"Bacterial chemoautotrophic communities in the deep Atlantic Ocean"
Danksagung


„Etwas was du lernst kann dir niemand mehr nehmen und du lernst nur für dich.“ Diese Worte meiner Oma leiteten mich während meines Studiums. Danke!

Acknowledgements

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Abstract

The dark ocean is a harsh environment where available energy and organic carbon sources for microorganisms are scarce. However, a large pool of dissolved inorganic carbon (DIC) is present there that can be potentially fixed through chemosynthesis by bacteria leaving the question on the available energy sources. Several reduced inorganic substrates are potentially available as energy source to fuel dark bacterial autotrophic carbon fixation such as ammonia, hydrogen and sulfur. The aprA gene encodes for the alpha subunit of the adenosine-5′-phosphosulfate (APS) reductase, responsible for either oxidizing or reducing sulfur. The oxidizing pathway might represent an energy source for the Calvin-Benson-Basham (CBB)-cycle in the dark ocean. In this study, cbbM, encoding RubisCO form II in Bacteria, and aprA gene abundance were determined along a transect in the Atlantic from 64°N to 50°S to quantify the potential for bacterial chemoautotrophy throughout the water column from the epi- to the bathypelagic realm. Both cbbM and aprA gene abundance were highest in the mesopelagic zone (200 - 1000 m), coinciding with the oxygen minimum layer, and upper bathypelagic (1000 - 2000 m) throughout the Atlantic. Moreover, bacterial autotrophic genes were relatively more abundant in oligotrophic regions, such as the North and South Atlantic Gyre and the Western Tropical Atlantic, as compared to high latitude regions. The co-occurrence of RubisCO and aprA genes in the same bacterial lineages affiliated to Gammaproteobacteria and Deltaproteobacteria subphyla indicates the potential of using sulfur oxidation as an energy source for CO₂ fixation through the CBB cycle in these widespread groups. Taken together, our results provide evidence that DIC fixation by chemoautotrophic microbes represents a potential alternative source of organic carbon for the deep ocean heterotrophs, especially in the oligotrophic gyral and tropical regions.

Key words: sulfur oxidation, adenosine-5′-phosphosulfate (APS) reductase, RubisCO, chemoautotrophy, Bacteria
1 Introduction

The ocean is a heterogeneous environment, with microbial hotspots of activity and abundance throughout the water column (Reinthaler et al., 2010), associated to local inputs of biologically available organic matter, such as copepods' fecal pellets (Tang et al., 2005) or particles (Baltar et al., 2009). Organic carbon is a limiting resource for heterotrophic prokaryotic growth in the dark ocean (>200 m deep) since only 1-40% of the epipelagic primary production is exported into the dark ocean below 200 m depth (Ducklow et al., 2001). Indeed, the measured heterotrophic bacterial carbon demand of the deep ocean is larger than the estimated input from epipelagic particulate organic carbon (POC) (Banse, 1990; Reinthaler et al., 2006). Consequently, there must be other sources of energy and carbon available to sustain bacterial metabolism in the ocean’s interior. Recent studies indicate that dissolved inorganic carbon (DIC) fixation driven by chemical energy might represent an alternative, non-sinking source of organic carbon in the deep ocean (Reinthaler et al., 2010; Varela et al., 2011; Sintes et al., 2013).

Chemolithoautotrophic Bacteria are ubiquitously present in the dark ocean (Aristegui et al., 2009; Swan et al., 2011). The main pathway of chemoautotrophic carbon fixation is the Calvin-Benson-Basham (CBB) cycle (Tabita, 1988; 1999; Atomi, 2002). The key enzyme in this pathway is ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) forms I and II encoded by the cbbL and cbbM gene, respectively (Tabita, 1988; Cleland et al., 1998; Swan et al., 2011). The RubisCO form II is used in environments with high CO$_2$ and low O$_2$ levels, while RubisCO form I works better in low CO$_2$ and high O$_2$ concentration environments (Tabita, 1988; Badger and Bek, 2008).

Microbial chemosynthesis can potentially use a variety of energy sources, i.e., electron donors, such as ammonium, methane, reduced sulfur and hydrogen (Aristegui et al., 2009; Swan et al., 2011; Walsh et al., 2009; Reinthaler et al., 2010; Anantharaman et al., 2013; Sintes et al., 2013). Particularly, chemosynthesis based on the oxidation of reduced sulfur compounds is widely distributed among diverse marine environments such as hydrothermal vent plumes and oxygen minimum zones (OMZs) (Walsh et al., 2009; Anantharaman et al., 2013). In particular, the OMZs are widespread in the oceans, frequently expanding vertically for a few hundred meters in the mesopelagic zone (Ulloa et al., 2012). However, the extent to which reduced sulfur is used as an electron donor or energy source in the oxygenated oceanic water column remains poorly investigated. Reduced sulfur is low in concentration to
undetectable outside upwelling regions and the vicinity of hydrothermal vents (Walsh et al., 2009; Radford-Knoery et al., 2001). A potential source for reduced sulfur in the otherwise oxygenated water column might be marine snow where sulfide can be generated in oxygen depleted or anoxic microzones (Shanks and Reeder, 1993). Different forms of reduced sulfur compounds might be available as energy source for chemoautotrophic prokaryotes in the ocean (Ghosh and Dam, 2009).

