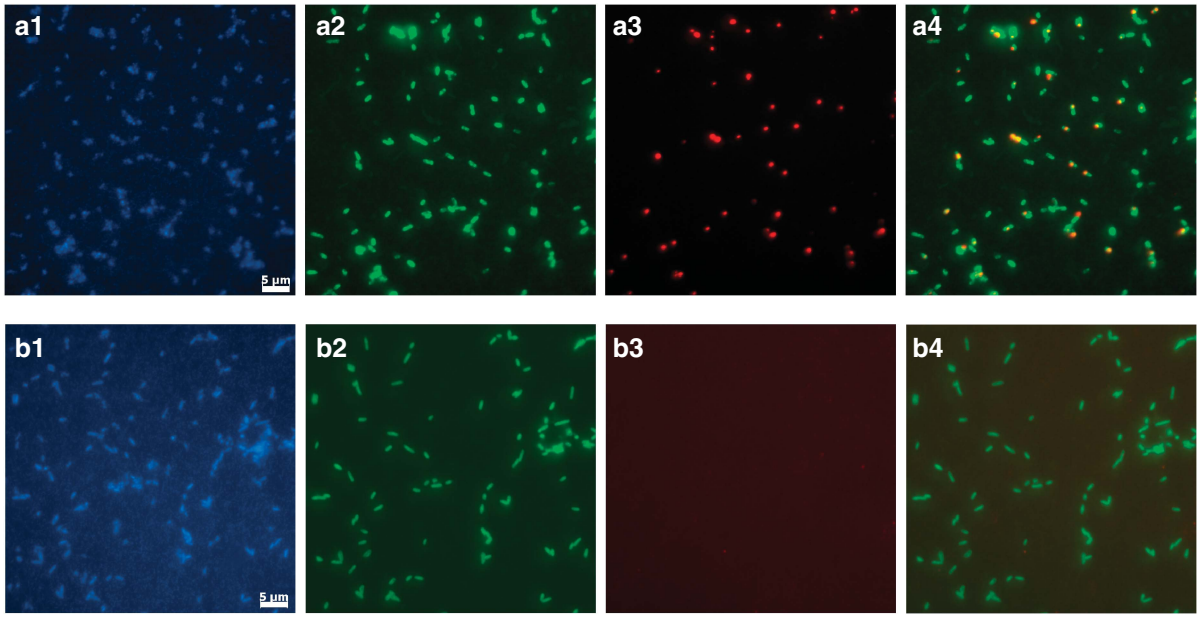

Figure 6 Epifluorescence microscopy images of RCB encoding the *dsrAB* gene from sulfidic enrichment culture. Dual color hybridization of the 16S rRNA of RCB stained in green (Alexa 488 tyramide) and the *dsrA* targeting polynucleotide probe stained in red (Alexa 594 tyramide). Overlay images demonstrate that the signal for *dsrA* (red, **a3** and **a4**) colocalizes with the 16S rRNA FISH signal for RCB (green, **a4**). Other bacteria in the enrichment (*Epsilon*- and *Gammaproteobacteria*) are stained with 4′,6-diamidino-2-phenylindole and are not fluorescent (**a1**). In addition, no fluorescent signals were obtained from hybridization of the NonPoly350 probe (**b3**) to the enrichment culture, which indicates the absence of nonspecific binding (**b1–b4**). **a1**, **b1**: DNA stains (4′,6-diamidino-2-phenylindole). **a2**: FISH signals of probe ROS537 targeting 16S rRNA (RCB specific). **b2**: FISH signals of probe GAM42a targeting 16S rRNA (*Gammaproteobacteria*). **a3**: FISH signals of *dsrA* gene-targeted probe. **b3**: negative control probe NonPoly350. **a4**: overlay images of 16S rRNA-FISH and *dsrA*-FISH. **b4**: overlay images of 16S rRNA-FISH and negative control probe NonPoly350. Scale bar corresponds to 5 µm.

complement early findings of Gonzalez and Moran (1999) and corrobate that RCB are also an integral part of the microbial community in coastal surface sediments.

Using several methods, we explored the RCB diversity in two tidal sediments to look for groups specific to marine sediments. We obtained 58 16S rRNA gene sequences indicative of a large diversity within the marine RCB group in tidal sediments. In several diversity studies, RCB-related sequences were recovered from sediments (Buchan *et al.*, 2005), but an analysis of available sequences did not reveal a dedicated RCB group that is exclusively found in sediments. Most of our sequences were related to those from bacterioplankton and algae or invertebrate biofilms, which rather mirrors the flexibility of RCB to thrive as free-living or attached to surfaces. In support of this, pelagic RCB were found to rapidly colonize submerged surfaces in marine waters (Dang and Lovell, 2002). Moreover, we detected RCB in high numbers in permanently anoxic layers and also enriched a facultatively anaerobic RCB from anoxic sediments. Previous results indeed showed that planktonic RCB in the open North Sea can assimilate glucose both under aerobic and anaerobic conditions (Alonso and Pernthaler, 2005). Taken together, these results strongly suggest that many pelagic RCB are able to survive and grow even in anoxic marine sediments.

Some RCB encode both the rDSR and the complete SOX pathway

Thiosulfate is a key intermediate in sulfur cycling in marine sediments (Jørgensen, 1990). As we recovered many and diverse *soxB* sequences related to RCB, we propose that RCB are important thiosulfate-oxidizing bacteria in Janssand tidal sediments. This is consistent with published data and genomic surveys indicating that known RCB encode the SOX pathway including *soxCD* subunits but not the rDSR pathway (Meyer *et al.*, 2007, Moran *et al.*, 2007). Moreover, in RCB the SOX pathway also appears to be involved in the oxidation of sulfur intermediates during breakdown of dimethylsulfoniopropionate (Rinta-Kanto *et al.*, 2011).

