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SHORT COMMUNICATION

Consuming algal products: trophic interactions of bacteria and a diatom species determined by RNA stable isotope probing

Melanie Sapp · Gunnar Gerdts · Marco Wellinger · Antje Wichels

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Abstract Heterotrophic marine bacteria utilise a wide range of carbon sources. Recently, techniques were developed to link bacterial identity and physiological capacity of microorganisms within natural communities. One of these methods is stable isotope probing (SIP) which allows an identification of active microorganisms using particular growth substrates. In this study, we present the first attempt to analyse bacterial communities associated with microalgae by rRNA-SIP. This approach was used to analyse bacterial populations consuming algal products of *Thalassiosira rotula* by applying SIP followed by reverse transcription of 16S rRNA and denaturing gradient gel electrophoresis. Generally, our results indicate that bacteria which consume algal products can be detected by isotope arrays coupled with fingerprinting methods.

Keywords Bacteria–phytoplankton interaction · Stable isotope probing · RT-PCR · DGGE

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M. Sapp (✉)
Centre for Environment,
Fisheries and Aquaculture Science, Pakefield Road,
Lowestoft, Suffolk NR33 0HT, UK
e-mail: Melanie.Sapp@cefas.co.uk

M. Sapp · G. Gerdts · A. Wichels
Biologische Anstalt Helgoland,
Alfred Wegener Institute Foundation for Polar
and Marine Research, PO Box 180,
27483 Helgoland, Germany

M. Wellinger
Swiss Federal Institute of Technology Zurich,
Zurich, Switzerland

Introduction

The association of bacteria and phytoplankton cells has been studied by a wide range of methods. Microscopy was applied to study the association on the level of single algal cells. In detail, a combination of tyramide signal amplification—fluorescent in situ hybridisation and confocal laser scanning microscopy was successfully introduced by Biegala et al. (2002) to identify and localise bacteria associated with phytoplankton. A deeper insight into the type of this attachment was achieved by scanning electron microscopy (Kaczmarska et al. 2005).

Associated bacteria were also studied by fingerprinting methods on the community level showing that distinct bacterial groups occur in different microalgal cultures (Schäfer et al. 2002; Grossart et al. 2005; Jasti et al. 2005; Sapp et al. 2007a). Furthermore, Grossart et al. (2005) performed experiments revealing that specific bacterial groups from natural communities colonise phytoplankton cells.

Although these findings indicate specific interactions of bacteria and microalgae, it is still a challenge to study these on a physiological level. Recently, techniques have been developed to link bacterial identity and physiological capacity. One of these is RNA-based stable isotope probing (RNA-SIP) of uncultured microorganisms consuming substrates enriched with stable isotopes (Neufeld et al. 2007). Generally, commercially available substrates with high enrichment of stable isotopes were used such as ^{13}C-labelled methanol and propionate (Lueders et al. 2004a, b) or phenol (Manefield et al. 2002).

We present the first attempt to study bacteria associated with the diatom *Thalassiosira rotula* by RNA-SIP. Incubation of algal cells with $NaH^{13}CO_3$ was carried out to label cellular compounds. Afterwards, ^{13}C-labelled rRNA

templates were separated by isopycnic centrifugation followed by reverse transcription of a 16S rRNA gene fragment. Associated bacterial communities of fractions with different buoyant densities were compared by denaturing gradient gel electrophoresis (DGGE).

To the best of our knowledge this is the first study combining enrichment of a natural substrate with ^{13}C and SIP of algal associated bacteria.

Methods

Algal cultures

Single cells of *T. rotula* Meunier were isolated as described by Sapp et al. (2007a). The xenic algae were cultivated in f/2 medium (Guillard 1975) pH 8.0, based on aged seawater of the mid North Sea stored in the dark after filtration for at least 1 year. It is suggested that microbial respiration had removed organic carbon completely from the seawater. Prior to the experiment *T. rotula* grew in 1 l culture flasks at 16°C and a 12:12 LD-cycle (45.0 µmol photons $m^{-2}s^{-1}$).

Experiment

The experiment included three treatments: (1) Incubation of the microalga with its associated bacterial community, (2) incubation of the algal culture with a natural bacterial seawater community, (3) control consisting of algal associated bacterial community without algal cells. Every treatment was carried out in two replicates.

