The ISME Journal (2013) 7, 199–209

www.nature.com/ismej

ORIGINAL ARTICLE

Biogeography of *Rhizobium radiobacter* and distribution of associated temperate phages in deep subseafloor sediments

Tim Engelhardt, Monika Sahlberg, Heribert Cypionka and Bert Engelen
Carl-von-Ossietzky Universität Oldenburg, Institut für Chemie und Biologie des Meeres, Oldenburg, Germany

Bacteriophages might be the main 'predators' in the marine deep subsurface and probably have a major impact on indigenous microbial communities. To identify their function within this habitat, we have determined their abundance and distribution along the sediment columns of two continental margin and two open ocean sites that were recovered during Leg 201 of the Ocean Drilling Program. For all investigated sites, viral abundance followed the total cell numbers with a virus-to-cell ratio between 1 and 10 in the upper 100 mbsf (meters below seafloor). An increasing ratio of about 20 in deeper layers indicated an ongoing viral production in up to 11 Ma old sediments. We have used *Rhizobium radiobacter* as the most frequently isolated organism from the deep subsurface with a high *in situ* abundance to identify the frequency of associated rhizobiophages. In this study, 16S rRNA gene copies of *R. radiobacter* accounted for up to 5.6% of total bacterial 16S rRNA genes (average: 0.75%) as detected by quantitative PCR. A distinctive distribution was identified for *R. radiobacter* as indicated by a site-specific arrangement of genetically similar populations. Whole genome information of rhizobiophage RR1-A was used to generate a primer system for quantitative PCR specifically targeting the prophage antirepressor gene, indicative for temperate phages. The quantification of this gene within various sediment horizons showed a contribution of temperate phages of up to 14.3% to the total viral abundance. Thus, the high amount of temperate phages within the sediments and among all investigated isolates indicates that lysogeny is the main viral proliferation mode in deep subsurface populations.
The ISME Journal (2013) **7,** 199–209; doi:10.1038/ismej.2012.92; published online 2 August 2012
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: deep biosphere; lysogeny; ODP Leg 201; rhizobiophages

Introduction

The marine deep biosphere potentially harbors the majority of all prokaryotic cells on earth (Whitman *et al.*, 1998). Their abundance was shown to be high in various marine sediments below the world's oceans (Parkes *et al.*, 1994). Even though the organisms within deep-sea sediments have to cope with severe substrate and nutrient depletion (Jørgensen and D'Hondt, 2006), a large proportion of the microbial inhabitants were shown to be alive (Schippers *et al.*, 2005; Morono *et al.*, 2011). Furthermore, geochemical characteristics of deeper sediments indicate microbial activity as several chemical transformations are linked to microbial processes (Parkes *et al.*, 2000; D'Hondt *et al.*, 2004). During the last decades we got insights into microbial community composition and diversity within the deep subsurface (Inagaki *et al.*, 2006; Sørensen and Teske, 2006; Webster *et al.*, 2006; Fry *et al.*, 2008; Lipp *et al.*, 2008; Teske and Sørensen, 2008; Durbin and Teske, 2011).

Owing to the mostly anoxic regime in highly compacted sediments, grazers are most likely absent in marine subsurface sediments. Whether viruses take over the role as the main controlling factor for indigenous microbial communities is an open question. The impact of viruses on shaping microbial communities and affecting carbon and nutrient cycling was shown by estimates of high viral activity at the seafloor (Danovaro *et al.*, 2008). In a previous study, we found viruses in high numbers within the marine subsurface as deep as 320 mbsf (meters below seafloor) (Engelhardt *et al.*, 2011). Nevertheless, their activity in deep subsurface sediments is not proven yet. An ongoing discussion about the activity and the origin of viruses in deeper marine sediments led to the suggestion that viruses were buried and are well preserved within sediment layers where microbial activity is low

Correspondence: B Engelen, Carl-von-Ossietzky Universität Oldenburg, Institut für Chemie und Biologie des Meeres, Carl-von-Ossietzky Straße 9-11, D-26129 Oldenburg, Germany.
E-mail: engelen@icbm.de
Received 21 February 2012; revised 8 June 2012; accepted 9 June 2012; published online 2 August 2012

(Bird *et al.*, 2001). A different view was given by Middelboe *et al.* (2011), who hypothesized that only a small fraction of the whole viral community was active in deeper sediments and that viral productivity may be restricted to microniches.

In the present study, we analyzed host populations of *Rhizobium radiobacter* and associated phages within various deep-sea sampling sites from the Peru margin area to the open ocean of the eastern equatorial Pacific. The sediments, drilled during Leg 201 of the Ocean Drilling Program (Supplementary Figure S1), cover representative aspects of the marine deep subsurface (D'Hondt *et al.*, 2004). The continental margin sites 1227 and 1230 are considered to be of higher microbial activity due to a higher content of organic carbon (1227: 1.05–10.84 wt%, average 5.14 wt%; 1230: 0.71–5.21 wt%, average 2.77 wt%). Sites 1225 and 1226 are more oligotrophic (1225: 0.01–0.85 wt%, average 0.18 wt%; 1226: 0.25–3.49 wt%, average 0.92 wt%) and represent sediments with lower microbial activity. Thus, the sampling sites comprises a broad range of characteristics in terms of microbial activity, chemical profiles and abundances of prokaryotes (Parkes *et al.*, 2000; D'Hondt *et al.*, 2004; Schippers and Neretin, 2006). Therefore, this area of the deep biosphere is well suited to study the role of viruses for the investigated sites and also allow extrapolating their impact to other marine subsurface habitats.

R. radiobacter was chosen to analyze host-phage relationships as it is the most frequently isolated and highly abundant representative of the marine deep subsurface. This species was detected to be a dominant member of microbial communities within deep-sea sediments of the Mediterranean by both, molecular investigations (5% of the total bacterial community) and a cultivation-based study (Süß *et al.*, 2004, 2006). In our culture collection from ODP Leg 201, *R. radiobacter* was the predominant isolate from various sites and sediment depths (Batzke *et al.*, 2007). In this study, we quantified *R. radiobacter* in the respective sediment layers to prove if the high number of isolates correlates with their *in situ* abundance.

Our aim was to identify the occurrence and diversity of temperate rhizobiophages associated with our isolates and to elucidate the relevance of lysogeny for deep subsurface populations. Thus, the so far deepest sediments ($\sim$381 mbsf) were analyzed for viral abundance. Two rhizobiophages (RR1-A and RR1-B) were subjected to whole genome sequencing. These phages were originally associated to a *R. radiobacter*-affiliated strain that was isolated from a depth of 198 mbsf at ODP site 1225. Based on the genome information of rhizobiophage RR1-A, we established a detection system to quantify their distribution with sediment depth. The characterization of this representative group of temperate phages within the host population and their quantification within the sediments will enhance our understanding of the viral impact on life and death in the marine subsurface.

Materials and methods

Origin of sediment samples

Sediment samples were collected during ODP Leg 201 of the Ocean Drilling Program from February to March 2002 with the R/V JOIDES Resolution (Supplementary Figure S1). A detailed description of the sampling sites at the Peru margin (ODP sites 1227–1230) and sites from the open Pacific ocean (ODP sites 1225, 1226 and 1231) and geochemical parameters are given by D'Hondt *et al.* (2002, 2004). Sediment samples for molecular analysis described in this study have been stored at $-80\,^{\circ}C$.

