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Virus-Like Particle derived from a Bacterium belonging to the Oldest Lineage of the Domain *Bacteria*

HIROSHI XAVIER CHIURA*[1], HIROYUKI YAMAMOTO[2], DAISUKE KOKETSU[1,4], HIROKI NAITO[1,5] and KENJI KATO[3]

[1] *Department of Biology, Division of Natural Sciences, International Christian University, Mitaka, Tokyo 181–8585, Japan*
[2] *Department of Microbiology, St. Marianna University School of Medicine, Kawasaki, Kanagawa 216–8511, Japan*
[3] *Department of Biology and Geosciences, School of Science, Shizuoka University, Shizuoka 422–8529, Japan*
[4] *Present address: Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bunkyo, Tokyo 113–8657, Japan*
[5] *Present address: Research and Development Department, GC Corporation, Itabashi, Tokyo 174–8585, Japan*

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Aquificales, a thermophilic bacterium of the deepest-branching lineage of the domain Bacteria produced virus-like particles (VLPs). Mature VLPs resided in 6.5±4.4% (n=341) of bacterial cells. A count of the free particles in water from geothermal hot spring showed high abundance ($1.41\pm1.70\times10^7$ VLP/ml, n=12). The molecular mass of the VLP-encapsulated DNA was 406.4±10.1 kb (n=13).

Key words: *Aquificales*, geothermal hot spring, thermophilic bacteria, virus-like particles (VLPs)

Free virus particles (virioplankton) are the most abundant class of plankton class in many aquatic environments[10,29]. However, information on this kind of virus is limited to those that are lytic[2], and little is known about lysogens[13,14,25] in natural aquatic environments[15,16]. It has been indicated that 40% to 75% of marine bacteria contain inducible prophages[11,12,19,20,29]. Their origin, biochemical nature, infectious activity, host specificity, gene transfer capability and fates are beyond the understanding of traditional concepts of viral ecology[17–20].

Currently, there is no reason to believe that so-called Virus-Like Particles (VLPs) share common characteristics with the traditional viruses that are associated with bacteria such as T4 or lambda phages for *Escherichia coli*. Some marine bacterial isolates have been shown to spontaneously release VLPs into the culture medium[4–7]. It should be also pointed out that those VLPs in natural environments[4–7] have not been well characterised. These VLPs contain DNA and have some morphological similarity to viruses[1]. However, in mode of formation, mechanism of bactericidal effect and gene transfer, they seem to be different from any of the well known types of lysogenic or virulent bacteriophages[4–7]. While information on virus-host interactions has accumulated mainly through studies of mesophilic microbial communities[10–25,28,29], there is a dearth of information about the distribution and ecological roles of viruses in thermal environments such as hot springs and hydrothermal vents. Although particulate DNA has been reported to exist in hydrothermal vents[27], nothing has been documented on the virus-host interactions in such an extreme environment. As for thermal environments, only a few viruses for Archaea have been described[31], because of the difficulty in culturing thermophilic bacteria and the fastidious growth of thermal viruses.

This communication describes an abundance of VLPs in a hot spring and a novel particle production scheme. Samples of water and microbial mats, (termed "sulphur turfs"[30]), were collected from a hot spring located at Nakanoyu in Nagano Prefecture, Japan (36°12′N, 137°36′E) in

* Corresponding author; E-mail: chiura@icu.ac.jp, Tel: +81–422–33–3244, Fax: +81–422–33–1449

