First evidence of virus-like particles in the bacterial symbionts of Bryozoa

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Bacteriophage communities associated with humans and vertebrate animals have been extensively studied, but the data on phages living in invertebrates remain scarce. In fact, they have never been reported for most animal phyla. Our ultrastructural study showed for the first time a variety of virus-like particles (VLPs) and supposed virus-related structures inside symbiotic bacteria in two marine species from the phylum Bryozoa, the cheilostomes Bugula neritina and Paralicornia sinuosa. We also documented the effect of VLPs on bacterial hosts: we explain different bacterial ‘ultrastructural types’ detected in bryozoan tissues as stages in the gradual destruction of prokaryotic cells caused by viral multiplication during the lytic cycle. We speculate that viruses destroying bacteria regulate symbiont numbers in the bryozoan hosts, a phenomenon known in some insects. We develop two hypotheses explaining exo- and endogenous circulation of the viruses during the life-cycle of B. neritina. Finally, we compare unusual ‘sea-urchin’-like structures found in the collapsed bacteria in P. sinuosa with so-called metamorphosis associated contractile structures (MACs) formed in the cells of the marine bacterium Pseudoalteromonas luteoviolacea which are known to trigger larval metamorphosis in a polychaete worm.

Viruses are found in all kingdoms of living organisms and are best studied in those that have an applied or medical value (1–4, https://talk.ictvonline.org/taxonomy/). In addition to harboring the viruses replicating in eukaryotic cells, all known animals (as well as other multicellular organisms) are associated with specific microbial communities that include viruses infecting their symbiotic microorganisms. Most of these viruses are bacteriophages. Although the bacteriophage communities (viromes) of vertebrates are much better studied (reviewed in refs 5–7), some data are also available for invertebrates. For example, the viral communities associated with the cnidian Hydra8,9 and certain scleractinian corals10,11 have been recently characterized using metagenomic methods. These studies showed that such communities are species-specific and may be significant for homeostasis of the animal hosts.

In addition to the complex microbiomes associated with invertebrate digestive systems or body surfaces, some species harbor specific bacterial symbionts that may be vertically transmitted. Although such symbionts are either intracellular or live in the host tissues (sometimes in special organs), they can also harbor bacteriophages. The best-known example is Wolbachia bacteriophage WO. The symbiotic bacteria Wolbachia exert a major influence on their arthropod hosts by manipulating their reproduction and providing increased resistance to infections12. In turn, the bacteriophage WO influences the bacterial titer in the host tissues13. This phage also encodes proteins important for bacterium interactions with the host14.

In the marine realm, the large variety of viruses are found across diverse taxa, including protists and various invertebrates such as sponges, cnidarians, flatworms, polychaetes, mollusks, crustaceans and echinoderms (reviewed in refs 15–19; see also20–26 and references therein). Being present in large numbers in the seawater27,28, viruses, among others, enter suspension-feeders (e.g.29). Indeed, by filtering enormous volumes of water, sea...
sponges acquire viruses that infect their cells\(^{30-32}\) as well as their bacterial symbionts\(^{33}\). Corals, together with their eukaryotic and prokaryotic symbionts, harbor a variety of viruses too (e.g.\(^{34-40}\)). Similarly, filter-feeders such as bivalve mollusks and tunicates and bacteria living in them also acquire viruses (reviewed in\(^{41-43}\)). Recently a bacteriophage was found to enhance the biofilm formation in the gut of an ascidian by interacting with its bacterial hosts\(^{44}\).

The phylum Bryozoa is comprised of active filterers that feed mainly on microscopic algae, gathering them out of seawater\(^{46-50}\). Together with sponges and cnidarians this group of colonial invertebrates is among the dominating foulers in manybottom communities from the intertidal zone to a depth of 8 km\(^{51-54}\). Although viruses were never reported from bryozoans before, symbiotic associations with bacteria are known in species from several families of the order Cheilostomata, the largest bryozoan group (e.g.\(^{55-61}\), reviewed in\(^{62}\)). The symbionts are reportedly vertically transmitted via the larval stage (\(^{63-69}\) and references therein). During our ongoing research on bryozoan prokaryotic symbionts, we discovered presumed virus-like particles and virus-related structures associated with bacteria in two bryozoan species from two different families and distant localities. This paper presents the first description of the VLP (supposedly bacteriophages) in Bryozoa and their effect on their bacterial hosts. We also discuss the possible ways of the virus transmission and circulation and their role in these symbiotic systems.