Enumeration of virus-like particles (VLPs)

Viruses were extracted according to the protocol described by Danovaro *et al.* (2001). Briefly, individual subsamples of 1 cm^3 of sediment were added to a 0.02-μm pre-filtered mixture of 3.5 ml ddH_2O and 1 ml sodium-pyrophosphate (55 mM). Slurries were mixed at 2,000 r.p.m. for at least for 15 min or until the sediment was completely dissolved. Samples were sonicated for 3 min with interruption of 30 s each minute and shaken at 2000 r.p.m. during interruptions. After centrifugation (700 *g*, 2–10 min), the supernatants were removed and 4 ml of TE-buffer (10 mM Tris, 1 mM EDTA, 0.02 μm filtered) was added to the sediment pellet. Slurries were shaken for 10 min after resuspension of sediment pellets, followed by centrifugation as described above. Supernatants of both extraction steps were pooled and filtered though polycarbonate filters (mesh 0.45 μm, Satorius, Göttingen, Germany).

For enumeration of VLPs, between 10 and 500 μl of virus extracts were filtered onto 0.02 μm anodisc filters (13 mm, Whatman, England) and washed with TE-buffer. Viruses were stained by applying 3.5 μl of a mounting solution (Lunau *et al.*, 2005) containing SybrGreenI (final concentration 1 $\times$). Counting of VLPs was done in 10–20 randomly chosen fields per sample by using an Olympus BX51 microscope (Hamburg, Germany). Between 100 and 350 VLPs were counted per sample.

DNA extraction from sediments

DNA extraction was performed according to a protocol described by Lueders *et al.* (2004) and Gabor *et al.* (2003). Briefly, 0.2 ml of $\sim$1:1 mixed 0.1 mm and 0.5 mm silica beads and 700 μl PTN-buffer (120 mM $NaPO_4$, 125 mM TRIS, 25 mM NaCl, pH 8) were added to 0.5 cm^3 sediment. This slurry was vortexed for 10 s. After adding 40 μl lysozyme (50 mg ml^{-1}) and 10 μl Proteinase K (10 mg ml^{-1}) the mixture was incubated at 37 °C with repeated shaking. Subsequently, 100 μl SDS (20%) was added and the mixture was incubated at 65 °C for 15 min.

DNA was purified by phenol/chloroform/isoamylalcohol (PCI, 25:24:1, pH 8) extraction including mechanical disruption of cells by beat beating. After addition of 100 µl of PCI, the mixture was treated by bead beating for 45 s, centrifuged (6000 *g*, 5 min, 4 °C) and the supernatant was collected separately. Subsequently, PTN-buffer (300 µl) was added to the slurry and after beat beating for 20 s the mixture was centrifuged again as described above. DNA was extracted from the pooled supernatants by adding one volume of PCI followed by a second step using one volume of chloroform/isoamylalcohol (24:1). Centrifugation was performed for 4 min, at 13 000 *g* and 4 °C. For precipitation, two volumes of PEG/NaCl (30% polyethylene glycol 8000, 1.6 M sodium chloride) were added to the final supernatant. Thereafter, the mixture was incubated for 2 h at 4 °C and the DNA pelletized (13 000 *g*, 30 min, 20 °C). The pellet was washed by adding 150 µl of 70% ethanol (−20 °C). After spinning (13 000 *g*, 5 min, 4 °C) ethanol was carefully removed. The pellet was dried and the DNA was eluted in 50 µl PCR-H_2O. DNA templates were stored at −20 °C.

Quantification of R. radiobacter

Quantification of *R. radiobacter* was performed by quantitative PCR using *R. radiobacter*-specific primers as described by Süß *et al.* (2006). Originally, the specificity the primers was tested with 19 non-target strains from the German culture collection (DSMZ) and 9 environmental strains. Only one isolate (*Bacillus* sp. J105) revealed a weak positive signal that was neglectable as 10^6 targets of strain J105 showed the same CT-value as 10 targets of *R. radiobacter*. Furthermore, in mixtures with *E. coli* DNA, *R. radiobacter* was accurately quantified over a broad range of target concentrations (0.0003–30%) (Süß *et al.*, 2006).

To generate standards for quantifying *R. radiobacter*, the almost complete 16S rRNA gene of strain P007 was amplified before quantitative PCR by using universal primers 8F (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGA CTT-3′) (Overmann and Tuschak, 1997). Amplification was carried out as described by Süß *et al.* (2004). The PCR amplicons were diluted from 1×10^8–1×10^0 gene copies per µl. The calibration standards were additionally amplified in every qPCR run for quantification.

Quantitative PCR was performed in a Rotor-Gene-3000 cycler (Corbett Research, Sydney, New South Wales, Australia). Each reaction mixture (total volume 25 µl) contained 12.5 µl of Maxima SybrGreen qPCR MasterMix (2×; Thermo Scientific, Karlsruhe, Germany), BSA, respective primers. Environmental DNA templates were added in 1:10 and 1:100 dilutions to identify putative inhibitions by co-extracted substances from the sediment matrix. Quantification was performed with an initial activation at 95 °C for 15 min followed by 50 cycles with a denaturation at 94 °C of 10 s, annealing at 54 °C for 20 s, and an elongation at 72 °C for 30 s. Fluorescence was measured at 72 and 82 °C. Subsequently, a melting curve was recorded by increasing the temperature from 50–99 °C with a temperature ramp of 1 °C per 10 s.

Phylogenetic analysis of R. radiobacter *isolates*

In total, 36 *R. radiobacter*-affiliated strains were obtained from ODP Leg 201 sediments. The isolation procedure and contamination control were described in detail by Batzke *et al.* (2007). The 16S rRNA genes of 36 strains of *R. radiobacter* were amplified by using the universal primers 8F and 1492R as described above. Amplified DNA was purified (Qiagen PCR Purification Kit, Qiagen, Hilden, Germany) and subsequently sequenced by GATC Biotech AG (Konstanz, Germany). Phylogenetic analysis was performed by using the ARB software package (Versalovic *et al.*, 1991; Ludwig *et al.*, 2004). The database Silva 106 (www.arb-silva.de) was used as a backbone-tree. Sequences obtained during this study were integrated by using the ARB Fast Aligner feature (www.arb-home.de). The accession numbers of all 16S rRNA gene sequences obtained in this study are deposited at the GenBank database under JX110581-JX110616.

The genetic diversity of the different *R. radiobacter* strains below the species level was determined by enterobacterial repetitive intergenic consensus (ERIC) PCR (Versalovic *et al.*, 1991). The ERIC-PCR mixture had a final volume of 50 µl and contained: Taq polymerase (0.02 U per µl; New England BioLabs, Ipswich, MA, USA), the 10-fold polymerase buffer according to manufacturers specification, BSA (0.4 mg ml^{-1}), dNTPs (0.2 mM each) and the ERIC primers ERIC1R (5′-ATGTAAGCTCCT GGGGATTCAC-3′) and ERIC2F (5′-AAGTAAGT-GACTGGGGTGAGCG-3′) (0.2 pmol per µl each). The ERIC-PCR amplification was performed with an initial denaturation (96 °C, 4 min), followed by 37 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 1 min), and elongation (72 °C, 8 min) with a single final elongation (72 °C, 10 min). PCR products were run on an acrylamid gel at 50 V for 18 h, stained with SYBRGold (Invitrogen, Carlsbad, CA, USA) and documented digitally (BioDocAnalyze, Biometra, Göttingen, Germany).

