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To cite this version:

Pierre Galand, Laura Alonso-Saez, Stefan Bertilsson, Connie Lovejoy, Emilio O. Casamayor. Contrasting activity patterns determined by BrdU incorporation in bacterial ribotypes from the Arctic Ocean in winter. Frontiers in Microbiology, Frontiers Media, 2013, 4, pp.118. <10.3389/fmicb.2013.00118>. <hal-01561031>

HAL Id: hal-01561031

https://hal.sorbonne-universite.fr/hal-01561031

Submitted on 12 Jul 2017

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Contrasting activity patterns determined by BrdU incorporation in bacterial ribotypes from the Arctic Ocean in winter

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The winter Arctic Ocean is one of the most unexplored marine environments from a microbiological perspective. Heterotrophic bacteria maintain their activity at a baseline level during the extremely low-energy conditions of the winter, but little is known about the specific phylotypes that have the potential to survive and grow in such harsh environment. In this study, we aimed at identifying actively growing ribotypes in winter Arctic Ocean seawater cultures by experimental incubations with the thymidine analog bromodeoxyuridine (BrdU), followed by immunocapturing, terminal restriction fragment length polymorphism fingerprinting, cloning, and sequencing the 16S rRNA gene. We incubated water collected at different months over the Arctic winter and showed that the actively growing bacterial fraction, taking up BrdU, represented only a subset of the total community. Among the BrdU-labeled bacterial taxa we identified the Flavobacteria Polaribacter, the Alphaproteobacteria SAR11, the Gammaproteobacteria Arctic 96B-16 cluster and, predominately, members of Colwellia spp. Interestingly, Colwellia sequences formed three clusters (90 and 97% pairwise 16S rRNA identity) that contributed in contrasting ways to the active communities in the incubations. Polaribacter, Arctic 96B-16 and one cluster of Colwellia were more abundant in the active community represented by the BrdU-labeled DNA. In contrast, SAR11 and two other Calwellia clusters were underrepresented in the BrdU-labeled community compared to total communities. Despite the limitation of the long incubations needed to label slow growing arctic communities, the BrdU approach revealed the potential for active growth in low-energy conditions in some relevant groups of polar bacteria, including Polaribacter and Arctic 96B-16. Moreover, under similar incubation conditions, the growth of different Colwellia ribotypes varied, suggesting that related clusters of Colwellia may have distinct metabolic features.

Keywords: Colwellia, bacteria, Arctic Ocean, BrdU, activity

INTRODUCTION

The winter in the Arctic Ocean is characterized by an almost complete lack of sunlight and water temperatures below zero degrees Celsius. These extreme conditions are expected to have a large impact on the composition and activity of marine microbes in surface waters down to the nitracline (i.e., the depth interval where nitrate concentration rapidly increase from depleted surface concentrations), as carbon and nutrient availability drastically changes when primary production is reduced or stops during darkness. Indeed, changes in the relative abundance of major bacterial groups over the course of the winter (Alonso-Sáez et al., 2008), as well as in their use of dissolved organic substrates (Nikrad et al., 2012), have been previously reported. Interestingly, despite the harsh environmental conditions, heterotrophic bacterial production is sustained at a baseline level during the entire winter (Garneau et al., 2008), and the single-cell activity of some abundant groups of higher taxonomic rank has been shown to be relatively high (Alonso-Sáez et al., 2008).

Identifying metabolically active ribotypes in low productive marine environments is challenging and information on the specific phylotypes that can actively grow in polar winter waters is scarce. Here, we address this challenge by using Bromodeoxyuridine (BrdU) incubations. Labeling of DNA with BrdU was first used in microbial ecology as an alternative to radioactive thymidine (TGR) to measure bacterial growth (Steward and Azam, 1999). BrdU is an analog of TdR that is incorporated into newly synthesized DNA. This feature was also used to identify the pool of growing bacteria within a community (Bornerman, 1999; Ulbach et al., 1999) and later coupled to microscopy to identify DNA-synthetizing cells by fluorescence in situ hybridization.
A significant advantage of the BrdU approach is that this method can potentially show differences in the activity of closely related phyotypes. In previous studies of Arctic waters, a high degree of diversity was found within the most abundant phylotypes, implying that many closely related ribotypes can co-exist when conditions are favorable. To what extent the specific metabolic characteristics of such related but ecologically distinct ribotypes differ has not yet been studied extensively. Ecological differences based on spatial and temporal distribution patterns have been described for phyotypes of major marine prokaryotic groups such as unicellular cyanobacteria (Rocap et al., 2003; Mou et al., 2007) or the SAR11 cluster (Brown et al., 2012), implying that many closely related ribotypes that may constitute ecotypes in this environment.