Materials and methods

Animal material collection, fixation and microscopy. Colonies of the cheilostome bryozoan Bugula neritina (Linnaeus, 1758) (Bugulidae) (Fig. 1A) were collected intertidally on Atlantic Beach, Jaycee Park, Morehead City, North Carolina, USA, in spring 2011. Paralicornia sinuosa (Canu & Bassler, 1927) (Candidae) (Fig. 1B) was collected by SCUBA-diving in the vicinity of the Lizard Island Research Station, Great Barrier Reef, Coral Sea, Australia, on 4, 5 and 10 October 2012 between 6 and 12 m depth.

Both species were collected and fixed aiming ultrastructural studies focused on the host-symbiont spatiotemporal interactions. No molecular work was planned in a frame of this project that is why no ethanol-fixations were made. For microscopical research colony fragments were fixed during 3–4 h after collecting in 2.5% glutaraldehyde (buffered in 0.1 M Na-cacodylate containing 10% sucrose (pH 7.4)). They were postfixed with a 1% solution of osmium tetroxide (OsO\(_4\)). Decalcification was conducted for 24 h in 2% aqueous solution of EDTA. After this step the fragments were dehydrated in a graded ethanol series (30–50–70–80–90–100%) and embedded in epoxy resin type TAAB 812. Postfixation, decalcification and embedding of bryozoan fragments to the resin were made in a less than one month period after fixation.

Embedded specimens were sectioned (70 nm thick) using a Leica EM UC7 microtome (Leica Microsystems, Wetzlar, Germany). To find the area for transmission electron microscopy (TEM), the histological sections (1.0 μm thick) were prepared for light microscopy and stained with Richardson's stain using standard methods\(^{75}\). We sectioned seven branches of B. neritina and four branches of P. sinuosa. Ultrathin sections were picked up with single slot copper grids with formvar support film and contrasted with uranyl acetate and lead citrate. Semithin sections were analyzed using an AxioImager.A1, Zeiss microscope (Zeiss, Oberkochen, Germany). Ultrathin sections were examined with a Jeol JEM-1400 microscope (JEOL Ltd., Japan).

Bacterial and bacteriophage strains and their cultivation. To compare the objects found in the bacterial symbionts of Bugula neritina and reminiscent the VLPs, we prepared a suspension of Escherichia coli cells infected by bacteriophage RB49 (a T4-related virus) and visualized them using the same fixative and following the same procedure as used for bryozoan samples.

E. coli C600 strain and bacteriophage RB49 were from the collection of the Laboratory of Microbial Viruses, Winogradsky Institute of Microbiology. For the infection experiment the overnight culture of E. coli F5 (10.1007/s00705-019-04371-1) was grown in LB medium at 37 °C with agitation. The culture was diluted 100-fold with the same medium and cultured under the same conditions up to OD\(_{600}\) 0.3. This optical density corresponds to 2 × 10\(^7\) c.f.u. ml\(^{-1}\). Phage RB49 lysate was added to 3 ml of the bacterial culture up to the multiplicity of infection of 5. The culture was further incubated 15 min and then the cells from 1 ml of the infected culture were spun down in the table-top centrifuge at 10 000 g for 40 s. The supernatant was removed and 200 μl of the same fixative solution that was used for the animal material was added, and the samples were then processed for thin sectioning and TEM study following the same protocol.

Results

Colonies of both studied species, Bugula neritina and Paralicornia sinuosa, are lightly calcified, erect and branched (Fig. 1A,B & inserts). Histological sections and TEM study of colony fragments showed the presence of symbiotic bacteria in the funicular system in both species (Fig. 1C–F). In B. neritina the densely packed bacterial cells were present in so-called ‘funicular bodies’, which are swollen areas of the transport funicular cords crossing the coelomic cavity of autozooids (Figs. 1C,D, 2 (box 1)), and in P. sinuosa they were present inside the funicular cords themselves (Fig. 1E,F). Also, in B. neritina, bacteria were additionally recorded in and between the epidermal cells of the tentacles (Fig. 2 (box 2)), between and inside the cells of the body wall (epithelium of introvert and peritoneal cells of ooeial vesicle) (Fig. 2 (boxes 3 & 4)) and in the presumed coelomocytes situated on the epithelial lining of the ooeial vesicle (Fig. 2 (box 4)) plugging an entrance to the brood chamber (ovicell). In both bryozoan species we detected intact (non-modified) as well as morphologically altered bacterial cells. Although the virions of bacteriophages are dimensionally and morphologically stable
and uniform, both fixation and TEM observations potentially could generate a 'false' visual diversity since the capsids are sectioned at different levels and viewed at different angles, and the tails (if present) may be hidden.

