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An atomic force microscopy investigation of cyanophage structure

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ABSTRACT

Marine viruses have only relatively recently come to the attention of molecular biologists, and the extraordinary diversity of potential host organisms suggests a new wealth of genetic and structural forms. A promising technology for characterizing and describing the viruses structurally is atomic force microscopy (AFM). We provide examples here of some of the different architectures and novel structural features that emerge from even a very limited investigation, one focused on cyanophages, viruses that infect cyanobacteria (blue-green algae). These were isolated by phage selection of viruses collected from California coastal waters. We present AFM images of tailed, spherical, filamentous, rod shaped viruses, and others of eccentric form. Among the tailed phages numerous myoviruses were observed, some having long tail fibers, some other none, and some having no visible baseplate. Syphoviruses and a podovirus were also seen. We also describe a unique structural features found on some tailed marine phages that appear to have no terrestrial homolog. These are long, 450 nm, complex helical tail fibers terminating in a unique pattern of 3 + 1 globular units made up of about 20 small proteins.

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1. Introduction

The realization that the oceans present a myriad of viral species has only dawned over the past 30 years. An effort is currently underway at the metagenomic level to classify and characterize the viruses genetically, but only the most preliminary attempts to catalog and describe their structural features have been initiated. Marine viruses, therefore, promise to provide a fertile field for future investigation. Marine organisms live in unique environments, offer an enormous diversity of hosts, and place demands on virus structure and function that are likely uncommon or nonexistent among terrestrial plants, animals, and microorganisms.

Structural studies to date have been largely restricted to viruses that infect cyanobacteria. This is principally due to the fact that cyanobacteria, or at least some species, can be grown in the laboratory, and through plaque selection, allow the isolation and purification of individual virus species. Thus, like *Escherichia coli*, they can provide a useful, reliable, and reasonably well characterized host. Viruses that infect cyanobacteria are worthy of study for another reason, and that is because they are ecologically important to the control of cyanobacterial populations in natural environments, particularly the oceans.

Blue-green algae, now known as Cyanobacteria, are among the most abundant natural host organisms for viruses in marine ecosystems. Two related genera of unicellular cyanobacteria, *Synechococcus* and *Prochlorococcus*, are estimated to contribute between a third to four-fifths of primary productivity in the oceans (Rocap et al., 2002). They photosynthetically fix carbon from CO_2 into organic matter such as sugars and thereby are crucial to the base of the marine food chain. *Synechococcus* spp. alone is thought to be responsible for 5–25% of the oceans' primary productivity (Breitbart et al., 2007).

Cyanophages of all varieties are commonly found in abundances greater than 10^7 particles per milliliter of seawater, and they are undoubtedly the most abundant members of marine ecosystems. It has been estimated that the world's oceans may contain on the order of 10^{30} virus particles (Suttle, 2005b). Marine viral genetic diversity is extremely high. Metagenomic studies reveal that 65–95% of marine viral sequences are unlike any previously reported sequences, indicating that most of this diversity remains to be described (Breitbart et al., 2007). Indeed, estimates based on these data suggest that there are hundreds of thousands of viral genotypes in the world's oceans (Angly et al., 2006). The vast genetic pool in turn implies the probability of discovering a wealth of new virus architectures and structural features that have otherwise escaped attention.

Structural analyses using electron microscopy have only scratched the surface in terms of identifying marine viral species and describing their morphologies. An alternative and

