Functional gene transfer towards a broad range of recipients with the aid of vector particles originating from thermophiles

Toshihiro Sugitate, and Hiroshi X. Chiura*
International Christian University, 3-10-2 Osawa, Mitaka, Tokyo 181-8585, Japan
*E-mail: hxc@icu.ac.jp

Abstract
Escherichia coli, a mesophilic bacterium, acquired viability above its permissive temperature with the aid of broad-host range gene transfer particle (VP) originating from Aquifex sp and Thermococcus kodakaraensis B41. Gene transfer capable VPs have likely mediated the transfer of a functional thermo-resistance gene. VPs are already known to transfer enzyme genes to rescue auxotrophic recipients. VP particles, as part of a group of ubiquitously distributing virus-like particles (VLPs), are likely to take part in gene flux that enables horizontal gene transfer that enhances biodiversity and evolution.

Keywords; broad-host range gene transfer particle (VP), virus-like particles (VLP), thermo-resistance gene

1. Introduction

Virus-Like Particles (VLPs) generally refers to those particles that showing similar morphology to viruses by electron microscopic observation. A high abundance of VLPs, ca 10-100 times higher than bacterial population are found in all aquatic environments [1-5]. The postulated roles for VLPs in the aquatic environment are bacterial population control and gene transfer in situ [6], however, their biological characteristics and process of generation are still almost unknown. Information on such viruses has been almost restricted to lytic populations [7], whose host range has been considered to be limited to a range of phylogenetically-specific organisms.

There are a number of types of VLPs, which are derived from various water columns such as surface sea water, hot spring, and submarine hydrothermal fluids, and which all showed lethal effects and gene transfer capability towards species distantly related from the original hosts [8-13]. Such VLP particle production occurs by budding without host cell lysis [13].

An example of particles with broad host-range so far examined is derived from the hyper-thermophilic anaerobic archaeon, Thermococcus kodakaraensis B41 classified in the Domain Archaea, which inhabits the Suiyo Seamount (Chiura et al, Abstr. Marine Biotech. Conf. 2003). The particles derived from this archaeon (referred to KD-VLPs) are able to mediate gene transfer to the auxotrophic and mesophilic recipient Escherichia coli AB1157, and transductants with restored amino acids deficiencies can be obtained (Chiura et al, Abstr. Marine Biotech. Conf. 2003). Such obtained transductants acquire particle production capability.

The purpose of this study is to examine thermo-resistance gene transfer towards mesophile recipients by KD-VLPs derived from the thermal-environment. T. kodakaraensis B41, a KD-VLP-producing microorganism that inhabits thermophilic environments at ca 250°C, is expected to have thermo-resistance of high quality, and VLPs derived from this strain might expect to contain thermo-resistance genes.

Gene transfer, other than for amino acid requirement, was tested in KD-VLP-mediated E. coli transductants. Another E. coli transductant, ST-E-trans [11, 13], which was transduced by a particle originating from Aquifex sp [14,15], a thermophilic bacterium, was also used. Transductants were cultured for 15 days at various temperatures (50, 56, and 70°C), which are above the permissive temperature for E. coli.

2. Materials and Methods
2.1 Source of vector particles

KD-VLPs: A hyper thermophilic archaeon, *Thermococcus kodakaraensis*, was successfully obtained from an APSK06 (28°34.313'N, 140°38.617'E, 1386 m deep) boring core, and cultivated in sulphur-supplemented medium at a temperature range of between 70 and 90°C by Hoaki. VLP production was observed under anaerobic culture condition in an elementary sulphur-supplemented medium in *T. kodakaraensis*, whose cell and particle abundance per ml at 480 h culture at 70°C was ca 3x 10^9 (Chiura et al, Abstr. Marine Biotech. Conf. 2003). The culture supernatant (30 litres) separated from cells by centrifugation (7,500 x g, 40 min, 4°C), which was passed through 0.2 μm membrane filter and concentrated to 20 ml using tangential flow concentration systems (Pellicon and Minitan, Millipore, Bedford, MA) as 0.2 μm > KD-VLPs > 100 kDa. Purified KD-VLPs (8.34 x 10^13 particles) were finally obtained as the specimen of the equilibrium CsCl density gradient centrifugation method (178,000 x g, 18h, 25°C), whose buoyant density (ρ25) was 1.3860 ± 0.0022 (n: 3). Encapsulated dsDNA was estimated to be 309 ± 8.65 kb (n: 3).