Comparison of *E. coli* cells infected by the phage RB49 showed that the bacteriophage heads and proheads exhibited a significant degree of apparent morphological variation (Fig. 3) similar to VLPs observed in bryozoans (Figs. 4, 5E,F, 6, 7C,D). The tails of RB49 particles could not be reliably seen inside the infected bacterial cells.
VLPs in bacterial symbionts of *Bugula neritina*. In *B. neritina* the virus-like particles were found in the symbiotic bacteria associated with the tentacle epidermis (Figs. 4, 5) and epithelium of the tentacle sheath (introvert), and those located inside the funicular bodies (Figs. 6, 7C,D, 8). In these three loci, bacteria were morphologically different (those in tentacles vs in introvert and funicular bodies), supposedly belonging to two different species. Else, in the tentacles and funicular bodies bacterial cells fall into three ‘ultrastructural types,’ presumably representing the successive stages of bacterial transformation/destruction during the viral lytic cycle. Solely ‘ultrastructural type II’ was recorded in the wall of the introvert. The VLPs became visible in the bacteria of ‘types’ II and III.

**VLPs and bacteria in tentacles.** The VLPs inside bacteria in the tentacle epidermal (ciliated) cells were predominantly oval and isometric, with diameters varying between 50 and 65 nm. Being either electron-dense or translucent, their content probably reflects different virion assembly intermediates (Fig. 4A,B). Some VLPs with relatively translucent content were detected on the surface of bacterial cells or between them and bryozoan epidermal cells (Fig. 4C, D). They were either oval or polygonal with clearly recognizable thick ‘cover/peripheral layer (Fig. 4D).

Bacteria of ‘type I’ represent non-altered cells with an ultrastructure typical for Gram-negative bacteria. These were cocoid or slightly elongated cells (Fig. 5A–C) with a diameter/length of about 0.5–0.7 μm. A well-defined electron translucent nucleoid zone is surrounded by a thin peripheral layer of electron-dense cytoplasm enveloped by two membranes. Some of the cells were obviously dividing. Bacteria were recorded either directly
in the cytoplasm or inside large vacuoles of the tentacle epidermal cells, individually or in groups. Some of them presumably occupied intercellular spaces. VLPs were not seen inside or between the ‘type I’ bacterial cells.

‘Type II’ bacterial cells (presumably next stage of their transformation) were rod-like and much larger than the coccoid form, reaching lengths of 4–5 μm and diameters of about 1.5 μm (Fig. 5C–E). The cytoplasm was granular

Figure 3. Thin sections of *E. coli* F5 cells infected by the bacteriophage RB49 (15 min post infection). Bacteriophage heads and proheads (both empty and partially filled) are visible (arrows on A). Note that the capsids do not appear exactly uniform and the tails are not visible.

Figure 4. Virus-like particles associated with symbiotic bacteria in the tentacles of *Bugula neritina*. (A,B) Electron-dense and electron-translucent VLPs inside the cytoplasm of the bacterial cell of ‘type III’. (C) VLP inside (left) and on the surface of the bacterial host. (D) Virions outside bacteria between epithelial cells of a bryozoan. TEM.
and electron-dense most of the volume, with the translucent nucleoid zone strongly reduced and fragmented. The cell wall was wavy. Some of these bacteria showed the presence of scant VLPs (Fig. 5E). The membranes of the vacuoles containing the ‘type II’ bacteria were often not clearly recognizable, possibly destroyed (Fig. 5).