### MATERIALS AND METHODS

#### SAMPLING, INCUBATIONS AND BrdU IMMUNOCAPTURE

Water was sampled in the Amundsen Gulf, western Arctic, on November 16th 2007 (Nov), January 10th 2008 (Jan), and February 11th 2008 (Feb) from the base of the nitracline to target a well-defined non-surface water mass also studied by Alonso-Sáez et al., 2010 (Table 1). Samples were immediately filtered through a 50 μm mesh, divided between 6 acid-rinsed 10 L polycarbonate bottles and incubated in the dark at 2°C for 30 days with 100 μmol L⁻¹ BrdU. Bacteria assimilating the thymidine analog BrdU into their newly synthesized DNA can then be identified by immunocapture. This subset of the community will then be considered the actively replicating portion of the community. BrdU incubations are usually short (Pernthaler et al., 2002), but due to the slow growth of bacteria during the Arctic winter, we chose to increase incubation times to ensure sufficient BrdU incorporation (see discussion).

For the November and January experiments one bottle was sacrificed after 5 days and ca. 10 days. For the February experiment, additional two bottles were sacrificed after 22 and 28 days. Five liters of water were filtered sequentially through a 3 μm pore size polycarbonate filter (Poretics) and a 0.2 μm pore size Durapore filter, using a peristaltic pump. Filters were immediately preserved by freezing at −80°C and DNA was extracted from the 0.2 μm filters as described earlier (Alonso-Sáez et al., 2009). BrdU-labeled DNA (BrdU), representing the active bacterial fraction, was then separated from the total DNA fraction (Tot) by immunocapture. Labeled DNA was captured with anti-BrdU primary antibodies (Mouse IgG monoclonal antibody clone BMC 9318, Roche) followed by isolation using magnetic beads with secondary antibodies (Dynabeads M-450 coated with goat anti-mouse IgG, Invitrogen). DNA was then precipitated with 3M sodium acetate (NaAc) and 99% EtOH. Non-BrdU controls (i.e., seawater cultures without addition of BrdU) were also incubated in the February experiment. For these controls no polymerase chain reaction (PCR) amplification was obtained after the immunocapture procedure. This result, together with the lack of amplification of BrdU labeled DNA after incubation times shorter than 10 days, suggests that no false positives (non-BrdU labeled DNA) was captured in our study.

#### TRFLP, CLONING-SEQUENCING, AND STATISTICAL ANALYSIS

Bacterial 16S rRNA sequences were amplified for terminal restriction fragment length polymorphism (TRFLP) analysis with primers 27f (Virgin et al., 1998) labeled with hexachlorofluorescein (HEX) at the 5 end, and 519r (Lane, 1991) as described earlier (Lymer et al., 2008). Triplicate PCR products were digested using three restriction enzymes in parallel: HaeIII, Hhal, and HindIII. Fragments were separated and detected with an ABI 3730 capillary sequencer together with internal size standards. TRFLP peaks were determined with GeneMarker 1.51 (SoftGenetics) following thresholds and criterions published earlier (Lymer et al., 2008). The different data sets from the three different restriction enzymes were grouped together and a matrix based on peak intensity was built to compare community composition between samples. Jaccard’s similarity coefficient was calculated to compare communities by non-metric multidimensional scaling (NMDS). Analysis of similarity (ANOSIM) statistics were used to verify the significance of the grouping by testing the hypothesis that samples from a same group were more similar in composition with each other than with communities in different group.

