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ORIGINAL ARTICLE

Ubiquitous *Gammaproteobacteria* dominate dark carbon fixation in coastal sediments

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Marine sediments are the largest carbon sink on earth. Nearly half of dark carbon fixation in the oceans occurs in coastal sediments, but the microorganisms responsible are largely unknown. By integrating the 16S rRNA approach, single-cell genomics, metagenomics and transcriptomics with ^{14}C-carbon assimilation experiments, we show that uncultured *Gammaproteobacteria* account for 70–86% of dark carbon fixation in coastal sediments. First, we surveyed the bacterial 16S rRNA gene diversity of 13 tidal and sublittoral sediments across Europe and Australia to identify ubiquitous core groups of *Gammaproteobacteria* mainly affiliating with sulfur-oxidizing bacteria. These also accounted for a substantial fraction of the microbial community in anoxic, 490-cm-deep subsurface sediments. We then quantified dark carbon fixation by scintillography of specific microbial populations extracted and flow-sorted from sediments that were short-term incubated with ^{14}C-bicarbonate. We identified three distinct gammaproteobacterial clades covering diversity ranges on family to order level (the *Acidiferrobacter*, JTB255 and SSr clades) that made up $>$50% of dark carbon fixation in a tidal sediment. Consistent with these activity measurements, environmental transcripts of sulfur oxidation and carbon fixation genes mainly affiliated with those of sulfur-oxidizing *Gammaproteobacteria*. The co-localization of key genes of sulfur and hydrogen oxidation pathways and their expression in genomes of uncultured *Gammaproteobacteria* illustrates an unknown metabolic plasticity for sulfur oxidizers in marine sediments. Given their global distribution and high abundance, we propose that a stable assemblage of metabolically flexible *Gammaproteobacteria* drives important parts of marine carbon and sulfur cycles.
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Introduction

Marine coastal sediments are global hot spots of carbon remineralization and burial (Hedges and Keil, 1995). In current models of oceanic carbon cycling, the sequestration of microbially altered organic matter is the major mechanism of carbon preservation in sediments (Parkes *et al.*, 1993; Burdige, 2007). Marine sediments are sites not only of carbon remineralization but also of carbon fixation. Recent estimates suggest that marine microbes fix inorganic carbon independent of light (chemolithoautotrophy) in amounts that are in the same order of magnitude as the annual organic carbon burial (Middelburg, 2011). Chemolithoautotrophic microorganisms in marine sediments fix up to 370 Tg C/year, which equals 48% of carbon fixed chemolithoautotrophically in the ocean (Middelburg, 2011). Thereof, 47% are fixed in shallow, near-shore sediments (175 Tg C/year). Near-shore sediments, therefore, contribute more to oceanic carbon fixation than pelagic oxygen minimum zones (OMZs) and hydrothermal vents (Middelburg, 2011). In recent years, chemolithoautotrophy in these marine systems has received much attention. The ecophysiology and genetic composition of key players of carbon (and sulfur) cycling in OMZs and hydrothermal vents, such as the gammaproteobacterial SUP05 clade, have been extensively studied (Lavik *et al.*, 2009; Canfield *et al.*, 2010; Reinthaler *et al.*, 2010; Swan *et al.*, 2011; Grote *et al.*, 2012; Anantharaman *et al.*, 2013; Mattes *et al.*, 2013). This cosmopolitan clade is expected to

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have an important role in attenuating atmospheric carbon dioxide concentrations, when OMZs expand in a warming climate (Hawley *et al*., 2014).

In contrast to pelagic OMZs, where the oxic-anoxic/sulfidic interface can be meters thick, in near-shore sediments this interface is only a few millimeters thick, and is characterized by steep biogeochemical gradients and approximately 1000-fold higher cell abundances per sample volume. Biogeochemical evidence indicates that sulfur oxidation is the dominant chemolithoautotrophic process in coastal sediments, while nitrification appears to play only a minor role (Middelburg, 2011; Boschker *et al*., 2014). Previous studies of benthic autotrophic sulfur oxidizers mostly focused on large, conspicuous sulfur bacteria such as *Beggiatoa*, which are widely distributed but occur in high abundances only in certain habitats (Salman *et al*., 2013; Ruff *et al*., 2015). Other *Gammaproteobacteria* affiliating with cultured sulfur oxidizers (*Acidithiobacillus, Thiohalophilus* and *Thiomicrospira*) or with uncultured symbiotic sulfur oxidizers among the *Chromaticaeae* and *Ectothiorhodospiracea* have been regularly found in marine and estuarine sediments (Bowman *et al*., 2005; Orcutt *et al*., 2011). Consistent with this, recent molecular and isotopic approaches suggest that some of these are indeed autotrophs (Lenk *et al*., 2011; Boschker *et al*., 2014; Vasquez-Cardenas *et al*., 2015).

Culture-independent molecular studies previously identified predominant carbon fixation pathways such as the Calvin-Benson-Bassham (CBB) cycle and the reductive tricarboxylic acid cycle in marine chemolithoautotrophic bacteria. The key genes encoding ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form I and form II (*cbbL*, *cbbM*) and the ATP citrate lyase (*aclAB*) in the reductive tricarboxylic acid cycle pathway have been frequently detected in environmental studies (reviewed by Hügler and Sievert, 2011). Likewise, genes encoding subunits of the reverse dissimilatory sulfite reductase (*dsrAB*), of the adenosine-5'-phosphosulfate reductase (*aprA*) and of the thiosulfate-oxidizing Sox-multienzyme complex (*soxB*) have been used to target the diversity of marine benthic sulfur oxidizers (Lenk *et al*., 2011; Thomas *et al*., 2014).

To understand how inorganic carbon at sediment surfaces is turned over and possibly buried, detailed knowledge of the microbes driving these processes is essential but currently still lacking. To fill this gap, we surveyed the diversity of candidate bacterial chemolithoautotrophs in 13 coastal surface sediments across Western Europe and Australia. Moreover, we studied whether these chemolithoautotrophic bacteria are also present in anoxic, 490-cm-deep subsurface sediments. We developed a new method to combine ^{14}C-bicarbonate labeling of cells with fluorescence *in situ* hybridization (FISH), fluorescence-activated cell sorting (FACS) and scintillography to quantify dark carbon fixation by distinct taxonomic groups. Meta- and single-cell genomics along with metatranscriptomics provided evidence for a largely sulfur-based carbon fixation in a selected tidal sediment. Metatranscriptomic reads were mapped against reference databases containing *cbbL*, *cbbM* and *aclAB* sequences to identify active carbon fixation pathways. Metatranscriptomic reads mapped against reference databases containing *dsrAB*, *aprA* and *soxB* sequences indicated sulfur oxidation pathways active *in situ*. This unique combination of molecular and isotopic approaches provided unprecedented insights into the ecology and ecophysiology of cosmopolitan microorganisms driving a major part of global dark carbon fixation.

