Applied and Environmental Microbiology, Sept. 2001, p. 4335–4337 Vol. 67, No. 9
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.9.4335–4337.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Lytic and Lysogenic Infection of Diverse *Escherichia coli* and *Shigella* Strains with a Verocytotoxigenic Bacteriophage

CHLOE E. JAMES,[1] KAREN N. STANLEY,[2] HEATHER E. ALLISON,[1] HARRY J. FLINT,[2] COLIN S. STEWART,[2] RICHARD J. SHARP,[3] JON R. SAUNDERS,[1] AND ALAN J. McCARTHY[1]*

School of Biological Sciences, University of Liverpool, Liverpool, L69 7ZB,[1] Gut Microbiology and Immunology Division, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB,[2] and The Centre for Applied Microbiological Research, Porton Down, Salisbury,[3] United Kingdom

Received 12 March 2001/Accepted 18 June 2001

A verocytotoxigenic bacteriophage isolated from a strain of enterohemorrhagic *Escherichia coli* O157, into which a kanamycin resistance gene (*aph3*) had been inserted to inactivate the verocytotoxin gene (vt_2), was used to infect *Enterobacteriaceae* strains. A number of *Shigella* and *E. coli* strains were susceptible to lysogenic infection, and a smooth *E. coli* isolate (O107) was also susceptible to lytic infection. The lysogenized strains included different smooth *E. coli* serotypes of both human and animal origin, indicating that this bacteriophage has a substantial capacity to disseminate verocytotoxin genes. A novel indirect plaque assay utilizing an *E. coli recA441* mutant in which phage-infected cells can enter only the lytic cycle, enabling detection of all infective phage, was developed.

Verocytotoxigenic *Escherichia coli* (VTEC) is a serious pathogen of considerable public health concern worldwide. Infection is usually characterized by bloody diarrhea and can be life threatening due to the subsequent development of hemolytic-uremic syndrome mediated by verocytotoxins (VTs), of which there are two forms, VT1 and VT2. In almost all cases, the VT genes are carried on temperate bacteriophages (VT phages). Although *E. coli* O157 is the most commonly isolated VTEC serogroup in the United Kingdom, North America, and Japan, more than 30 disease-causing non-0157 VTECs have been described (1) and over 100 serotypes are capable of producing VT (6). VT production has been observed in other members of the *Enterobacteriaceae*, including *Enterobacter cloacae* (8) and *Citrobacter freundii* (12), but was first described in *Shigella dysenteriae* as Shiga toxin (3). The localization of *vt* genes on a bacteriophage was first described by Smith et al. (13), but their acquisition by pathogenic *E. coli* strains remained anomalous because only nonpathogenic (rough) *E. coli* strains could apparently be infected with VT phage. Previously, the vt_2 gene of a bacteriophage ($\phi 24_B$), isolated from an *E. coli* O157 strain, had been inactivated by insertion of a selectable marker (kanamycin resistance) (10). This provided an ideal opportunity to investigate the host range of a lysogenic VT bacteriophage and thus its potential to transfer the ability to produce VT between *E. coli* and related gram-negative bacteria.

The host range of this recombinant VT2 phage ($\phi 24_B$::Kan) was determined by infection of pathogenic and commensal strains of *E. coli* and other *Enterobacteriaceae* strains from human and animal sources. Lysogens were detected by spreading phage-infected cultures of the host bacteria (100 μl) onto Luria-Bertani Miller (LB) agar (Difco) plates containing kanamycin (50 $\mu g\ ml^{-1}$).

As it is clear that some phage infections create lysogens and do not result in a lytic infection, plaque assays may not necessarily detect all infectious phage particles. Induction of the VT phage lytic cycle is RecA dependent (7). RecA plays a central role in the SOS response of *E. coli*, during which phage-mediated lysis is induced. The *recA441* mutant *E. coli* K-12 strain, DM1187 (5), was used to detect all free phage particles. This mutation results in constitutive activation of RecA protease in the absence of induction. This leads to inactivation of the phage immunity repressor, preventing maintenance of lysogeny and forcing the phage into the lytic cycle. Phage stocks were prepared in this strain and stored at 4°C. Strain DM1187-rif was created by successive passage in increasing rifampin concentrations (5 to 500 $\mu g\ ml^{-1}$). An indirect plaque assay was developed using this DM1187-rif strain, enabling the detection of all free infectious phage particles in any infection mix. Briefly, DM1187-rif was added, as an indicator, to a culture previously infected with phage. Rifampin (300 $\mu g\ ml^{-1}$) was incorporated in the soft agar overlay to select for the indicator host. Lysogens from this infection mix, which could not be of DM1187 origin, could be detected on LB agar (Difco) containing kanamycin (50 $\mu g\ ml^{-1}$). This allowed the detection of all free infective particles. Strain MC1061, an *E. coli* K-12 strain possessing wild type RecA, was susceptible to both lysis and lysogeny and was used as a control throughout.