For the experiment, $NaH^{13}CO_3$ (98% ^{13}C, ISOTEC, SIGMA-ALDRICH, USA) was added to the medium resulting in a concentration of 2 mM $NaH^{13}CO_3$. Incubation was performed for 15 days. For treatment (1) a volume of 100 ml *T. rotula* culture (early stationary phase) was added to 10 l of medium (K1, K2). Treatment (2) consisted of 100 ml filtrate of a seawater sample [<3 µm, through membrane filters (Millipore, Germany)] and 100 ml *T. rotula* culture both added to 10 l of ^{13}C-medium (Sea 1, Sea 2). The control (treatment 3) consisted of 100 ml of a filtrate (<3 µm, through membrane filters) of the algal culture in 10 l of ^{13}C-medium to analyse which bacteria might incorporate labelled carbonate autotrophically (C1, C2). Incubation was carried out at 16°C and a 12:12 LD-cycle (45.0 µmol photons $m^{-2}s^{-1}$).

Isotope ratio

Two hundred millilitre of each treatment were filtered through GF/C filters (1.2 µm, Whatman, UK) in duplicate. One of these was used to determine the carbon mass, the other was used to measure the isotope ratio. Determination of carbon mass was carried out with an Elemental Analyser EA1108 (Fisons Instruments, Italy). Acetanilid served as internal standard. For stable isotope analyses parts of the GF/C filters with an amount of 100–200 µg carbon were put into tin cups (Lüdi, Switzerland). These were oxidised and the isotope ratio was measured in a Delta C mass spectrometer coupled with a Conflo II-Interface (both Thermo-Finnigan, Germany) and an elemental analyser Fisons1106 (Fisons Instruments) with acetanilid as standard. Isotope ratios are expressed using the standard Delta notation $\delta^{13}C$ [‰] and ^{13}C atom%. A GF/C filter (Whatman) served as blank and revealed no signal within the isotope analysis. The natural ratio of $^{13}C/^{12}C$ of *T. rotula* in culture was revealed as 1.08 ^{13}C atom%. Stable isotope analyses were performed by the competence centre of stable isotopes (KOSI), Georg August University of Göttingen, Germany.

RNA extraction

Biomass was filtered through 10 µm (treatments 1, 2) or 3 µm (treatment 3) and 0.22 µm membrane filters (Millipore) in succession. Filters were stored at −70°C until nucleic acid extraction. Filters with 0.22 µm pore size were cut and transferred to sterile and RNAse free 2 ml vials. Biomass was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and cell lyses were performed at 64°C for 2 min by adding lysozyme (20 µg ml^{-1}) and SDS (1%). Sodium acetate (0.05 M, pH 5.2) and acid phenol–chloroform–isoamylalcohol (25:24:1) were enclosed; incubation was carried out at 64°C for 6 min. Further purification was carried out by addition of chloroform. Precipitation was achieved by addition of ethanol and sodium acetate buffer (0.15 M NaOAc, 50 µM EDTA, pH 5.2). The quality of nucleic acid extracts was analysed by gel electrophoresis on 1% agarose gels (90 min at 100 V in 1× TBE (10× TBE: 0.89 M Tris, 0.89 M boric acid, 0.025 M EDTA)) and ethidium bromide staining (0.5 mg l^{-1}). Imaging was performed with a ChemiDoc XRS System (BioRad, Germany).

Digestion of co-extracted DNA was carried out with RNAse free DNAse I (Ambion, UK) according to the manufacturer's protocol. Re-extraction with acid phenol–chloroform–isoamylalcohol (25:24:1) and chloroform (see above) provided pure RNA which was finally kept in RNA storage solution (Ambion) and stored at −70°C until further analysis.

Isopycnic centrifugation and gradient fractionation

Polyallomer Quick-Seal tubes (5.1 ml, Beckman, Germany) were used for density gradient centrifugation in an Optima TL ultracentrifuge using a TLA-100.4 fixed angle rotor

(Beckman, Germany). Cesium trifluoroacetate (CsTFA, GE Healthcare, UK) gradients with an average density of 1.80 g ml^{-1} were prepared according to Lueders et al. (2004a). A maximum amount of 500 ng RNA was resolved in centrifugation media consisting of CsTFA stock solution, gradient buffer (0.1 M Tris–HCl, 0.1 M KCl, 1 mM EDTA, pH 8.0) and formamide (AppliChem, Germany). Centrifugation was carried out at 20°C and 55,000 rpm (126,000g_{av}) for 66 h. Fractionation was performed with a programmable syringe pump (TSE Systems, Germany) resulting in 12 fractions per gradient. An aliquot of 100 µl of each fraction was used to determine the density of each fraction using an Abbe refractometer (Zeiss, Germany). Precipitation of RNA of fractions was done by addition of ethanol including a washing step with 80 % ethanol. RNA was finally kept in RNA storage solution (Ambion) and stored at −70°C.

