Hunting for Phages in the Anoxic World

Microbial Diversity Course
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Introduction

Viruses are by far the most abundant biological entities on the planet, and their importance in shaping bacterial communities and influencing the global biogeochemical cycles is unquestioned (Suttle, 2007). In the oceans, stretching the estimated 10^{30} viral particles end to end would span farther than the nearest 60 galaxies. Viruses are not exclusively present in the oceans, but they are widely distributed and abundant wherever a life form is found (Suttle, 2005).

In aquatic environments, viral ecology studies have been focusing mainly on the virioplankton, while little information is available for the viriobenthic fraction. In particular, viral diversity and community structure in sediments is still at the pioneering stage (Danovaro *et al.*, 2008 and references therein), and until now no prokaryotic virus (phage) has been isolated for anaerobic microorganisms, common inhabitants of anoxic sediments.

This project aims at the isolation of lytic viruses (phages) affecting anaerobic microorganism. Sulfate-reducing bacteria (SRB) will be isolated in pure cultures from a number of brackish and marine sediments from marshes and ponds in the surroundings of Woods Hole. SRB isolates will be then used as hosts in spot assays with phages collected from the same sediments.

Material and methods

Sampling sites. Sediment samples were collected in 5 sites in the surroundings of the Marine Biological Laboratory (MBL; Woods Hole, MA, USA), *i.e.* Trunk River Marsh (brackish), School Street Marsh (brackish), Eel Pond (marine), Sippewissett Marsh (marine), Salt Pond (marine). Samples were collected and kept under anoxic conditions during transportation. Enrichments were started immediately upon arrival at the lab working in the anaerobic chamber.

Culture media for sulfate-reducing bacteria (SRB). Sea water base (SWB) medium composition was as follow: per liter, 20 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$,

0.5 g KCl. One milliliter of 0.1% resazurin, 20 mL 1M MOPS pH 7.2, 10 mL 1000X trace element solution (see below), 10 mL 1000X vitamin mix (see below), 5.9 g $NaHCO_3$, were added to 1 L of SWB and the solution was boiled for 10 minutes under a flow of N_2/CO_2 (80%/20%). Into the anaerobic chamber, 0.27 g NH_4Cl, 1 mL anoxic 1 M potassium phosphate buffer pH 6.8, 0.07 g Cys-HCl, 4.09 g Na_2SO_4 and 2.0 mL anoxic 0.2 M Na_2S were added to the anoxic solution.

The trace element solution (1000X) had the following composition: 20 mM HCl, 7.5 mM $FeSO_4 \cdot 7H_2O$, 0.48 mM H_3BO_3, 0.5 mM $MnCl_2 \cdot 4H_2O$, 6.8 mM $CoCl_2 \cdot 6H_2O$, 1.0 mM $NiCl \cdot 6H_2O$, 12 µM $CuCl_2 \cdot 2H_2O$, 0.5 mM $ZnSO_4 \cdot 7H_2O$, 0.15 mM $Na_2MoO_4 \cdot 2H_2O$, 2.0 mM $NaVO_3$, 75 µM $Na_2WO_4 \cdot 2H_2O$, 23 µM $Na_2SeO_3 \cdot 5H_2O$.

The vitamin mix solution (1000X) had the following composition: 10 mM MOPS pH 7.2, 1.0 mg/ml riboflavin, 0.3 mg/ml biotin, 1.0 mg/ml thiamine HCl, 1.0 mg/ml L-ascorbic acid, 1.0 mg/ml d-Ca-pantothenate, 1.0 mg/ml folic acid, 1.0 mg/ml nicotinic acid, 1.0 mg/ml 4-aminobenzoic acid, 1.0 mg/ml pyrodixine-HCl, 1.0 mg/ml lipoic acid, 1.0 mg/ml NAD, 1.0 mg/ml thiamine pyrophosphate, 1.0 mg/ml cyanocobalamin.

The medium was dispensed in 30 mL aliquots into 100 mL serum bottles, sealed with butyl rubber stoppers and aluminum crimps, and autoclaved (121°C, 60 min). After cooling, for growth on lactate, acetate, and propionate, the headspace gas was exchanged with N_2/CO_2 (80%/20%), while for growth on H_2/CO_2 the headspace was exchanged with H_2/CO_2 (80%/20%). In both cases, headspace gas exchange was performed with three cycles of vacuum/pressurization. Just before inoculation, anoxic and filter-sterilized lactate, acetate, and propionate solutions were added to the serum bottles to a final concentration of 40 mM. No electron acceptor was added to the medium for growth on H_2/CO_2.