A range of *E. coli* strains from ruminants, pigs, and humans and a number of representatives of other enteric bacterial genera were screened for susceptibility to lysis and/or lysogeny by $\phi 24_B$::Kan. The strain groups and sources are listed in Table 1 along with the proportion of susceptible strains in each group. All of the wild-type strains were shown to be vt_2-negative by application of the PCR protocol described below for confirming lysogens after $\phi 24_B$::Kan infection. Primers VT2A3′ (5′-TCTGTTCAGAAACGCTGC-3′) and VT2A5′ (5′-TACTGTGCCTGTTACTGG-3′) were designed from the

* Corresponding author. Mailing address: School of Biological Sciences, Life Sciences Building, University of Liverpool, Crown Street, Liverpool L69 7ZB, United Kingdom. Phone: 44-151-794-4413. Fax: 44-151-794-4401. E-mail: aj55m@liverpool.ac.uk.

TABLE 1. Susceptibility of *Enterobacteriaceae* hosts to infection with bacteriophage $\phi 24_B$::Kan

Organism	Source	Strain or descriptor[a]	Total no. of strains	No. of strains infected by $\phi 24_B$::Kan
E. coli	Laboratory	K-12	7	7[e]
	Sheep rumen[b]	Commensal	24	7
	Cattle rumen[b]	Commensal	12	6
	Cattle feces[b]	Commensal	26	2
	Pig feces[c]	ETEC	4	2
	Pig feces[d]	K88	2	2
	Human feces[d]	EHEC	12	2
		EPEC	1	0
		EIEC	1	1
		Commensal	1	0
	Human rectum[d]		3	0
	Human urine[d]		1	1
	Human CSF[d,f]		1	0
	Human[d]		8	1[e]
S. flexneri[d]	Human feces	PT 2a	2	2
		PT 6	1	1
S. sonnei[d]		PT36	1	1
E. cloacae[d]			3	0
C. freundii[d]			1	0
Serratia[d]			2	0

[a] ETEC, enterotoxigenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; PT, phage type.
[b] Strains supplied by The Rowett Research Institute.
[c] Strains supplied by The Robert Koch Institute, Berlin, Germany.
[d] Strains supplied by The University of Liverpool.
[e] Susceptible to lysis.
[f] CSF, cerebrospinal fluid.

published sequence of the 933W VT2 phage (GenBank accession number NC_000924) (9) to amplify the vt_2A subunit gene. All $\phi 24_B$::Kan-susceptible strains were shown to be sensitive to kanamycin (50 μg ml^{-1}) and lacked detectable norfloxacin or UV-inducible prophages.

To test the ability of $\phi 24_B$::Kan to infect wild-type strains, cultures were grown in LB broth containing 0.01 M $CaCl_2$ to mid-exponential phase (optical density at 600 nm, ca. 0.5). Phage suspensions were added to a final concentration of 10^7 PFU ml^{-1}. Infection mixtures were incubated at 37°C with shaking at 120 rpm. Duplicate samples (100 μl) were taken after 2 h and spread on LB agar containing 50 μg of kanamycin ml^{-1} and incubated overnight at 37°C to select for putative lysogens. Control infections in which bacteriophage was omitted were conducted in parallel to confirm that kanamycin resistance was dependent on phage infection. Susceptibility of the strains to lytic infection by $\phi 24_B$::Kan was determined by conventional plaque assay.