Materials and methods

Sediment sampling and characteristics

Between October 2012 and December 2014, we sampled 10 tidal and 3 sublittoral sandy sediments

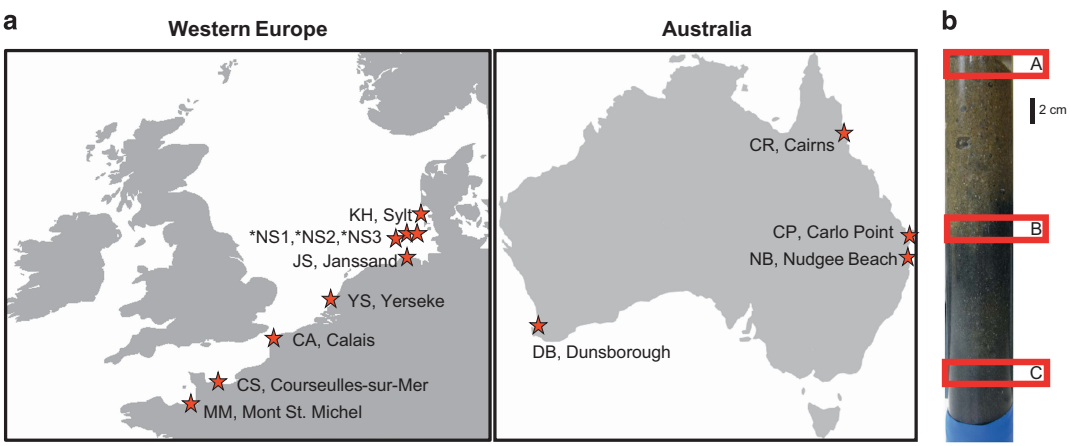

Figure 1 Sampling sites of the 16S rRNA gene survey (**a**). Example for a typical stratification of a sediment core from coastal sandy sediments (**b**). A = uppermost sediment layer, B = sulfide transition zone and C = sulfidic layer refer to the different sampling depths in this study. During sampling, the sediment colors were used as an indicator for the presence of iron sulfide (dark gray to black). Asterisk indicates samples from sublittoral sediments.

in Western Europe and Australia (Figure 1 and Supplementary Table S1). The 10 tidal sediments were sampled during low tide using polyacryl-cores or cutoff syringes of up to 25 cm length. The three sublittoral, coastal sandy sediments were sampled during cruise He417 with the RV Heincke in March 2014 in the German Bight using multi- and boxcorers. At each site, two to three different sediment layers were selected for molecular analyses (16S rRNA gene amplicon sequencing, catalyzed reporter deposition (CARD)-FISH). Sediment for ^{14}C incubations was collected from sites Calais, Courseulles-sur-Mer and from Janssand (Supplementary Table S1). Sediment colors served as a proxy for redox state and active sulfide formation and oxidation (Figure 1). For most of the sites, sediment (i) from the uppermost cm (brownish sediment, sulfide-free), (ii) from the sulfide transition zone (brown to gray, reflecting the presence of iron sulfides) and (iii) from sediment of the sulfidic layer was sampled for molecular analyses (Supplementary Table S1). Sulfide concentrations in pore waters were measured for sites Calais and Courseulles-sur-Mer using the methylene blue method (Cline, 1969). More details on the biogeochemistry of sulfide and oxygen at the study sites Janssand and Königshafen in the German Wadden Sea have been published previously (de Beer *et al.*, 2005; Billerbeck *et al.*, 2006; Jansen *et al.*, 2009). In addition, in April 2005 we sampled a 490-cm-deep subsurface core at site Janssand as described in detail by Gittel *et al.* (2008). Sulfate and methane concentration profiles and lithological data from this core have been described previously (Gittel *et al.*, 2008; Seidel *et al.*, 2012).

DNA extraction for barcoded 16S rRNA gene amplicon sequencing

For all intertidal and subsurface sediment samples from Europe and Australia, DNA was extracted from 200 to 250 µl sediment recovered from distinct layers using the PowerSoil DNA isolation kit (MoBio Laboratories, Solana Beach, CA, USA). DNA from sites NoahA, NoahB and CCPδ was extracted from 5 g of homogenized surface sediments according to Zhou *et al.* (1996) including Proteinase K treatment for improved cell lysis.

Barcoded 16S rRNA gene amplicon sequencing

The bacterial diversity in all sediment samples was determined by analyzing the hypervariable V3–V4 region of the 16S rRNA gene using Roche 454 pyro- or Illumina MiSeq-sequencing of barcoded amplicons. Barcoded amplicons from all surface sediments were prepared using primers 341f/785rev (Herlemann *et al.*, 2011; Klindworth *et al.*, 2012). Barcoded amplicons from the 490 cm-deep subsurface sediment from site Janssand were prepared by replacing primer 785rev with 907rev (Muyzer *et al.*, 1998; Klindworth *et al.*, 2012). This primer covers a similar bacterial diversity as the reverse primer 785rev used for surface sediments (see above, Figure 2), but it is known to bias against some phyla that are, however, low abundant or absent in marine sediments (Klindworth *et al.*, 2012). In total, 311 196 bacterial 16S rRNA reads were kept for taxonomic classification using the SILVAngs pipeline v115 (Quast *et al.*, 2013) with a clustering at 98% identity. Details on PCR conditions, sequencing and processing are given as Supplementary Information.

Sediment incubations with ^{14}C-bicarbonate

Sediment for ^{14}C-DIC incubations was collected from sites Calais and Courseulles-sur-Mer in July 2013 and from Janssand in April 2014 (Supplementary Table S1). These cores were kept at *in situ* temperature (15–20 °C) and used for ^{14}C-DIC incubations within 48 h after sampling. After slicing, 2 ml of the uppermost sediment layer and from the sulfide transition zone was transferred into 10 ml glass vials. In all, 1 ml of sterile filtered seawater and 1 ml of artificial seawater containing 1.5 mM ^{14}C bicarbonate (specific activity 54.7 mCi mmol^{-1}, Hartmann Analytic, Braunschweig, Germany) were added. Vials were sealed with lab-grade butyl rubber stoppers (GMT Inc., Ochelata, OK, USA) leaving 6 ml of headspace (air). Then, vials were incubated for 20 h with mild agitation (100 r.p.m.) at *in situ* temperature in the dark. In parallel incubations 1 mM thiosulfate was added to slurries from Courseulles-sur-Mer and Calais. Slurry incubations were performed in duplicates (Calais, Courseulles-sur-Mer) or in triplicates (Janssand). Dead controls were included for each site by adding formaldehyde (2%, final concentration) before the incubation.

CARD-FISH and sample preparation for flow cytometry

For CARD-FISH, sediment from sites Calais, Courseulles-sur-Mer and Janssand was fixed immediately after core retrieval as described in Lenk *et al.* (2011). Cells were detached from 100 to 200 µl sediment by ultrasonic treatment as described previously (Lenk *et al.*, 2011). Permeabilization and CARD-FISH were performed as described by Pernthaler *et al.* (2002) with the modifications detailed in Supplementary Information. Tyramides labeled with Alexa488 fluorescent dye (Molecular Probes, Eugene, OR, USA) were used for CARD signal amplification. An overview of oligonucleotide probes used in this study is shown in Supplementary Table S2. Novel oligonucleotide probes were designed for the JTB255 group using ARB and the SILVA 16S rRNA reference database release 117 (Pruesse *et al.*, 2007). Please note that the *Xanthomonadales,* which includes the JTB255 clade, are not targeted by probe GAM42a, specific for most *Gammaproteobacteria* (Siyambalapitiya and Blackall, 2005). In line with