June 1997. The sulphur-turf bacterium is still uncultivable, however, a 16S rRNA gene phylogenetic technique revealed its abundance[30]. Sulphur-turf mats were fixed in 4% paraformaldehyde in 3×phosphate-buffered saline [PBS: 0.8% NaCl, 0.02% KCl, 0.115% Na_2PO_4, 1.15 g, 0.02% $NaHPO_4$, (pH 7.3–7.6)] for 6 to 12 h. Fixed cells were washed three times in 1×PBS, and stored in 50% (v/v) ethanol in 1×PBS at −20°C until use. A small portion of the fixed samples was smeared onto gelatine-coated glass slides, air-dried, sequentially rinsed with an ethanol series (50, 80, and 99%), and mounted in hybridisation buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS, 20% formamide], containing 5 ng/μl fluorescein isothiocyanate (FITC)-labelled oligonucleotide probe (Takara, Japan). In situ hybridisation was carried out in a humid box at 46°C for 1.5 h, then the slides were rinsed with a washing buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS] at 48°C for 15 min. Finally, the slides were stained with 4′6-diamidino-2-phenylindole (DAPI, Sigma, USA, 0.1 μg/ml in distilled water), and rinsed with distilled water. An oligonucleotide probe (5′ GTC GCC AGC ACT ATT ACC 3′; S-*-ST-0089-a-A-18) complementary to the 16S rRNA sequences of representative clones (NAK and GANI) of sulphur-turf bacteria belonging to the phylogenetic lineage of *Aquificales* was used[30]. The sulphur-turf mats typically have a white ruffled fur or turf-like mass of filaments, and consist of bundles of large bacteria (1 μm wide and 5 to 20 μm long), as shown in Fig. 1. Cells of *Escherichia coli* and *Bacillus subtilis* were employed as negative controls of hybridisation, and none of the specific probe hybridised to the negative-control bacteria (data not shown).

A sulphur-turf bacterium-specific DNA probe emitting FITC fluorescence showed that the population was almost completely composed of *Aquificales* [95.7±15.6% (n=7)]. The cell density in the hot spring water sample was $1.70\pm2.73\times10^5$ (n=7) cells per ml, and free-VLP abundance was $1.41\pm1.70\times10^7$ (n=7) particles per ml. Mature VLPs in the sulphur-turf bacterial cells were observed in up to 11% (6.5±4.4%, n=341 eye fields) of the population in the mat, and appeared morphologically similar to free VLPs. All the free VLPs, which were found in the hot spring water column, had a spherical shape ranging in diameter from 43 to 140 nm without a tail structure (*cf.* Fig. 2A).

The concentration of free VLPs in the hot spring water sample (12 litres) was measured with a tangential-flow system[4] (A Minitan Ultrafiltration system equipped with a 30-kDa cut-off membrane, Millipore, USA). The treatment was done at ambient temperature, which brought about the eduction of colloidal sulphur on the membrane, and caused

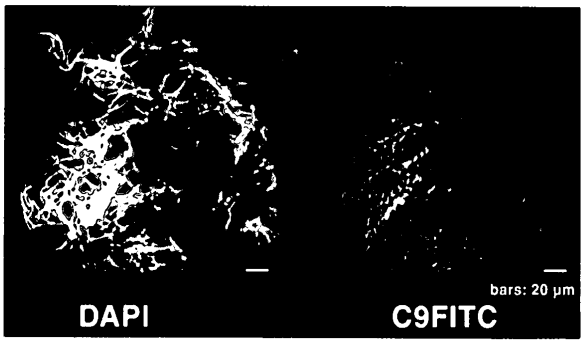

Fig. 1. Epifluorescence microscopic image of sulphur-turf mat cells taken in June 1997. Left panel, cells stained with DAPI; right panel, FITC-labelled *Aquificales*-specific probe (C9FITC). The population was almost completely composed of *Aquificales*. The microscopic images were obtained with a charge-coupled device equipped camera (model C5910: Hamamatsu Photonics, Hamamatsu, Japan).

