Broad-Host Range Gene Transporter Particles Produced by *Aliivibrio fischeri*

HIROSHI XAVIER CHIURA^{1,2*}, NAMI UCHIYAMA^{1†}, and KAZUHIRO KOGURE¹

¹Marine Microbiology Laboratory, Department of Marine Ecosystems Dynamics, Ocean Research Institute, University of Tokyo, 1–15–1 Minamidai, Nakano-ku, Tokyo 164–8639, Japan; and ²Arbeitsgruppe Allgemeine Mikrobiologie, Zentrum für Anatomie und Zellbiologie, Medizinische Universität Wien, Währingerstr. 10, Wien A-1090, Austria

(Received August 5, 2009—Accepted October 20, 2009—Published online November 12, 2009)

Aliivibrio fischeri NCIMB1281^T (basonym, *Vibrio fischeri*) spontaneously started broad-host range vector particle (AfVP) production by budding from the logarithmic phase, and stabilised at around 7.0×10^{10} – 7.4×10^{11} particles mL⁻¹ without any accompanying change in the host population. AfVPs had a spherical shape and varied in diameter from 18.1 to 159.2 nm [median±SD, 58.4±11.9 nm, $n=528$], with 95.1% between 30.2 and 84.6 nm in diameter exhibiting a normal distribution. Their buoyant density and DNA content ranged from 1.3607 to 1.3980 g cm⁻³, and 17.3 to 95.3 kbp, respectively. Regardless of UV treatment, AfVPs enhanced the efficiency of plating 116–136% at a multiplicity of infection of *ca.* 140 in *Escherichia coli* AB1157. Generalised transduction was observed with a frequency of between 10^{-4} and 10^{-6} cells per AfVP without UV treatment. Upon infection, the particle membrane remained outside the recipient cell, and a string-like structure coated with a fibrous proteinaceous-like material was present. The growth of the *E. coli* transductant (AfV-E-trans) reached a maximum of *ca.* 415% that of the parental *E. coli* recipient. AfV-E-trans acquired the ability to produce budding particles.

Key words: broad-host range, horizontal gene transfer (HGT), serial transduction, *Aliivibrio fischeri*, VLP(s)

In environmental samples, particles similar in morphology to viruses are called virus-like particles (VLPs). Although viroplankton is the most abundant class of plankton in a variety of aquatic habitats (2, 12, 21, 23, 30, 32, 33), studies on its behaviour in the environment are scarce. VLP-mediated transduction among microbes has become of interest to researchers in microbial ecology (12, 33, 34), because of the possible impact VLPs could have on horizontal gene transfer (HGT). Virus-mediated HGT is considered one of the most important components of evolution and the genetic diversity in natural microbial communities, since virus-mediated gene transfer has had more effect on microbial populations than was previously considered (18). HGT for various gene clusters, such as drug resistance genes, has recently been reported based upon sequence analyses (16, 20). However, assessment of the degree of transduction in natural environments is still limited by insufficient experimental systems. The development of a technique of isolating VLPs from the environment may lead to new findings regarding gene transfer mediators (28).

We have demonstrated that VLPs released spontaneously into culture medium from some marine bacterial isolates show a gene transfer capability, with a lethal effect on recipients distantly related to their original host (3–5). These VLPs contain DNA, and are similar morphologically to viruses (3–5). We have proposed calling such particles “broad-host-range vector particles” (VPs) because of their extraordinary properties. VPs only become apparent on electron microscopic (EM) observation, which reveals budding particles

generated using a recipient different from the sampled habitat. Hence, the proportion of VPs in the VLP fraction of environmental samples is unclear. VPs are postulated to randomly encapsulate a certain length of host chromosome through serial transductions (9). Regardless of UV irradiation, VPs exhibit a mildly lethal effect (maximum, 10%) towards a taxonomically broad range of recipients without plaque formation. VPs (3–10) do not fit the current concept of a “virus” with a narrow host range (1). Although the DNA-encapsulating system of VP is still unclear, these characteristics support the possibility that VP-mediated transduction enhances microbial differentiation and evolution.

Recent developments in genome science have revealed that virus-mediated transduction is not only a phenomenon observed in the laboratory, but contributes to microbial evolution and differentiation.

A comparison of gene sequences between *luxA* and its accessory fluorescent protein together with multilocus housekeeping genes among luminous bacteria of the genus *Aliivibrio*, done by Yoshizawa (35), has made clear that luciferase-related genes in some luminous strains were acquired from the genus *Photobacterium*. These findings suggest the spectral diversity in light emission to be generated by both divarication of the luciferase gene at the genus level and the acquisition of accessory fluorescent proteins. Although HGT would not be responsible for the speciation of luminous bacteria, it would probably be involved in the diversification of emitting light wavelengths shared at the order level. As is predicted from the sequence data (15, 30, 35), HGT must have occurred for the spectral diversity in light emission to have evolved. All the factors necessary for the expression of functional genes in phylogenetically distant species must have been transferred. However, it is impractical to expect the transfer of all functional gene clusters to a

* Corresponding author. E-mail: hxc@ori.u-tokyo.ac.jp; Tel: +81–3–5351–6535; Fax: +81–3–5351–6482.