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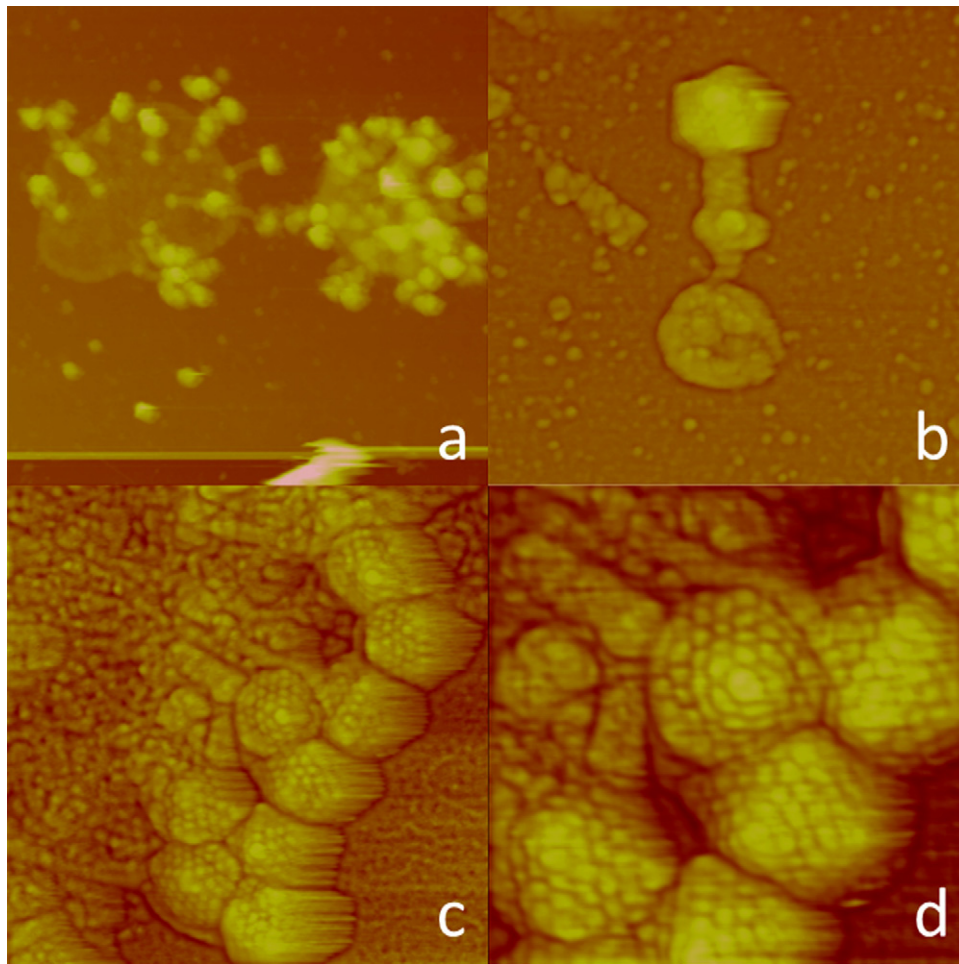
Fig. 1. In (a) large numbers of myoviruses attach to large fragments of lysed cyanobacteria. In (b) a single S-CAM4 phage with fully contracted tail sheath, but still full head, is seen attached to a small fragment of *Synechococcus*. In (c) clusters of bacteriophage are attached to a host cell. In the higher magnification image in (d) capsomeres on the icosahedral heads of the particles are visible. Scan areas are (a) 2.5 μm × 2.5 μm, (b) 500 nm × 500 nm, (c) 500 nm × 500 nm and (d) 250 nm × 250 nm. The height ranges for (a) through (d) are respectively 300 nm, 250 nm, 200 nm and 120 nm.

complementary approach to achieving the same end is the application of atomic force microscopy (AFM) to marine viruses. AFM provides another means of visualizing virus structure at nanometer resolution, and one that provides no less information, in general, than electron microscopy. In addition, it can also be carried out rapidly and in a liquid media, a distinct advantage when dealing with marine organisms. Scanning in fluid undoubtedly preserves structural features that are lost upon dehydration and staining.