Here, we provide evidence that some RCB also possibly employ the rDSR pathway for sulfur oxidation. First, we identified a RCB-related metagenomic fragment that harbored a nearly complete set of genes of both the SOX and the rDSR pathway. Second, we enriched a RCB population in a sulfidic, nitrate-respiring culture, in which we simultaneously visualized the 16S rRNA and the *dsrA* gene in single cells. Congruent phylogenies of DsrAB and 16S rRNA and denaturing gradient gel electrophoresis analysis supported the presence of only one *dsrAB*-encoding RCB in the enrichment culture. This organism most likely also encoded SoxCD as we could also amplify a *soxCD* gene fragment from the enrichment culture that was closely related to *soxCD* on fosmid ws101A12.

Extended sulfur oxidation potential of RCB from tidal sediments

The co-occurrence of *dsr* and *soxCD* loci is novel, as both are mutually exclusive in known SOP genomes (Meyer *et al.*, 2007; Frigaard and Dahl, 2009; Gregersen *et al.*, 2011). In s*oxCD*-lacking organisms, the rDSR pathway substitutes the sulfur dehydrogenase activity of SoxCD and the sulfane–sulfur transiently accumulates as elemental sulfur before it is further oxidized to sulfite (Hensen *et al.*, 2006; Grimm *et al.*, 2008). In contrast, a complete pathway including SoxCD allows the direct oxidation of thiosulfate to sulfate (Friedrich *et al.*, 2001; Rother *et al.*, 2001). It was presumed that the rDSR pathway conserves more energy than the SOX pathway (Gregersen *et al.*, 2011), which would explain the loss of s*oxCD* in all yet described rDSR-encoding SOP.

However, we propose that such an extended genomic repertoire provides an increased metabolic flexibility. A fast, direct oxidation of thiosulfate using only the SOX pathway including SoxCD is beneficial, when electron donor and acceptor are available in excess. In turn, the rDSR pathway likely catalyzes the oxidization of elemental sulfur that has been formed from ambient sulfide or thiosulfate under electron acceptor-limiting conditions (Van den Ende and Van Gemerden, 1993); for instance, during tidal fluctuations (Jansen *et al.*, 2009). Unfortunately, the enrichment culture could not be maintained in our lab. Hence, it remains to be shown under which conditions these RCB employ the SOX and rDSR pathways.

A novel subunit in the proteobacterial DSR operon

On fosmid ws101A12, we identified a previously undescribed ORF (ORF 23) that is located upstream of *dsrAB* in most of our *dsr*-encoding metagenomic fragments and in several SOP genomes. Such a conserved gene neighborhood and the typical dioxygenase motifs among many different phyla are strongly indicative of a catalytic activity in the rDSR pathway. Thus we suggest the designation '*dsrQ*' for this ORF. The actual function of the deduced protein is unclear, but different scenarios are conceivable. The DsrQ protein may catalyze the release of sulfite during oxygenolytic breakdown of intracellular (Franz *et al.*, 2009) or ambient sulfonates, similar to the function of taurine dioxygenase in *E. coli* (Eichhorn *et al.*, 1997). This is supported by the fact that the derived proteins of ORFs 17–19 are homolog to a recently proposed novel-type sulfite dehydrogenase SoeABC (Lehmann *et al.*, 2012). Mutation studies have shown that this enzyme is crucially involved in sulfite oxidation and in taurine degradation by *R. pomeroyi* (Lehmann *et al.*, 2012; S. Lehmann, personal communication). Moreover, homologs enzymes are possibly involved in dimethylsulfoniopropionate breakdown by RCB (Rinta-Kanto *et al.*, 2011).

Conclusions

We derived potential ecophysiological traits of RCB in coastal sediments from FISH counts, metagenomics and analyses of an enrichment culture. Some of these RCB possess an extended genetic repertoire for sulfur oxidation that is novel among RCB and all known sulfur-oxidizing microorganisms. Using the enrichment culture, we monitored growth of RCB by FISH upon supply of different electron donors and oxygen or nitrate as electron acceptors. Although RCB could have relied on metabolites released by other organisms in the enrichment culture, the observed growth of RCB on different sulfur compounds is consistent with our genomic data and supports a sulfur-based energy metabolism. Our substrate tests in the enrichment culture indicated a potential to grow on organic and inorganic sulfur compounds of the *dsrAB*-containing RCB phylotype. Such a unique metabolic flexibility is particularly useful in highly fluctuating environments, for example, at oxic–anoxic interfaces in tidal sediments and may allow occupying new ecological niches. These RCB may oxidize sulfur compounds via two different pathways and could overcome oxygen limitation by respiring nitrate. Unlike most known sulfur-oxidizing bacteria, they are probably not thioautotrophic as we did not observe any carbon fixation by RCB in a previous microautoradiography experiment using ^{14}C bicarbonate (Lenk *et al.*, 2011). This is in line with the general absence of CO_2-fixing pathways in RCB (Moran *et al.*, 2007; Newton *et al.*, 2010). During lithoheterotrophic growth, the use of reduced inorganic sulfur compounds as additional energy source would provide a competitive advantage over autotrophic SOP (Sorokin, 2003; Moran *et al.*, 2004) that are present in our tidal sediments (Lenk *et al.*, 2011). Because of their ability to switch between planktonic and a surface-adapted lifestyle, RCB most likely persist in sediments by vertical migration, active settlement and growth, and may therefore contribute to the pelagic–benthic coupling. Considering their high cell abundances, RCB may significantly contribute to sulfur cycling not only in the marine water columns, but also in coastal surface sediments.

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