The ISME Journal (2010) 4, 1581–1590

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ORIGINAL ARTICLE

High bicarbonate assimilation in the dark by Arctic bacteria

Laura Alonso-Sáez[1], Pierre E Galand[2], Emilio O Casamayor[2], Carlos Pedrós-Alió[3] and Stefan Bertilsson[1]

[1]*Limnology/Department of Ecology and Evolution, Uppsala University, Uppsala, Sweden;* [2]*Group of Limnology-Department of Continental Ecology, Centre d'Estudis Avançats de Blanes, CEAB-CSIC, Blanes, Spain and* [3]*Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, Barcelona, Spain*

Although both autotrophic and heterotrophic microorganisms incorporate CO_2 in the dark through different metabolic pathways, this process has usually been disregarded in oxic marine environments. We studied the significance and mediators of dark bicarbonate assimilation in dilution cultures inoculated with winter Arctic seawater. At stationary phase, bicarbonate incorporation rates were high (0.5–2.5 μg C L^{-1} d^{-1}) and correlated with rates of bacterial heterotrophic production, suggesting that most of the incorporation was due to heterotrophs. Accordingly, very few typically chemoautotrophic bacteria were detected by 16S rRNA gene cloning. The genetic analysis of the biotin carboxylase gene *accC* putatively involved in archaeal CO_2 fixation did not yield any archaeal sequence, but amplified a variety of bacterial carboxylases involved in fatty acids biosynthesis, anaplerotic pathways and leucine catabolism. *Gammaproteobacteria* dominated the seawater cultures (40–70% of cell counts), followed by *Betaproteobacteria* and *Flavobacteria* as shown by catalyzed reporter deposition fluorescence *in situ* hybridization (CARDFISH). Both *Beta-* and *Gammaproteobacteria* were active in leucine and bicarbonate uptake, while *Flavobacteria* did not take up bicarbonate, as measured by microautoradiography combined with CARDFISH. Within *Gammaproteobacteria, Pseudoalteromonas-Colwellia* and *Oleispira* were very active in bicarbonate uptake (ca. 30 and 70% of active cells, respectively), while the group Arctic96B-16 did not take up bicarbonate. Our results suggest that, potentially, the incorporation of CO_2 can be relevant for the metabolism of specific Arctic heterotrophic phylotypes, promoting the maintenance of their cell activity and/or longer survival under resource depleted conditions.
The ISME Journal (2010) **4,** 1581–1590; doi:10.1038/ismej.2010.69; published online 17 June 2010
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: Arctic; bacteria; carbon; fixation; seawater

Introduction

Virtually all microorganisms incorporate CO_2 as part of their metabolism. However, while photosynthetic fixation of CO_2 has received considerable attention, the significance of the light-independent or dark CO_2 assimilation for microbial metabolism is poorly understood. Inorganic carbon (C) represents the main or even the only C source for chemoautotrophs, such as ammonia or sulfur oxidizers. These microorganisms fix CO_2 mainly via the Calvin-Benson-Bassham cycle (Bassham and Calvin, 1957), or, as recently shown for *Archaea*, the 3-hydroxypropionate/4-hydroxybutyrate pathway (Berg *et al.*, 2007). Additionally, heterotrophic bacteria rely on organic compounds for C supply, but also incorporate CO_2 via a variety of carboxylation reactions as part of their central or peripherical metabolic pathways (Dijkhuizen and Harder, 1984). These pathways, which also contribute to the CO_2 assimilation by chemoautotrophs, are involved in anaplerotic reactions, and the synthesis of fatty acids, nucleotides and amino acids. Hence, dark CO_2 incorporation may be relevant for a wide range of metabolic groups, including chemoautotrophs, heterotrophs, and even phototrophs that can maintain CO_2 fixation activity in the absence of light (Casamayor *et al.*, 2008).

In marine systems, dark C fixation has mostly been studied at oxic/anoxic interfaces (Sorokin, 1972; Detmer *et al.*, 1993; Casamayor *et al.*, 2001), which are favorable environments for chemoautotrophic activity. Recently, a few studies have also reported dark C fixation rates for deep oceanic samples and related them to the presence of chemoautotrophic archaeal populations (Agogué *et al.*, 2008), suggesting an active autotrophic CO_2 fixation in the deep ocean (Herndl *et al.*, 2005;

Correspondence: L Alonso-Sáez, Centro Oceanográfico de Xixón, Instituto Español de Oceanografía, Camín del L'Arbeyal s/n, E-33212 Xixón, Spain.
E-mail: laura.alonso@gi.ieo.es
Received 7 December 2009; revised 23 April 2010; accepted 23 April 2010; published online 17 June 2010

Könneke *et al.*, 2005). However, the possibility that dark CO_2 assimilation is substantial in the widespread heterotrophy-dominated marine environments has usually been disregarded, even if some studies have reported substantial dark CO_2 uptake in marine surface waters (Prakash *et al.*, 1991; Li *et al.*, 1993; Markager, 1998), and positively correlated it with the activity of heterotrophic bacteria (Li and Dickie, 1991).

