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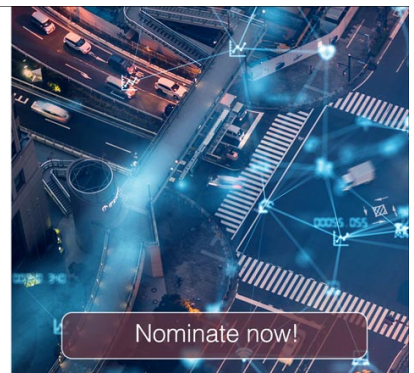
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Isolation protocol of jumbo phage from winter grass soil

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Abstract. Jumbo phage can isolated by two different methods and protocols. It depends on size, phage species, substrate sources, environment, media and tools. Each method has positive and negative results within each factors. The research aimed to determine the suitable protocol to isolate the jumbo phages from winter grass soil during winter conditions, utilizing two main protocols, the enrichment protocol and non-enrichment protocol. In Sequent, research was conducted with several steps, including bacterial isolation, jumbo phage isolation, plaque test in *Escherichia coli* colony, bacterial genus identification, phage test in soil bacteria, protocol evaluation, DNA extraction and electrophoresis. Based on the plaque size and plaque total, each protocol did not significantly differ on plaque total among the *E. coli* or each isolated soil bacteria. On the *E. coli* test, the plaque total average was 65 plaque, nor 45 plaque emerge on each isolated soil bacteria. The significant difference on each protocol was on the clarity of plaque. Based on visual observation, the plaques with enrichment protocol were clearer. It was obvious that the isolation of jumbo phage from the winter grass during winter conditions can be done without enrichment protocol. It can be isolated with standard isolation protocol.

1. Introduction

Bacteriophage or phage is a virus that infect bacteria [1]. It can effectively kill bacteria, high specific, abundant in nature, and with minimal risk of host toxicity [2]. Phage can utilize on broad area, from human health to food technology. For human, phage can be utilized as an alternative to tackle antibiotic resistance, a critical therapeutic problem [3]. Most antibiotics are non-specific, not only targeting specific pathogen, but also against other bacteria naturally present in the environment or plant and animal microflora [4]. In agriculture, phages offer a non-antibiotic method to improve food production and safety. Moreover, bacteriophage are increasingly being recognized as a potential solution to the bacterial pathogen detection, start/stop agents in fermentation of, and biocontrol agents of various undesirable bacteria or infectious diseases in animal food and crops [5].

Phages have many families, types, sizes, and mode of actions. More than 90% of bacteriophages have large or jumbo, double-stranded DNA genomes, with tails of varying lengths. They belong to there major morphological groups of bacteriophages. These are Myoviridae (with long, rigid, contractile tails), Siphoviridae (with long, flexible, noncontractile tails) and Podoviridae (with short, noncontractile tails) [6]. Phages exhibit extensive variation in genome size, ranging from ~10 kbp to >500 kbp [7]. Large phages with 200- to 500-kbp genomes have been defined as “jumbo phage”[8]. Many phage has lytic pathway to infected bacterial host depending on toxin production [9].



Each phage has different requirement for isolation protocol. Some of them would be perfectly isolated on one protocol rather than other. Location, isolation source, phage type and size are independent factors on protocol purposing [10]. Two main protocols, mainstream protocol and enrichment protocol reported can be used for isolate jumbo phage [11,12]. However, most articles not yet been focused or discuss about which method is suitable to isolate soil phage during winter condition. Due that why, our research aimed to determine the suitable protocol for isolation of jumbo phage from soil in winter condition.

2. Material and Methods

2.1. Material

The research material were soil from two different location and altitude around Higashiroshima, Hiroshima, Japan under the healthy grasses in winter condition. The TOP Agar, The LB Medium, Chloroform, Agarose H, Steradisk Cellulose Filter Paper 0,45 μ m and the SM buffer (50 mM TriseHCl [pH 7.5], 100 mM NaCl, 10 mM MgSO₄, and 0.01% gelatin). The tools were use in this research are Hitachi Himax Centrifuge, the Gel electrophoresis tools set and several microbiology laboratories equipment.

2.2. Methods

The main methods of the research were conducted with following the Saad *et al* [12] and comparison with the enrichment protocol by Carvalho *et al* [11]. Soil/Rhizospheres bacteria and soil phages were isolated from two different sites (Table 1), with different soil temperatures and altitudes, under common grasses in Higashishiroshima location, Japan.