Figure 5. Symbiotic bacteria inside tentacles of Bugula neritina. TEM. (A,B) coccoid symbiotic bacteria of ‘type I’ inside the cytoplasm (A, arrows) and a large vacuole (B) in the ciliated cells of the tentacles (sectioned kinetosomes and cilia are visible in the lower half of the image (A). (C) groups of smaller coccoid bacteria of ‘type I’ (arrows) and the larger rod-like bacteria of ‘type II’. (D,E), ‘type II’ bacterial cells without (D) and with (E) few electron-dense virus-like particles (little arrows). (F) symbiotic bacteria of ‘type III’ inside with viral particles in their cytoplasm. TEM. Abbreviations: bl, basal lamina; ci, cilia; m, mitochondria; n, nucleus.
‘Type III’ bacteria were rod-like or ovoid. Their length was the same as in ‘type II’ cells, but the diameter increased up to 2–2.5 microns. There were no traces of the nucleoid zone in ‘type III’ cells, and the cytoplasm was filled with abundant VLPs (Fig. 5F).

The ‘type III’ bacterial cells were usually detected closer to the apical parts of the tentacles, and their numbers were significantly lower than the number of intact coccoid bacteria (‘type I’) distributed through the entire tentacle length.

**VLPs and bacteria inside autozooid.** The VLPs associated with the bacteria found in the epithelium of the introvert and filling the funicular bodies (swollen parts of the funicular system, Figs. 1C,D, 2) of *B. neritina* were oval-polygonal, isometric, with a capsid diameter of 50–70 nm and thick peripheral layer (Fig. 6C). Most virions were detected in the spaces between bacterial cells (in both introvert and funicular bodies) (Figs. 6B,C, 7C,D), but individual VLP were also visible in the cytoplasm of bacteria (in the funicular bodies) (Fig. 6A).

As was the case in the tentacles, the three bacterial ‘ultrastructural types’ (but of presumably different species, see above) were also detected inside autozooids. Intact Gram-negative bacterial symbionts (‘type I’) were found in the presumed coelomocytes and in the peritoneal cells of the ooecial vesicle wall (Fig. 7A,B). They were rod-like (length 2.0–2.5 μm and diameter 0.4 μm), had two outer membranes, a well-defined nucleoid zone and a thin layer of cytoplasm. They were present in groups in the cytoplasm as well as in the vacuoles. No VLPs were recognizable in them.

The ‘type II’ bacterial cells found in the epithelium of the introvert, and in the funicular bodies had a wide oval, often irregular shape (length up to 4.0–4.5 μm, width up to 1.5–1.7 μm) (Figs. 6A, 7C, 8A,B). Their cytoplasm was electron-dense and their nucleoid was fragmented (Fig. 7C). In some of these bacterial cells, ‘tube’-like structures 40–50 nm thick were detected in the cytoplasm, being separated or assembled into groups (Fig. 8A,B). The walls of these ‘tubes’ were morphologically and dimensionally similar to the membranes of the bacterial cell wall. In some cases these ‘tubes’ and cell membranes were connected.

The shape and size of the ‘type III’ bacterial cells found in the funicular bodies (Figs. 7D, 8C–F) were about the same as in ‘type II’. The cytoplasm was relatively translucent and mostly homogeneously flocculent. Some of the ‘type III’ bacteria showed two well-recognizable, albeit deformed, cell membranes (Figs. 6B,C, 7D). Some of them, similar to the ‘type II’ bacteria (Fig. 8A,B), contained ‘tube’-like structures. In addition, only one cell membrane was often recognizable in the ‘type III’ bacteria that presumably were on the late stage of degradation (Fig. 8C–F). They contained large, twisted or curled electron-dense bodies with a spiral arrangement of parallel membranes. The bodies were up to several micrometers long, while their thicknesses varied, sometimes reaching 135 nm. The shape of these bodies strongly varied—we detected rings, U-shaped figures, complex multiple
loops and other configurations. When the bacterial cells were destroyed, these bodies were released in the space between the bacteria.

Funicular bodies contained bacteria of either ‘type II’ or ‘type III’ separately, but the funicular bodies with different bacterial ‘types’ were detected in the same zooids.