Heterotrophic CO_2 assimilation was first described by Werkman and Wood (1942). This process was found to be widespread and tightly coupled to cell growth and has been used as a measure of bacterial metabolic activity (Romanenko, 1964; Roslev *et al.*, 2004; Hesselsoe *et al.*, 2005). However, its use as a proxy for bacterial heterotrophic production was ruled out due to the large uncertainty in the ratio of inorganic C fixed to total C assimilated (Overbeck and Daley, 1973). The importance of CO_2 assimilation varies with the type of organic substrate used for growth (Romanenko, 1964; Sorokin, 1966), the metabolic state of the organisms (Overbeck, 1979) and even between different bacterial species, which incorporate CO_2 to replenish distinct biomass components (Feisthauer *et al.*, 2008). Interestingly, the proteorhodopsin-containing flavobacterium *Polaribacter* sp. MED152 showed light-stimulated CO_2 incorporation, suggesting that anaplerotic pathways had a significant role in the life strategy of this marine heterotroph (González *et al.*, 2008). Yet, very few studies have assessed the relevance of dark CO_2 incorporation for environmental bacteria and thus, the factors regulating this process *in situ*, and the significance of CO_2 assimilation for different taxa remains unknown.

The objective of this study was to identify key mediators of dark CO_2 assimilation in Arctic seawater. We hypothesized that the low availability of labile organic C could favor the uptake of bicarbonate by chemoautotrophic microorganisms or heterotrophs undergoing metabolic imbalances. Thus, the study was carried out during the winter, a season characterized by low availability of fresh photosynthesis-derived C. Our experimental set-up consisted of seawater cultures (SWCs) containing 3-months aged Arctic seawater in order to promote further depletion of labile organic C and to analyse the potential relevance of dark CO_2 assimilation under resource-limited conditions.

Materials and methods

Preparation of SWCs

Dilution cultures were prepared with seawater collected on 16th November 2007 in the Amundsen Gulf, western Arctic (69° 52.104 N, −126° 30.031 W) from two different depths: surface (10 m) and the base of nitracline (20 m). Seawater was filtered through 0.7 μm GF/F filters with a peristaltic pump, placed in acid-rinsed 25 L polycarbonate carboys, and aged at 2 °C in the dark for 3 months to promote consumption of labile organic C. On February 11th 2008, the aged water was filtered through 0.2 μm pore size polycarbonate filters (Millipore) and used for preparing the SWCs. Three sets of SWCs were inoculated with freshly collected samples (71° 4.236 N, −124° 47.135W) from 3 depths: surface (12 m), temperature inversion (50 m) and base of nitracline (60 m). The inocula (0.1 L) were gravity filtered (0.7 μm, GF/F filters) and added to 1 L of aged-seawater in triplicate bottles. The surface sample was inoculated in 10 m aged-seawater, whereas the 50 and 60 m-samples were inoculated in 20 m aged-seawater. The prokaryotic assemblages present after the 3 months aging period were used as inocula in a set of parallel SWCs (aged SWCs, ASWCs), in order to analyse the CO_2 assimilation by long-term nutrient- and energy-depleted prokaryotes. Finally, a replicate series of SWCs were spiked with ammonium at the beginning of the experiments to test whether the addition of reduced nitrogen could favor the growth of nitrifiers. For that purpose, the seawater was initially amended with 160 μM N (NH_4Cl) and 12.5 μM P (Na_2HPO_4) after collection in the carboys, and was spiked again with 160 μM N (NH_4Cl) at the start of the experiments. In total, 24 SWCs were incubated at 2 °C in the dark for 25 days. At stationary phase, samples for analysis of leucine uptake as an estimate of bacterial heterotrophic production, bicarbonate uptake and microautoradiography combined with CARDFISH (MAR-CARDFISH) were collected.

Bacterial abundance

Samples for bacterial abundance were collected every 3–4 days until stationary phase. Samples (1 mL) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.), flash frozen in liquid N and kept at −80 °C until analysis. Bacterial abundance was analyzed by flow cytometry (FACS, Becton and Dickinson) after staining with Syto13 (Molecular probes, 2.5 μM final conc., del Giorgio *et al.*, 1996).

Bulk uptake of bicarbonate

Incubations with radiolabeled bicarbonate ($NaH^{14}CO_3$; final conc. 0.15 μCi mL^{-1}; Amersham CFA3, 56 mCi mmol^{-1}) were carried out in the dark, in ice-cold seawater. For each sample (50 mL), 3–4 replicates and one killed control were analysed. Killed controls were preserved with formaldehyde (3.7% final conc.) prior to bicarbonate addition. After incubation (22–28 h), samples were filtered through 0.22 μm polycarbonate filters (Millipore). Filters were placed in vials and exposed to HCl fumes overnight. Scintillation cocktail (Fisher Scientific, Scintiverse BD cocktail SX18-4) was added to each filter (4 mL), and the radioactivity

was measured in a Packard Liquid Scintillation analyzer Tri-Carb 2900 TR on board. Calculations of C assimilation were made taking into account the added radiolabeled bicarbonate and the total amount of dissolved inorganic C (DIC) in the samples. DIC content was determined by coulometric determination (Johnson *et al.*, 1993) with calibration against certified reference material provided by A. Dickson (Scripps Institution of Oceanography).

Bacterial heterotrophic production (BHP)
The [^{3}H]-leucine incorporation method (Kirchman *et al.*, 1985), modified as described by Smith and Azam (1992) was used to determine BHP. For each sample, triplicate aliquots (1.2 mL) and one killed control were incubated with 10 nM leucine (Perkin Elmer, NET460A005, 140 Ci mmol^{-1}) for about 4 h in the dark, in ice-cold seawater. The incorporation was stopped by adding 120 μL of cold 50% trichloroacetic acid (TCA) to the vials and samples were kept frozen at −20 °C until processing.

