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Morphological diversity of virus-like particles within the surface microlayer of scleractinian corals

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ABSTRACT: Transmission electron microscopy was employed to determine the morphological diversity of virus-like particles (VLPs) associated with the coral surface microlayer (CSM) of *Acropora muricata* and *Porites* spp. from the Great Barrier Reef, Australia. VLPs were assigned to one of 17 sub-groups within 5 major morphological groups including tailed bacteriophages, polyhedral, filamentous and lemon-shaped VLPs. Polyhedral VLPs in the 30 to 60 nm size class dominated the CSM of *A. muricata* and *Porites* spp., comprising 29.4 and 26.9% of total VLPs, respectively. Tailed bacteriophages comprised <6% of total VLPs within the CSM of both *A. muricata* and *Porites* spp. Filamentous VLPs (FVLPs) of varying lengths and widths accounted for up to 19.9% of total CSM VLPs, with no significant difference between the CSM samples and overlying water. Unique VLPs, which could not be classified into any known viral morphological group, accounted for 1.2 to 11.7% of total VLPs within the CSM and were absent from overlying water. While some exchange of VLPs likely occurred between the CSM and overlying water, our results suggest that the majority of CSM morphotypes were specific to the CSM micro-niche. The similarity of many of these VLPs to previously described viruses suggests that a range of potential hosts exist in the CSM, including bacteria, archaea, cyanobacteria, fungi, algae (possibly including zooxanthellae) and the coral animal. Research on coral–microbial interactions and their role in coral health and functioning is in its infancy and the present study provides important information on the largely unstudied viral component of the coral microbiota.

KEY WORDS: Coral surface microlayer · CSM · Virus-like particles · VLP · Transmission electron microscopy · TEM

INTRODUCTION

Viruses in aquatic environments

Viruses are ubiquitous within aquatic ecosystems worldwide, typically reaching concentrations of 10^6 to 10^8 ml^{-1} in seawater, and are likely to be the most diverse component of the planktonic community (Wommack & Colwell 2000). It is likely that viruses infect all cellular organisms (Fuhrman 1999) and viral infections have been reported in many ecologically important groups of marine microbial communities, including heterotrophic bacteria (Proctor & Fuhrman 1990), archaea (Zillig et al. 1996), cyanobacteria and phytoplankton (Suttle et al. 1990). Viruses are important regulators of microbial communities, influencing horizontal gene transfer, population dynamics, community structure, and nutrient cycling (Jiang & Paul 1998, Weinbauer & Höfle 1998, Middelboe et al. 2001)

The decline of coral reefs on a global scale has been attributed predominantly to coral bleaching (Hoegh-Guldberg 1999) and coral disease (Sutherland et al.

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2004). In addition to viruses being pathogens of planktonic communities, viruses have been identified as agents of disease in a range of higher marine organisms from molluscs to mammals. It is therefore surprising that the composition and dynamics of the viral community associated with coral reefs remains largely unstudied (Munn 2006). In contrast, an increasing number of studies have investigated the bacterial (Rohwer et al. 2001, 2002, Frias-Lopez et al. 2002) and archaeal communities (Kellogg 2004, Wegley et al. 2004) associated with corals. The first study of viruses in a coral reef environment revealed abundant concentrations of virus-like particles (VLPs) (~2×10^6 ml^{-1}) in waters overlying coral reefs (Paul et al. 1993). More recently, Seymour et al. (2005) and Patten et al. (2006) provided evidence for the abundant and dynamic nature of the VLP community within close proximity to coral surfaces, with VLPs often coupled to spatially dynamic bacterioplankton communities.