**Virus-related structures in bacterial symbionts of Paralicornia sinuosa.** In *P. sinuosa* the cytoplasm of morphologically altered bacterial cells found in the funicular cords (Fig. 1E,F) contained spherical particles consisting of the cylindrical/tube-like elements evenly radiating from the central double-walled ‘core’ (Fig. 9). The diameter of these ‘sea urchin’-shaped structures was about 300 nm, while the individual cylinders/tubes were about 120 nm long and about 20 nm thick. In cross-section the core with the bi-layered darker periphery (core wall?) and lighter central part were recognizable at high magnification (Fig. 9B). The cylinders/tubes were apparently interconnected by the electron-dense layer slightly below their distal tips. The proximal ends of the tubes also appear connected, forming the above-mentioned ‘double-walled’ core of the particle that has a diameter of 50–60 nm. In the medial sections of the particles about 20–21 cylinders/tubes are visible (Fig. 9B and insert), leading to an estimated 400–450 of them in one particle. The particle diameter seems to be stable/identical, suggesting that the size and/or the number of the cylinders/tube-like elements is tightly controlled during particle development.

Similar to *Bugula neritina*, closer examination of the morphology of bacterial cells also revealed three different ‘ultrastructural types’, which we again interpret as corresponding to the stages of the virus-like particle development before bacterial lysis. ‘Type I’—presumably intact or slightly altered Gram-negative bacteria. Such cells were rod-like, 3–4 μm long and about 1 μm wide (Fig. 9C, insert) The central part of the cell is occupied by filaments of nucleoid surrounded by an electron-translucent zone that often included electron-dense areas of various size and shape. The peripheral cytoplasm is electron-dense, granular, without inclusions, surrounded by a plasma membrane that was poorly recognizable in some cases.
Bacterial cells of ‘type II’ presented moderate alterations in the cell ultrastructure presumably corresponding to the initial phase of VLP development. Cell length and width were increased, sometimes doubled (up to 8 and 2 μm, respectively), and the cell wall often took on a wavy appearance. The nucleoid fibrils disappeared, and most of the cytoplasm became homogeneously electron-dense. The viral particles and paracrystalline structures became visible inside the bacteria (Fig. 9D). In ‘type III’ cells the VLPs filled most of the cell volume (Fig. 9E). The paracrystalline bodies became more numerous, acquiring very different shapes, from irregular polygons to long rods with a length of up to 8–10 μm (Figs. 1E,F, 9E,F). At high magnification the densely packed parallel fibrils were visible in them (Fig. 9F, insert).

After destruction of bacteria, both the viral particles and paracrystals were freely distributed inside the cavity of the funicular cords. In some occasions we observed them in the zooidal coelom too, obviously following disintegration of the funicular cord wall.

**Discussion**

Detection of new viruses is often a serendipitous event during ultrastructural studies (e.g.20,23,30). In our case we found VLPs when studied of symbiotic associations of bacteria with an invertebrate. We explain the different bacterial ‘ultrastructural types’ detected in the tissues of two bryozoan species as stages in the gradual destruction of bacterial cells caused by the virus development.

Figure 8. ‘Tube’-like structures in the bacterial cells found in the funicular bodies of Bugula neritina. (A,B), bacteria of ‘type II’ with tube-like structures in the beginning of their formation. (C–F), fully-formed ‘tube’-like structures in the bacteria of ‘type III’ and in destroyed bacteria (in F).
Figure 9. (A,B) Structures presumably derived from viruses in bacterial symbionts of *Paralicornia sinuosa*. Insert: cross-section of radial cylinders showing darker periphery and lighter central part (also shown by arrow in B) with a dark central ‘spot’. (C–F, insert), various stages in the lytic cycle of bacteriophages in the symbiotic bacteria of *P. sinuosa*. (C) and insert, longitudinal and cross-sections of non-/less altered bacteria (‘type I’ cells) inside funicular cord. (D) VLP (in circles) becoming visible inside bacteria predominantly filled with electron-dense cytoplasm (‘type II’ cells), nucleoid is fragmented and poorly recognizable; insert shows paracrystalline body at higher magnification. (E) bacteria (‘type III’ cells) filled with viruses and paracrystalline structures. (F) bacteriophages and paracrystals inside the cavity of the funicular cord after bacterial destruction. TEM. Abbreviations: f, cells of funicular cord.
The two variants of VLPs found in the symbiotic bacteria of *Bugula neritina* (compare Figs. 4 and 6) were similar to the capsids of tailed bacteriophages. No tails were observed, however. We suggest that the tail appendages are probably present but indiscernible in the TEM-images, as, for example, in the case of podoviruses with their short tails. Nonetheless, we cannot exclude that the VLPs belong to non-tailed phages that possess the virions of similar size such as, e.g., in the family *Tectiviridae*.

