

Isolation and Partial Characterization of a New *Escherichia coli* Bacteriophage E3

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Bacteriophages were screened from aquatic environment around the campus of Seoul National University. Among several phages isolated, one bacteriophage infecting *Escherichia coli* produced unusually large plaques upon overnight incubation. Since we planned to screen phages with interests in microbial researches, this phage was referred to as E3 and characterized morphologically and physiologically. The new bacteriophage E3 has very similar morphology to lambdoid. Other characteristics studied so far clearly showed that it is rather likely to have E3 as a member of Styloviridae (15). From electron microscopic studies, it was found that E3 has icosahedral head of 40~45 nm in width and 155~160 nm long flexuous tail. E3 has host specificity to *E. coli* only, and its genome was identified as a double stranded linear DNA with 43 kb. SDS-polyacrylamide gel electrophoresis showed that there are more than seven polypeptide bands as structural proteins, and all were actively stained with polyclonal antibodies conjugated with horse radish peroxidase (HRP). The most abundant protein in detected amount was of 33 Kd m.w. Some restriction endonucleases cut E3 DNA into several fragments (*EcoRI*, >18; *RsaI*, >19; *HpaII*, >21; and *HinfI*, >21), while many others in common use such as *BamHI*, *HindIII*, *AvaiI*, *KpnI*, *PvuII*, and *SaII* have no reactive site at all. Studies on genetic analysis and physical map construction are still under investigation in the hope to find out some possibilities for the application of E3 to microbial genetic researches in identification of new strong promoters, in application of bacteriophages for bacteriological control in environment, or in basic studies on host parasite relationship.

KEY WORDS □ new bacteriophage, *E. coli*, promoter, plaque

There have been accumulated reports on finding new phage systems for various purposes in line with unique goals of their researches (6, 9, 10, 11, 13, 18, 20, 21, 22). It is believed that the availability of bacteriophages for some studies could lead others interested in applied fields including basic ones such as the somatic receptor sites for the phage infection (18).

Until this moment, many bacteriophages infecting *E. coli* were steadily reported. Morphological traits, structural composition, and even genomic nature, however, were quite different from one to another. Electron microscopic studies had revealed that all T even-numbered phages possess contractile tails and elongated heads, while T odd-numbered ones have noncontractile tail with octahedral heads. Lambda phage, however, possesses icosahedral head and noncontractile tail, with 47 kb long

double stranded DNA genome (5). While there is increasing requirement to develop simple experimental systems for microbial researches especially in the field of microbial genetic manipulation, bacteriophage has been regarded to be the most suitable model system for simple and easy way for the application to gene expression of various interests. As it was necessary to screen a new phage system for our researches including development of efficient vector systems with strong promoters, bacterial pollution control, and other related studies, we examined several aquatic sites for screening of phages fulfilling our requirement, finding a new *E. coli* bacteriophage referred to as E3. We, hereby, report isolation of a new *E. coli* phage, E3, with partial characterization for future studies.

MATERIALS AND METHODS

Phage isolation

Water samples were collected in sterilized 10

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ml screw-capped tubes from several sites around the campus of Seoul National University. Before filtration (0.22 μ m, GV type, Millipore), each sample was centrifuged at 12,000 rpm for 10 min at 4°C (Sorvall Instrument RC-5C, SA600 rotor). Filtrates were transferred to the plates previously spread with host bacteria, either directly or diluted in Tris-Magnesium (TM) buffer (Tris 50 mM, MgSO₄ 10 mM, pH 7.4). Host cells were actively growing when infected with filtrates. Chemicals were purchased from Sigma Co. unless otherwise mentioned.

Phages were isolated according to the agar-overlaying technique (2). Total volume of 100 μ l of each filtrate was added to the same volume of host bacterial cells prepared fresh. Then this mixture was resuspended in 3 ml of top agar and poured onto Luria-Bertani (LB) agar plate (1.8% agar) (14). Top agar was prepared with 1% tryptone, 0.5% yeast extract, 1% NaCl, and 0.7% Bacto-agar (Difco). Plates were incubated overnight at 37°C.

