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Morphological, Host Range, and Genetic Characterization of Two Coliphages

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Two coliphages, AR1 and LG1, were characterized based on their morphological, host range, and genetic properties. Transmission electron microscopy showed that both phages belonged to the *Myoviridae*; phage particles of LG1 were smaller than those of AR1 and had an isometric head 68 nm in diameter and a complex contractile tail 111 nm in length. Transmission electron micrographs of AR1 showed phage particles consisting of an elongated isometric head of 103 by 74 nm and a complex contractile tail 116 nm in length. Both phages were extensively tested on many strains of *Escherichia coli* and other enterobacteria. The results showed that both phages could infect many serotypes of *E. coli*. Among the enterobacteria, *Proteus mirabilis*, *Shigella dysenteriae*, and two *Salmonella* strains were lysed by the phages. The genetic material of AR1 and LG1 was characterized. Phage LG1 had a genome size of 49.5 kb compared to 150 kb for AR1. Restriction endonuclease analysis showed that several restriction enzymes could degrade DNA from both phages. The morphological, genome size, and restriction endonuclease similarities between AR1 and phage T4 were striking. Southern hybridizations showed that AR1 and T4 are genetically related. The wide host ranges of phages AR1 and LG1 suggest that they may be useful as biocontrol, therapeutic, or diagnostic agents to control and detect the prevalence of *E. coli* in animals and food.

Lytic bacteriophages (phages) can provide a natural and nontoxic method for detecting and controlling the growth of human pathogens. Phages are parts of both gastrointestinal and environmental ecosystems (1). Historically, phages have been employed as therapeutic agents, in diagnostic tests to detect bacterial pathogens, and as biological control agents to reduce and eliminate food-borne pathogens in food (21, 24, 26, 33, 40).

Upon their discovery, phages found immediate use as antimicrobial agents to treat cholera infections. However, while the initial results were encouraging, the use of phages as therapeutic agents for microbial infections did not progress due to poorly defined experimental conditions, which led to irreproducible results. More importantly, the advent of antibiotics in the 1940s reduced interest in the use of phages to cure bacterial disease (3). Recently, a renewed interest in phage therapy has surfaced with the advent of antibiotic resistance (3). Phages have also been found useful as diagnostic tools for rapid detection of such pathogens as *Mycobacterium tuberculosis*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* (5, 28, 29, 36). These diagnostic methods make use of phages that have been genetically modified to carry a reporter gene. These reporter phages transduce the reporter gene to the target bacteria, producing a highly detectable phenotype. The reporter genes employed in reporter gene assays include the *lux* and *luc* genes, the *inaW* gene, and the *lacZ* gene (9). Alternatively, phages may be fluorescently labeled and used in a manner analogous to antibodies, to specifically tag and identify the target organism (8, 14).

Phages have been employed as biocontrol agents capable of eliminating or reducing the levels of bacterial pathogens in or on food by taking advantage of the ability of virulent phages to lyse, and thereby kill, the target organism (19, 21, 24).

This study describes the morphological, host range, and genetic characterization of two bacteriophages, AR1 and LG1, that are capable of infecting a wide range of *E. coli* strains. First described by Escherich in 1885 (7), *E. coli* is the most abundant facultative anaerobe of the normal intestinal flora and is responsible for producing what is perceived as the widest spectrum of disease of any bacterial species (18). Therefore, phages that are capable of infecting a wide range of *E. coli* strains would be potentially valuable as therapeutic and diagnostic agents for the control and detection of this bacterial species.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. A total of 246 bacterial strains (Tables 1 to 4) were used for host range studies and phage propagation. The strains included 133 wild-type strains representing 33 serogroups and 46 serotypes of *E. coli* (Table 1). The specificity of the two phages was tested on the *E. coli* reference (ECOR) collection (Table 2), in order to estimate the specificity of the phages to the whole species of *E. coli*. Twenty-one non-*E. coli* strains representing members of the *Enterobacteriaceae* including *Salmonella* spp., *Shigella* sp., *Enterobacter* sp., *Serratia* sp., *Escherichia* sp., and *Klebsiella* sp. were tested to determine whether any of these bacteria could be infected by the phages (Table 3). A series of *E. coli* K-12 outer membrane protein (OMP) mutants and a series of *E. coli* lipopolysaccharide (LPS) outer core oligosaccharide (OS) mutants were used for AR1 receptor studies (Table 4). Four pairs of *E. coli* K-12 isogenic mutants were obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University. These mutants were isogenic with respect to OmpF, OmpA, FadL, and tsx. Additionally, two LamB strains that were not isogenic but had mutations that did not affect the outer membrane were evaluated for phage

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TABLE 1. Wild-type STEC strains used for the host range specificity study[a]

