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NOTE

Formation of submicron colloidal particles from marine bacteria by viral infection

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ABSTRACT: We tested the hypothesis that viral lytic infection leads to the formation of submicron-sized colloidal particles originating from marine bacteria. Laboratory experiments were performed using a marine bacterium, *Vibrio alginolyticus*, and and its infectious phage. A particle counter was used to determine abundance and size distribution of particles. We found that the non-living submicron sized particles (size range from 0.38 to 0.7 µm in diameter) increased rapidly along with a decrease of bacteria and an increase of phage, indicating that these particles are cell debris originating from bacteria. These particles were stained faintly by acridine orange but were not countable due to the amorphous shape. These results show that amorphous submicron particles are produced by viral lysis of bacteria. This process may be one of the major pathways of colloid formation associated with microbial food webs in the sea.

KEY WORDS: Virus · Phage · Marine bacteria · Submicron particles · Marine colloids

Recently, non-living colloidal particles (approximate size range, 0.005 to 1 µm) have been found to be abundant in marine environments (Koike et al. 1990, Wells & Goldberg 1991, 1994, Longhurst et al. 1992, Sieracki & Viles 1992). Their sources and sinks are interesting from an ecological and biogeochemical point of view, because high molecular weight dissolved organic carbon (HMW-DOC) including such colloids (1) represents a significant fraction of the bulk DOC (Benner et al. 1992, Ogawa & Ogura 1992, Guo et al. 1994), and (2) is important as reactive components of DOC (Moran & Buesseler 1992, Amon & Benner 1994, Santschi et al. 1995).

Little is known about the actual mechanism of colloid formation in natural seawater. Distribution of the submicron-sized colloids determined using a particle counter was found to be correlated with bacterial concentration and some other biological parameters, suggesting that their formation is associated with biological activities (Koike et al. 1990). Microorganisms including phytoplankton, flagellates and bacteria have been suggested as the sources of colloids using laboratory culture experiments (Decho 1990, Passow et al. 1994). The egestion of bacteria by mixotrophic and heterotrophic flagellates have been reported as one important process of colloid formation (Koike et al. 1990, Nagata & Kirchman 1992, Tranvik 1994). Microbial processes, especially mortality, seem to be one of the key factors in the formation of marine colloids.

Observations with transmission electron microscopy (TEM) have clarified that free-living virus-like particles are abundant in aquatic environments (Bergh et al. 1989, Bratbak et al. 1990). The presence of viral particles within bacteria, cyanobacteria and small eucaryotes suggests significant mortality due to viral infection (Proctor & Fuhrman 1990, 1991). It can be hypothesized that bacterial lysis by viruses may result in the release of colloids such as macromolecules and small cellular debris in seawater (Bratbak et al. 1990, Heldal & Bratbak 1991, Proctor & Fuhrman 1991, Wells & Goldberg 1992, 1994, Zweifel & Hagström 1995). However, whether or not this virus pathway results in the formation of marine detrital colloids has not yet been clarified.

In order to confirm the formation of colloidal particles by viral infection of bacteria, we conducted laboratory experiments with phage infectious to a marine bacterium, *Vibrio alginolyticus*. The results suggest the formation of colloidal particles associated with viral lysis of bacteria in natural seawater. In this work, we define submicron particles as those ranging from 0.38 to 0.7 µm determined by the particle counter. This size range practically excludes bacterial cells.

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Materials and methods. ***Isolation of phage:*** A bacteriophage named VA1 was isolated from surface water in Aburatubo Inlet, Kanagawa, Japan, on May 1, 1991. The seawater sample was serially filtered through Millipore filters (pore-size, 0.65 and 0.22 µm) and the host bacterium *Vibrio alginolyticus* (NCMB 1903) was inoculated with 0.25% yeast extract. After incubation for 3 d at 20°C in a glass bottle, enriched phage infectious to *V. alginolyticus* was isolated by the standard plaque assay (Suttle 1993) using ½ strength ZoBell 2216E medium.