2.2 Employed microbial strains and culture conditions

Mesophilic and auxotrophic mutant *Escherichia coli* AB1157 (F; thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 metL1 proA2 his-4 argE3 rpsL31 tsx-33 supE44) was exclusively used as the recipient of KD-VLP-mediated gene transfer. The strain was first grown in 1.5% agar supplemented LB plate at 37°C overnight, and generated colonies were subjected to marker check using amino acid requirements as described [9]. Appropriate single colony with confirmed auxotrophic mutation was grown to mid-exponential phase (6.25 x 10^8 cells/ml) in LB liquid medium at 30°C, and then glycerin was added to the culture to make 7% as the final concentration, dispensed as 1.0 ml aliquots, frozen in LN2, and stored in a deep freezer at –80°C until use.

leu<sup>+</sup> ST-E-trans: The particle, referred to ST-E-VP, was obtained and purified from a ST-VLP-mediated gene transferred auxotrophic *E. coli* AB1157 (ST-E-trans) as described [11], whose buoyant density (ρ<sub>25</sub>) was 1.3356 ± 0.0165 (n: 3) with protein/nucleic acid ratio of 17.78 ± 2.47, and encapsulated nucleic acid as linear DNA was 373.33 ± 23.1 kb (n: 49) [11]. This strain showed prototrophic for amino acid requirement, while it was originally selected as leucine revertant at multiplicity of infection (MOI) of 5.48.

Generated ST-VP-mediated leu<sup>+</sup> *E. coli* transductants at MOI of 0.16 were used for the examination of thermo-resistance gene transfer. The leu<sup>+</sup> transductants were prepared through 3 generations by infecting the particles from the generated transductants to recipient *E. coli*. At preparation of the 3<sup>rd</sup> generation transductant, recipient *E. coli* AB1157 (1.52 x 10^8 cells) was infected with STEVP (1.58 x 10^8 particles) produced by the 2<sup>nd</sup> transductant in TBT buffer [9] at 30°C according to the method described [9], and amino acid maker transfer was examined. A portion of the cell population was also applied to thermo-resistance acquisition test.

The leu<sup>+</sup> transductant was grown to ca 4 x 10^7 cells/ml in 240 ml of leu<sup>+</sup> Davis’s minimal medium (leu<sup>+</sup> MM) [0.2% KH<sub>2</sub>PO<sub>4</sub>, 0.7% K<sub>2</sub>HPO<sub>4</sub>, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05% NaCl, 0.01% MgSO<sub>4</sub>, 0.02 % glucose, 1 μg/ml thiamine, 20 μg/ml threonine, 20 μg/ml arginine, 20 μg/ml histidine, 20 μg/ml proline, pH 7.0], harvested by centrifugation (7,800 x g, 40 min, 4°C), and then subjected to sequential rinsing with the same medium, and centrifugation (12,000 x g, 5 min, 4°C). Washed cells were finally suspended in 7% glycerin supplemented leu<sup>+</sup> MM to make 4.61 x 10^8 cells/ml, dispensed as 1.0 ml aliquots, frozen in LN2, and stored in a deep freezer at -80°C until use.

2.3 VLP-mediated gene transfer and examination of acquisition of thermo-resistance

Gene transfer experiment was done as described [9] using KD-VLP at multiplicity of infection (MOI) of 0.1. Recipient *E. coli* AB1157 was grown to 4.3 x 10^8 cells/ml and concentrated by centrifugation (8,000 x g, 5 min, 4°C). Concentrated cells (3.43 x 10^8 cells) were suspended in 3.3 ml of LB [16] liquid medium, and mixed with KD-VLP (3.51 x 10^8 particles) suspended in 60 μl of TBT buffer. The mixture was stood undisturbed for 15 min at 30°C.
For examination of lethal effect, the same procedure for the gene transfer as described above was applied. The cells were diluted to make 1,000,000 folds dilution with TBT buffer, which was plated in LB plates in triplicate, and incubated for 2 days at 30°C. As for controls, recipient cells added with 60 μl of TBT instead of KD-VLP with/without UV treatment were used.

For the examination of thermo-resistance, KD-VLP-infected E. coli (2.60 x 10⁸ cells) was inoculated in LB-Gelrite plates supplemented 0.7% Gelrite (Wako Pure Chemical Ind., Ltd., Osaka Japan) and 0.1% MgSO₄ instead of agar, and incubated at 50, 56 and 70°C. In every single temperature condition, examination was done in triplicate. The formed colonies as transductants in these plates referred to KD-E-trans, which were grown in LB liquid medium to 4 x 10⁸ cells/ml at 30°C. Then, 1.04 x 10⁸ cells were transferred to 10 ml of LB liquid medium, and incubated at 50, 56 and 70°C, respectively.