Serotype of *E. coli*[b]	No. of strains tested	Plaque assay result (no. of positive results/ no. of strains tested)	
		LG1	AR1
O2:H5	1	0/1	0/1
O2:H6	2	0/2	2/2
O3:H2	1	1/1	0/1
O4:H5	1	0/1	1/1
O5:H$^-$	1	0/1	1/1
O6:H$^-$	1	0/1	0/1
O8:H14	1	1/1	0/1
O15:H$^-$	4	4/4	3/4
O18:H7	1	0/1	1/1
O22:H8	2	2/2	2/2
O26:H$^-$	6	6/6	5/6
O26:H11	5	5/5	5/5
O38:H21	1	0/1	0/1
O45:H2	2	2/2	1/2
O48:H21	4	4/4	0/4
O55:H$^-$	1	1/1	0/1
O55:H6	1	0/1	0/1
O55:H7	3	3/3	3/3
O55:H9	1	0/1	1/1
O91:H$^-$	5	5/5	0/5
O91:H21	4	3/4	1/4
O103:H2	4	4/4	4/4
O111:H$^-$	5	5/5	0/5
O111:H8	5	5/5	0/5
O112:H2	1	1/1	1/1
O113:H2	4	4/4	1/4
O113:H4	1	0/1	0/1
O114:H4	1	0/1	0/1
O116:H2	1	1/1	0/1
O117:H4	5	0/5	0/5
O118:H1	1	0/1	0/1
O118:H3	1	1/1	1/1
O119:H6	1	1/1	0/1
O121:H1	4	4/4	1/4
O123:H$^-$	1	0/1	1/1
O128:H$^-$	3	2/3	2/3
O128:H2	5	5/5	0/5
O139:H$^-$	1	0/1	0/1
O145:H$^-$	5	0/5	5/5
O153:H2	1	1/1	1/1
O157:H$^-$	5	5/5	5/5
O157:H1	2	1/2	1/2
O157:H2	1	0/1	0/1
O157:H7	18	16/18	18/18
O163:H1	4	3/4	0/4
O165:H2	1	1/1	0/1
OR:H$^-$	1	1/1	0/1
OR:K48:H$^-$	1	1/1	0/1
OR:H7	1	1/1	1/1
OR:H11	1	1/1	0/1

[a] All strains were obtained from the Canadian Research Institute for Food Safety.
[b] "R" in a designation denotes a rough strain.

receptor specificity. Two pairs of OmpC isogenic mutants were included as negative controls (Table 4).

Twelve *E. coli* LPS outer core OS mutant strains were obtained from Chris Whitfield (Department of Microbiology, University of Guelph, Guelph, Ontario, Canada) and were used to assess the usefulness of *E. coli* LPS as a receptor for phage AR1 (Table 4). Four strains represented four of the five different core types (R1 to R4) (13) of *E. coli*. These strains are deficient in the O antigen and were used to assess the usefulness of the outer core OS region of the LPS as a receptor for bacteriophage AR1. *E. coli* K-12 was also included in the host range study. This strain represents the fifth core type (K-12) of *E. coli* (13). The remaining eight strains were produced to be sequentially deficient in the genes (the *waa* operon) responsible for producing the enzymes that add carbohydrates to the growing outer core OS region of the outer membrane LPS. These strains were used to determine which carbohydrate residue(s) could serve as a receptor for AR1. The construction of the LPS mutant strains is described elsewhere (2, 12, 42). Stock cultures were maintained in 30% glycerol and were frozen at −70°C. Fresh bacterial host cultures for use in experiments were produced by inoculating frozen stock cultures onto Luria-Bertani (LB) agar plates (Difco Laboratories, Detroit, Mich.) and incubating the plates overnight at 37°C. For growth experiments, the inocula consisted of stationary-phase cells that were obtained by inoculating LB broth with cells from an overnight LB agar plate and incubating the preparations overnight with shaking at 37°C.

The bacteriophages used in this study, LG1 and AR1, have been previously described (8, 32). Both phages were propagated on their host, *E. coli* O157:H7 strain EC920333. Bacteriophage T4 ATCC 11303B4 and its host *E. coli* B 11303 were obtained from the American Type Culture Collection (Manassas, Va.).

Phage propagation. To amplify the phages, 10 ml of an overnight culture of *E. coli* O157:H7 EC920333 and *E. coli* B 11303 (10^9 CFU/ml) and 1 ml of the phage (LG1, AR1, or T4) (10^{11} PFU/ml) were added to 500 ml of LB broth and incubated with shaking at 37°C for 5 h. After incubation, during which time lysis occurred, the bacterium-phage suspension was treated with 10 ml of chloroform to release any progeny phage which may still have been in the host cells, and the suspension was incubated for an additional 10 min with shaking at 37°C. To remove bacterial debris, the suspension was centrifuged at 5,000 × *g* for 15 min, in a Beckman J2-MC centrifuge (Beckman Instruments Incorporated, Palo Alto, Calif.), and the supernatant was withdrawn and filtered through 0.2-μm-pore-size VacuCap 60 PF sterile vacuum filtration devices (Pall Corporation, Mississauga, Ontario, Canada). The phage lysates were stored at 4°C.

Host range determination. In order to determine their host ranges and the nature of the AR1 receptor(s), phages LG1 and AR1 were tested against 246 bacterial strains (Tables 1 to 4). These strains consisted of 133 Shiga toxin-producing *E. coli* (STEC) isolates; the ECOR collection (72 *E. coli* strains); 21 non-*E. coli* strains representing members of the *Enterobacteriaceae* including *Salmonella* spp., *Shigella* sp., *Enterobacter* sp., *Serratia* sp., *Escherichia* sp., and *Klebsiella* sp.; and 20 *E. coli* K-12 outer membrane (OMP) and LPS mutant strains. Bacteriophage lysis assays were conducted based on a modified procedure of the traditional double-layer plaque technique (15). Lysis assays were conducted in 10-cm-diameter petri plates (Fisher Scientific, Nepean, Ontario, Canada). The top agar layer consisted of 1% (wt/vol) tryptone (Difco), 0.8% (wt/vol) sodium chloride (Fisher Scientific), and 0.5% agar. For each strain tested, 3 ml of top agar was steamed for approximately 10 min and allowed to cool to 47°C. One hundred microliters of an overnight culture of the bacterium to be tested was added to the top layer, and the mixture was vortexed and poured onto the bottom layer (LB agar). The top agar was allowed to solidify at room temperature, and 20 μl of a bacteriophage suspension (10^{11} PFU/ml) was pipetted onto the top agar layer. The plates were inverted, incubated at 37°C overnight, and then examined for the presence of clear zones of lysis.