As an example of the kinds of information regarding marine viruses that can be obtained from AFM, we report here a preliminary investigation of viruses isolated by plaque selection from cultures of *Synechococcus* created from isolates taken from California coastal waters between Laguna Beach and Long Beach. We have identified, based on morphological features alone, more than a dozen different viruses. These include a number of tailed bacteriophages, principally myoviruses, but also syphoviruses, and a
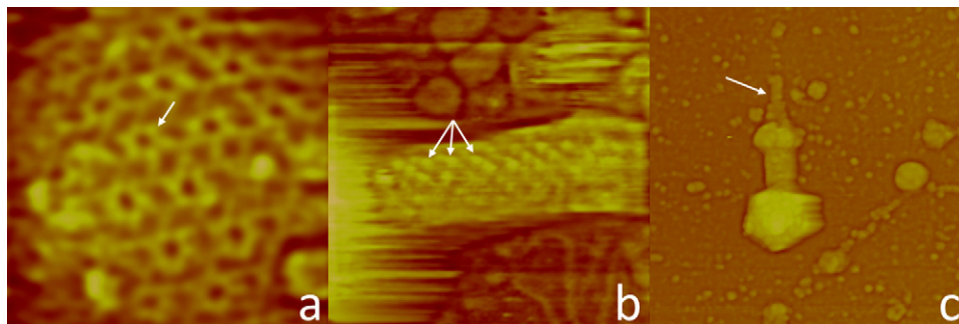
Fig. 2. In (a) hexameric capsomeres making up the *T* = 16 icosahedral head of an S-CAM4 cyanophage. Individual protein subunits comprising the capsomeres (indicated by an arrow) can in some clusters be discriminated. The center to center distances of the hexameric capsomeres is 16 nm. In (b) is a high magnification AFM image of a phage tail sheath where individual protein subunits (arrows) that comprise the contractile assembly can be seen. In (c) the arrow indicates the short, 50 nm long tail fibers directly responsible for attachment of the virus to the host cell. The scan areas are (a) 76 nm × 76 nm, (b) 150 nm × 150 nm and (c) 500 nm × 500 nm. The height ranges for (a)–(c) respectively are 100 nm, 150 nm and 250 nm.
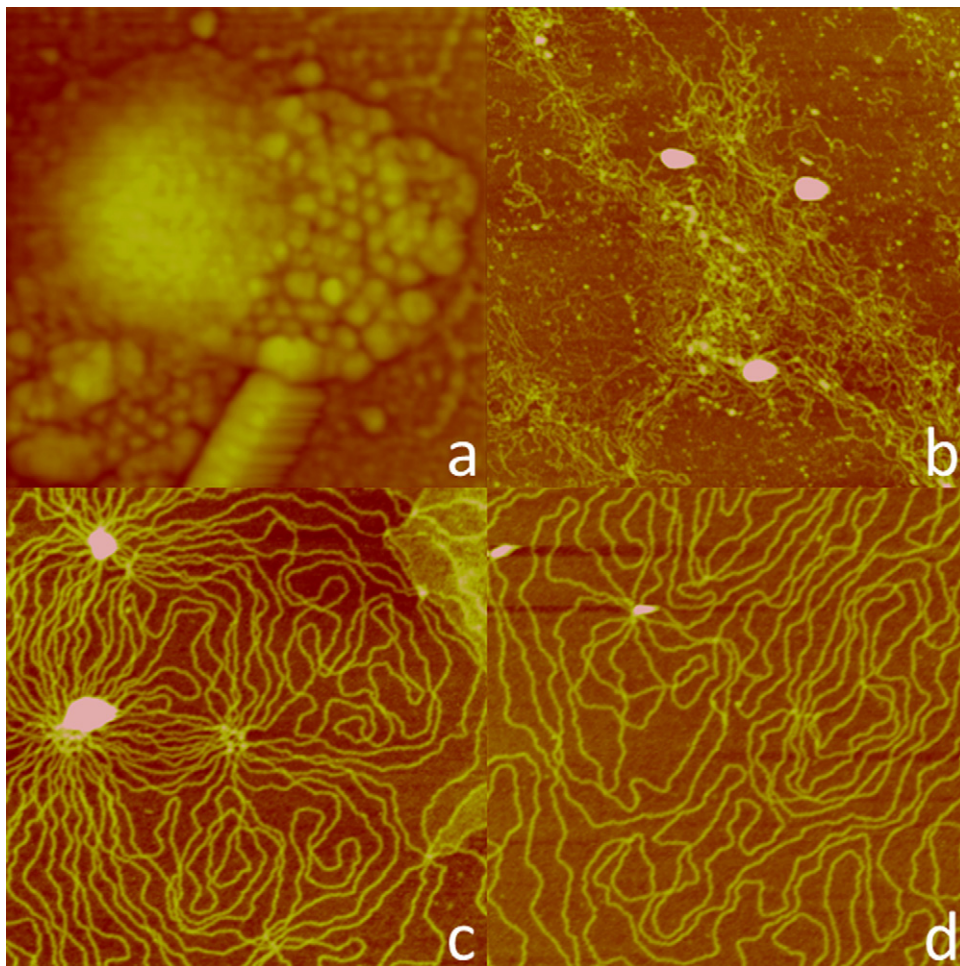
Fig. 3. In (a) a phage head burst by osmotic shock has expelled its bolus of DNA (bright, spherical mass to the left) onto the AFM substrate. In (b)–(d) are increasingly images of phage DNA spread on the substrate. The scan areas are (a) 250 nm × 250 nm, (b) 1 μm × 1 μm, (c) 1 μm × 1 μm and (d) 1 μm × 1 μm. The height ranges for (a)–(d) respectively are 50 nm, 5 nm, 4 nm and 5 nm.