Purification of phages

Phages from single plaques were collected in TM buffer. After removal of cellular debris phages were stored at 4°C with a few drops of chloroform added. Titration of phage particles was carried out in the same way as for phage isolation.

Preparation of E3

After E3, a new bacteriophage infecting *E. coli* JM109, was isolated from unusually large plaques. E3 was cultured in large scale. E3 was infected, at the concentration of 1 moi, into the host culture in LB broth at the time of logarithmic phase (4.5×10^8 cells/ml). Host cells were lysed after overnight culture at 37°C.

Culture supernatant was centrifuged at 10,000 rpm for 20 min at 4°C. Phage particles were recovered from the pellet in TM buffer. Phage preparations were treated with Deoxyribonuclease I at 37°C for 2 hrs at the final concentration of 10 μ g/ml. They were further purified by CsCl step density gradient centrifugation ($\rho = 1.450 - 1.708$ g/ml) at 5,000 rpm for 18 hrs at 4°C (Beckman Instrument L7-55, SW50.1 rotor) (4). Purified E3 was dialysed against TM buffer and stored at 4°C after titration.

Host specificity of E3

Since E3 was subsequently isolated from the plate of *E. coli* JM109 among phages infected to 12 different host bacteria, host specificity of E3 was subsequently tested with the above mentioned host cells. In addition, several other strains of *E. coli* than JM109 were also examined.

Electron microscopy

One drop of E3 preparation, 10^{12} pfu/ml, was placed onto a carbon-coated grid and stained with 2% (w/v) phosphotungstic acid, pH 6.8. Specimens were examined using Hitachi H-800 electron microscope.

Determination of nucleic acid from E3

To determine whether the nucleic acid of E3 is RNA or DNA, nucleic acid from E3 particles was isolated by phenol extraction methods (10, 14) with slight modification. Phenol-saturated buffer was added to the equal volume of phage preparation with vigorous mixing at room temperature. Extraction was repeated twice collecting the aqueous phase. The nucleic acid from E3 was further purified and concentrated with ethanol precipitation with 3 M sodium acetate, and resuspended in TE buffer (Tris-HCl 0.01 M, EDTA 1 mM, pH 7.6).

Nature of the nucleic acid was evaluated either with DNase I (Boehringer Mannheim GmbH) at final concentration of 2 μ g/ml or with 0.1 M NaOH. It was further identified with S1 nuclease and exonuclease III (Kosco).

Digestion of E3 DNA with restriction endonuclease

Purified DNA from E3 phages was digested with restriction endonuclease such as *Bam*HI, *Eco*RI, *Hind*III, *Av*II, *Bsp*HI, *Kpn*I, *Pvu*II, *Rsa*I, *Sal*I, *Sma*I, *Hpa*II, and *Hin*FI, which were purchased either from Boehringer Mannheim or Kosco. Fragments of DNA were analyzed under the condition described by Maniatis *et al.* (14).

One-step growth experiment for E3

Different phases of phage infection cycles and the burst size of E3 infection were evaluated (8). Host cells were infected with E3 at 1 moi and kept in 37°C water bath for the convenience of experimental procedure. Small aliquot of culture was collected every min and plated for evaluation. Burst size was calculated as (pfu/ml at maximum plateau)/(pfu/ml at minimum plateau).

Coat proteins and Western blotting

Structural polypeptides from E3 were analyzed by SDS-polyacrylamide gel electrophoresis (12). Proteins were identified by staining with Coomassie brilliant blue R250 (BioRad).

Mouse polyclonal antibodies developed against intact particles of E3, confirmed by double immunodiffusion assay, reacted with proteins transferred to nitrocellulose papers (BA85, Schleicher & Schuell) and were further recognized by anti-mouse IgG antibodies conjugated with HRP (Cappel Laboratory).