The adenosine-5’-phosphosulfate pathway involves the enzymes adenosine-5’-phosphosulfate (APS) reductase and adenosine triphosphate (ATP) sulfurylase (Kappler and Dahl, 2011). APS reductase, comprising an alpha- and a beta-subunit (Lampreia et al., 1994; Hipp et al., 1997; Fritz et al., 2000; Meyer and Kuever, 2007), is the key enzyme in the sulfate reduction pathway of sulfate-reducing prokaryotes. However, this enzyme is also found in sulfur-oxidizing prokaryotes, where it catalyzes the transformation of sulfite to sulfate (Hipp et al., 1997; Meyer and Kuever, 2007).

The aim of this study was to determine the distribution and phylogenetic affiliation of DIC-fixing Bacteria in the Atlantic Ocean from 64°N to 50°S throughout the water column and hence covering several Atlantic oceanographic provinces. We also explored the potential of these chemoautotrophic Bacteria to use reduced inorganic sulfur as an energy source. Consequently, the study focused on the functional genes cbbM and aprA (APS reductase alpha subunit) using quantitative polymerase chain reaction (q-PCR). Also, the phylogeny of DIC-fixing sulfate reducing or oxidizing Bacteria in the water column was analyzed by cloning and sequencing.

2 Materials and Methods

Characteristics of the sampling area and sample collection – Water samples were collected on three research cruises (GEOTRACES 1 - 3) along a latitudinal transect in the Atlantic Ocean from 64°N to 50°S (Fig. 1). Water samples were taken using R/V Pelagia (GEOTRACES 1 - 2) during April and July 2010 and RSS James Cook (GEOTRACES 3) during February - March 2011 and a CTD (conductivity-temperature-depth;
Seabird, Bellevue, WA, USA) rosette sampler equipped with 25 L Niskin bottles and sensors for chlorophyll fluorescence, turbidity and oxygen.

Water samples for microbial DNA analysis (2 - 10 L) were collected at 6 to 8 depths per station, filtered onto 0.2 μm pore-size polycarbonate filters, immediately frozen in liquid N₂ and stored at -80°C. Samples for contextual abiotic and biotic parameters were taken at 24 depths per station. Inorganic nutrient concentrations and prokaryotic cell abundance were determined as described previously (De Corte et al., 2012; Sintes et al., 2013).

Four pelagic zones were distinguished throughout the depth profiles: epipelagic (50 - 200 m) (E), mesopelagic (200 - 1000 m) (M), upper bathypelagic (1000 - 2000 m) (UB) and lower bathypelagic (2000 - 4000 m) (LB). Moreover, six oceanic provinces were encountered and discriminated along the latitudinal transect following Longhurst (1998): North Atlantic Arctic (ARCT; 70°N - 55°N), North Atlantic Drift (NADR; 55°N - 40°N), North Atlantic Gyre (NAG; 40°N - 12°N), Western Tropical Atlantic province (WTRA; 12°N - 6°S), South Atlantic Gyre (SATL; 6°S - 40°S), and Subantarctic (SANT; 40°S - 55°S) (Fig.1).

**DNA extraction and amplification** – DNA was extracted from the filters using the Mobio UltraClean Soil DNA Isolation Kit following the manufacturer’s protocol.

Two cbbM genes encoding for Rubisco form II named cbbM65 and cbbM68 (Swan et al., 2011), and one aprA gene encoding for the alpha subunit of APS reductase were amplified using polymerase chain reaction (PCR). The primer set used to amplify the aprA gene targeted both sulfate-reducing as well as sulfur-oxidizing prokaryotes (Blazejak et al., 2006; Meyer and Kuever, 2007).

The PCR amplification mix consisted of the specific primer pair (Table 1) for each gene (1 mM), 1 μL of DNA extract (9 - 25 ng of DNA), MgCl (2.5 mM), PicoMax Polymerase (1 U), 1x PicoMax polymerase buffer, bovine serum albumin (BSA) (0.2 μM), deoxyribonucleoside triphosphate (dNTPs) (0.2 mM) and sterile water filled up to a total volume of 25 μl. The PCR cycles consisted of an initial denaturation step (94°C for 5 min) followed by 30 cycles of denaturation at 94°C for 1 min, annealing at the specific primer temperature (Table 1) for 1 min, and extension at 72°C for 1 min. The PCR was finalized with a final extension at 72°C for 10 min and cooling down at 4°C.
Cloning and sequencing of cbbM65, cbbM68, aprA gene – The microbial DNA from three different depth layers (mesopelagic, upper bathypelagic and lower bathypelagic) of station 33 (Fig. 1) was used to characterize the RubisCO- and aprA-harboring bacterial community. The amplified PCR products obtained as described above were checked on a 2% agarose gel. Thereafter, they were purified using 5 Prime PCRExtract Mini Kit, and the purified DNA concentration was determined on a Nanodrop 2000 spectrophotometer. Subsequently, an adenine-tail extension was conducted on the purified DNA at 72°C for 15 min using Taq Polymerase (Fermentas). The amplified DNA was inserted in a vector and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Approximately 50 colonies were picked randomly after blue-white screening. These colonies were checked by gel electrophoresis in order to ensure the proper size of insert after PCR amplification using the vector primers M13F/M13R (Table 1). Subsequently, the clones containing the inserts of cbbM and aprA genes were submitted to GATC Biotech for Sanger sequencing.

