Evidence of bacteriophage-mediated horizontal transfer of bacterial 16S rRNA genes in the viral metagenome of the marine sponge *Hymeniacidon perlevis*

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Marine sponges have never been directly examined with respect to the presence of viruses or their potential involvement in horizontal gene transfer. Here we demonstrate for the first time, to our knowledge, the presence of viruses in the marine sponge *H. perlevis*. Moreover, bacterial 16S rDNA was detected in DNA isolated from these viruses, indicating that phage-derived transduction appears to occur in *H. perlevis*. Phylogenetic analysis revealed that bacterial 16S rDNA isolated from sponge-derived viral and total DNA differed significantly, indicating that not all species are equally involved in transduction.

INTRODUCTION

Viruses are the most numerous and diverse biological entities in not only the marine environment, with $10^6$ to $10^9$ particles (ml seawater)$^{-1}$ (Kristensen *et al.*, 2010) and $10^{30}$ unique viral genotypes (Suttle, 2007), but also on Earth (Angly *et al.*, 2006). Viruses are extremely important life forms known to play a crucial role in microbial ecosystems. They are essential for maintaining and influencing the diversity of all microbial communities both by affecting them directly (by controlling numbers of bacterial cells) and by horizontally transferring genetic material to the host via the transduction process (Lohr *et al.*, 2005). Phages can also affect microbial evolution as killing certain dominant bacteria may allow other related strains that are resistant to the phage to become dominant (Angly *et al.*, 2006) in what is known as the ‘kill the winner’ hypothesis. This may help explain microbial diversity and the changes that can be observed within bacterial community composition (Rohwer *et al.*, 2009). However, this interaction between phages and their bacterial hosts has remained largely under studied, especially with respect to marine sponge ecosystems.

Microbial communities of marine sponges are a major focus of current research, as sponge-associated microbes have been shown to produce a plethora of novel bioactive metabolites (Taylor *et al.*, 2007). Due to the advent of high throughput sequencing, numerous metagenomic studies have facilitated the identification of sponge-associated bacterial groups, which have revealed remarkable levels of bacterial diversity, with 26 major phyla to date having been found to be present in close association with sponge species worldwide (Kennedy *et al.*, 2008; Webster & Taylor 2012; Jackson *et al.*, 2012). Some sponges have a low microbial abundance, with a microbial range similar to that of
seawater (Gerçe et al., 2011). However, some sponges, termed bacteriospences, contain up to 10^{10} bacteria per gram wet weight, which surpasses seawater concentrations by two to four orders of magnitude (Hentschel et al., 2006), and can account for up to 40% of the sponge tissue volume (Grozdanov & Hentschel, 2007). It has been estimated that in most environments, there are 10 virus-like particles for every microbial cell. Hence it can be expected that bacteriospences could contain up to ~10^{11} virus-like particles per gram wet weight. This would exceed the concentration of viruses typically present in seawater by up to four orders of magnitude (Kristensen et al., 2010). While there are few reports on the presence of viruses within sponges, recent metagenomic studies of sponge-associated bacteria have demonstrated a high number of clustered regularly interspaced short palindromic repeats (CRISPRs) which are indicative of the presence of horizontal transfer by e.g. phages (Webster & Taylor, 2012). However, to the best of our knowledge, viruses isolated from, and likely to be associated with, bacterial gene transduction have never been reported in a marine sponge. Therefore, we aimed to determine the presence of viruses in marine sponges. In order to estimate the extent of bacteriophage-mediated horizontal gene transfer that may occur within the sea sponge *Hymeniacidon perlevis* and to compare it to the surrounding seawater, 16S rDNA bacterial genes incorporated into the bacteriophages were analysed. For the first time to our knowledge, it was shown that viruses are present in *H. perlevis*. Bacterial genes were successfully amplified from these viruses, with a greater diversity of bacterial DNA being found in viral DNA than in total DNA.

**METHODS**

**Collection of *H. perlevis* and seawater samples.** Samples of the sponge *H. perlevis* and the surrounding seawater were identified and collected from an intertidal zone of Ballyhenry Island, 5°34'31.07"W 54°23'36.55"N, Strangford Lough, County Down, in June 2010 (summer sample) and March 2011 (spring sample). Approximately 2 kg (wet weight) of *H. perlevis* was collected from exposed rocks, cut into small pieces, combined and suspended in 4 l filtered (0.2 μm pore size) and autoclaved seawater which had previously been collected from the same location. Adjacent seawater (21 l) was also collected and transported to the laboratory, kept at 4 °C and processed within 24 h. Due to the fact that viral genomes are much smaller than bacterial genomes, a subset of ~20 g of sponge and 1 l of seawater was taken from the collection and stored separately for total DNA extraction. All samples were transported to the laboratory within two hours of collection.