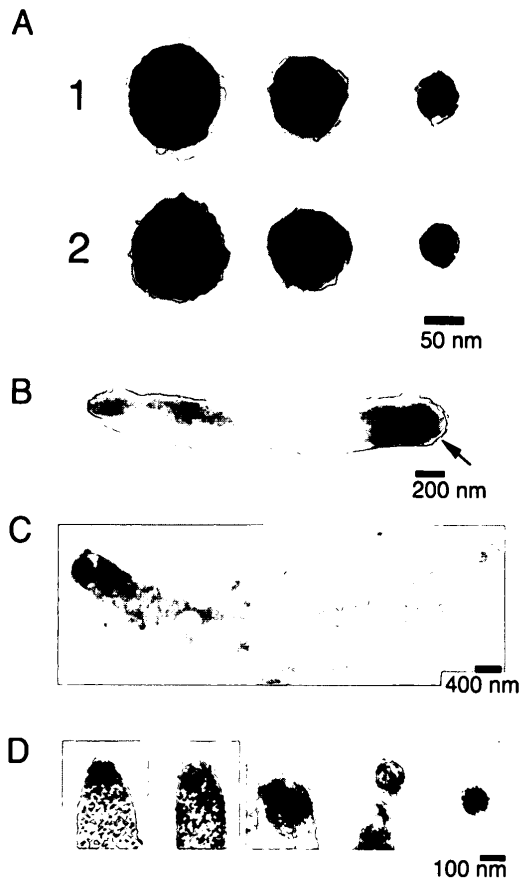

Fig. 2. Electron microscopic images of virus-like particles. A: 1, Hot spring water sample; 2, Cell lysate sample; B: Sulphur-turf cells under particle induction. Arrow indicates VLP in the cell. C: Ultrathin section of a sulphur-turf cell under particle induction. An inclusion body can be seen at the left end of the cell. D: "Budding-like" particle production of the sulphur-turf cell. Particle production can be observed at the end of cell from the left to right panel.

a reduction in the yield of the concentrate to 33.1% (5.59×10^{11} particles). The final yield of purified sulphur-turf derived VLPs (ST-VLPs) from the hot spring water sample as determined by CsCl-density equilibrium ultracentrifugation, amounted to 5.55×10^{11} particles, which corresponds to 99.2% of the amount applied. ST-VLPs (1.92×10^{11} particles) were successfully extracted and purified from the cell lysate of sulphur-turf bacteria (9.06 g).

For preparation of intracellular VLPs, cells were homogenised, washed with TE buffer (0.1 mM Tris-HCl, 1 mM EDTA, pH 7.5) and centrifuged twice (9,000×*g*, 15 min). The packed cells were then dehydrated through placing Spectra/Por (SPECTRUM, molecular weight cut-off: 12,000–14,000) tubing and dialysed against CS_2 (200 volumes of the cell mass). Dehydrated cells were re-hydrated again in a methanol series, TE buffer, and 25% sucrose-50 mM Tris-HCl (pH 7.0). Lysozyme (10 mg/ml, Sigma, USA) was added, and the cells were incubated at 37°C for 5 h. Then Proteinase K (1.0%, Boehringer Mannheim, Germany) and sodium dodecylsulphate (SDS, 0.4%, Nakarai, Japan) were added, and incubation was continued at 65°C overnight. The tubing was placed in 0.4 N NaOH, and finally neutralised with CH_3COONa. The dialysed solution was centrifuged at 17,000×*g* for 90 min at 4°C. The recovered supernatant was again placed in tubing in TBT (100 mM Tris-HCl, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4) buffer. After three changes of this buffer, RNase A (1.0%, Sigma, USA) was added, and treated at 37°C with dialysis against TBT for 17 h. Extracted VLPs in the dialysed solution were purified with CsCl-density equilibrium ultracentrifugation[4]. The buoyant density (ρ^{25}) of the purified VLP was measured by refractometry with an Abbe's refractometer (Atago, Japan). VLP abundance and particle size distribution in the respective bands were examined with electron microscopy. Contents of protein (Pro) and nucleic acid (NA) were determined photometrically.

Intra- and extracellular VLPs obtained from the water and cells gave comparable results for all the examined criteria: the buoyant densities (ρ^{25}) of bands, proportion, particle size, and protein/nucleic acid ratio, are summarized in Table 1.