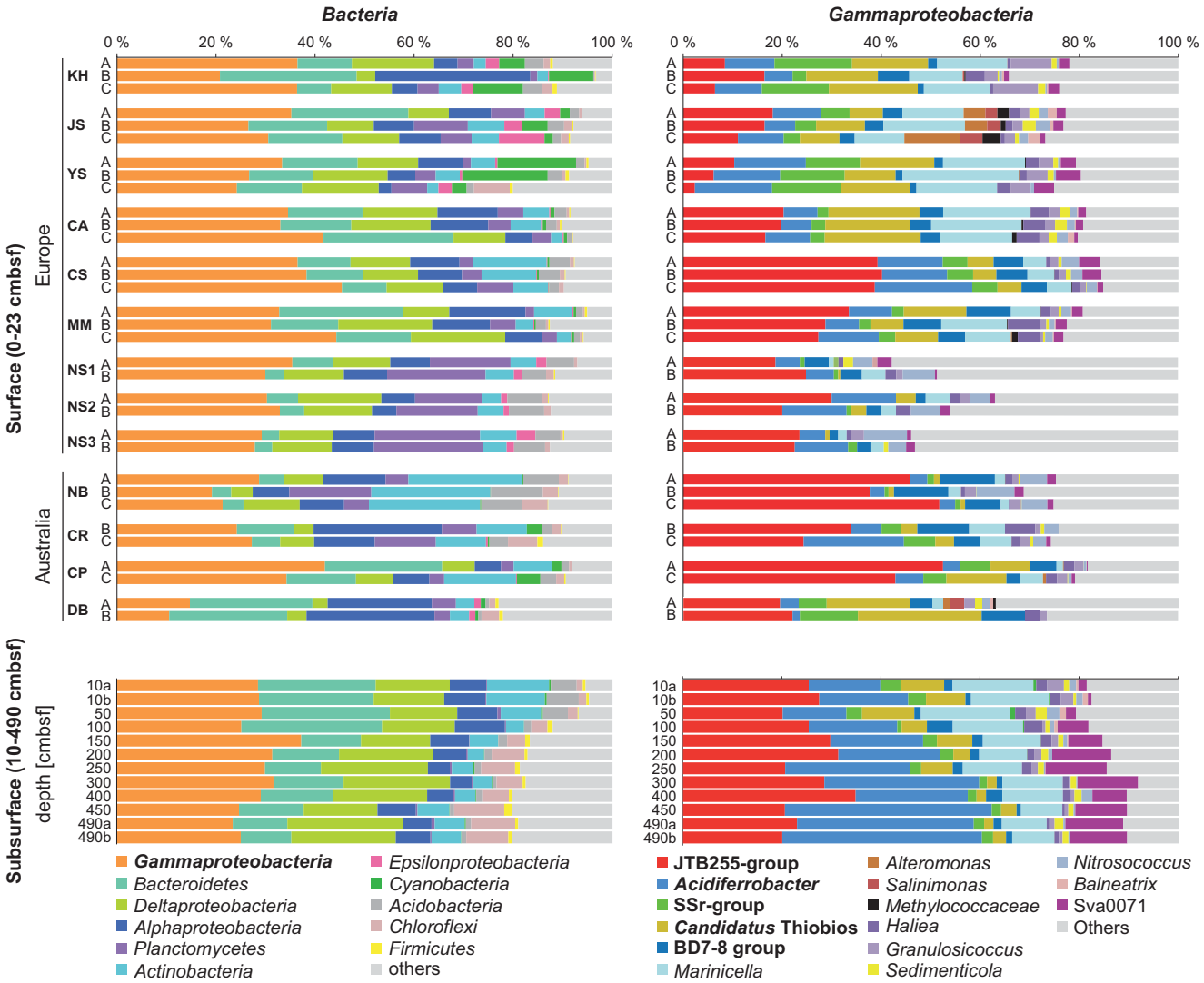

Figure 2 Relative abundances of the bacterial 16S rRNA gene sequence (V3–V4 regions) in the 13 coastal sediments. Left panels: relative sequence abundance of most frequent bacterial phyla and classes. Right panels: relative sequence abundance of most frequent taxonomic groups (family to order level) within the class of *Gammaproteobacteria* according to the taxonomy of SILVA release 117 (Pruesse *et al*., 2007). The five candidate chemolithoautotrophic clades are given in bold. Upper panels: relative sequence abundance in 13 coastal surface sediments from 0 to 23 cm below surface (cmbsf) max. A = uppermost sediment layer (0.5 or 1 cmbsf), B = sulfide transition zone, C = sulfidic layer. For the detailed depth ranges, see Supplementary Table S1. Lower panels: relative sequence abundance in subsurface sediments at site Janssand (JS) from 50 to 490 cmbsf. For comparison, samples from 10 cmbsf are included. Sediment samples from 10 and 490 cmbsf were run in duplicate (10ab, 490ab). All but one amplicons were pyrosequenced, samples from DB were sequenced via the Illumina MiSeq platform.

this, the JTB255 clade could not be detected with probe GAM42a in double hybridizations with the JTB-probe mix (Supplementary Table S2). Therefore, we summed up FISH counts of the JTB255-probe mix and of probe GAM42a to yield the total relative abundance of FISH-detectable *Gammaproteobacteria*.

FACS and scintillography of sorted cells

We developed a novel protocol to quantify bulk assimilation of radiolabelled substrates in a defined number of cells phylogenetically identified via CARD-FISH before for flow sorting. To minimize cell loss, the filters were handled extremely carefully during the CARD-FISH procedure. Cells were scraped off from membrane filters by using a cell scraper or membrane filters were vortexed in 5 ml of 150 mM NaCl containing 0.05% Tween-80 according to Sekar *et al.* (2004). Before flow cytometry, large suspended particles were removed by filtration through 8-µm pore-size filter (Sartorius, Göttingen, Germany) to avoid clogging of the flow cytometer.

Flow sorting of cells that were fluorescently labeled by CARD-FISH was performed using a FACSCalibur flow cytometer equipped with cell sorter and a 15-mW argon ion laser exciting at 488 nm (Becton Dickinson, Oxford, UK). Autoclaved Milli-Q water was used as sheath fluid. Cell sorting

was done at low flow rate of 12 ± 3 µl min^{-1} or med flow rate of 35 ± 5 µl min^{-1} with single-cell sort mode to obtain the highest purity. The event rate was adjusted with a fluorescence threshold, and sorting was performed at a rate of approximately 25–100 particles s^{-1}. Hybridized cells were identified on scatter dot plots of green fluorescence versus 90° light scatter (Supplementary Figure S1). Sediment background such as clay particles was determined by flow-cytometric analysis of sediment hybridized with a nonsense probe (NON338) (Supplementary Figure S1). For subsequent measurements, 50 000 cells were sorted and filtered onto 0.2-µm polycarbonate filters (GTTP, Millipore, Eschborn, Germany). Dead controls of ^{14}C incubated sediments did not show any measurable assimilation of ^{14}C, and were thus not used for cell sorting. Unspecifically adsorbed label in live samples caused only minor radioactive background as determined by spiking experiments with fluorescent beads and *Escherichia coli* cells (Supplementary Figure S2). Fluorescent beads (yellow-green, 1.0 µm, Polyscience, Warrington, PA, USA) or hybridized *E. coli* cells were flow-sorted from of a sample hybridized with the nonsense probe to determine radioactive background. *E. coli* cells were hybridized with EUBI-III probe beforehand. Beads and *E. coli* cells were mixed with the sample in approximately the same quantity as the target populations (10–20% of total cells). Two to three repeated sortings were applied to confirm the technical reproducibility from duplicate or triplicate incubations. Collected cell batches on polycarbonate filters were directly transferred into 5 ml scintillation vials and mixed with 5 ml Ultima-Gold XR (Perkin-Elmer, Boston, MA, USA) scintillation cocktail. Radioactivity of sorted cell batches was measured in a liquid scintillation counter (Tri-Carb 2900, Perkin-Elmer).

The purity of flow-cytometric enriched target cells was >93%, and was manually analyzed under an Axioplan epifluorescence microscope (Zeiss, Jena, Germany). For microscopic analysis, filters were counterstained with 1 µg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) and at least 1000 DAPI-stained cells were examined for CARD-FISH. Our approach was technically highly reproducible, and the radioactivity linearly increased with the number of sorted cells (Supplementary Figure S2).

For calculation of average cell-specific carbon fixation rates in our slurry experiments, we assumed a background concentration of dissolved inorganic carbon of 2 mM (Billerbeck *et al.*, 2006) as we used local seawater for our experiment (see Supplementary Information for calculations). The relative abundance of assimilating gammaproteobacterial cells in the sulfide transition zone from Calais, Courseulles-sur-Mer and Janssand sediments was determined by microautoradiography (MAR). MAR was performed according to Alonso and Pernthaler (2005) and Lenk *et al.* (2011) with an exposure time of 2 days. Relative abundance of MAR-positive cells was manually determined under an Axioplan epifluorescence microscope (Zeiss).