Quantification of RNA was performed by fluorometric assays using the stain Ribo Green (Invitrogen, Germany) with RNA of *Escherichia coli* (Ambion) as standard as recommended by the manufacturer's protocol. Measurements were carried out in a Cary Eclipse Fluorescence Spectrophotometer with Microplate Reader (VARIAN, Germany).

Reverse transcription was carried out in two steps using the sensiscript reverse transcription kit of QIAGEN (Germany). A maximum amount of 50 ng RNA was incubated with 2 µl of Hexanucleotide mix (Roche, Germany) at 85°C for 3 min (Muyzer et al. 2004). For transcription each 20 µl reaction contained 2 µl 10× RT-buffer, 2 µl dNTP mix (5 mM each dNTP), 1 µl sensiscript reverse transcriptase (all QIAGEN) and 10 U Protector RNAse inhibitor (Roche). The reaction was carried out at 37°C for 60 min as recommended by the manufacturer. Reverse transcription was performed in an Eppendorf Mastercycler (Germany) including negative and positive controls the latter consisting of *E. coli* RNA (Ambion).

Amplification of 16S rRNA gene fragments was performed using the primers 341f with a 40-bp GC-rich sequence at the 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and 907rm (5'-CCG TCA ATT CMT TTR AGT TT-3′) (Sapp et al. 2007b). PCR reaction mixtures with a volume of 100 µl contained 10 µl 10× Taq buffer (500 mM KCl, 100 mM Tris–HCl, 15 mM $MgCl_2$, pH 8.3, Eppendorf), 20 µl 5× Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer), 0.2 µM of each primer, 2 U Taq DNA Polymerase (Eppendorf), and 20 µl RT-reactions. The touchdown PCR started with a denaturing step at 94°C for 5 min. Every cycle consisted of three steps of 1 min, i.e., 94°C, annealing temperature, and 72°C. The initial annealing temperature of 65°C decreased by 0.5°C per cycle until a touchdown of 55°C, at which temperature 12 additional cycles were carried out. Final primer extension was performed at 72°C for 10 min followed by 22 cycles starting at 71°C decreasing by 1°C per cycle to avoid heteroduplices. PCR reactions were performed in an Eppendorf Mastercycler. PCR products were inspected on 1.2% (w/v) agarose gels.

Denaturing gradient gel electrophoresis (DGGE)

Community analyses were performed with a Bio-Rad DCode system as described by Sapp et al. (2007b). Fragments were resolved in 6% (w/v) polyacrylamide gels in 0.5× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA) with denaturing gradients of 15–70% urea/formamide (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was run at 60°C and 150 V for 10 h. DGGE gels were stained with SYBRGold and imaging was performed with a ChemiDoc XRS System (BioRad).

Results

Isotope signatures

Isotope ratio mass spectrometry revealed labelling of all treatments with slightly different signatures (Table 1). Isotope signatures ranged from 31.18 to 45.40 ^{13}C atom%. Generally, this finding shows that enrichment of algal and bacterial biomass could be achieved. In detail, the isotope signatures of biomass of treatment 3 were slightly lower than signatures of biomass of treatments 1 and 2.

Isopycnic centrifugation

Average densities of gradient fractions ranged from 1.72 to 1.87 g ml^{-1}. Fractions retrieved from treatment 1

Table 1 Isotope signatures of different treatments

	Treatment					
	1		2		3	
	K1	K2	Sea 1	Sea 2	C1	C2
Atom% ^{13}C	45.03	45.40	41.78	43.10	31.18	40.39
$\delta^{13}C$ [‰]	72284.55	73382.18	63174.92	66755.65	39521.37	59611.38

displayed a distribution of RNA within the density range of 1.72–1.85 g ml^{-1}, gradient fractions retrieved from treatment 2 displayed slightly different distributions of RNA with buoyant densities ranging from 1.75 to 1.87 g ml^{-1}. Fractions with highest ratios of maximum quantities of RNA of treatment 3 were detected with a buoyant density of 1.74 g ml^{-1}, also fractions with higher buoyant densities were found. A comparison of RNA distribution in gradients of treatments 1–3 is shown in Fig. 1. Treatment 1 displayed some fractions with a high ratio of maximum quantities above 0.6 at buoyant densities of 1.81, 1.80 and 1.77 g ml^{-1}. Other fractions with lower ratios of maximum quantities were retrieved over the whole gradient.