podovirus. In addition, we have recorded images of a tailless, spherical virus having a unique icosahedral capsid structure, and several varieties of filamentous or cylindrical phages. Finally we describe an unusual and previously unreported tail fiber structure of some cyanophages that suggest a function probably unique to marine viruses.

2. Materials and methods

Viruses were isolated from the surf zones between Laguna Beach, CA and Long Beach, CA on *Synechococcus* sp. WH7803 by dilution-to-extinction in 48-well microtiter plates. The lysate taken from one well was further purified by two rounds of plaque selection on soft agar plates. In coastal waters of southern California, *Synechococcus* spp. commonly attain concentrations above 10^5 cells/ml (Tai and Palenik, 2009). Cyanophage titers infecting WH7803 reach over 10^3 ml^{-1} of seawater during the summer and often drop to less than 10^1 ml^{-1} in the winter and spring (Martiny, unpublished).

For AFM experiments, samples of virus or virus infected cells were spread on poly-L-lysine coated slips of cleaved mica and fixed by *in situ* exposure for fifteen minutes to 5% glutaraldehyde. The substrates were then either dried and imaged, or washed two to three times with water before analysis. Fixation with glutaraldehyde has been shown in previous studies not, to the resolution of the AFM technique, to perturb the surface structure of viral particles, and poly-L-lysine insures adherence of cells, virus particles,

and their components (Kuznetsov et al., 2001, 2002, 2003, 2004, 2005a).

For AFM imaging in air the sample was dried in a stream of nitrogen gas before imaging. AFM analysis was carried out using a Nanoscope III multimode instrument (Veeco Instruments, Santa Barbara, CA). Imaging procedures were fundamentally the same as described for previous investigations of viruses (Kuznetsov et al., 2001, 2002, 2003); and RNA (Kuznetsov et al., 2005b; Kuznetsov and McPherson, 2006). For scanning in air, silicon tips were employed. Hydrated samples were scanned at 25 °C using oxide-sharpened silicon nitride tips in a 75 μl fluid cell containing buffer. In all cases images were collected in tapping mode (Hansma and Hoh, 1994; Hansma and Pietrasanta, 1998) with an oscillation frequency of 9.2 kHz in fluid and 300 kHz in air, with a scan frequency of 1 Hz.

