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UC-1, a New Bacteriophage that Uses the TonA Polypeptide as Its Receptor

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We characterized UC-1, a previously undescribed *Escherichia coli* phage. UC-1 was observed to have an icosahedral head and a long, flexible, noncontractile tail; its genome consisted of linear double-stranded DNA having a molecular weight of 34×10^6. The product of the *tonA* gene served as at least part of the receptor for UC-1. *E. coli tonA* strains neither plated nor adsorbed UC-1 well, *tonA* mutants were selected on the basis of UC-1 resistance, and ferrichrome, a siderophore which utilizes TonA as its receptor, blocked infection. Restriction analyses, DNA-DNA hybridization experiments, and guanine-plus-cytosine determinations demonstrated that UC-1 DNA was unrelated to that of other phages (T1, T5, and φ80) which employ TonA as a receptor. Also, mutants specifically resistant to UC-1 were isolated. UC-1 may be useful as a probe for investigating TonA, which functions as a receptor for more ligands than any other membrane protein. Study of the resistant mutants may improve our understanding of how phage DNA penetrates the cell envelope.

Bacteriophage-resistant mutants of *Escherichia coli* K-12 fall into a relatively small number of groups, even when their isolation is accomplished with a great variety of virulent bacteriophages (12). The TonA resistance group is a particularly interesting example of one such class; *tonA* mutants are resistant to phages T1, T5, and φ80, colicin M, and the antibiotic albomycin. The product of the *tonA* gene, TonA, is a 78,000-dalton outer membrane protein (4, 7) which serves as a receptor for the lethal ligands listed above (20). TonA functions in iron uptake as a receptor for the hydroxamate siderophore ferrichrome, and the biosynthesis of TonA is regulated by the amount of available iron (19). Because this role in iron assimilation is the only known physiological function of the protein, it has been proposed that *tonA* be renamed *fhuA* (ferric hydroxamate uptake) (18).

Here we describe UC-1, a new bacteriophage that uses the TonA polypeptide for at least part of its receptor. The phage is similar in structure to T1, T5, and φ80 but is genetically unrelated to any of these well-known phages.

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* AB259 *thi-1 relA1 spoT1 supQ80*, obtained from B. Bachmann of the Coli Genetic Stock Center, was routinely used as the host organism. Colicin M was isolated by standard procedures from cultures of Plasmid Reference Center strain PRC 442 pColM. Sources and relevant characteristics of other *E. coli* K-12 strains are shown in Table 1.

Bacteriophages T5 and T6 were from the collection of this laboratory. T1 was obtained from H. Drexler, and φ80 and λ were supplied by J. R. Walker, as was φX174 RFII DNA. UC-1 was isolated from local sewage by the Microbial Genetics group at the University of Oklahoma, Norman, Oklahoma.

Medium and growth conditions. Cells were typically grown at 37°C with aeration in L broth, which consisted of 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of distilled water; the pH was adjusted to 7.0 with NaOH. L broth was supplemented with 0.75 and 1.5% agar for top agar and plates, respectively. MM medium has been described previously (16).

Enzymes and chemicals. DNase I and RNase were obtained from Worthington Diagnostics, Freehold, N.J. Restriction endonucleases were purchased from New England Biolabs, Beverly, Mass.

Ethidium bromide and $H_3{}^{32}PO_4$ were obtained from Sigma Chemical Co., St. Louis, Mo., and New England Nuclear Corp., Boston, Mass., respectively; ferrichrome and ferrichrome A were obtained from Porphyrin Products Inc., Logan, Utah.

Purification of phages. Bacterial debris was removed from lysates by centrifugation, and the phages were concentrated from the resulting supernatants by precipitation with 12% polyethylene glycol (PEG6000) (44). After centrifugation, phages were resuspended in 3 ml of phage buffer (10 mM sodium phosphate [pH 7.0]–1 mM $MgSO_4$–0.5 M NaCl) and treated with 200 U of DNase and 3,000 U of RNase. Phages were purified by sedimentation into CsCl density layers of 1.6, 1.5, 1.4, and 1.3 g/ml at 35,000 rpm for 2.5 h in a Spinco 50.1 rotor. After collection, phages were cen-