† Present address: Quality Assurance Department, Q.P. Corporation, 2–5, Sengawa-cho, Chofu-shi, Tokyo 182–0002, Japan

recipient with the aid of conventional gene transfer schemes, since the maximum size of a dsDNA insert in existing gene transfer vectors such as plasmids and coliphage lambda is <25 kbp (plasmid: insert DNA maximum size=15 kbp, phagemid: insert=25 kbp) (1). Thus, an aid capable of transferring a substantial functional gene cluster would be necessary. The possibility of specific particle production having a gene transfer capability was examined in a luminal marine bacterial type strain, *Aliivibrio fischeri* (29). Furthermore, the contribution of the HGT of chromosomal genes to the recipient *Escherichia coli* AB1157 at a high multiplicity of infection (MOI) was examined. The strain was shown to be capable of producing a broad-host gene transfer particle: AfVP, as described above.

Materials and Methods

Bacterial Strains, media, culture conditions, and cell and particle enumeration

Aliivibrio fischeri (NCIMB 1281^T=ORI No. 194), originally isolated from a light organ of *Euprymna scolopes*, was selected from our laboratory collection. *A. fischeri* was cultured in ZoBell Marine Broth 2216E (Difco, Becton Dickinson, Sparks, MD, USA) and ZoBell Marine Agar 2216E (Difco) at 20°C. The inoculum comprised 3.6×10^6 cells mL⁻¹, and was incubated at 20°C for 315 h with shaking at 60 rpm. The total volume of the culture was 3.8 L.

Cells were enumerated using samples fixed in 2% (v/v) neutral-buffered formaldehyde (Merck, Darmstadt, Germany) on AnodiscTM 47 membrane filters (pore size 0.02 µm; Advantec Toyo Kaisha, Tokyo, Japan) and stained with SYBR[®] Gold (Molecular Probe, Invitrogen, Carlsbad, CA, USA), under an epifluorescence microscope (E600; Nikon, Tokyo, Japan) equipped with a super high-pressure mercury lamp with B excitation (450–490 nm) without an ND filter, at a magnification of $\times 1,000$. The number of bacteria was determined from a count exceeding four hundred individuals or 40 eye-fields.

Particles were enumerated by fixing a portion of sample with 10 mM EDTA containing 2.5% (v/v) glutaraldehyde (Wako Pure Chemical Industries, Osaka, Japan) overnight at ambient temperature, and applying it to a carbon and parlodion-coated copper grid (150 mesh; Stork Veco International, Eerbeek, Netherlands) placed in an Epok 812 (Shell Chemicals, Harris County, TX, USA)-embedded-flat-bottomed ultracentrifugation tube and centrifuging at $46,000 \times g$ for 90 min at 20°C (2) using a Beckman Preparative Ultracentrifuge L8M with a SW 55.2 Ti rotor (Beckman-Coulter, Fullerton, CA, USA). Grids were stained with 2% (w/v) uranyl acetate (Merck) for 30 s, and then sequentially washed for 15 s with 0.2 µm-filtered deionised and distilled water, and 95% (v/v) ethanol (Wako) twice.

Particle counts were made under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV and magnification of $\times 50,000$ as described (2, 7).

Preparation and purification of virus-like particles

The culture was spun down at $7,500 \times g$ for 40 min at 4°C using a Kubota RT20000 refrigerated centrifuge equipped with a RA-6 rotor (Kubota Medical Appliance Supply, Tokyo, Japan) to separate the cells and the supernatant. The cell pellet was subjected to serial rinsing with TBT [100 mM Tris-HCl, 100 mM NaCl, and 10 mM MgCl₂, pH 7.4; Wako] buffer twice, and centrifugation ($8,000 \times g$, 15 min, 4°C), then stored in a deep freezer at -85°C until *in situ* lysis for Crossfield gel electrophoresis. The rinsing obtained as the supernatant was combined with the above the culture supernatant (total volume, ca. 4 L), filtered with a 0.2-µm membrane filter (CCS-020-E1H, Advantec), and concentrated with Minitan Ultra-filtration Systems using a 100 kDa cut-off membrane (Minitan;

Millipore, Bedford, MA, USA), to ca. 15 mL. Free nucleic acid and protein levels were monitored by reading absorbance at 260 nm and 280 nm using a spectrophotometer (Type UV260; Shimadzu, Kyoto, Japan). The concentrated specimen was dialysed against 6 changes of 0.25 L of 10 mM potassium phosphate buffer saline [5 mM MgCl₂, and 200 mM NaCl supplemented phosphate buffer, pH 7.0], then treated with 10 µg mL⁻¹ each of DNase I and RNase A (Sigma-Aldrich, St. Louis, MO, USA) at 25°C overnight, and finally placed at 80°C for 15 min to inactivate DNase. The treated specimen was spun down at $88,000 \times g$ for 40 min at 4°C (Beckman Preparative Ultracentrifuge L8M equipped with a 55.2Ti rotor) to obtain a particle pellet, which was re-suspended in 500 µL of TBT buffer, and dispersed in 5 mL of 35% (w/v) CsCl (Boehringer Mannheim, Mannheim, Germany) in TBT. After CsCl-density gradient equilibrium ultracentrifugation at $178,000 \times g$ for 18 h at 4°C, bands containing particles were recovered by a side puncture technique (17). Buoyant densities (ρ^{25}) of the bands were determined with an Abbe-refractometer (Atago Seisakusho, Tokyo, Japan), and then specimens were placed in Spectra/Por 4 (molecular weight cut off, MWCO=12,000–14,000; Spectrum Chemicals Mfg, Houston, TX, USA) tubes, and dialysed against 6 changes of 0.25 L of 10 mM phosphate buffer (pH 7.0) at 4°C to remove CsCl. This purified particle is referred to as AfVP, and was used for subsequent experiments.