Culture conditions: Bacteria + phage mixed culture was grown in 1/20 strength ZoBell 2216E medium based on artificial seawater (NaCl, 30 g; $MgSO_4 \cdot 7H_2O$, 4 g; KCl, 0.8 g; $CaCl_2$, 1.2 g; HEPES (pH 7.6), 11.92 g; in 1 l distilled water). The medium was filtered (Millipore, pore size, 0.22 µm) before autoclaving to remove the particles in the submicron size range. We also prepared bacterial pure culture as a control in the same culture condition. The bacterial cells (2 to 3×10^5 cells ml^{-1}) and phage VA1 [1 to 2×10^4 PFU (plaque forming units) ml^{-1}] were inoculated to the medium and then incubated at 20°C with gentle shaking. Subsamples were taken, and those for determination of bacteria and particle size distribution were fixed with 0.22 µm prefiltered formaline (final concentration, 2%). The experiments were repeated to confirm the reproducibility.

Enumeration of bacteria and phage: Bacterial cells were counted under an epifluorescence microscope (Olympus, BH2-RFCA) using the acridine orange direct counting method (Hobbie et al. 1977). At least 300 cells and 10 fields were counted. PFU of phage VA1 were counted by the standard plaque counting method (Suttle 1993) using ½ strength ZoBell 2216E medium after 2 d of incubation at room temperature. Chloroform (final concentration 1%) was used to kill phage-resistant bacterial cells immediately after sampling.

Determination of particle size distribution: Submicron particles were counted with the Elzone Particle Counter 80XY (Particle Data Inc., USA), equipped with a 12 µm orifice counting tube (Kogure & Koike 1987). The countable size range using this tube was 0.38 to 4.03 µm equivalent spherical diameter. The current and gain were set at 1.5 and 1.0, respectively. The conductivity of the samples was normalized for each one. Samples were diluted with 0.22 µm (Millipore filter) filtered 3.5% NaCl solution which was prepared with fresh distilled water. All samples were analyzed within 30 min after sampling. Measurements were repeated consecutively at least 3 times and the average values are shown as results. The coefficient of variation was less than 10%.

Results and discussion. In the mixed culture, the abundance of phage VA1 started to increase soon after the start of incubation and reached ca 1×10^8 PFU ml^{-1} after 4 h (Fig. 1). Bacterial abundance decreased with the increase of PFU, indicating that bacterial cells were lysed by viral infection. With the decrease of bacterial abundance, fragile and amorphous materials which were weakly stained with acridine orange on the 0.2 µm filters were seen. These were, however, not countable because of the faint staining and amorphous shape. Considering the shapes, these materials seem to be different from 'ghost cells', which are non-nucleoid-containing bacteria-like particles suggested by Zweifel & Hagström (1995).

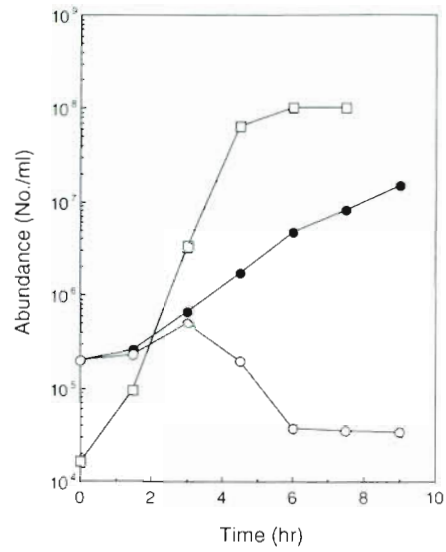

Fig. 1 Change in abundance in *Vibrio alginolyticus* + phage culture (mixture) and *V. alginolyticus* pure culture (control). (○) Bacterial abundance in mixture. (●) Bacterial abundance in control. (□) Phage abundance counted as plaque forming units in mixture

Under the same culture conditions, the change of particle size distribution was determined using the particle counter (Fig. 2). A single peak representing the size of bacterial cells appeared in the control during the incubation, although the size of the peak shifted slightly during incubation. In the mixed culture, considerable change in the particle size distribution was observed. The abundance of the submicron particles (size, 0.38 to 0.7 µm), which were apparently different from the size of bacterial cells, increased after 3 h and became dominant after 4.5 h. These particles are not phage themselves, because TEM observation indicate that phage VA1 particles are less than 0.2 µm in length (data not shown), a size which is not detectable by the particle counter. At 4.5 h, the size distribution peak of the bacteria and the submicron particles were 1.5 and 0.51 µm, respectively. If there was no loss in the volume, this suggests that 1 bacterial cell might be broken into 27 pieces. Considering the possible dispersion of soluble fractions, however, this number should be the maximum estimation (see below).

