Physiological Characterization of SP816 Bacteriophage

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SP816 박테리오파아지의 생리적 특성

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Abstract: Some of the physiological properties of SP816 bacteriophage of *Bacillus subtilis* SNU816 were characterized. It could form plaques on either *B. subtilis* SNU816 or *B. natto* 8102, but not on any other bacillus strains investigated. Its plaque morphology was circular with a diameter of less than 1.0 mm and had a narrow halo surrounding the clear center. Its latent period was 34-36 min and had a burst size of 547. It was most stable at pH 6.0, and rapidly inactivated at 60°C with a initial death rate of $-0.216\ min^{-1}$. Host range, thermal inactivation rate at 60°C, pH stability, and UV sensitivity revealed that SP816 was quite different from any other phages investigated together but seemed to be rather related to *B. natto* phages.

Key Words: SP816 bacteriophage, phage stability, latent period, burst size.

SP816 is a virulent bacteriophage of *Bacillus subtilis* SNU816, originally described by Lee (1978). This phage was known to be detrimental to soybean fermentation with *B. subtilis* SNU816 (Lee, 1978). Some of its characteristics such as electron microscopical morphology (Ahn *et al.*), optimum temperature and pH for phage reproduction (Lee, 1978), and change of reproducibility during the growth and sporulation of its host (Lee and Lee, 1984) were previously reported. Here I report additional properties of SP816 including; i) plaque morphology, ii) host range, iii) dose-response titration, iv) one-step growth characteristics, v) burst size, vi) pH stability, vii) thermal inactivation, and viii) inactivation by ultraviolet (UV) irradiation. These are the criteria advanced by Adams (1953, 1959) as most useful for phage classification. During this investigation, some of other bacillus phages were compared with SP816.

MATERIALS AND METHODS

Bacterial and Bacteriophage strains

Bacillus subtilis SNU816 isolated by Lee (1974), and *B. subtilis* CU1050 kindly provided by Dr. H.E. Hemphill were used as hosts for phage SP816 and all the other phages used, respectively. The bacteriophage SP816 was firstly described by Lee (1978). SP01, SP82, and ϕe are virulent group 1 phages containing 5-hydroxymethyluracil (HMU) in place of thymine (Kallen *et al.*, 1962). ϕ1, β22, and SPP1 are group 2, group 3, and group 5 phages respectively. $SP02_{c1}$ and $\phi 105_{c1}$ are clear-plaque mutants of temperate phage SP02 and ϕ105, respectively. ϕ3T, ρ11, and $SP\beta_{c1}$ are related temperate phages, where $SP\beta_{c1}$ is a clear-plaque mutant of SPβ. All of these phages except SP816 were obtained from Dr. H.E. Hemphill.

Media

M broth (10g tryptone, 5g yeast extract, 10g NaCl, 5 mM $MgCl_2$, and 0.1 mM $MnCl_2$ per liter) was used for the propagation of all the bacteria and phages except β22 for which 5mM $CaCl_2$ was additionally supplimented. For ordinary propagation of SP816, commercial nutrient broth (Difco) was used without any suppliment. Bottom layer and soft agars for plating contained 1.5 and 0.6% agar in above broths, respectively. Nutrient sporulation medium phosphate (Fortnagel and Freese, 1968) was used for preparation of *B. subtilis* SNU816 spores.

Preparation of spores

B. subtilis SNU816 cultured in nutrient sporulation medium phosphate (NSMP) broth was plated on NSMP agar and incubated for 4 days at 37°C. Spores were collected in 10m*l* of sterile D.W. per plate and preserved at 4°C until use.

Preparation of phage lysate and assay of phage concentration

Procedures for the preparation and purification of high-titer phage lysates were described previously (Lee and Lee, 1984), except that purification of phage lysate was achieved by filtration through Millipore membrane filter (0.45 μm pore size). Phage concentrations were assayed by the conventional agar-layer method (Adams, 1959) using *B. subtilis* SNU816 spores and vegetatively growing *B. subtilis* CU1050 as indicator cells for SP816 and all the other phages, respectively. Plaque morphology was frequently observed during these plaque assays.

