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# Isolation and Characterization of a Bacteriophage Infectious to an Extreme Thermophile, *Thermus thermophilus* HB8

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A bacteriophage ($\phi$YS40) infectious to an extreme thermophile, *Thermus thermophilus* HB8, was isolated and characterized. $\phi$YS40 grows over the temperature range of 56 to 78 C, and the optimum growth temperature is about 65 C. The phage had a latent period of 80 min and a burst size of about 80 at 65 C. The phage has a hexagonal head 0.125 $\mu$m in diameter, a tail 0.178 $\mu$m long and 0.027 $\mu$m wide, a base plate and tail fibers. The phage is thermostable in broth but rather unstable in a buffer containing 10 mM Tris, 10 mM $MgCl_2$, pH 7.5. The addition of Casamino Acids (1%), polypeptone (0.8%), yeast extract (0.4%), NaCl (0.1 M) or spermidine (1 mM) to the buffer restores the thermostability of $\phi$YS40 to the same degree as in broth. The phage is also thermostable in water of the hot spring from which this phage was isolated. The nucleic acid of $\phi$YS40 is a double-stranded DNA and has a molecular weight of $1.36 \times 10^8$. The guanine plus cytosine content of the DNA was determined to be about 35% from chemical determinations, buoyant density (1.693 g/cm$^3$ in CsCl), and melting temperature (83.5 C in 0.15 M NaCl plus 0.015 M sodium citrate).

There have been many reports on bacteriophages infectious to thermophilic bacteria (1, 5-8, 14, 16, 17, 19, 20). Most of these are the bacteriophages of *Bacillus stearothermophilus* or its related species which grow above 40 to 42 C and below 72 to 76 C. Properties of these phages and their DNAs have been studied to some extent, but little is known on the mechanism of thermostability of these phages. For further understanding on the growth mechanism of thermophilic organisms, we started the studies on bacteriophages of thermophilic bacteria. This paper reports the isolation and the characterization of the bacteriophage infectious to an extreme thermophile, *Thermus thermophilus* HB8, which can grow at 85 C (11, 12). As far as we know, this is the first description of a bacteriophage of extreme thermophiles.

## MATERIALS AND METHODS

**Bacterial strain.** The host bacterium *T. thermophilus* HB8 (ATCC 27634) has been previously reported (11, 12).

**Media.** HB8 broth consists of 0.8% polypeptone (Kyokuto Seiyaku, Tokyo), 0.4% yeast extract (Difco), 0.2% NaCl, and 0.05% basal elements solution. The broth is adjusted to pH 7.2 with NaOH. The basal elements solution contains 25 g of $MgCl_2 \cdot 6H_2O$, 5 g of $CaCl_2$, 2 g of $MnSO_4 \cdot 6H_2O$, 0.5 g of $ZnSO_4 \cdot 7H_2O$, 0.5 g of $H_3BO_3$, 5 g of $FeCl_3 \cdot 6H_2O$, 15 mg of $CuSO_4$, 25 mg of $Na_2MoO_4 \cdot 2H_2O$, 50 mg of $CoNO_3 \cdot 6H_2O$, 20 mg of $NiNO_3 \cdot 6H_2O$, 80 mg of pyridoxine hydrochloride, 10 mg of folic acid, 0.5 ml of $H_2SO_4$, 100 mg of thiamine hydrochloride, 40 mg of riboflavin, 80 mg of nicotinamide, 80 mg of *p*-aminobenzoate, 10 mg of biotin, 0.4 mg of cyanocobalamine, 80 mg of panthotheic acid, 20 mg of lipoic acid, 200 mg of inositol, 50 mg of cholinchloride, 50 mg of orotic acid, 100 mg of spermidine per liter. Top and bottom agar contain 0.7% and 1.5% agar, respectively, in HB8 broth.

**Phage isolation.** Samples from the hot springs were plated with the culture of *T. thermophilus* HB8. After incubation at 65 C overnight, plaques were picked with toothpicks and single plaque isolation was repeated several times.

**Phage growth.** *T. thermophilus* HB8 was grown to a concentration of $2 \times 10^9$/ml in HB8 broth and infected with $\phi$YS40 at a multiplicity of 0.5. After incubation at 65 C for 10 min, the culture was diluted 10-fold with HB8 broth and the phage was grown for several hours with shaking at 65 C. The final clear lysate contained $10^{10}$ to $5 \times 10^{10}$ PFU/ml of $\phi$YS40.