As for controls, recipient cells added with 60 μl of TBT buffer instead of KD-VLP, with/without UV treatment was used. For UV treatment, KD-VLP suspension (5.86 x 10⁹ particles/ml) was placed in a quartz container with water column thickness as 2 mm, and irradiated with a 12 W UV sterilizing lamp (Cosmo Bio Co, Tokyo, Japan) for 15 min from 10 cm distance. Under the same condition, coliphage T4 (ca 3 x 10⁹ particles) reduced its plaque forming ability by seven orders of magnitude.

For examination of leu⁺ ST-E-trans, the transductants were grown in 5 ml LB liquid medium with shaking at 60 rpm at 30°C to 4.20 x 10⁸ cells/ml. Cells were then concentrated (8,000 x g, 5 min, 4°C) and of which 1.05 x 10⁹ cells were inoculated in LB-Gelrite plates in triplicate, and incubated at 50, 56 and 70°C. At the same time, 4.20 x 10⁸ cells of ST-E-trans were inoculated in 10 ml LB liquid medium and also incubated at 50, 56 and 70°C, respectively.

2.4 Methods for cell and particle count

Approximate number of cells was determined by a Thoma’s haemocytometer at appropriate dilution with TBT buffer at a magnification of x 400 with a phase-contrast optical microscope (Olympus BX50, OLYMPUS Co. Ltd, Tokyo, Japan).

The number of VLP was determined according to Børsheim [7], with modification [9]. Grids were stained for 30 sec with 0.5% uranyl acetate (Merk KGaA, Darmstadt, Germany), and then sequentially washed respectively for 15 sec with distilled water, and 95% ethanol twice. Enumeration was done with a JEM-1200EX transmission electron microscope (JEOL Inc., Tokyo, Japan) at a magnification of x 50,000 at an accelerating voltage of 80 kV. At least 50 eye fields were selected for counting.

The total and viable cell number of the transductants in culture broth at high temperature was examined by the epifluorescence staining method, using LIVE/DEAD (Molecular Probes, Eugene, OR). Fixed cells with 2% neutral-buffered formaldehyde (Merck) were subjected to rinsing with 1.0 ml of 0.2 μm filtered sterilized distilled water (SDW) and centrifugation (8,000 x g, 5 min, 4°C) for five times, and suspended in 1 ml of SDW. Then, the cells were stained with LIVE/DEAD at the final concentration of 0.3% for 15 min under dark at ambient temperature, filtered on a polycarbonate membrane filter (Advantec type K020N025A, TOYO ROSHI, Tokyo, Japan), and examined through an oil immersion epifluorescence microscope at a magnification of x 1,250 equipped with super high pressure mercury lamp with B excitation (Eclipse E600, Nikon Corporation Ltd, Tokyo, Japan). The number of bacteria/particles per millilitre were determined from a count exceeding four hundreds individuals.

3. Results and Discussion

3.1 Lethal effect on an auxotrophic E. coli AB1157 recipient, and particle-mediated thermo-resistance gene transfer

Table 1 shows the efficiency of plating (EOP) after exposure to KD-VLP at MOI of 0.1 on recipient cells, E. coli AB1157. Formed colony number in plates with TBT buffer instead of particles was regarded as 100%. Although the addition of KD-VLPs to the recipients did not give any plaque formation, KD-VLPs had a lethal effect on the recipients by reducing the EOP. It is notable that reduction of EOP was observed regardless of UV treatment of the particle. Lethality in the recipients caused by KD-VLP is not in line with the accepted concept of viruses [17-19], since the killing effect was observed regardless of UV-treatment of the particles.
In transduction, no preferential marker transfer was recorded, hence, the mode of gene transfer mediated by KD-VLPs was likely to be generalised transduction, sharing the same feature observed in the preceding studies [8-12]. The gene transfer frequency for similar “broad-host range gene transfer particles” has been shown to be between $10^{-6}$ and $10^{-3}$ transductants per particle [8-12,20]. A possibility is that a “broad-host range gene transfer particle” could be common in thermal water columns. Hereafter, the particles produced by *T. kodakaraensis* are referred to KDVPs. KD-E-trans acquired particle production (Chiura et al. Abstr. Marine Biotech. Conf. 2003).