In the AFM images presented here, height above substrate is indicated by increasingly lighter color. Thus points very close to the substrate are dark and those well above the substrate white. Because lateral distances are distorted due to an AFM image being the convolution of the cantilever tip shape with the surface features scanned, quantitative measures of object size were based either on heights above the substrate, or on center to center distances on particle surfaces. The AFM instrument was calibrated to the small lateral distances by imaging the 1 1 1 face of a thaumatin protein crystal and using the known lattice spacing (Ko et al., 1994; Kuznetsov et al., 1999; Kuznetsov et al., 1997) as standard.
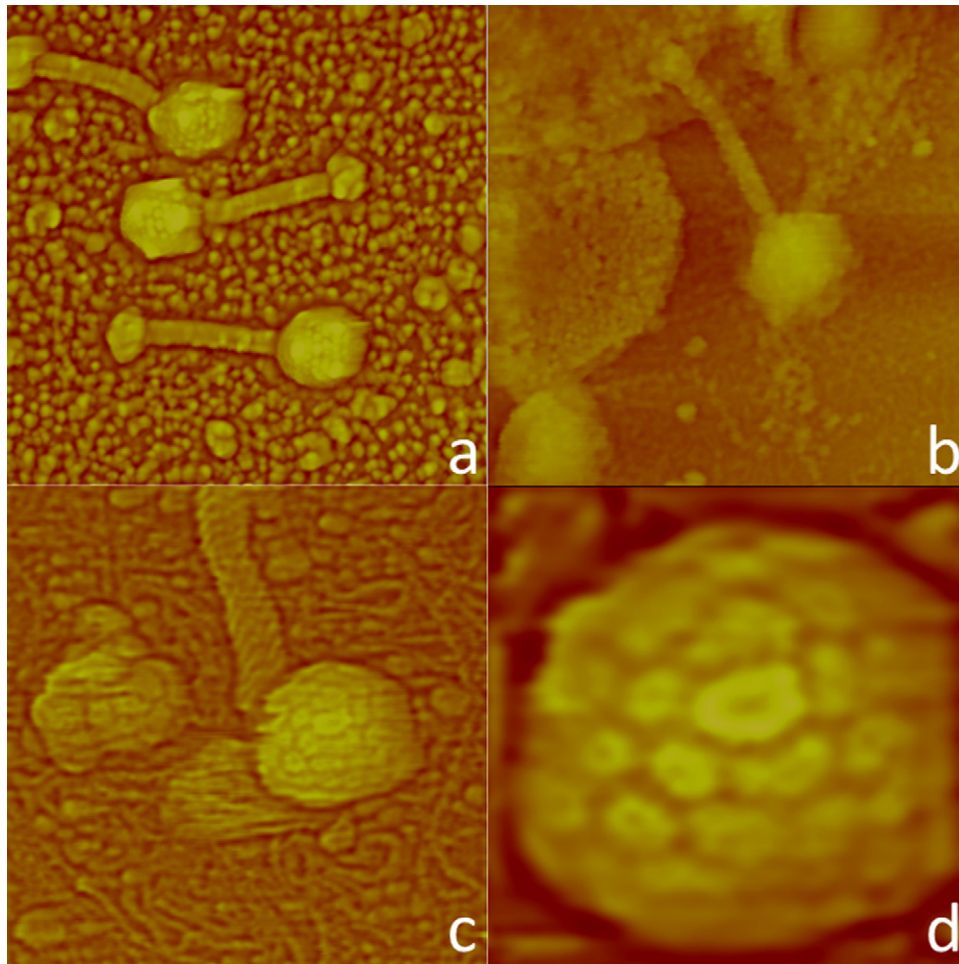
Fig. 4. In (a) are three syphophages with their extended tail apparatuses isolated on the AFM substrate. In (b) is a syphophage attached to a cyanobacteria surface. In (c) and (d) are images of the heads of the syphoviruses showing the icosahedral distribution of capsomeres. The scan areas are (a) 600 nm × 600 nm, (b) 500 nm × 500 nm, (c) 300 nm × 300 nm and (d) 100 nm × 100 nm. The height ranges for (a)–(d) respectively are 250 nm, 150 nm, 200 nm and 120 nm.

3. Results and discussion

We present here a selection of AFM images of a number of marine bacteriophages, and some details of their various substructures. This is not intended to be an exhaustive description of our investigations, but to provide examples of the kinds of viruses we encountered, and perhaps more importantly, to illustrate the kinds and level of structural information that AFM can provide. We emphasize, particularly, architectural features that are ordered, form patterns, or are symmetrical, and those that have recognizable homologous structures on more well characterized, terrestrial particles. We also include some examples of truly unique structural features, possibly specific to marine viruses alone that have not, so far as we know, been previously observed.

The only marine bacteriophage that has been thoroughly characterized by AFM is S-CAM4 (Kuznetsov et al., 2010) a myovirus similar in most respects to the terrestrial T4 bacteriophage that infects *E. coli*. Some AFM images of S-CAM4 are shown in Fig. 1. In Fig. 1a, masses of the phage, which exhibit prominent heads, tail assemblies, baseplates, are seen attacking fragments of lysed host cells. In Fig. 1b is an isolated S-CAM4 attached through its baseplate and tail fibers to a small cellular fragment. The tail sheath is fully contracted, but the head remains full, the DNA undelivered. We know this because empty phage heads collapse upon drying, giving a very different appearance. This phage head, imaged in air, retains its icosahedral form.

In Fig. 1c and d clusters of phage are seen attached to cell surfaces. Even at relatively modest magnification, capsomeres exhibiting an icosahedral distribution can be clearly seen on the phage heads. In this regard, S-CAM4 and most of the other marine bacteriophages we have visualized differ from T4. As shown elsewhere (Kuznetsov et al., 2011), the heads of native T4 exhibit no ordered icosahedral network from the exterior because the heads are coated with two small, flexible proteins known as hoc and soc (Ishii and Yanagida, 1977; Yanagida, 1977) that obscure the underlying capsomeres and their symmetrical arrangement.