The nucleic acid and protein contents of the specimens were determined photometrically as above. Protein content was also determined using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to its specifications for micro assays.

Preparation of the recipient, *Escherichia coli* AB1157, for the gene transfer experiment

Seed cultures of the recipient *E. coli* AB1157 (F⁻; *thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44*), obtained from the National Institute of Genetics, Shizuoka, Japan, were prepared as described previously (10). After confirmation of respective markers, the recipient was grown to ca. 4.0×10^8 CFU mL⁻¹, and subjected to sequential rinsing with TBT buffer and centrifugation ($7,500 \times g$, 40 min, 4°C) twice. The rinsed cells were re-suspended in 7% (v/v) glycerine (Merck)-containing TBT buffer, dispensed as 1-mL aliquots, immediately frozen in liquid nitrogen, and stored at -85°C prior to use.

AfVP-mediated gene transfer to *Escherichia coli* AB1157

The transduction experiment was carried out as described (5). Briefly, the selection medium was based upon the minimal medium of Davis [MM (% w/v): 0.2% KH₂PO₄, 0.7% K₂HPO₄, 1.0% (NH₄)₂SO₄, 0.05% sodium citrate (Kyowa Hakko Kogyo, Tokyo, Japan), 0.01% MgSO₄, 0.2% glucose and 1 µg mL⁻¹ thiamine, 1.5% (w/v) agar (Wako, reagent grade)] supplemented with three out of four amino acids [final concentration: 20 µg mL⁻¹ leucine, proline, histidine, arginine]. All media used for the cultivation of *E. coli* AB1157 were supplemented with 100 µg mL⁻¹ of streptomycin (Meiji Seika, Tokyo, Japan).

Recipient cells, *E. coli* AB1157 (5.33×10^7), were mixed with AfVPs to obtain a multiplicity of infection (MOI) of 10, and left undisturbed for 30 min at 30°C. A portion of the mixture withdrawn at 15 min after initiation was fixed with 2.5% (v/v) glutaraldehyde, and particles and cells were counted by EM to determine the practical MOI.

For the examination of lethality, 100 µL of a 10^5 -fold dilutant with TBT buffer was plated in triplicate in LB plates (17) with the over layer method with 0.6% TBT agar, and incubated for 1 d at 30°C.

For the examination of gene transfer, the original mixture (100 µL) was plated in the selection medium and incubated for 5 d at 30°C. Two controls were included: 1, recipient cells with TBT buffer instead of AfVPs to determine the spontaneous revertant rate; 2, ultraviolet light (UV)-treated AfVPs and recipient cells. For UV treatment, 400 µL of an AfVP suspension (4.0×10^{11} particles) was

placed in a quartz container with a water column thickness of 2 mm, and irradiated with a 12-W UV sterilising lamp (CosmoBio, Tokyo, Japan) for 15 min from a distance of 10 cm. As a reference, the same UV irradiation resulted in a reduction in the plaque-forming ability of coliphage T4 (2×10^9 PFU mL⁻¹) by seven orders of magnitude (2×10^2 PFU mL⁻¹).

Colonies generated in the respective selection medium were transferred with a sterile tooth pick to all other unselected marker plates, and cultured for 5 d at 30°C, from which results co-ordinated marker transfer was determined. The gene transfer potential of AfVPs could be estimated from this result, since a single infection cannot result in colonies with co-ordinated marker transfer. Furthermore, the gene transfer capability of AfVP can be inferred in relation to the surface exclusion (1) at such a high MOI.

The growth profile of generated colonies was examined in LB broth at 30°C with shaking at 60 rpm.

Electrophoretic analysis of nucleic acid species encapsulated in AfVPs

AfVPs and *A. fischeri* cells were embedded in 1.0% (w/v) agarose (SeaKem ME; FMC, Philadelphia, PA, USA) gel plugs. An *in situ* lysis method (8) was adopted, and Crossfield agarose gel electrophoresis was conducted (Crossfield AE-6800; ATTO, Tokyo, Japan). The gel plug was embedded in 1.5% (w/v) agarose, and the gel was run using 0.5×TBE buffer (45 mM Tris-borate, 2 mM EDTA, pH 8.0; Wako) at 14.3 V cm⁻¹; 200 mA; alternate angle, 110°; alternate time, 30 s; at 12°C for 14 h. Nucleic acid species were visualized by staining the gel with a 10,000-fold dilution of SYBR® Gold stock solution in 0.5×TBE buffer at 20°C for 1 h, then illuminated on a Spectroline Trans Illuminator Model TC-365A (Spectronics, Westbury, NY, USA) at 365 nm. Results were recorded with an Olympus E1 single-lens reflex digital camera (Olympus, Tokyo, Japan).