Induction of prophages from R. radiobacter *isolates*

Cultures of each *R. radiobacter* strain were grown in 250 ml LB-medium at 20 °C and shaking speed of 100 r.p.m. Growth was monitored by measuring the optical density at 580 nm. When reaching the exponential growth phase, 50 ml of the culture were separated and used as a respective control. The remaining culture was treated for 30 min with mitomycin C (final concentration: 0.5 µg ml^{-1}), washed by centrifugation (10 000 *g*, 10 min, 20 °C) and resuspended in fresh media. After a drop in cell

density of the induced culture in comparison with the control, the cells were pelletized (10 000 *g*, 10 min, 20 °C). To remove remaining cells and cell debris, the supernatant was filtered through a polycarbonate filter (mesh 0.45 μm, Sartorius stedium biotech GmbH, Göttingen, Germany). For further analyses, extracted phage particles were concentrated by a polyethylene glycol treatment.

Preparation of phage concentrates and viral DNA purification

To concentrate phage particles from induction experiments and sediment samples, a mixture of polyethylene glycol and sodium chloride (final concentration: 10% PEG 8000, 1 M NaCl) was added to the phage extract. After incubation for 1 h on ice, the phages were pelletized (10 000 *g*, 12 min, 4 °C). Finally, the pellet was resuspended in 400 μl of SM buffer.

To remove non-viral DNA, 88.5 μl of the phage concentrates were added to 10 μl DNAseI-buffer (10 ×) and 1.75 μl DNAseI (New England BioLabs). After incubation for 90 min at 37 °C, 1 μl ES-buffer (0.5 M EDTA, 0.5% w/v *N*-lauroylsarcosin) was added. To release encapsulated viral DNA, the mixture was incubated for 15 min at 75 °C. Subsequently, viral DNA was purified by using the Wizard PCR Preps DNA Purification System (Promega GmbH, Mannheim, Germany). DNA was quantified with NanoDrop 2000c Spectrophotometer (Thermo Scientific).

Pulsed-field gel electrophoresis (PFGE) to determine phages genome sizes

Pulsed-field gel electrophoresis was performed on a 0.8% agarose gel using the Rotaphor v6 system (Biometra, Göttingen, Germany). Purified viral DNA (150 ng per lane) was separated by applying a pulse ramp of 2–15 s, a linear decreasing electrophoresis rate of 5.3–4.6 V cm^{-1} with an included angel ramp of 120–130 °C. PFGE was performed for 22.5 h at 14 °C. The Low Range PFG Marker (New England Biolabs) was used for size assignment.

Whole genome sequencing of rhizobiophage RR1-A and RR1-B

Rhizobiophage RR1-A (Acc. no. PRJNA47553) and RR1-B (Acc. no. PRJNA60463) were induced from the host strain P007 (isolated from ODP site 1225, 198 mbsf). Sequencing, finishing and gene prediction of the viral genome was conducted in the frame of the Marine Phage Sequencing Project by the Broad Institute. For more Information see (www.broadinstitute.org/annotation/viral/Phage).
Manual gene annotation was performed using the BLASTp sequence analysis service of the NCBI online platform (http://blast.ncbi.nlm.nih.gov).

Establishing a detection system for temperate rhizobiophages

Two primer pairs were developed by using the pDraw32 software package (www.acaclone.com) to target the prophage antirepressor gene (ORF 65) in the genome of Rhizobiophage RR1-A. Antirepressor genes of phages are involved in the switch from lysogeny to lytic proliferation. Because of their ability to operate with non-cognate repressors, they have the capacity to coordinate a multiple prophage induction. Thus, viral antirepressor genes are unique for temperate phages. Therefore, they can be used as marker genes for temperate phages. The first primer pair RR1-A-F (5′-GGAAAAGCCAGTCCG CGC-3′) and RR1-A-R (5′-GGTGTGAGGATGCGGAT GC-3′) was designed to amplify the almost complete antirepressor gene (length: 624 bp). PCR was performed with an initial denaturation at 94 °C for 10 min, followed by 30 cycles with a denaturation at 94 °C of 30 s, annealing at 56 °C for 45 s, elongation at 72 °C for 1 min and a final elongation for 10 min at 72 °C. The resulting PCR product was used to generate standards for qPCR analysis. The second primer pair, RR1As-F (5′-GCTTGCCAACCGGCTCG G-3′) and RR1As-R (5′-TTGCGGATGCCCACTGTTG G-3′), was developed to amplify a shorter fragment of the gene (124 bp) for detection and quantification of the antirepressor gene. PCR was performed with an initial denaturation at 94 °C for 10 min, followed by 30 cycles with a denaturation at 94 °C of 30 s, annealing at 56 °C for 45 s, elongation at 72 °C for 45 s and a final elongation for 10 min at 72 °C.

Quantification of temperate rhizobiophages in sediment samples

The 624 bp long fragment of the almost complete antirepressor gene was used as a standard for quantitative PCR. The amplicons were purified (Qiagen PCR Purification Kit, Qiagen) and the amount of DNA was quantified with a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The number of gene targets was calculated from the length of the DNA fragment, the amount of DNA and the average weight of base pairs (1.1×10^{-21} g). A standard curve was generated by a 10-fold dilution in a range from 10^2–10^7 antirepressor gene targets.

Prior to quantification, extracts of viral particles were subjected to DNAse digestion to remove free DNA and purified as described above. Quantitative PCR was performed by using the primer set RR1As-f/RR1As-r in a Rotor-Gene-3000 cycler (Corbett Research, Sydney, Australia). Each reaction mixture (total volume 25 μl) contained 12.5 μl of Maxima SybrGreen qPCR MasterMix (2 ×; Thermo Scientific), BSA, respective primers. Viral DNA templates in 1:10 and 1:100 dilution were added to identify putative inhibitory effects. Quantification was performed with an initial activation at 95 °C for 15 min followed by 50 cycles with a denaturation at 94 °C of 10 s, annealing at 56 °C for 20 s, and an elongation at

72 °C for 30 s. Fluorescence was measured at 72 and 80 °C. Subsequently, a melting curve was recorded by increasing the temperature from 50–99 °C with a temperature ramp of 1 °C per 10 s.

Results

Total counts of cells and viruses

The abundance of cells and viruses was determined in deep-subsurface sediments of the Peru margin (ODP sites 1227 and 1230) and the eastern equatorial Pacific (ODP sites 1225 and 1226). Cell numbers were between 10^7 and 10^8 cells cm^{-3} in the upper most sediment samples and continuously decreased by two orders of magnitude in the deepest sediment layer at 381 mbsf (Figure 1a). The virus counts were between 10^7 and 10^8 per cm^3 at 1 mbsf and decreased with depth by one order of magnitude, only. Generally, the virus-to-cell ratios increased with sediment depth (Figure 1b). For example, at the Peru margin site 1230, the ratio between viruses and cells in the upper hundred meters were determined to be rather low (4–1.7). A higher ratio of 17 was detected for the deepest sediment layer at a depth 257 mbsf. This was also found at the open ocean site 1225, where the virus-to-cell ratios increased from about 2 at 1 mbsf to 21 at 320 mbsf (Supplementary Table S1).

Abundance and phylogenetic diversity of R. radiobacter

The 16S ribosomal RNA gene of *R. radiobacter* was specifically detected by quantitative PCR (qPCR) at all investigated sites and sediment layers except for site 1227 at 34 mbsf (Figure 1c). The proportion of *R. radiobacter*-related 16S rRNA gene copies increased with depth in comparison with the abundance of bacterial 16S rRNA gene copies. The relative amount of *R. radiobacter* ranged between 0.0004% at 1 mbsf of site 1230% and 5.6% at 198 mbsf of site 1225 (Supplementary Table S1). On an average, the relative abundance of *R. radiobacter*-affiliated 16S rRNA genes was 0.76%.

