

Isolation and Characterization of an *Escherichia coli* Bacteriophage Requiring Cell Wall Galactose

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A new coliphage, designated U3, has been selected for the ability to discriminate the presence of galactose in the cell wall of *Escherichia coli*. U3 attacks *E. coli* K-12 cells that are able to incorporate galactose into their cell walls, but mutants blocked in the synthesis of uridine diphosphogalactose, the precursor of cell wall galactose, are completely resistant to the phage. U3 is a small, tail-less, approximately spherical phage resembling ϕ X174 in its physical properties. Its diameter by electron microscopy is 21 to 22 nm, and its particle weight is approximately 4×10^6 daltons. Like ϕ X174, U3 appears to have a single-stranded deoxyribonucleic acid genome and has at least four cistrons.

Many bacteriophages serve as sensitive indicators of specific cell wall variants in populations of bacterial cells. For example, the phage P22 attacks only *Salmonella* cells that have incorporated galactose into the lipopolysaccharide of their cell wall; galactose-deficient cells are completely resistant to the phage (3). Phages such as P22 that recognize the presence of galactose in the bacterial coat are especially useful in the diagnosis and selection of mutations affecting cell wall biosynthesis. Among the extant coliphages we have surveyed, only phage C21 was able to distinguish the presence of galactose in *Escherichia coli* cell walls. The host range of this phage is the reverse of that shown by P22, for it attacks *E. coli* K-12 cells deficient in cell wall galactose (5) but not *E. coli* K-12 cells that contain cell wall galactose. A coliphage whose host range parallels that of P22, that is, a phage which attacks only cells with galactose in their cell walls, would be useful in the selection and diagnosis of mutants. A phage with this property has now been isolated.

This communication describes the isolation and preliminary characterization of this new phage, designated U3. The evidence indicates that U3 is a small, single-stranded deoxyribonucleic acid (DNA) phage rather similar to ϕ X174 in its physical properties.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used are listed and described in Table 1. A T4-resistant mutant of strain SA242 was isolated in addition. Phage strains used were λ , T4B (an rII mutant), C21, and ϕ X174, and were obtained from J. Weigle, A. Hershey, H. Wu, and I. Tessman, respectively.

Media. Defined growth medium was M9 (1) with 0.1% glycerol as the carbon source and supplemented with 1 μ g of thiamine per ml and 20 μ g of methionine per ml when required. Broth contained 1% tryptone (Difco) and 0.5% NaCl. PAM, modified from Guthrie and Sinsheimer (4), contained 0.8% nutrient broth (Fisher Scientific Co.), 1% Casamino Acids, 10% sucrose, and 0.2% MgSO₄ (added after autoclaving). Solid agar medium consisted of tryptone broth with 1.5% agar added; soft agar used for overlay was either tryptone broth with 0.65% agar added or, for experiments with spheroplasts, PAM with 0.8% agar added. Dilution media consisted of 10 mM MgSO₄, 10 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 7.4) and 0.006% gelatin.

Chemicals. Deoxyribonuclease I (electrophoretically purified), ribonuclease B, and lysozyme were from Worthington Biochemicals (Freehold, N.J.). Sterile 35% bovine serum albumin (BSA) was from Mann Research Laboratories (New York, N.Y.).

Isolation of U3. Raw sewage (provided by the Buffalo Sewer Authority) was clarified by centrifugation followed by filtration through a membrane filter (type HA, 0.45 μ m pore size, Millipore Corp., Bedford, Mass.). The filtrate contained approximately 140 plaque-forming particles/ml when plated with cells of strain W3110. These plaques varied greatly in size and appearance. Phages whose adsorption did not require the presence of cell wall galactose were depleted by adsorbing the filtrate with 6×10^9 cells per ml of a *gal* deletion strain (SA242). To satisfy possible adsorption cofactor requirements, the adsorption mixture contained tryptone (1%), yeast extract (0.5%), NaCl (0.5%), glucose (0.1%), MgSO₄ (1 mM) and CaCl₂ (0.25 mM). After 8 min at 37 C, the suspension was centrifuged, and the pellet of cells containing any phage they had adsorbed was discarded. To select phages whose adsorption requires cell wall galactose, 1.6×10^9 *gal*⁺ cells per ml (strain W3110)