Coral-associated microbes within the coral surface microlayer

In contrast to the nutrient-depleted waters in which coral reefs exist, the coral surface microlayer (CSM) is a highly productive, mucus-rich zone that extends a few millimeters above the surface of the coral (Paul et al. 1986). The CSM plays an important role in a variety of processes fundamental for coral health and functioning, including protection from ultraviolet damage and desiccation, removal of sediments, facilitation of feeding through mucociliary transport and defence against pathogens (Brown & Bythell 2005 and references therein). Bacteria are a dominant component of the CSM and exhibit elevated activity when compared with bacteria from surrounding water (Paul et al. 1986). Archaea have also been shown to be abundant and diverse within the CSM, and distinct from archaea within the water column (Kellogg 2004). While little is known of the diversity, functioning, and interactions of coral-associated microbes, the bacterial component has been hypothesised to be important in processes such as nutrient cycling (Sorokin 1973) and the production of antibiotics (Ritchie 2006).

Bacteria, fungi, cyanobacteria and dinoflagellate algae (zooxanthellae) within the CSM and coral tissue have been proposed to be important components of the coral-holobiont model (Rohwer et al. 2002), yet the presence and subsequent role(s) of viruses within this model have yet to be conclusively established. However, in other aquatic ecosystems, viral abundance has been shown to be correlated with bacterial production (Heldal & Bratbak 1991). Therefore it may be reasonable to suggest that viruses will also be abundant within the CSM, either through non-discriminate accumulation from surrounding waters, as has been shown for human-sourced enteroviruses occurring within the CSM of near-shore coral communities (Lipp et al. 2002), or alternatively forming distinct communities within the CSM in response to the abundance and distribution of their hosts. Previous studies using transmission electron microscopy (TEM) have shown the presence of VLPs within the tissues (Wilson & Chapman 2001) and zooxanthellae (Wilson et al. 2001) of 2 species of temperate anemone, as well as aquaria-maintained scleractinian corals and their zooxanthellae (both freshly isolated and *in situ*) (Wilson et al. 2005, Davy et al. 2006). These studies suggest the induction of a latent virus infecting zooxanthellae and/or their host with increased seawater temperatures in laboratory settings. The presence of VLPs within zooxanthellae isolated from corals infected by yellow-blotch disease has also been reported (Cervino et al. 2004), although clear evidence of viral infection of tropical corals and their symbiotic partners in natural systems has yet to be shown.

Viral morphology as a tool for investigating viral diversity

There are 2 factors that hinder attempts to identify and measure the community composition and dynamics of marine viruses in natural systems. Firstly, while the hosts of the majority of marine viruses are bacteria, approximately only 1% of these can be cultured using standard techniques (Fuhrman & Campbell 1998). Secondly, unlike bacterioplankton, viruses do not have universally conserved genetic elements and therefore it is currently not possible to use a monitoring approach for determining viral diversity that is analogous to ribosomal DNA for profiling species diversity (Edwards & Rohwer 2005). In addition, species delineation is currently based primarily on percent DNA–DNA hybridisation (Coenye & Vandamme 2004). However, the range of viral nucleic acid types (double-stranded DNA, single-stranded DNA, double-stranded RNA and single-stranded RNA) makes this system of classification redundant for viruses. While the use of primers specific for sub-sets of viral communities is helping to overcome some of these limitations (Chen & Suttle 1995, Breitbart et al. 2004), classification of viruses is currently based on a combination of the following criteria: host cell type, viral nucleic acid type, morphology of viral capsids and the presence/absence of associated appendages (e.g. tails, lipid envelope). TEM provided the first evidence for highly abundant and morphologically diverse viral assemblages in marine systems (Proctor & Fuhrman 1990). Moreover,

examination of aquatic viruses with TEM is the most common method of recording virioplankton diversity (Wommack & Colwell 2000). In the present study, TEM was used to determine the morphological diversity and relative abundances of VLPs within the complex microbial community of the CSM. As this is the first investigation of its type, important information is presented on the viral communities associated with scleractinian coral surfaces.