All 36 *R. radiobacter*-affiliated isolates from ODP Leg 201 (Batzke *et al.*, 2007) were phylogenetically analyzed in detail. Based on their 16S rRNA gene similarities, all isolates showed a close affiliation to the type strain of *R. radiobacter* (Figure 2). Differences in 16S rRNA gene sequences were below 0.1%, only. Most strikingly, sequence similarities were highest among strains isolated from the same sampling sites. This was further proven by genome fingerprinting of the *R. radiobacter*-affiliated strains using ERIC-PCR. According to the ERIC-PCR pattern analysis, the different isolates arranged in three site-specific subclusters (Figure 3).

Detection of rhizobiophages in isolates of R. radiobacter

To analyze the presence of temperate rhizobiophages, the prophage-inducing agent mitomycin C was used in stimulation experiments with all 36 isolates affiliated to *R. radiobacter*. A drop in cell density in the mitomycin C-treated cultures in comparison with the non-induced controls

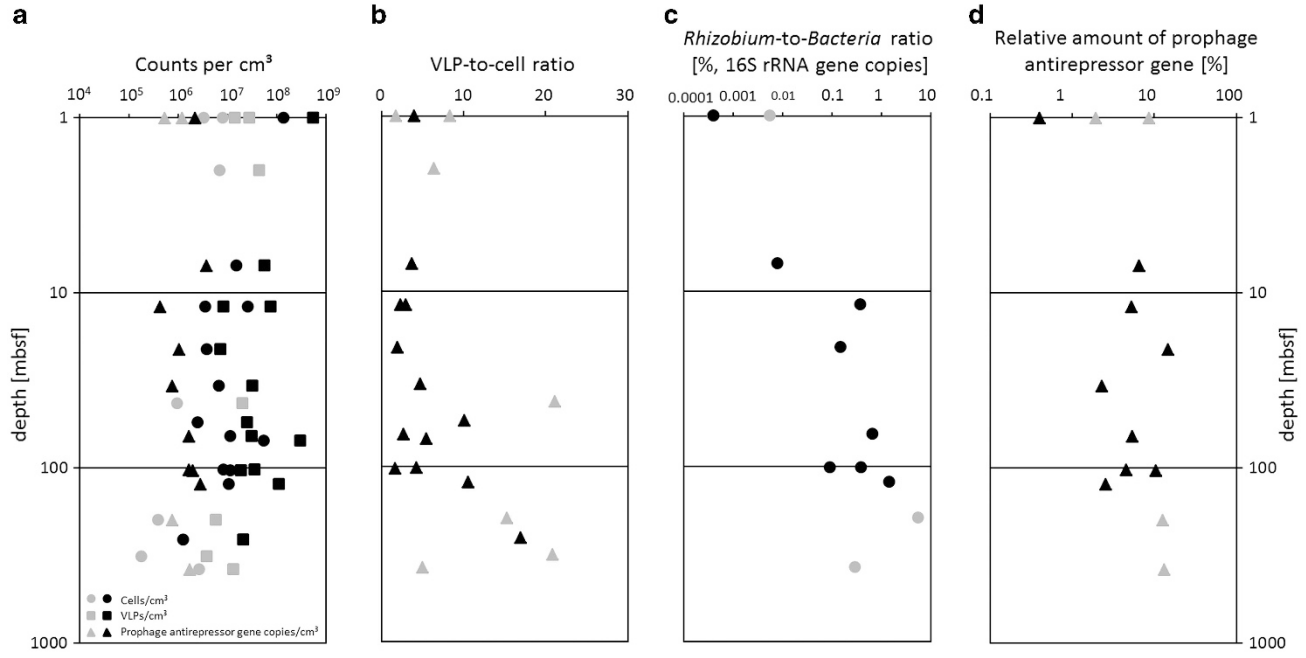


Figure 1 (**a**) Abundances per cm^3 of cells (circles), VLP (squares) and copies of prophage antirepressor gene (triangles) within ODP Leg 201 sediments. Peru margin sites and open ocean sites are represented by black and gray symbols, respectively. (**b**) VLP-to-cell ratio. (**c**) Relative amount of *R. radiobacter* compared with total *Bacteria* based on 16S rRNA gene copies. (**d**) Relative amount of prophage antirepressor gene copies compared with total viral abundance.

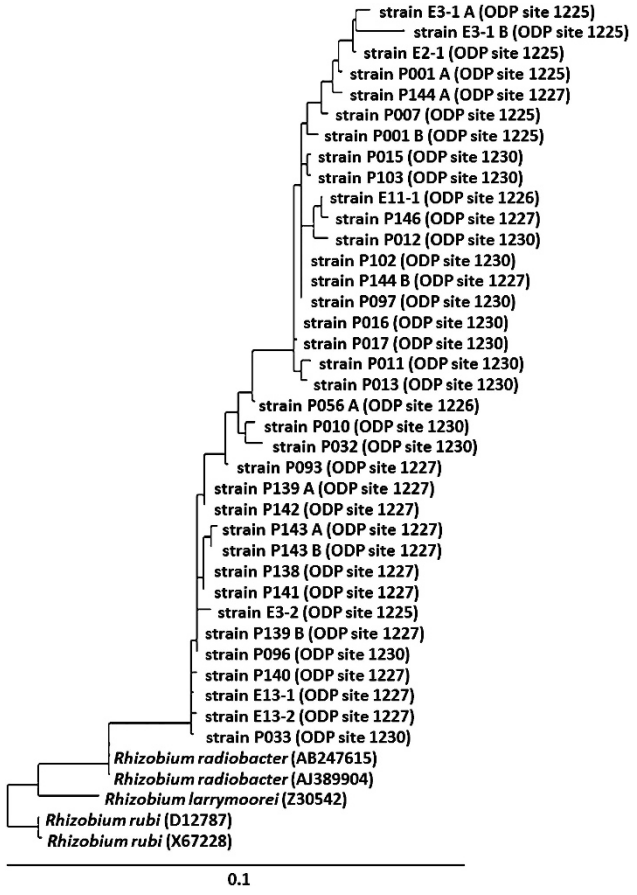


Figure 2 Phylogenetic tree based on the 16S rRNA gene, showing the affiliation of various strains of *R. radiobacter*. Differences in 16S rRNA genes are indicated, bar = 0.1%.

indicated a cell lysis due to the release of phage particles (Engelhardt *et al*., 2011). Pulsed-field gel electrophoresis (PFGE) revealed the presence of phages with two different genome sizes within the population of *R. radiobacter* (Figure 3). The genome sizes were ~52 and 37 kb, respectively. Nine of the strains showed a multiple prophage infection as both phages were induced simultaneously. Several strains showed individual bands with 10 kb in size, which may suggest the presence of phage-like gene transfer agents, which are known to be widespread in Alphaproteobacteria (Lang and Beatty, 2007).

Genome annotation of rhizobiophage RR1-A and RR1-B

The genome of rhizobiophage RR1-A was determined to be of circular conformation and 52 641 kb in length with an average G + C content of 57.4% (Figure 4a). The genome sequence comprised a total number of 67 predicted open reading frames (Supplementary Table S2). Considering only E-values smaller than $1e^{-30}$ as significant hits, about 70% of the predicted genes were unknown. The other 30% were identified as core genes of viral genomes found in members of the order Rhizobiales. Among those, nine genes were supposed to encode for structural modules, such as capsid- and tail-forming proteins. Ten genes were related to DNA processing and phage assembly. Additionally, a detected prophage antirepressor gene was highly similar (96% query coverage, E-value $2e^{-91}$, maximum identity 55%) to that found in *Rhizobium vitis* (formally named *Agrobacterium vitis*; Young *et al*., 2001). Due to its function of switching from lysogeny to lytic reproduction, this gene is indicative for temperate phages.