**Viral particle purification.** Sponge samples were pretreated prior to viral particle purification. For this, sponge samples were shaken overnight in 4 l sterile seawater at 4 °C, to release loosely attached viral particles. Following this, sponges were homogenized gently between two paddles moving at 160 r.p.m. for 1 min in a stomacher (Steward) with sterile seawater to release more viruses. The homogenized sponge mixture was then centrifuged at 13000 g and the supernatant was used for further purification.

Viral particles were isolated from seawater and pre-treated sponge samples by tangential flow filtration (TFF), as previously described by Thurber et al. (2009). In brief, samples were filtered through a 0.2 μm TFF membrane using the Centramate TFF system (Pall Life Sciences) in order to remove bacteria, and then a second filtration step using a 100 kDa cut-off TFF membrane was performed to concentrate the viral particles. The final volume of both sea and sponge viral concentrates was 40 ml and these preparations were further filtered with a 0.2 μm syringe filter and stored in phosphate buffer (0.1 M NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris/Cl pH 7.5) at 4 °C. Viral concentrates were then purified via 0.75 g cesium chloride ml⁻¹ continuous ultracentrifugation in the Beckman Optima TLX ultracentrifuge (18 h at 127 600 g in the TLN-100 rotor). As environmental samples could be expected to contain viruses of different densities, the ultracentrifugation tube was equally fractionated into eight different fractions (Sambrook & Russell, 2001) in order to identify the fraction(s) in which the majority of viruses had settled.

**Epifluorescence microscopy.** Epifluorescence microscopy was used to identify the fraction(s) containing the viruses and to ensure that no bacteria were present. Viral concentrate (100 μl) was diluted in 400 μl sterile H₂O and viruses were trapped on a 0.02 μm Anodisc filter (Whatman). This filter was then stained with 10 μl 2 × SYBR Gold (Invitrogen), and incubated for 20 min in the dark, after which it was washed twice with 15 μl sterile water and allowed to dry briefly on filter paper. The 0.02 μm filter was then placed on a clean microscope slide; 10 μl mounting solution [100 ml 10% ascorbic acid, 4.9 ml PBS (pH 7.4), 5 ml glycerol filtered through a 0.02 μm syringe filter] was applied, and samples were immediately visualized via a 630 x magnification using a Leica DMR-HR fluorescent microscope (Thurber et al., 2009). A minimum of 20 fields of view were analysed to ensure samples were free of bacterial contamination. *Pseudomonas putida* Pgg1 was used as a bacterial control.

**Transmission electron microscopy (TEM) of seawater and sponge phages.** Aliquots of seawater and sponge phage concentrate were analysed using TEM. For this, carbon films (approx. 3 × 3 mm in size) were floated from mica-plates into 100 μl of phase concentrate. After an adsorption time of 5–10 min, the samples were washed twice for a few seconds in drops of distilled water. Samples were finally transferred into drops of 2% uranyl acetate and allowed to dry briefly on a slide; 10 μl mounting solution [100 ml 10% ascorbic acid, 4.9 ml PBS (pH 7.4), 5 ml glycerol filtered through a 0.02 μm syringe filter] was applied, and samples were immediately visualized via a Leica DMR-HR fluorescent microscope (Thurber et al., 2009). A minimum of 20 fields of view were analysed to ensure samples were free of bacterial contamination. *Pseudomonas putida* Pgg1 was used as a bacterial control.

**DNase treatment.** Viral fractions were treated with DNase (50 U DNase per 1 μl sample) in order to degrade any external bacterial DNA that may have passed through the 100 kDa TFF filter. A 16S rDNA PCR, as previously described using 63F and 1387R primers, was performed on the phase particle suspension to test whether the DNase treatment had been successful (Marchesi et al., 1998).

**Extraction of viral DNA.** DNA was extracted from eight different samples taken over a 2 year period (Table 1): seawater total DNA June 2010 (ST10 or iST10) and March 2011 (SV10), seawater viral DNA June 2010 (SV10 or iSV10), seawater DNA June 2011 (SpV11) and March 2011 (SpT11), sponge viral DNA March 2011 (SpV11) and sponge viral DNA March 2011 (SpT11). Viral DNA was extracted from seawater and sponge phase concentrates via the procedure described by Casas & Rohwer (2007). DNA concentration was measured using a PerkinElmer Victor® Fluorometer and analysed by using PerkinElmer WorkOut 2.5 Data analysis software. DNA concentrations were determined by comparing the fluorescence of bound SYBR Green, which binds to double stranded DNA of known standards from which a standard
The solution was centrifuged at ~5000