Our observations indicate that the phages of symbiotic bacteria of *B. neritina* act as pathogenic agents, destroying bacteria in the course of their multiplication in the lytic cycle.

The situation observed in bacteria from the funicular system of *B. neritina* is special. We suggest that the ‘tubes’ visible in the cells of the second ‘morphotype’ are invaginations of membranes of the bacterial cell wall, and can later cluster as the bacteria are lysed, forming complex structures in the cytoplasm. Subsequently, elongation and spiral twisting of these ‘tubes’ could result in the formation of flexible ‘rods’. Further curling of these rods yields structures of various shapes. A similar effect is observed in the action of lactocins and of the viruses on Gram-positive bacteria of the genus *Lactobacillus*.

Bacteria discovered in the tentacles and in the introvert wall and the funicular system of *B. neritina* have a different morphology (coccoid vs rod-like), suggesting different (and independent) ways of their acquisition. The former potentially could penetrate into tentacle tissues directly from the surrounding water. This kind of interaction between bryozoan hosts, bacteria and phages might be common, but more extensive ultrastructural research on bryozoan tentacles is required to show whether this mechanism of infection is widespread. A supporting argument in this case is the discovery of similar symbionts and phage particles in the tentacles of *B. neritina* collected in the Mediterranean Sea on the coast of Spain (Vishnyakov, Schwaha, Souto, Ostrovsky, unpublished data).

The symbiotic bacteria occupying funicular bodies in the *B. neritina* zooidal cavity have been identified as *Candidatus Endobugula sertula* responsible for production of bryostatins—cyclic polyketides presumably participating in the regulation of bryozoan oogenesis and protection of free larvae against predation, and also having antitumor activity being considered as potential anti-cancer drug. These bacteria are vertically transmitted through the larva and are thus inherited by the first zooid (ancestrula) during metamorphosis. Zooloidal budding then results in colony formation and the spread of the symbionts through it. Occupying the transport funicular system and utilizing the nutrients from it, the symbionts multiply, triggering the formation of the funicular bodies that was recently shown in another chelostome bryozoan. Bacteria partly stay in these bodies and partly move to the ooecial vesicle of the brood chamber by an unknown mechanism. Woolacott and Zimmer showed the presence of bacteria inside the lacunae of the funicular cords of *B. neritina*, which could be the pathway for the symbionts traveling through zooids. Our data indicate an opportunity for bacterial transport to the ooecial vesicle by coelomocytes, but more research is needed to evaluate this hypothesis. The bacterial transmission to the wall of the tentacle sheath is enigmatic. These bacteria are very similar/identical to those found in the funicular bodies and possess the VLPs of the same morphology. Their position between/inside epithelial cells, however, is similar to bacteria found in the tentacles.

The available data are insufficient to conclude whether the VLP production in symbiotic bacteria results from infection by externally acquired bacteriophage particles or is due to induction of the prophages or prophage-like elements present in the genomes of these bacteria. The infection hypothesis seems more plausible for bacteria found in *B. neritina* tentacles, but the scenario potentially could be different in case of the funicular body symbionts. We suggest that in this species the phages circulate in the bryozoan host population together with bacteria transported to the ooecial vesicle by coelomocytes, but more research is needed to evaluate this hypothesis. The infection hypothesis seems more plausible for bacteria found in the tentacles, however, is similar to bacteria found in the tentacles.

The next step is a transfer of infected bacteria from the ooecial vesicle through its wall to the brood cavity. Moosbrugger with co-authors showed the presence of bacteria inside an ovicell containing an embryo in another bugulid species (see also). Accordingly, if our model is correct, prophages are transferred to the brood chamber with their bacterial hosts finally reaching the larvae. This enables them to be transmitted to the next bryozoan generation. Potentially, the phages and bacteria found in the tentacles might circulate in the host in a similar way, but it remains unclear how they could reach larvae in the brood chamber. The only imaginable strategy is their transfer during oviposition when the tentacle crown delivers the zygote to the brood cavity (its mechanism reviewed in). This way, however, appears less probable.