Transmission electron microscopy. To observe bacteriophage morphology, 100 μl of each bacteriophage suspension (10^{11} PFU/ml) was placed on a separate piece of Parafilm, and a Formvar-carbon-coated copper grid (200 mesh) (Marivac Ltd., Halifax, Nova Scotia, Canada) was floated on top of each sample for 5 min. The copper grids were blotted dry, were floated sample side down on a drop of 2% (wt/vol) aqueous uranyl acetate (Fisher Scientific) for 3 min, and again blotted dry. The samples were examined in a Philips EM300T transmission electron microscope (Philips Electrical Corp., New York, N.Y.) at 60-keV accelerating voltage.

One-step growth curve. One-step growth curves were performed in order to determine the burst sizes and latent periods of phages AR1 and LG1 at 37°C, as described by Ellis and Delbruck (6). The resulting time series data were plotted by using Sigma Plot 5.0 (SPSS Inc., Chicago, Ill.), and a sigmoidal line of best fit was calculated for each plot. The average burst size per infected host and the average latent period were then calculated from the sigmoidal curves.

Isolation of bacteriophage DNA and pulsed-field gel electrophoresis (PFGE). Bacteriophage DNA was extracted and purified from phage lysates by a proteinase K method, followed by resuspension in Tris-EDTA buffer after isopropanol precipitation as described by Maniatis et al. (22).

For PFGE, 20 μl of each DNA sample was mixed with 10 μl of 3× DNA loading buffer (MBI Fermentas, Flamborough, Ontario, Canada) and loaded into a separate well of a 1% Bio-Rad agarose (Bio-Rad Laboratories) running gel. The gel was placed in a CHEF-DR II electrophoresis cell (Bio-Rad Laboratories) and submerged in 0.5× Tris-borate-EDTA buffer equilibrated to 14°C. Bacteriophage lambda DNA concatemers (48.5 to 1,018.5 kb; Bio-Rad Labora-

TABLE 2. ECOR strains used in the host range specificity study

Serotype of *E. coli*	ECOR no.	Origin	Plaque assay result		Serotype of *E. coli*	ECOR no.	Origin	Plaque assay result	
			LG1	AR1				LG1	AR1
ON:HN	1	USA[a]	−	−	ON:HN	37	USA	+	+
ON:H32	2	USA	−	−	O7:H$^-$	38	USA	−	−
O1:H$^-$	3	USA	−	−	O7:H$^-$	39	Sweden	−	+
ON:HN	4	USA	−	+	O7:H$^-$	40	Sweden	−	−
O79:H$^-$	5	USA	−	−	O7:H$^-$	41	Tonga	+	+
ON:H$^-$	6	USA	−	+	ON:H26	42	USA	+	−
O85:H$^-$	7	USA	−	+	ON:HN	43	Sweden	−	−
O86:H$^-$	8	USA	−	+	ON:HN	44	USA	−	+
ON:H$^-$	9	Sweden	−	−	ON:H$^-$	45	Indonesia	+	+
O6:H10	10	Sweden	−	−	O1:H6	46	USA	−	−
O6:H10	11	Sweden	−	−	ON:H18	47	New Guinea	−	+
O7:H32	12	Sweden	−	+	ON:HN	48	Sweden	−	+
ON:HN	13	Sweden	−	−	O2:H$^-$	49	Sweden	+	−
ON:HN	14	Sweden	−	+	O2:HN	50	Sweden	+	−
O25:H$^-$	15	Sweden	−	+	O25:HN	51	USA	+	−
ON:H10	16	USA	−	+	O25:H1	52	USA	+	+
O106:H$^-$	17	Indonesia	−	−	O4:HN	53	USA	+	+
O5:H$^-$	18	USA	−	+	O25:H1	54	USA	−	+
O5:HN	19	USA	−	−	O25:H1	55	Sweden	−	+
O89:HN	20	Bali	−	−	O6:H1	56	Sweden	−	+
O121:HN	21	Bali	−	−	ON:H$^-$	57	USA	−	−
ON:HN	22	Bali	−	+	O112:H8	58	USA	−	−
O86:H43	23	USA	−	+	O4:H40	59	USA	−	−
O15:H$^-$	24	Sweden	−	−	O4:HN	60	Sweden	−	−
ON:HN	25	USA	−	−	O2:H$^-$	61	Sweden	+	−
O104:H21	26	USA	−	+	O2:H$^-$	62	Sweden	+	+
O104:H$^-$	27	USA	−	+	ON:H$^-$	63	Sweden	+	+
O104:H$^-$	28	USA	−	−	O75:H$^-$	64	Sweden	−	+
O150:H21	29	USA	−	−	ON:H10	65	USA	+	−
O113:H21	30	Canada	−	+	O4:H40	66	USA	−	+
O79:H43	31	USA	−	−	O4:H43	67	Indonesia	−	−
O7:H21	32	USA	−	+	ON:H$^-$	68	USA	−	−
O7:H21	33	USA	−	+	ON:H$^-$	69	USA	+	+
O88:H$^-$	34	USA	+	+	O78:H$^-$	70	USA	+	+
O1:H$^-$	35	USA	−	+	O78:H$^-$	71	Sweden	−	+
O79:H25	36	USA	−	+	O144:H8	72	Sweden	−	−

[a] USA, United States.

tories) were used as size standards and served as a control for the run parameters of the CHEF-DR II unit. The run parameters were as follows: initial pulse, 50 s; final pulse, 90 s; voltage, 200 V (6 V/cm); time, 22 h; and temperature, 14°C. Following electrophoresis, the gels were stained with ethidium bromide (Fisher Scientific) solution (0.5 mg/ml in H_2O) for 60 min and then destained for 60 min in deionized water. Gels were then visualized under UV transillumination and photographed.