TABLE 1. *E. coli* strains used and their relevant characteristics

Strain	Relevant properties	Source (reference)	Efficiency of plating for UC-1
AB259	Hfr	Coli Genetic Stock Center (2)	1.0
PC2283 (CE1040)	$OmpC^-$ $OmpF^-$ $OmpA^-$ heptose-deficient lipopolysaccharide	B. Lugtenberg (38)	1.0
PC2281 (CE1037)	$OmpC^-$ $OmpA^-$ heptose-deficient lipopolysaccharide	B. Lugtenberg (38)	0.94
PC2281-9	*tonA*	T1- and T5-resistant derivative of PC2281 isolated for this study	$\leq 10^{-10}$
AB1515 (13-6)	*tonA*	Coli Genetic Stock Center (35)	8.3×10^{-10}
MH522	*tonA*	M. M. Howe	$\leq 10^{-10}$
MH259	*tonB*	M. M. Howe	$\leq 10^{-10}$
M107	*tonB*	M. M. Howe (17)	$\leq 10^{-10}$
W3110AD8	*tonB*	C. Yanofsky (36)	3.3×10^{-5}

trifuged to equilibrium in CsCl (density, approximately 1.5) at 35,000 rpm in a Spinco 50.1 rotor for at least 24 h. The purified phages were dialyzed against phage buffer and stored at 4°C.

Preparation of phage DNA. For hybridization and restriction experiments, DNA from purified phages was extracted by the phenol method of Mandell and Hershey (26). For electron microscopy, phages were disrupted with 50% formamide in 10 mM Tris–1 mM EDTA (pH 8.0) at 25°C for 3 h. This solution was then dialyzed against 0.5 M NH_4 acetate–10 mM Tris (pH 7.5)–1 mM EDTA to remove the formamide (39).

Electron microscopy. Purified UC-1, diluted with distilled water, was applied to 300-mesh copper grids coated with Parlodion by the droplet method of Leonard et al. (21). The phages were stained with 1% uranyl acetate, and the excess stain was removed with filter paper. The grids were shadowed with platinum-paladium.

For contour length measurements, phage DNA was spread onto grids by the aqueous technique (39). Photographs of phage and phage DNA molecules were taken with a Hitachi 7H electron microscope, and contour lengths were measured with a Hewlett-Packard calculator equipped with a digitizer.

DNA-DNA hybridization. Hybridizations were performed with 4 μg of DNA immobilized on nitrocellulose filters (no. BA85; Schleicher & Schuell Co., Keene, N.H.) (40). [^{32}P]DNA (0.4 μg) dissolved in 1 ml of 50% formamide in 4× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) (8) was added to the filters bearing the appropriate immobilized DNA. The reaction mixtures were incubated at 37°C for 24 h and then washed four times in 2× SSC containing 50% formamide and four times in 2× SSC. The filters were dried at 80°C for 3 h and then counted with a Beckman LS-250 liquid scintillation system.

Restriction endonuclease digestion of phage DNA. Purified phage DNA was digested with *Eco*RI and *Eco*RI* as described elsewhere (33). *Hin*dIII digestion was performed as described by Old et al. (31). The conditions for *Sal*I digestion were the same as those for *Eco*RI, and the conditions for *Hae*II digestion were the same as those for *Hin*dIII.

Agarose gel electrophoresis of phage DNA. Electrophoresis of digested phage DNA was done in 0.7% agarose (Bio-Rad Laboratories, Richmond, Calif.). The running buffer consisted of 0.1 M Tris, 5 mM EDTA, 0.1 M boric acid, and 25 mM ethidium bromide. Electrophoresis was carried out at 30 mA under constant-current conditions for 6 h. The restriction fragments were visualized under UV illumination and photographed with a Polaroid MP-3 Land camera (37).

Polyacrylamide gel electrophoresis. Electrophoresis of UC-1 structural proteins was carried out with 11% polyacrylamide (Eastman Kodak Co., Rochester, N.Y.) slab gels (14 by 10 by 1.5 mm) as described by Lugtenberg et al. (25). Phage were disrupted by boiling for 5 min in 0.0625 M Tris-hydrochloride (pH 6.8) containing 2% sodium dodecyl sulfate. Staining and destaining were done as described previously (9). Densitometer scans were performed with a Joyce-Loebl Chromoscan 200 densitometer. Molecular weights were calculated from standard molecular weight markers by the method of Weber and Osborn (42).