Phylogenetic analysis – All phylogenetic and evolutionary analyses were conducted using MEGA5 software (Tamura et al., 2011). The PCR amplified sequences were uploaded to ExPASy (Expert Protein Analysis System, Swiss Institute of Bioinformatics (SIB)) to trim and decrease redundancy. Gene sequences with ≥ 97% similarity were clustered into operational taxonomic units (OTUs) (Bairoch and Apweiler, 2000). The representative sequences of the OTUs were aligned together with known published sequences from NCBI using ClustalW. The evolutionary distances and history were computed using Maximum Composite Likelihood and the neighbor-joining method, respectively (Tamura et al., 2011). The bootstrap consensus tree was inferred from 100 replicates (Felsenstein, 1985). The resulting phylogenetic tree was generated with iTOL (Leutnic and Bork, 2006 & 2011).
Quantitative-PCR (q-PCR) – Q-PCR was conducted in triplicate for all functional genes on a LightCycler 480 SW 1.5 (Roche). Internal standards for the different genes were prepared using plasmids from the clone libraries containing the target gene fragments using the primers and PCR conditions as given in Table 1. A serial dilution from $10^7$ to $10^2$ copies of the purified PCR product was prepared for the different genes based on the DNA concentration and the fragment size. One µL of standards, blanks and DNA extract were added to a 96-well q-PCR plate (Bio-Rad) containing 9 µL of reaction mix per well. The well was covered with an optical tape (Bio-Rad). The reaction mix for each well contained: 1x Mastermix (LightCycler 480 SYBR Green I Master, Roche), the specific primers (Table 1) (0.5 µM) and enough sterile DNase-free water to give a total volume of 9 µl. The q-PCR for cbbM was composed of an initial denaturation step at 95°C for 10 min and 50 amplification cycles consisting of denaturation at 95°C for 5 s, annealing at the specific primer temperature (Table 1) for 5s, extension at 72°C for 15 s and plate read at 76°C for 3 s. The average efficiency of the q-PCR for the cbbM65 gene was 73% and for cbbM68 gene 91%. The q-PCR cycling for aprA consisted of an initial denaturation step at 95°C held for 10 min, followed by 50 amplification cycles consisting of denaturation at 95°C for 5 s, annealing at 60°C for 15s, extension at 72°C for 30 s and plate read at 80°C for 3 s. The average efficiency of the q-PCR for aprA gene was 81%. The specificity of the q-PCR reaction for the different genes was checked with melting curve analyses (65 - 95°C).

The contribution of chemoautotrophic Bacteria containing cbbM or aprA genes to the bulk bacterial community was assessed by determining the ratio between the abundance of these functional genes and the abundance of the gene recA. The recA gene is encoding for recombinase A, an enzyme involved in DNA repair, and it is present in all known prokaryotic cells in one single copy (Miller and Kokjohn, 1990; Lin et al., 2006). The recA gene abundance was measured on a different aliquot of extracted DNA from the same samples used for quantifying cbbM and aprA (Steiner, 2013). Due to the possibility that the extraction efficiency between replicate DNA extracts vary, the results presented here should be taken with caution. The recA gene abundance will be determined on the same DNA extracts as used for functional gene abundance in the near future.

Statistical analysis – A one-way ANOVA followed by a post hoc Dunn’s test on ranks was conducted to elucidate the differences between the different biogeographic provinces and depth layers for the two genes (cbbM and aprA). The results were considered
to be statistically significant at \( p<0.05 \). The Spearman Rank Order correlation was carried out using the SigmaPlot11 software to examine the influence of the different biotic and abiotic factors on the distribution of the \( cbbM \) and \( aprA \) genes. The distance-based multivariate analysis for a linear model using forward selection (DISTML forward) was applied to test the relationship of the genes \( cbbM \) and \( aprA \) with the biotic and abiotic factors.

### 3 Results

**\( cbbM65, cbbM68 \) and \( aprA \) gene abundance determined by q-PCR**

In the epipelagic waters, maximum gene abundance of \( cbbM65 \) was found in the SANT with an average of \( 5.97 \times 10^2 \pm 1.01 \times 10^3 \text{ mL}^{-1} \) (Fig. 2A, Fig. 6A). The gene abundance of \( cbbM65 \) in the NADR province (\( 6.20 \times 10^1 \pm 5.29 \times 10^1 \text{ mL}^{-1} \)) was not significantly different from that in the SATL (\( 4.82 \pm 5.50 \text{ mL}^{-1} \)) (one way ANOVA on rank, \( p>0.05 \)). The lowest abundance of \( cbbM65 \) genes was detected in the NAG province (\( 1.06 \pm 7.70 \times 10^{-1} \text{ mL}^{-1} \)) (Fig. 2A, Fig. 6A).