Restriction endonuclease analysis. DNA samples from phages AR1, LG1, and T4 were tested with the restriction endonucleases *Eco*RV, *Ssp*I, *Nde*I, and *Taq*I (Gibco BRL), according to the suppliers' recommendations. DNA fragments were separated by electrophoresis in 0.8% agarose gels containing ethidium bromide (0.5 μg/ml) in 1× Tris-borate-EDTA buffer, at 80 to 100 V with a Bio-Rad agarose gel electrophoresis system (Bio-Rad Laboratories). Fragment sizes were determined from comigrating *Hin*dIII digests of lambda DNA (MBI Fermentas).

Southern blot analysis of bacteriophage DNA. For Southern blot analysis, DNA samples were digested with *Eco*RV and *Nde*I, and the resulting fragments were separated as described above. DNA was transferred to a nylon membrane by a simplified downward alkaline transfer method (23). Following the transfer of DNA to the membrane, the membrane was removed, neutralized in 2× standard saline citrate (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) for 15 min, and baked at 60°C for 1 h, or until dry. Whole-genomic *Nde*I- or *Eco*RV-digested AR1 DNA was used as the probe for the Southern analysis (under high-stringency conditions), and the probe was generated by using a Random Prime labeling kit (Roche) and visualized by using alkaline phosphatase direct imaging as specified by the supplier.

Random sequence analysis of bacteriophage LG1 genome. *Eco*RV-digested phage LG1 DNA fragments were randomly ligated into the *Sma*I site of vector pTRUEBLUE (Genomics One, Laval, Quebec, Canada), with a rapid kit (Roche) as specified by the supplier. Ligation products were introduced into *E. coli* strain JM109 by electroporation, and transformants were selected by plating on agar plates containing ampicillin (50 μg/ml). Transformants were analyzed, and sequence determination was accomplished with the oligonucleotides F (5′-TCTGCAGCGTCGCGACTGGGAAAACCC-3′) and R (5′-GCTAATCGATT CAATTGGGTAACGCCC-3′). An ABI Prism DNA sequencing apparatus (Guelph Molecular Supercenter, University of Guelph) was employed to determine the sequence of the DNA samples. Sequence data were collected and stripped of poor-quality data, selected clones were scanned for homologues with the BLASTN (2.0) program, and data were compared with available sequences in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST/).

Nucleotide sequence accession number. Four sequences have been submitted to GenBank (accession numbers AF465471 to AF465474).

RESULTS

Host range. Bacteriophages LG1 and AR1 were characterized according to host range. Both phages were tested against 205 *E. coli* strains (Tables 1 and 2). Phage LG1 lysed 98 of 133 (73.6%) STEC strains (Table 1) and 16 of 72 (22%) ECOR strains (Table 2). AR1 lysed 68 of the 133 (51%) STEC strains

TABLE 3. Strains of non-*E. coli* bacteria used in the host range specificity study[a]

Taxon[b]	Plaque assay result	
	LG1	AR1
Edwardsiella tarda	−	−
Enterobacter aerogenes	−	−
Enterobacter agglomerans	−	−
Enterobacter cloacae	−	−
Escherichia hermannii	+	−
Morganella morganii	−	−
Pantoea agglomerans	−	−
Proteus mirabilis	+	−
Proteus vulgaris	−	−
Providencia rettgeri	−	−
Providencia stuartii	−	−
Salmonella Arizonae	−	−
Salmonella Choleraesuis	−	+
Salmonella Enteritidis	−	+
Salmonella Gallinarum	−	−
Salmonella Pullorum	−	−
Salmonella Typhimurium	−	−
Salmonella Typhimurium	−	−
Salmonella Urbana	−	−
Serratia marcescens	−	−
Shigella dysenteriae	+	+

[a] All strains were obtained from the Canadian Research Institute for Food Safety.
[b] *Salmonella* taxa are serovars of *S. enterica.*

(Table 1) and 38 of 72 (53%) ECOR strains (Table 2). In addition to the *E. coli* strains, phages LG1 and AR1 were tested against non-*E. coli* members of the *Enterobacteriaceae* to determine whether any of these bacteria could be lysed (Table 3). Phage LG1 formed plaques on *Proteus mirabilis*, and AR1 lysed *Salmonella enterica* serovar Choleraesuis and *S. enterica* serovar Enteritidis. Both phages lysed *Shigella dysenteriae*.

Bacteriophage morphology. Transmission electron microscopy was employed to observe the morphology of phages AR1 and LG1 (Fig. 1). Phage particles of LG1 were smaller than those of AR1 and had an isometric head 68 nm in diameter and a complex contractile tail of 111 by 15 nm (Fig. 1A). Tail fibers were not observed. Transmission electron micrographs of AR1 show phage particles consisting of an elongated isometric head of 103 by 74 nm and a complex contractile tail of 116 by 16 nm. Phage AR1 bears a striking resemblance to phage T4. Base plates and tail fibers were visible on most phage AR1 particles (Fig. 1B). Based on their morphology, both LG1 and AR1 belong to the *Myoviridae*; phage AR1 belongs to the T4-like genus, while LG1 is unassigned to a genus within the family.

One-step growth experiment. One-step growth curves were determined for phages AR1 and LG1 with mid-logarithmic-phase host cells. LG1 had a burst size of 177 and a latent period of 52 min. Phage AR1 had a burst size of 38 and a latent period of 40 min. Calculation of the percentage of adsorption indicated that more than 90% of both phages had adsorbed to the host cells within the first 10 min.