MATERIALS AND METHODS

Site and collection. Field sampling was conducted on SCUBA in April 2006 at Heron Reef, on the Southern Great Barrier Reef, Australia (23° 27′ S, 151 °55′ E). CSM were collected at depths of 6 to 10 m from the branching coral *Acropora muricata* (n = 9) and from massive poritids later identified as *Porites lutea*, *P. lobata* and *P. australiensis* (n = 7). Using sterile 25 ml needle-less syringes, CSM were carefully removed from coral surfaces. Care was taken to ensure that the syringe tip remained in contact with the coral surface at all times and syringes were then capped underwater. As such, the amount of surrounding seawater within samples was kept to a minimum. Overlying water was also collected 10 cm from the surfaces of a subset of the sampled coral colonies (n = 3 for *A. muricata*; n = 3 for *Porites* spp.) using sterile 50 ml centrifuge tubes. Samples were transported to the laboratory and processed within 2 h of collection. CSM and water samples (15 ml) were fixed in EM-grade glutaraldehyde (2% final concentration) and stored in the dark at 4°C until analysed.

Concentration of virus-like particles. To eliminate large particles (including suspended coral tissue, particulate matter and most bacteria), fixed CSM and water samples were centrifuged at 3000 rpm (1500 × *g*) for 10 min at 4°C in a Sigma 3K15 benchtop centrifuge. The supernatant (13 ml) was transferred to polypropylene centrifuge tubes and VLPs concentrated by ultracentrifugation for 2 h at 29 000 rpm (146 000 × *g* at 4°C, Beckman Optima L-XP ultracentrifuge, SW41Ti rotor). The resultant viral pellet was resuspended in 100 µl of supernatant and stored in the dark at 4°C.

Transmission electron microscopy. Pioloform-coated 200 µm mesh copper grids were carbon coated and glow-discharged prior to floating on 20 µl subsamples of the viral suspensions for 1 h. Grids were then washed in ultrapure water for 2 s, excess water wicked away with filter paper (Whatman™) and negative stained for 30 s with 3% uranyl acetate. Excess stain was wicked away and grids air dried while covered. Grids were viewed on a JEOL JEM1010 TEM (80 kV) at 40 000 to 100 000× magnification. Images were captured using the Megaview III soft imaging system and the size and morphology of 70 to 100 VLPs per sample were determined from digital images at 40 000× magnification using Image J analysis software (http://rsb.info.nih.gov/ij/).

Statistical analysis. For viral morphotypes present within all sample groups (*Acropora muricata*, *Porites* spp. and overlying water), data were log transformed and 1-way ANOVAs performed in SPSS. When a viral morphotype was absent from one sample group, a *t*-test was run on log transformed data from 2 groups. No statistical tests were performed when a viral morphotype was absent from 2 groups.

RESULTS AND DISCUSSION

TEM analysis revealed that VLPs associated within the CSM were morphologically diverse and abundant (Figs. 1 to 3), suggesting a wide range of different hosts. VLPs were assigned to one of 17 sub-groups within 5 major groups on the basis of morphology (Table 1), with some bearing close resemblance to previously characterised groups including tailed bacteriophages (families *Myoviridae*, *Podoviridae*, *Siphoviridae*) (Fig. 1) and lemon-shaped viruses (family *Fuselloviridae*) (Ackermann 2001) (Fig. 3F). Polyhedral and spherical VLPs exhibiting bilateral symmetry, which may include untailed bacteriophages as well as viruses infecting eukaryotes, could not be distinguished using TEM alone and therefore this group was sub-divided according to capsid size alone (Fig. 2, Table 1).

Collectively, bacteriophages designated as *Myoviridae, Podoviridae,* or *Siphoviridae*-like groups comprised <6% of total VLPs for each of *Acropora muricata* and *Porites* spp. (Table 1). Supporting these results, the DNA sequence of a T-7 like Podovirus has also been extracted from the CSM of a Caribbean coral (Breitbart et al. 2004). In contrast to other natural marine viral communities, which show a dominance of tailed phages (e.g. Cochlan et al. 1993), the low frequency of tailed phages observed here may be explained by VLPs with very short tails being overlooked during analysis or incorrectly identified as a result of some tails becoming detached from capsids during the ultracentrifugation process (Proctor 1997). However, the resolution of the electron micrographs was clear and revealed the fine structure of many phages, with base plates, fibres, collars and helical-symmetry of tails apparent on these particles (Fig. 1A–F), suggesting that the method used in this study successfully preserved virus structure. Further explanations for low numbers of tailed phages may be that within the CSM there is a high concentration of