Determination of host range

Host range was determined by the spotting method (Romig and Brodetsky, 1961) on M agar plate.

Dose-response titration of SP816

To determine whether a single virion is sufficient to infect a cell, phages were serially diluted and counted for plaque forming units (Ellis and Delbrück, 1939).

One-step growth experiment

One-step growth experiment was performed to determine the latent period of SP816 according to the method described by Brodetsky and Romig (1965) except that antiserum inactivation procedure for the elimination of unadsorbed phages was replaced by brief centrifugation (10,000 × g, 5min) followed by high-fold dilution (Ellis and Delbrück, 1939) assuming about 30% of phages were adsorbed within a given period allowed to adsorb. During incubation at 37°C with gentle shaking, samples were withrawn and assayed for plaque forming units until 80 min had elapsed.

Determination of burst size

Average burst size was determined by single burst experiment (Ellis and Delbrück, 1939). For details, see legend of Table 3.

Effect of temperature on the stability of SP816

For determination of the stability of phage against heat, phage suspensions were treated at various temperatures, and samples were withrawn at intervals for plaque assay.

Inactivation by UV irradiation

The UV source used was a 15-w Shin-Kwang Electric germicidal lamp. Phage suspensions were diluted in 1% NaCl and irradiated in petri dishes under UV light source 50cm apart.

pH stability of SP816

Phage suspensions were diluted in either 0.01M potassium phosphate buffer or M broth adjusted to desired pH values with HCl and NaOH. After incubation at 37°C for 1hr, surviving phages were plaque assayed.

RESULTS AND DISCUSSION

Plaque morphology

Morphology of the plaques formed by SP816 on *B. subtilis* SNU816 was circular with a diameter of less than 1.0mm and it often had a narrow halo surrounding the clear center (Fig. 1). It has been reported that halos formed on T_2-infected *Escherichia coli* were resulted from the action of phage lysozyme (Koch and Dryer, 1958). Brodetsky and Romig (1965) also reported that some of the group 1 phages such as SP6 and SP13, which didn't form halos on *B. subtilis* Marburg strain, could form halos when strains SB19 and 168B were used as hosts. The appearance of

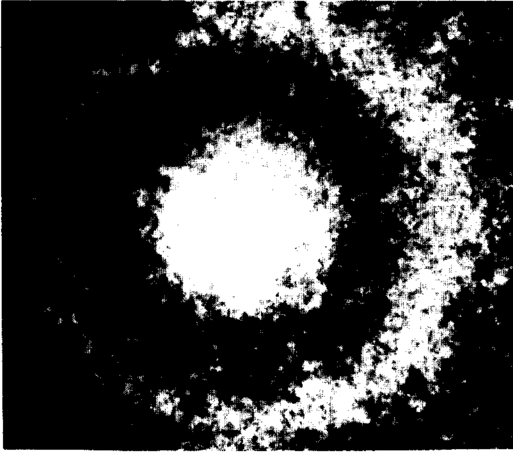

Fig. 1. *Plaque morphology of SP816.*

halos seemed to be even more conditional for SP816 since halos often failed to appear even the same medium and same host were used. Therefore it seems likely that the use of halo forming ability as a criteria for characterization of a phage requres some precautions.

Host range

The determination of host range of a given phage has a notable significance for characterization of both phage and host because of high specificity of phage to its host. As is shown in Table 1, SP816 could form plaques only on *B. natto* 8102 but not on any other bacillus strains investigated, suggesting close relatedness of *B. subtilis* SNU816 to *B. natto* 8102. Furthermore, of the phages investigated only β22 and ϕ1 could form plaques on either *B. subtilis* SNU 816 or *B. natto* 8102 (Table 2). Therefore is was concluded that both SP816 and *B. subtilis* SNU 816 were closely related to *B. natto* strain.