Incubation for 2 h at 70°C

H. perlevis

Program (Lozupone et al., 2011). Community comparisons were carried out using the Unifrac (data not shown). For bootstrap analysis, 1000 replications were generated. Sequences were also aligned without common alignment gaps characters, and the resulting neighbour-joining trees were identical. In order to analyze the viral particles in more detail, TEM was performed. Two different phage morphotypes were observed in the seawater samples, a phage with a 20 nm thick contractile tail (length 95 nm) of the isometric-headed (head diameter 75 nm) which is indicative of it belonging to the Myoviridae (Fig. 1a, ii), and a phage with a 140 nm long and apparently flexible tail with similar head size which is indicative of Siphoviridae phages (Fig. 1a, iii).

Members of these Siphoviridae phages were also detected in the sponge sample. A typical phage particle with an isometric head (diameter 55 nm) and a long flexible tail (~240 nm) was observed (Fig. 1b, iii) and an isometric-headed phage (head diameter 87 nm) with a short tail (35 nm) representing Podoviridae phages was also detected (Fig. 1b, ii) (Veesselaar & Cambillau, 2011; Ackermann, 1996; Suttle, 2005).

The broad-host-range Myoviridae and Siphoviridae viruses are some of the most commonly isolated phages from marine viral communities (Huang et al., 2012; Suttle 2005). Podoviridae, which have a narrow host range, are, however, less frequently isolated from seawater (Suttle, 2005; Huang et al., 2010). Sponge-derived bacteria have been shown to possess a high number of CRISPRs, indicative of the presence of phages (Webster & Taylor, 2012). However, to our knowledge, this is the first report of the presence of viral particles in marine sponges.

**H. perlevis and seawater phages contain bacterial genes**

After removal of potentially contaminating external DNA, phage DNA was isolated and subjected to 16S rDNA PCR

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**Table 1. Details of samples of seawater and the sponge H. perlevis that were collected and analysed**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample name</th>
<th>GenBank accession nos</th>
<th>DNA concentration (ng μl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater total DNA June 2010</td>
<td>ST10</td>
<td>JQ435913–JQ435941</td>
<td>25.5</td>
</tr>
<tr>
<td>Sponge total DNA June 2010</td>
<td>SpT10</td>
<td>JQ435942–JQ435952</td>
<td>1594.5</td>
</tr>
<tr>
<td>Seawater viral DNA June 2010</td>
<td>SV10</td>
<td>JQ435953–JQ435983</td>
<td>24.8</td>
</tr>
<tr>
<td>Sponge viral DNA June 2010</td>
<td>SpV10</td>
<td>JQ435984–JQ436010</td>
<td>71.6</td>
</tr>
<tr>
<td>Seawater total DNA March 2011</td>
<td>ST11</td>
<td>JQ436092–JQ436120</td>
<td>17.4</td>
</tr>
<tr>
<td>Sponge total DNA March 2011</td>
<td>SpT11</td>
<td>JQ436011–JQ436031</td>
<td>1741.3</td>
</tr>
</tbody>
</table>

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**RESULTS AND DISCUSSION**

**Microscopy reveals the presence of viruses in H. perlevis**

Viral fractions that had been obtained following caesium chloride ultracentrifugation from samples of the surrounding seawater and of the sponge H. perlevis were visualized by epifluorescence microscopy to determine whether virus-like particles were present in the sponge and the surrounding seawater. Virus-like particles were identified in fractions 4 and 5 in both the seawater (Fig. 1a, i) and sponge (Fig. 1b, i) samples, while no bacterial contamination was observed.

In order to analyse the viral particles in more detail, TEM was performed. Two different phage morphotypes were observed in the seawater samples, a phage with a 20 nm thick contractile tail (length 95 nm) of the isometric-headed (head diameter 75 nm) which is indicative of it belonging to the Myoviridae (Fig. 1a, ii), and a phage with a 140 nm long and apparently flexible tail with similar head size which is indicative of Siphoviridae phages (Fig. 1a, iii).

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**H. perlevis and seawater phages contain bacterial genes**

After removal of potentially contaminating external DNA, phage DNA was isolated and subjected to 16S rDNA PCR.
analysis in order to detect bacterial 16S rRNA genes in the phages. 16S rRNA genes were successfully amplified, demonstrating that phages isolated from seawater and sponge samples contained 16S rDNA bacterial genes in their genomes (Fig. 2). It has previously been shown that environmental phages in wastewater, sludge and soil contain 16S rRNA genes of bacterial origin (Sander & Schmieger, 2001, Del Casale et al., 2011a, b; Parsley et al., 2010; Ghosh et al., 2008).