Adsorption assay. Phage were added to early log-phase cultures (absorbancy at 540 nm, 0.25) at a multiplicity of infection of 0.1. Samples were removed at 5-min intervals, diluted in the presence of chloroform, and assayed for unadsorbed phage.

Inhibition of plaque formation by ferrichrome. *E. coli* AB259 was grown in L broth to mid-log phase, washed once with 1 mM $MgSO_4$, and resuspended in 1 mM $MgSO_4$. A 0.1-ml volume of siderophore or 0.1 ml of distilled water was added to the bacteria 10 min before the addition of phage. This mixture was assayed for plaque formation 10 min after the addition of phage to the host-ferrichrome suspension.

Mapping by gradient of transmission. The approximate locations of mutations conferring UC-1 resistance were determined essentially as described by

Miller (28), except matings were for 100 min and the selections and screenings were on appropriately supplemented MM medium.

RESULTS

Growth and purification of coliphage UC-1. UC-1 infection of early exponential-phase cultures of *E. coli* produced lysates with the highest titer. Addition of five UC-1 particles per cell when AB259 was at this growth stage in L broth resulted in an approximately 80% decrease in turbidity in 20 min. The lysates contained about 5×10^9 PFU/ml. Subsequent steps to concentrate and purify the phages were done as described in Materials and Methods.

Morphology of UC-1. Figure 1 shows UC-1 as it appeared under the electron microscope. The head appears to be an icosahedron with a diameter of 45 nm. The tail, which is approximately 150 nm long and 10 nm in diameter, assumed a variety of configurations (Fig. 1B), indicating extreme flexibility. We detected no evidence of tail contraction, and no base-plates, spikes, or collars were observed, which places UC-1 in bacteriophage group B of Bradley (3).

Protein composition of UC-1. At least seven phage structural proteins were detected by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (Fig. 2). The polypeptides, which had molecular weights of 14,000, 33,000, 39,000, 54,000, 56,000, 70,000, and 130,000, were designated VP7 through VP1, respectively.

Characterization of UC-1 DNA. UC-1 particles contained linear double-stranded DNA (Fig. 3). The DNA did not have cohesive ends, as no circles were observed when the DNA was heated to 45°C for 1 h before spreading and staining (1). The molecular weight of UC-1 DNA was calculated to be 34×10^6 by comparing its contour length with that of φX174 replicative form (molecular weight, 3.56×10^6 [34]).

The guanine-plus-cytosine content of UC-1 DNA was approximately 45% as determined by both cesium chloride equilibrium centrifugation and high-resolution thermal denaturation studies. UC-1 DNA does not contain significant amounts of any unusual bases (M. Mandel, personal communication).

Identification of receptor for UC-1. Studies to identify the host components required for UC-1 attachment were initiated by screening a number of *E. coli* strains with known envelope defects for UC-1 resistance; some representative strains tested and their relative plating efficiencies are shown in Table 1. Resistant strains had lesions in either the *tonA* or *tonB* locus. UC-1-resistant derivatives of sensitive strains could be readily obtained by introducing *tonA* mutations. Thus,