TABLE 1. *Bacterial strains and host range of phage U3^a*

Strain designation	Description	Efficiency of plating U3 ^b	Source
W3110	K-12	1.0	J. Weigle
W3350	K-12, <i>galK⁻ galT⁻</i>	1.0	J. Weigle
W4597	K-12, <i>galU⁻</i>	<10 ⁻⁹	T. Fukasawa
C600	K-12, <i>suII⁺</i>	0.6	J. Weigle
SA242	K-12, $\Delta gal_{\lambda 2}$	<10 ⁻⁹	S. Adhya
SA242 (λdg)	SA242 transduced to <i>gal⁺</i> with λdg	1.2	This laboratory
SA242 (<i>gal⁺</i>)	SA242 transduced to <i>gal⁺</i> with P ₁	0.4	This laboratory
S165 (λ)	K-12, Δgal	<10 ⁻⁹	S. Adhya
S165 (λ , λdg)	S165(λ) transduced to <i>gal⁺</i> with λdg	0.8	This laboratory
PL2	K-12, <i>galE⁻ met⁻</i>	0.6	A. Rapin
Q81	K-12, F ⁻	0.5	A. Campbell
W52	<i>Escherichia coli</i> B	<10 ⁻⁹	J. Weigle
W53	<i>E. coli</i> C	<10 ⁻⁹	J. Weigle
Q176	<i>E. coli</i> W	<10 ⁻⁹	L. Pizer
Q172	<i>Shigella</i> 16	<10 ⁻⁹	S. Luria
LT2	<i>Salmonella typhimurium</i>	<10 ⁻⁹	S. Luria
W3350 (P1kc)	W3350 lysogenic for P1kc	0.3	This laboratory

^a Symbols used are: K-12, *E. coli* K-12; *galK⁻*, *gal*-actokinase deficient; *galT⁻*, uridine diphosphate (UDP)-galactose-transferase deficient; *galE⁻*, UDP-galactose-4-epimerase deficient; *galU⁻*, UDP-glucose pyrophosphorylase deficient; Δgal , deletion including most or all of the galactose operon; λdg , defective phage λ carrying genes of the galactose operon; *suII⁺*, carries amber suppressor II; *met⁻*, nutritional requirement for methionine.

^b Indicates the ability or inability of a bacterial strain to support U3 plaque formation on tryptone broth-agar plates relative to strain W3110.

were then added to the supernatant fluid and adsorption was allowed to proceed for another 8 min at 37 C. This suspension was centrifuged, the pellet of cells and adsorbed phage was resuspended in dilution medium, and samples were plated with a mixed indicator designed to identify phages whose adsorption required cell wall galactose. This indicator contained approximately 1% *gal* deletion cells (strain SA242) and 99% *gal⁺* cells (strain W3110). Phages unable to adsorb to cells lacking cell wall galactose form speckled plaques on this indicator; phage U3 was isolated from one of several such speckled plaques on these plates.

Single-cycle growth curve. Log-phase cells of strain W3110 grown in tryptone broth were infected with U3 at a multiplicity of infection (MOI) of less than one in the presence of 2.5 mM NaCN. After more than 95% of the phage had been adsorbed, the mixture was diluted with fresh broth and allowed to grow at 37 C with aeration. Samples were titered for infective centers at various times after dilution.

Adsorption kinetics. Growth of log-phase cells in M9 minimal medium with and without added galactose was arrested by the addition of NaCN to 2.5 mM. U3 phage particles were added at an MOI of less than one, and at various times samples were removed,

diluted, treated with a few drops of CHCl₃ to kill infected cells, and plated with strain W3110 as the host. The fraction of free phage remaining was calculated for each time. The presence or absence of galactose or methionine after the addition of NaCN had no effect on the adsorption kinetics. In similar experiments with amber mutants of U3, the addition of CHCl₃ was unnecessary as the mutant phage did not proliferate in the nonpermissive hosts to which the phage were adsorbed. In this case, free mutant phage remaining were determined by plating on strain C600, a permissive host.