In order to taxonomically identify TRFLP peaks we amplified, cloned (TOP10 TA kit, Invitrogen) and sequenced bacterial 16S rRNA sequences with primers 27f-1492r from the total DNA fraction for the three incubations. Theoretical terminal restriction fragments were then generated for each enzyme with the program TRFLPlex (Jumier et al., 2008) with the 159 cloned sequences and with 81 sequences from incubated sea water published earlier from the same region (Alonso-Sáez et al., 2010), and environmental TRFLP peaks were identified by comparison with theoretical TRFLP peaks based on a 454 pyroseqencing database provided by the Pernthaler lab (Pernthaler et al., 2002; Pernthaler and Pernthaler, 2005). More recently BrdU incubations have been combined with 16S rDNA-based analysis to study the spatial distribution of active communities (Taniguchi and Hamasaki, 2007) or identify active bacteria degrading dissolved organic compounds (Mou et al., 2007).

### Table 1: Position of sampling stations and environmental characteristics of the water used for BrdU incubations.

| Station ID | Date       | Longitude | Latitude | Depth (m) | Temperature (°C) | Salinity |
|-----------|------------|-----------|----------|-----------|------------------|----------|
| D14       | 2008-01-08 | 125° 35.766 W | 71° 31.584 N | 80 | −1.5 | 32.3 |
| D19       | 2008-02-11 | 124° 47.136 W | 71° 4.236 N | 50 | −1.35 | 32.6 |
| D117      | 2007-11-16 | 126° 30.031 W | 69° 52.104 N | 20 | −0.9 | 33.8 |
RESULTS AND DISCUSSION

BrdU-labeled DNA (BrdU), representing the actively dividing bacterial fraction, was successfully separated from the total DNA fraction (Tot) by immunocapture in incubations of winter Arctic seawater. The BrdU method had been successfully applied in different freshwater and marine environments, but its application in the extremely low productive winter Arctic water was challenging. Therefore, we increased the concentration of BrdU and the incubation time as compared to previous studies. While short incubation times are generally desirable in order to obtain results representative of in situ conditions, we found that only BrdU-labeled DNA incubated for at least 10 days was successfully amplified by PCR in the winter Arctic seawater incubations. According to estimates based on the different phases of cell cycles, a BrdU incubation time of 65% of the doubling time would label 100% of all growing cells (Pernthaler and Pernthaler, 2003). In winter waters of the Amundsen Gulf, a prokaryotic growth rate of 0.1 d\(^{-1}\) was measured in dilution cultures (Alonso-Sáez et al., 2010), which indicates a doubling time of ca. 7 days. Therefore, theoretically, the labeling of actively growing Arctic communities would take ca. 4.5 days. However, growth rates in our incubations were probably lower as we incubated the water without dilution. That may explain that insufficient BrdU-labeled DNA could be extracted from samples collected during the first week of incubation. In the experiment carried out in February, additional BrdU samples were analyzed after 22 and 28 days of incubation. For all the samples, the active and total community DNA was amplified and analyzed by TRFLP (Figure 1).

As shown by the TRFLP analysis, BrdU-labeled communities were different from the total communities, and NMDS grouped all labeled samples away from non-labeled samples (Figure 1; ANOSIM, \(p < 0.01\)). This indicated that the actively growing bacterial fraction (BrdU) represented only a subset of the total community. Interestingly, during the February experiment the composition of the total bacterial community changed little from 13 to 28 days of incubation, while differences in the active communities were detected over the course of the experiment, particularly between 22 to 28 days (Figure 1). The active bacterial communities after ca. 10 days of incubation in November and January showed higher similarity, but were different to the active community in the experiment of February after 13 days. As incubation conditions were strictly similar for the three experiments, our results suggest that Arctic communities contained different bacterial phylotypes that varied in their potential for growth during the course of the winter.