**16S rRNA genes from sponge virome distinct from seawater virome**

In order to identify the origin of the 16S rDNA found in the phage particles isolated from sponge and seawater samples, 16S rDNA clone libraries were constructed from sponge viral DNA and seawater viral DNA, in 2010 and 2011. Clones were sequenced and subsequently grouped using the FastGroup program, followed by phylogenetic analysis. Firstly, the 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) which were defined by a 3% sequence dissimilarity cut-off.

In both 2010 and 2011 samples, seawater and sponge libraries contained many of the same species; however the relative abundances of these species were distinct in both environments (Fig. 3). Comparisons between the two communities were made via statistical analysis using the Unifrac program (Lozupone et al., 2006) which showed that 16S rDNA seawater virome libraries were significantly different from 16S rDNA sponge virome libraries (P-value <0.01) in 2010 and 2011. Environmental distance matrix analysis, also carried out using the Unifrac program, showed that the sponge 16S rDNA viral communities from 2010 and 2011 were more similar to one another than either was to the seawater 16S rDNA viral community. Seawater 16S rDNA viral communities were also seen to be more similar to one another than either was to sponge 16S rDNA viral communities. This further indicates that *H. perlevis* may contain a unique microbiota distinct from the surrounding seawater.

**Bacterial biodiversity differs between *H. perlevis* and seawater**

To investigate whether bacterial DNA found in the phage particles corresponded with the bacterial diversity found in the seawater and sponge samples, total 16S rDNA clone libraries were constructed. These libraries were then subjected to sequence and phylogenetic analysis. A dramatic difference between the two libraries was seen in the content of Bacteroidetes in 2010, which, while composing a third of the seawater total DNA library, was not detected from the sponge total DNA 16S rRNA gene library (Supplementary Fig. S1, available with the online version of this paper). A similar trend was noticed in 2011, when Bacteroidetes composed ~10% of ST11, but only 3% of SpT11 (Fig. 3b). Although statistical analyses revealed that in both 2010 and 2011, there was a significant difference between total rDNA found in seawater and in *H. perlevis* (P-value, 0.01), indicating their distinct bacterial biodiversity, environmental distance matrix analysis carried out using the Unifrac program showed that similar differences were found between seawater samples from 2010 and 2011, and indeed sponge samples from 2010 and 2011. Moreover, the rarefaction curves did not demonstrate complete saturation (Fig. S2). Although a more extensive study would need to be carried out to fully examine the bacterial diversity in *H. perlevis*, this is the first report showing the bacterial diversity of this sponge. In general, the diversity found in *H. perlevis* seems to be in
**Fig. 2.** Rooted neighbour-joining phylogenetic trees based on (a) 16S rRNA gene sequences amplified from viral DNA retrieved from seawater in June 2010 (SV10) (GenBank accession nos JQ435953–JQ435983) and (b) 16S rRNA gene sequences amplified from viral DNA isolated from *H. perlevis* in June 2010 (SpV10) (GenBank accession nos JQ435984–JQ436010). Trees display one representative clone per OTU. The numbers next to the clone names indicate total number of replicates of the clone. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Phylogenetic analyses were conducted in MEGA5.
line with previously reported studies on other marine sponges.

**Viral DNA displays greater bacterial gene diversity than total DNA**

Finally, assessment of the diversity of 16S rRNA genes found in viral and total bacterial DNA libraries showed that 16S rRNA gene sequences derived from phage metagenomes did not mirror the total bacterial community in the environment under study. Statistical analysis showed that 16S rDNA retrieved from total DNA and viral DNA was significantly different in both sponge and seawater environments (P-value <0.01).

Although the full bacterial biodiversity of *H. perlevis* remains to be determined, substantial differences in relative abundance were observed. Betaproteobacteria, which have previously been found in both sponges and seawater (Meyer & Kuever, 2008; Zinger et al., 2011), were seen to be relatively abundant in seawater and sponge viral DNA in both 2010 and 2011. However, they were not detected in the total DNA libraries from these environments. Similar findings were obtained for Acidobacteria (Figs 2 and 3).

A similar trend was previously reported by Del Casale et al. (2011a), which suggests that not all species are equally involved in transduction – i.e. bacteriophages of bacterial groups present in small numbers may be actively involved in transduction.

In conclusion, this is the first report to demonstrate the presence of viral particles in marine sponges to our knowledge. These viruses not only are ubiquitous in the marine sponge *H. perlevis* but also contain bacterial 16S rRNA genes, indicating that phage-derived transduction appears to occur in this environment, as well as in the surrounding seawater.

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**REFERENCES**


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