Of the total 113 strains tested, 30 of the 103 *E. coli* strains and all 4 of the *Shigella* strains were susceptible to lysogenic infection by $\phi 24_B$::Kan, indicated by growth in the presence of kanamycin after infection. In all cases, lysogens were confirmed by detection of the inactivated vt_2A subunit gene (vt_2A::*aph3*) in the kanamycin-resistant colonies by PCR amplification. The primers VT2A3′ and VT2A5′ were used to amplify the vt_2A::*aph3* gene from bacterial colonies and genomic DNA preparations from putative lysogens, to yield a 2.2-kb product in all cases. The *Taq* polymerase (MBI Fermentas) system was used according to the manufacturer's instructions in the presence of 1.5 mM $MgCl_2$. Cycling conditions were comprised of a 94°C denaturation step (4 min), a 56°C annealing step (30 s), and a 72°C extension step (2 min 45 s) for 35 cycles with the GenAmp PCR System 2400 (Perkin-Elmer). PCR products were visualized by gel electrophoresis (0.75% agarose containing 0.4 μg of ethidium bromide ml^{-1}).

Lysogens were induced to release infective phage particles by exposing mid-exponential-phase cultures of representative kanamycin-resistant colonies to norfloxacin (1 μg ml^{-1}) (Sigma) (4) for 1 h or to UV light (256 nm) for 40 s. Induced cultures were allowed to recover by subculture (1 ml) in fresh LB broth containing $CaCl_2$ (0.01 M) (2 h). Released phage particles were detected by the indirect plaque assay with DM1187-rif described above. These data are summarized in Table 1.

E. coli strains from both animal and human sources were susceptible to lysogenic infection by the phage, and this included a large number of strains isolated from the rumen. The other strains of *Enterobacteriaceae* species studied were not susceptible to the phage, with the exception of the *Shigella sonnei* and *Shigella flexneri* strains, whose susceptibility was not

TABLE 2. Description of non-K-12 *E. coli* and *Shigella* strains found to be susceptible to lysogeny by bacteriophage $\phi 24_B$::Kan[a]

Species	Source	Strain(s)	Serotype[b]
E. coli	Sheep rumen[c]	F315, F318	O162
	Sheep rumen[c]	F38, F39, F310, H312	O rough
	Sheep rumen[c]	2374D1(1)	O5
	Cattle rumen[c]	CR1/2, CRW1/1, CRW2/1	O170
	Cattle rumen[c]	CR2/2	NT[f]
	Cattle rumen[c]	COW957D2(2), COW957D2(3)	Unknown
	Cattle feces[c]	CF11	NT
	Cattle feces[c]	CF18	O46
	Pig feces (ETEC)[d,h]	CDC63-57	O139
	Pig feces (ETEC)[d]	A1	O149
	Pig feces[e]	E56	O149
	Pig feces[e]	E61	NT
	Human[e]	F172	O107[g]
	Human urine[e]	E545	O21
	Human (EIEC)[e,i]	D435	O124
	Human (EHEC)[e]	E164	O118
	Human (EHEC)[e]	E635	O rough
S. flexneri	Human[e]	E713	PT 2a
	Human[e]	E406	PT 6
	Human[e]	E398	PT 2a
S. sonnei	Human[e]	Sson	PT 36

[a] Susceptibility to lysogeny was scored positive only if infective phage particles were released upon induction of putative lysogens (i.e., kanamycin-resistant colonies) using norfloxacin or UV induction and if the expected 2.2-kb fragment could be amplified using VT2A primers.
[b] All strains were typed by Colindale Public Health Laboratories, London, United Kingdom.
[c] Strains supplied by The Rowett Research Institute.
[d] Strains supplied by The Robert Koch Institute.
[e] Strains supplied by The University of Liverpool.
[f] NT, nontypeable.
[g] Strain F172 (O107) was also susceptible to lysis.
[h] ETEC, enterotoxigenic *E. coli*.
[i] EIEC, enteroinvasive *E. coli*.

unexpected in view of their relationships to VTEC (2). Of the seven *E. coli* K-12 strains tested, six were susceptible to both lysogenic and lytic infection; the expected exception was strain DM1187, which is the RecA mutant susceptible only to lytic infection.