Approximately 3 g of sediment were inoculated into 30 mL of liquid medium for the primary enrichments in liquid medium. For the secondary enrichments, 100 µL of culture were transferred into new medium. After inoculation, serum bottles were stored in an incubator at 35°C until growth was visible by increased turbidity of the solution and formation of a black precipitate. Sulfide production was determined using the Hach Hydrogen Sulfite Test Kit (Hach Company, Loveland, CO, USA; cat.# 223801).

For plates, 14 g agar were added to 1 L of medium before autoclaving. After cooling,

electron donors were added as above, and the medium was poured onto plates in the anaerobic chamber. One hundred microliters of secondary enrichment were spread on plate for isolation of single colonies. Single colonies were picked with a sterile toothpick and streaked on a new plate. After inoculation, plates were stored at 35°C in an incubator under strictly anaerobic $H_2S/CO_2/N_2$ atmosphere (0.1%/20%/79.9%, 1000 ppm) until colonies were visible on the agar surface.

Visual characterization of SRB isolates. Colony morphology was determined by visual inspection of the agar plates, while light microscopy was used to study SRB isolates cell morphology and motility. A drop of liquid culture was spotted on a microscope glass slide and examined using a Zeiss Axio Scope equipped with an AxioCam MR3 camera (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Phylogenetic characterization of SRB isolates. SRB isolates were also characterized phylogenetically using 16S rRNA as marker gene. A colony PCR was performed for each SRB isolate; a colony sample was collected from the plate with a sterile toothpick and resuspended into 20 µL of sterile 0.05% Nonidet P40 aqueous solution (Roche Diagnostics GmbH, Mannheim, Germany; cat.# 11754599001). Cells were lysed and bacterial enzymes inactivated by incubation at 100°C for 5 minutes. Two microliters of the lysate were added to 23 µL of Promega Master Mix (Promega Corporation, Madison, WI, USA; cat.# M7505) containing the universal bacterial primers 8F and 1492R (Lane, 1991) in concentration of 0.6 µM each. Positive control, *i.e.* purified *16S* PCR product from *E. coli*, and negative control without DNA were always included in PCR amplification experiments. The reaction was carried in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA, USA). The cycling conditions were as follows: hotstart at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 46 °C for 30 s, and elongation at 72 °C for 1.5 min. The cycling was completed by a final elongation step at 72 °C for 5 min. Five microliters of PCR product were cleaned for sequencing using the ExoSAP-IT enzymes mixture (USB Corporation, Cleveland, Ohio, USA; cat.# 78202) according to manufacturer's instructions. Sequencing was carried on at the MBL sequencing facility using 3 µL of the ExoSAP-IT reaction.

Recovered 16S rRNA sequences were initially compared to GenBank database (Benson *et al.*, 2005) for preliminary identification using the programs BLASTN ([http://www.ncbi.nml.nih.gov/BLAST]). The alignment and treeing software of the ARB package (Ludwig *et al.*, 2004; [http://www.arb-home.de]) were used for the phylogenetic analyses. Nucleotide sequences were added to the Silva database ([http://www.arb-silva.de]) and aligned using the Fast Aligner tool. Alignments were refined by visual inspection. Phylogenetic inference was performed with sequences of at least 650 nucleotides in length, by using the maximum likelihood tool (AxML+FastdnaML) and appropriate filters (pos_var_Bacteria_94).

Phages collection from sediment samples. Viral particles were collected from sediment samples following the protocol described by Danovaro *et al.* (2001). A volume of anoxic sediment equal to 15 mL was collected from all five sampling sites on the day of the plaque assay, mixed with sodium pyrophosphate to a final concentration of 10 mM and incubated for 15 min. After incubation, samples were treated with ultrasounds in an ice bath to prevent overheating. Sonication was carried on for a total of 3 min, with an interruption of 30 s every minute, during which time the samples were shaken manually. Samples were then centrifuged (10,000 rpm, 15 min) and the surnatant containing the viral particles filtered over a sterile 0.2 μm pore size filter (Corning Incorporated, Acton, MA, USA, cat.# 431219). The filtered 0.2 μm fraction was sealed in a 100 mL serum bottle, made anoxic by changing the headspace gas with 5 cycles of vacuum/pressurization, and used as inoculum for the spot assay.