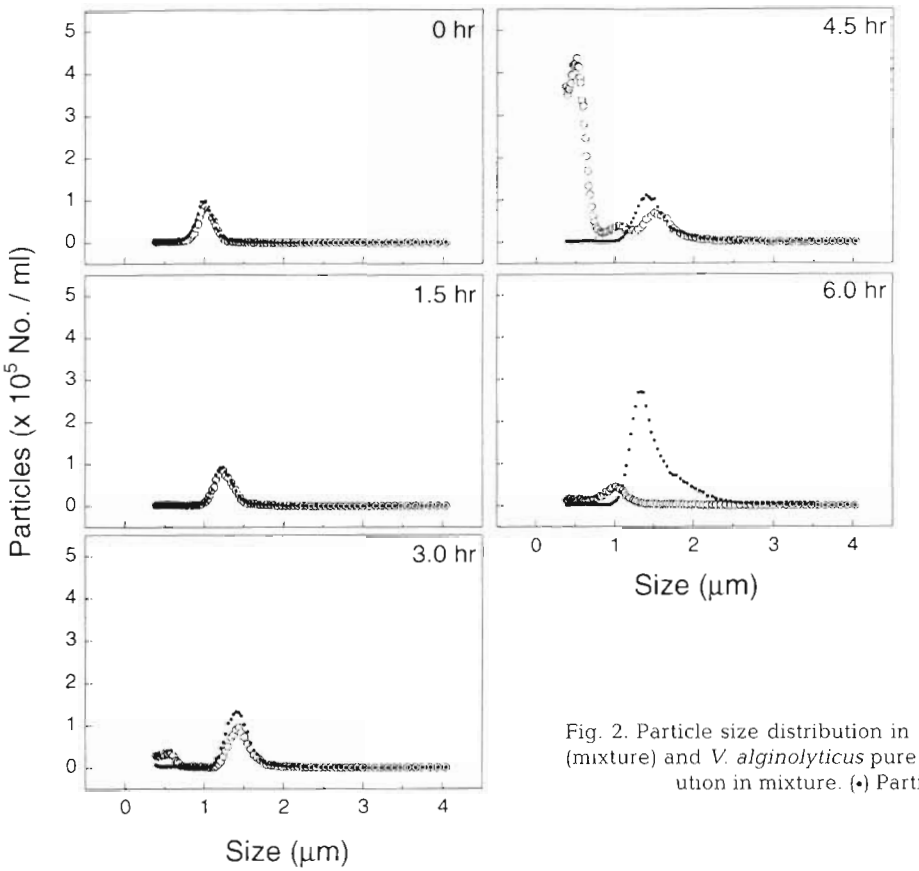

Fig. 2. Particle size distribution in *Vibrio alginolyticus* + phage culture (mixture) and *V. alginolyticus* pure culture (control). (○) Particle distribution in mixture. (•) Particle distribution in control

The change of particle abundance during incubation is shown in Fig. 3. The total particles (size, 0.38 to 4.03 μm) are those measured by the particle counter, and should include both bacteria and other particles. Bacterial abundance was obtained by epifluorescent microscopy. In the control, the increase of the total particles was due to bacterial growth. In contrast, the submicron particles started to increase sharply after 2 h in the mixed culture, and made up the largest part of the total particles after 3.75 h. The submicron particles increased with the production of phage (Figs. 1 & 3), indicating that this submicron-sized bacterial debris was produced by viral infection. We tried to estimate the number of submicron particles originating from 1 bacterial cell. From 3.75 to 4.5 h, the net decrease of bacteria in the mixed culture was 0.5×10^6 cells ml^{-1} and the net increase of submicron particles was 5.1×10^6 ml^{-1}. Therefore, roughly 10 particles might be released from 1 bacterium. Because gross production of submicron particles might be larger than apparent increase (Fig. 3), this should be a minimum estimation. Together with the estimation stated above, it is reasonable to assume that roughly 20 submicron particles were formed from a lysed bacterium.