Results

Aliivibrio fischeri cell growth and particle production

As shown in Fig. 1, *A. fischeri* entered a logarithmic phase of growth for up to 12 h immediately after the initiation of culture showing a generation time of *ca.* 1.84 h, and then entered a stationary phase, in which cell abundance stabilised at *ca.* 1.2×10^9 cell mL⁻¹ until the end of the culture. Initiation the production of particles, referred to as AfVPs, by budding (Fig. 1E) deduced from the particle per cell ratio and particle induction frequency (Fig. 1B and D) occurred *ca.* 15 h after initiation. The numbers of free particles continued to increase up to *ca.* 50 h after initiation, and then stabilised at 1.20×10^{10} particles mL⁻¹. Three sharp peaks (24 h, 100 h, and 200 h) of induction were recorded, while no marked change in the cell population was observed to the end of culture. The average number of mature AfVP particles per cell was *ca.* 3 (average±SD, 3.0 ± 0.9 , $n=475$; see Fig. 1C). As shown in Fig. 1E, cells with budding particles and other particles were heavily surrounded by mucoidal substances. Budding particles from the cells showed a clear boundary of dense inclusion bodies.

Characteristics of AfVPs observed during purification

From 315 h into the culture of *A. fischeri*, 3.40×10^{15} particles in total (100%) were recovered from the broth supernatant by centrifugation (7,800×g, 40 min, 4°C). AfVP production was accompanied by mucoidal substances (Fig. 1E, Dull shape of particles surrounding the cell), a trait observed in marine strains (3, 5), which reduced the rate of recovery

Fig. 1. Growth profile of *Aliivibrio fischeri* with specific reference to particle per cell ratio and induction frequency. (A) Growth profile: ●, cells mL⁻¹; ○, particles mL⁻¹. (B) ◆, Change in particle per cell ratio during the culture. (C) ■, Change in "burst size" during the culture. Average number of particles produced is *ca.* 3 (mean±SD, 3.0 ± 0.9 , $n=475$). (D) ○, Change in induction frequency. Induction frequency is given as the fraction of AfVP-bearing cells in the total cell population by EM count. (E) Budding production of AfVPs from a *ca.* 170 h incubated *A. fischeri* cell surrounded by mucoidal substances, in which free particles are embedded. Scale bar, 500 nm.

using a tangential flow concentration system ($0.2 \mu\text{m} > \text{particle} > 100 \text{ kDa}$). Namely, 1.08×10^{15} particles were recovered, corresponding to 39% of the initial number. CsCl density-gradient equilibrium ultracentrifugation resulted in 1.07×10^{15} particles (28%). Nucleic acid and protein contents of AfVP in the final specimen were 4.010 mg and 101.764 mg, which corresponded to 29.8% and 35.8% of the initial values, respectively. The ratio of protein per nucleic acid was 24.9, comparable to values reported to date (3, 5–10). Another feature of AfVP is its coagulating behaviour. As often found under EM, AfVPs formed clusters, which could be dispersed by adding EDTA, whose titration point to release entire particles was 6.7 mM.

The buoyant density (ρ^{25}) of AfVP was between 1.3443 and 1.3980 g cm⁻³ (Fig. 2A). AfVPs had a spherical structure (Fig. 1E), 18.1 to 159.2 nm ($n=627$) in diameter. The range between 30.2 and 84.6 nm (95.1% of the overall population) showed a standard probability curve (statistic: D'Agostino-Pearson and Jaque-Bera, significance level, 5%) with a con-

Fig. 2. CsCl density gradient equilibrium ultracentrifugation profile of AfVPs produced by *Aliivibrio fischeri* NCIMB1281^T. (A) Superimposed photo placed above shows the result of ultracentrifugation. Trend curve shows relation between ultracentrifuge tube's distance from bottom and buoyant density (right axis). Relative intensity (left axis) of particle sedimentation profile of the photo as a Gray value obtained by subtracting the background using ImageJ, which is available from the public domain (<http://rsbweb.nih.gov/ij/>), draws single normal probability distribution. (B) Size distribution of AfVPs showed a standard probability curve (statistic, D'Agostino-Pearson and Jaque-Bera, significance level=5%) with a continual increase in diameter. (C) Relationship between buoyant density versus particle diameter, and encapsulated dsDNA in a particle. Capacity of the particle was obtained at a known packed ratio of dsDNA such as Coliphages T4, lambda and p1 (1, 5). The trend line was drawn by combining other findings for VP (3, 5–6, 8, 10) with the present data. ○, particle diameter in nm; ●, DNA content of the particle in kbp.

tinual increase in diameter. The major proportion consisted of particles 40.3 to 76.6 nm in diameter [84.2% of the overall particle population (median±SD, 58.4±11.9 nm, *n*=528), Fig. 2B], whose estimated DNA content in kbp would vary between 14.6 and 135.7 (Fig. 2C and 3A). The distribution of particle abundance in the sedimentation profile exhibited a similar continual increase with size, an apparent difference from findings concerning the size distribution of particles showing discrete bands, which were composed of several subpopulations (10).

The trend curve showing the relation between the distance of the ultracentrifuge tube from the bottom and buoyant density (Fig. 2A) is given as:

$$f_{\text{Buoyant density}}(d) = -0.0004d^3 + 0.0087d^2 - 0.0978d + 1.5605$$

$$(R^2 = 0.999)$$

Where: *d* stands for distance from the bottom in cm. As an extension (Fig. 2C), particle diameter and content as dsDNA would be expressed as the following functions of buoyant density (*ρ*).

$$f_{\text{Particle diameter}}(\rho) = 14,027\rho^3 - 53,695\rho^2 + 67,125\rho - 27,197$$

$$(R^2 = 0.999)$$

$$f_{\text{Particle DNA content}}(\rho) = -571,110\rho^3 + 2,394,972\rho^2 - 3,347,855\rho + 1,560,453$$

$$(R^2 = 0.999)$$

Results of a nucleic acid type analysis using Crossfield agarose gel electrophoresis (Fig. 3A) gave bands in kbp between 7.4 and 125.8 from *A. fischeri* cells and 17.3 and 95.3 from AfVPs as encapsulated molecular mass as dsDNA (Fig. 3B), which accords well with that predicted from the empirical functions (see above, Fig. 2C). The major propor-

Fig. 3. Molecular type analysis of AfVP encapsulated nucleic acids, and translocation of AfVP content to the recipient cell.