RESULTS

Isolation and purification of a new bacteriophage infecting *E. coli* JM109

A number of bacteriophages were screened using 12 different host bacteria from water samples collected from ponds and stream around the campus of Seoul National University. The phages isolated are listed in Table 1 with the host used for isolation. Most of them were typical phages on the view point of plaque formation.

One sample, however, from a small pond located near College of Engineering attracted attention producing plaques with unusual features. Plaques after overnight culture were very large with clear edge, larger than 10 mm in diameter. This new bacteriophage with the host specificity to *E. coli* was referred to as E3 and morphological and physiological characteristics were examined. Purified E3 after CsCl step density gradient centrifugation was confirmed whether it still could make large plaques. Plaques developed by E3 infection was growing so fast that they could be easily detectable even with 4 hr incubation only.

Morphological characteristics of E3

Electron microscopy revealed that E3 has an icosahedral head with the diameter of 40~45 nm and a long flexuous tail about 155~160 nm long. Fig. 1 shows the typical morphology of E3.

One-step growth of E3

Inasmuch as E3 multiplies very fast, one-step growth experiment was carried out to examine the infection cycle and burst size. As shown in Fig. 2, the latent period after E3 infection was as short

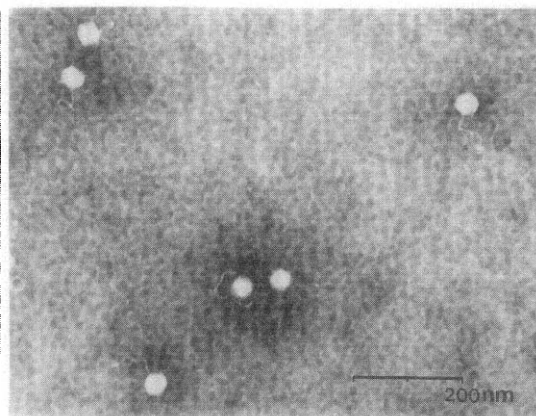


Fig. 1. E3, a newly isolated bacteriophage infecting *E. coli* stained with 2% phosphotungstic acid, pH 6.8.

These phage particles are of icosahedral head (43 nm in diameter) and very flexuous tail (156 nm long).

Table 1. Bacteriophages isolated with unique host specificities.

Host Bacteria	Phage	Description of plaque
<i>Citrobacter freundii</i> ATCC 6750	—*	
<i>Enterobacter aerogenes</i> ATCC 13048	—	
<i>Escherichia coli</i> K12 JM109	E3	large, clear, smooth margin
	E4	small, clear, smooth margin
<i>Klebsiella pneumonia</i> ATCC 10031	—	
<i>Proteus vulgaris</i> ATCC 6059	—	
<i>Pseudomonas aeruginosa</i> ATCC 25619	—	
<i>Salmonella typhimurium</i> ATCC 14028	E1	small, turbid, rough margin
	E2	small, turbid, rough margin
<i>Salmonella paratyphi</i> ATCC 11511	—	
<i>Serratia marcescens</i> ATCC 27117	—	
<i>Shigella flexner</i> ATCC 9199	—	
<i>Staphylococcus epidermidis</i> ATCC 12228	—	

* No phage isolated.

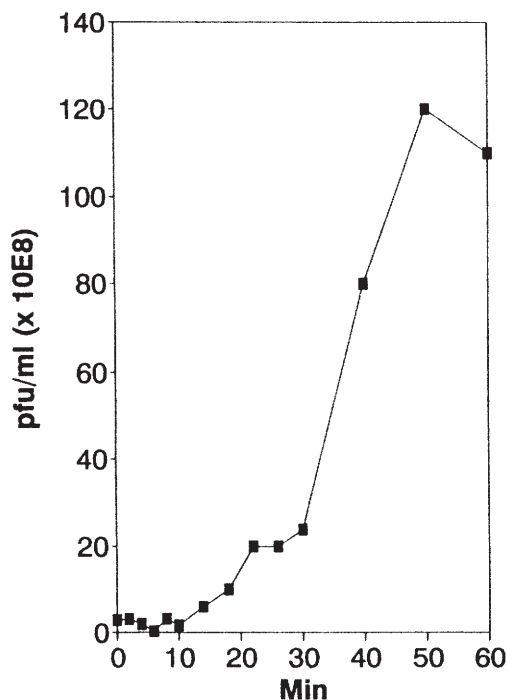


Fig. 2. One step growth curve for E3 infection to *E. coli* JM109.