DNA extraction
Microbial biomass was collected by sequentially filtering 1 L of sample through a 3 μm pore size polycarbonate filter (Poretics) and a 0.2 μm pore size Durapore filter, using a peristaltic pump. The filters were preserved with 1.8 mL of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) in microcentrifuge tubes and kept at −80 °C. Microbial biomass was treated with lysozyme (1 mg mL^{-1} final conc.) for 45 min at 37 °C, and proteinase K and sodium dodecyl sulphate (0.2 mg mL^{-1} and 1% final conc., respectively) for 60 min at 55 °C. The nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, by vol.) and the residual phenol was removed once with chloroform-isoamylalcohol (24:1, v/v). Nucleic acids were purified, desalted and concentrated with Amicon Ultra-4 (Millipore).

Clone libraries and sequencing
16S rRNA genes were amplified by PCR with the universal primers 27f and 1492r as previously described (Eiler and Bertilsson, 2004). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) genes (form I) were amplified by *cbbL* primers as described in Alfreider *et al.* (2003). Archaeal biotin carboxylase subunit genes (*accC*) were amplified by primers ACAC254f and ACAC720r (Auguet *et al.*, 2008). Products from 3 individual PCR reactions (20 μL) were pooled and purified with the QIAGEN PCR purification kit and cloned using the TOPO-TA cloning kit as described in Eiler and Bertilsson (2004). Potential chimeric sequences were identified by using Bellerophon (Huber *et al.*, 2004) and BLAST search targeting different regions in the sequences. Functional gene annotation was carried out with Blast2go (Conesa *et al.*, 2005). One third of the 42 clones obtained with *cbbL* primers were unspecific products not encoding a Rubisco and were omitted from further analyses. 16S rRNA, *cbbL* and *accC* gene sequences were deposited in Genbank under accession numbers GU249366-249504, GU249304-GU249332, and GU249333-GU249365, respectively.

CARDFISH
The abundance of different bacterial groups was analysed by CARDFISH (Pernthaler *et al.*, 2004). Samples were fixed with formaldehyde (3.7% final conc., overnight at 4 °C) and cells were permeabilized with lysozyme (1 h) and achromopeptidase (30 min) at 37 °C. We used the following horseradish peroxidase labeled probes: PSA184 (Eilers *et al.*, 2000), Arctic96B16-196 (Malmstrom *et al.*, 2007), CFB563 (Weller *et al.*, 2000), GAM42a, BET-42a, and EUBI-III. The probe OLE232 (5′-AGCTAATCTCACT CAGGC-3′) was designed in this study to target a cluster affiliated with *Oleispira*, and used with the competitor OCE232 (5′-AGCTAATCTCACGCAGG C-3′, Eilers *et al.*, 2000) at 60% of formamide. Probes CREN554 and EURY806 were used to test the presence of *Archaea* in the samples. The non-specific probe NON338 detected < 1% of cells. The filters sections were counter-stained with DAPI (1 μg mL^{-1}). At least 300 DAPI cells in a minimum of 10 fields were counted in a Nikon eclipse E600 epifluorescence microscope.

MAR-CARDFISH
Samples (20 mL) were incubated with [^{3}H]-leucine (0.5 nM, Perkin Elmer, NET460A005, 140 Ci mmol^{-1}) or $NaH^{14}CO_3$ (3 μCi mL^{-1}, Amersham CFA3, 56 mCi mmol^{-1}) in the dark, in ice-cold seawater. Incubation times were 7 and 24 h for leucine and bicarbonate samples, respectively. After incubation, samples were fixed with formaldehyde (1.8%, overnight at 4 °C), and filtered on 0.2-μm polycarbonate filters (Millipore). Samples killed with formaldehyde before the addition of the radioactive compounds were used as controls. Filters were hybridized following the CARDFISH protocol, and subsequently processed for microautoradiography as previously described (Alonso-Sáez *et al.*, 2008). Optimal exposure times were determined for each treatment and compound (1 and 13 days for leucine and bicarbonate, respectively). Filters were counted in a Nikon eclipse E600 epifluorescence microscope. Replicates were counted for ca. 25% of the filters.

Results

Bacterial growth and bulk bicarbonate and leucine uptake rates
The growth of prokaryotes in SWCs inoculated with coastal Arctic seawater freshly collected from

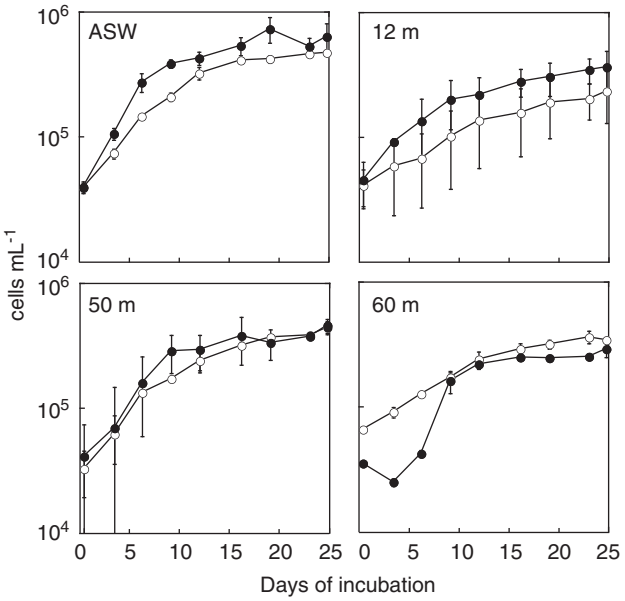

Figure 1 Growth kinetics of unamended (open circles) and nutrient amended (closed circles) dilution cultures inoculated with seawater from 12-, 50- and 60-m depths and 3-months aged seawater (ASW) bacterial communities. Error bars indicate the standard deviation from replicate seawater cultures.