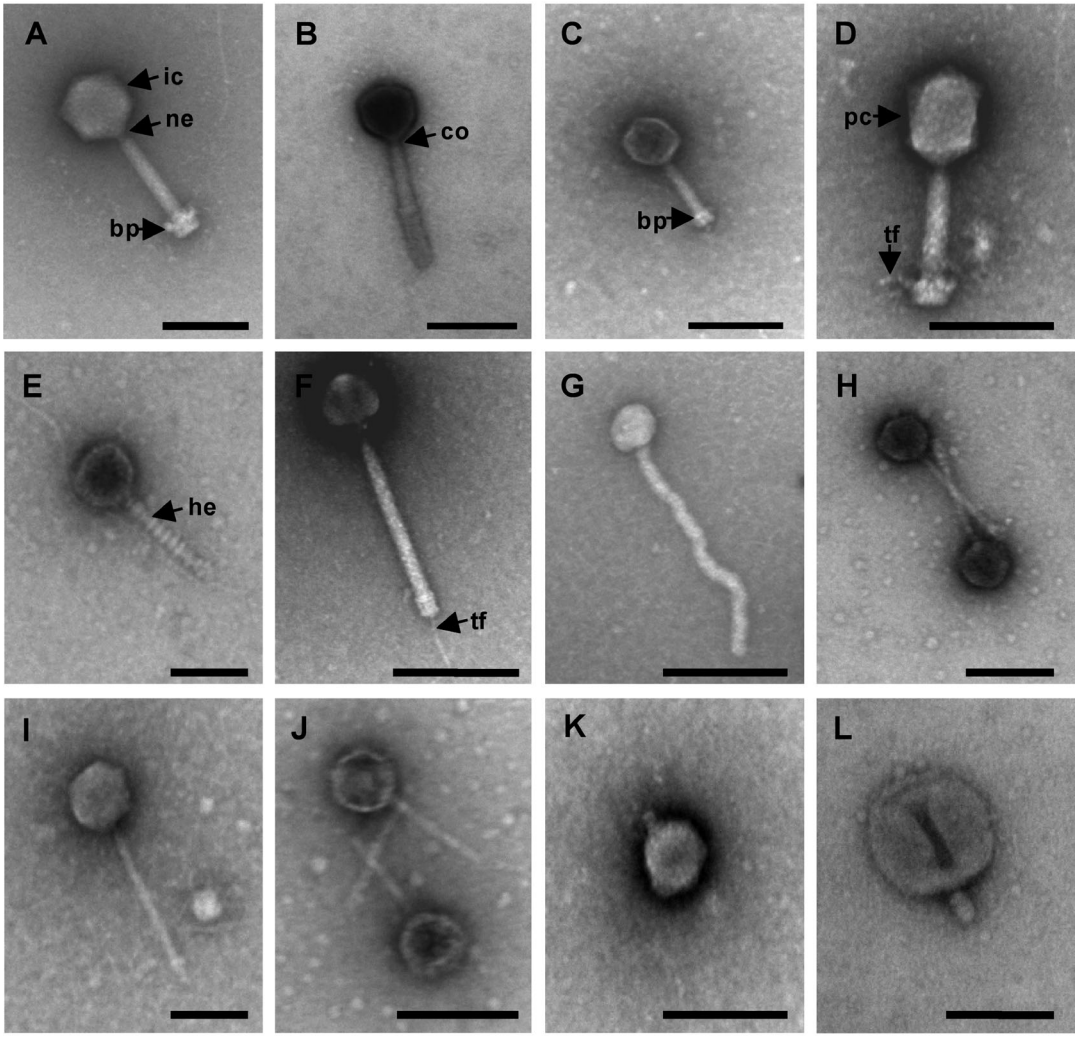

Fig. 1. Virus-like particles (VLPs) classified as bacteriophages within coral surface microlayer (CSM) samples from *Acropora muricata* and *Porites* spp. (A–F) Myovirus-like bacteriophages; arrows indicate a variety of morphological features: icosahedral symmetry of capsid (ic), prolate capsid (pc), neck (ne), collar (co), baseplate (bp), tail fibres (tf), helical symmetry of contractile tail sheath (he). (G–J) Siphovirus-like bacteriophages with filamentous, non-contractile tails and isometric capsids. (K,L) Podovirus-like VLPs with short non-contractile tails. Scale bars = 100 nm

eukaryotic hosts or that a percentage of the bacteria are in a lysogenic cycle, with the phage genome incorporated into the host's genetic material (Weinbauer 2004).

Polyhedral VLPs in the 30 to 60 nm size class dominated the CSM, accounting for 29.4 ± 4.2% and 26.9 ± 4.0% of total VLPs for *Acropora muricata* and *Porites* spp. respectively (Table 1). Dominance of the 30 to 60 nm size VLP size class has been reported from other aquatic habitats (e.g. Bergh et al. 1989). Particles in the >100 nm size range were also common (16.8 ± 2.0% and 14.4 ± 3.0% for *A. muricata* and *Porites* spp., respectively) (Fig. 2A,B, Table 1) and these VLPs may be representative of eukaryotic algal viruses, which typically