(A) Nucleic acid species encapsulated in AfVPs: Lane 1, Recipient, *Escherichia coli* AB1157, as a negative control; Lane 2, 72 h cultured *Aliivibrio fischeri* cell; Lane 3, AfVP; Lane λl, Lambda ladder [48.5 kbp–1.2 Mbp, FMC]; Lane Sc, *Saccharomyces cerevisiae* chromosome [220 kbp–1 Mbp, Nippon Gene, Tokyo, Japan]; and Lane λh, λ/HindIII [Nippon Gene]. (B) Trend line of dsDNA molecular mass for AfVP. Specific migration distance was obtained by comparing with the mass standards on the electrophoretogram using a densitogram created by ImageJ (<http://rsbweb.nih.gov/ij/>).

tion of AfVPs, obtained as $\rho^{25}=1.3560\text{--}1.3929\text{ g cm}^{-3}$, was used as the material to examine the lethal effect and gene transfer capability.

AfVP-mediated gene transfer to an auxotrophic Escherichia coli

The marker *thr-1* was not used because of its high rate of spontaneous reversion (10^{-7}) (5). Fig. 4A shows an EM image of the AfVP particle content to be under translocation to recipient *E. coli* cytosol. Multiplicity of infection (MOI), the number of particles per cell, was originally designed to be 10, but the observed MOI was actually 140.3. Despite such an extremely high MOI, no lethality in recipient *E. coli* AB1157 cells was observed. Results were expressed as efficiency of plating (EOP), with the number of colonies formed in TBT control plates taken as 100% (average $\pm$ SD, $100\pm 11\%$, $n=9$). The EOP of UV-treated AfVPs was $136\pm 14\%$ ($n=9$). That of untreated AfVPs was $116\pm 39\%$ ($n=9$). In other words, AfVP was not lethal to the recipients regardless of UV-treatment. As a result of gene transfer mediated by AfVPs, amino acid deficiencies in the recipient *E. coli* were successfully restored. Transduction frequency is given as fraction of the generated colony number in each selected marker plate in the total applied particles (1.81×10^5 particles per plate). Spontaneous reversion frequencies were found to be below the level of detection for the markers employed. No gene transfer was detected in UV-irradiated AfVPs used as mediators. On the contrary, colonies formed in the respective selection medium were: *leu*⁺, 27; *pro*⁺, 24; *his*⁺, 1; *arg*⁺, 48; and MM plate without any amino acid requirements, 29. Result of co-ordinated marker determination revealed that every colony generated in any selected

marker plates exhibited growth in any unselected marker plates. Consequently, 129 transductant colonies, whose amino acid auxotrophy was totally restored, were obtained. Transduction frequency for the respective marker is estimated to be: *leu*⁺, 1.5×10^{-4} ; *pro*⁺, 1.3×10^{-4} ; *his*⁺, 5.5×10^{-6} ; *arg*⁺, 2.6×10^{-4} ; and all amino acid requirements restored, 1.6×10^{-4} CFU per particle.

Particle production from the transductants, referred to as AfV-E-trans, grown in LB broth at 30°C was confirmed. AfV-E-trans acquired budding particle production as shown in Fig. 5B. Maximum growth, corresponding to *ca.* 415% of that of the parental *E. coli* recipient, was recorded at 170 h incubation.

Discussion

To extend our knowledge of “broad-host range gene transfer particles”, a luminal marine bacterium, *Aliivibrio fischeri*, was examined for its ability to produce a specific particle having a gene transfer capability. This study may initiate effects to elucidate HGT, which is predicted from the sequence data (35).

The oscillating nature of the induction frequency was different from the particle production observed for other microbes taken from various water columns (3–8). The *ca.*

Fig. 4. Translocation of content from infecting AfVPs to recipient *Escherichia coli* with specific reference to budding particles from *Aliivibrio fischeri* and AfV-E-trans.

(A) Diagrammatical representation of particle budding from the cell surface. A discrete boundary can be seen around the budding particle as indicated by an arrow. (B) AfVP budding from *A. fischeri*. (C) Particle budding from AfV-E-trans, a transductant *E. coli* generated with the aid of AfVP-mediated transduction. (D) and (E) Translocation of content from infecting AfVPs to recipient *E. coli*. On transduction, a portion of AfVP and recipient mixture was fixed with 2.5% (v/v) glutaraldehyde at 15 min after initiation and stood undisturbed for 3 h at ambient temperature to determine practical MOI. (D) shows EM image of UV-AfVP infecting the recipient, and (E) is a diagrammatical representation of the translocation of UV-AfVP content into the recipient. Scale bars, 100 nm.

Fig. 5. Growth profile of AfVP-mediated *Escherichia coli* transductant (AfV-E-trans) with specific reference to parental *E. coli* AB1157 (number per mL $\pm$ SD, $n=3$). (A) AfV-E-trans was cultured in LB medium at 30°C with shaking at 60 rpm. ●, AfV-E-trans cells mL⁻¹; ○, particle mL⁻¹; ▲, *E. coli* AB1157 cells mL⁻¹. (B) Photo represents budding production of particles from *ca.* 48 h cultured AfV-E-trans cells. Scale bar, 500 nm.