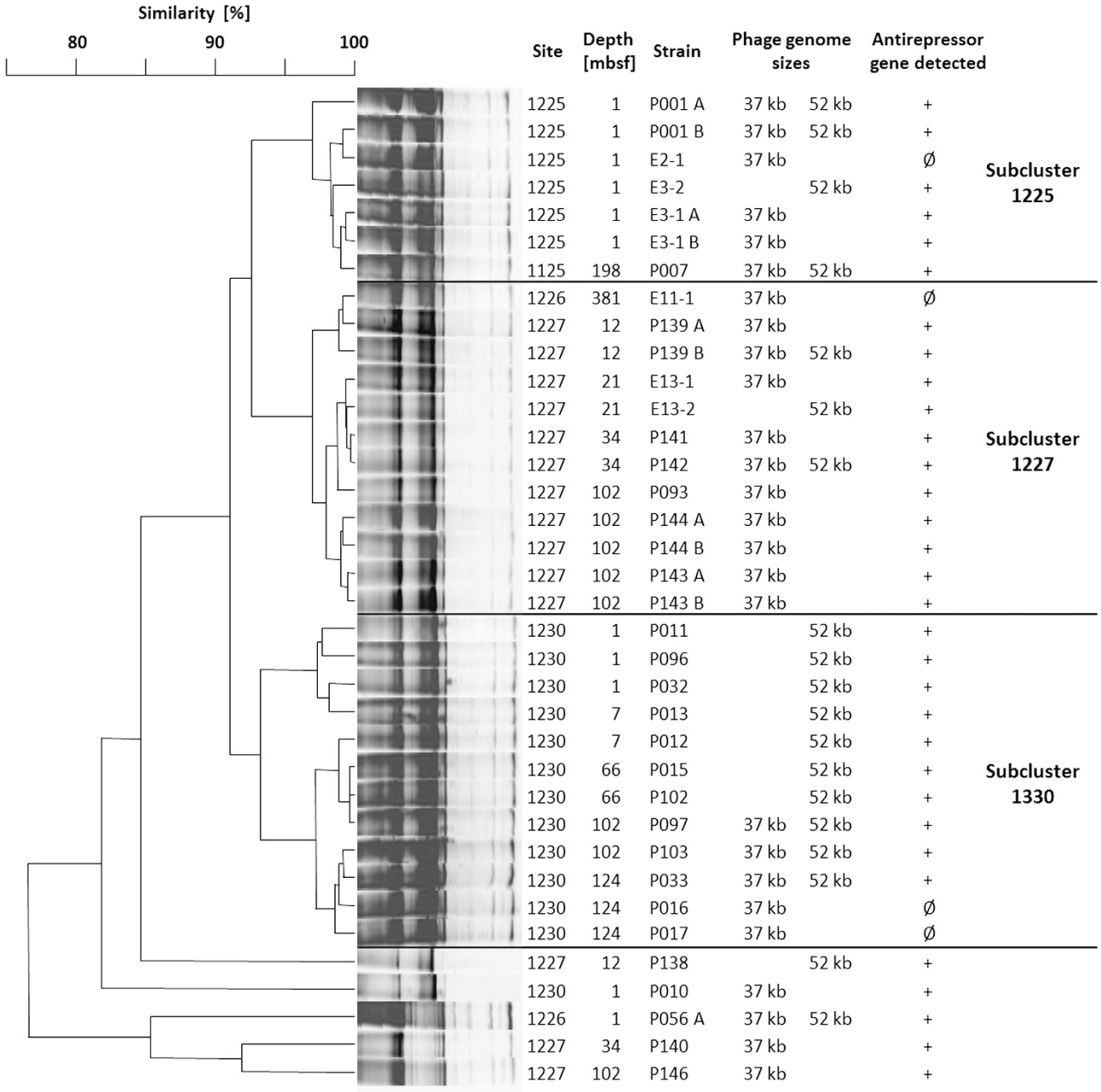
Rhizobiophage RR1-B comprised 37 378 kb and 52 predicted ORFs. The average G + C − content was 58.54% (Figure 4b). 75% of the predicted genes had an unknown function (e-value $>e^{-30}$). Three of these genes resulted in no hit at all. BLASTP analysis indicated six genes to be involved in DNA processing and eight predicted genes encode for structure proteins of phage particle (Supplementary Table S3). The antirepressor gene from prophage RR1-A was not detected.

Detection of the prophage antirepressor gene and quantification in sediment samples

A protein BLASTp analysis using the NCBI database revealed only one hit with a sufficient query coverage for the antirepressor gene of *R. vitis* strain S4 as described above. The next closest protein sequence derived from *Pseudovibrio* sp. strain FO-BEG1 (query coverage 70%; maximum identity 38%), other protein sequences showed query coverages below 61% and maximum identities of < 57%. Available pure cultures *R. radiobacter* (36 strains) and virus extracts from environmental samples were tested for the presence of the antirepressor gene by using the primer set RR1As-f/RR1As-r. No unspecific amplification was observed for all investigated DNA templates (Figure 5). A diversity analysis of this gene fragment across all sites and sediment depths by denaturing gradient gel electrophoresis revealed only one band, indicating a high degree of conservation (data not shown). In total, 32 isolates of *R. radiobacter* were tested positively. Thirteen of these strains did not show a respective 52 kb band in the PFGE analysis (Figure 3), which might be due to defective prophages. For four other isolates, neither the PFGE band nor the the antirepressor gene was detected.

The BLASTp analysis, the specific PCR with isolates and environmental viral DNA extracts, and the denaturing gradient gel electrophoresis analysis of the respective PCR products indicate the specificity of the molecular approach to detect temperate rhizobiophages. This gene was used for the specific quantification of temperate rhizobiophages within the fraction of free viral particles by qPCR as a DNAse treatment of virus extracts before quantification excluded the quantification of prophages within bacterial genomes.

Gene copy numbers were 10^5–10^6 genes cm^{-3} of sediment. The number of free phages containing this



Site	Depth [mbsf]	Strain	Phage genome sizes		Antirepressor gene detected	
1225	1	P001 A	37 kb	52 kb	+	
1225	1	P001 B	37 kb	52 kb	+	
1225	1	E2-1	37 kb		Ø	Subcluster 1225
1225	1	E3-2		52 kb	+	
1225	1	E3-1 A	37 kb		+	
1225	1	E3-1 B	37 kb		+	
1125	198	P007	37 kb	52 kb	+	
1226	381	E11-1	37 kb		Ø	
1227	12	P139 A	37 kb		+	
1227	12	P139 B	37 kb	52 kb	+	
1227	21	E13-1	37 kb		+	
1227	21	E13-2		52 kb	+	
1227	34	P141	37 kb		+	Subcluster 1227
1227	34	P142	37 kb	52 kb	+	
1227	102	P093	37 kb		+	
1227	102	P144 A	37 kb		+	
1227	102	P144 B	37 kb		+	
1227	102	P143 A	37 kb		+	
1227	102	P143 B	37 kb		+	
1230	1	P011		52 kb	+	
1230	1	P096		52 kb	+	
1230	1	P032		52 kb	+	
1230	7	P013		52 kb	+	
1230	7	P012		52 kb	+	
1230	66	P015		52 kb	+	Subcluster 1330
1230	66	P102		52 kb	+	
1230	102	P097	37 kb	52 kb	+	
1230	102	P103	37 kb	52 kb	+	
1230	124	P033	37 kb	52 kb	+	
1230	124	P016	37 kb		Ø	
1230	124	P017	37 kb		Ø	
1227	12	P138		52 kb	+	
1230	1	P010	37 kb		+	
1226	1	P056 A	37 kb	52 kb	+	
1227	34	P140	37 kb		+	
1227	102	P146	37 kb		+	