In general, only lytic infection results in virus replication and death of the host cell (Hayes 1976). If a bacterium is attacked by several of the phage, then a phenomenon known as 'lysis from without' occurs in which the phage weaken the bacterial cell wall to the point where lysis occurs without virus production (Delbrück 1940). In the present experiments, 'lysis from without' was not responsible for the increase of the submicron particles, because bacteria were much more abundant

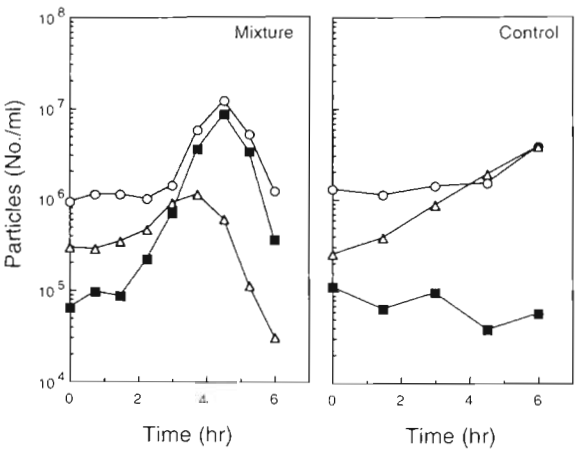

Fig. 3. Change in particle abundance in *Vibrio alginolyticus* + phage culture (mixture) and *V. alginolyticus* pure culture (control). (○) Total particles (size, 0.38 to 4.03 μm). (△) Bacteria. (■) Submicron particles (size, 0.38 to 0.7 μm)

than phage in the mixture for the first 1.5 h and the particles still continued to increase with the production of phage VA1 (Figs. 1 & 3). These results indicate that the increase in the submicron particles was caused by typical viral lysis.

After 4.5 h, the abundance of the submicron particles decreased rapidly in the mixed culture (Fig. 3), indicating the removal of the particles from the detectable size range (0.38 to 4.03 µm) of the particle counter. This rapid decrease was repeatedly observed under the experimental conditions of the present study. Although we have no further data, some possible hypethetical cases can be considered. First, fragmentation of the submicron particles into even smaller ones that are below the detection limit is a possible explanation for the decrease in submicron particles. Bacteria contain various ectoenzymes and cytoplasmic enzymes that might degrade those particles. Second, adsorption of these particles into the glass wall of the flask is also possible, because glass is known to adsorb biopolymers such as proteins. Third, aggregation of particles larger than 4.03 µm might occur. Hydrophobic interaction of bacterial membrane components (for example, proteins and lipids) is probably an important factor in the case of adsorption and aggregation (Schulz & Schirmer 1979, Kirchman et al. 1989) Although it is difficult to identify the reason for the decrease in submicron particles, our result indicates the unstable nature of the particles. More work is definitely needed to clarify the actual process causing this phenomenon.

Recent studies show that the viral contribution to bacterial mortality is similar to that of protists (Fuhrman & Noble 1995, Steward et al. 1996) or that the former even occasionally prevails over the latter (Weinbauer & Peduzzi 1995). If we assume that (1) 20 submicron particles are formed from 1 bacterial cell after viral lysis (disscussed above), (2) 1×10^5 bacterial cells ml^{-1} were present, and (3) 10 to 20% of bacteria are lysed by viruses on a daily basis (Suttle 1994), then 2 to 4×10^6 colloidal particles ml^{-1} d^{-1} will be produced through viral lytic infection in natural seawater. Thus, this virus-pathway is likely to be important for the production of marine colloids in addition to the flagellate-pathway suggested previously (Koike et al. 1990, Nagata & Kirchman 1992, Tranvik 1994).

In conclusion, our hypothesis that viral lysis leads to the formation of submicron-sized colloidal particles originating from host marine bacteria was confirmed. This suggests that viruses probably have the role as colloidal particle producers in the marine ecosystem. Although the actual contribution of viruses towards producing colloidal particles in natural seawater remains to be clarified, this process seems to have a considerable importance for the fate of dissolved organic matter in the ocean.

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