Table 1. *Reproducibility of SP816 bacteriophage on various bacillus strains.*

Strains	Reproducibility*
B. subtilis SNU816	+
B. subtilis ATCC 6633	−
B. subtilis 168	−
B. subtilis PCI129	−
B. subtilis CU1050	−
B. natto 8102	+
B. cereus T	−
B. megaterium	−

*+ : phage growth, − : no phage growth

Dose-response titration

To determine whether viral infection follows one hit kinetics, SP816 phages were diluted serially until reached to 0 concentration and plaque assayed. As the dose-response curve represents (Fig. 2), the number of plaques was linearly proportional to the concentration of SP816, indicating a single particle is sufficient to infect its host.

One-step growth characteristics

One-step growth experiment was done to determine the latent period. A typical growth curve is shown in Fig. 3. The latent period of SP816 was about 34-36min, relatively short when compared with those of other *B. subtilis* phages (Hemphill and Whitely, 1975).

Burst size

Though the average burst size can be determined by one-step growth experiment, burst size of SP816 was determined by single burst experiment because too many progeny particles were produced during one-step growth experiment. From the data presented in Table 3, average burst size of SP816 was determined to be 547 particles per infected cell. Although ϕ29, one of virulent

Table 2. *Susceptibility of* ***Bacillus subtilis*** *SNU-816 and* ***B. natto*** *8102 to several* ***B. subtilis*** *phages*

Bacteriophages	Susceptibility*	
	B. subtilis SNU816	*B. natto* 8102
SP816	+	+
SPO1	−	−
SP82	−	−
ϕe	−	−
ϕ1	+	+
β22	+	+
SPP1	−	−
$SPO2_{c1}$	−	−
$\phi 105_{c1}$	−	−
ϕ3T	−	−
ρ11	−	−
$SP\beta_{c1}$	−	−

*+ : phage growth, − : no phage growth

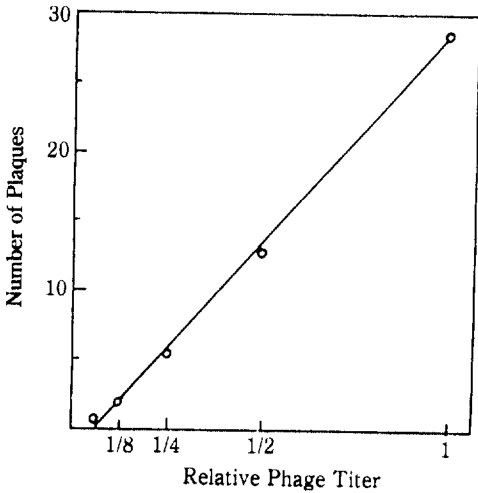

Fig. 2. *Dose-response curve of SP816.*

group 6 phages of *B. subtilis*, has as much as 570 burst size (Schachtele *et al.*, 1970), this value is considerably high. *B. subtilis* SNU816 seldom grows in a single cell state in exponential growth phase but rather in cells attached each other to form a long thread-like structure. Therefore it is possible that such a large burst size might be resulted from the consecutive infections of neighboring cells in chain. However, considering that even the lowest value from the productive tube (culture number 30) was 463, it is somewhat reasonable to accept this value as correct one.

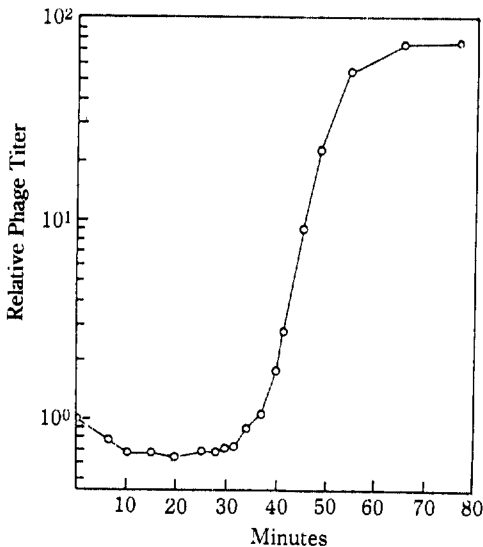

Fig. 3. *One-step growth curve of SP816.*

Effect of pH

Effect of pH on phage stability was examined and the results obtained are shown in Table 4. In contrast to majority of *B. subtilis* phages which are most stable at neutral pH, SP816 was more stable at acidic pH either in salt buffer (0.01M potassium buffer) or in complete M medium. It had optimum stability at pH 6.0 in both solutions with slight differences in occurrence of complete loss of infectivity at extreme pH region and in surviving fractions at regions near optimum pH. Rhee (1985) has reported that although RK temperate phage of *B. cereus* had a optimum stability at pH 7.0 in