It should be emphasized that the studied communities do not directly represent in situ active bacterial communities, as incubations may promote or limit the growth of some bacteria from the natural assemblages, particularly when long incubation periods are required and high concentrations of potentially inhibiting compounds such as BrdU are applied. Other potential pitfalls in our approach are that the fingerprinting techniques such as TRFLP may miss some bacterial taxa, and that the BrdU substrate could potentially be toxic for some bacteria. For example, early studies have shown that a strain of Bacillus subtilis had reduced sporation and growth under BrdU incubation (Binnie and Coote, 1986). We were, however, able to detect different bacteria in the BrdU fraction, such as SAR11, Polaribacter, Arctic96B–16 and Colwellia, which are known to be environmentally relevant in Arctic waters (Bano and Hollibaugh, 2002; Alonso-Sáez et al., 2010). Our results thus show that even though some Arctic microorganisms may have been inhibited, a large diversity of them take up BrdU at the concentration added.
The major components of the bacterial communities were identified by cloning and sequencing bacterial 16S rRNA genes from the three experiments, and we used these data to identify the TRFLP peaks by using the program TRiFLe. We obtained 41, 57, and 61 sequences (ca. 950 bp long) from the libraries Tot_Nov_6d, Tot_Jan_10d, and Tot_Feb_13d, respectively. Diversity was low in all three libraries, which were dominated by the genus Colwellia that represented >88% of the sequences. Colwellia is a psychrophilic marine bacteria, which is well adapted to life in cold environments (Methé et al., 2005). Members of Colwellia have been detected in a wide range of marine habitats, including polar regions (Deming and Junge, 2005), and appear to be important to carbon and nutrient cycling in the cold marine environments (Methé et al., 2005). Colwellia was also abundant in previous Arctic winter water incubations, particularly in samples taken from the nitracline (i.e., 38% of clones; Alonso-Sáez et al., 2010). Clone libraries from in situ samples collected at the same location in winter showed communities dominated by Gammaproteobacteria and Alphaproteobacteria, a composition that is typical for the Arctic Ocean (Lovejoy et al., 2011). The Arctic 96B cluster and Polaribacter were also present while Colwellia represented 4% of the sequences (Alonso-Sáez et al., 2010).
Arctic winter waters in an earlier study (11 sequences from clusters 1 and 2) were successfully resolved based on their TRFLP profiles using the software TRiFle (Bowman et al., 1998; Junge et al., 2002). The three clusters had a minimum of 93% identity. The three clusters fell into three separate clusters (Figure 2). Colwellia psychrerythraea 34H, sequences from clusters 2 and 3, and SAR11 (significant difference between means between total and active communities, p < 0.05) had a minimum of 97% identity and sequences from cluster 2 and 3 had a minimum of 93% identity. The three clusters are very diverse, including the presence of closely related ribotypes. Yet, the ecological significance of their diversity, and the ecological role of different Colwellia clusters remain unclear. In our Arctic seawater incubations, Colwellia showed intra-genus diversity and sequences fell into three separate clusters (Figure 2). Most of the cloned sequences belonged to cluster 3 (123 sequences), represented by the Colwellia strain ZS4-15, while fewer fell into cluster 2 and 1 (6 and 12 sequences, respectively). The least abundant cluster 2 was closest to C. psychrerythraea 34H. Sequences from clusters 2 and 3 had a minimum of 97% identity and sequences from cluster 2 and 1, a minimum of 93% identity. The three Colwellia clusters were detected in clone libraries from seawater dilution cultures with Arctic winter waters in an earlier study (11 sequences from cluster 1, 1 from cluster 3 and 1 from cluster 2, Alonso-Sáez et al., 2010), and were earlier shown to be selectively enriched in North Sea seawater incubations (Eilers et al., 2000) and able to outcompete other bacteria during bottle incubations. Thus, Colwellia probably dominated in our experiment because they were able to take advantage of the incubation conditions. However, in contrast to previous studies, it is remarkable that this phylotype was able to grow in winter Arctic waters, as the availability of fresh organic substrates is very low. Colwellia species have typically been cultured on heterotrophic media and grow from ~1 to 10^5 CFU (Huston et al., 2000). An analysis of the genome of the psychrophile Colwellia psychrerythraea 34H has revealed the potential for this strain to degrade fatty acids and polyamines, and suggests a possible C1 metabolism (Methé et al., 2005). This type of metabolism could be related to the fact that Colwellia cells showed active uptake of bicarbonate during incubations in Arctic winter seawater, and may be interpreted as a strategy to deal with starvation under resource depleted conditions (Alonso-Sáez et al., 2010).