A single-cell genome of the SSr clade from Janssand sediment

In January 2011, the upper two centimetres of Janssand sediment were sampled for extraction and sorting of single bacterial cells for whole-genome amplification. After extraction, cells were cryopreserved with N,N,N-trimethylglycine ('glycine betaine') (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 4% according to Cleland *et al.* (2004), stored at −80 °C and shipped overseas. Single-cell sorting and whole-genome amplification via multiple displacement amplification were performed at the Bigelow Laboratory Single Cell Genomics Center (https://scgc.bigelow.org) as described by Swan *et al.* (2011). A single amplified genome (SAG) encoding a single, high quality 16S rRNA gene sequence affiliating with the SSr clade was sent to Max Planck Genome Centre (MP-GC) Cologne for MiSeq (Illumina) sequencing yielding 9 557 547 PE reads. The SAG assemblies were auto-annotated using the Joint Genome Institute IMG-ER pipeline (Markowitz *et al.*, 2012). Details on cell extraction, sequencing and quality control of the assembled genomic data are given as Supplementary information.

cDNA libraries and metatranscriptomic mapping

In April 2013, sediment was sampled from the sulfide transition zone at site Janssand and immediately frozen on dry ice. Total RNA was extracted from sediment in triplicates (one ml each) by the Vertis Biotechnologie AG (Freising, Germany), and bacterial rRNA was depleted with the Ribo-Zero Magnetic Kit (for *Bacteria*) (Epicentre, Madison, WI, USA). Barcoded RNA TrueSEQ libraries were constructed from RNA extractions and paired-end sequenced using Illumina HiSeq2000 (MP-GC, Cologne, Germany). After quality trimming at a Phred score 28 using Nesoni clip v.0.115 (http://www.vicbioinformatics.com/software.nesoni.shtml), reads were mapped to reference databases of nucleotide sequences encoding key genes for sulfur oxidation (Sox-multienzyme complex, *sox*B; reverse dissimilatory sulfite reductase, *dsr*AB; adenosine-5′-phosphosulfate reductase, *apr*A; uptake [NiFe]-hydrogenase, *hup*L; ammonia monooxygenase, *amo*A, ribulose-1,5-bisphosphate carboxylase/oxygenase form I, *cbbl,* and form II, *cbbm*; ATP citrate lyase, *acl*AB) and to the SAG using Bowtie2 (Langmead and Salzberg, 2012). Details on program settings and normalization are given as Supplementary information.

Nucleotide accession numbers

All nucleotide sequences obtained in this study have been deposited in GenBank. Sequences of 16S rRNA and hydrogenase gene libraries are available under

accession numbers KR824952–KR825244 and KR534775–KR534844, respectively. Amplicon sequences from the 16S rRNA gene surveys were deposited in NCBI BioProjects PRJNA283163 and PRJNA285206. All cDNA reads are available in BioProject PRJNA283210. Fosmid end sequences are available in NCBI's Genome Survey Sequences database (GSS) with the accession numbers KS297884–KS307053. The genome sequence of the SAG WSgam209 is accessible under the IMG Genome ID 2609459745 through the Joint Genome Institute portal IMG/ER (https://img.jgi.doe.gov/cgi-bin/er/main.cgi), the metagenomic bin *Acidiferrobacter*-a7 is accessible under the IMG Genome ID 2616644801.

Results and Discussion

Identification of candidate chemolithoautotrophs in coastal surface sediments

To identify candidate chemolithoautotrophs in coastal sediments, we studied the bacterial diversity in 10 tidal and 3 sublittoral sandy sediments from Western Europe and Australia (Figure 1 and Supplementary Table S1). We sequenced the V3–V4 region (>300 bp) of tagged 16S rRNA gene amplicons. After quality trimming, 311 196 Illumina- and 454-tag reads were recovered. Taxonomic classification revealed that *Gammaproteobacteria* were consistently among the most abundant clades on class to phylum level, accounting for 12–45% of sequences regardless of sampling site, sediment depth or season (Figure 2). These data were supported by CARD-FISH, which showed that *Gammaproteobacteria* make up 19–22% of all bacteria at sites Janssand, Calais and Courseulles-sur-Mer (Supplementary Table S5). At all sites, we observed a recurring diversity pattern also at the family to order level within the *Gammaproteobacteria*. We consistently identified candidate chemoautotrophs most closely related to: (1) *Acidiferrobacter thiooxydans* of the family *Ectothiorhodospiraceae*, (2) symbionts of the siboglinid tubeworms such as *Oligobrachia* spp. (henceforth designated as *Siboglinidae* Symbionts related, SSr, see Supplementary Figures S3 and S4), (3) ciliate symbiont *Candidatus* Thiobios zoothamnicoli and (4) BD7-8 clade, including the γ3 symbiont of the marine gutless oligochaete *Olavius algarvensis* (Woyke *et al.*, 2006). Sulfur-dependent chemolithoautotrophy for cultured or symbiotic relatives of these clades has been shown before (Rinke *et al.*, 2006; Lösekann *et al.*, 2008; Hallberg *et al.*, 2011; Kleiner *et al.*, 2012). *Acidiferrobacter thiooxydans* can also grow autotrophically with ferrous iron (Hallberg *et al.*, 2011). Moreover, we previously showed carbon fixation by the SSr- and the *Acidiferrobacter*-related clades, and determined relative cell abundances of up to 8% at site Janssand in the German Wadden Sea (Lenk *et al.*, 2011).

Strikingly, in all tested sediments up to 52% of gammaproteobacterial sequences grouped with the uncultured JTB255 clade. This clade is affiliated with the order *Xanthomonadales* and accounted for the largest fraction of gammaproteobacterial sequences at 10 out of 13 sites (Figure 2). In line with the sequence data, CARD-FISH targeting members of the JTB255 clade revealed rod-shaped cells (Supplementary Figure S5) that made up 3–6% of total cell counts in Janssand, Calais and Courseulles-sur-Mer sediments (Supplementary Table S6). So far, the exact environmental function of the JTB255 clade is unknown;however, a sulfur-oxidizing activity has been hypothesized (Bowman and McCuaig, 2003). In summary, we identified five candidate chemolithoautotrophs that accounted for 28–75% of *Gammaproteobacteria* and for 8–31% (average = 17%) of all bacterial sequences across all sites. Other potentially autotrophic populations such as sulfur-oxidizing *Epsilonproteobacteria*, anoxygenic phototrophs, the BD1-5/SN-2 clade, nitrifiers and cyanobacteria were found in low abundance or were patchily distributed (Figure 2).

Gammaproteobacteria in subsurface sediments

Because of the high sedimentation rates of >3 mm/year in the German Wadden Sea at site Janssand (Ziehe, 2009), a yet unknown fraction of the surface microbial community including the chemolithoautotrophic *Gammaproteobacteria* is buried into the anoxic subsurface. To study, how these organisms are affected by such a burial, we also analyzed the distribution of chemolithoautotrophic *Gammaproteobacteria* in a 490-cm-deep subsurface core from site Janssand, spanning a sedimentation record of 1000–2000 years (Ziehe, 2009). This sediment core displayed a typical sulfate-methane-transition zone in 150–200 cm below surface (cmbsf) (Gittel *et al.*, 2008), reflecting the changes in the major metabolic pathways that are active along this depth range. Surprisingly, *Gammaproteobacteria* including the chemolithoautotrophic gammaproteobacterial clades also accounted for a dominant fraction of 16S rRNA gene pyrotags over the entire depth range (21–37% of all sequences, Figure 2). This observation was supported by 16S rRNA gene libraries from 200 and 490 cmbsf (Supplementary Figure S4), in which these clades accounted for 95 out of 289 clones (33%). To gain PCR-independent support for the dominance of *Gammaproteobacteria* in subsurface sediments, we fosmid-cloned large metagenomic fragments of ~40 kb in size from 490 cmbsf and taxonomically classified the end sequences (Supplementary Table S7), as FISH is commonly too insensitive to comprehensively target subsurface organisms with very low ribosome content (Schippers *et al.*, 2005). In support of our 16S rRNA gene data, 24% of all prokaryote-affiliated fosmid end sequences ($n = 4052$) showed best hits to *Gammaproteobacteria*, while only approximately 1% affiliated with *Archaea* (Supplementary Table S7).