Fractions of treatment 2 displayed highest ratios of maximum quantities at buoyant densities of 1.83 and 1.82 g ml^{-1}. Fractions with a ratio of maximum quantities of 0.2 and lower could be detected at buoyant densities ranging between 1.80 and 1.75 as well as 1.87 g ml^{-1}.

Fractions with high ratios of maximum quantities of the control were detected at buoyant densities of 1.74 g ml^{-1}. Other fractions of treatment 3 displayed buoyant densities of 1.77 and 1.79 g ml^{-1}.

Community structure

RT-PCR was performed for unfractionated RNA samples and for respective gradient fractions. Summarised are results of all samples for which both RT-PCR and amplification of the 16S rRNA gene fragment could be achieved. Fingerprints are shown for treatment 1 (Fig. 2). The communities of the duplicates varied slightly. It became apparent in duplicate one (K1) that the majority of bands appeared in both the unfractionated sample and in the fractions. Nevertheless, differences in the fingerprints occurred. Bands combined in K1_1 were detected in the unfractionated sample and in a fraction with the buoyant density of 1.81 g ml^{-1}. Other fractions of lower buoyant densities did not display those bands. Band K1a1 could be detected in the unfractionated sample and in fractions with buoyant densities of 1.81 and 1.78 g ml^{-1}. Band K1b1 was detected solely in a fraction with the buoyant density of 1.80 g ml^{-1}. Major bands assigned to duplicate 2 (K2) occurred in the unfractionated sample and in its fractions. Beside these similarities band K2a1 appeared solely in the unfractionated sample and in a fraction with the buoyant density of 1.79 g ml^{-1}. Band K2b1 occurred in fractions with buoyant densities of 1.78, 1.77 and 1.75 g ml^{-1}. Band K2b2 appeared in fractions with buoyant densities of 1.78 and 1.75 g ml^{-1}. In treatments 2 and 3 major bands were detected for both duplicates and their fractions. Some bands occurring in the unfractionated sample of treatment 2, duplicate 1, were not detected in the fractions. Within the DGGE profiles of duplicate 2 the unfractionated sample and its fractions displayed identical patterns except of the appearance of an additional single band in the fraction with the buoyant density of 1.78 g ml^{-1}. Some bands assigned to treatment 3, duplicate 1, could not be detected within the fractions. Duplicate 2 and its fraction (buoyant density 1.74 g ml^{-1}) displayed identical DGGE profiles.

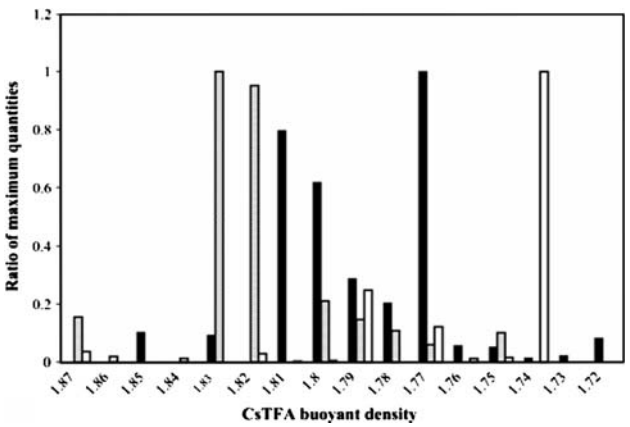

Fig. 1 Comparison of fractions: CsTFA density gradient centrifugation of RNA samples and quantitative evaluation of nucleic acid distribution within gradient fractions (*black*: treatment 1, *grey*: treatment 2, *white*: control). RNA was detected fluorometrically