In the mesopelagic layer, \( cbbM65 \) gene abundance in the WTRA (\( 2.18 \times 10^3 \pm 1.27 \times 10^3 \text{ mL}^{-1} \)) was similar to that in the SANT province (\( 7.76 \times 10^2 \pm 9.16 \times 10^2 \text{ mL}^{-1} \)) (one way ANOVA on rank, \( p>0.05 \)) as shown in Fig. 2B-D and Fig. 6A. The \( cbbM65 \) gene abundance was significantly higher in the mesopelagic of WTRA and SANT provinces than in the ARCT, NADR, NAG and SATL provinces (one way ANOVA on ranks, \( p<0.05 \)) (Fig. 2B, Fig. 6A). In the bathypelagic layers, lower \( cbbM65 \) gene abundances were found than in the mesopelagic realm in all regions, except in the upper bathypelagic layers of SANT and SATL (Fig. 2B-D, Fig. 6A).

The \( cbbM68 \) gene abundance was low in the epipelagic layer of all six provinces (< \( 30 \text{ mL}^{-1} \), Fig. 3A). In the mesopelagic layer, the \( cbbM68 \) gene abundance was significantly higher in the WTRA province (\( 1.5 \times 10^2 \pm 7.99 \times 10^1 \text{ mL}^{-1} \)) than in the ARCT, NADR, NAG, SATL and SANT province (one way ANOVA on rank, \( p<0.05 \)) (Fig. 3B, 6B). Similar \( cbbM68 \) gene abundances were found in the mesopelagic layers of the NAG, SATL and SANT province (one way ANOVA on rank, \( p>0.05 \)) (Fig. 3B). The same latitudinal distribution pattern of the \( cbbM68 \) gene abundance was observed for the mesopelagic and the upper bathypelagic
layer, albeit generally lower in the bathypelagic than in the mesopelagic waters (Figs. 3C and 6B). No significant latitudinal differences in the cbbM68 gene abundance were apparent in the lower bathypelagic communities (one way ANOVA on rank, p>0.05, Fig. 3D, 6B).

The ratio of cbbM65:cbbM68 generally decreased significantly from high to low latitudinal provinces (Spearman Rank Order correlation, p<0.05) (Table 2; Fig. 4) and was significantly higher in the mesopelagic than into epipelagic, upper bathypelagic and lower bathypelagic layers (one way ANOVA, p>0.05) (Fig. 5).

The aprA gene abundance in the epipelagic realm was similar throughout all the oceanic provinces (one way ANOVA, p>0.05) except the ARCT province (Figs. 6C and 7A). The abundance of the aprA gene increased towards the mesopelagic layer, except in the NADR (Fig. 6B). There was a significant difference in aprA gene abundance between the mesopelagic communities of the NAG (6.20x10^2 ± 4.29x10^2 mL^-1), WTRA (6.00x10^2 ± 2.96x10^2 mL^-1) and SATL province (5.95x10^2 ± 3.17x10^2 mL^-1) as compared to the ARCT (8.28x10^1 ± 5.48x10^1 mL^-1) and SANT (1.41x10^2 ± 1.59x10^2 mL^-1) based on one-way ANOVA on ranks p<0.05. In the NADR province, the aprA gene abundance (2.45x10^2 ± 1.14x10^2 mL^-1) decreased from the epipelagic to the mesopelagic layer. A general decrease in the aprA gene abundance was determined towards the bathypelagic as compared to the mesopelagic realm in all provinces, except the ARCT (Figs. 6C and 7).

The distribution of the recA gene abundance along this longitudinal transect has been previously described (Steiner, 2013). The relative contribution of bacterial cells harboring the gene for RubisCO (cbbM65 and cbbM68) was high in the mesopelagic realm of the WTRA and SANT province. The proportion of Bacteria harboring the cbbM65 gene decreased towards the bathypelagic realm (Fig. 8A). Bacterial cells containing cbbM68 were relatively abundant in meso- to bathypelagic waters throughout the Atlantic (Fig. 8B). The aprA:recA ratio showed a similar distribution to that of cbbM68:recA (Fig. 8B, C).

**Phylogenetic affiliation of aprA-, cbbM65- and cbbM68-harboring Bacteria**

The phylogenetic affiliation of aprA and cbbM harboring Bacteria was determined in three depth layers at Station 33 located in the WTRA province (Fig.1). In total, 148 clones of cbbM65 were sequenced. All cbbM65 sequences obtained were putatively associated to the Gammaproteobacteria subphylum. One OTU (D1) was abundant in all pelagic layers...
(represented by 29, 38 and 50 clones from the mesopelagic, upper bathypelagic and lower bathypelagic, respectively) (Fig. 9).

Only 35 clones of cbbM68 were effectively sequenced, due to the low efficiency of the cloning reaction with this specific gene. All the sequences of cbbM68 corresponded to the same OTU, putatively assigned to the Deltaproteobacteria subphylum.