Isolation of phages. All actions were performed into the anaerobic chamber. SRB isolates were grown in liquid medium; 1 mL of log phase host cells culture was mixed with 3 mL of 0.6% agar medium kept molten on a heat block at 45°C, briefly vortexed, immediately evenly poured on a solidified 1.5% agar medium bottom layer, and let solidify for 15 min. Three different dilutions (1:1, 1:10, 1:100) of the phage-containing filtrates from all sampling sites were spotted (10 μL) in an arrayed order on the top agar layer and let adsorb for 15 min. Plates were incubated inverted.

Results and discussion

The goal of this study was at the isolation of phages affecting anaerobic microorganisms. Sulfate-reducing bacteria (SRB) were chosen as model host organisms, as generally faster growing compared to other anaerobes. The experimental scheme is depicted in Fig. 1. Briefly, pure cultures of the SRB host were obtained from brackish and marine sediments through a series of enrichments in liquid medium and isolation on agar plates. Four different electron donors were used in enrichment cultures in order to increase the spectrum of potential SRB isolates. Morphologically and phylogenetically characterized isolates were then tested for susceptibility to viral attack in spot assays with phages collected from the same sediments, and from any of the other sediments used for the anaerobic enrichments.

Incubation time on liquid/solid medium varied depending on electron donor: 4-5 days were necessary to observe growth on lactate, while growth on acetate, propionate, and H_2/CO_2 required at least 5-10 days (incubation time was longer for growth on solid medium). For this reason, although primary and secondary enrichments were successful on all electron donors, in the three weeks available for the experiment SRB host isolates were obtained only from the enrichments on lactate. In addition, isolates were obtained in time only from the brackish sediments of Trunk River Marsh and the marine sediments collected from Eel Pond.

Thirty-eight SRB strains were isolated from each of these two sites. On agar medium, both isolates grew in milky white colonies (Fig. 2), with circular shape and sticky consistency. All SRB from Trunk River Marsh were characterized by vibrioid (Fig. 3a) and motile cells and were affiliated with the *Desulfovibrionaceae* line of descent, in particular with *Desulfovibrio alaskensis* (NCBI, 98-99% sequence identity) and *Desulfovibrio ferrireducens* (Silva, Fig. 4), strains isolated from a soured oil well (Beech *et al.*, 2004) and a permanently cold fjord sediment (Vandieken, 2006), respectively. Instead, according to both databases, all isolates from Eel Pond were motile slightly curved cells (Fig. 3b) that showed high sequence identity (99%) with *Desulfoluna butyratoxidans*, a mesophilic SRB belonging to the family *Desulfobacteraceae* isolated from an estuarine sediment (Suzuki *et al.*, 2008) as shown in Figure 4.

As the susceptibility to viral infection may vary between strains, all 76 isolates from Trunk River and Eel Pond were used as host in spot assays. However SRB strains could not be grown as lawns on agar plates due to technical problem, making the spot assay impossible to perform. Given to the slow SRB growth and limited time available, the experiment could not be completed during the course time frame.

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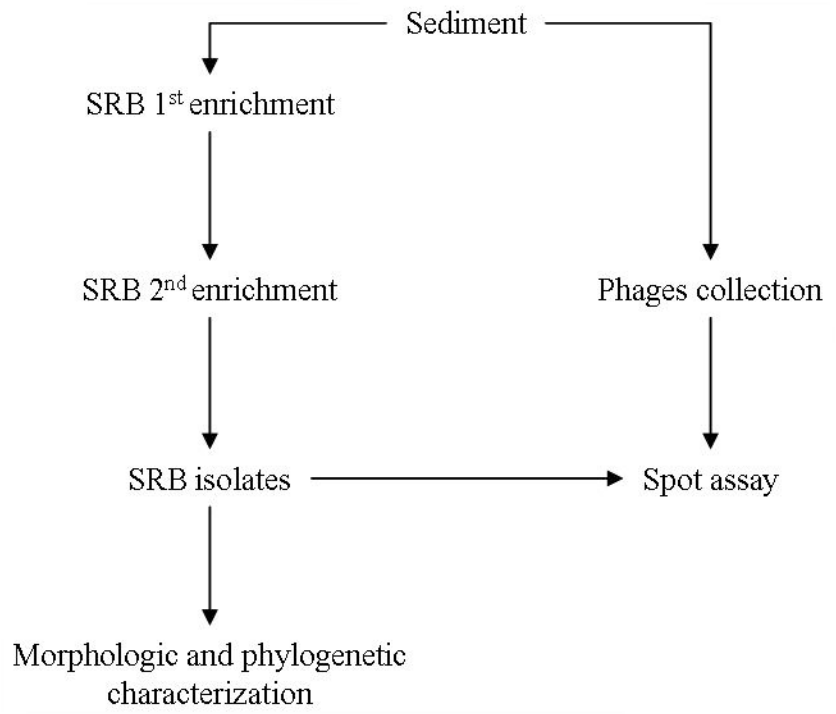