**Phage purification.** After treatment with pancreatic DNase (10 $\mu$g/ml) and pancreatic RNase (1 $\mu$g/ml), the lysate was cleared by centrifugation for 10 min at 5,000 $\times$ *g*. To 1,000 ml of supernatant liquid, 27 g of NaCl and 100 g of polyethelene glycol 6000 were added. After standing overnight at 4 C, the precipitate was harvested by centrifugation and suspended in 40 ml of a buffer containing 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$. After insoluble materials were removed by centrifugation for 10 min at 5,000 $\times$ *g*, each 3 ml of the supernatant fraction was applied

onto 10 to 30% sucrose gradient centrifugation in Beckman SW27 for 20 min at 20,000 rpm. Sucrose solution contained 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 0.1 M NaCl. Phage were found around the middle of the gradient. Purification by CsCl equilibrium density gradient centrifugation was unsuccessful because the phage is sensitive to high concentrations of salt as described later. Also, the recovery of phage was poor after pelleting phages by ultracentrifugation.

**Radioactive phages.** $^{32}P$-labeled $\phi$YS40 was grown in HB8 broth containing [$^{32}P$]orthophosphate (5 $\mu$Ci/ml) and purified as described above. $^3H$-labeled T4: *Escherichia coli* B *thy*$^-$ was infected with T4 at a multiplicity of 0.5 and grown in M9 Casamino Acids medium (14.7 g of $NaHPO_4 \cdot 12H_2O$, 3 g of $KH_2PO_4$, 5 g of Casamino Acids [Difco], and 4 mg of thymidine $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgCl_2$, 0.3 mg of $FeCl_3 \cdot 6H_2O$, 5 g of Casamino Acid (Difco), and 4 mg of thymidine per liter) containing [$^3H$]thymidine (4 $\mu$Ci/ml). The phage was purified with differential centrifugation.

**Extraction of DNA.** Phage DNA was extracted by the method of Mandell and Hershey (9) and dialyzed against SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). When necessary, DNA was precipitated by 2 volumes of cold ethanol.

**Enzymatic digestion.** Pancreatic RNase A: The reaction mixture (5 ml) contained 0.25 mmol of Tris (pH 7.5), 250 $\mu$g of $\phi$YS40 DNA, and 0.3 mg of RNase A (Sigma Chem. Co.). S1 nuclease: the method of Ando (2) was used. Pancreatic DNase: The reaction mixture (5 ml) contained 50 $\mu$mol of Tris (pH 7.5), 50 $\mu$mol of $MgCl_2$, 50 $\mu$g of $\phi$YS40 DNA, and 10 $\mu$g of DNase I (Sigma Chem. Co.). In every case, the reaction was carried out at 37 C for 60 min and stopped by adding 0.5 ml of 0.25% uranyl acetate in 25% perchloric acid. After standing at 0 C for 10 min, the precipitate was removed by centrifugation and the optical density at 260 nm of the supernatant liquid was measured.

**Base composition.** The DNA was hydrolyzed with 98% formic acid for 30 min at 175 C or 6 N HCl for 3 h at 100 C. After drying, the hydrolysis products were dissolved in 0.1 N HCl and analyzed by the JEOL nucleic acid analyzer JLC-6UH.

**Melting temperature of DNA.** This was measured with a Gilford spectrometer 2400S.

**Buoyant density of DNA.** Buoyant density was determined by CsCl equilibrium centrifugation in a Hitachi 282 analytical centrifuge. Centrifugation was done at 40,000 rpm for 24 h at 25 C. Phage SPO1 DNA ($\rho$ = 1.742) and T7 DNA ($\rho$ = 1.710) were used as density markers.

**Spectrum.** UV and circular dichroism spectra of $\phi$YS40 DNA were obtained with a Cary 17 and a JASCO J-20 Spectro-polarimeter, respectively.

**Molecular weight.** $^{32}P$-labeled $\phi$YS40 DNA was mixed with $^3H$-labeled T4 DNA ($1.3 \times 10^8$ daltons) and applied onto 5 to 20% sucrose gradient centrifugation in Beckman SW50.1 at 30,000 rpm for 2.5 h at 15 C. The positions of $\phi$YS40 and T4 DNA were determined by measuring $^{32}P$ and $^3H$ radioactivity, respectively, with a Packard liquid scintillation spectrometer 3385 in scintillation fluid containing 1,000 ml of toluene, 500 ml of Triton X-100, 5 g of 2,5-diphenyloxazole, and 0.3 g of 2,2-*p*-phenylenbis(4-methyl-5-phenyloxazole). The molecular weight was determined by using the equation obtained by Burgi and Hershey (4): $D_2/D_1 = (M_2/M_1)^{0.35}$.