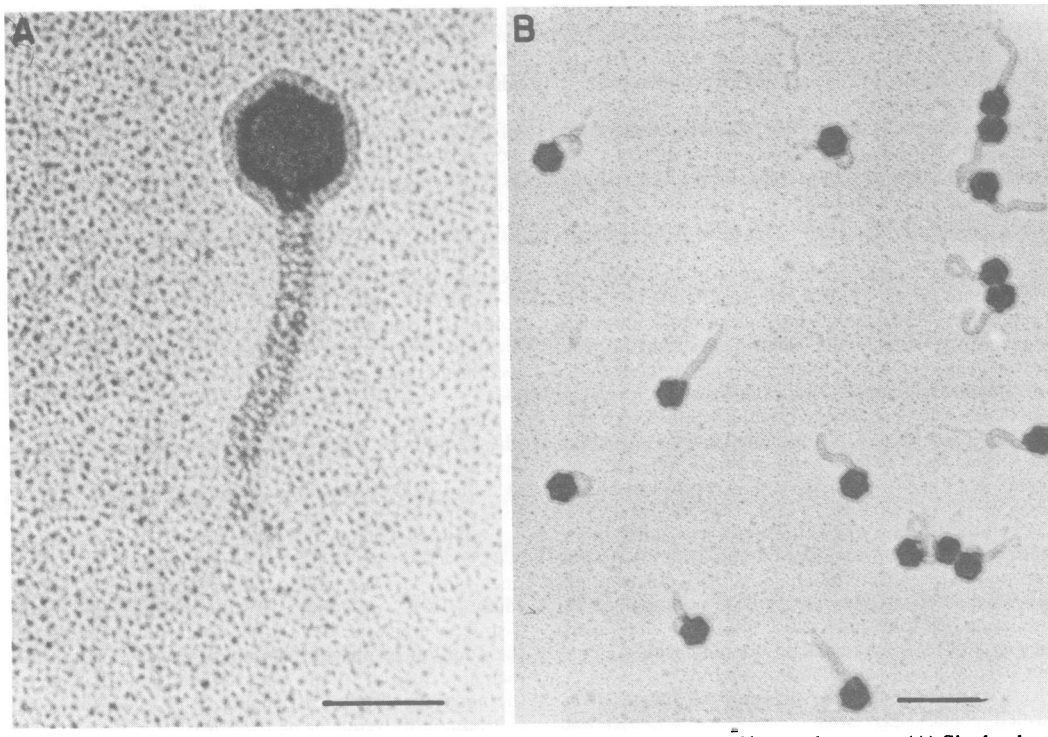

FIG. 1. Electron micrographs of coliphage UC-1 negatively stained with 1% uranyl acetate. (A) Single phage particle (bar = 50 nm). (B) Field of UC-1 virions showing flexibility of the tail (bar = 150 nm).

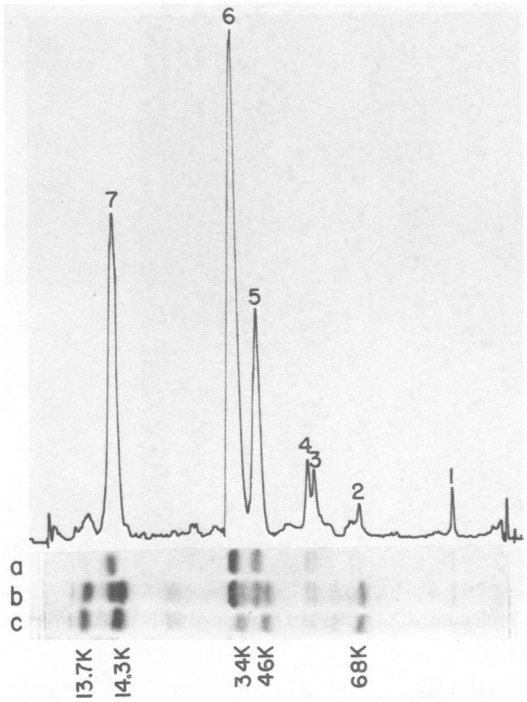

FIG. 2. UC-1 structural proteins. Electropherograms of UC-1 proteins (lane a), UC-1 proteins mixed with molecular weight standards (lane b), and molecular weight standards (bovine serum albumin [68,000], ovalbumin [46,000], DNase [34,000], lysozyme [14,300], and RNase [13,700]; lane c), are shown. Electrophoresis samples contained 20 μg of protein each, and the anode is to the right. A densitometer scan of lane a is shown above the electropherogram.

PC2281-9, a *tonA* derivative of sensitive strain PC2281 obtained by selection for T1 and T5 resistance (32), was resistant to UC-1.

Forty-five UC-1-resistant mutants of AB259 (R1 through R45) were isolated, and their patterns of resistance to colicin M and three phages (T1, T5, and φ80) known to use TonA as a receptor were analyzed (Table 2). (All remained sensitive to phage T6, whose receptor is a 26,000-dalton outer membrane protein [27].) Five classes of resistant mutants were found; 21 isolates were resistant to colicin M and all four phages (class I), and 19 were resistant only to UC-1 (class II). Three mutants (class V) were sensitive only to T5 and are presumably *tonB*. Classes III and IV were represented by only one isolate each.

The adsorption kinetics of UC-1 to three class I mutants (R6, R7, and R8) and to two mutants specifically resistant to UC-1 (R11 and R16) are shown in Fig. 4. The class II mutants adsorbed UC-1 almost normally, whereas the three class I mutants were obviously defective in adsorption. As is the case with phages T1 and φ80, adsorption of UC-1 to *tonB* cells was reversible (5; data not shown).