Colwellia was previously isolated from a variety of polar habitats (Bowman et al., 1998; Jureg et al., 2002) and Arctic 96B-16 were more abundant in the active community represented by the BrdU-labeled DNA, indicative of a high potential for growth in Arctic winter waters. In contrast, Colwellia cluster 3, SAR11, and Colwellia cluster 2 were underrepresented in the BrdU-labeled community compared to total communities (significant difference between means between total and active communities, p < 0.05). Our observation that Polaribacter was more frequently found in the active fraction is in agreement with a recent study which found that Polaribacter had higher leucine uptake rates during winter compared to summer in the Beaufort Sea (Nilkov et al., 2012). It should be noted that other microorganisms present in the incubations might have been overlooked by the TRFP fingerprinting techniques. The abundant Colwellia clusters 3 and 1 showed marked differences in activity in the experiments based on BrdU labeling (Figure 3). A SIMPER analysis conducted on TRFLP peaks confirmed that Colwellia cluster 1 contributed more than cluster 3 to the active communities across all incubations (Table 2). A SIMPER analysis identified the phylotypes that contributed most to the difference between BrdU labeled and total community.

| Phylotypes | Contribution | Cumulative % | Mean abundance BrdU | Mean abundance Total |
|------------|--------------|--------------|---------------------|---------------------|
| Colwellia cluster 3 | 4.20 | 6.61 | 0.27 | 0.41* |
| Colwellia cluster 1 | 1.70 | 24.62 | 0.11 | 0.04* |
| SAR11 | 1.33 | 33 | 0.01 | 0.06* |
| Colwellia cluster 2 | 0.73 | 46.28 | 0.05 | 0.02 |
| Polaribacter | 0.70 | 55.76 | 0.06 | 0.02** |

Cumulative % represents the added contributions of individual phylotypes to the difference between groups; * indicates a significant difference between means. ** Significant difference in February only.

Additional analyses identified other abundant TRFLP peaks, including the Gammaproteobacteria, Arctic 96B-16 cluster, Polaribacter, and SAR11 (Figure 2). Colwellia cluster 1, Polaribacter, and Arctic 96B-16 had significant differences between means between total and active communities (p < 0.05) as calculated with a Wilcoxon test for paired values.

FIGURE 2 | Proportion of bacterial groups present in the total and the active (BrdU-labeled) fraction of the community. Data obtained by TRFLP represents averages across all three experiments. Colwellia cluster3, cluster1, and SAR11 had significant differences between means between total and active communities (p < 0.05) as calculated with a Wilcoxon test for paired values.
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**ACKNOWLEDGMENTS**

We thank Ramon Terrado for carrying out a portion of the experimental sampling, Carmen Gutierrez for laboratory assistance, Maria Hellman for her advice with the BrdU protocol and José M. González for help with the program T-RFPred. We also thank our fellow scientists, officers and crew of the CCGG Amundsen for their support during the cruise. This work is a contribution to the International Polar Year – Circumpolar Flaw lead system study (IPY-CFL 2007/2008) and ArcticNet. Pierre E. Galand and Laura Alonso-Sáez were supported by Marie Curie International European Fellowship grants CRENARC MEIF-CT-2007-004247 and CHEMOAR CIEF-GA-2008-221121, respectively. Connie Lovejoy was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) SRO and Discovery grants and ArcticNet. The work done in Emilio O. Casamayor lab was supported by grant FIRENA CGL2009-13318 from the Spanish Ministerio de Ciencia e Innovacion (MICINN) and the molecular work carried out in Uppsala was funded by grants from the Swedish Research Council to Stefan Bertilsson.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 January 2013; accepted: 26 April 2013; published online: 20 May 2013.

Citation: Galand PE, Alonso-Sáez L, Bertilsson S, Lovejoy C and Casamayor EO (2013) Contrasting activity patterns determined by BrdU incorporation in bacterial ribotypes from the Arctic Ocean in winter. Front. Microbiol. 4:118. doi: 10.3389/fmicb.2013.00118

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