Figure 3 Genetic similarities below species level of various *R. radiobacter*-affiliated strains as analyzed by enterobacterial repetitive intergenic consensus (ERIC) PCR. Site-specific clustering of *R. radiobacter* subpopulations regarding to their ERIC-pattern is shown. Genome sizes of inducible prophages given here are referred to the genome sizes of phages RR1-A and RR1-B. Detection of the prophage antirepressor gene of Rhizobiophage RR1-A is indicated by + (detected) and Ø (not detected).

gene varied with depths, but constituted no clear trend (Figure 1a). However, the relative amount of temperate rhizobiophages in relation to the total virus counts increased slightly with sediment depth (Figure 1d). For example, in near-surface sediments of site 1230, the relative amount of these phages accounted for 0.4% of the total viral numbers in contrast to 10.2% at 103 mbsf (Supplementary Table S1). At site 1225, a comparably high amount of temperate rhizobiophages of 8.4% was already determined in near-surface sediments and increased slightly to 12.4% at 198 mbsf.

Discussion

All isolates of *R. radiobacter* analyzed in this study turned out to be lysogens. The large number of associated temperate phages in the sediments points towards an ongoing viral production presumably originating from natural induction events. Thus, lysogeny appears to be a predominant factor for host populations and the viral communities in the deep subsurface. We hypothesize that even the slow growing subsurface microbial communities are capable to support the production of new phages.

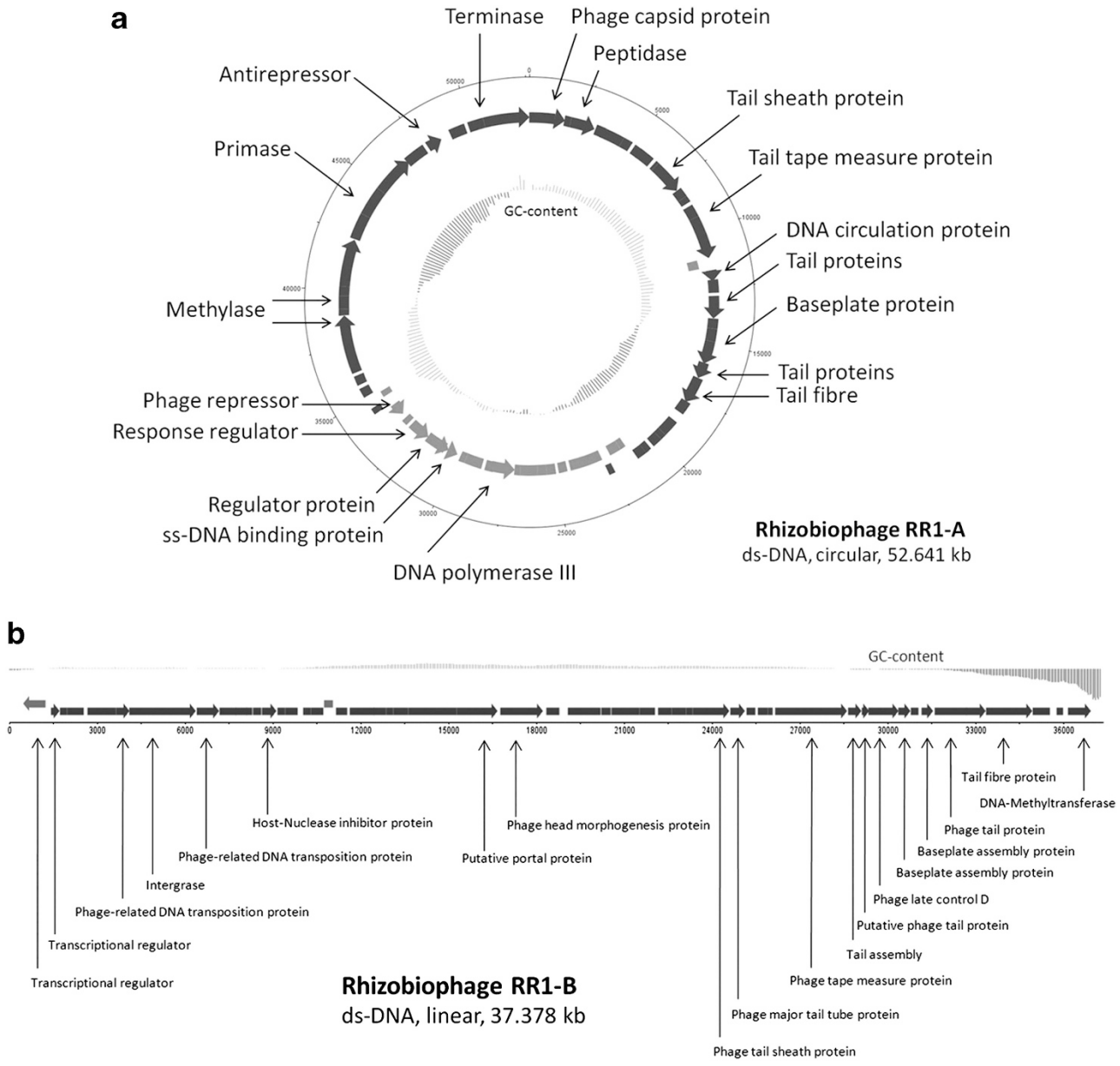


Figure 4 Gene cards of Rhizobiophage RR1-A and RR1-B. Predicted gene functions are indicated according to the manual annotation (Supplementary Tables S2 and S3).

The high virus-to-cell ratio in deep sediment layers indicates viral production

The virus-to-cell ratio between 1 and 10 we found for the upper ~100 mbsf was also described for subsurface sediments down to a depth of ~110 mbsf for a high sedimentary region of the Saanich Inlet (Bird *et al.*, 2001) and the eastern margin of the Porcupine Seabight (Middelboe *et al.*, 2011). The latter authors interpret this ratio as a result of a better preservation of viruses within deep sediments due to the absence of exoenzymes. These enzymes are known to affect inactivation and degradation of viruses (Noble and Fuhrman, 1997). In addition, the stabilization of viral structures after absorption to inorganic particles could support this effect (Dowd *et al.*, 1998).

In general, viral abundance in sedimentary habitats is likely the sum of preservation, degradation and ongoing production of viruses. The ratio between viruses and cells was shown to increase up to 21 in deeper sediments layers. As the deepest sediments are ~11 Ma old, it appears unlikely, that this high number of viruses can be preserved over such a long time scale. Therefore, an ongoing production of phages within an active host population along the investigated sediment columns might contribute to a higher degree to the viral abundance than preservation. At ODP sites 1225 and 1230, for example, near-basement sediment layers are affected by a deep intrusion of electron acceptors from below (D'Hondt *et al.*, 2004). For similar hydrogeological settings (IODP site 1301), it was shown