Restriction analysis, Southern hybridization, and genome size. Restriction enzyme analysis performed with four enzymes confirmed that phages LG1 and AR1 were distinct. The restriction enzymes *Ssp*I, *Nde*I, and *Taq*I completely digested AR1 DNA, and these results coincided with those observed when the same enzymes were used to digest T4 DNA. Phage LG1 DNA was partially degraded by *Nde*I, and completely digested by *Eco*RV, *Ssp*I, and *Taq*I. That both AR1 and LG1 DNA were readily digested by several restriction enzymes indicates that both phages possess double-stranded DNA. PFGE data indicated that phage LG1 had a genome size of 48.5 kb and that phage AR1 had a genome size of approximately 150 kb.

TABLE 4. *E. coli* K-12 OMP and LPS mutants used in the host range receptor study

Strain	Relevant properties	Origin[a]	Plaque assay result
F470	*E. coli* R1 prototype; R-LPS[b] derivative of O8:K27	A	+
F632	*E. coli* R2 prototype; R-LPS derivative of O100:K? or -H2	A	−
F653	*E. coli* R3 prototype; R-LPS derivative of O111:K58	A	+
F2513	*E. coli* R4 prototype; R-LPS derivative of O14:K7	A	+
CWG311	*waaV*::*aacC1* derivative of F470; Gm^r	A	−
CWG310	*waaW*::*aacC1* derivative of F470; Gm^r	A	+
CWG309	*waaT*::*aacC1* derivative of F470; Gm^r	A	+
CWG308	*waaO*::*aacC1* derivative of F470; Gm^r	A	−
CWG303	*waaG*::*aacC1* derivative of F470; Gm^r	A	−
CWG297	*waaQ*::*aacC1* derivative of F470; Gm^r	A	+
CWG350	*waaJ*::*aacC1* derivative of F653; Gm^r	A	−
CWG346	*waaF*::*aacC1* Gm^r	A	−
CS1230	λ^- *ompC161 atoS298*::Tn*10 gyrA261* (*Nal*^{r}) *rph-1*	B	−
CS1231	λ^- *atoS298*::Tn*10 gyrA261* (Nal^r) *rph-1*	B	−
JF701	*lacY29 proC24 tsx-63 purE41* λ^- *aroA357 his-53 ompC264 rpsL97* (Str^r) *xyl-14 metB65 cycA1 cycB2*? *ilv-277*	B	−
JF568	*lacY29 proC24 tsx-63 purE41* λ^- *aroA357 his-53 rpsL97* (Str^r) *xyl-14 metB65 cycA1 cycB2*? *ilv-277*	B	−
RE107	*proA23 lac-28 ompF625 trp-30 his-51 rpsL101* (Str^r)	B	−
RC712	*proA23 lac-28 trp-30 his-51 rpsL101* (Str^r)	B	−
JF700	*lacY29 proC24 tsx-63 purE41* λ^- *ompF254 ompA256 his-53 rpsL97* (Str^r) *xyl-14 metB65 cycA1 cycB2*? *ilv-277*	B	−
JF703	*lacY29 proC24 tsx-63 purE41* λ^- *ompF254 his-53 rpsL97* (Str^r) *xyl-14 metB65 cycA1 cycB2*? *ilv-277*	B	−

[a] A, Chris Whitfield, Department of Microbiology, University of Guelph. B, *E. coli* Genetic Stock Center, Yale University.
[b] R-LPS, rough LPS.

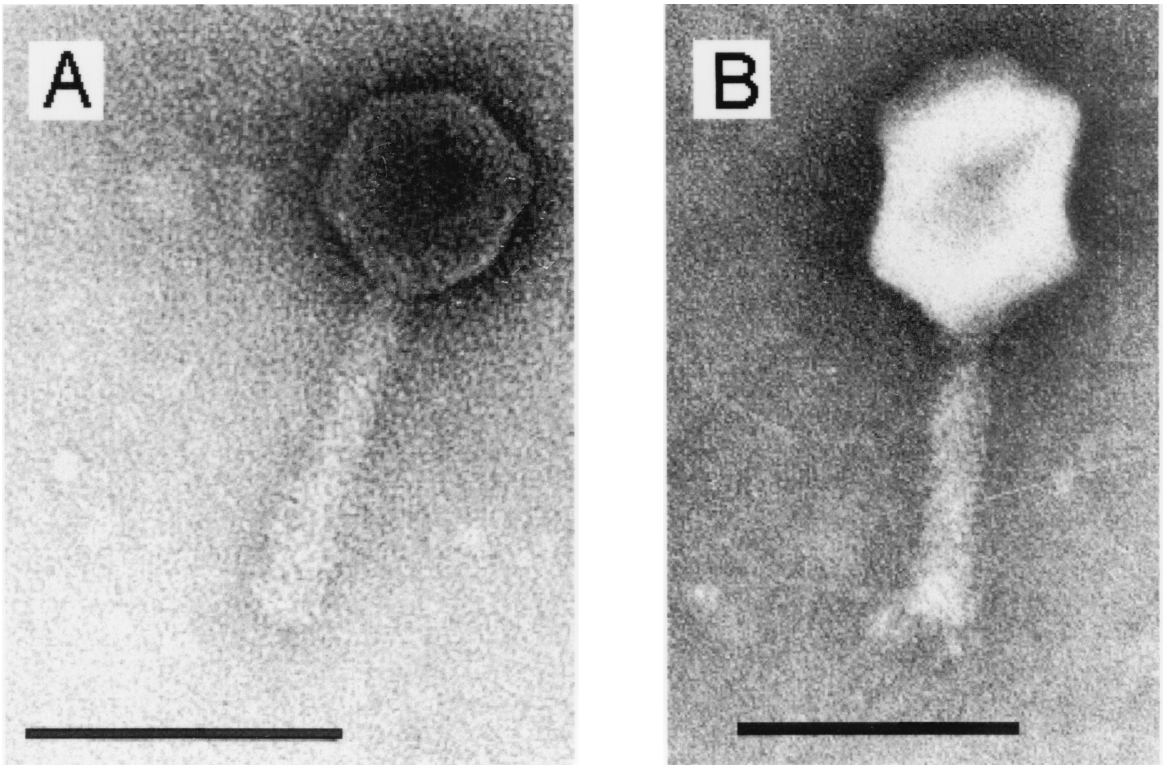

FIG. 1. Transmission electron micrographs of bacteriophages LG1 (A) and AR1 (B). Bars, 100 nm. Magnifications, ×377,910.