Intra- and extra-cellular particles showed marked similarity in each other of the criteria examined, except size distribution. Hence, intracellular and extracellular particles are of the same origin. The protein/nucleic acid ratio was estimated by reading absorbance at 260 and 280 nm, to be used an index during phage purification. A precise chemical determination of the protein and nucleic acid content needs at least 10^{12} particles, which is impractical.

Hot spring water was fixed with 2.5% glutaraldehyde, and the free ST-VLPs and bacteria were enumerated by electron microscopy[3]. For the examination of VLP production in the bacterial cells, a portion of sulphur-turf taken from the hot spring was used to prepare thin sections as previously described[5]. Grids were examined at ×75,000 for VLPs and ×20,000 for bacteria at an accelerating voltage of 80 kV with a JEM-1200EX electron microscope (JEOL Inc., Tokyo Japan).

Electron microscopic images of typical VLPs purified from the spring water and cell lysate are shown in Fig. 2, in which large "sausage-shaped" cells under particle induction are shown (Fig. 2B and C).

Upon particle production, a condensation of the inclusion bodies occurred at the end of the cell, and a "budding-like" extrusion of the particle was observed (Fig. 2D). Intracellular particles numbered 2.5±1.5 (n=60 cells), hence "burst size" was estimated as 3. The mode of production of VLP by some transductants of marine origin was observed to be "budding-like" (Chiura unpublished data 1998), which showed a similarity to this ST-VLP. As reported[4–7] in marine bacteria, no plaque formation was observed in transduction experiments using their VLPs. Such a "budd-

Table 1. Buoyant density of ST-VLP, recovered amount, proportion, particle size, and protein/nucleic acid ratio from hot spring water and cell.

Band	ρ^{25}±SD, g/cm^3 (n=3)	Amount	Proportion, %	Size, nm (n=50)	Protein/NA ratio
Hot Spring water: Recovery upon original abundance in water=33.1%					
upper	1.2595±0.0109	1.84×10^{11}	33.2	127.7±18.4	17.54
middle	1.2892±0.0188	2.23×10^{11}	40.1	104.4±9.2	23.93
lower	1.3168±0.0088	1.48×10^{11}	26.7	56.3±12.4	22.01
Cell lysate: Particles extracted from 9.06 g of cell sampled in June 1997					
upper	1.2601±0.0298	2.09×10^{10}	10.9	127.7±16.6	17.95
middle	1.2949±0.0184	1.02×10^{11}	53.0	104.4±10.4	27.12
lower	1.3366±0.0171	6.93×10^{10}	36.1	56.3±10.1	26.71

ing-like" particle production would not induce cell lysis.

Nucleic acid extraction from the VLP genome was carried out in an agarose gel plug (in situ lysis gel), which was examined electrophoretically. ST-VLPs purified from the spring water and the cells were embedded in the gel plug. Procedures for the preparation of agarose gel plugs followed those recommended by the manufacturer (InCert Agarose, For preparation of Gel Plugs useful in electrophoretic separation of chromosomal DNA, 1988 FMC BioProducts, Rockland, ME). A biased Sinusoidal Field Gel Electrophoresis (BSFGE)[26] system (Genofield, ATTO) was employed to run a gel in 0.5×TBE buffer at room temperature for 30 h under conditions of 1.6 V/cm DC; 9.6 V/cm AC; start frequency 0.01 Hz; end frequency 0.3 Hz; with a logarithmic ramp. Nucleic acid species were stained with a 1/10,000 SYBR GREEN II stock solution (Molecular probe, USA), and then illuminated on a SPECTROLINE Trans Illuminator Model TC-365A (Spectronics, USA) at 360 nm. Results were recorded with a Canon PowerShot Pro 70 digital camera. The amount of nucleic acid was determined from the images using the public domain NIH Image programme (http://rsb.info.nih.gov/nih-image/).