## RESULTS

**Growth characteristics.** Several phages infectious to *T. thermophilus* HB8 were isolated from Mine and Atagawa hot springs, Japan. One of these, named $\phi$YS40, was further studied. This phage is a virulent phage because it makes a clear plaque on the plate. Efficiency of adsorption to the host was about 50% in HB8 broth after incubation at 60 C for 15 min with a bacterial concentration of $2 \times 10^9$ cells/ml. $\phi$YS40 grew over the temperature range of 56 to 78 C and the optimum growth temperature was about 65 C, at which temperature the burst size was about 80. A one-step growth experiment showed that $\phi$YS40 has a latent period of about 80 min at 65 C. The phage growth was very poor below 55 C and above 78 C. The growth temperature range of $\phi$YS40 was narrower than that of the host (50 to 85 C).

Infectivity of $\phi$YS40 to previously reported thermophiles (3, 15) was tested (Table 1). *Thermus aquaticus* YT-1 isolated from Yellowstone Park in the United States was insensi-

TABLE 1. *Host range of $\phi$YS40*

| Bacterial strains | Reference | Sensitivity | Plating efficiency[a] |
|---|---|---|---|
| *Thermus flavus* BS1 | 15 | +[b] | 0.41 |
| *Thermus flavus* BS2 | 15 | – | |
| *Thermus flavus* AT61 | 15 | +[b] | 0.14 |
| *Thermus flavus* AT62 | 15 | – | |
| *Thermus flavus* BK1 | 15 | +[b] | 0.05 |
| *Thermus aquaticus* YT1[c] | 3 | – | |
| *Thermus* HB26 | U[d] | + | 1.0 |
| *Thermus* HB27 | U[d] | + | 0.01 |
| *Thermus* HA23 | U[d] | – | |
| *Thermus* 110 | U[d] | – | |
| *Thermus* 111 | U[d] | – | |
| *Bacillus stearothermophilus* IAM1035 | O[e] | – | |

[a] $\phi$YS40 was plated with these bacteria at 65 C. Plating efficiency on *T. thermophilus* HB8 was assumed to be 1.0.

[b] $\phi$YS40 makes a turbid plaque on these strains for unknown reasons.

[c] Grown in the medium described by Brock and Freeze (3).

[d] U, Unpublished data, T. Oshima.

[e] O, Obtained from Institute of Applied Microbiology, University of Tokyo, Japan.