It takes only 25 min till progeny phage particles appear in the medium after infection to host cells at the concentration of 1 moi. The burst size has been calculated to be 1,170. The average size of plaques produced by E3 after overnight incubation was larger than 10 mm in diameter.

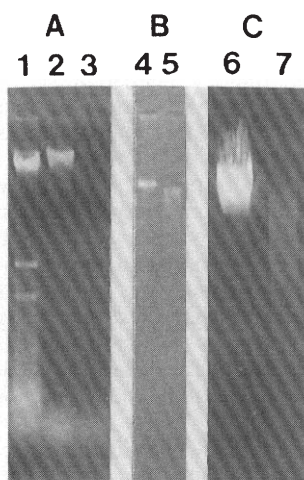


Fig. 3. Nucleic acid E3 contains is double stranded linear DNA.

A. Nucleic acid from E3 and digested DNA fragments with DNase I and 0.1 M NaOH (lanes 1, 2, and 3, respectively).

B. S1 nuclease treatment, lanes 4 (without) and 5 (with).

C. Treatment of exonuclease III, lanes 6 (without) and 7 (with).

as 6 min followed by a rise period of 18 min. The burst size 1,170 was obtained from the calculation of $(2 \times 10^9 \text{ pfu/ml}) / (1.7 \times 10^8 \text{ pfu/ml})$.

Nature of nucleic acid from E3

The nucleic acid E3 contains was digested by DNase I and exonuclease III, while affected neither by 0.1 M NaOH nor by S1 nuclease (Fig. 3). It is quite evident that the nucleic acid of E3 was linear and double stranded DNA. The size of genomic DNA from E3 was compared with other DNAs of known size, and it was determined to be 43 kb.

More than half of restriction endonucleases listed in Materials and Methods cut down E3 DNA into a number of fragments except six restriction enzymes such as *Bam*HI, *Hind*III, *Kpn*I, *Pvu*II, *Ava*II, and *Sal*I (Fig. 4). Reactive enzymes cut E3 DNA into many fragments; *Eco*RI 17, *Rsa*I 18, *Hpa*II 20, *Hin*II 20, and *Bsp*HI 11.

Coat proteins

Structural polypeptides from E3 were analyzed on 12.5% SDS-polyacrylamide gel electrophoresis. At least seven different proteins could be detected after Coomassie blue staining as shown in Fig. 5. The major protein in amount was detected at the site of molecular weight 33,000 dalton.

Polyclonal antibodies against intact E3 were developed to find out the location of each structural proteins in phage particles. All seven bands stained by Coomassie blue were reactive

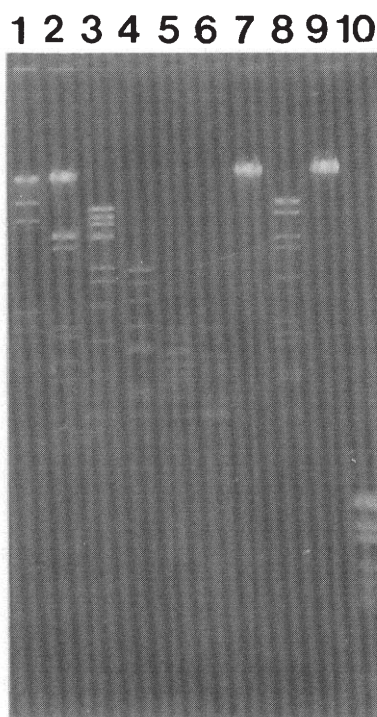


Fig. 4. E3 DNA digested with restriction endonucleases.