The *Nde*I and *Eco*RV restriction digests of phage LG1, AR1, and T4 DNA were subsequently Southern blotted and probed with digoxigenin-labeled *Nde*I- or *Eco*RV-digested AR1 genomic DNA. Probing with *Nde*I-digested AR1 DNA showed that the level of homology between AR1 and T4 was particularly high (see Fig. 3). Phage LG1 shared no homology with AR1 or T4. However, when *Eco*RV-digested AR1 genomic DNA was used as the probe, a small amount of genetic homology between AR1 and LG1 (<3 kb) was observed (data not shown).

Random sequence analysis of LG1 genome. Random sequence analysis of phage LG1 DNA indicated limited homology to anything in the database. One sequence (accession number AF465474) showed 99% homology to the terminal 207 bp of the 3′ end of open reading frame (ORF) 17 of the *Salmonella* bacteriophage Felix O1.

DISCUSSION

Bacteriophages represent the largest of all virus groups (1). They occur in archaea and bacteria and are found in enormous numbers in many diverse natural habitats (1, 41). Phages are easy to isolate and inexpensive to purify. The isolation of two bacteriophages (LG1 and AR1) that infect *E. coli* provided an opportunity to characterize the bacteriophages and to determine their host range, life cycle, and genetic characteristics.

Based upon their contractile tails, phages LG1 and AR1 (Fig. 1) belong to the *Myoviridae*. The taxonomic structure of the *Myoviridae* consists of six genera; the phage AR1 head and tail morphology, the size of the phage genome, and the host range place phage AR1 within the T4-like virus genus. The morphological similarity to phage T4 (type species of the T4-like virus genus) and the high amount of genetic homology between AR1 and T4 as evidenced by Southern hybridizations (see Fig. 3) provide additional proof that AR1 belongs in this genus.

There are no genera within the *Myoviridae* that have characteristics similar to those observed for phage LG1. Therefore, phage LG1 is unassigned within the family and may constitute the formation of a new genus within the *Myoviridae*.

The *Myoviridae* contain many phages that infect members of the *Enterobacteriaceae* (17). In this study, the host ranges of AR1 and LG1 were examined (Tables 1 to 4). Both phages were shown to infect many serogroups of *E. coli*, as well as several other members of the *Enterobacteriaceae*. Additionally, to investigate the extent to which LG1 and AR1 could infect a population of *E. coli* strains representative of the whole species, both phages were tested for their ability to form plaques on the 72 isolates of the ECOR collection, a compilation of isolates that is considered to be broadly representative of the genetic diversity of the species (27). *E. coli* is thought to be a highly clonal species (37), and it has been reported that, while commensal microflora is multiclonal (38), individual clones or groups of clones are found in isolates from intestinal infections, such as enterohemorrhagic *E. coli* (39). While no clonal information was available for the 133 STEC strains evaluated, testing LG1 and AR1 for their ability to infect the ECOR strains ensured an evaluation of the ability of these phages to infect different clonal populations of *E. coli*. Phage LG1 in-

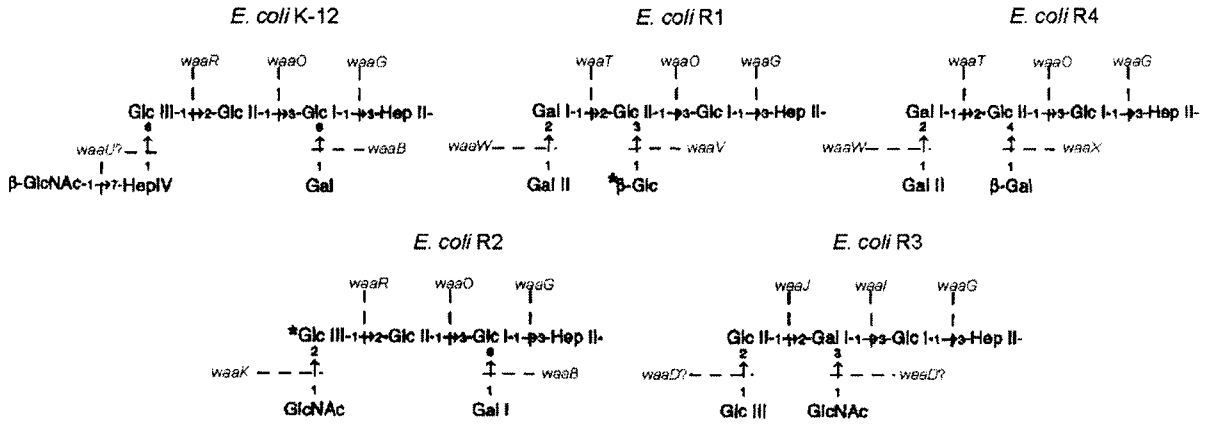

FIG. 2. Structures of the five known outer core OSs from the LPS of *E. coli*. The genes whose products catalyze formation of each linkage (the *waa* operon) are indicated. The figure was adapted from the work of Amor et al. (2).

fected 16 of 72 (22%) ECOR strains, while AR1 was able to form plaques on 38 of the 72 (53%) ECOR strains (Table 2).