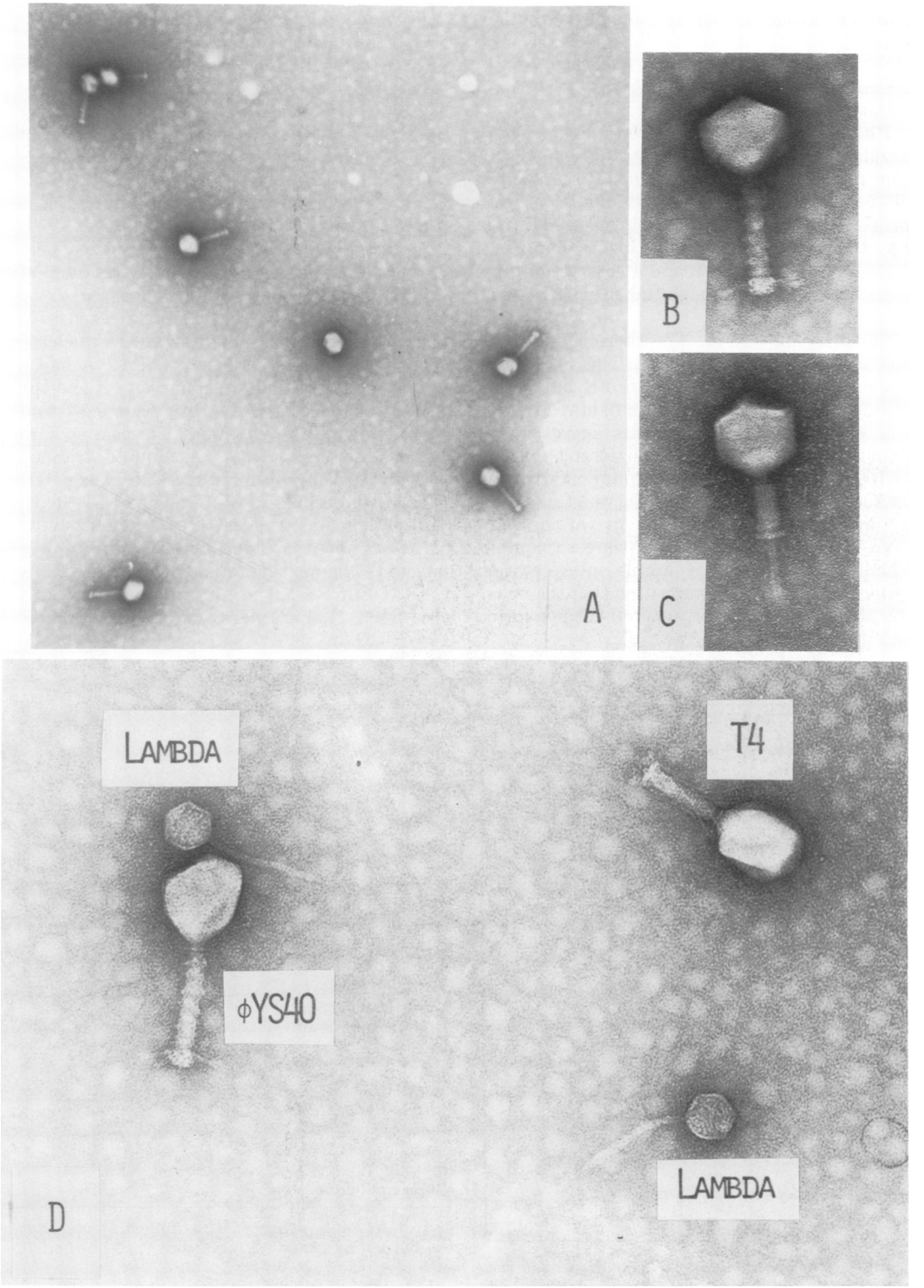


FIG. 1. *Electron micrographs of φYS40 negatively stained with uranyl acetate. (A) ×30,000, (B) ×90,000. (C) A shrunken particle; ×90,000. (D) Samples containing phage T4 and lambda for comparison; ×90,000.*

tive to this phage, whereas some of the thermophiles isolated from hot springs of Japan were sensitive. φYS40 was not infectious to *B. stearothermophilus*, *E. coli* K-12, or *Pseudomonas aeruginosa*.

**Phage morphology.** Electron micrographs of negatively stained φYS40 are shown in Fig. 1. The phage has a hexagonal head 0.125 μm in diameter, a tail 0.178 μm long and 0.027 μm wide, a base plate, and tail fibers. As shown in Fig. 1D, φYS40 appears to be bigger than phage T4 or lambda. A sheath and a core structure of the tail are visible in a shrunken particle (Fig. 1C).

**Stability of φYS40.** The thermostability of φYS40 was studied (Fig. 2). The phage was fairly thermostable in HB8 broth but rather unstable in a buffer containing 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$. This suggests that something in HB8 broth stabilized the phage at high temperatures. To obtain more information on such a stabilizing factor(s), we studied the thermostability of the phage in various solutions (Table 2). The results were so complicated, however, that a simple interpretation cannot be made. It is interesting that the phage was thermostable in the water of the hot spring from which this phage was isolated. The factor(s) contained in this water is thought to be a natural stabilizing factor(s) for this phage.

The phage was sensitive to high concentrations of salt such as NaCl or CsCl; it was easily inactivated in the presence of 2 M NaCl. At high concentrations of salt, phage DNA appeared to be released from a phage particle because the phage suspension became viscous in the absence of DNase.