A total of 151 clones containing the aprA gene fragment were successfully sequenced. The aprA gene primer pair used in this study is targeting either sulfate reducing bacteria (SRB) or sulfur oxidizing bacteria (SOB) (Meyer and Kuever, 2007). The most abundant OTU (A3) was present in the upper and lower bathypelagic (Fig. 10), and was putatively assigned to SOB Gammaproteobacteria. The next most abundant OTU (B3, Fig. 10) was obtained from the upper bathypelagic, and was presumably related to a SRB Deltaproteobacteria. Some OTUs were putatively associated to Alphaproteobacteria and Betaproteobacteria SOB (Fig. 10).

**Distribution of Bacteria harboring cbbM and aprA and the influence of abiotic and biotic factors**

Some background abiotic and biotic factors have already been analyzed including leucine incorporation as a proxy for prokaryotic heterotrophic production (De Corte et al., 2012). In the present work, we specifically explored the relationships between these abiotic and biotic parameters and the functional genes aprA and cbbM related to chemoautotrophy.

The ratio of cbbM65:cbbM68, indicative of the dominance of different cbbM containing Bacteria, was positively correlated with the prokaryotic heterotrophic production, temperature and bacterial abundance (Table 2). A negative correlation was obtained between the ratio cbbM65:cbbM68 and salinity and depth (Table 2).

The relative contribution of cells harboring chemoautotrophic genes to the total bacterial abundance, represented by the ratios of cbbM65:recA, cbbM68:recA and aprA:recA (Table 2), was negatively correlated with latitude, temperature, heterotrophic production, bacterial abundance and salinity. All these parameters were positively correlated with depth (Table 2).
Additionally, we explored the explanatory value of different environmental factors on the variability of genes indicative of chemoautotrophy (Table 3). The variability of the $cbbM65:cbbM68$ ratio was mainly explained by depth, temperature, salinity, bacterial abundance and latitude, together explaining 45.8% of the variation in this gene ratio (Table 3). The variability of $cbbM65:recA$ ratio was mainly explained by salinity (31.8%), depth (12.5%), bacterial abundance (6.3%) and temperature (1.8%), together explaining 52.4% of the variation. The variability of $cbbM68:recA$ ratio was mainly explained by bacterial abundance (42.9%), latitude (3.9%), salinity (3.6%), bacterial activity (2.3%) and temperature (1.9%). The variability of $aprA:recA$ ratio was mainly explained by bacterial abundance and latitude, explaining 47.0% of the variation (Table 3).

4 Discussion

**Latitudinal and depth related trends in Bacteria harboring $cbbM$ or $aprA$**

The total prokaryotic abundance in the ocean decreases with depth (Patching and Eardly, 1997; Nagata et al., 2000; Reinthaler et al., 2006; De Corte et al., 2012). The $recA$ gene encodes the recombinase A protein, responsible for DNA repair mechanisms; this gene is present in all prokaryotes in one copy per cell (Miller and Kokjohn, 1990; Lin et al., 2006). Thus, it can be used as a proxy for bacterial abundance (Steiner, 2013). Indeed, the $recA$ gene abundance decreases from epipelagic to lower bathypelagic waters in all Atlantic Provinces (Steiner, 2013) and correlated with the prokaryotic abundance measured by flow cytometry (Steiner, 2013). Thus, the ratio of a functional gene, such as $cbbM$ or $aprA$, to the $recA$ gene is an indicator of the relative abundance of the Bacteria harboring a specific gene compared to the bulk bacterial community.

Prokaryotic abundance and heterotrophic production was highest in the ARCT and decreased towards the NAG and WTRA (De Corte et al., 2012), related to the input of biologically available organic matter from phytoplankton production in these oceanic provinces (Sathyendranath et al., 1995). The DIC fixation in the dark ocean by chemolithoautotrophic Bacteria and Achaea is of the same order of magnitude as heterotrophic biomass production (Herndl and Reinthaler, 2013). Thus, the activity of
chemolithoautotrophic Bacteria and Archaea represents another potential source of organic carbon for the meso- and bathypelagic ocean microbes (Alonso-Saez et al. 2010; Herndl and Reinthaler, 2013). Bacteria harboring either cbbM65, cbbM68 or aprA were more abundant in meso- to bathypelagic waters than in epipelagic layers throughout the Atlantic indicating that the potential for chemosynthesis and sulfur oxidation is widespread among bacteria in the dark ocean, even in the well-oxygenated layers of the mesopelagic realm. Furthermore, the higher contribution of bacteria harboring chemoautotrophy-related genes in regions with lower primary production (NAG, SATL, WTRA) supports the idea that, in these environments, chemoautotrophy can provide a complementary source of organic carbon for heterotrophic organisms.

The bacterial heterotrophic activity explains most of the variability of the genes cbbM68 and aprA, 42.87% and 41.31%, respectively (Table 3). Together with the negative correlation obtained between the leucine incorporation and the contribution of these two genes to the bulk bacterial community, this finding further supports the potential importance of the chemoautotrophic pathways in low productivity regions, such as the oligotrophic gyres and particularly the dark ocean.

