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A generalized transducing phage for the murine pathogen *Citrobacter rodentium*

Nicola K. Petty¹, Ana L. Toribio², David Goulding², Ian Foulds¹, Nicholas Thomson², Gordon Dougan², and George P. C. Salmond¹

¹Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, UK

²Wellcome Trust Sanger Institute, Hinxton CB10 1SA, UK

Abstract

A virulent phage (ϕ CR1) capable of generalized transduction in *Citrobacter rodentium* was isolated from the environment and characterized. *C. rodentium* is a natural pathogen of mice, causing transmissible murine colonic hyperplasia. Sequencing of its genome has recently been completed and will soon be fully annotated and published. *C. rodentium* is an important model organism for infections caused by the human pathogens enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC). ϕ CR1 uses a lipopolysaccharide receptor, has a genome size of approximately 300 kb, and is able to transduce a variety of markers. ϕ CR1 is the first reported transducing phage for *C. rodentium* and will be a useful tool for functional genomic analysis of this important natural murine pathogen.

INTRODUCTION

Citrobacter rodentium is a natural mouse pathogen. It is the causative agent of transmissible murine colonic hyperplasia, and is responsible for high mortality in suckling mice (Barthold *et al.*, 1978; Schauer *et al.*, 1995). As a member of the attaching and effacing (AE) family of bacterial pathogens, and the only known AE pathogen to naturally infect mice, it is a valuable model organism for the study of pathogenesis of the clinically significant human pathogens, enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Luperchio & Schauer, 2001; Mundy *et al.*, 2005; Wales *et al.*, 2005). EPEC is an important cause of infantile diarrhoea, particularly in developing countries, whilst EHEC causes haemorrhagic colitis and haemolytic uraemic syndrome (Nataro & Kaper, 1998). The genome of *C. rodentium* has recently been sequenced, and will soon be fully annotated and published (http://www.sanger.ac.uk/Projects/C_rodentium/). However, there are currently limited genetic tools available for the study of this bacterium, and it would be useful to expand this repertoire to enable a comprehensive functional genomics programme of the pathogen.

Transducing bacteriophages are useful as genetic tools for studying their host bacteria (Smith & Rees, 1999), but to our knowledge, there are no known phages capable of transduction in *C. rodentium*. Generalized transduction can be used for a range of simple genetic manipulations, for example in mutant strain construction and movement of genetic markers between strains for studies of gene regulation, proteomics and molecular pathogenesis. Here we report the identification and characterization of phage ϕ CR1, which

is capable of generalized transduction in *C. rodentium*, and a useful tool for enabling functional genomics.

METHODS

Culture conditions

C. rodentium strains were grown at 37 °C in Luria-Bertani (LB) medium. For solid medium 1.5 % agar was added, and 0.35 % agar was used in the soft medium overlay (top agar). When required, chloramphenicol (Cm) was added to LB to a final concentration of 25 µg ml⁻¹ for selection. Auxotrophic strains were identified on M9 glucose minimal medium (M9) agar (Sambrook *et al.*, 1989), lactose and galactose mutants on MacConkey lactose medium (Mac) agar (Oxoid MacConkey Agar No. 3), and lactone mutants on top lawns of the *Chromobacterium violaceum* biosensor strain CV026. Phage buffer was composed of 10 mM Tris/HCl pH 7.4, 10 mM MgSO₄, and 0.01 % (w/v) gelatin. Chloroform used in this study was saturated with sodium hydrogen carbonate.

Phage isolation and lysate preparation

The final treated effluent was collected from a sewage treatment plant in Milton, Cambridge. Phages able to infect *C. rodentium* were isolated from chloroform-treated effluent, mixed with overnight culture of the host strain and LB top agar, and overlaid on a LB agar plate as previously described (Petty *et al.*, 2006). Lysates were prepared for each plaque-purified phage isolated (Petty *et al.*, 2006).

Host range determination

Host range was determined by spotting 10 µl of high-titre phage lysate onto lawns of test strains alongside a 10 µl phage buffer control, and the plates incubated overnight. Any clearance observed was followed up by full titration of ϕCR1 on that strain to distinguish plaque formation from other forms of bacterial lysis, such as that caused by bacteriocinogeny or lysis from without.

Generation of DBS100 transposon mutants

Random transposon mutagenesis of *C. rodentium* wild-type strain DBS100 was performed using a Cm resistance transposon from pDS1028 (Smith, 2005), to isolate mutant strains with easily identifiable secondary phenotypes, that could be transduced to confirm generalized transduction. DBS100 and *E. coli* BW20767(pDS1028) were patch mated, and DBS100 transposon insertion mutants were selected on LBA plates containing 25 µg Cm ml⁻¹ plus 50 µg nalidixic acid (Nal) ml⁻¹, then screened for mutant phenotypes on M9 or Mac agar, or CV026 top lawns. Two auxotrophic transposon mutants were identified, NKP01 and NKP14, and mutants appearing white and pink (NKP04 and NKP07 respectively) on Mac, as opposed to the wild-type red, were isolated. In addition, a mutant (IF232) not producing a purple halo on CV026 was also isolated. The insertion point of the transposon in each mutant was identified using random-primed PCR (Fineran *et al.*, 2005) and sequencing. Flanking sequence from both sides of the transposon was compared to the current assembly of the *C. rodentium* genome (http://www.sanger.ac.uk/Projects/C_rodentium/), to identify the gene disrupted by the transposon for each mutant. Sequence analysis revealed that the transposon in NKP01 had inserted in a homologue of *purC* encoding an enzyme involved in purine metabolism, and in NKP14, it had inserted into a homologue of *thrA*, a gene encoding bifunctional aspartokinase I/homoserine dehydrogenase 1, involved in the metabolism of aspartate-family amino acids. NKP04 had a transposon insertion in *lacY*, which codes for lactose permease, therefore affecting lactose metabolism, and NKP07 had the insertion in a homologue of *galE*, which codes for UDP

glucose 4-epimerase, and is thus affected in galactose metabolism. The transposon in IF232 had inserted in *croI*, causing loss of *N*-acylhomoserine lactone production.