The host range results for AR1 contradict the findings of other researchers. Originally, it was reported that phage AR1 was specific for *E. coli* O157:H7 and *S. dysenteriae* (32). In that study, the authors tested 14 *E. coli* O157:H7 strains and 5 other non-O157:H7 *E. coli* strains, in addition to 10 non-*E. coli* bacteria consisting of members of the *Enterobacteriaceae*. Our results suggest that there may be more receptor determinants for phage AR1 than originally described or that the receptor determinants reside in a component of the *E. coli* outer membrane that is not specific to *E. coli* O157:H7 strains. Yu et al. (43) used transposon mutagenesis to identify possible AR1 receptors in the *E. coli* O157:H7 OmpC protein and in the *waaJ* gene product of *E. coli* O157:H7 LPS. This gene is involved in the biosynthesis of *E. coli* R3-type outer core OS, and the R3 outer core OS type has been found in many serotypes of STEC including O157:H7 (2). Therefore, a receptor for AR1 located in the outer core OS region would explain the wide host range of this phage. We decided to evaluate whether other OMPs besides OmpC and outer core OS types of *E. coli* besides R3 could serve as receptors for AR1. To determine if phage AR1 uses proteinaceous components of the cell besides OmpC as a receptor, a series of *E. coli* K-12 OMP isogenic mutants were tested for phage AR1 susceptibility (Table 4). Phage AR1 did not infect any of the isogenic mutants, showing that AR1 cannot use *E. coli* K-12 OMPs as receptors. Phage AR1 was tested for its ability to form plaques on four *E. coli* LPS mutant strains, devoid of the O antigen and representing four core OS types (R1 to R4). AR1 was also tested for its ability to infect the fifth core OS type of *E. coli*, K-12-type core OS. AR1 lysed the R1, R3, and R4 strains but could not infect the K-12 and R2 core OS strains (Table 4). The R2 and K-12 core OS both contain a terminal α1,2-*N*-acetylglucosamine (GlcNAc) side branch (Fig. 2) (13). These results suggested that a carbohydrate residue in the *E. coli* outer core OS may be recognized by AR1 as a cellular receptor in a manner analogous to phage T4, which recognizes *E. coli* B LPS when at least one of the two terminal glucose (Glc) residues is present (31).

To determine which component of the core OS serves as a receptor, AR1 was plated on six R1 core OS mutants, produced to be sequentially deficient in the genes (the *waa* operon) responsible for producing the enzymes that add carbohydrates to the growing R1 core OS region (Fig. 2). AR1 formed plaques on *waaW* and *waaT* mutants but could not form plaques on *waaV*, *waaO*, or *waaG* mutants. *waaW* adds an α1,2-Gal residue to the Gal I position of the outer core OS, while *waaT* is responsible for adding α1,2-Gal to the Glc II position (Fig. 2) (13). AR1 also formed plaques on a *waaQ* mutant, but *waaQ* is responsible for adding heptose residues to the inner core of the OS (13), and its absence does not cause any structural deformation of the LPS. Based on these results, it seems that phage AR1 can utilize *E. coli* outer core OS that has a terminal Glc residue or Gal residue but not core OS types that have a terminal GlcNAc residue. *E. coli* LPSs that have core types R1 (Gal), R3 (Glc), and R4 (Gal) can be used by AR1 as a receptor, but K-12 (GlcNAc), and R2 (GlcNAc) core type LPSs are not recognized by AR1. To test the theory that AR1 cannot recognize outer core OS with a terminal GlcNAc residue, phage AR1 was plated on an R3 *waaJ* mutant (*waaJ* mutants result in a truncation of the outer core OS at Gal I, leaving a terminal GlcNAc side branch attached to the Gal I position [Fig. 2]). AR1 was not able to form plaques on this mutant, which agrees with the *waaJ* transposon-inactivated results reported by Yu et al. (43). However, it appears that truncation of the outer core OS can eventually result in *E. coli* resistance to AR1 infection, regardless of the exposed carbohydrate residue. Phage AR1 could not form plaques on *waaO* or *waaV* mutants, even though there are still terminal Glc residues in these mutants (Fig. 2). Additionally, AR1 cannot form plaques on a *waaG* mutant. The loss of phage sensitivity in *E. coli* strains with a truncated outer core OS may be due to a conformational change in the LPS to such an extent that AR1 binding is not possible.

The perceived discrepancies between our results and published reports dictated that the sequence of the host range genes of phage AR1 should be confirmed. The adsorption specificity of phages in the T4-like virus genus is determined by the protein sequence near the tip of the long tail fibers (the distal tail fiber locus) (34). The tail fiber adhesin domains are located within different genes in closely related phages of the T-even type. For example, in phage T4, the adhesin sequence

is encoded by the C-terminal domain of the large tail fiber gene (gene 37), while phage T2 encodes the adhesin as a separate gene product (gene 38) that binds to the tip of the T2 tail fibers (34). Three groups of T-even phages that possess the tail fiber organization of phage T2 are identified, the T2-like, T6-like, and Ac3-like sequences (34). The sequences of genes 37 and 38 vary to differing degrees among these phages, and an analysis of the sequences of the corresponding AR1 host range genes shows that the AR1 genes 37 and 38 are most closely related to Ac3 (43). A 4.5-kb AR1 DNA segment consisting of genes 36, 37, and 38 and the 5′ end of gene *t* (lysis protein) was amplified and cloned, and the 5′ and 3′ ends of the fragment were sequenced. The 5′ sequence includes the entire gene 36 sequence and the first 450 nucleotides of gene 37 (accession number AY167110). BLASTN analysis showed that the sequence had 99% homology to the GenBank AR1 sequence (accession number AF208841) of the same region. Sequence analysis of the terminal 409 nucleotides of the 3′ end of gene 38 (accession number AY167111) indicated discrepancies between the AR1 sequence derived from this study and the sequence in GenBank (accession number AF208841). The sequences were found to be 98% homologous at the DNA and protein levels. An analysis of the protein sequences shows that none of the nucleotide differences resulted in a major amino acid change. The differing sequences observed in gene 38 are noteworthy, since in phage AR1 it is likely that gp 38 is bound to the tip of the tail fibers for receptor recognition (43).