Lanes 1 & 2, λ 4DNA cut with *Hind*III, and *Eco*RI and *Hind*III; 3~9, E3 DNA with *Exo*RI, *Rsa*I, *Hpa*II, *Hin*II, *Hinc*II, *Bsp*HI, and *Xho*I; 10, pUC18 with *Hpa*II.

with the antisera, detectable with naked eyes even though very vague in the picture.

DISCUSSION

This study was initiated to screen bacteriophages with microbial research interests such as identification of new promoters, application of bacteriophages in bacteriological control in environment, or basic studies in host parasite relationship. We could isolate several bacteriophages infecting different host bacteria according to their host specificity. Among these, one bacteriophage referred to as E3 infecting *E. coli* produced unusually large plaques only with overnight incubation. The average plaque size was larger than 10 mm in diameter implying either their very fast reproduction or the extraordinarily large burst size. One step growth experiment showed that E3 phages infected and released progeny particles in 25 min with the burst size of slightly less than 1,200. We further characterized E3 in the hope that this newly isolated phage could be used for development of a new

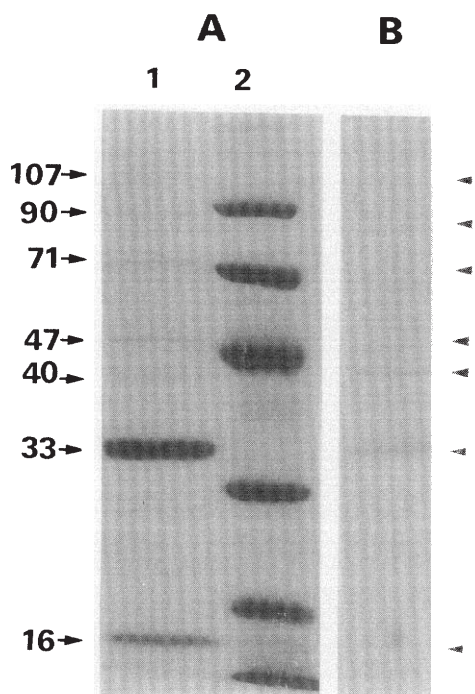


Fig. 5. Structural proteins from E3.

A. Seven polypeptide bands were recognized by Coomassie brilliant blue staining (lane 1). B. All of these peptides were blotted with polyclonal antibodies developed against intact E3 phages in mice, even though they were stained vague. Molecular weights were calculated in kd as compared with molecular markers.

vector system very useful in microbial genetics with every strong promoter.

As previously mentioned, E3 was screened with *E. coli* JM109 which belongs to K12 strain, and no other bacteria were infected by E3 showing strong specificity to *E. coli*. Other strains of *E. coli* such as B and C were also very susceptible with similar plaque formation and burst size (data not shown), which implies that E3 is not male specific at all. There are still possibilities to control coliform bacterial pollution in natural environment, and preliminary studies carried out revealed that they are natural mutants resistant to E3 infection occurring in the same rate as other phage infections, identified as phage receptor mutants (6, 19).

Nucleic acid of E3 had been analyzed and it was known that E3 contains double stranded linear DNA genome of 43 kb long. Electron microscopy showed this phage has icosahedral head 40~45 nm in diameter and very flexuous

tail 155~160 nm in length (Fig. 1). It is much likely that E3 should be a member of lambdoid. Other characteristics investigated for classification made it clear that E3 was not a member of lambdoid family (3), as lambda phage is different in head size, 62 nm in diameter, and in genome size, 47 kb. One important evidence came from that E3 successfully infected JM101 reconstructed with immunity region from λ 200 (imm²¹Pr-trpA0-lacZ fusion) (16) and JM101Nr69 with immunity region from λ , which are immune to lambda infection, meaning that these two phages do not share their receptors for infection. In addition, some morphological characteristics and restriction endonuclease digestion patterns were also different as well.