Isolation of amber-suppressible mutants. The method used was adapted from the mixed indicator technique of Thomas et al. (8). U3 phage were irradiated with ultraviolet light to a survival frequency of 7×10^{-4} . The irradiated phage together with the permissive host, strain C600, were plated in soft agar on plates that already contained another soft agar overlay, seeded with the nonpermissive host strain W3110, which had been preincubated for 1 to 2 hr. After an additional 8 hr of incubation at 37 C, the plates were scanned for semiclear plaques in which the top layer of strain C600 was lysed, but the underlying layer of strain W3110 was not. Such plaques were picked, tested for growth on strains C600 and W3110, and cloned twice on strain C600 if they were unable to make plaques on strain W3110. Suppressible mutants were isolated with an average frequency of one per 10⁸ phage surviving irradiation.

Antiserum against U3. Rabbits were injected intravenously nine times over a period of 3 weeks with 8×10^9 viable particles of U3 per injection. Antisera from three rabbits were collected by bleeding from the ear once a week for several weeks starting 1 week after the last injection. All sera were pooled.

Complementation tests. Cells of strain W3110 were infected with two amber mutants of U3 at an MOI of between 2 and 10 for each mutant. After allowing 15 min for adsorption of phage to cells (in 2.5 mM NaCN to prevent lysis), the infected cells were diluted 10-fold into dilution medium containing antibody against U3 to eliminate free phage. The infected cells were then further diluted and plated with strain W3110 as the host. Only cells that had been infected with two complementing mutants were capable of phage multiplication and produced wild-type recombinants able to form plaques on the W3110 host. In this manner, several U3 mutants were assigned to complementation groups.

Kinetics of ultraviolet killing. U3, T4B, and λ phages were mixed in dilution medium at about 3×10^7 per ml each. A 10-ml amount of this mixture was irradiated at a distance of 61 cm from two 15-w germicidal lamps (General Electric). The energy dosage was estimated at 7.5 ± 2.5 ergs per mm² per sec. Irradiation was interrupted at various times to take samples which were diluted and plated on three different host strains of bacteria. Strain SA242 (λdg) supported only the growth of U3, strain W52 supported only T4B, and a T4-resistant mutant of strain SA242 supported only λ . A similar experiment comparing U3 and ϕ X174 was done by using strains W3110 and

W53 as the mutually exclusive hosts. These experiments were done in the presence of yellow light to minimize photoreactivation.

Centrifugation. The buoyant density of U3 was estimated by centrifuging 5×10^9 particles in 3 ml of a CsCl solution for 23 hr at $91,000 \times g$ in an SW39 rotor. Fractions were collected through a hollow needle piercing the bottom of the tube. The density of every sixth fraction was determined by refractive index, and the remaining fractions were titrated for viable phage by using strain W3110 as the host.

Sedimentation velocity experiments were carried out in linear 5-ml sucrose gradients containing 3 to 20% sucrose (w/v) dissolved in dilution medium. A mixture of U3 and ϕ X174 in 0.2 ml of dilution medium was layered on top of a gradient which was then centrifuged for 45 min at $44,000 \times g$ in an SW39 rotor. Through a hollow needle piercing the bottom of the tube, 0.1-ml fractions were collected into 1-ml volumes of dilution medium. The number of viable phage in each fraction was determined by titrating on strain W3110 for U3 and on strain W53 for ϕ X174.

U3 nucleic acid. A 400-ml lysate of U3 was centrifuged at low speed with 1 mg of celite per ml and then passed through a membrane filter to remove cells and cell debris. The lysate was then centrifuged for 90 min at $54,000 \times g$ in a Spinco 30 rotor; the pellets were resuspended and pooled in 30 ml of 0.05 M Tris (pH 7.4); the phage were collected once more by centrifugation; and finally they were resuspended in 3 ml of Tris buffer. The concentrated phage suspension was extracted twice with equal volumes of cold, redistilled phenol (saturated with Tris buffer) and dialyzed to remove dissolved phenol. Extracted U3 nucleic acid was stored at -20°C .