Collectively, these molecular data indicate a relatively stable community structure over the 5-m depth range, despite the measured strong biogeochemical gradients. These data are consistent with similar total cell abundances and a similar composition of major phospholipids over the entire 490 cm depth in the same sediment core (Gittel *et al*., 2008; Seidel *et al*., 2012).

Whether these gammaproteobacterial populations are active despite very different biogeochemical settings in the subsurface, or whether they are simply surviving in subsurface sediments with little or no turnover, are currently unclear. Upon burial, marine microbial cells may survive in the subsurface over geological time scales without significant growth (Jørgensen, 2011). Energy for maintenance and survival could be supplied by fermentation of refractory organic matter and by the slow transport and diffusion of dissolved organic compounds to subsurface sediments, which has been demonstrated for the subsurface at site Janssand (Røy *et al*., 2008; Seidel *et al*., 2012).

Global occurrence of candidate chemolithoautotrophic Gammaproteobacteria

Our 16S rRNA diversity and FISH data agree well with numerous studies showing a substantial contribution of *Gammaproteobacteria* to microbial communities in diverse marine surface sediments (Hunter *et al*., 2006; Kim *et al*., 2008; Schauer *et al*., 2009; Orcutt *et al*., 2011; Gobet *et al*., 2012; Ruff *et al*., 2015). To examine the geographic distribution of the five candidate chemoautotroph groups in more detail, we did a meta-analysis of 16S rRNA gene sequence data from 65 diversity studies of the sea floor (Figure 3). Although these published data sets hardly covered the extent of microbial diversity at the studied sites, sequences related to the *Acidiferrobacter*, SSr and to a lesser extent *Ca.* T. zoothamnicoli and BD7-8 groups were found in all types of benthic habitats ranging from intertidal sediments to deep-sea hydrothermal chimneys (Figure 3). Intriguingly, the JTB255 clade was detected in 92% of all studies (Figure 3) and accounted for the most frequent sequence group among *Bacteria* in Arctic, Antarctic and tropical deep-sea as well as shallow coastal sediments (Wang *et al*., 2013; Zheng *et al*., 2014; Liu *et al*., 2014; Emil Ruff *et al*., 2014). In temperate Tasmanian and in cold Antarctic coastal sediments, 16S rRNA gene copies of the JTB255 clade accounted for 6–9% of total bacterial 16S rRNA gene sequences (Bowman *et al*., 2005). In summary, our biogeographic survey shows that these five clades are important members of microbial communities in marine surface sediments worldwide, and clearly, the JTB255 clade is one of the most successful bacterial lineages in marine surface sediments.

Measuring dark carbon fixation by Gammaproteobacteria in sediments

Our 16S rRNA gene survey suggested that sulfur-oxidizing *Gammaproteobacteria* are potentially the major carbon fixers in dark coastal sediments.

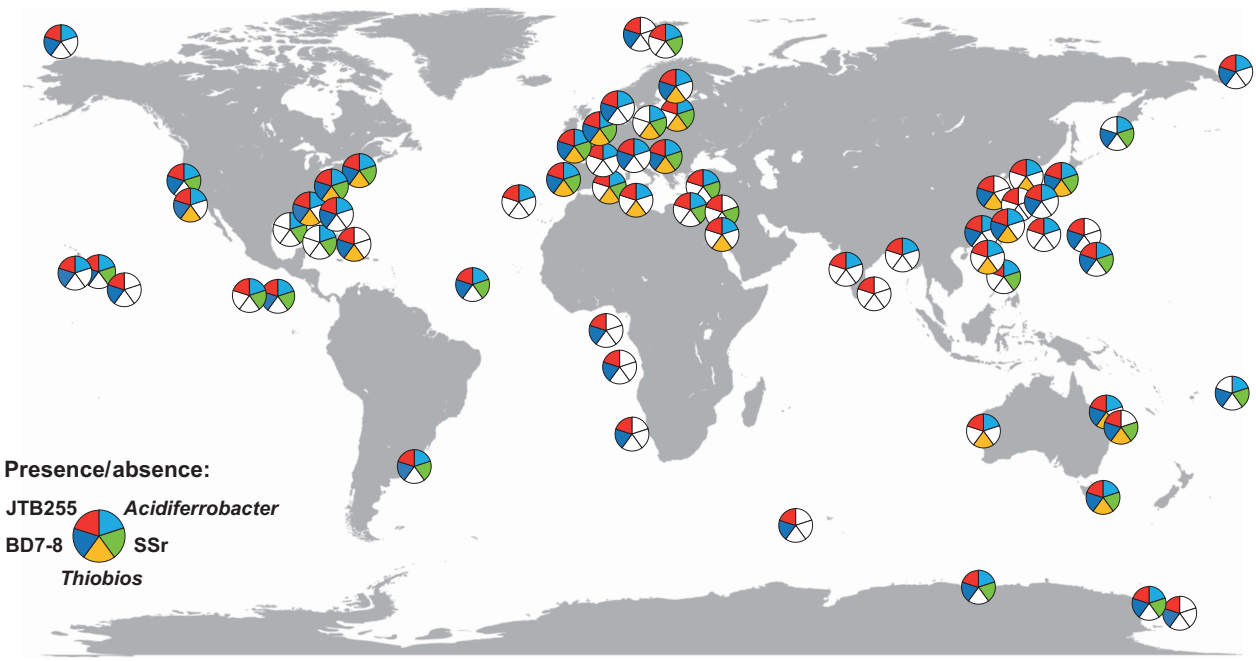

Figure 3 Biogeographic survey of the major chemolithoautotrophic gammaproteobacterial clades identified in this study. Only clone sequences (presence/absence) from bacterial diversity studies from 65 marine sea floor surfaces have been considered, deposited in the SILVA database release 117 (Pruesse *et al*., 2007).

However, quantitative data on carbon fixation by distinct bacterial populations in marine sediments are lacking. To determine the contribution of *Gammaproteobacteria* to dark carbon fixation in sediments, we developed a novel approach to quantify assimilation of a radiolabeled compound by specific populations. Previous FACS experiments with autofluorescent, radiolabeled marine bacterioplankton and subsequent scintillography of sorted populations prompted us to use FISH signals instead of autofluorescence or unspecific DNA staining to identify and enrich populations from sediments (Zubkov *et al.*, 2003; Jost *et al.*, 2008). We incubated aerobic sediment slurries prepared from surface and from sulfide transition zone sediments from sites Calais, Courseulles-sur-Mer and Janssand with ^{14}C-labeled bicarbonate and for 20 h in the dark. After detachment of cells from sand grains and CARD-FISH, we sorted fluorescently labeled *Bacteria* (probe EUBI-III) or *Gammaproteobacteria* (probe GAM42a). To account for potentially nitrifying autotrophic *Archaea*, we also sorted cells targeted by the archaeal probe Arch915. Fifty thousand cells were sorted per population, and bulk radioactivity was measured. This workflow allowed us to accurately quantify the bulk assimilation of radiolabeled substrates by a defined population in high throughput. At the analysis level of populations and communities it thereby overcomes limitations in throughput and precision of other methods such as MAR-FISH, HISH-SIMS and stable isotope probing (Boschker *et al.*, 1998; Lee *et al.*, 1999; Radajewski *et al.*, 2000; Manefield *et al.*, 2002; Musat *et al.*, 2008).