3 depths (12, 50 and 60 m) and aged seawater (ASW, see Materials and Methods) was followed for 25 days, until they reached stationary phase (Figure 1). Maximum growth rates were calculated based on increases in cell numbers during exponential phase. In unamended incubations, 12- and 60-m-depth SWCs showed growth rates of 0.10 d^{-1}, while ASW and 50 m SWCs showed growth rates of ca. 0.23 d^{-1}. After nutrient enrichment, growth rates were similar in the 50 m SWC (i.e. 0.22 d^{-1}), while they were 0.33 and 0.17 d^{-1} in ASW and 12 m SWC, respectively. The addition of nutrients to the 60 m depth SWC produced an initial inhibition of growth, but thereafter, the maximum growth rates were 0.45 d^{-1}.

At stationary phase, Leucine and bicarbonate uptake rates were significantly correlated (Spearman $Rho = 0.74$, $P = 0.001$, $n = 16$). The addition of nutrients resulted in higher leucine uptake rates in all SWCs (Wilcoxon signed-rank test, $P < 0.007$, n ranged from 5 to 6, Figure 2), while bicarbonate uptake was only substantially increased in ASW and 12-m-depth SWCs.

Phylogenetic analysis of the bacteria growing in the seawater cultures

The initial bacterial assemblages at 12 and 50 m depth were dominated by *Gamma-* and *Alphaproteobacteria* (ca. 30% and 20% of clones, respectively), followed by *Flavobacteria* (10% of clones, Figure 3a). At 50 m depth, *Verrucomicrobia* and *Deltaproteobacteria* were also significant, together making up >10% of the total clones (Figure 3a). *Gammaproteobacteria* were affiliated

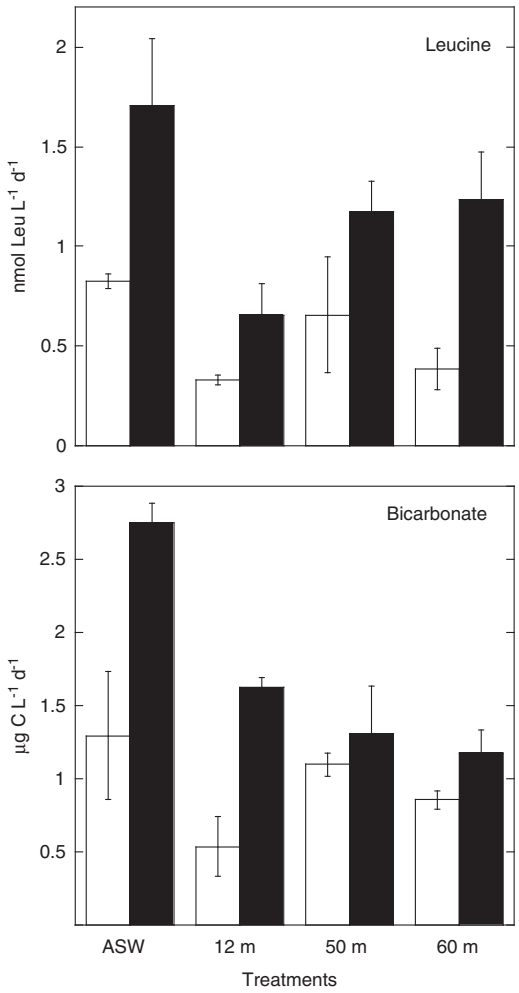

Figure 2 Bacterial leucine and bicarbonate uptake estimates in unamended (white bars) and nutrient amended (black bars) treatments, measured at stationary phase, after 23 days incubation. Error bars indicate the standard deviation from replicate seawater cultures. Abbreviation: ASW, aged seawater.

with *Alteromonadales*, Arctic96B-16, and some unidentified taxa (Supplementary Figure S1). Most *Alphaproteobacteria* were affiliated with SAR11 (Supplementary Figure S1). *Archaea* contributed a minor fraction of the communities (<5% of cell counts, as analyzed by CARDFISH).

In the stationary phase, *Gammaproteobacteria* dominated the assemblages (70–80% of clones) and the contribution of *Alphaproteobacteria* was strongly reduced to 2–5% of the clones (Figure 3a). Within *Gammaproteobacteria*, most clones were affiliated with a group of *Alteromonadales* related to *Colwellia* sp. and *Pseudoalteromonas* sp. (*PSA-Colwellia,* up to 38% of clones), a group of *Oceanospirillales* related with *Oleispira sp.* (15% of clones), and two clusters of unidentified *Gammaproteobacteria* related to freshwater Antarctic clones (Supplementary Figure S1). While *Betaproteobacteria* were not detected in the inocula, their representation increased up to 10% of clones in