**Properties of phage DNA.** The nucleic acid of φYS40 was extracted by the method of Mandell and Hershey (9). It appeared to be double-stranded DNA because it was sensitive to pancreatic DNase but resistant to nuclease S1(2) and pancreatic RNase. The DNA had a maximum absorption at 259 nm ($A_{259}$) and the ratios of $A_{280}/A_{260}$ and $A_{230}/A_{260}$ were 0.545 and 0.434, respectively. Circular dichroism spectra showed this was a typical double-stranded DNA. Base composition of the DNA was determined as described in Materials and Methods. The guanine plus cytosine (G+C) content of the DNA was about 35% and no unusual base was detected. The melting temperature of the DNA was 83.5 C in SSC. The buoyant density in CsCl was 1.693. Using the equation G+C(%) = $2.44(T_m\text{-}69.3)$ (10) and $\rho$ = 1.660 + (G+C%)/1,000 (18), the G+C content of the DNA was estimated to be 34.6 and 33.0%, respectively. These estimated G+C contents agree with those obtained from chemical deter-

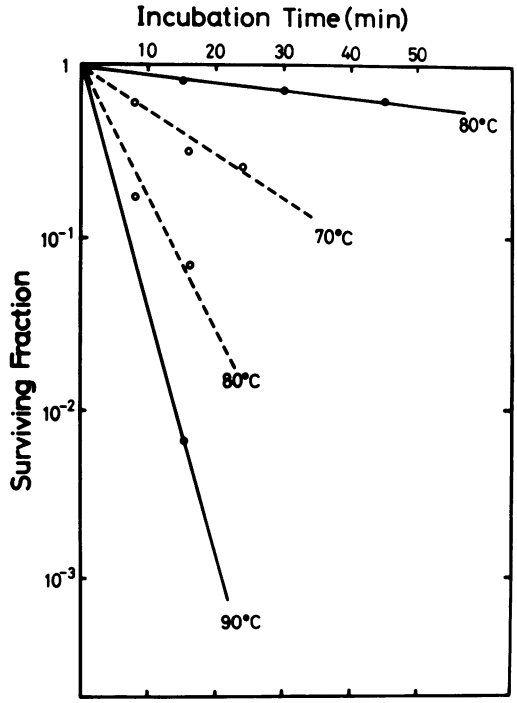


FIG. 2. *Thermostability of φYS40. φYS40 was heated at the indicated temperatures for the indicated periods in HB8 broth (●) or a buffer containing 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$ (○).*

TABLE 2. *Thermostability of φYS40 in various solutions*

| φYS40 suspended in:[a] | Survival (%)[b] |
|---|---|
| HB8 broth | 80 |
| Tris-$Mg^{2+}$ [c] | 0.5 |
| Peptone (0.8%) | 36 |
| Yeast extract (0.4%) | 51 |
| Casamino Acids (1%) | 70 |
| Basal element solution (0.05%) | <0.1 |
| Amino acid mixture[d] | <0.1 |
| NaCl (0.1 M) | 50 |
| Glycerol (5%) | 0.4 |
| Gelatine (0.1%) | 0.8 |
| Putrescine (1 mM) | <0.1 |
| Cadaverine (1 mM) | 2.2 |
| Spermidine (1 mM) | 35 |
| Spermine | 1.9 |
| Water from mine hot spring | 80 |

[a] All the solutions except HB8 broth and water from the hot spring contain 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$.

[b] Survival of φYS40 after heating at 80 C for 30 min.

[c] 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$.

[d] 0.5 mg of each amino acid per milliliter.

minations. The G+C content of the phage DNA is remarkably different from that of the host bacterial DNA (69%) (13). The molecular weight of the phage DNA was determined to be $1.36 \times 10^8$, as described in Materials and Methods, using $^3$H-labeled T4 DNA ($1.3 \times 10^8$ daltons) as an internal marker.

## DISCUSSION

A bacteriophage infectious to the extreme thermophile *T. thermophilus* HB8 was isolated and characterized. As expected, the phage ($\phi$YS40) is thermophilic and stable at high temperatures. The thermostability of $\phi$YS40, however, depends upon the composition of the solution in which the phage is suspended. This suggests that a certain factor(s) is required to stabilize the phage at high temperatures. We think that studies on such a factor(s) may be interesting because it may provide an insight into the mechanism of the thermostability of thermophilic organisms. In this context, it is interesting that the phage is quite thermostable in water of the hot spring from which the phage was isolated, because the factor(s) contained in this water is probably a natural one for stabilizing the phage at high temperatures. A study of the effective factor(s) contained in this water is now in progress.

There is no evidence suggesting that the phage DNA needs some special properties to exist at high temperatures. Since a G+C content of $\phi$YS40 DNA is 35%, phage DNA probably contains highly adenosine-plus-thymidine-rich regions which melt at lower temperatures. Therefore, such regions of $\phi$YS40 DNA may have unique properties to avoid the melting at high temperatures. Further study is required, however, to clarify this problem.

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