Although the total amount of fixed carbon in sorted populations varied between sites and samples, the ^{14}C-assimilation by sorted *Gammaproteobacteria* ranged from 2.4 to 10.3 Bq and was 2.5- to 5-fold higher than those of sorted *Bacteria* (0.5–2.1 Bq) (Figure 4a). At all three sites, the relative abundance of ^{14}C-assimilating *Gammaproteobacteria* was approximately 40–50% as determined by MAR (Supplementary Table S8; Lenk *et al.*, 2011) and is similar to the relative sequence frequency of chemoautotrophic subpopulations (Figure 2). The ^{14}C-radioactivity of 50 000 archaeal cells accounted for 1.4–3.8 Bq, ranging between the average assimilation by *Bacteria* and *Gammaproteobacteria* (Figure 4b). Addition of nitrate did not stimulate ^{14}C-assimilation in anoxic slurry incubations. The addition of 1 mM thiosulfate doubled the total carbon fixation by *Gammaproteobacteria* in the uppermost surface sediments, but not in the sulfide transition zone at Calais and Courseulles-sur-Mer (Figure 4a). Likewise, thiosulfate did not stimulate carbon fixation in the sulfide transition zone from site Janssand (Zerjatke, 2009). The oxidized surface sediments are possibly limited in electron donors, while the sulfide transition zone contained sufficient reduced sulfur compounds such as free and iron sulfides as energy sources for carbon fixation (Jansen *et al.*, 2009; Supplementary Table S1).

Dark carbon fixation in Gammaproteobacterial clades

To quantify carbon fixed by the candidate chemolithoautotrophic clades, we used the FISH probes available for three of the five clades for cell sorting and subsequent scintillography. Our FISH probes for the SSr and *Acidiferrobacter* clades mostly target sequences retrieved from site Janssand (Supplementary Figure S4; Lenk *et al.*, 2011); therefore, we measured carbon assimilation by specific subpopulations at this site. The *Acidiferrobacter* clade showed the highest ^{14}C-assimilation (6.1–10 Bq), while the SSr clade assimilated 2.7–6.9 Bq (Figure 4b

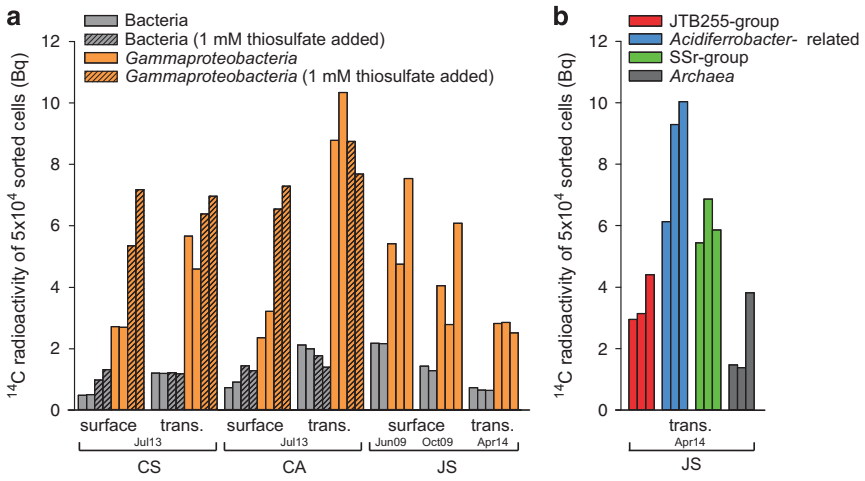

Figure 4 ^{14}C carbon fixed by *Bacteria, Gammaproteobacteria* and *Archaea* in three coastal sediments. Carbon fixation by flow-sorted populations of *Bacteria, Gammaproteobacteria* and *Archaea* from the uppermost sediment layer (surface, 0–1 cmbsf) and from the sulfide transition zone (trans.) incubated with ^{14}C bicarbonate. ^{14}C-assimilation by *Bacteria* and *Gammaproteobacteria* at three sampling sites (Courseulles-sur-Mer, Calais and Janssand) and in different seasons (**a**). ^{14}C-assimilation by three gammaproteobacterial clades and *Archaea* (**b**). Batches of 50 000 cells were sorted per measurement. The ^{14}C carbon activity is given in Becquerel (Bq).

and Supplementary Figure S6). This is consistent with the chemolithoautotrophic potential encoded in the corresponding genomes (Supplementary Table S5), and is confirmed by the previously detected ^{14}C bicarbonate assimilation by single cells of both clades (Lenk *et al.*, 2011).

Our probes for the ubiquitous JTB255 clade display a wider target range; therefore, we used these probes to sort JTB255 cells from Janssand, Calais and Courseulles-sur-Mer. The ^{14}C-assimilation by the JTB255 clade ranged from 3 to 4.4 Bq in Janssand sediment to up to 10.7 Bq in Calais sediment (Figure 4b and Supplementary Figure S6). The ^{14}C-assimilation by the JTB255 clade was slightly less than that of the SSr clade. The addition of thiosulfate did not stimulate the ^{14}C-assimilation by the JTB255 clade in Courseulles-sur-Mer but did slightly stimulate it in Calais sediments, which is consistent with the hypothesized thiotrophy of members of this clade (Bowman and McCuaig, 2003).

The ^{14}C-assimilation by the three gammaproteobacterial clades was up to 10-fold higher than the ^{14}C-assimilation by the entire bacterial community (Figures 4a and b), which largely consisted of heterotrophic bacteria (Figure 2). Using the carbon assimilated by the bulk bacterial community (targeted by probes EUBI-III) as an approximate reference for heterotrophic, anaplerotic carbon fixation (Wood and Werkman, 1936; Li, 1982; Roslev *et al.*, 2004), we conclude that heterotrophic carbon fixation was minor. Moreover, we calculated average carbon fixation rates per sorted gammaproteobacterial cell based on all sorted *Gammaproteobacteria* (1.1–3.0 fg C cell^{-1} day^{-1}, Supplementary Table S8). Average carbon fixation rates per cell and for each of the three individual subpopulations ranged from 1.1 to 3.5 fg C cell^{-1} day^{-1}. These rates are consistent with those of autotrophic freshwater green sulfur bacteria (1.4–5.8 fg C cell^{-1} day^{-1}) (Musat *et al.*, 2008), and are in the lower range of rates measured for autotrophic marine bacterioplankton (3.5–24.7 fg C cell^{-1} day^{-1}) (Jost *et al.*, 2008). Collectively, these data strongly support an autotrophic carbon fixation by the *Acidiferrobacter*, SSr and JTB255 clades.

Gammaproteobacteria dominate dark carbon fixation in coastal sediments

The relative contribution of carbon fixed by the *Acidiferrobacter*, SSr and JTB255 clades amounted to 77% of gammaproteobacterial and to 50–62% of bacterial dark carbon fixation at Janssand (Figure 5). Although they make up only 19–22% of the total microbial community, *Gammaproteobacteria* in total accounted for 70–86% of the microbial dark carbon fixation irrespective of sampling site, season and sediment depth (Figure 5 and Supplementary Figure S7).