The results presented here raise questions regarding the genetic basis for an extended host range in AR1. Several bacteriophages are capable of altering their host range via a site-specific recombination system that inverts the sequence that determines the host range (30). For example, bacteriophage Mu is a broad-host-range phage capable of forming plaques on several bacterial species (11). The Mu genome contains tail fiber gene S, which encodes a site recognized by a Mu-encoded invertase, with a second site beyond the gene in inverted orientation. Depending on the orientation of the genomic segment between the two sites, the tail fiber has alternative carboxyl termini that encode different specificities (30). T-even phages such as AR1 use an alternative strategy to obtain adhesin diversity. The T-even phages exchange their adhesin domains with related ones from other phages, possibly via a specialized recombination system (30). Homologous regions within tail fiber genes have been identified in phage Mu; phages P1 and P2; the T-even phages TuIa, TuIb, and T4; and phage lambda (10). The similarities in the tail fiber genes of phages from different families provide evidence that recombination events are occurring between unrelated bacteriophages, an observation that is compatible with the modular theory of phage evolution (4).

The morphological and genome size similarities between AR1 and T4 suggested that these phages would share some degree of homology. Whole-genomic, *Nde*I-digested AR1 DNA was used to probe a Southern blot containing *Nde*I digests of AR1, LG1, and T4 DNA (Fig. 3). Only the digests of AR1 and T4 hybridized, indicating that AR1 is homologous to phage T4. When whole-genomic *Eco*RV-digested AR1 was used to probe a Southern blot containing *Eco*RV digests of AR1, LG1, and T4, in addition to the cross hybridization observed between AR1 and T4, there was a small amount of homology (<3 kb) between AR1 and LG1. This is not unexpected, since different phages that infect the same host have been shown to share varying amounts of homology (25).

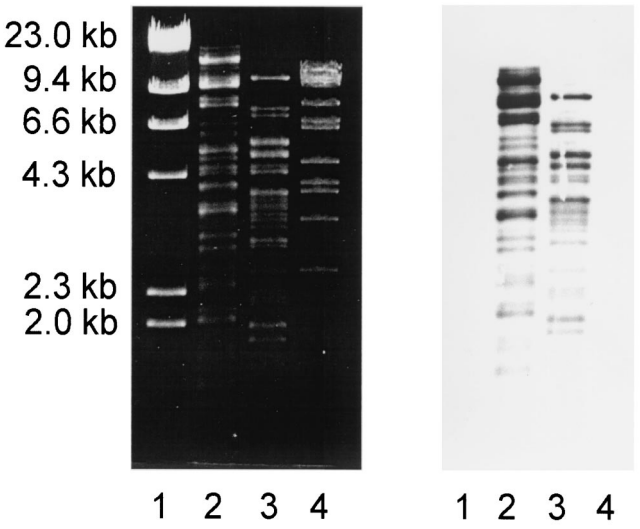

FIG. 3. Agarose (0.8%) gel (left) and corresponding Southern blots (right) of AR1, T4 ATCC 11303B4, and LG1 *Nde*I-digested genomic DNA. Whole-genomic, digoxigenin-labeled, *Nde*I-digested AR1 DNA was used as the probe for the Southern analysis. Lanes 1, lambda *Hin*dIII molecular size markers (2.0 to 23.0 kb); lanes 2, *Nde*I-digested AR1 genomic DNA; lanes 3, *Nde*I-digested T4 ATCC 11303B4 genomic DNA; lanes 4, *Nde*I-digested LG1 genomic DNA.

Due to the low homology observed between LG1 and AR1, random genomic sequencing of the LG1 genome was performed in an attempt to obtain an overview of the LG1 genome structure. The inserts of 13 independently isolated hybrid plasmids were then partially sequenced, and BLASTN searches showed that 12 of 13 sequences had no significant homology to anything in the database. One sequence (accession number AF465474) showed 99% homology (26% overall) to the terminal 207 bp of the 3′ end of ORF 17 of the bacteriophage Felix O1. Phage Felix O1 is a *Salmonella*-specific phage, capable of infecting 98 to 99.5% of the species (16). The function of ORF 17 is unknown.

This study characterized two bacteriophages, AR1 and LG1, that are capable of infecting many different isolates of *E. coli*. The wide host ranges of these phages suggest that they could be employed as biocontrol agents to eliminate the growth of pathogenic *E. coli* on minimally processed foods such as vegetables. Alternatively, phages AR1 and LG1 may be utilized as therapeutic agents to control the growth of pathogenic *E. coli* within humans and animals.

The similarities between AR1 and T4 are advantageous; it is likely that a phage-based diagnostic assay for *E. coli* could be constructed based on phage AR1. The genome of T4 has been completely sequenced (20), and many of the gene products have been elucidated. Such an assay would be extremely useful in the food industry as a test for the quality of a given food. The U.S. Department of Agriculture has mandated the use of generic *E. coli* testing in slaughterhouses as a means to evaluate the quality of raw meat (35).

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