On the basis of physiological as well as morphological characteristics, it is much likely that E3 belongs to Styloviridae (15). According to Ackermann and his colleagues as well as Bradely's group, E3 is of B1 morphological features (1, 7, 11, 17). For the distinctive classification, we believe that we still need some more comparative data such as physical mapping, mode of infection, protein expression in host cells, and many other ones supporting our present analysis.

Genomic analysis with some restriction endonuclease digestion disclosed very peculiar features, showing there are more than 17 restriction sites for *EcoRI* in E3 DNA even though E3 infects *E. coli* without any disturbance. E3 was screened using JM109 strain, but other B or C strains were also as much susceptible as JM109. It is believed that there are some genomic modification in E3 not to be cut by normal *EcoRI*. In addition, there were no restriction site for some restriction enzymes such as *BamHI*, *HindIII*, *AvaII*, *KpnI*, *PvuII*, and *SalI*, which is also unusual in comparison with its genomic size. We are currently carrying out studies on genetic analysis and physical map construction as well as on receptor for E3 infection in the hope that we might be able to find out some specific details explaining such unusual features of E3 DNA.

There were at least seven structural protein bands from intact E3 particles on SDS-polyacrylamide gel (12.5%) electrophoresis (Fig. 5). The most abundant polypeptide was of 33 Kd in molecular weight, probably the major coat protein of E3. When these proteins were blotted with polyclonally developed antibodies, developed in mice, all detectable protein bands were stained implying they were structural proteins protruding outside viral capsid. Lundrigan *et al.* (13) described very similar *E. coli* phage to E3 on the basis of the number of structural proteins and its nucleic acid as double stranded linear DNA, but it was not identical to E3.

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REFERENCES

1. Ackermann, H.-W., A. Audurier, L. Berthiaume, L. A. Jones, J.A. Mayo, and A.K. Viraver, 1978. Guidelines for bacteriophage characterization. *Adv. Virus Res.* **23**, 1-24.
2. Adams, M.H., 1959. Bacteriophages, p. 450-456. Interscience Publishers, New York.
3. Allet, B., P.G.N. Jeppesen, K.J. Katagiri, and H. Delius, 1973. Mapping the DNA fragments produced by cleavage of Lambda DNA with endonuclease R1. *Nature (London)* **241**, 120-123.
4. Bancroft, F.C. and D. Freifelder, 1970. Molecular weights of coliphage and coliphage DNA: I. Measurements of the molecular weights of bacteriophage T7 by high speed equilibrium centrifugation. *J. Mol. Biol.* **54**, 537-546.
5. Birge, E.A., 1981. Bacterial and bacteriophage genetics, p. 67-151. Springer-Verlag, New York.
6. Bigby, D. and A.M.B. Kropinski, 1989. Isolation and characterization of a *Pseudomonas aeruginosa* bacteriophage with a very limited host range. *Can. J. Microbiol.* **35**, 630-635.
7. Bradely, D.E., 1967. Ultrastructure of bacteriophages and bacteriocins. *Bact. Rev.* **31**, 230-314.
8. Eisenstark, A., 1967. Bacteriophage techniques, p. 449-524. In K. Maramorosch and H. Koprowski (ed.), *Methods in virology*, Vol. 1. Academic Press, New York.
9. Elsas, J.D. and E.G.C. Penido, 1982. Characterization of a new *Bacillus megaterium* bacteriophage, MJ-1, from tropical soil. *Antonie van Leeuwenhoek* **48**, 365-371.
10. Kikuchi, A., D. Elseriers, and L. Gorini, 1975. Isolation and characterization of Lambda transducing bacteriophages for *argF*, *argI*, and adjacent genes. *J. Bacteriol.* **122**, 727-742.
11. Kuroda, K., R. Kagiya, and M. Kageyama, 1983. Isolation and characterization of a new bacteriophage, KF1, immunologically cross-reactive with F-type pyocins. *J. Biochem.* **93**, 61-71.
12. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685.
13. Lundrigan, M.D., J.H. Lancaster, and C.F. Earhart, 1983. UC-1, a new bacteriophage that uses the Ton A polypeptide as its receptor. *J. Virol.* **45**, 700-707.
14. Maniatis, T., F.H. Fritsch, and J. Sambrook, 1982. Molecular cloning, a laboratory manual, p. 2.3-2.8 and 2.60-2.80. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
15. Mathews, R.E.F., 1982. Classification and nomenclature of virus; Fourth report of the international committee on taxonomy of viruses. *Intervirology* **17**, 1-179.
16. Meyer, B.J., R. Maurer, and M. Ptashne, 1980. Gene regulation at the right operator (O_R) of bacteriophage λ II, O_{R1} , O_{R2} , and O_{R3} : Their roles in mediating the effects of repressor and *cro*. *J. Mol. Biol.* **139**, 163-194.
17. Polisky, B., P. Greene, D.E. Graf, B.J. McCarthy, H.M. Goodman, and H.W. Boyer, 1975. Specificity of substrate recognition by the *EcoRI* restriction endonuclease. *Proc. Natl. Acad. Sci. USA* **72**, 3310-3314.
18. Preissner, W.C., S. Maier, H. Volker, and P. Hirsch, 1987. Isolation and partial characterization of a bacteriophage active on *Hyphomicrobium* sp. WI-926. *Can. J. Microbiol.* **34**, 101-106.
19. Reanet, D.C. and H.-W. Ackermann, 1982. Comparative biology and evolution of bacteriophage. *Adv. Virus Res.* **27**, 205-280.
20. Schreier, H.J., E.K. Vonada, R.E. Yasbin, and R. W. Bernlohr, 1982. Isolation and characterization of a bacteriophage for *Bacillus licheniformis* A5. *Current Microbiol.* **7**, 103-106.
21. Temple, G.S., P.D. Ayling, and S.G. Wilkinson, 1986. Isolation and characterization of a lipopolysaccharide-specific bacteriophage of *Pseudomonas aeruginosa*. *Microbiol.* **45**, 81-91.
22. Trevor, K.E., R.A. Holley, and A.G. Kempton, 1983. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage isolated from a meat starter culture. *J. Appl. Bacteriol.* **54**, 281-288.