Table 3. *Single burst experiment of SP816 bacteriophage.*

Culture number	Number of plaques	Culture number	Number of plaques
1	0	21	0
2	0	22	1
3	0	23	1
4	0	24	1
5	0	25	1
6	0	26	1
7	0	27	850
8	0	28	687
9	0	29	838
10	0	30	463
11	0	31	526
12	0	32	588
13	0	33	532
14	0	34	993
15	0	35	630
16	0	36	1741
17	0	37	1533
18	0	38	1795
19	0	39	1602
20	0	40	1325

Exponentially growing cells of *B. subtilis* SNU816 were infected with a dilute phage suspension, allowed for 5 min to adsorb, and then washed by centrifugation. After further dilution to a appropriate value, aliquots of 0.1m*l* were placed in 40 tubes and incubated for 80 min. The entire contents of each tube was then used in a plaque assay.

Table 4. *Effect of pH on SP816 bacteriophage**

pH	Recovery of viable phage**	
	M broth	phosphate buffer
3.5	0	0
4	0	6
5	41	68
6	100	100
7	75	60
8	19	30
9	2	6
10	0	0

*Phages were suspended in either M broth or 0.01 M phosphate buffer. After incubation at 37℃ for 1 hr, surviving phages were plaque assayed.
**Per cent of plaque-forming units recovered from pH 6 broth.

nutrient broth, optimum pH was variable according to the salts used to make buffer because of the effects of ions desolved. It is not clear, however, whether such a ion effect had caused SP816 to have a acidic pH optimum.