20% higher induction frequency shown by *A. fischeri* should have a positive effect on the survival of the strain, since the induction frequency for some marine strains of *Alphaproteobacteria* fell between 4 and 9% under comparable condition (5). As for the “burst size” of AfVPs, *ca.* 3 mature particles were observed in an induced cell (Fig. 1C), which is comparable to previous findings (9, 10). As no disrupted particles were observed during the periods corresponding to the decrease in particle per cell ratio (Fig. 1B), it is likely that particles in the broth would again be incorporated into the cells. Similar phenomena were observed for *E. coli* transductants (10) generated with the aid of particles collected from a geothermal vent at the Toyoha mine (19).

Predicted values for AfVP encapsulated dsDNA were comparable to that obtained from gel electrophoresis (Fig. 2C and 3).

In AfVP-mediated transduction, translocation of AfVP content to recipient *E. coli* cytosol was observed by EM as shown in Fig. 4D. It is likely that the particle membrane remains outside of the recipient cell, and the string-like structure coated with a proteinaceous material was unravelled as traced in Fig. 4E. Empirical findings make it possible to distinguish infecting particles from budding particles, since the boundary of infecting particle content is unclear. In contrast, budding particles show a clear boundary of its dense content (Fig. 4A–C). An observation done for similar particle production in a cell revealed that the host chromosomal DNA string was coated with a proteinaceous material, which was confirmed using a gold-labelled antibody (11, 31).

As for transduction frequency in marine environments, although the transduction frequency is intricate to estimations *in situ*, virus-mediated HGT has been documented in studies of freshwater and marine systems (13, 22, 26). Jiang *et al.* (13) made an effort constructing an applicable transduction studies model system in marine environments. Consequently, applying several lysogenic phage-host systems isolated from Hawaiian coastal waters, transduction frequencies ranging from 1.3×10^{-7} transductant PFU⁻¹ (for laboratory assays) to 3.7×10^{-8} transductant PFU⁻¹ (for natural communities) either from wild-type strains or concentrates of natural marine bacterial communities as recipients (14) were found. Transduction frequencies reported to date, evaluated using either natural bacterial or viral communities, have varied from 10^{-8} to 10^{-5} per transducing particle (33), of which the highest was documented by the authors (5, 6). We have suggested that such broad-host range VPs transferred genes through generalised transduction, since VPs from different sources showed similar gene transfer efficiency with respect to the genetic marker rescued (3–7, 10). From findings to date, the VP-mediated transductants acquired the ability to produce particles, and regenerated particles from the transductants had a lethal effect and were capable of gene transfer into the *E. coli* recipient (3–7, 10). In contrast, the current findings endorsed a further impact for HGT on marine environments because AfVPs were non-lethal to recipients of distant phylogeny together with inter-generic VP-mediated gene transfer. The transduction frequency of AfVPs was found to be higher by 2 to 3 orders of magnitude than that reported for naturally isolated transducing phages (12, 24, 27, 33), and almost comparable to our previous findings in marine

environments (5, 6). This is the first demonstration of VP-mediated HGT achieved experimentally between marine *Aliivibrio* and enteric bacteria. Furthermore, extremely high co-ordinated marker transfer frequencies, implicated in concomitant gene translocation through multiple infection, would provide a clue for understanding the gene flux in marine environments.

The current results suggest that gene transfer occurred as generalised transduction, because comparable marker transfer frequencies were observed. Co-ordinated marker transfer examined for transductant colonies generated in selection media (100 colonies in total) revealed that all the transductants formed colonies on all other unselected marker plates, namely, the co-ordinated marker transfer frequency was 100%. Apparently, supplementary genes, which were additionally translocated to the recipient by AfVP multiple infection, were not excluded, but successfully expressed in the transductant (1). In principle, the analysis of genetic co-ordinated marker transfer has arisen from classical genetics of monozygotic crosses (1). Transduction-like gene transfer mediated by AfVPs is somewhat unusual, because additional incorporation in multiple particles seems to promote recipient viability. To estimate the occurrence of transductants with prototrophic reversion of all the four markers adopted, the following approximation was attempted. On the basis of particle volume and coliphage T4 DNA packed ratio (2.34×10^{-24} L bp⁻¹), AfVPs would encapsulate linear dsDNA from 1.3 kbp to 902.7 kbp. A major proportion (*ca.* 84%) of the particle would encapsulate 14.7 kbp to 100.5 kbp, whose diameter varied from 40.3 nm to 76.6 nm. Although AfVPs varied in size, supposing that the major proportion consisted of a median particle size of 58.4 nm, the dsDNA content of the particles is estimated at 44,665 bp. Distance in bp between adopted respective *E. coli* biochemical marker orthologues on *A. fischeri* chromosome 1 exceeds the content, which could be encapsulated in one particle: *argE3-hisG4*=1,478,467; *hisG4-proA2*=313,679; *proA2-leuB6*=508,637; and *leuB6-argE3*=606,441 according to *V. fischeri* ES114 genome sequence (25). Consequently, it could be hardly expect that a substantial proportion of AfVPs applied would be particles with genetic resources covering all the biochemical markers to be restored in a recipient at a single infection. However, current transduction experiments resulted in transductants with all the adopted markers restored to prototropy. Therefore, additional incorporation in multiple “infections” of a recipient without any hindrance during transduction is indispensable for generating transductants.