fall within the 100 to 180 nm size range (Van Etten et al. 1991). Fig. 2C shows a polyhedral VLP that resembles an algal virus from the family *Phycodnaviridae*. Zooxanthellae, filamentous algae, and plankton have all been recorded to occur in coral mucus aggregates (Wild et al. 2004) and therefore represent potential hosts for this type of virus. Fig. 2F shows a VLP resembling viruses from the family *Geminiviridae*, consisting of 2 polyhedral subunits with a constricted waist. These VLPs occurred in low abundances (<2% of total VLPs) with no difference between *A. muricata* and *Porites* spp. (Table 1).

Filamentous VLPs (FVLPs) exhibiting a range of morphologies, lengths and widths were commonly observed, accounting for 20.8 and 16.3% of total VLPs in *Acropora muricata* and *Porites* spp., respectively (Fig. 3A–E, Table 1). The majority of FVLPs were approximately 10 nm

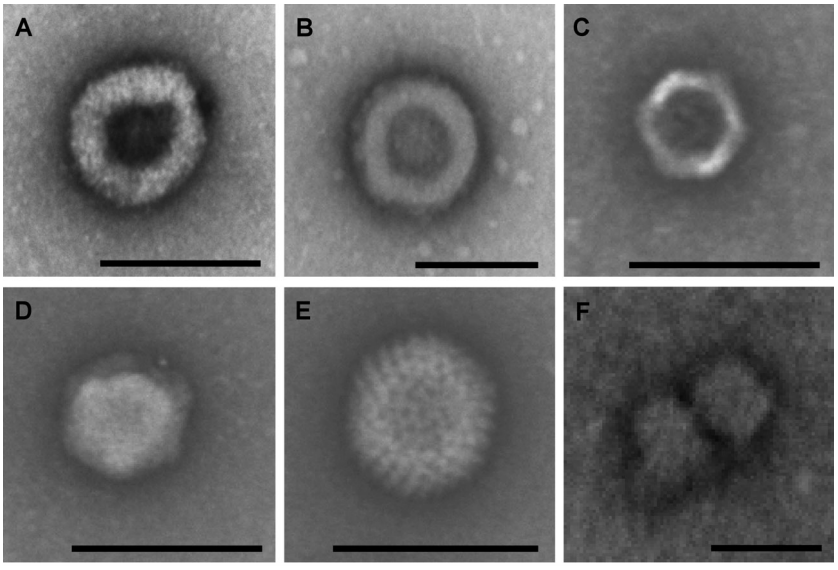

Fig. 2. Polyhedral and spherical virus-like particles (VLPs) within coral surface microlayer (CSM) samples from *Acropora muricata* and *Porites* spp. (A,B) Enveloped, spherical VLPs with electron-dense cores. (C) Enveloped, isometric VLP. (D) Non-enveloped, isometric VLP. (E) Isometric VLP with evident capsomeres. (F) Geminate VLP, comprising 2 quasi-isometric particles with a constricted waist. Scale bars = 100 nm