**Phylogenetic community composition of cbbM- and aprA-harboring Bacteria**

The RubisCO genes cbbM65 and cbbM68 were found in several members of the phylum Bacteria, at least at the sequencing level reached in this study. Deltaproteobacteria were harboring the cbbM68 gene and Gammaproteobacteria possessed the cbbM65 gene (Fig 9). The different phylogenetic assignment of these two cbbM genes can help to explain the large difference in abundance of the genes (cbbM65>>cbbM68) in general with a distinct geographic distribution pattern of the two cbbM harboring groups. According to previous studies members of the Deltaproteobacteria have been reported to be more abundant in oxygen minimum zones (Wright et al., 1997; 2012), while Gammaproteobacteria have been reported to be more abundant in deep waters (Lopez-Garcia et al., 2001a).

The SAR324 clade of the class Deltaproteobacteria is ubiquitously present in the dark ocean (Brown and Donachi, 2007) and harbors cbbM and aprA genes (Swan et al., 2011). Although we could not find members of this subphylum with our Sanger sequencing approach, the
limited sequencing effort does not preclude that this group can be found by a more in-depth sequencing approach.

Based on the topology of the phylogenetic tree, the aprA gene of sulfur-oxidizing and sulfate-reducing bacteria form two phylogenetic lineages (Meyer and Kuever, 2007). The majority of sulfate-reducing bacterial sequences were related to Deltaproteobacteria sequences, while the majority of sulfur-oxidizing bacterial sequences were affiliated to Gammaproteobacteria, in agreement with previous studies (Hügler et al., 2010). Members of the Gammaproteobacteria subphylum have been shown to increase in relative abundance with depth in the ocean (Lopez-Garcia et al., 2001a). As shown in this study, most of the aprA harboring bacteria were affiliated to Gammaproteobacteria. Taken together, these findings might indicate an adaptation of some members of this group to dark ocean conditions (Swan et al., 2011), and reflect metabolic versatility (Walsh et al., 2009). The co-occurrence of RubisCO and aprA genes in the same bacterial lineages of Gammaproteobacteria supports the possibility of using sulfur oxidation as an energy source for CO$_2$ fixation through the CBB cycle in this group of organisms, in agreement with previous studies (Swan et al., 2011; Hügler et al., 2010).

Overall, this study showed that dark carbon fixation is widespread among bacteria in the oceanic water column although oxygen minimum zones are not pronounced in the Atlantic.
5 Conclusion

The biomass and activity of dark ocean prokaryotic communities is constrained by the amount of organic carbon availability. However, the ocean contains a large pool of DIC that can potentially be fixed by Bacteria and Archaea through chemosynthesis, sulfur being one of the most probable energy sources (Swan et al., 2011). In this study, we found genes related to carbon fixation (cbbM) and sulfur oxidation (aprA) down to bathypelagic waters, and extending from the polar to the tropical and gyral systems. More importantly, the relative contribution of bacteria harboring these genes increases with depth, and towards the low productive low latitude regions. The most abundant microbe with co-occurrence of aprA and cbbM genes was putatively affiliated to Gammaproteobacteria. Taken together, these results indicate that the potential to carry out chemosynthesis is widespread, not only in oxygen minimum zones and hydrothermal vents, but throughout the oxygenated water column and under diverse environmental conditions.
Graphs and Figures

Table 1. Primers used to amplify the two cbbM genes, subsequently named cbbM65 and cbbM68, and aprA gene fragments for q-PCR, cloning and sequencing and annealing temperature.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Fragment length</th>
<th>Target</th>
<th>Annealing temp.</th>
<th>Ref.</th>
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</thead>
<tbody>
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<td>Plank1</td>
<td>cbbM65f</td>
<td>~204 bp</td>
<td>cbbM</td>
<td>59°C</td>
<td>Swan et al., 2011</td>
</tr>
<tr>
<td>Plank1</td>
<td>cbbM68r</td>
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<td>cbbM</td>
<td>57°C</td>
<td>Swan et al., 2011</td>
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<tr>
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<td>cbbM</td>
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<td>Swan et al., 2011</td>
</tr>
<tr>
<td>Plank2</td>
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<td>~202 bp</td>
<td>cbbM</td>
<td>57°C</td>
<td>Swan et al., 2011</td>
</tr>
<tr>
<td>M13F</td>
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<td>Cloning Seq.</td>
<td>60°C</td>
<td>Meyer and Kuever, 2007</td>
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<tr>
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<tr>
<td>aprA-1F</td>
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<tr>
<td>aprA-5R</td>
<td>5’-GCGCCAACYGGRCCRTA-3’</td>
<td></td>
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</tbody>
</table>

Table 2. Results of a Spearman Rank Order Correlation between the contribution of functional genes (cbbM and aprA) to the bulk community (as indicated by recA) or relative cbbM contribution (cbbM65:cbbM68) and environmental parameters (BA-bacterial abundance determined by recA, leucine-leucine incorporation into bacterial biomass as a proxy for heterotrophic bacterial production).