The *E. coli* transductant successfully acquired the ability to produce particles (Fig. 5). It is notable that the extent of transductant growth was much enhanced in comparison with that of the mother strain, *E. coli* AB1157. A distinctive difference is apparent for the transducing particles of typical bacteriophages, which contain a maximum of 1% of the host genome in frequency of 10^{-5} to 10^{-7} cells per particle and exhibits nearly 100% lethality (1) in recipient. The “production and incorporation trait” of the particle during the growth certainly endorsed the non-virulence of AfVPs in the transduction experiment. According to the growth profile, the proportion of free particles standing alone was rather scarce in

the particle population, whereas the major proportion was observed as clustering bodies to make assemblages of more than 100 individuals. When such clustered particles were exposed to EDTA, the proportion of free particle increased as described above. In other words, gene transfer experiment under 10 mM Mg²⁺ may enhance the chance of the recipient incorporating multiple numbers of AfVPs. Under the circumstances, a high MOI (140) was applied in the gene transfer, which resulted in 129 transductants with all the markers reverted across the phylogenetic order from *Vibrionales* to *Enterobacteriales* with a considerably high transduction frequency.

Concerning HGT in *Vibrionaceae*: Kasai *et al.* (15) deduced from a *lux* operon sequence analysis that the operon and related gene clusters would have been horizontally transferred from *Aliivibrio* to *Schwanella*. Urbanczyk and colleagues (30) examined *lux* gene horizontal transfer in *Vibrionaceae*, and concluded that HGT routes based upon results of phylogenetic analyses were not substantially clear, and the contribution of HGT to the speciation would have been inadequate. Despite the prediction, sufficient updated findings are possible to upward revision. Phylogenetic analyses of the *lux* operon and related gene clusters, done by Yoshizawa (35), elucidated more frequent HGT than that ever considered in *Vibrionaceae*. In relation to this contemporary knowledge, accomplishment of HGT for *lux* operon and related gene clusters would be an inevitable consequence of a gene transfer scheme capable of huge gene cluster translocation with relaxed restrictions.

The current results suggest the horizontal transfer of regulatory gene assemblages to be accomplished by concomitant gene translocation and expression with the aid of non-virulent particles at a high MOI in nature. Together with non-inhibitory effects of additional gene incorporation to the recipient, the trait of AfVP is separate from the conventional virus concept, and is likely to be an apparatus aimed for preservation of the host's chromosomal genes. As we reported, various functional gene clusters such as thermo-resistance was transferred to the *E. coli* mediated by VP (10). VP-mediated serial transduction supports an indispensable role in microbial differentiation and evolution as mentioned above.

Acknowledgements

This paper is dedicated to the memory of Mari Segawa, who thrived on arduous tasks as a student member of JSME at the beginning of VP-mediated HGT study.

The authors are grateful to S. Yoshizawa, B. Velimirov, R.W. Ridge, M. Nishimura, K. Inoue and 2 anonymous peer reviewers for critical reading, advice, thoughtful comments, and useful suggestions on the manuscript. The authors' research presented here was supported in part by Grant-in-Aid for Scientific Research No. 16310031 from the Japan Society for the Promotion of Science (JSPS), and funded partly by Donations to Encourage Research by Kyowa Hakko Kogyo Ltd and Life Cycle Association Ltd to hxc. Last and certainly not least, thanks are given to the authors' families.