wide with variable lengths from 90 nm to >1 µm. Other FVLPs were approximately 25 nm wide and up to 1.6 µm in length (Fig. 3C,D). Previously, FVLPs have only been observed in a small number of other aquatic environments, such as estuarine sediments (Middelboe et al. 2003), alpine lakes (Hofer & Sommaruga 2001), and in the seawater immediately surrounding thermally stressed, aquarium-maintained *Acropora formosa* (Davy et al. 2006). Using TEM analysis alone, we cannot confirm if the filaments seen in the present study are indeed all viruses, but the similarity of several particles to known filamentous viruses suggests that a real and diverse assemblage of FVLPs occurs within the CSM.

Fig. 3F depicts a lemon-shaped VLP, morphologically similar to the Fusellovirus SSV1 that is known to infect archaea from extreme envi-

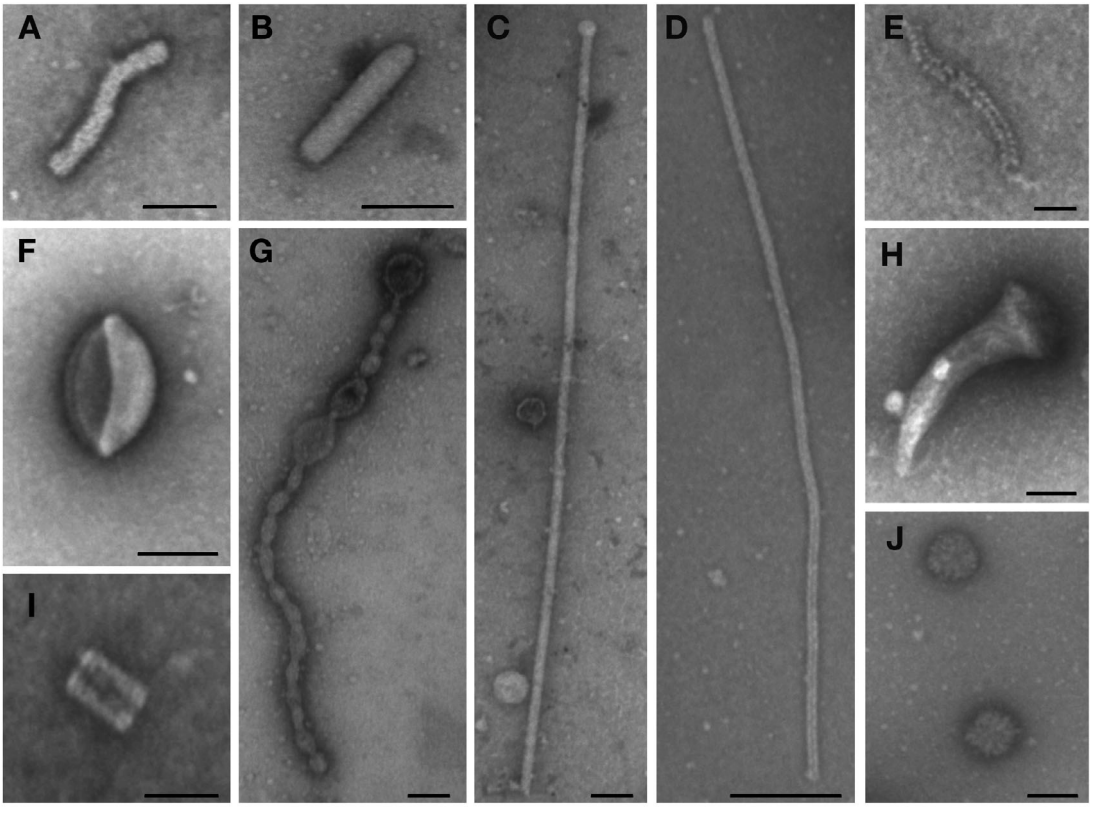

Fig. 3. Filamentous virus-like particles (FVLPs) within coral surface microlayer (CSM) samples from *Acropora muricata* and *Porites* spp. (A,B) Rod-like VLPs. (C) Filamentous VLP with head. (D) FVLP. (E) FVLP with clear helical structure and central groove. (F) Lemon-shaped VLP. (G,H) Unique hook VLPs. (I) Unique cubic VLP with central groove. (J) Incomplete virion. Scale bars = 50 nm

Table 1. Morphological diversity and relative abundances (mean ± SE) of virus-like particles (VLPs) within coral surface microlayer (CSM) samples from *Acropora muricata* (n = 9) and *Porites* spp. (n = 7) and in 10 cm distant overlying water (OW) (n = 6). *Significant differences ($p < 0.03$) between overlying water and CSM from *Porites* spp. and *Acropora muricata*. **Significant difference ($p = 0.012$) between overlying water and CSM from *Porites* spp.. ***Significant difference ($p = 0.023$) between overlying water and CSM from *Acropora muricata*. Total number of VLPs analysed for the determination of the mean% VLPs were 900 for *Acropora muricata*, 700 for *Porites* spp. and 435 for overlying water. –: viral morphotype was not present in the sample