Although they can be important autotrophs in organic-poor deep-sea sediments (Molari *et al.*, 2013)

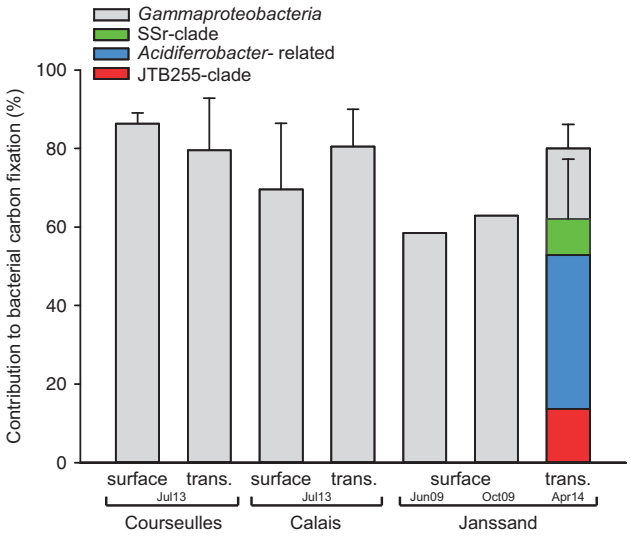

Figure 5 Relative contribution of *Gammaproteobacteria* to total bacterial dark carbon fixation in three coastal sediments. The relative contribution of carbon fixed by total *Gammaproteobacteria* and by the *Acidiferrobacter*, SSr and JTB255 clade to total bacterial dark carbon fixation was calculated by integrating the ^{14}C-carbon assimilation per population (dark gray bar) and the relative cell abundances of the respective populations in the sediments. Error bars represent the standard deviation (s.d.) of 3–4 replicate incubations. Values from 2009 (site Janssand) are depicted as average of duplicates only. Note that for experiments in 2009 ^{14}C-assimilation by the JTB255 clade was not measured (for details, see Supplementary Information).

nitrifying *Archaea* have a minor role in dark carbon fixation in the sediments we measured. In our study, *Archaea* occurred at low relative abundances and assimilated less ^{14}C than *Gammaproteobacteria* (Figures 4a and b and Supplementary Figure S7).

Genomics suggests thioautotrophy in uncultured Gammaproteobacteria

We previously showed that key genes of sulfur oxidation in Janssand sediments are mainly affiliated with *Gammaproteobacteria* (Lenk *et al.*, 2011). To further investigate the metabolism of the candidate chemolithoautotrophic *Gammaproteobacteria*, we sequenced the amplified genomic DNA of a single cell of the SSr clade from Janssand sediment. In addition, we recovered a metagenomic bin of a member of the *Acidiferrobacter* clade from a deep-sea hydrothermal chimney that displayed 91% sequence identity to 16S rRNA gene sequences from site Janssand (Supplementary Figure S4).

The assembled SAG of the SSr cell ('WSgam209') consists of 1.9 Mbp on 311 scaffolds (Supplementary Table S3). In addition to the cytochrome c oxidase for oxygen respiration, the genome encoded a reverse dissimilatory adenosine-5′-phosphosulfate (APS)-reductase subunit A (AprA), a widely distributed enzyme catalyzing the oxidation of sulfite to sulfate (Meyer and Kuever, 2007). Similar to the 16S rRNA gene also the AprA is affiliated with the

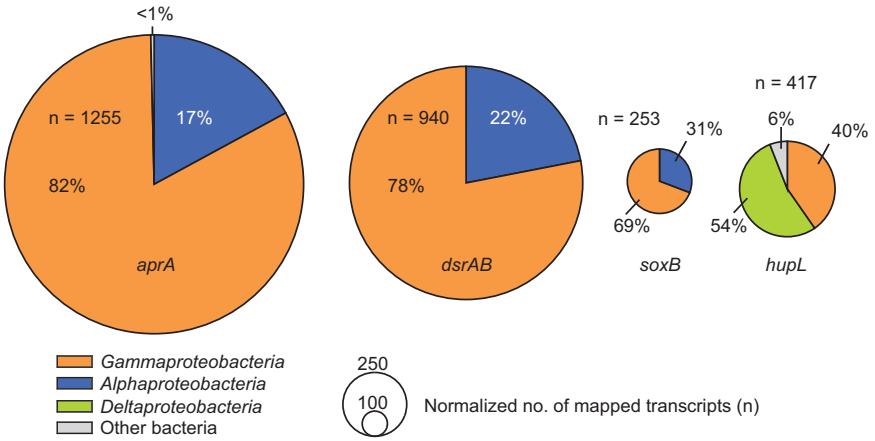

Figure 6 Taxonomic affiliation of metatranscriptomic reads of key genes from sulfur and hydrogen oxidation pathways. Metatranscriptomic reads from the sulfide transition zone (Janssand surface sediments 2–3 cmbsf) were mapped to key genes of sulfur and hydrogen oxidation genes (*aprA*, adenosine-5′-phosphosulfate reductase; *dsrAB*, reverse dissimilatory sulfite reductase; *soxB*, Sox-multienzyme system; uptake [NiFe]-hydrogenase). Metatranscriptomes were sequenced in triplicates, and the average values are displayed. Transcript abundance is normalized for gene length and number of reads per data set.

sulfur-oxidizing symbionts of *Oligobrachia haakonmosbiensis* (Supplementary Figures S4 and S8), indicating a congruent phylogeny of both phylogenetic markers. Moreover, it encoded the large and small subunits of the RuBisCO form I (CbbL, CbbS) (Supplementary Table S5).

The metagenomic bin of the *Acidiferrobacter*-clade organism ('*Acidiferrobacter*-a7') consists of 2 Mbp on 66 scaffolds (Supplementary Table S3). It contained genes for thiosulfate oxidation (Sox-multienzyme complex, *soxABXYZ*), sulfite oxidation (*soeABC*) and carbon fixation (RuBisCO form I subunits *cbbL* and *cbbS*) (Supplementary Table S4). Together with the measured carbon fixation of SSr and the *Acidiferrobacter* clades at site Janssand, the identification of sulfur oxidation and carbon fixation genes in both clades provides the genetic background of their chemolithoautotrophic potential.

Metatranscriptomics underscores the role of Gammaproteobacteria in thioautotrophy

To further test, whether *Gammaproteobacteria* in these sediments are the major active chemolithoautotrophs, we sequenced triplicate metatranscriptomes from the sulfide transition zone of Janssand sediments. Transcript reads were mapped to reference sequences from the GenBank database, functional gene libraries, metagenomic fragments and the SSr-single cell genome recovered from site Janssand (this study; Lenk *et al.*, 2011, 2012).

To identify the expressed carbon fixation pathways, we mapped the metatranscriptomic reads to gene sequences of RuBisCO form I and form II (*cbbL*, *cbbM*) and of ATP citrate lyase (*aclAB*) from cultured representatives and environmental sequences from diverse aquatic environments including sediments. All transcripts of RuBisCO genes, on average 112 reads, mapped to those of sulfur-oxidizing *Gammaproteobacteria,* confirming active carbon fixation through the CBB-cycle in Janssand sediments. Only very few transcripts ($n \leqslant 6$) could be mapped on genes encoding an epsilonproteobacterial ATP citrate lyase (Supplementary Table S4), reflecting the low relative abundance and low activity of sulfur-oxidizing *Epsilonproteobacteria* in these sediments (Figure 2; Lenk *et al.*, 2011).

To identify the expressed sulfur oxidation pathways and the respective organisms in the sulfur transition zone in Janssand sediments, we mapped the metatranscriptomic reads to references databases containing gene sequences encoding SoxB, AprA and subunits of the reverse dissimilatory sulfite reductase, DsrAB. The major fraction (69–82%) of transcripts mapped to *dsrAB, soxB* and *aprA* sequences that are affiliated with *Gammaproteobacteria* (Figure 6 and Supplementary Figure S8), further supporting their central role in chemolithoautotrophy. Here, *dsrAB* transcripts were fourfold higher than *soxB* transcripts. The identification of the sulfur oxidation pathway prevailing in marine sediments has important implications for modeling of carbon budgets, as the reverse Dsr (rDsr) pathway may allow a higher ATP gain that can be used for carbon fixation than the complete Sox-multienzyme complex (Klatt and Polerecky, 2015). In the rDsr pathway electrons finally enter the electron transport chain at the level of quinone (Holkenbrink *et al.*, 2011), while the Sox-multienzyme complex donates electrons to cytochrome c (Kelly *et al.*, 1997), probably resulting in higher energy yields for rDsr-encoding sulfur oxidizers. However, further *in situ* studies are essential to confirm this observation.