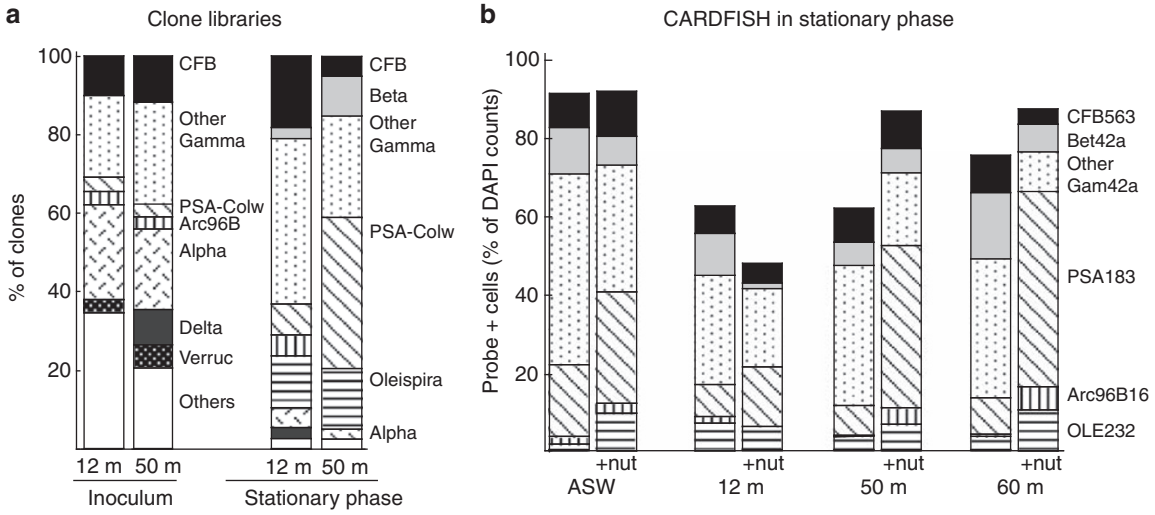

Figure 3 Proportion of bacterial 16S rRNA clones (**a**) and DAPI counts by CARDFISH (**b**) affiliated with different groups: *Flavobacteria* (CFB, probe CFB563), *Alphaproteobacteria* (Alpha), *Betaproteobacteria* (Beta, probe Bet42a), *Gammaproteobacteria* (Gamma, probe Gam42a), *Deltaproteobacteria* (Delta), *Pseudoalteromonas-Colwellia* (*PSA-Colw*, probe PSA183), *Oleispira* (probe OLE232), Arctic96B-16 (probe Arctic96B16-196), and *Verrucomicrobia* (Verruc). Abbreviations: ASW, aged seawater; +nut, nutrient enriched.

the 50 m depth SWC (Figure 3a), mainly due to an increase in sequences affiliated with *Methylophylaceae* (Supplementary Figure S1). *Flavobacteria* increased in the 12 m depth SWC (up to 18% of clones), and decreased in the 50 m depth SWC (Figure 3a).

The three main general phyla in the SWCs (i.e., *Gamma-*, *Betaproteobacteria* and *Flavobacteria*) and three specific groups within *Gammaproteobacteria* (i.e., *PSA-Colwellia*, *Oleispira* and Arctic96B-16) were targeted by CARDFISH in the stationary phase (Figure 3b). In accordance with the data from the clone libraries, *Gammaproteobacteria* were dominant, contributing from 41 to 76% of cells counts. *PSA-Colwellia* was generally the most abundant of the *Gammaproteobacteria* groups tested, and their relative abundance was higher in nutrient-amended cultures (up to 49% of DAPI counts, Figure 3b). *Oleispira* (1–10% of DAPI counts) and Arctic96B-16 (0–6% of DAPI counts) also tended to be more abundant under nutrient enrichment conditions. *Flavobacteria* and *Betaproteobacteria* (4–12% and 1–17% of DAPI counts, respectively) did not show any consistent growth response to the nutrient enrichment (Figure 3b).

Cloning of Rubisco and biotin acetyl-CoA carboxylase genes

With the aim of detecting and describing mediators of dark C fixation, we analyzed the diversity of the *cbbL* gene encoding the Rubisco enzyme and the *accC* gene encoding the archaeal biotin acetyl-CoA carboxylase. These two enzymes are involved in the main CO_2 fixation pathways of chemoautotrophic *Bacteria* and *Archaea*, respectively. Three different clusters were identified for the *cbbL* gene (at >95% amino acid similarity, Table 1). The two first clusters (*cbbL* I and *cbbL* II) were closely related (ca. 94% amino acid similarity) and were affiliated with a sulfur oxidizer (*Allochromatium vinosum)* and a methylotroph (*Methylococcus capsulatus)*. The first cluster (*cbbL* I) included all sequences from the inoculum, while the cluster *cbbL* III, which was affiliated with *Bradyrhizobium* sp. and *Nitrosomonas europaea,* only included sequences from the end of the incubation (Table 1).

The amplification of the *accC* gene at the stationary phase resulted in more deeply divergent sequences and clusters were defined at 70% amino acid similarity level. The amplified sequences encoded not only acetyl-CoA carboxylases, but also other types of biotin carboxylases such as methylcrotonyl-CoA or propionyl-CoA carboxylases. Only bacterial sequences were retrieved, affiliated with genes from a broad range of species, with *Ehrlichia chaffeensis* and *Colwellia psychrerythraea* being the most common examples (Table 1).

Single-cell identification of active heterotrophs and dark bicarbonate fixers

Gamma- and *Betaproteobacteria* showed activity in both leucine and bicarbonate uptake, while *Flavobacteria* only incorporated leucine, as detected by MAR-CARDFISH (Figure 4a). In unamended SWCs, the three groups showed similar percentages of cells active in leucine uptake (range 14–37%), although the highest values were generally found for *Gammaproteobacteria* (20–34% of active cells, Figure 4a). The addition of nutrients produced a striking effect on the leucine uptake in the later group (increasing the active cells to 83%), whereas no noticeable increase was observed for *Betaproteobacteria* or *Flavobacteria*. For bicarbonate uptake, the percentages of active cells were similar for *Gamma-* and

Table 1 Functional gene clusters containing sequences with amino acid identities larger than 95% and 70% for *cbbL* and *accC* gene, respectively