Viral morphotype	Mean% VLPs		
	A. muricata	*Porites* spp.	OW
Tailed phage			
Podovirus-like	2.9 ± 0.7	2.1 ± 0.9	0.7 ± 0.7***
Myovirus-like	1.2 ± 0.3	1.6 ± 0.5	–
Siphovirus-like	1.2 ± 0.6	0.9 ± 0.3	0.6 ± 0.6
Polyhedral/Spherical			
30–60 nm	29.5 ± 4.2	26.8 ± 4.0	49.2 ± 7.1
60–80 nm	11.3 ± 1.6	8.9 ± 1.5	18.3 ± 2.6**
80–100 nm	8.0 ± 0.7	9.3 ± 2.1	8.5 ± 0.8
>100 nm	16.8 ± 2.0	14.4 ± 3.0	6.0 ± 3.0*
Geminivirus-like	1.0 ± 0.3	1.9 ± 0.4	–
Lemon-shaped			
<200 nm	5.1 ± 1.3	4.1 ± 1.5	–
>200 nm	0.9 ± 0.4	2.0 ± 0.5	0.7 ± 0.7
Filamentous			
<100 nm	0.9 ± 0.4	0.7 ± 0.4	1.2 ± 0.8
100–500 nm	14.7 ± 3.4	11.6 ± 2.2	8.3 ± 1.4
>500 nm	5.3 ± 1.2	4.0 ± 0.8	6.5 ± 2.2
Unique			
Hook	0.3 ± 0.2	–	–
Cubic	0.9 ± 0.7	–	–
Incomplete virion	–	11.3 ± 3.1	–
Other	–	0.4 ± 0.4	–

ronments (Zillig et al. 1996). However, not all archaea are extremophiles, with widespread occurrence of archaea known from coastal and oceanic environments (Delong 1998). Ubiquitous archaeal distributions may also apply to coral-associated archaea as, unlike bacteria, no species-specific associations between archaea and individual coral species have been shown (Kellogg 2004, Wegley et al. 2004).

Unique VLPs that did not resemble members of any known group of viruses comprised 1.2 and 11.7% of total CSM VLPs for *Acropora muricata* and *Porites* spp., respectively. Both 'hook' and 'cubic' VLPs (Fig. 3H,I) were found only in samples from *Acropora muricata*. An unknown type of VLP accounting for 11.3 ± 3.1% of total VLPs occurred exclusively within CSM from *Porites* spp. (Fig. 3J, Table 1). It is possible that these particles are incomplete virions, as polyhedral symmetry is evident in some cases, and the size range is approximately 75 to 110 nm. Incomplete virions have also been observed in fish infected with an iridovirus (Paperna et al. 2001).