Gradient of transmission experiments (28) were performed to roughly map the loci responsible for UC-1 resistance in representatives of the phenotypic classes. RW193, a *tonA*$^+$ (UC-1-susceptible) derivative of AB1515 (22), was used as the recipient, and both Leu$^+$ and Leu$^+$ Pro$^+$ Trp$^+$ recombinants were selected and screened. The results were consistent with classes I, III, and IV arising from mutations in *tonA*. The locus or loci conferring the class II phenotype did not map in the first quadrant (97- to 27-min region) of the chromosome, as all RW193 recombinants remained UC-1 susceptible.

Inhibition of plaque formation by ferrichrome. The siderophore ferrichrome uses TonA as a receptor, as do phages φ80, T5, and T1 and colicin M. By competing for their common receptor, ferrichrome has been shown to protect cells from φ80 (41), T1 (14), and colicin M (13, 41) and to inhibit binding of T5 to partially purified TonA in vitro (24). Accordingly, the ability of ferrichrome to inhibit infection by UC-1 was tested (Table 3). Ferrichrome inhibited UC-1 plaque formation to approximately the same extent as it did that of T1. Phage φ80 appears to be most sensitive to inhibition of plaque formation by ferrichrome.

Comparison of the UC-1 genome with that of T1, T5, and φ80. UC-1 is morphologically similar to T1, T5, and φ80 (3, 43), and all four phages use the same receptor protein. It was of interest to determine the genetic relatedness of UC-1 to these well-known phages. This was done by restriction endonuclease digestion (30) and DNA-DNA hybridization experiments.

Endonucleases *Eco*RI, *Hin*dIII, and *Sal*I failed to produce any detectable cutting of phenol-extracted UC-1 DNA (data not shown). In

TABLE 2. Phenotypes of UC-1-resistant mutants of AB259

Class	Resistance[a]				No.
	T1	T5	φ80	ColM	
I	R	R	R	R	21
II	S	S	S	S	19
III	P	P	R	R	1
IV	R	P	R	S	1
V	R	S	R	S	3

[a] R, Resistant; S, sensitive; P, partially resistant. Sensitivity to phages was tested by spotting bacterial lawns with each of the phages. Resistant, sensitive, and partially resistant strains exhibited no clearing, complete clearing, and a turbid zone of lysis after application of 10^7, 10^3, and 10^5 PFU, respectively. Colicin M-sensitive strains gave clear spots after application of 10λ of a crude colicin preparation.

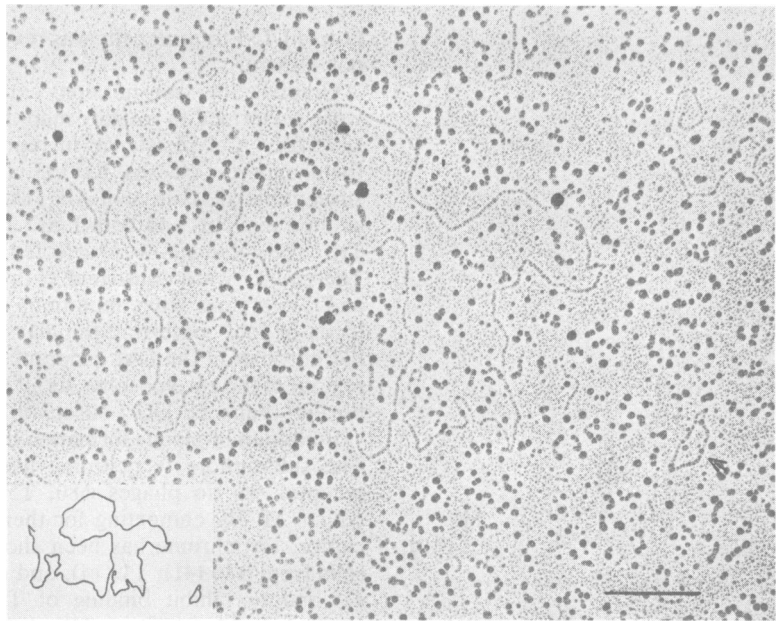

FIG. 3. Electron micrograph of an intact UC-1 DNA molecule. φX174 RFII DNA (arrow) is included as a length standard. Bar = 1 μm.

the same experiment, *Hae*III cleaved UC-1 DNA into at least 23 fragments, and λ DNA, which served as a control, was cut into appropriate fragments by each enzyme. Comparison of the phages with *Eco*RI* proved more useful (Fig. 5), as UC-1 DNA was cut into at least eight discrete fragments. The endonuclease cleavage patterns of φ80, T1, and T5 clearly differ from that of UC-1.