Although, recipient strains did not grow at all the high temperature conditions examined, KD-E-trans grew in LB-Gelrite plate at 50°C, and gave 4 transductant colonies through gene transfer. When UV irradiated particles were used as the gene mediator, no transductant was detected. Transduction frequency is given as a fraction of generated colony number in LB-Gelrite plates at high temperature to the total applied particle number (7.81 x $10^7$ particles). Transduction frequency at 50°C in terms of the acquisition of thermo-resistance was estimated to be $5.12 \times 10^{-8} \text{ cfu/particle}$. Under the higher temperature conditions (56 and 70°C), none of transductants colony was generated. Comparing with the marker transfer frequency summarised in Table 1, acquisition of thermo-resistance trait for the mesophile would be difficult to establish showing that frequency were observed to be one and two orders of magnitude smaller than the amino acid marker transfer.

Amino acid marker transfer was observed for ST-E-trans (STEVP MOI: 1.03) of the 3rd generation as shown in Table 1, while no transductant with colony forming ability was obtained under the high temperature conditions examined. Consecutive transfer of genes relating to the thermo-resistance might have been deteriorated through serial transduction, which would have brought about the loss of colony forming ability to the generated transductants at the 3rd generation.

Table 1. EOP (± SD, n: 21) and amino acid maker transfer by KDVPs and STEVPs on *E. coli* AB1157

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV treatment a</th>
<th>EOP±SD, % b</th>
<th>Marker transfer frequency c</th>
<th>leu+</th>
<th>pro+</th>
<th>his+</th>
<th>arg+</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDVP</td>
<td>-</td>
<td>63.9±8.1</td>
<td>0.33±0.58</td>
<td>0.98±1.00</td>
<td>2.94±1.00</td>
<td>2.23±1.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>79.9±4.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>STEVPd</td>
<td>-</td>
<td>66.2±1.9</td>
<td>1.23±1.18</td>
<td>8.76±8.74</td>
<td>3.16±0.65</td>
<td>6.00±1.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>67.8±1.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a, VLP without (-) and with (+) UV irradiation. Condition is given in the text.
b, colony number formed with TBT buffer instead of VLP was regarded as 100%.
c, expressed per $10^6$ particles; ND: not detected.
d, particles produced by the second generation *E. coli* transductants.

3.2 Incubation profile of transductants with specific reference to parental recipient *E. coli* AB1157

At all the high temperature conditions, parental recipient, *E. coli* AB1157, decreased its population to smaller than a few percent of the inocula by 48 h, and then living cells became undetectable by LIVE/DEAD staining. It is known that “heat shock response” would be activated [21] when *E. coli* cells are transferred from optimal growth temperature to high temperature, however, such a response cannot afford to rescue the situation exceeding 50°C [22]. Hence, prolonged treatment condition, above 50°C, would bring about the disorganisation of cell structure resulting in cell death [23]. Profile observed in this study apparently exhibited the consequence of the heat treatment on the parental recipient, *E. coli* (Fig. 1).

Obtained KD-E-trans was inoculated as the initial population size as $1.23 \times 10^7$ cells/ml. The cells decreased to $6.31 \times 10^5$ cells/ml (ca 5% of the inocula) at day 5, and then cells grew to $7.48 \times 10^7$ cells/ml (ca 6 folds of inocula) at day 13 at 50°C. Generation time of the transductant in the period of propagation was estimated to be ca 28 hours. At 56°C condition, the cells decreased to $6.31 \times 10^7$ cells/ml (ca 25% at day 5, then cells grew to $1.95 \times 10^7$ cells/ml (ca 1.6 folds of the inocula), whose generation time in the growing period was estimated to be ca 73 hours. At 70°C, the cells decreased to $1.53 \times 10^7$ cells/ml (ca 12% of the inocula) at day 3, and then grew to $2.43 \times 10^7$ cells/ml (ca 2 folds of the inocula) at day 13, of
which generation time during the propagation was estimated to be ca 266 hours. Results are shown in Fig.1.

Change in population under high temperature incubation for ST-E-trans is also given in Fig.1. As for ST-E-trans (initial population size: 4.20 x 10^7 cells/ml), the population was observed to decrease to ca 2 x 10^7 cells/ml (ca 50%) towards the 2nd day, and maintained this level for another 4 days, then the cells changed to grow to ca 6 x 10^9 cells/ml (ca 140 folds of the inocula) in the following 2 days, and finally the population became stable. Generation time of the transductant during the growth was estimated to be ca 6 hours. At 56 °C, the cell number at day 2 was observed as ca 2 x 10^7 cells/ml (ca 50%), and its population became constant afterward. At 70°C, ca 2 x 10^7 cells/ml (ca 50%) of cells were observed at day 2, and its population became constant afterward.