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초 록: 대장균에 특이적으로 감염하는 새로운 세균성바이러스 E3의 분리와 특성

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서울대학교 구내의 연못으로 부터 대장균에 감염하는 세균성 바이러스를 분리하였다. 이들이 대장균에 감염하여 유도되는 플라크가 보통의 경우와는 달리 대단히 크고 빠르게 만들어지고 있음을 관찰하였다. 저자들은 이들이 독특한 특징을 지니고 있다고 판단하고 E3라 명명된 뒤 이들의 생리화학적 특성을 연구하였다. 형태학적으로 E3는 lambda phage와 유사한 모양을 하고 있는데, 다른 여러가지 형태학적인 특성을 고려한다면 오히려 Styloviridae와 유사하다고 생각된다. 전자현미경 관찰로부터 이들의 머리 부분이 대략 40~45 nm, 그리고 꼬리 부분은 155~160 nm의 길이를 가지고 있다고 측정하였다. E3는 오직 대장균에 대해서만 감염하고 있으며, 43 kb 크기의 double stranded DNA를 가지고 있다. 단백질 분석에서 7개 이상의 단백질이 확인되었으며, 이들은 생쥐에서 얻은 항혈청에 대하여 모두 반응하였고, 가장 양적으로 많이 발현되는 단백질의 분자량은 대략 33 kd이었다. 제한효소를 사용한 제한효소지도 작성에서도 흔히 사용하고 있는 *Bam*HI, *Hind*III, *Ava*II, *Kpn*I, *Pvu*II, 그리고 *Sal*I 등의 효소에 대해서는 전혀 작용점을 가지고 있지 아니하였으며, *Eco*RI, *Rsa*I, *Hpa*II, *Hin*II 등의 효소로는 각각 18, 19, 21, 21개 이상의 절편을 생산하였다.

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