The induction hypothesis appears to be the only possibility to explain the formation of the structures observed in *Paralicornia sinuosa*. These structures do not resemble any known bacteriophages and appear to be too large to be interpreted as the virions of a novel bacteriophage family. At the same time the formation of these structures is clearly associated with cell destruction by lysis. We should add here that after submission of this paper very similar structures were encountered in another chelostome bryozoan *Paracribellina cribrraria* (from the distant family Catenicellidae) collected near New Zealand in 2009.

We speculate that the particles observed in *P. sinuosa* symbionts are similar to the metamorphosis-associated contractile structures (MACs) recently discovered in the free-living bacteria *Pseudoluteromonas luteoviolacea*.
MACs are encoded by prophage-like elements in the P. luteoviolacea genome, and their formation is associated with host cell death and lysis. Structurally, MAC is an assemblage of multiple contractile systems related to the contractile tails of myoviruses (tailed bacteriophages with contractile tails) (reviewed by [66,67]). The tail-like structures in MAC are assembled in a sea urchin-like pattern that fills almost the entire host cell. The baseplates of the individual contractile systems point outwards and are interconnected by a network formed by the homologs of the tail fibers [68]. Interestingly, the formation of MAC in P. luteoviolacea was associated with the formation of 2D paracrystalline arrays that were tentatively interpreted as the crystals of the sheath protein [31]. In P. sinuosa bacterial cells, the sea-urchin-like structures are also associated with large protein crystals (Fig. 9), adding to the similarity of these two systems. Although the formation of similar crystalline bodies was previously reported in certain cells infected by viruses [64], the physiology of this process and the possible function of these structures are poorly understood.

The MACs of P. luteoviolacea function to deliver a protein signal into the cells of the larvae of the tubeworm Hydroides elegans. The delivery takes place when the larva contacts the biofilm containing the MACs. This causes the contraction of the tail-like structures and the penetration of the tail tube tips into the animal cells [85]. The delivery of the protein signal induces larval metamorphosis [81,85]. It is currently unclear whether bacteria can benefit from this effect.

The features of the structures that we detected in P. sinuosa symbionts are compatible with a presumed MAC-like nature. The length of their cylindrical elements is about 120 nm, i.e. comparable to the phage tail of the bacteriophage T4 contractile tail (114 nm long) [66]. Although no data are currently available on a potential involvement of MAC-related structures in interspecies communications between bacterial symbionts and their P. sinuosa host, it seems a plausible hypothesis. In this regard it is important that small (compared to P. luteoviolacea MAC) particles released by the lysed symbiont cells inside spongozoan host may move from its funicular system to the zooidal coelomic cavity and further to the surrounding water via coelomocytes [60].

Interestingly, the onset of the MACs or VLPs production appears to occur almost simultaneously in multiple bacterial cells within one funicular body (Figs. 7C,D, 9). This suggests the triggering of induction by some cue generated by bacteria or by the animal host.

We speculate that maintaining an excess of symbiotic bacteria in the funicular bodies becomes energetically disadvantageous at a certain point, and viruses destroying bacteria thus act as regulators of their numbers. Such phages are termed mutualistic, and their participation in indirectly regulating the number of symbiotic bacteria and processes carried out by symbionts has been described for aphids and some other insects [13,87,88]. This relationship as also been assumed for the symbiotic associations of cnidarians with their microbial symbionts [49,90].

Our study is the first step towards future research on viruses of the bacterial symbionts of Bryozoa. The next step should include determination of the systematic status of both the viruses and their bacterial hosts. The wide range of bioactive substances identified from spongozoans (reviewed in [84], see also [85]) suggests a wide distribution of symbionts inside these invertebrates. These symbionts, in turn, could host various viruses—but this research has just begun.
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Author contributions

A.V., A.L. and A.O. designed the study, analysed the data and wrote the manuscript. A.L. and PI performed the experiments. A.O. and G.L. collected material. G.L. contributed to data interpretation and writing the manuscript. T.S. processed fixed material and contributed to data interpretation. A.V. and N.K. performed microscopical study. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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