U3 DNA infection of spheroplasts. Infection was carried out after the method described by Guthrie and Sinsheimer (4) and modified by Strauss (7). To make spheroplasts, a 40-ml culture of strain SA242 in tryptone broth was harvested by centrifugation at a cell density of about 4×10^8 per ml. The pellet was resuspended in 0.35 ml of 1.5 M sucrose and then the following were added in sequence: 0.17 ml of 35% BSA, 0.02 ml of lysozyme (2 mg/ml in 0.25 M Tris, pH 8.1) and 0.04 ml of 4% EDTA. After 10 min of incubation at room temperature, 10 ml of PAM was added. To 0.1 ml of this spheroplast preparation, 0.1 ml of buffer (10 mM Tris, pH 7.4, and 10 mM MgSO_4) with or without deoxyribonuclease was added followed by 0.7 ml of U3 DNA in PAM. After 10 min of incubation at 37°C , U3 antiserum and NaCN (to a concentration of 2.5 mM) were added to eliminate any free phage and to inhibit premature lysis of infected cells. After another 20 min, samples were diluted in PAM and plated with strain W3110 in PAM soft agar.

RESULTS

Growth characteristics. U3 plaques were clear and large, about 6 mm in diameter after overnight incubation at 37°C . In single-cycle growth curves (Fig. 1), the latent period in broth medium was 15 to 17 min, and the burst size averaged 100 to 200 phage particles per cell.

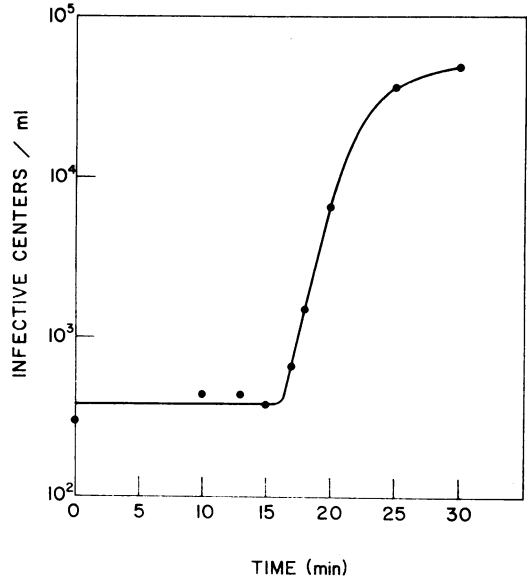


FIG. 1. Single-cycle growth curve.

Lysates of U3 were unstable, even at 4°C and after the removal of bacterial cell debris by centrifugation with celite and filtration through a membrane filter. The half-life of the phage in lysates so treated was about 2 days. U3 could be stabilized either by addition of NaCl to a concentration of 1 M or by freezing.

Host specificity. The host range of U3 is indicated in Table 1. Only *E. coli* K-12, and not *E. coli* B, C, or W, was sensitive to U3. Among the K-12 strains tested, only those capable of synthesizing uridine diphosphogalactose, a precursor of bacterial cell wall lipopolysaccharide, proved hosts for U3. An apparent exception was strain PL2; however, as demonstrated below, strain PL2 becomes sensitive to U3 when grown in the presence of low concentrations of galactose. Presumably, the small amounts of galactose present in commercial agar together with that liberated during autoclaving were sufficient to make strain PL2 sensitive to U3 on agar plates.

Most coliphages are sensitive to P1 restriction, that is, the efficiency of plaque formation on a host lysogenic for phage P1 is much lower than on a nonlysogenic host. Exceptions to P1 restriction are the ribonucleic acid phages, single-stranded DNA phages, glycosylated DNA phages, and phage T5 (2). The efficiency of plaque formation of U3 on strain W3350 (P1kc) was only slightly less than on the parental strain W3350 (Table 1), implying that U3 belongs to one of the classes of phage that are resistant to P1 restriction.

U3-receptor sites. Galactose-deficient strains

are resistant to U3 because they lack functional receptor sites for this phage. At cell concentrations of 10^8 per ml, strain W3110 rapidly adsorbed phage U3, whereas strain SA242, which is deleted for the *gal* operon and cannot synthesize uridine diphosphogalactose from uridine diphosphoglucose, did not adsorb U3 significantly (Fig. 2a). Cells of strain SA242 grown in the presence of galactose also failed to adsorb U3 since they are unable to synthesize uridine diphosphogalactose from exogenously supplied galactose.