<table>
<thead>
<tr>
<th></th>
<th>Latitude</th>
<th>Depth</th>
<th>Temp.</th>
<th>Salinity</th>
<th>BA</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbbM65:cbbM68</td>
<td>r=0.282</td>
<td>r=0.405</td>
<td>r=0.238</td>
<td>r=0.299</td>
<td>r=0.608</td>
<td>r=0.472</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>cbbM65:recA</td>
<td>r=0.0141</td>
<td>r=0.346</td>
<td>r=0.353</td>
<td>r=0.492</td>
<td>r=0.322</td>
<td>r=0.448</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>cbbM68:recA</td>
<td>r=0.421</td>
<td>r=0.490</td>
<td>r=0.432</td>
<td>r=0.327</td>
<td>r=0.613</td>
<td>r=0.647</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>aprA:recA</td>
<td>r=0.440</td>
<td>r=0.464</td>
<td>r=0.313</td>
<td>r=0.158</td>
<td>r=0.651</td>
<td>r=0.659</td>
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<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3. Results of the multivariate multiple regression analysis with forward selection (DISTML forward) to explain the variability of the contribution of the functional genes to the bacterial community. The response variable was log transformed and the resulting data converted in Euclidean distance similarity matrices. The Pseudo-F and the p-value were obtained by permutation (n=999). For abbreviations see Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Latitude</th>
<th>Depth</th>
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<th>Salinity</th>
<th>BA</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cbbM65:cbbM68</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prop</td>
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<td>0.1717</td>
<td>0.0588</td>
<td>0.1104</td>
<td>0.0366</td>
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<tr>
<td>p</td>
<td>0.001</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
<td>0.004</td>
<td>0.634</td>
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<tr>
<td><strong>cbbM65:recA</strong></td>
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<tr>
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<tr>
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<td>0.001</td>
<td>0.001</td>
<td>0.084</td>
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<tr>
<td><strong>cbbM68:recA</strong></td>
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<td></td>
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<tr>
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<td>0.0072</td>
<td>0.0193</td>
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<tr>
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<td>0.016</td>
<td>0.001</td>
<td>0.001</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>aprA:recA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>prop</td>
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<td>0.0024</td>
<td>0.0017</td>
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<td>0.0047</td>
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<tr>
<td>p</td>
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<td>0.725</td>
<td>0.389</td>
<td>0.483</td>
<td>0.001</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Figure 1. Sampling stations (red dots) along the latitudinal transect in the Atlantic. Dashed horizontal lines denote the borders between the 6 oceanic provinces (Longhurst, 1998). The encircled dot indicates the station where microbial communities were collected for cloning and sequencing of $cbbM$ and $aprA$ genes. ARCT - 70°N - 55°N, NADR - 55°N - 40°N, NAG - 40°N - 12°N, WTRA - 12°N - 6°S, SATL - 6°S - 40°S, SANT - 40°S - 55°S.
Figure 2. Distribution of the cbbM65 gene abundance [mL⁻¹] throughout the different provinces in the Atlantic for A) epipelagic, (B) mesopelagic, (C) upper bathypelagic and (D) lower bathypelagic realm. Abbreviations of the oceanic provinces are given in Fig. 1.
Figure 3. Distribution of the cbbM68 gene abundance [mL⁻¹] throughout the different provinces of the Atlantic in the (A) epipelagic, (B) mesopelagic, (C) upper bathypelagic, (D) lower bathypelagic realm. Abbreviation of oceanic provinces is given in Fig. 1.
Figure 4. Ratio of $cbbM65:cbbM68$ gene abundance in the different oceanic provinces of the Atlantic. Abbreviation of oceanic provinces is given in Fig. 1.

Figure 5. Ratio of $cbbM65:cbbM68$ gene abundance in different depth layers throughout the Atlantic. EPI—epipelagic (0-100 m), MESO—mesopelagic (100-1000 m), UB—upper bathypelagic (1000-2000 m) and LB—lower bathypelagic (2000-4000 m) realm.
Figure 6. Distribution of the gene abundance [mL⁻¹] along the latitudinal transect and oceanic provinces versus depth: cbbM65 gene abundance mL⁻¹ (A), cbbM68 gene abundance mL⁻¹ (B) and aprA gene abundance mL⁻¹ (C). Note the different scales for the different genes. Vertical dashed lines denote the limits between oceanographic regions. Abbreviation of oceanic provinces is given in Fig. 1.
Figure 7. Distribution of the aprA gene abundance [mL⁻¹] throughout the different oceanographic regions in the Atlantic for (A) epipelagic, (B) mesopelagic, (C) upper bathypelagic, (D) lower bathypelagic.
Figure 8. Proportion of bacterial cells harboring cbbM65 (A), cbbM68 (B) and aprA (C) along the latitudinal transect in the Atlantic and through the depth profile. The relative contribution of the cells containing the specific genes is given as the ratio between the abundance of the respective functional gene and recA gene abundance.
Figure 9. Neighbor–joining phylogenetic tree of the $cbb$M65 gene sequences at 97% similarity. The bar indicates the number of clones obtained from different depth layers (color code) represented by one sequence.
Figure 10 Neighbor–joining phylogenetic tree obtained by the aprA gene sequences at 97% similarity. The bar indicates the number of clones obtained from different depth layers (colour code) represented by one sequence. *Euryarchaeota* sequences obtained from NCBI were used to root the tree. Putative SRB (sulfate reducing bacteria) and SOB (sulfate oxidizing bacteria) are also indicated in brackets.
7 References