For determination of the type of nucleic acid contained in the VLP genome, "bands of ST-VLP genome" were incised from the gel after BSFGE, and each was placed in a Falcon tube. DNase I (10 mg/ml, Sigma) in TM (50 mM Tris-HCl pH 7.4, 20 mM $MgSO_4$) buffer and RNase A (10 mg/ml, Sigma) in deionised and distilled water (DDW) were applied to the gel pieces contained in the Falcon tubes. Four VLP genome blocks were treated with DNase I, RNase A, DDW, and TM buffer overnight at 37°C. The genome blocks and the original gel plugs of ST-VLP were again subjected to BSFGE for 16 hours and the results recorded as described above.

The nucleic acid encapsulated in VLP derived from the water and cell lysate gave a single band in the electrophoretogram, regardless of particle size. The estimated molecular mass of ST-VLP nucleic acid, as a linear dsDNA, was 406.4±10.1 kb (n=13) (Fig. 3). The amount of nucleic acid obtained from 3.1×10^{10} ST-VLP was 1.24 μg. The nucleic acid content of the VLP was subjected to nuclease treatment to distinguish whether the genome would be DNA or RNA. As shown in Fig. 3, the band disappeared after DNase treatment, while RNase A treatment had no effect, therefore the content consisted of DNA.

In molecular mass, the nucleic acid of ST-VLP purified from the hot spring water resembled that found in the cell. Therefore, the ST-VLP recovered from the hot spring is likely to be obtained from the sulphur-turf cells belonging to *Aquificales*.

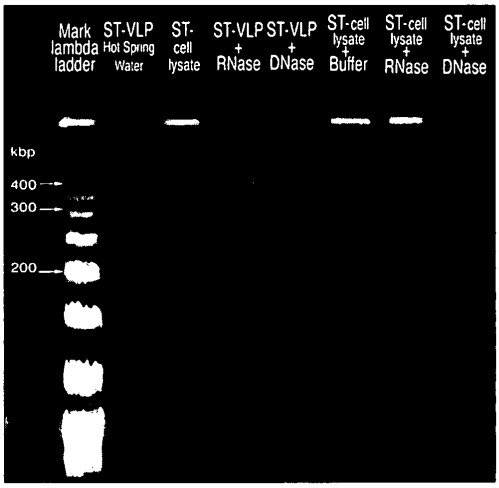

Fig. 3. Determination of molecular mass and type of nucleic acid encapsulated in VLP. Mark, Lambda ladder: 48.5 kbp~1.2 Mbp; ST-VLP hot spring water, ST-VLP purified from hot spring water; ST-cell lysate, Sulphur-turf cell treated with in situ agarose gel technique; ST-VLP+RNase, ST-VLP purified from hot spring water treated with in situ agarose gel technique, then nucleic acid treated with RNase; ST-VLP+DNase, ST-VLP purified from hot spring water treated with in situ agarose gel technique, then nucleic acid treated with DNase; ST-cell lysate +buffer, Sulphur-turf cell treated with in situ agarose gel technique, then nucleic acid treated with the buffer used for enzyme solvent; ST-cell lysate+RNase, Sulphur-turf cell treated with in situ agarose gel technique, then nucleic acid treated with RNase; ST-cell lysate+DNase, Sulphur-turf cell treated with in situ agarose gel technique, then nucleic acid treated with DNase.

Never before has a virus with such a large nucleic acid content been reported[9]. The inner volume of coliphage T4 is reported as 4.01×10^{-16} ml, and a chromosome of 171 kb is packaged at a density of 0.5 g/ml (2.34×10^{-18} ml/kb)[8]. Most of the particles used to determine the DNA were *ca* 120 nm in diameter: their volume is estimated to be 9.0×10^{-16} ml corresponding *ca* 400 kb of content. Although based upon circumstantial evidence, the VLP characterised in this study is likely to have originated from cells belonging to *Aquificales*. This is the first finding of a VLP derived from a thermophilic chemolithotrophic bacterium of *Aquificales*, the oldest lineage of domain Bacteria.

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