Very few reads mapped to bacterial ($n \leqslant 2$) and archaeal ($n \leqslant 5$) *amoA* genes encoding the ammonia monooxygenase subunit A (AmoA) (Supplementary Table S1). This is consistent with the minor role of *Archaea* in carbon assimilation measured in our

study (Supplementary Figure S7), and is also consistent with the very low nitrification rates measured at site Janssand (Marchant *et al.*, 2014). Overall our data confirm previous biogeochemical models suggesting a low impact of nitrification on chemolithoautotrophic production in coastal sediments (Middelburg, 2011; Boschker *et al.*, 2014).

Hydrogen is likely an alternative energy source for sulfur-oxidizing Gammaproteobacteria

Recently, uptake [NiFe]-hydrogenase genes were found in metagenomic bins of *Gammaproteobacteria* from estuarine sediments, indicating alternative energy sources for dark carbon fixation under oxic to suboxic conditions (Baker *et al.*, 2015). As the ability to oxidize hydrogen has been shown to confer metabolic plasticity to symbiotic and pelagic sulfur-oxidizing bacteria (Petersen *et al.*, 2011; Anantharaman *et al.*, 2013; Hansen and Perner, 2015), we tested whether hydrogen could serve as an alternative energy source also for gammaproteobacterial sulfur oxidizers in marine sediments, for example, to respire sulfur under anoxic conditions (Laurinavichene *et al.*, 2007). To overcome the lack of reference sequences from marine sediments, we constructed an uptake [NiFe]-hydrogenase gene library from Janssand sediments. The recovered hydrogenase gene diversity comprised different physiological groups from phyla such as *Proteobacteria* and *Bacteroidetes* (Supplementary Figure S9). A substantial fraction (40%) of all hydrogenase transcripts were assigned to sequences of diverse *Gammaproteobacteria,* in particular to those grouping with sulfur-oxidizing bacteria (Figure 6 and Supplementary Figure S9). Expression levels of hydrogenase genes were lower than those of sulfur oxidation genes, but were in the same order of magnitude (Figure 6).

To link a potential hydrogen-oxidizing activity with sulfur-oxidizing *Gammaproteobacteria*, we searched for co-localization and co-expression of key genes of both pathways. First, we identified a metagenomic fragment from Janssand, affiliated with *Gammaproteobacteria,* which encoded both the uptake [NiFe]-hydrogenase HupSL and the rDsr operon (Supplementary Figure S10). Notably, the SAG of the SSr-group recovered from site Janssand also encodes an uptake [NiFe]-hydrogenase gene in addition to *aprA* and *cbbLS*. Moreover, these genes were among the top 20 transcribed genes out of 2008 identified genes (Supplementary Figure S11).

Collectively, our single-cell genomic, metagenomic and metatranscriptomic data indicate that *Gammaproteobacteria* in marine surface sediments may use both reduced sulfur species and hydrogen as energy sources for carbon fixation. In fact, the thioautotroph *Sulfurimonas denitrificans* grows more efficiently with hydrogen than with thiosulfate, when the electron acceptor nitrate is limiting (Han and Perner, 2014). Hence, hydrogen oxidation could be a hitherto overlooked energy source for carbon fixation in marine sediments.

Key functions of ubiquitous chemolithoautotrophic Gammaproteobacteria in sediments

Overall, our molecular and ^{14}C-assimilation data suggest that rather than a single group, a stable assemblage of *Gammaproteobacteria* drives dark carbon fixation in coastal surface sediments. In particular, we showed that members of the *Acidiferrobacter*, the JTB255 and the SSr clade occur in sediments worldwide and fix carbon in rates similar to those of uncultured sulfur-oxidizing bacteria from other aquatic habitats. Our genomic and metatranscriptomic evidence supports the previous assumption that chemolithoautotrophy in marine surface sediments is mainly driven by sulfur oxidation (Middelburg, 2011). However, the expression of uptake [NiFe]-hydrogenases in the SSr clade and other *Gammaproteobacteria* suggests that these organisms may also use hydrogen as an energy source for carbon fixation. On the basis of our data, we cannot exclude the possibility that other chemolithoautotrophic pathways such as ferrous iron oxidation also contributed to dark carbon fixation, but these are probably minor in organic- and sulfide-rich systems. Molecular, isotopic and physiological studies will be critical to determine, how other chemoautotrophic processes such as nitrification, metal oxidation, sulfur disproportionation (Jørgensen, 1990) and possibly also hydrogen-dependent sulfate respiration (Boschker *et al.*, 2014) contribute to dark carbon fixation in marine sediments. Intriguingly, sulfur-oxidizing, autotrophic *Gammaproteobacteria* including members of the SSr clade were recently shown to be associated with heterotrophic, electrogenic 'cable bacteria'. These were hypothesized to use cable bacteria as an electron sink during autotrophic sulfur oxidation (Vasquez-Cardenas *et al.*, 2015). Considering their cosmopolitan distribution, their metabolic lifestyle and their ecological importance, the *Acidiferrobacter*-, the JTB255-, and the three symbiont-related clades may be benthic counterparts to the gammaproteobacterial SUP05 clade, key organisms for sulfur and carbon cycling in hydrothermal plumes and OMZs (Canfield *et al.*, 2010; Wright *et al.*, 2012; Anantharaman *et al.*, 2013; Glaubitz *et al.*, 2013).

Role of chemolithoautotrophic gammaproteobacteria in carbon cycling

Coastal sediments are global hot spots of carbon cycling. The importance of marine vegetation such as sea grass, salt marshes and mangroves for carbon sequestration is already well established (Duarte *et al.*, 2005), but the role of coastal sediments as hot spots of microbial dark carbon fixation was only recently realized (Middelburg, 2011). According to our most conservative estimate, 70% of dark carbon

fixation in coastal sediments are driven by chemolithoautotrophic *Gammaproteobacteria* (Figure 5). These could fix 122 Tg C/year in Earth's coastal sediments (assuming a total of 175 Tg/year, from Middelburg 2011), which is similar to the 111 Tg carbon buried yearly by marine vegetated habitats worldwide (Duarte *et al.*, 2005). It is still unclear whether significant amounts of carbon fixed in the dark are buried into subsurface sediments. However, because identical/almost identical chemolithoautotrophic *Gammaproteobacteria* are frequently found in surface and subsurface sediments, they may have the potential to trap inorganic carbon and survive for centuries in subsurface sediments by tapping yet unknown sources of energy. Understanding whether the buried populations are in a state of dynamic equilibrium or whether they merely survive will be essential for assessing their role as a carbon sink.

Even though chemolithoautotrophy in shelf sediments mostly represents a 'secondary production', as it is ultimately based on energy from recycled organic matter (Middelburg, 2011), it may mitigate carbon (and sulfide) emissions from re-mineralized organic matter already at sediment surfaces. As marine sediments are the main site of global carbon sequestration, it is imperative to understand the processes and microorganisms that govern rates of burial of organic and inorganic carbon in these habitats. Here, our study provides first detailed insights into the microbiology of a largely overlooked aspect of the marine carbon cycle and highlights the environmental importance of widely distributed chemolithoautotrophic, most likely sulfur-oxidizing *Gammaproteobacteria*. As hypoxic events will expand and intensify in a warming ocean, sulfur-dependent carbon cycling will be more prevalent not only in pelagic OMZ, but also in organic-rich coastal sediments. Thus, sulfur-oxidizing and carbon-fixing microorganisms may have an increasingly important role in attenuating the rising emissions of sulfide and inorganic carbon to ocean waters and ultimately to the atmosphere.

Conflict of Interest

The authors declare no conflict of interest.

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