Tabel 1. Bacterial and Phage Isolate Sources Information

Category	Loc. A	Loc. B
Common Name Location	JICA Chugoku International Center, Higashihiroshima, Japan	Hiroshima University Campus, Higashihiroshima, Japan
Coordinate	34°24'03.0"N 132°44'01.3"E	34°24'05.5"N 132°43'01.1"E
Soil Type	Sandy-Clay	Sandy-Clay
Elevation (a.s.l)	350 \pm 5 m	200 \pm 5 m
Soil Temperature (when taken)	-2 \pm 2 °C	-3 \pm 2 °C

2.3 Bacterial and Phage Isolation.

Each genus of soil bacteria were cultivated in LB medium at different temperatures[13]. Isolation of Jumbo Phage was follow saad *et al.* [12]. A 400 ml of each sample was centrifuged at 5000g for 10 min at room temperature to remove most particulates, unwanted debris and microbial cells and followed by re-centrifugation of the supernatant at 15,000g for 1 h at 4 °C, all the pellets were suspended by vortexing in 5 ml of SM buffer (50 mM Tris HCl [pH 7.5], 100 mM NaCl, 10 mM MgSO₄, and 0.01% gelatin). To remove small phages, this centrifugation cycle was repeated three times. Accordingly, an equal volume of chloroform was added to the final suspension to kill any residual contaminating bacteria. The detection of jumbo phages was used by standard plaque assays were modified as follows: Bacterial cultures (*Escherichia coli* strain BL-21 and isolated bacterial) were prepared in 4.5 ml LB broth and adjusted to OD 600 $\frac{1}{4}$ 0.25. A 100ml phage sample was mixed with 250 ml bacterial culture and shake for 30 min at room temperature. With 4.5 ml of molten LB top agar (0.35%), the mixture was poured onto an LB agar plate (1.5% agar). The plates were incubated for 24 h at 23 °C. Small plaques that appeared on the plates were selectively picked for further purification and enrichment. Single small plaque purification was repeated three times to confirm the plaque was derived from only one kind of phage

2.4 Host range test

The host range of Jumbo phages was done according to Kutter [14], by spot tests and plaque-forming assays. The overnight cultures of bacterial strains prepared in LB medium were sub cultured in 500ml LB broth and adjusted to OD 600 $\frac{1}{4}$ 1. 100ml of each bacterial subculture was mixed with 3 ml of molten LB top agar (0.35%), poured onto the surface of an LB agar plate (1.5% agar) and left to dry for 10 min. Then, 1ml of each phage preparation (with titer $\sim 10^8$ plaque forming units/ml) was spotted onto the bacterial overlay, left for 15 min to dry, and then incubated at 30 °C for 24 h. When a lysis zone appeared, efficiency of plating was determined for each strain as the host.

2.5 Genome Size Analysis

The phage genome size was determined by Pulsed Filed gel electrophoresis followed by Higashiyama and Yamada [15] after purification of phage particles by sucrose gradient (20-40%) centrifugation at 40,000 x g for 1 h, they were embedded in 1.7% low-melting-point agarose (Agarose H). Phage-containing plugs were treated by proteinase K (1 mg/ml; Merck Ltd., Tokyo, Japan) and 1% sarkosyl.

3. Results and Discussion

Phage or bacteriophage are the most abundant viruses in the world. Living together with their host, Bacteria. Mostly for jumbo type of phage live in aquatic and soil. According to Holtapless *et al* [16] in the future phages can be integrated with smart farming system especially for biocontrol of bacterial phytopathogen. Duhaime *et al* [17], Viruses influence the ecology and evolutionary trajectory of microbial communities.

Phage populations in the environment are mostly following their bacterial host populations, as affect the phage living mechanism, Lytic obligate. According to Sasani *et al* [18], lytic obligates mean the obligate parasites which replicate within a particular bacterial host and cause rapid lysis and cell death within a short period. Based on that, the isolation of phage became the first important activity to develop the phage as biocontrol agents for pathogenic bacteria. Hypothetically, the abiotic factor-like climate, temperature, elevation, and relative humidity became independent factor to choose appropriate isolation protocol. The different abiotic factor may allow the different isolation protocol.