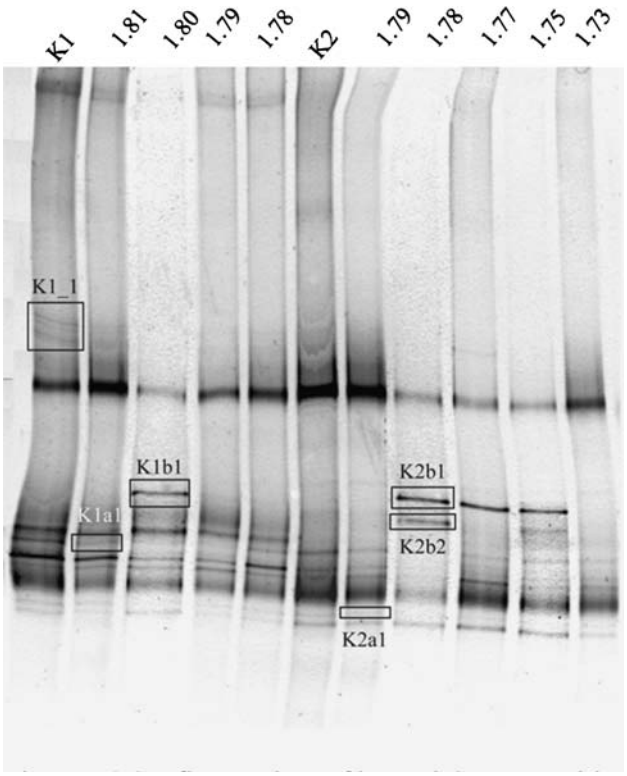

Fig. 2 DGGE fingerprints of bacterial communities retrieved from treatment 1 resolved on 6% polyacrylamide gels with denaturing gradient of 15–55% urea/formamide. Specific phylotypes or groups of phylotypes are indicated

Discussion

In this study, we investigated if rRNA-based stable isotope probing can generally be applied to study bacterial communities associated with phytoplankton. Overall, algal biomass was labelled with ^{13}C, and different fractions of CsTFA gradients containing bacterial phylotypes could be separated by isopycnic centrifugation. This indicates that stable isotope probing can be used to link phytoplankton and associated bacteria.

Phylotypes retrieved from different fractions of RNA samples could be found in most of the respective analysed fractions. This suggests that enrichment with ^{13}C occurred in RNA of associated bacteria with different $\delta^{13}C$ ratios indicating consumption of labelled products of microalgae by bacteria. Nevertheless, it remains unclear if DGGE bands occurring solely in one fraction display a natural buoyant density of the RNA template or if it was labelled as it is still unknown which buoyant density can be ascribed to certain bacterial species (Lueders et al. 2004a). Apart from this, it is assumed that differences in the $\delta^{13}C$ ratio within the same RNA template result from insufficient labelling of algal cells. Unfortunately, it was not possible to analyse the respective $\delta^{13}C$ ratio of gradient fractions by isotope ratio mass spectrometry due to insufficient biomass.

However, small concentrations of RNA of gradient fractions are hard to measure (Lueders et al. 2004a) possibly masking a broader distribution of RNA within the gradients. A contamination amongst gradient fractions due to interactions of different rRNA molecules (Lueders et al. 2004a) might have occurred but did not prevent a strong separation of different gradient fractions observed in this dataset. Furthermore, it has to be taken into account that some phylotypes occurred solely within a certain fraction but not within respective unfractionated samples. It is assumed that rRNA templates with low abundance in unfractionated samples were insufficiently transcribed in RT-PCR what might have caused underestimated diversity before fractionation. However, the different treatments indicate that associated bacteria as well as those introduced by a pristine seawater sample consumed labelled algal biomass.

Additionally, it was observed that labelling of biomass was achieved within the control (treatment 3) indicating assimilation of ^{13}C by autotrophic bacteria. Allgaier et al. (2003) could already show that certain phylotypes associated with microalgae are able to live autotrophically. As the comparison of DGGE profiles revealed phylotypes in fractions of high buoyant density in treatments 1 and 2 which could not be detected in the control treatments, consumption of ^{13}C-labelled algal products by associated bacteria is indicated.

Despite of difficulties in labelling of algal cells above 45 atom% ^{13}C, SIP could be applied successfully to study bacteria–phytoplankton associations. Nevertheless, more experiments are needed to ensure that stable isotope probing can be used to study the consumption of naturally mixed substrates. Applying this technique will lead to deeper insights into the physiological capacities of bacteria in the future.

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