The *gal*-negative strain PL2, which carries a point mutation in the epimerase gene, is also unable to convert uridine diphosphoglucose to uridine diphosphogalactose and therefore synthesizes a galactose-deficient cell wall lipopolysaccharide (5). Strain PL2 when grown in M9-glycerol was as unable to adsorb U3 as was strain SA242. However, when cells of strain PL2 were allowed to grow for a generation in the presence of galactose, uridine diphosphogalactose could be accumulated directly through the action of galactokinase and uridine diphosphoglucose-galactose transferase. Once able to synthesize uridine diphosphogalactose, strain PL2 became capable of adsorbing U3 (Fig. 2b). PL2 cells accumulate lethal levels of uridine diphosphogalactose when exposed to external galactose concentrations exceeding 2×10^{-5} M. At this concentration of galactose, the conversion of PL2 cells to a U3-

sensitive form is limited, and the PL2 cells never become as capable of adsorbing U3 as cells of the wild-type strain W3110.

These experiments demonstrate that among K-12 strains the host range of phage U3 is determined at the level of phage adsorption rather than at some later stage in the phage cycle. Also, the synthesis of functional U3 phage-receptor sites requires the prior synthesis of uridine diphosphogalactose.

Genetics of U3. Of 20 amber-suppressible mutants isolated, 9 have been assigned to complementation groups, 2 do not yield recombinants with any of the mutants tested, and the remaining 9 either have too high a reversion rate to wild type, or produce lysate titers too low to yield reliable complementation data. Table 2 indicates a set of U3-am crosses and whether the members of each cross did or did not complement and recombine to form wild-type phage. The following complementation groups were assigned: (A) 3, 7, 19; (B) 10, 13; (C) 2, 14, 20; and (D) 12. Since these nine mutants were not a random sample of the 20 mutants isolated, and since the method of mutant isolation was probably nonrandom with respect to all of the U3 cistrons, one cannot assume a Poisson distribution to estimate the total number of U3 cistrons. Nevertheless, the fact that nine mutants were distributed among only four complementation groups suggests that the U3 genome contains only a few cistrons.

Physical properties of U3. Ultraviolet killing curves for U3 were compared to curves for λ , T4B, and ϕ X174 to estimate the size of the U3 genome. The results (Fig. 3) indicate that U3 is either a double-stranded phage of a size intermediate between λ and T4, or a much smaller, single-stranded phage very similar in size to ϕ X174. Indeed, the curves for U3 and ϕ X174 are indistinguishable. Because single-stranded DNA is several times more sensitive to ultraviolet radiation than double-stranded DNA, single-stranded DNA phages have high rates of inactivation despite their small physical sizes.

The buoyant density of U3 was determined by equilibrium centrifugation in CsCl. Most of the phage were recovered in fractions having densities around 1.40 (Fig. 4); however, there was a significant and reproducible minor peak at a density of 1.365. These values are lower than the buoyant densities of most double-stranded DNA phages and are very similar to the densities of single-stranded phages.

Electron micrographs of U3 purified by CsCl centrifugation and negatively stained with phosphotungstic acid showed particles that were tail-

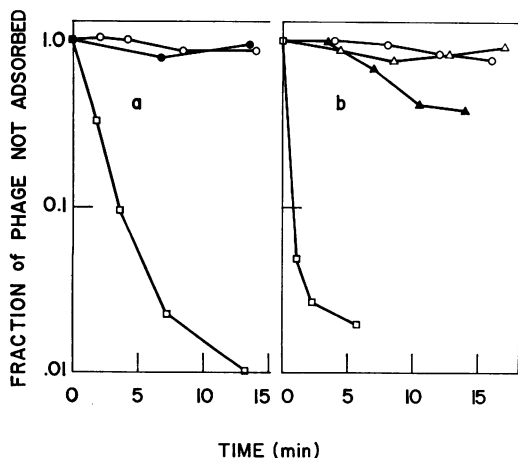


FIG. 2. Adsorption kinetics of phage U3 to cells. (a) U3⁺ phage adsorbed to strains W3110 (□), SA242 (○), and SA242 grown for several generations in the presence of 5 mM galactose (●). These kinetics are based on cell concentrations of 10^8 per ml. (b) U3^{am14} phage adsorbed to strains W3110 (□), SA242 (○), PL2 (△), and PL2 grown for one generation in the presence of 0.02 mM galactose (▲). These kinetics are based on cell concentrations of 2×10^9 per ml.