Cluster	*Blastx Hits ACC*	*ID(%)*	*Phylogenetic affiliation*	*Sequence description*	*Enzyme codes (EC)*	N *SWC*	N *Inoculum*
cbbL I	ABY77423(11) P24672 (4) ZP_04773652(5)	>94	*Allochromatium vinosum/Alvinoconcha hessleri* symbiont	Ribulose-bisphosphate carboxylase	4.1.1.39	2	18
cbbL II	AAL40972 (5) ABY77423 (1)	>94	*Methylococcus capsulatus*	Ribulose-bisphosphate carboxylase	4.1.1.39	6	0
cbbL III	YP_001204346(2) ABY77424 (1)	>92	*Bradyrhizobium* sp/ *Nitrosomonas europaea*	Ribulose-bisphosphate carboxylase	4.1.1.39	3	0
accC I	YP_507303 (11)	>80	*Ehrlichia chaffeensis*	Pyruvate/propionyl-CoA carboxylase (carbamoyl phosphate synthase)	6.4.1.1; 6.4.1.3; 6.3.4.14	11	ND
accC II	YP_268342 (9)	91	*Colwellia psychrerythraea*	Methylcrotonyl-CoA carboxylase	6.4.1.4; 6.3.4.14	9	ND
accC III	YP_548992 (3) YP_003059875(1)	>80	*Polaromonas* sp/ *Hirschia baltica*	Propionyl-CoA carboxylase (carbamoyl phosphate synthase)	6.4.1.3	4	ND
accC IV	ZP_05725266(1) YP_267695 (1)	>80	*Colwellia psychrerythraea/ Dickeya dadantii*	Acetyl-CoA carboxylase	6.4.1.2; 6.3.4.14	2	ND
accC V	YP_266148 (2)	83	*Pelagibacter ubique*	Acetyl-CoA carboxylase	6.4.1.2	2	ND
accC VI	ABZ05899 (2)	>79	Uncultured marine microorganism	Acetyl/propionyl-CoA carboxylase (carbamoyl phosphate synthase)	6.4.1.2; 6.3.4.14	2	ND
accC VII	YP_659905 (2)	85	*Pseudoalteromonas atlantica*	Acetyl/propionyl-CoA carboxylase (carbamoyl phosphate synthase)	6.4.1.2; 6.3.4.14	2	ND
accC VIII	ZP_05127626 (1)	80	NOR5-3 gammaproteobact.	Acetyl/propionyl-CoA carboxylase	6.4.1.2; 6.3.4.14	1	ND

Accession numbers (ACC, numbers in brackets indicate number of clones), identity values (ID, % positive amino acids) and phylogenetic affiliation of top *blastx* hits are shown for each cluster. The number (N) of clones contained in each cluster in the seawater cultures (SWCs: 50 m depth for cbbL and 12 m depth for accC) or the *inoculum* (only for *cbbL* gene) are reported. Functional sequence description and enzyme codes were obtained by automatic annotation with blast2go software. ND: not determined.

Betaproteobacteria (7–32%), and their proportions were not affected by nutrient addition (Figure 4a).

Within *Gammaproteobacteria*, dramatic differences in uptake activities were observed for the three groups studied (*Oleispira*, *PSA-Colwellia* and Arctic96B-16, Figure 4b). While *Oleispira* showed higher activity in bicarbonate uptake (ca. 75% of active cells) compared to leucine uptake (Wilcoxon signed-ranked test, $n=4$, $P=0.02$), *PSA-Colwellia* were more active in leucine uptake (Wilcoxon signed-ranked test, $n=4$, $P=0.03$, Figure 4b). Between 13 and 70% of *PSA-Colwellia* cells were also active in bicarbonate uptake (Figure 4b). For Arctic96B-16, around 40% of the cells were taking up leucine while bicarbonate uptake activity was generally very low. In unamended incubations, the activity of the three gammaproteobacterial groups in bicarbonate uptake was highest in SWCs inoculated with ASW. In general, the addition of nutrients did not affect the activity of these groups to any major extent, except for the decrease in *Oleispira* cells taking up leucine (Figure 4b).

Discussion

Dark CO_2 assimilation has been usually assumed to be insignificant in oxygenated marine waters. However, rates of dark CO_2 uptake have seldom been properly assessed and interpreted and thus, the relevance of this process remains largely unknown. In this study, we report surprisingly high rates of bicarbonate uptake in the stationary growth phase of Arctic SWCs (0.5–2.3 $\mu g\,C\,L^{-1}\,d^{-1}$), being in the same range as BHP rates commonly found in marine field studies (Ducklow and Carlson, 1992). Using the theoretical conversion factor of 1.5 $kg\,C\,mol\,Leu^{-1}$, estimates of BHP based on leucine uptake would equal rates of bicarbonate uptake in our experiments (Figure 2). This ratio (1:1) is much higher than what is commonly assumed for heterotrophic bacterial growth (i.e. bicarbonate assimilation representing 1–8% of total biomass production, Romanenko, 1964; Roslev *et al.*, 2004). However, these findings do not necessarily imply that most bacterial biomass production was derived from bicarbonate assimilation, as there is a large uncertainty in the biomass conversion factors for these measurements. This is emphasized in our experiments as, in fact, total cell counts remained rather stable during the period of bicarbonate and leucine uptake rate measurements (Figure 1), and thus, the uptake of these compounds did not produce any significant net increase in biomass.