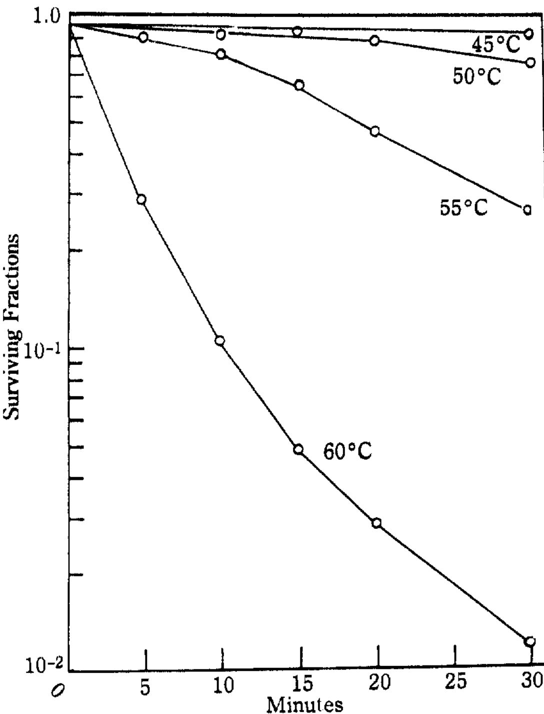

Fig. 4. *Thermal inactivation of SP816 at various temperatures.*

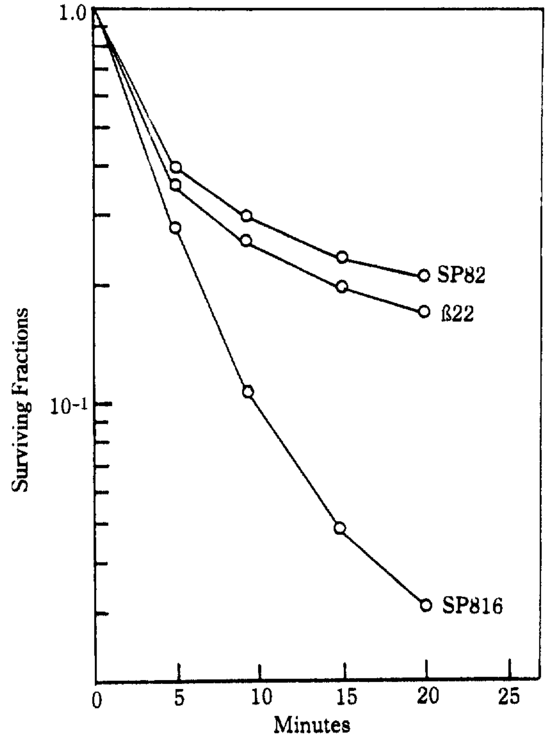

Fig. 5. *Thermal inactivation of SP816, SP82, and ß22 at 60°C.*

Effect of temperature

Examination of the sensitivity of phage to heat yielded results shown in Fig. 4. SP816 was relatively stable up to 50°C, but rapidly inactivated at 60°C with a initial death rate of $-0.216 min^{-1}$. Because SP816 was morphologically similar to SP82 (Lee, 1978) and *β*22 could reproduce on *B. subtilis* SNU816, both of these phages were compared with SP816 in thermal inactivation characteristics at 60°C. As is revealed in Fig. 5, SP816 was more sensitive to heat than SP82 and *β*22, suggesting unrelatedness of SP816 to either of these phages.

UV sensitivity

Inactivation kinetics of SP816 by UV irradiation is shown in Fig. 6. When it was compared with SP82, SP816 was quite sensitive to UV irradiation and more than 99% of phages were inactivated within 30 sec of irradiation. SP82 was highly resistant to UV irradiation, characteristic of HMU-containing group 1 phages of *B. subtilis* (Brodetsky and Romig, 1965). Wacker (1963)

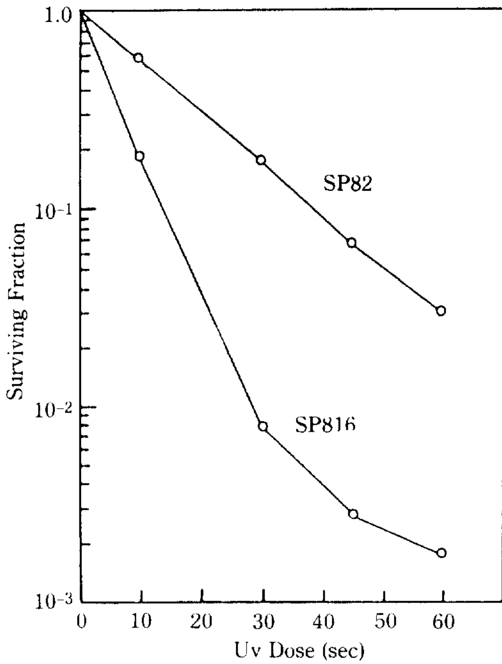

Fig. 6. *Inactivation of SP816 and SP82 by ultraviolet irradiation.*

reported that irradiation of 5-hydroxymethyluracil gave rise to a smaller amount of dimerization than irradiation of either thymine or uracil, causing HMU-containing phages greater resistant to UV induced injury. Because SP816 was quite sensitive to UV irradiation, SP816 may then have no HMU base substitution.

In conclusion, SP816 was quite virulent in that single virion could successfully cause host cell lysis yielding large progeny particles within relatively short periods, and also quite different from those phages investigated together judging from host range, pH stability, thermal inactivation kinetics, and UV sensitivity. SP816 seemed to be rather related to *B. natto* phages. This conclusion concerning the classification of SP816, however, requires more evidences such as G+C content, phage density, DNA morphology and molecular weight, DNA restriction patterns, and direct comparison with *B. natto* phages. These are subjects of subsequent investigation.