References

1. Birge, E.A. 1994. Bacterial and bacteriophage genetics. 3rd ed. Springer-Verlag, New York, USA.
2. Børshiem, K.Y., G. Bratbak, and M. Haldal. 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Appl. Environ. Microbiol.* 56:352–356.
3. Chiura, H.X., and J. Takagi. 1994. Phage-like particles production and gene transfer by marine bacteria. *Bull. Jap. Soc. Microb. Ecol.* 9:74–90.
4. Chiura, H.X., K. Kato, and J. Takagi. 1995. Phage-like particles released by a marine bacterium. *Wien. Mitteil.* 128:149–157.
5. Chiura, H.X. 1997. Generalized gene transfer by virus-like particles from marine bacteria. *Aquat. Microb. Ecol.* 13:74–85.
6. Chiura, H.X., B. Velimirov, and K. Kogure. 2000. Virus-like particles in microbial population control and horizontal gene transfer in aquatic environments, p. 167–173. In C.R. Bell, M. Brylinsky, and P. Johnson-Green (ed.), *Microbial Biosystems: New Frontiers*. Atlantic Canada Society for Microbial Ecology, Halifax, Canada.
7. Chiura, H.X. 2002. Broad host range xenotrophic gene transfer by virus-like particles from a hot spring. *Microbes Environ.* 17:53–58.
8. Chiura, H.X., H. Yamamoto, D. Koketsu, H. Naito, and K. Kato. 2002. Virus-like particle derived from a bacterium belonging to the oldest lineage of the domain bacteria. *Microbes Environ.* 17:48–52.
9. Chiura, H.X. 2004. Novel broad-host range gene transfer particles in nature. *Microbes Environ.* 19:249–246.
10. Chiura, H.X., and M. Umitsu. 2004. Isolation and characterisation of broad-host range gene transporter particles from geo-thermal vent of Toyoha Mine. *Microbes Environ.* 19:20–30.
11. Chiura, H.X., and B. Velimirov. 2007. New serial transducing particles: Are we still missing the Essential?, p.45. In *Concurrent Meeting: ISME Asian 2007 and the Annual Meeting of the Japanese Society Microbial Ecology*, Matsuyama, Japan.
12. Fuhrman, J.A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature* 399:541–548.
13. Jiang, S.C., C.A. Kellogg, and J.H. Paul. 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Hawaii. *Appl. Environ. Microbiol.* 64:535–542.
14. Jiang, S.C., and J.H. Paul. 1998. Gene transfer by transduction in the marine environment. *Appl. Environ. Microbiol.* 64:2780–2787.
15. Kasai, S., K. Okada, A. Hoshino, T. Iida, and T. Honda. 2007. Lateral transfer of the *lux* gene cluster. *J. Biochem.* 141:231–237.
16. Kobashi, Y., A. Hasebe, M. Nishio, and H. Uchiyama. 2007. Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. *Microbes Environ.* 22:44–51.
17. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Purification of bacteriophage λ , p. 80–84. In Maniatis, T., E.F. Fritsch, and J. Sambrook. (ed.), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, USA.
18. Miller, R.V., and G.S. Saylor. 1992. Bacteriophage-host interactions in aquatic system, p. 176–193. In E.M.H. Wellington and J.D. Van Elsas (ed.), *Gene interactions among microorganisms in the natural environment*, Pergamon Press, Oxford, UK.
19. Nakagawa, T., S. Hanada, A. Maruyama, K. Marumo, T. Urabe, and M. Fukui. 2002. Distribution and diversity of thermophilic sulfate-reduction bacteria within a Cu-Pb-Zn mine (Toyoha, Japan). *FEMS Microbiol. Ecol.* 1378:1–11.
20. Nonaka, L., K. Ikeno, and S. Suzuki. 2007. Distribution of tetracycline resistance gene, *tet(M)*, in gram-positive and gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes Environ.* 22:355–364.
21. Paul, J.H., J.B. Rose, S.C. Jiang, and C. Kellogg. 1993. Distribution of viral abundance in the reef environment of Key Largo, Florida. *Appl. Environ. Microbiol.* 59:718–724.
22. Paul, J.H. 1999. Microbial gene transfer. *J. Mol. Microbiol. Biotechnol.* 1:45–50.
23. Proctor L.M., and J.A. Fuhrman. 1990. Viral mortality of marine bacteria and cyanobacteria. *Nature* 343:60–62.
24. Replicon, J., A. Frankfater, and R.V. Miller. 1995. A continuous culture model to examine factors that affect transduction among *Pseudomonas aeruginosa* strains in freshwater environments. *Appl. Environ. Microbiol.* 61:3359–3366.
25. Ruby, E.G., M. Urbanowski, J. Campbell, *et al.* 2005. Complete genome sequence of *Vibrio fischeri*: A symbiotic bacterium with

- pathogenic congeners. Proc. Natl. Acad. Sci. USA 102:3004–3009.
26. Saye, D.J., and R.V. Miller. 1989. The aquatic environment: Consideration of horizontal gene transmission in a diversified habitat. p. 223–259. In S.B. Levy and R.V. Miller (ed.), *Gene transfer in the environment*. MacGraw-Hill, New York, USA.
27. Saye, D.J., O.A. Ogunseitan, G.S. Sayler, and R. V. Miller. 1990. Transduction of linked chromosomal genes between *Pseudomonas aeruginosa* strains during incubation in situ in a freshwater habitat. Appl. Environ. Microbiol. 56:140–145.
28. Takashima, Y., T. Yoshida, A. Kashima, S. Hiroishi, and K. Nagasaki. 2007. Cryopreservation of a myovirus infecting the toxin-producing cyanobacterium *Microcystis aeruginosa*. Microbes Environ. 22:297–299.
29. Urbanczyk, H., J.C. Ast, M.J. Higgins, J. Carson, and P.V. Dunlap. 2007. Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov., Int. J. Syst. Evol. Microbiol. 57:2823–2829.
30. Urbanczyk, H., J.C. Ast, A.J. Kaeding, J.D. Oliver, and P.V. Dunlap. 2008. Phylogenetic analysis of the incidence of *lux* gene horizontal transfer in *Vibrionaceae*. J. Bacteriol. 190:3494–3504.
31. Velimirov, B., H.X. Chiura, and K. Kogure. 2005. New transducing particles in the sea or are we still missing the essential? p. 249–250. In M. Che, G. Drazic and S. Fidler (ed.), *Proceedings of 7th Multinational Congress on Microscopy*. Slovene Society for Microbiology, Ljubljana, Slovenia.
32. Weinbauer, M.G., D. Fuks, and P. Peduzzi. 1993. Distribution of viruses and dissolved DNA along a coastal trophic gradient in the Northern Adriatic Sea. Appl. Environ. Microbiol. 59:4074–4082.
33. Weinbauer, M.G., and F. Rassoulzadegan. 2003. Are viruses driving microbial diversification and diversity? Environ. Microbiol. 6:1–11.
34. Wommack, K.E., and K.K. Colwell. 2000. Virioplankton: viruses in aquatic systems. Microbiol. Mol. Biol. Rev. 64:69–114.
35. Yoshizawa, S. 2009. Diversity of light emission spectra of marine luminous bacteria. Ph.D. thesis, University of Tokyo, Tokyo, Japan.