It is well known that many pathways involving heterotrophic CO_2 incorporation, e.g. fatty acids

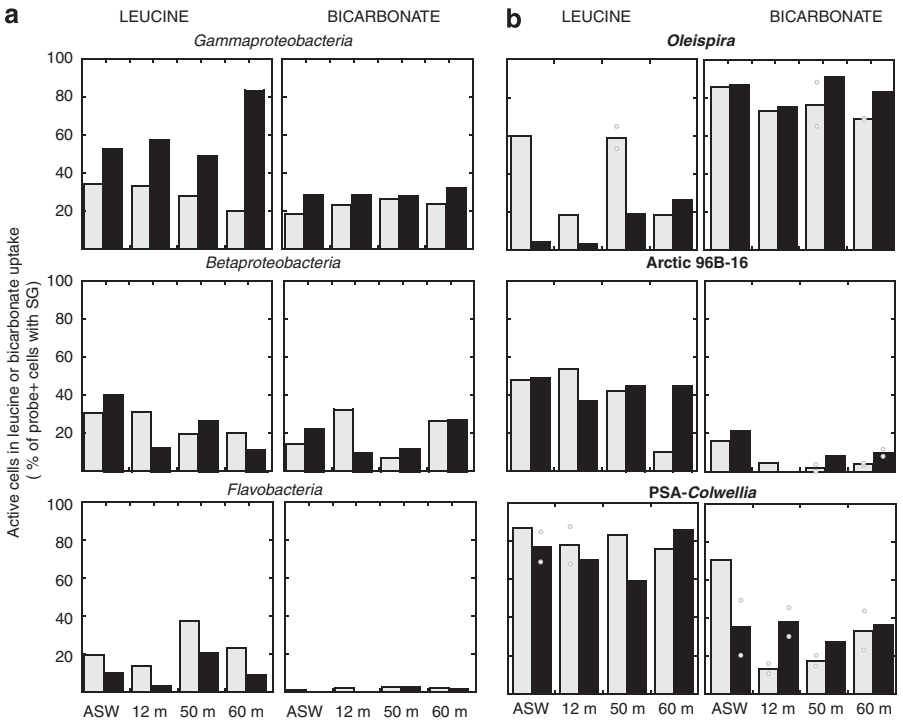

Figure 4 (**a**) Percentage of probe positive cells affiliated with *Gamma-*, *Betaproteobacteria* and *Flavobacteria* active in the uptake of leucine (left panel) or bicarbonate (right panel), and (**b**) percentage of probe positive cells affiliated with *Oleispira*, Arctic 96B-16 and *Pseudoalteromonas-Colwellia (PSA-Colwellia)* active in the uptake of leucine (left panel) or bicarbonate (right panel) as detected by MAR-CARDFISH. Grey and black bars represent values for unamended and nutrient enriched treatments, respectively. Dots represent individual measurements of replicate filters. Abbreviations: ASW, aged seawater inoculum; SG, silver grains.

biosynthesis and anaplerotic reactions, do not lead to any net C assimilation. Leucine-to-carbon conversion factors are also largely unconstrained due to processes such as intracellular protein turnover or leucine catabolism, which has been shown to intensify under oligotrophic conditions (Kirchman *et al.*, 1986, Alonso-Sáez *et al.*, 2007). In general, the metabolic changes undergone by cells during the stationary phase have been poorly characterized, but our results suggest that the incorporation of CO_2 could be of great importance for the metabolism of such resources-depleted bacteria.

Due to the artificial situation created by the dilution culture experimental set-up the high bicarbonate uptake activities only represent a potential for dark CO_2 assimilation but, interestingly, similarly high CO_2 assimilation rates have been reported in environmental oceanic samples (Prakash *et al.*, 1991; Li *et al.*, 1993). In the Arctic site of study, *in situ* bicarbonate uptake rates were usually one order of magnitude lower than in the SWCs (average $0.09\,\mu g\,C\ L^{-1}\,d^{-1}$, L. Alonso-Sáez unpublished results). However, these *in situ* bicarbonate assimilation rates were comparable to winter and spring BHP estimates in this Arctic region (Garneau *et al.*, 2008). This suggests that dark CO_2 assimilation might also be relevant in the environment in polar systems, as was shown in a recent study in Antarctic waters (Manganelli *et al.*, 2009). A MARFISH study in the western Arctic also showed that the *in situ* number of *Bacteria* active in bicarbonate uptake was generally $<10\%$, but increased up to 20% in shelf waters (Kirchman *et al.*, 2007).

In the present study, seawater was collected during the Arctic winter and incubations were maintained in the dark. Hence, any potential effects of phototrophs on CO_2 fixation would be negligible. Part of the detected bicarbonate incorporation could be due to chemoautotrophic microorganisms, however, only a small fraction of potential chemoautotrophs was detected by 16S rRNA cloning, mainly affiliated with *Nitrospinaceae*, *Hydrogenophaga* and *Methylophilus*. The genetic analysis of the *cbbL* gene suggested the presence of relatives of *Nitrosomonas europaea* (with 76% identity at the nucleotide level), a well-known betaproteobacterial nitrifier (Chain *et al.*, 2003). However, the addition of ammonia did not have a major effect on the bicarbonate uptake activity of *Betaproteobacteria* (Figure 4), and the typically slow growth of ammonia oxidizers would not support the high rates of bicarbonate fixation detected in our experiments.

Instead, the significant correlation between bicarbonate uptake rates and BHP indicates that most of the dark C assimilation can be attributed to heterotrophs. The heterotrophic demand for bicarbonate can be the result of multiple cellular processes, such as anaplerotic reactions to replenish TCA cycle intermediates, synthesis of amino acids or nucleic acids precursors, and biosynthesis of fatty acids.