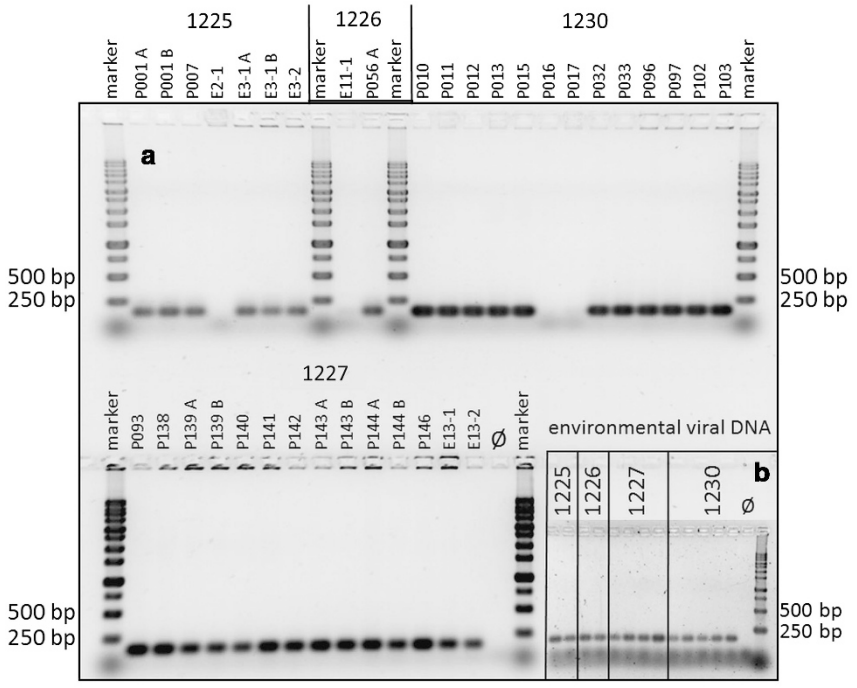


Figure 5 Detection of prophage antirepressor gene of Rhizobiophage RR1-A. A fragment (124 bp) of the prophage antirepressor gene of RR1-A was amplified by using the primers RR1As-f and RR1As-r. DNA from the different isolates of *R. radiobacter* or purified viral DNA extracts from various sediment samples obtained from ODP Leg 201 were used as template DNA. *R. radiobacter* strain P007 served as a positive control. Ø denotes non-template control.

that microbial communities in up to 265 m deep sediments are stimulated by fluids from the oceanic crust (Engelen *et al.*, 2008). Such environmental conditions promote cell proliferation and probably result in enhanced viral production. Thus, the high abundance of viruses throughout all investigated sediment layers indicates that even low microbial activity may support viral production.

R. radiobacter *as an abundant subseafloor inhabitant*

R. radiobacter is a unique example of a highly abundant organism in the deep subsurface that can be detected by both, cultivation and molecular techniques. *R. radiobacter* was shown to contribute up to 5% to the bacterial community in the Mediterranean subsurface (Süß *et al.*, 2006). In our study, their relative abundance even accounted for up to 5.6% of total bacterial 16S rRNA genes in ODP Leg 201 sediments (average: 0.75%). Furthermore, their increasing abundance with depth indicates that the environmental conditions within the deep subsurface were selective for this species. The constant degradation of organic matter during sediment accumulation leads to a limited substrate availability for indigenous microorganisms. Thus, metabolically versatile species such as *R. radiobacter* (Stowers, 1985; Batzke *et al.*, 2007) might take advantage on this situation. A further adaptation factor for *R. radiobacter* to the conditions in the deep subsurface might derive from their associated prophages. It was shown that genes carried by prophages affect host metabolism by providing metabolic facilities (Jiang and Paul, 1998) and might increase host cell fitness and survivability. For example, the integration of prophages in bacterial genomes can shut down host genes (Brussow *et al.*, 2004) and might help to save energy, which appears to be of critical importance for survival in subsurface sediments (Morono *et al.*, 2011).

Site-specific development of distinct subpopulations of R. radiobacter *indicates activity and evolution*

Site-specific arrangement of genetically similar isolates of *R. radiobacter* shows the development of separate subpopulations at different sites due to geological segregation. This provides first evidence of a biogeographical distribution of an organism in the marine deep subsurface. The isolation caused by the geographical distance between continental margin and open ocean sites is the prerequisite for individual evolutionary processes. In addition, this might be promoted by the various physical and geochemical parameters contributing to the site-specific characteristics. Evolution of a subpopulation within a particular habitat only occurs when these organisms are actively growing. Nevertheless, in the deep subsurface, these processes can be expected to be very slow as the turnover times of prokaryotic communities were estimated to be several years for sediments from ODP Leg 201 (Schippers *et al.*, 2005; Biddle *et al.*, 2006).

Lysogeny appears to be characteristic for the deep subsurface

Previous studies addressing lysogeny in marine habitats mainly focused on pelagic environments (Weinbauer and Suttle, 1996; Cochran *et al.*, 1998; Jiang and Paul, 1998; Leitet *et al.*, 2006; De Corte *et al.*, 2012). The lysogenic fraction is often shown to be rather low (Wilcox and Fuhrman, 1994; Weinbauer and Suttle, 1996). Concurrently, the contribution to total viral production by natural induction of prophages is calculated to be only 0.02% (Jiang and Paul, 1998). In contrast, unfavorable growth conditions for host organisms were shown to promote lysogeny. In the study by Williamson *et al.* (2002), they demonstrated lysogeny to occur in the water column mainly during winter month simultaneously to lowest microbial activity. As prokaryotes living in the marine deep subsurface are mainly prone to starvation (Jørgensen and D'Hondt, 2006), our study demonstrates the link between lysogeny and extreme environments such as the energy-poor marine subsurface. Other extreme habitats like energy-rich deep-sea hydrothermal vents can even be dominated by lysogens (Williamson *et al.*, 2008). Previously, we found prophages in a variety of different phylogenetic groups isolated from the marine deep subsurface (Engelhardt *et al.*, 2011). In the present study, we could show that lysogeny is characteristical for all investigated isolates of *R. radiobacter*. As this species accounts for a significant fraction of bacteria in this environment and rhizobiophages are found to be highly abundant, lysogeny appears to be a common feature for the marine deep biosphere.

Viral production in the deep subsurface is presumably due to a natural induction of prophages

The strict occurrence of lysogeny among *R. radiobacter* is reflected by the high amount of temperate rhizobiophages found in the investigated sediments. A production of phages can be assumed due to the generally high amount of free phages in deeper sediment layers. As the mobility of phages and host cells is limited in highly compacted sediments, it appears unlikely that lytic viral reproduction is a dominant mechanism for viral proliferation. In addition, the host cell density might be too low to support a lytic phage reproduction. Thus, viral production may result predominantly from a natural induction of prophages which was exemplarily shown for *R. radiobacter* cultures that were not treated by mitomycin C (Engelhardt *et al.*, 2011). To what extent viral activity may have a role regarding the viral shunt and horizontal gene transfer within the deep subsurface sediments is yet unknown.

Acknowledgements

We like to thank Regina Gohl and Nawras Ghanem for their help during phage-induction experiments and the ODP Leg 201 personnel and shipboard scientists for sampling and data acquisition. Barry Cragg is acknowledged for providing additional cell counts. This research used samples and data provided by the Integrated Ocean Drilling Program (IODP) and was funded in part by the Gordon and Betty Moore Foundation (GBMF) through a grant to the Broad Institute. Phages RR1-A and RR1-B were sequenced and assembled at the Broad Institute. Main funding for this research was provided by a grant of the German Research Foundation (DFG).

References

Batzke A, Engelen B, Sass H, Cypionka H. (2007). Phylogenetic and physiological diversity of cultured deep-biosphere bacteria from equatorial Pacific Ocean and Peru Margin sediments. *Geomicrobiol J* **24**: 261–273.

Biddle JF, Lipp JS, Lever MA, Lloyd KG, Sørensen KB, Anderson R *et al.* (2006). Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proc Natl Acad Sci USA* **103**: 3846–3851.