Reinthaler, T, van Aken, H., Veth, C., Arístegui, J., Robinson, C., Williams, PjleB, Lebaron,


Tang, Kam W. (2005). Copepods as microbial hotspots in the ocean: effects of host feeding


Appendix

8 Abstract

The dark ocean is a harsh environment where available energy and organic carbon sources for microorganisms are scarce. However, a large pool of dissolved inorganic carbon (DIC) is present there that can be potentially fixed through chemosynthesis by bacteria leaving the question on the available energy sources. Several reduced inorganic substrates are potentially available as energy source to fuel dark bacterial autotrophic carbon fixation such as ammonia, hydrogen and sulfur. The aprA gene encodes for the alpha subunit of the adenosine-5′-phosphosulfate (APS) reductase, responsible for either oxidizing or reducing sulfur. The oxidizing pathway might represent an energy source for the Calvin-Benson-Basham (CBB)-cycle in the dark ocean. In this study, cbbM, encoding RubisCO form II in Bacteria, and aprA gene abundance were determined along a transect in the Atlantic from 64°N to 50°S to quantify the potential for bacterial chemoautotrophy throughout the water column from the epi- to the bathypelagic realm. Both cbbM and aprA gene abundance were highest in the mesopelagic zone (200 - 1000 m), coinciding with the oxygen minimum layer, and upper bathypelagic (1000 - 2000 m) throughout the Atlantic. Moreover, bacterial autotrophic genes were relatively more abundant in oligotrophic regions, such as the North and South Atlantic Gyre and the Western Tropical Atlantic, as compared to high latitude regions. The co-occurrence of RubisCO and aprA genes in the same bacterial lineages affiliated to Gammaproteobacteria and Deltaproteobacteria subphyla indicates the potential of using sulfur oxidation as an energy source for CO₂ fixation through the CBB cycle in these widespread groups. Taken together, our results provide evidence that DIC fixation by chemoautotrophic microbes represents a potential alternative source of organic carbon for the deep ocean heterotrophs, especially in the oligotrophic gyral and tropical regions.
9 Zusammenfassung

Für Mikroorganismen ist die Tiefsee ein spezielles Habitat, in dem sie sich adaptieren und auf verschiedene Nischen spezialisieren müssen. Bei Prokaryoten ist die Nutzung chemosynthetischer Prozesse weit verbreitet und kann in der gesamten Wassersäule nachgewiesen werden, insbesondere auch in sauerstoffarmen Zonen, hydrothermalen Tiefseespalten und bei unterschiedlichen Umweltbedingungen.


Das Schlüsselenzym bei diesem Vorgang ist die Ribulose-1,5-Bisphosphat-Carboxylase / -Oxygenase (RubisCO) in Form I und II, kodiert von cbbL- beziehungsweise cbbM-Genen. Das Gen aprA, die Alpha-Einheit der Adenosine 5'-Phosphosulfat Reduktase, ist in der Lage Schwefel zu reduzieren oder zu oxidieren. Untersucht wird der Anteil der autotrophen Gene aprA und cbbM in der gesamten Wassersäule.

Im Rahmen der Arbeit wurden Wasserproben analysiert, die zwischen dem 64. nördlichen und dem 50. südlichen Breitengrad vom Epipelagial bis zum Bathypelagial im offenen Atlantik genommen wurden. Untersucht wurde, ob die oben genannte Energiegewinnung für die Chemosynthese im Fall der vorliegenden Wasserproben zutrifft. Die Proben wurden mithilfe eines CTD - „conductivity-temperature-depth“ Rosetten-Sammlers eingeholt, der mit 25 Liter Niskinflaschen, sowie mit Sensoren für Chlorophyllfluoreszenz, Strömung und Sauerstoff ausgestattet ist. Die Proben (2 - 10 Liter) für die DNA-Analysen wurden an 51 Stationen gezogen. Dabei wurden jeweils Meerwasserproben aus sechs bis acht unterschiedlichen Tiefen entnommen, die durch Polykarbonatfilter mit einer Porengröße von 0,2 µm gefiltert und anschließend sofort in flüssigem molekularen Stickstoff (N₂) eingefroren und bei -80°C bis zur weiteren Untersuchung gelagert wurden.

Methodisch stützt sich die Arbeit auf Polymerasekettenreaktionen (PCR) und quantitative Polymerasekettenreaktionen (q-PCR). Letztere ist eine Hochdurchsatzmethode, die dazu


Curriculum vitae

Johanna Tiroch

Education

2011-2014  MSc curriculum Ecology with specialization in “Marine Biology” at the University of Vienna
2007-2011  Bachelor study “Biology” at the University of Vienna
1999-2007  Secondary School in Neunkirchen/Lower Austria

Marine courses

2012  Marine biological field course on the Mediterranean fauna and flora; Centre for Marine Research (Rovinj, Croatia)
2012  Practical course in marine biology, Royal Netherlands Institute for Sea Research, Texel/The Netherlands