In Fig. 2 are some examples of the level of detail that can be observed, in the best of cases, on bacteriophage. In Fig. 2a hexagonal capsomeres arranged in an icosahedral net are clearly evident on the heads of S-CAM4, and it is even possible to deduce the triangulation number, which is 16. The image allows good estimation of center to center distances, and it reveals the toroid shapes of the hexameric protein clusters. In Fig. 2a, on some capsomeres, even the individual capsid protein subunits can be resolved. Fig. 2b is an image of a phage tail assembly. The helical nature of the tail is evident, but close inspection even allows discrimination of the individual protein units that form the sheath. In Fig. 2c we see the short, 50 nm tail fibers, indicated by an arrow, that are responsible for attachment of the virus to a host cell surface.

On the phage particle in Fig. 2c there are no long tail fibers as are found on T4. Indeed, myoviruses from *Synechococcus* cultures were observed that possessed long tail fibers, some that lacked such
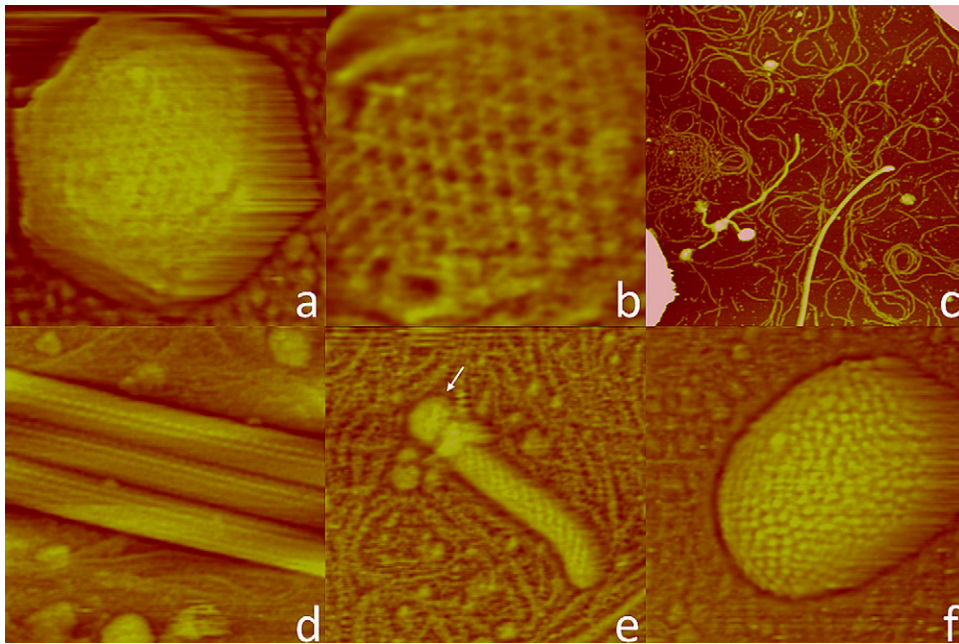
Fig. 5. In (a) is an AFM image of a spherical virus of about 125 nm diameter, and in (b) a higher magnification image showing the mesh-like appearance of the icosahedral distribution of capsomeres on the shell. In (c) are filamentous virions of indeterminate length and only a few nanometers diameter. In (d) are rod shaped viruses of helical architecture and diameters of about 20 nm. In (e) is an unusual viral form comprising a complex helical body of about 30 nm width and 200 nm length that is capped by some modification. In (f) is another enigmatic viral form, ovoid in shape with major axes of about 200 nm × 150 nm × 75 nm. The scan areas are (a) 150 nm × 150 nm, (b) 80 nm × 80 nm, (c) 2 μm × 2 μm, (d) 150 nm × 150 nm, (e) 250 nm × 250 nm and (f) 250 nm × 250 nm. The height ranges for (a)–(f) respectively are 250 nm, 150 nm, 20 nm, 30 nm, 60 nm and 250 nm.

fibers, and others on which a baseplate was absent or found only in some abbreviated form (data not shown). Presumably, each of these modifications is representative of a distinct strain.