![Fig. 1 Incubation profiles of KD-E-trans (upper row) and ST-E-trans (lower row) with specific reference to parental E. coli AB1157. Conditions are given in the text. Inoculum size (cells/ml) for KD-E-trans was 1.23 x 10^7, and 4.20 x 10^7 for ST-E-trans. Cell count under the test run was done by the epifluorescence LIVE/DEAD staining method on a polycarbonate membrane filter through an oil immersion epifluorescence microscope equipped with B excitation.](image)

When KD-E-trans was cultured in LB liquid medium at 37°C with shaking, the transductant showed a comparable period of the logarithmic phase to that of parental recipient, while attained maximum growth population was up to ca 10% of the parental recipient (data not shown). This trait is as well observed for ST-E-trans [12]. In addition, TY-E-trans, which is a transductant generated by VLPs originating from underground geo-thermal vent fluid (63.5°C) [12,13], exhibited comparable trait. TY-E-trans generated colonies at 50°C, whose thermo-resistance acquisition frequency was estimated to be 1.27 x 10^-7 cfu/particle [13].

Two transductant strains examined in this experiment underwent different processes before culturing at high temperatures. KD-E-trans was cultured at high temperature immediately after gene transfer from T. kodakaraensis. Therefore if the transductants received the genes for thermo-resistance, these genes would
have been kept and expressed. ST-E-trans cells were grown at 30°C to the mid-logarithmic phase, and then stored at -80°C. Followed by thawing, they were applied to the high temperature culture. Therefore, thermo-resistance gene may have been selectively discarded during culturing at a mesophilic temperature. Moreover, ST-E-trans is 3rd generation of *E. coli* transductant generated by the particles originating from *Aquifex* sp. Due to the fact that these strains were exposed to different conditions prior to culturing at high temperatures, it must be taken into consideration that each population of the strains might have original host gene in different extent.

4. Conclusion

*E. coli* is a mesophile, and under high temperature condition above permissive growth, cell is suffering from damages [23-25] and having to maintain a balance between the protective effect of heat shock protein and the metabolic cost by molecular mechanisms [26-29] as the countermeasure [21,30,31]. It is generally accepted that fragment size would be less than 40 kb in virus-mediated transductions [16], however fragment size deduced from thermo-resistance acquisition phenomenon may emerge as another gene transfer scheme in nature. The present study revealed thermo-resistant trait transfer, which could be evidence that the content of VP is a considerably large fragment of host chromosomal genes. The major molecular mass of DNA encapsulated was found ca 373 kb for STEVPs; and ca 310 kb for KDVPs, of which DNA content is large among the virus genomes [18]. A thermo-resistant or thermophilic trait shown by the transductants implies that broad-host range gene transfer particles (VPs) originating from thermophiles are capable of transferring genes towards distantly related recipients other than single enzyme genes. Gene transfer capable VPs have likely mediated the transfer of functional genes to take part in gene flux that enables horizontal gene transfer that enhances biodiversity and evolution.

Acknowledgments

The authors are grateful to N. Inaba, H. Naito D. Nakata and other Lab members at ICU for helpful collaboration and assistance with the experiments. Thanks also to H. Hoaki, Y. Kurusu, J. Ishibashi, A. Maruyama, T. Kakegawa, T. Urabe, Shinseimaru, Shinryumaru for assistance with sampling. Sincere thanks to H.W. Morgan and R.W. Ridge for critical reading, advice, useful comments, and suggestions on the manuscript. The research presented here was supported in part by Grant-in-Aid for Scientific Research No. 10490012, No. 12490009, No. 14208063, No. 14405016, No. 15310007 and No. 16310031, from the Ministry of Culture, Science, Sports and Education, Tokyo, Japan, and Japan Society for the Promotion of Science (JSPS), and also funded partly by Ministry of Education, Science, and Technology (MEXT), Japan, through a Special Co-ordination Fund "Archaea Park Project: International Research Project on Interaction between Sub-Vent Biosphere and Geo-Environments", and Donations to Encourage Research by Kyowa Hakko Kogyo Ltd to hxc. This article is completed in the Academic Frontier Project (2003-2007), International Christian University, supported by Ministry of Education, Culture, Sports, Science and Technology. Last and certainly not least, thanks are given to the authors' family.

5. References