There was no significant difference in the viral assemblage from overlying water sampled above *Acropora muricata* or *Porites* spp. (data not shown) and therefore these results were combined to compare with VLPs in the CSM. VLPs in the 30 to 60 nm size range were dominant in overlying water and exceeded VLPs within the CSM by up to 1.8-fold (Table 1). Significantly higher relative abundances of VLPs within the 60 to 80 nm size class occurred in overlying water compared with the CSM from *Porites* spp. ($p = 0.0012$), while VLPs in the >100 nm size class in the CSM exceeded those in overlying water by up to 2.8-fold ($p < 0.03$) (Table 1). The observation of similar abundances of FVLPs from water overlying corals compared to the CSM suggests some exchange of viral communities between the CSM and overlying water. This is reasonable given that overlying water was sampled within 10 cm of the surface of the coral colony and that viral abundance has been shown to exhibit decreasing trends from close to the coral surface to more distant water (Patten et al. 2006). The absence of VLPs from 7 of the 17 subgroups in overlying water and significant differences between the relative abundances of some viral morphotypes suggest however that the majority of VLPs within the CSM were specific to the CSM micro-niche and not an artefact from contamination with overlying water. In support of this, TEM analysis of coral tissue sections and crushed coral slurry from *Porites lutea* and *P. australiensis* has revealed the presence of VLPs that appear identical to some of those reported in the present study (J. E. Davy unpubl. data). Furthermore, flow cytometry analysis has revealed significantly lower VLP concentrations in water sampled 1 m from *Acropora muricata* colonies than in the CSM of this species (N. L. Patten unpubl. data).

Compared to other methods employed to investigate viral diversity within natural aquatic samples (e.g. pulsed field gradient gel electrophoresis and metagenomic analysis), which require concentration of large volumes of water (tens to hundreds of litres) to obtain sufficient yields of nucleic acid, the nature of the CSM (extending only millimetres from the coral surface) makes collecting volumes greater than tens to hundreds of millilitres difficult. For this reason, TEM was employed in a first attempt to explore viral diversity within this micro-niche. By categorising viral morphs (Table 1), it is likely we have underestimated the true diversity of VLPs associated with the CSM of the stud-

ied coral species. The potential for extensive viral diversity within marine environments has been recently demonstrated by metagenomic analysis, where more than 7000 distinct viral types were estimated in one 200 l marine water sample with the most abundant viral type comprising only 2 to 3% of the total viral community (Breitbart et al. 2002).

The range of VLP morphotypes shown in this study indicates a suite of likely hosts including bacteria, archaea, cyanobacteria, fungi, algae (possibly including zooxanthellae) and the coral animal. While this study was not able to determine the specific roles these VLPs may play in coral health and functioning it can be hypothesised that bacteriophages could directly influence coral health through infection of specialised bacteria residing within the CSM, thereby opening niches for opportunistic pathogenic bacteria. Alternatively, viruses could directly infect zooxanthellae or coral host (Wilson et al. 2001, 2005, Davy et al. 2006). In other marine environments, the abundance and virulence of viruses are influenced by environmental parameters including temperature and nutrient concentrations (Jiang & Paul 1994). As such, destabilisation of CSM viral-host dynamics due to changes in environmental conditions may further exert influence directly or indirectly on the health and functioning of corals. A loss of antibiotic activity and a switch from γ-proteobacteria dominance to *Vibrio* spp. dominance in the CSM from apparently healthy coral during a coral bleaching period provides some support for destabilisation of microbes within the CSM (Ritchie 2006). Researchers are increasingly aware for the need to further define the roles of microbes in coral ecosystems. Given our still limited understanding of the factors responsible for the decline in health of many of the world's coral reefs, studies of the viral component of the coral microbiota should be considered a vital part of future investigations.

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