TABLE 2. Complementation between U3 amber mutants

U3 am	U3 am										
	2	3	7	9	10	12	13	14	18	19	20
2	0	+	+	0	+	+	+	0	0	+	0
3		0	0	0	+	+	+	+	0	0	+
7			0	0	+	+	+	+	0	0	+
9			0	0	0	+	+	+	0	0	0
10				0	0	0	0	+	0	+	+
12						0	+	+	0	+	+
13						0	0	+	0	+	+
14							0	0	0	+	0
18								0	0	0	0
19									0	0	+
20											0

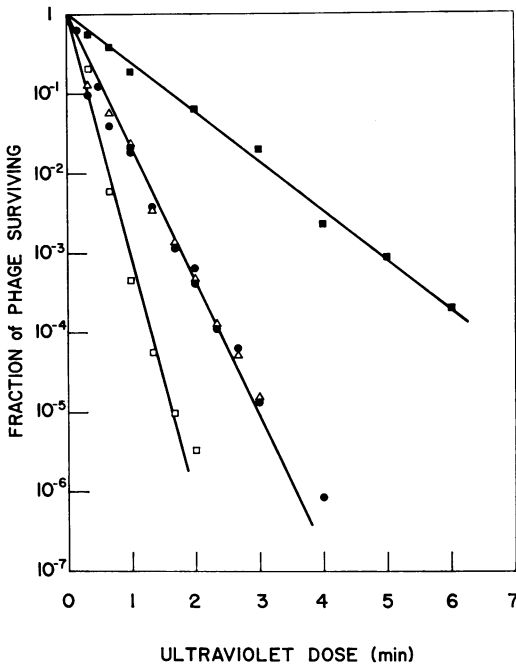


FIG. 3. Ultraviolet inactivation of phages λ (■), U3 (●), ϕ X174 (Δ), and T4B (\square).

less and approximately spherical with a diameter of 21 to 22 nm.

U3 sediments more slowly in a sucrose gradient than does ϕ X174 (Fig. 5). The sedimentation coefficients of the peak fractions for ϕ X174 and U3 were 114 and 83S, respectively. These values when combined with the known particle weight of ϕ X174 [6.2×10^6 daltons, determined by light scattering (6)] suggest that U3 has a particle weight of approximately 4×10^6 daltons.

U3 nucleic acid. Phenol-extracted U3 nucleic acid was sensitive to degradation by deoxyribo-

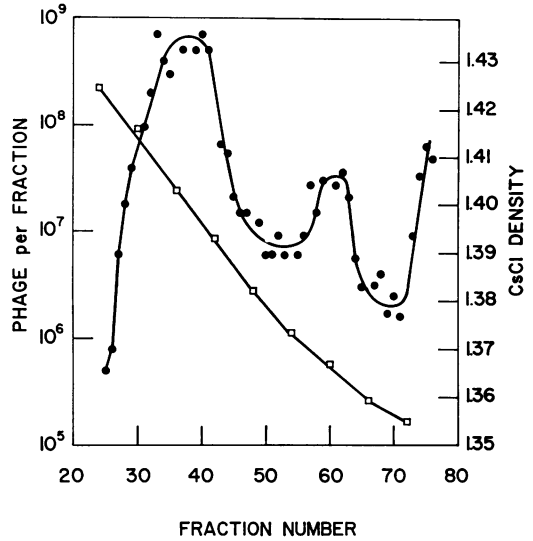


FIG. 4. Equilibrium centrifugation of U3 in CsCl. Density gradient of CsCl (\square) and U3 titers per fraction (\bullet) are indicated.