Figure 1. Experimental scheme.

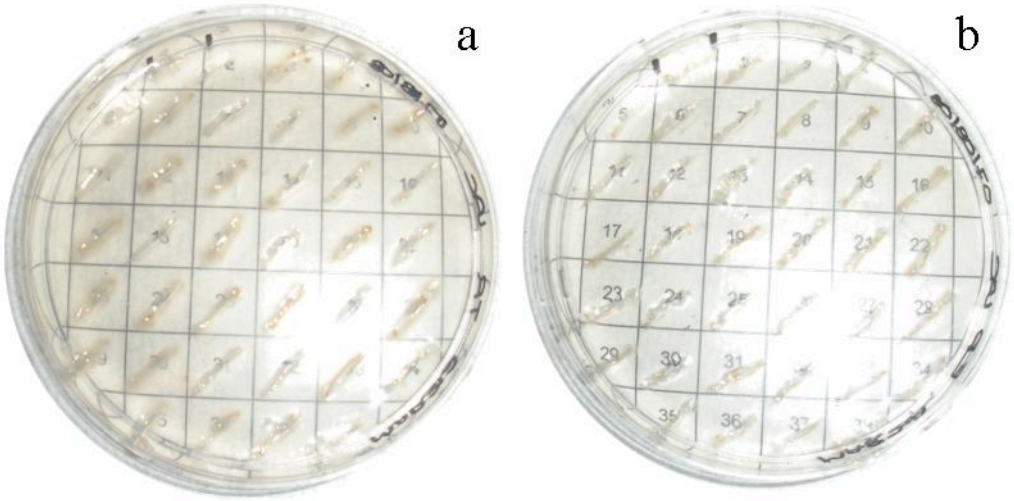

Figure 2. Growth on agar plates for the SRB isolates from Trunk River Marsh (a) and Eel Pond (b).

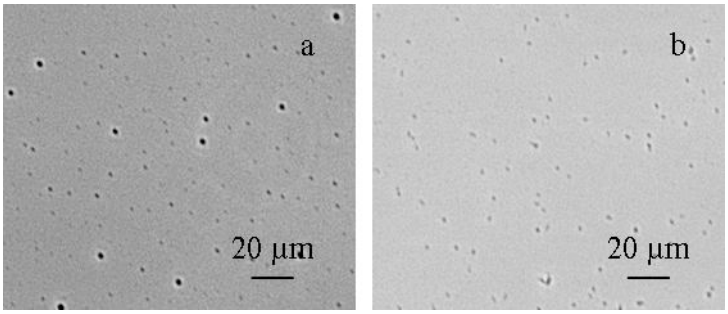

Figure 3. Phase-contrast photomicrography of a representative SRB strain from Trunk River Marsh (a) and Eel Pond (b).