The precise sources and serotypes of the wild-type strains susceptible to $\phi 24_B$::Kan are shown in Table 2. The most important feature of these data is the range of *E. coli* serotypes represented, including smooth strains with intact lipopolysaccharide. It has been suggested previously that VT phages can infect only rough strains of *E. coli* and *S. sonnei* (13), inferring that the phage receptor(s) is masked by lipopolysaccharide O side chains. This can now be refuted, since a smooth strain, F172, was susceptible to lytic infection (Table 2). Although the rough *E. coli* K-12 strains were very susceptible to both lysis and lysogeny by this VT phage, one smooth strain (serotype O107) was equally susceptible to both. Schmidt et al. (11) studied the host range of a different VT phage using a chloramphenicol resistance gene insert to inactivate *vt* and also found that VT genes could potentially be disseminated to different *E. coli* strains. However, their study examined only human isolates and a limited range of serotypes. The overwhelming preference for lysogeny rather than lysis among susceptible *E. coli* isolates is, nevertheless, a feature of both the data reported here and those of Schmidt and coworkers (11), with the exception of strain F172.

VT phage host range is an important indicator of the potential for *vt* gene transfer in clinical and agricultural environments. The VT phage used here exhibited a broad host range among *E. coli* and *Shigella* isolates, but susceptibility, i.e., the number of lysogens generated within 2 h, varied between strains (data not shown). This is evident because an enrichment step was not used following phage infection. While the reasons for this are not clear, it suggests that lysogen formation, and not just production of an observable plaque, is an important indicator of infectivity and should be an integral part of future studies on the epidemiology of temperate bacteriophages. The *recA441* mutation carried by *E. coli* DM1187 provides a convenient tool to enable the detection of every infective particle of such lambdoid phages, including those that would otherwise be destined for lysogeny.

This work was funded by the Ministry of Agriculture Fisheries and Food, the Biotechnology and Biological Sciences Research Council, and the Scottish Executive Rural Affairs Department, Edinburgh, United Kingdom.

We thank C. A. Hart of the Department of Medical Microbiology, University of Liverpool, for supplying clinical isolates and T. Cheasty of PHLS, Colindale, United Kingdom, for serotyping the susceptible strains.

REFERENCES

1. **Acheson, D. W., J. Reidl, X. Zhang, G. T. Keusch, J. J. Mekalanos, and M. K. Waldor.** 1998. In vivo transduction with Shiga toxin 1-encoding phage. Infect. Immun. **66:**4496–4498.
2. **Johnson, J. R.** 2000. *Shigella* and *Escherichia coli* at the crossroads: machiavellian masquerades or taxonomic treachery? J. Med. Microbiol. **49:**583–585.
3. **Keusch, G. T.** 1998. The rediscovery of Shiga toxin and its role in clinical disease. Jpn. J. Med. Sci. Biol. **51:**S5–S22.
4. **Matsushiro, A., K. Sato, H. Miyamoto, T. Yamamura, and T. Honda.** 1999. Induction of prophages of enterohemorrhagic *Escherichia coli* O157:H7 with norfloxacin. J. Bacteriol. **181:**2257–2260.
5. **Mount, D. W.** 1977. A mutant of *Escherichia coli* showing constitutive expression of the lysogenic induction and error-prone DNA repair pathways. Proc. Natl. Acad. Sci. USA **74:**300–304.
6. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
7. **Neely, M. N., and D. I. Friedman.** 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of Shiga-like toxin and lysis genes suggest a role for phage function in toxin release. Mol. Microbiol. **28:**1255–1267.
8. **Paton, A. W., and J. C. Paton.** 1996. *Enterobacter cloacae* producing a Shiga-like toxin II-related cytotoxin associated with a case of hemolytic-uremic syndrome. J. Clin. Microbiol. **34:**463–465.
9. **Plunkett, G., III, D. J. Rose, T. J. Durfee, and F. R. Blattner.** 1999. Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. J. Bacteriol. **181:**1767–1778.
10. **Saunders, J. R., M. J. Sergeant, A. J. McCarthy, K. J. Mobbs, C. A. Hart, T. S. Marks, and R. J. Sharp.** 1999. Genetics and molecular ecology of *Escherichia coli* O157, p. 1–25. *In* C. S. Stewart and H. J. Flint (ed.), *Escherichia coli* O157 in farm animals. CABI Publishing, Wallingford, Oxon, United Kingdom.
11. **Schmidt, H., M. Bielaszewska, and H. Karch.** 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage ϕ3538 isolated from *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **65:**3855–3861.
12. **Schmidt, H., M. Montag, J. Bockemühl, J. Heesemann, and H. Karch.** 1993. Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from humans and beef samples. Infect. Immun. **61:**534–543.
13. **Smith, H. W., P. Green, and Z. Parsell.** 1983. Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. J. Gen. Microbiol. **129:**3121–3137.