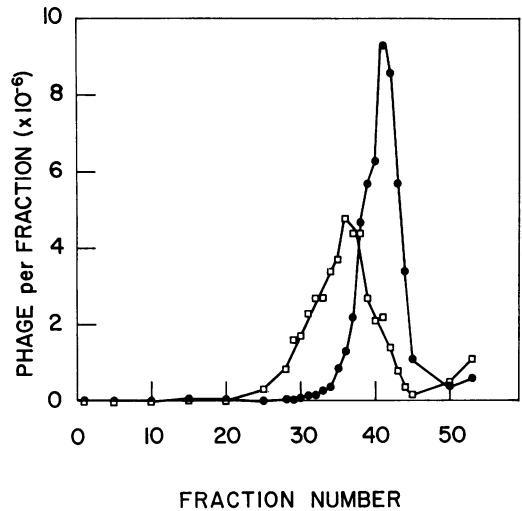


FIG. 5. Sedimentation of U3 and ϕ X174 in a sucrose gradient. U3 titers per fraction (\bullet) and ϕ X174 titers per fraction (\square) are indicated. The direction of sedimentation is right to left.

nuclease but not by ribonuclease (Fig. 6). Thus U3 is a DNA phage. When U3 DNA ($8.8 \mu\text{g/ml}$ in 0.05 M Tris, pH 7.4) was heated for 5 min in a boiling-water bath and then rapidly cooled, the absorption at 260 nm increased only 2%, suggesting that this DNA is single-stranded.

The extracted U3 DNA was able to infect spheroplasts of strain SA242 and produce normal phage particles. The efficiency of infection (num-

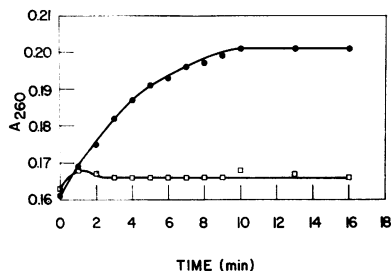


FIG. 6. Enzymatic degradation of U3 nucleic acid. Hyperchromic effects caused by incubation with deoxyribonuclease (●) and with ribonuclease (□) are indicated.

ber of infected cells/number of phage genomes extracted) was 2×10^{-6} . As expected, U3 DNA infection was inhibited by deoxyribonuclease (Table 3).

DISCUSSION

In its physical properties, U3 resembles the small, spherical, single-stranded DNA phages such as ϕ X174 and S13. Bouyant density, sedimentation velocity, and electron microscopy suggest that U3 is a small, tail-less phage, approximately spherical, with a diameter of 21 to 22 nm and a weight of approximately 4×10^6 daltons. Nucleic acid extracted from U3 loses its infectivity and displays hyperchromicity when exposed to deoxyribonuclease, but not when exposed to ribonuclease. This suggests that U3 contains a DNA genome rather than a ribonucleic acid genome. The kinetics of ultraviolet killing, the lack of sensitivity to P1 restriction, and the absence of a hyperchromic effect after boiling the extracted DNA all suggest that the DNA of U3 is single-stranded. The small number of cistrons found among the complementing amber mutants of U3 is consistent with the conclusion that U3 is a small, single-stranded DNA phage.

U3 differs from ϕ X174 and S13, and indeed from all other coliphages we have tested, in its host range. Among *E. coli* strains tested it infects only K-12 and among the K-12 family of strains, only those capable of synthesizing uridine diphosphogalactose. Presumably, galactose must be transferred from uridine diphosphogalactose and incorporated into the bacterial cell wall to make active U3 phage-receptor sites.

These properties make U3 quite useful for the diagnosis or selection (or both) of certain phenotypic variants and mutants. For example, among 69 spontaneous U3-resistant mutants of strain W3110, 11 proved to be galactose nonfermentors.

TABLE 3. Effect of deoxyribonuclease on the ability of extracted U3 nucleic acid to infect spheroplasts

Deoxyribonuclease (μ g/ml)	Infected cells	Fraction of control
0	2.5×10^4	1.0
0.01	8.4×10^3	0.34
0.1	1.7×10^3	0.068
1.0	2×10^2	0.008
10	10	0.0004

An indication that these mutants had lost the ability to synthesize uridine diphosphogalactose was provided by the observation that all 11 had become sensitive to phage C21, which only attacks K-12 strains unable to incorporate galactose into their cell wall (5).

U3 has also been used to measure the kinetics of receptor site formation when *gal* deletion cells are infected by λ dg transducing phage carrying a normal copy of the *gal* operon.

Analogously, the use of U3 has permitted the demonstration that functional U3-receptor sites can be reconstituted from spheroplasts of U3-resistant cells and lipopolysaccharide extracted from U3-sensitive cells (Nature, *in press*).

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