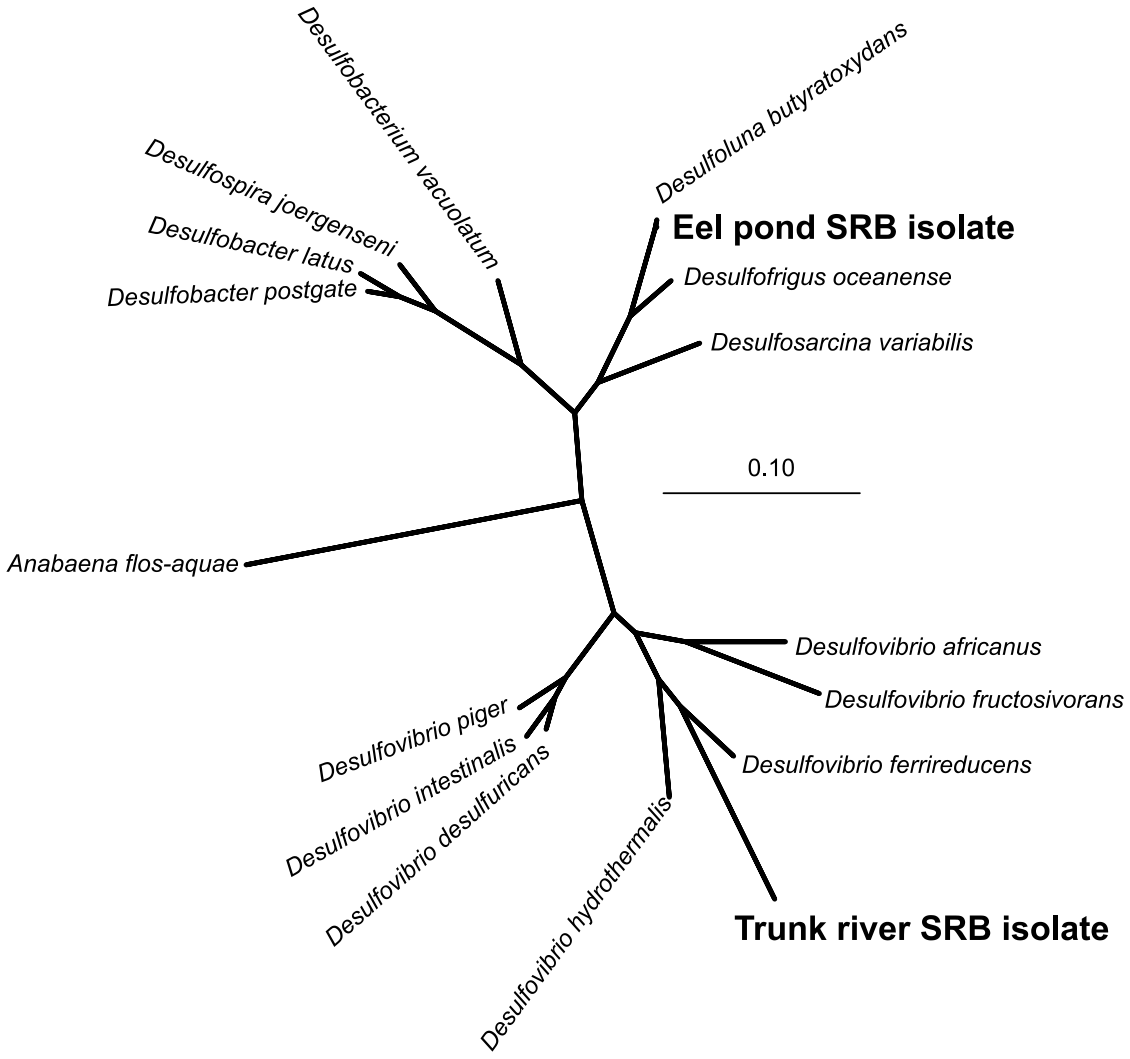

Figure 4. 16S rRNA-based phylogenetic tree showing the affiliation of a representative SRB sequence from Trunk River and Eel Pond to reference microorganisms included in the Silva database. Accession numbers for reference SRB strains are the following: *Desulfoluna butyratoxydans* (AB110540), *Desulfofrigus oceanense* (AF099064), *Desulfosarcina variabilis* (M34407), *Desulfovibrio africanus* (X99236), *Desulfovibrio fructosivorans* (AF050101), *Desulfovibrio ferrireducens* (AJ582755), *Desulfovibrio hydrothermalis* (AF458778), *Desulfovibrio desulfuricans* (AF081579), *Desulfovibrio intestinalis* (Y12254), *Desulfovibrio piger* (AF192152), *Anabaena flos-aquae* (AF247597), *Desulfobacter postgatei* (AF418180), *Desulfobacter latus* (AJ441315), *Desulfospira joergensenii* (X99637), *Desulfobacterium vacuolatum* (AF418178). Bar indicates 10% sequence divergence.