In addition to the exterior structural details, interior viral components can also be visualized if the phage particles are disrupted or systematically degraded. In Fig. 3a, for example, a phage head has been shattered by osmotic shock as water was flowed over the AFM substrate. The bright, spherical, diffuse mass to the left of the broken head, which can be seen shedding capsomeres, is the bolus of nucleic acid that was contained within. It is now free to slowly unravel and spread on the AFM substrate. The ejected, dispersed DNA is seen in Fig. 3b through d. Note that the DNA from bacteriophages is uncomplexed with proteins and exists as naked strands.

The tailed bacteriophages that were investigated by AFM were not only myoviruses, but included syphoviruses as well. Like the myoviruses, they varied in the presence and forms of the long tail fibers and the base plates. At least one podovirus was also seen. In Fig. 4 are images of an, as yet, unnamed marine syphovirus with its characteristic extended tail assembly. In Fig. 4a the phage particles are isolated on the AFM substrate while that in Fig. 4b is attached through its baseplate to a cell surface. In Fig. 4d, viewed more or less along a fivefold direction, a pentameric vertex can be seen surrounded by five hexameric capsomeres. Like S-CAM4, mapping of the distribution of the capsomeres on the surface of the head reveals an icosahedral net with triangulation number 16.

Fig. 5 presents a variety of cyanobacterial viruses that are not tailed phages, but have unusual, but by no means unique, architectures. In Fig. 5a is a spherical virus of about 125 nm diameter, considerably larger than the head of a tailed phage. Higher magnification images, as in Fig. 5b, allow the icosahedral network to emerge. The mesh like appearance of the capsid, and the number and distribution of the capsomeres is greater and more complicated than is seen on the heads of tailed phages. Nonetheless, it was possible to assign a triangulation number of 19, though other

possibilities could not be eliminated definitively. The lattice is clearly a skew lattice in contrast to those seen for the tailed phages.

In Fig. 5c and d are examples of filamentous and rod shaped virions respectively, the latter clearly helical in structure. The rod shaped virions have a diameter of about 20 nm, which is close to that of the classical, terrestrial tobacco mosaic virus. Thus it seems likely to have a similar structure, *i.e.* a single RNA molecule coated by small proteins arranged according to a helical architecture. It was not possible to clearly resolve by AFM the individual protein units making up these virus coats, likely due to the small size and close packing of the subunits. In Fig. 5e is a virus of eccentric shape and structure. It has a thick body of about 200 nm length and 35 nm diameter that is coated by a helical arrangement of protein units, but is at one end capped by some unusual structure that is different in design from the rest of the particle. In Fig. 5f is a puzzling ovoid form that appeared only infrequently and about which we can say little.

As we noted earlier, the diversity of marine viruses offers the opportunity to expand our knowledge of virus structures and substructures. Different environments call for new functions, which in turn demand unique structures. Fig. 6 contains AFM images of one of these structures that has not been seen on terrestrial bacteriophages. We explored the structure and possible function of these features in more detail elsewhere (submitted), but it is noteworthy and appropriate here as well.

The myoviruses, which have icosahedral heads of triangulation number 7, seen in Fig. 6 all have up to four, though generally no more than two long (450 nm), multistranded, helical fibers that terminate in unusual arrangements of roughly spherical particles of about 35 nm diameter. The tassels exhibit a distinctive 3 + 1 pattern of 3 equally spaced terminal spheres plus another at a fixed distance of 135 nm upstream. The fibers are attached to the phages either at the baseplate, as the phage in Fig. 6b, or extend from the collar of the phage as in Fig. 6c.