The results of DNA-DNA hybridization between the genomes of the phages are summarized in Table 4. Lack of significant hybridization indicates that UC-1 is not genetically related to T1, T5, or φ80.

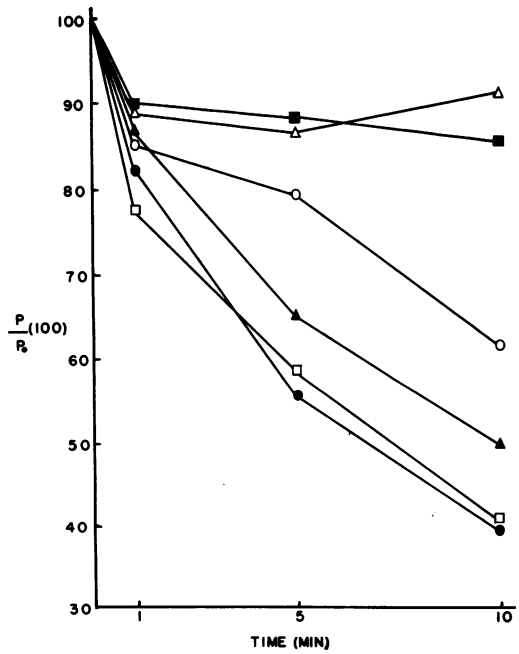

FIG. 4. Adsorption of UC-1 by *E. coli* AB259 and several UC-1-resistant derivatives of AB259. Symbols and phenotypic classes of the mutants are as follows: ●, AB259; ○, R6 (I); ■, R7 (I); △, R8 (I); ▲, R11 (II); □, R16 (II).

DISCUSSION

Coliphage UC-1 has a long, flexible, noncontractile tail and an isometric head containing double-stranded DNA. Morphologically, UC-1 thus belongs to bacteriophage group B of Bradley (3). In addition, the *tonA* and *tonB* products were necessary for UC-1 infection, which places UC-1 among the T1-like phages, according to

TABLE 3. Percent inhibition of plaque formation by ferrichrome

Phage	% Inhibition with ferrichrome concn (μM) of[a]:			
	8	80	400	800
UC-1	2.5	67	95	100
T1	7	66	97	100
φ80	44	79	100	100
T5	15	20	23	19

[a] Percent inhibition = (titer with ferrichrome/titer with water) × 100. Numbers are averages of three experiments.

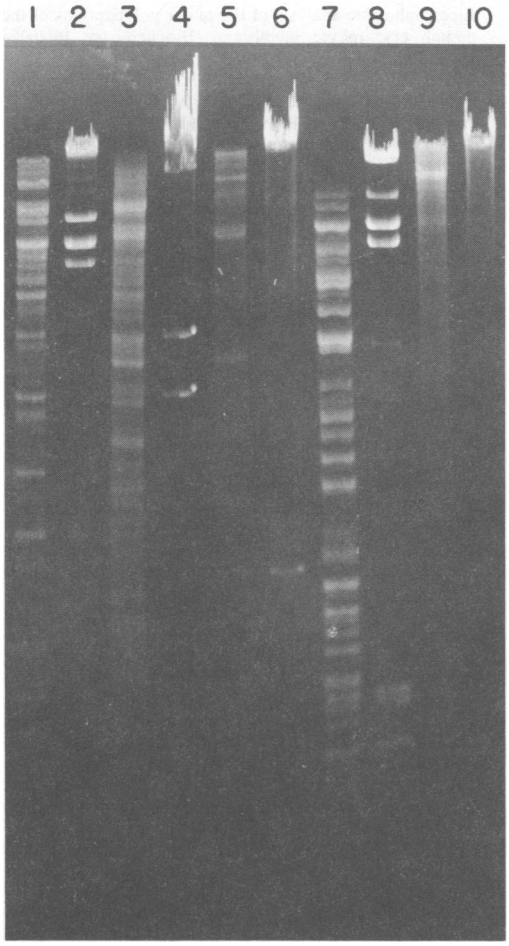

FIG. 5. Comparison of agarose gel electrophoresis fragment patterns of UC-1 (lanes 9 and 10), φ80 (lanes 7 and 8), T1 (lanes 5 and 6), T5 (lanes 3 and 4), and λ (lanes 1 and 2) DNA generated by either *Eco*RI (even-numbered lanes) or *Eco*RI* (odd-numbered lanes) activity.