Phage isolation should be followed by the isolation of their host bacteria, to ensure the availability of the bacteriophage host. The absence of the bacteria host form same environment causes the disruption of the bacteriophage isolation process. According to the test result, several bacterial genera were isolated (Table 2). Bacillus became the most isolated bacterial genus on both locations based on morphological identification and colony counting. According to the Nair *et al* [19], that genus become most abundance bacterial genera in the soil, and that population will be changed according to the soil microenvironment under the influence of different climatic conditions

Table 2. The isolated bacterial genus and their number of colonies

Bacterial Genus	Loc. A	Loc. B
Bacillus	350±15 x 10 ²	320±12 x 10 ²
Escherichia	20±6 x 10 ²	35±6 x 10 ²
Clostridium	16±3 x 10 ²	13±5 x 10 ²
Agrobacterium	80±17 x 10 ²	76±18 x 10 ²
Unknown genus	120±26 x 10 ²	80±16 x 10 ²

The main differences of normal systematic protocol and enrichment protocol on isolation of bacteriophage are in the culturable bacteria. In the enrichment protocol, before phage isolation by following the Saad *et al* [12], the soil sample was cultured overnight and grown in TSB [20]. The culturable sample in the enrichment protocol aimed to increase the number of bacterial host and expected to increase the number of isolated bacteriophages and make it easier to take the next step on

phage development. However, that aim, and purposes influenced by independent factors like abiotic factors and phage type.

Based on this research, the enrichment protocol did not have significant difference among the normal systematic protocols, especially on the plaques number and the plaque sensitivity. This result appeared to be the same at both locations. Conformable with that, the different elevation, soil temperature and bacteriophage type did not become dependent factor to the bacteriophage isolation process. The jumbo phage in both locations can isolated properly although without enrichment protocol.

Table 3. The Number of appeared plaque on each bacterial genus

Bacterial Genus	Loc A		Loc B	
	Enrichment	Non-Enrichment	Enrichment	Non Enrichment
<i>E coli BL21</i>	65±0.8 a	60±0.9 a	58±0.5 a	49±0.6 a
Bacillus	40±0.3 a	39±0.2 a	30±0.2 a	27±0.4 a
Escherichia	26±0.2 a	24±0.3 a	29±0.4 a	23±0.4 a
Clostridium	0±0	0±0	0±0	0±0
Agrobacterium	11±0.3 a	12±0.3 a	13±0.0 a	12±0.1 a
Unknown genus	20±0.6 a	18±0.3 a	19±0.1 a	18±0.4 a

Note: the different annotation was significantly different on the DMRT test (5%)

The sensitivity of bacterial host against the isolated jumbo phage was quite different among both protocols. To determine phage host range or host sensitivity used a spot titration protocol and determine the host range and relative phage killing (EOP) [21,22]. The different result showed if the sensitivity of the Bacillus become more sensitive to collected jumbo phage among other protocol. It's indicated two fact. 1. The selected phage had earlier contact with their bacterial host. The earlier contact makes the bacteriophage able to be replicated faster than other. The fastest replication of jumbo phage will allow more bacterial host became lysed. The lysed bacteria make more bacterial colony became more sensitive 2. The selected phage can't reach the bacterial host species on non-enrichment protocol, the bacterial host have produced the resistance metabolite before the selected jumbo phage colonizing the bacterial cell.

Moreover, all collected jumbo phage have more than 400 kbp by electrophoresis analysis. Its indicated that all collected phages are jumbo phage type. Furthermore, the utilization of jumbo phage considered as potential future treatments against antibiotic-bactericide-resistant bacterial infection on broad range area.

Table 4. The sensitivity of host range (genus)

Bacterial Genus	Enrichment						Non-Enrichment					
	E1	E2	E3	E4	E5	E6	E1	E2	E3	E4	E5	E6
<i>E coli E22</i>	+	+	+	+	+	+	+	+	-	+	-	+
Bacillus	+	+	+	-	+	+	-	-	-	+	-	+
Escherichia	+	+	+	+	+	+	+	+	-	+	-	+
Clostridium	-	-	-	+	-	-	-	-	-	+	-	-
Agrobacterium	-	-	-	-	-	-	-	-	-	-	-	-
Unknown genus	+	-	-	-	+	-	+	-	-	-	+	-

sensitivity: + sensitive (EOP>10² PUF/plate); - resistant (EOP<10⁶ PFU/plate).

4. Conclusion

Based on the plaque size and plaque total, each protocol did not significantly differ on plaque total among the *E. coli* BL 21 or each isolated soil bacteria. On the plaque test, the plaque total average was 65 plaques, nor 45 plaques emerged on each isolated soil bacteria. The significant difference on each protocol was on the clarity of plaque. Based on visual observation, the plaques with enrichment

protocol are clearer with indicated on sensitivity of bacterial host. It was showed that the isolation of jumbo phages from the winter grass during winter conditions can isolated without the enrichment protocol. It can isolate with standard isolation protocol.

5. References

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