As seen in Fig. 6c and d, the globular units making up the tassels sometimes spontaneously disintegrate, revealing them to be
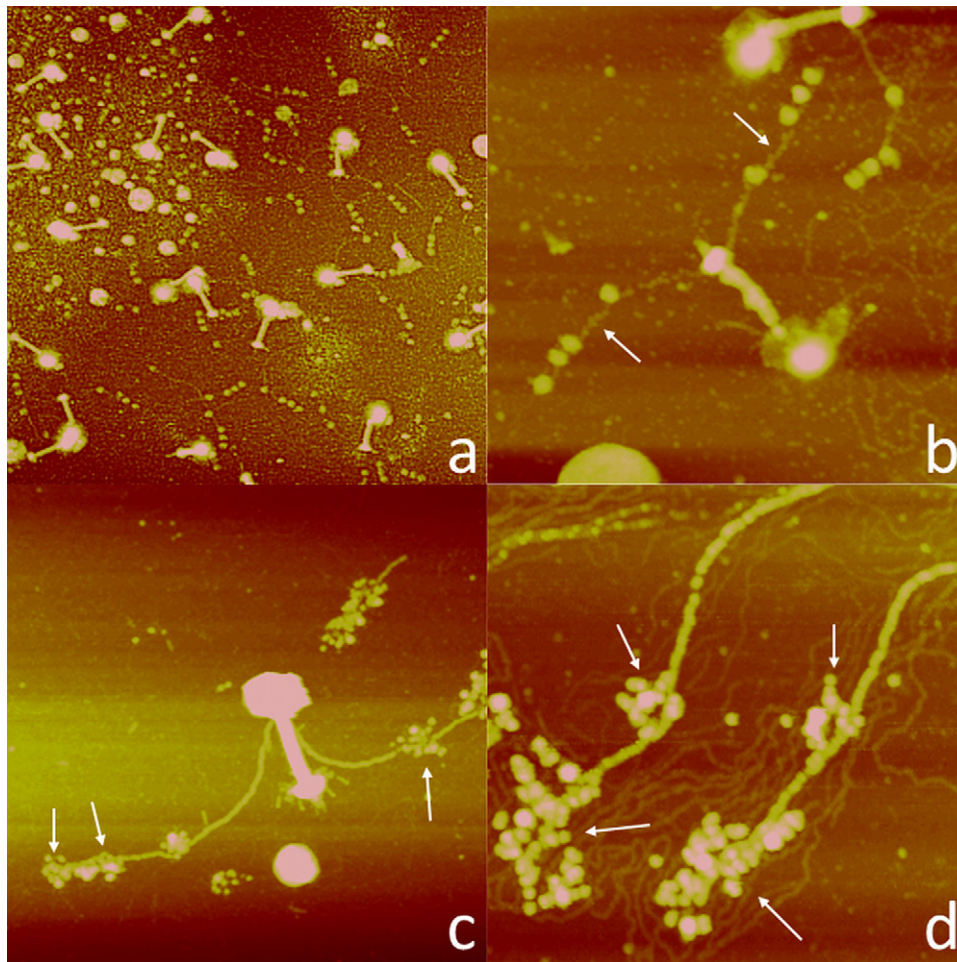
Fig. 6. In (a) the field shows many phage particles, all attached to extended fibers of length 450 nm terminated by tassels comprised of four roughly spherical masses. The tassels all exhibit a characteristic 3 + 1 pattern. The fibers are attached to the body of the phage at either the baseplates or the collars, and in some cases both on the same particle. In (b) is a single phage with two fibers attached through its baseplate. In (c) the spherical bulbs of the fibers are beginning to disintegrate and in (d) can be seen to be composed of large clusters of individual proteins. The scan areas are (a) 3 μm × 3 μm, (b) 1 μm × 1 μm, (c) 1 μm × 1 μm and (d) 500 nm × 500 nm. The height ranges for (a)–(d) respectively are 100 nm, 125 nm, 50 nm and 30 nm.

composed of about twenty individual proteins having more or less the same size. These have no homologs among terrestrial bacteriophages, which suggests that they may arise as a consequence of ecological factors unique to marine environments. Indeed, we have speculated that they are necessary or advantageous because cyanobacterial hosts do not form colonies, but are dispersed in a marine environment, in contrast to the dense masses of terrestrial host cells found, for example, in the gut. The long fibers may, therefore, be necessary to sweep a larger volume of space in search of a susceptible host cell.

4. Conclusions

In the images presented above, we have attempted to explore, admittedly in a very limited way, the diversity of a single kind of marine virus, those that infect Synechococcus. Even among this restricted set, the range of structural details and virus architectures is unique, and impressive. Two conclusions, we believe, are justified by the investigation presented here. The first is that AFM is, and will remain an important technology for analyzing and describing virus structure, including new structures from marine environments. The second is that marine organisms and marine viruses in particular will meet our expectations for accelerating discovery of new and fascinating structures at the nanometer level.

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