the nomenclature of Hancock and Reeves (12). The following four findings indicated that TonA functions as the receptor for UC-1: (i) UC-1 had a low plating efficiency on *tonA* strains, (ii) *tonA* mutants were selected on the basis of UC-1 resistance, (iii) UC-1 failed to adsorb to *tonA* mutants, and (iv) ferrichrome inhibited UC-1 infections.

UC-1 was distinct in a number of ways from other well-studied phages which use TonA as a receptor. T5 does not require an active *tonB* product for infection, and UC-1 differed from T1, T5, and φ80 in guanine-plus-cytosine content and by restriction endonuclease analyses and DNA-DNA hybridization tests. Also, bacterial mutants specifically resistant to UC-1 (class II) were isolated.

The discovery of another phage which utilizes TonA as a receptor serves to emphasize the fact that this protein has more receptor roles than any other *E. coli* outer membrane protein. Why TonA should be so frequently used as a receptor is unclear, as is the reason why TonA-specific phage all have similar morphologies. Available evidence suggests that TonA binding sites for its various specific ligands exhibit overlap but that they are not identical (5, 14). The UC-1-resistant strains with class III and IV phenotypes provide further support for this idea.

On the basis of their sensitivity to albomycin (10), it can be anticipated that most enteric bacteria will have polypeptides similar to TonA, and in several *Salmonella* species, proteins related to TonA have been studied. The *sid* gene of *Salmonella typhimurium* specifies a polypeptide necessary for ferrichrome and albomycin uptake, but it does not serve as a receptor for T5 or colicin M (6, 11, 23). *Salmonella paratyphi* B produces a protein close in function to that of TonA in that, although it does not absorb T1, it adsorbs T5 and colicin M (11). UC-1, like T1, was found to infect only *E. coli* strains (J. Belford, M.S. thesis, University of Oklahoma, Norman, 1968).

The existence and ease of isolation of the class II resistant mutants are of special interest. They suggest that a host factor is required specifically for UC-1 replication, the receptor for UC-1 is bipartite, or a specific cytoplasmic membrane constituent is necessary for entry of UC-1 DNA. The first possibility cannot be eliminated but it seems unlikely, a priori, that UC-1, whose

TABLE 4. Hybridization of UC-1 DNA with T1, T5, and φ80 DNA

Exp	Input DNA	Immobilized DNA	cpm Bound	% Homologous reaction
1	UC-1	UC-1	2,549	100
	UC-1	T1	61	2.4
	UC-1	T5	23	1
	UC-1	None	0	0
	T1	T1	511	100
	T1	UC-1	5	1
	T1	None	0	0
	T5	T5	2,138	100
	T5	UC-1	10	0.5
	T5	None	1	0
2	UC-1	UC-1	1,210	100
	UC-1	φ80	18	1.5
	UC-1	None	20	1.6
	φ80	φ80	793	100
	φ80	UC-1	27	3.4
	φ80	None	13	1.6

genome is similar in molecular weight to those of T1 and φ80, should require a unique bacterial factor for replication. The possibility that UC-1 requires a two-component receptor is unlikely for several reasons. Phages with bipartite receptors, such as T4 (15, 29), usually have a more elaborate tail structure. Also, complex receptors generally consist of a protein and lipopolysaccharide, and the lipopolysaccharide-deficient strains tested were good hosts for UC-1. Lastly, adsorption of UC-1 to class II strains was not greatly altered (Fig. 4). There is a precedent for the third possibility. Kadner et al. (18) speculated that *fhuB*, a gene mapping close to *tonA*, specifies a cytoplasmic membrane transport component that functions along with the *tonA* and *tonB* products for uptake of hydroxamate siderophores. The preliminary mapping experiments show that the mutation(s) giving rise to the class II phenotype cannot be in *fhuB*, but the relevant gene(s) could function in a way similar to that suggested for *fhuB*. UC-1 thus offers an additional approach to the study of TonA receptor functions and, more generally, to the mechanism by which phage DNA penetrates the bacterial envelope.

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