적 요

Bacillus subtilis SNU816을 숙주로 하는 SP816 박테리오파아지의 몇가지 생리적 특성을 조사하였다. 이 파아지의 숙주역을 조사해 본 결과 7가지 *bacillus* 균주 중에서 오직 *B. natto* 8102만이 이 파아지에 역시 감염되었으며, 조사된 여타 *sublilis* 파아지 중에서는 ϕ1, β22만이 *B. subtilis* SNU816에 감염을 일으켰다. 이 파아지의 용균반은 1.0mm이하의 크기를 갖는 원형이었으며 이를 둘러싸고 있는 이중원 형태의 halo가 보이는 것이 특색이었다. 이 파아지는 한 개의 virion으로도 감염을 일으킬 수 있었으며 잠복기가 34-36분, burst size가 547개로 높은 독성이 있음을 보였다. pH 6.0에서 가장 안정성을 보였으며 50℃까지는 비교적 안정하였으나 60℃에서는 사멸계수가 $-0.216min^{-1}$로 급격히 사멸하였고 SP82, β22파아지보다 더 열에 민감하였다. UV조사에 의해서도 SP82보다 훨씬 민감하였다. 이상과 같은 조사를 통해서 SP816은 *B. subtilis*의 독성파아지들과는 달리 오히려 *B. natto*계 파아지와 관련이 있는 것으로 사료되었다.

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REFERENCES

1. Adams, M.H., 1953. Criteria for a biological classification of bacterial virus. *Ann. N.Y. Acad. Sci.* **56:** 442-447.
2. Adams, M.H., 1959. Bacteriophages. Interscience Publishers, Inc., New York.
3. Ahn, K.J., Z.S. Lee, and W.J. Lee, 1982. Electron microscopical observation on the phage of *Bacillus subtilis* SNU816. *Kor. J. Election Microscopy* **12:** 69-73.
4. Brodetsky, A.M., and W.R. Romig, 1965. Characterization of *Bacillus subtilis* bacteriophages. *J. Bacteriol.* **90:** 1655-1663.
5. Ellis, E.L., and M. Delbrück, 1939. The growth of bacteriophage. *J. Gen. Physiol.* **22:** 365-384.

6. Fortnagel, M., and E. Freese, 1968. Analysis of sporulation mutants II. Mutants blocked in the citric acid cycle. *J. Bacteriol.* **95:** 1431-1438.
7. Hemphill, H.E., and H.R. Whiteley, 1975. Bacteriophages of *Bacillus subtilus. Bacteriol. Rev.* **39:** 257-315.
8. Kallen, R.G., M. Simon, and J. Marmur, 1962. The occurrence of a new pyrimidine base replacing thymine in a bacteriophage DNA: 5-hydroxymethyluracil. *J. Mol. Biol.* **5:** 248-250.
9. Koch, G., and W.J. Dryer, 1958. Characterization of an enzyme of phage T_2 as a lysozyme. *Virology* **6:** 291-293.
10. Lee, O.H., and Z.S. Lee, 1984. Susceptibility of *Bacillus subtilis* SNU816 to bacteriophage SP816 during growth and sporulation. *Kor. J. Microbiol.* **22:** 111-118.
11. Lee, Z.S., 1974. Studies on the *Bacillus subtilis* var. 816. Report of Research to Ministry of Education.
12. Lee, Z.S., 1978. Studies on the isolation and characterization of bacteriophage of *Bacillus subtilis* var. 816. *Kor. J. Microbiol.* **16:** 71-78.
13. Rhee, T.W., 1985. Studies on the RK-temperate phage of *Bacillus cereus. Kor. J. Microbiol.* **23:** 129-137.
14. Romig, W.R., and A.M. Brodetsky, 1961. Isolation and preliminary characterization of bacteriophages for *Bacillus subtilis. J. Bacteriol.* **82.** 135-141.
15. Schachtele, C.F., R.W. Orman, and D.L. Anderson, 1970. Effect of elevated temperature on deoxyribonucleic acid synthesis in bacteriophage ϕ29-infected *Bacillus amyloliquefaciens. J. Virol.* **6:** 430-437.
16. Wacker, A., 1963. Molecular mechanism of radiation effects, p367-399. In J.N. Davison and W.E. Cohen [ed.], Progress in nucleic acid research, Vol. 1. Academic Press, Inc., New York.

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