Provided that some of these carboxylations require energy, the incorporation of large amounts of bicarbonate by heterotrophic bacteria does not seem a reasonable metabolic strategy. However, demand for bicarbonate has previously been shown to increase during starvation and slow growth in the bacterial model *Escherichia coli* (Merlin *et al.*, 2003). A potential explanation for this response could be that cells undergo major shifts in protein and lipid composition during stationary phase, as it is known that lipid synthesis can increase the demand for CO_2 (Merlin *et al.*, 2003). Anaplerotic reactions to replenish TCA cycle intermediates or to synthesize amino acids and nucleotides may also be intensified when the extant organic precursors are not available to satisfy cellular requirements (Dijkhuizen and Harder, 1984).

Additionally, carboxylation reactions are involved in the degradation pathways of some organic compounds, such as leucine. Specifically, the enzyme methylcrotonyl-CoA carboxylase, which was detected in the *accC* clone library, catalyzes the carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA during leucine degradation (Massey *et al.*, 1976). While *accC* primers were originally designed for the amplification of the archaeal biotin acetyl CoA carboxylase, they can also amplify many bacterial biotin-carboxylase genes putatively involved in fatty acid biosynthesis, anaplerotic pathways (Auguet *et al.*, 2008) and, as we show in this study, in leucine catabolism. An active degradation of this amino acid would support the idea of high turnover of cellular components and/or leucine catabolism in resource-limited bacteria.

Another novel finding in our experiments is the differential dark CO_2 assimilation among distinct bacterial phylotypes. *Flavobacteria* were not active in bicarbonate uptake despite having a high fraction of heterotrophically active cells. Similarly, the gammaproteobacterial group Arctic96B-16 (Bano and Hollibaugh, 2002) was active in leucine uptake, in agreement with results by Malmstrom *et al.* (2007), but generally did not take up bicarbonate. Conversely, the gammaproteobacterial groups *Oleispira* and *PSA-Colwellia* were highly active in the uptake of bicarbonate (Figure 4b).

The group *Oleispira* is closely related to a group of Arctic environmental clones that have been suggested to be typical for the winter season (Arctic 95B, Bano and Hollibaugh, 2002). A psychrophilic strain of this group (*Oleispira antarctica)* does not take up common carbohydrates and amino acids as sole C sources, and shows a preference for aliphatic hydrocarbons (Yakimov *et al.*, 2003). This metabolic preference could explain the low leucine uptake for this group seen in the autoradiograms. The reasons why *Oleispira* showed such high activities in bicarbonate uptake (over 70% of active cells) are still unclear, but could be related to the observation that some pathways of anaerobic hydrocarbon degradation include carboxylation reactions (Zhang and Young, 1997).

PSA-Colwellia was the most abundant and heterotrophically active group within *Gammaproteobacteria* (Figure 4b). The high bicarbonate incorporation for this group could be related to the detection of a variety of *accC* sequences encoding different putative biotin carboxylases affiliated with a psychrophilic strain of this group: *Colwellia psychrerythraea* 34H (Table 1). The analysis of the genome of this bacterium has shown that it can metabolize complex organic substrates and possibly C1 compounds (Methé *et al.*, 2005), and the metabolic use of C1 compounds is known to enhance CO_2 assimilation (Doronia and Trotsenko, 1985). A similar rationale could apply to the betaproteobacterial phylotypes identified in the clone libraries affiliated with *Methylophylales,* which also use C1 compounds in their metabolism, explaining the high number of bicarbonate-incorporating *Betaproteobacteria* detected in the autoradiograms.

In summary, our results show that heterotrophic CO_2 assimilation was actively taking place at nutrient-depleted stationary phase in Arctic SWCs. Previous work with marine strains suggests that under oligotrophic conditions, anaplerotic CO_2 incorporation can play an important role for compensating metabolic imbalances (González *et al.*, 2008). Considering the resource-limited nature of most of the ocean, we hypothesize that dark CO_2 assimilation may have more relevance than previously assumed for some marine bacterial taxa, and deserves a closer look under different oceanic environmental regimes. We have shown that bicarbonate uptake varied markedly among Arctic heterotrophs, and we identified active groups closely related to *Oleispira*, and *PSA-Colwellia*. The ability to use bicarbonate as a key substrate involved in different metabolic reactions could potentially promote these groups for maintenance of cell activity and/or longer survival under resource depleted conditions.

Acknowledgements

We thank Helmuth Thomas (Dalhousie University) for his assistance with the DIC measurements onboard, Roxane Maranger (Université de Montreal) and Connie Lovejoy (Université Laval) for sharing lab equipment, and our fellow scientists, officers and crew of the CCCG *Amundsen* for their support during the cruise. J. González is acknowledged for valuable comments on the manuscript. This work is a contribution to the International Polar Year – Circumpolar Flaw Lead system study (IPY-CFL 2007/2008) lead by D. Barber (University of Manitoba) supported through grants from the Canadian IPY Federal Program Office, the National Sciences and Engineering Research Council, grant BOREAL (CLG2007-28872-E/ANT) from the Spanish Ministry of Science and Innovation to C.P.-A., and grants from the Swedish Research Council to S.B and L.A.S. L.A.S. was supported by a Marie Curie Intraeuropean Fellowship (CHEMOARC PIEF-GA-2008-221121), E.O.C by the Spanish grant CGL2009-13318-BOS, and P. E. G by a Marie Curie grant (CRENARC MEIF-CT-2007-040247). We would like to acknowledge the helpful contributions of three anonymous reviewers.

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