Bird DF, Juniper SK, Ricciardi-Rigault M, Martineu P, Prairie YT, Calvert SE. (2001). Subsurface viruses and bacteria in Holocene/Late Pleistocene sediments of Saanich Inlet, BC: ODP Holes 1033B and 1034B, Leg 169S. *Marine Geol* **174**: 227–239.

Brussow H, Canchaya C, Hardt WD. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* **68**: 560–602.

Cochran PK, Kellogg CA, Paul JH. (1998). Prophage induction of indigenous marine lysogenic bacteria by environmental pollutants. *Mar Ecol Prog Ser* **164**: 125–133.

D'Hondt S, Rutherford S, Spivack AJ. (2002). Metabolic activity of subsurface life in deep-sea sediments. *Science* **295**: 2067–2070.

D'Hondt S, Jørgensen BB, Miller DJ, Batzke A, Blake R, Cragg BA *et al.* (2004). Distributions of microbial activities in deep subseafloor sediments. *Science* **306**: 2216–2221.

Danovaro R, Dell'anno A, Trucco A, Serresi M, Vanucci S. (2001). Determination of virus abundance in marine sediments. *Appl Environ Microbiol* **67**: 1384–1387.

Danovaro R, Dell'Anno A, Corinaldesi C, Magagnini M, Noble R, Tamburini C *et al.* (2008). Major viral impact on the functioning of benthic deep-sea ecosystems. *Nature* **454**: 1084–1087.

De Corte D, Sintes E, Yokokawa T, Reinthaler T, Herndl GJ. (2012). Links between viruses and prokaryotes throughout the water column along a North Atlantic latitudinal transect. *ISME J* **6**: 1566–1577.

Dowd SE, Pillai SD, Wang SY, Corapcioglu MY. (1998). Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. *Appl Environ Microbiol* **64**: 405–410.

Durbin AM, Teske A. (2011). Microbial diversity and stratification of South Pacific abyssal marine sediments. *Environ Microbiol* **13**: 3219–3234.

Engelen B, Ziegelmueller K, Wolf L, Koepke B, Gittel A, Cypionka H *et al.* (2008). Fluids from the oceanic crust support microbial activities within the deep biosphere. *Geomicrobiol J* **25**: 56–66.

Engelhardt T, Sahlberg M, Cypionka H, Engelen B. (2011). Induction of prophages from deep-subseafloor bacteria. *Environ Microbiol Reports* **3**: 459–465.

Fry JC, Parkes RJ, Cragg BA, Weightman AJ, Webster G. (2008). Prokaryotic biodiversity and activity in the deep subseafloor biosphere. *Fems Microbiol Ecol* **66**: 181–196.

Gabor EM, de Vries EJ, Janssen DB. (2003). Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *Fems Microbiol Ecol* **44**: 153–163.

Inagaki F, Nunoura T, Nakagawa S, Teske A, Lever M, Lauer A *et al.* (2006). Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments, on the Pacific Ocean Margin. *Proc Natl Acad Sci USA* **103**: 2815–2820.

Jiang SC, Paul JH. (1998). Significance of lysogeny in the marine environment: Studies with isolates and a model of lysogenic phage production. *Microb Ecol* **35**: 235–243.

Jørgensen BB, D'Hondt S. (2006). Ecology A starving majority deep beneath the seafloor. *Science* **314**: 932–934.

Lang AS, Beatty JT. (2007). Importance of widespread gene transfer agent genes in alpha-proteobacteria. *Trends Microbiol* **15**: 54–62.

Leitet C, Riemann L, Hagstrom A. (2006). Plasmids and prophages in Baltic Sea bacterioplankton isolates. *J Mar Biol Assoc UK* **86**: 567–575.

Lipp JS, Morono Y, Inagaki F, Hinrichs KU. (2008). Significant contribution of Archaea to extant biomass in marine subsurface sediments. *Nature* **454**: 991–994.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al.* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Lueders T, Manefield M, Friedrich MW. (2004). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73–78.

Lunau M, Lemke A, Walther K, Martens-Habbena W, Simon M. (2005). An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* **7**: 961–968.

Middelboe M, Glud RN, Filippini M. (2011). Viral abundance and activity in the deep sub-seafloor biosphere. *Aquatic Microb Ecol* **63**: 1–8.

Morono Y, Terada T, Nishizawa M, Ito M, Hillion F, Takahata N *et al.* (2011). Carbon and nitrogen assimilation in deep subseafloor microbial cells. *Proc Natl Acad Sci USA* **108**: 18295–18300.

Noble RT, Fuhrman JA. (1997). Virus decay and its causes in coastal waters. *Appl Environ Microbiol* **63**: 77–83.

Overmann J, Tuschak C. (1997). Phylogeny and molecular fingerprinting of green sulfur bacteria. *Arch Microbiol* **167**: 302–309.

Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA *et al.* (1994). Deep bacterial biosphere in Pacific Ocean sediments. *Nature* **371**: 410–413.

Parkes RJ, Cragg BA, Wellsbury P. (2000). Recent studies on bacterial populations and processes in subseafloor sediments: A review. *Hydrogeol J* **8**: 11–28.

Schippers A, Neretin LN, Kallmeyer J, Ferdelman TG, Cragg BA, Parkes RJ *et al.* (2005). Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. *Nature* **433**: 861–864.

Schippers A, Neretin LN. (2006). Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. *Environ Microbiol* **8**: 1251–1260.

Sørensen KB, Teske A. (2006). Stratified communities of active archaea in deep marine subsurface sediments. *Appl Environ Microbiol* **72**: 4596–4603.

Stowers MD. (1985). Carbon metabolism in *Rhizobium* species. *Ann Rev Microbiol* **39**: 89–108.

Süß J, Engelen B, Cypionka H, Sass H. (2004). Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. *Fems Microbiol Ecol* **51**: 109–121.

Süß J, Schubert K, Sass H, Cypionka H, Overmann J, Engelen B. (2006). Widespread distribution and high abundance of *Rhizobium radiobacter* within Mediterranean subsurface sediments. *Environ Microbiol* **8**: 1753–1763.

Teske A, Sørensen KB. (2008). Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J* **2**: 3–18.

Versalovic J, Koeuth T, Lupski JR. (1991). Distribution of repetitive DNA-sequences in *Eubacteria* and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**: 6823–6831.

Webster G, Parkes RJ, Cragg BA, Newberry CJ, Weightman AJ, Fry JC. (2006). Prokaryotic community composition and biogeochemical processes in deep subseafloor sediments from the Peru Margin. *Fems Microbiol Ecol* **58**: 65–85.

Weinbauer MG, Suttle CA. (1996). Potential significance of lysogeny to bacteriophage production and bacterial mortality in coastal waters of the Gulf of Mexico. *Appl Environ Microbiol* **62**: 4374–4380.

Whitman WB, Coleman DC, Wiebe WJ. (1998). Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA* **95**: 6578–6583.

Wilcox RM, Fuhrman JA. (1994). Bacterial-viruses in coastal seawater - Lytic rather than lysogenic production. *Mar Ecol Prog Ser* **114**: 35–45.

Williamson SJ, Houchin LA, McDaniel L, Paul JH. (2002). Seasonal variation in lysogeny as depicted by prophage induction in Tampa Bay, Florida. *Appl Environ Microbiol* **68**: 4307–4314.

Williamson SJ, Cary SC, Williamson KE, Helton RR, Bench SR, Winget D *et al.* (2008). Lysogenic virus-host interactions predominate at deep-sea diffuse-flow hydrothermal vents. *ISME J* **2**: 1112–1121.

Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H. (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* **51**: 89–103.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)