Electron microscopy

Phage suspensions were prepared for electron microscopy as reported previously (Demczuk *et al.*, 2004). Briefly, ϕ CR1 virions were sedimented by ultracentrifugation at 25 000 *g* for 60 min, followed by two washes in 0.1 M ammonium acetate (pH 7.2). Five microlitres of this suspension was added to hydrophilic, freshly glow-discharged 200-mesh copper grids, with carboncoated Formvar film, and left to adsorb for 30 s. This was followed by negative staining with 5 μ l of 3 % ammonium molybdate (pH 7) for 10 s. Grids were then blotted to remove excess liquid and allowed to air dry before examination on a FEI Tecnai G² Spirit BioTWIN transmission electron microscope at an acceleration voltage of 120 kV. Images were taken with a Tietz TemCam-F415 digital camera.

DNA isolation

ϕ CR1 DNA for restriction enzyme digestion studies was isolated from high-titre lysates obtained from agarose top lawn infections, to reduce impurities from agar, using the Phase Lock Gel (PLG) kit from Eppendorf.

PFGE

To determine genome size, ϕ CR1 DNA was prepared in agarose blocks using a Bio-Rad CHEF Mammalian Genomic DNA Plug Kit. Blocks were loaded directly onto a 1 % PFGE-certified agarose gel (Bio-Rad) in 0.5 $\times$ Tris/borate/EDTA. A CHEF-DR II system (Bio-Rad) was used to perform electrophoresis, with a voltage of 6 V cm⁻¹ for 16 h at 12 °C, using ramped pulse times from 0.1 to 40 s. DNA was visualized under UV illumination after ethidium bromide staining.

Phage adsorption

Adsorption assays to study the interaction between ϕ CR1 and DBS100 were performed following methods described previously (Petty *et al.*, 2006).

Transduction assay

Initial transductions were performed as previously described (Petty *et al.*, 2006), using phage lysates prepared on donor strain IF232. Phages were tested for their ability to transduce the transposon from IF232 into wild-type DBS100. Transduction was measured by the production of Cm^R colonies and confirmed by co-inheritance of loss of lactone production. M.o.i., temperature and time of incubation for transduction were optimized.

To test for generalized transduction and determine transduction efficiencies, transductions were performed using the optimized assay; 10⁹ p.f.u. of ϕ CR1 from a donor strain lysate was added to 10¹⁰ cells from an overnight culture of DBS100 (approx. 5 ml), to give an m.o.i. of 0.1, mixed for 5 s and left static at room temperature for 30 min. Tubes were then incubated on a tube roller at 37 °C for 20 min, followed by centrifugation at 4 °C for 10 min at 2220 *g*. The pellet was resuspended in 300 μ l LB and 150 μ l spread onto each of two LBA plates containing Cm. Plates were incubated, together with controls for spontaneous mutation to drug resistance and lysate contamination, at 37 °C. Transductants usually appeared after 24 h, and transduction was confirmed by screening Cm^R colonies for either lack of growth on M9, colour change on Mac, or loss of a purple halo on CV026.

RESULTS AND DISCUSSION

Isolation of ϕ CR1

Phages able to infect *C. rodentium* were isolated from chloroform-treated sewage effluent, and each phage was tested for transduction ability. Out of 28 phages tested, only ϕ CR1 was able to transduce the test transposon from a mutant *C. rodentium* strain into the wild-type strain. ϕ CR1 was further investigated and characterized.

ϕ CR1 gives small, clear plaques of less than 0.5 mm diameter on 0.35 % agar top lawns. No difference in plaquing ability was seen at different temperatures, and so 37 °C, the optimum temperature for *C. rodentium*, was used for all incubations. Tests for lysogeny of ϕ CR1, using methods described previously (Petty *et al.*, 2006), showed no evidence of lysogen formation, and we therefore conclude that ϕ CR1 is most likely a virulent phage. In viability studies conducted over a period of 1 year, ϕ CR1 lysates were shown to retain maximum viability (70% of original titre) when stored in glass vials without chloroform at 4 °C, compared to storage in plastic vials at 4 °C, or in 15% glycerol at -20 °C or -80 °C (data not shown). The addition of chloroform adversely affected ϕ CR1 viability, as less than 5% of phages remained viable when stored over a few drops of chloroform, under otherwise identical conditions.

Host range

Other Gram-negative bacteria tested for susceptibility to ϕ CR1 infection included *Citrobacter freundii*, 21 *E. coli* strains, 28 *Serratia* strains, 5 strains of *Salmonella enterica* serovar Typhimurium, and strains of *Salmonella enterica* serovar Typhi, *Erwinia carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, *Yersinia enterocolitica*, *Salmonella enterica* serovar Enteritidis, *Pseudomonas aeruginosa*, and the Gram-positive *Staphylococcus aureus*. ϕ CR1 showed no signs of plaque formation on any bacteria other than *C. rodentium*.

ϕ CR1 was able to infect a spectrum of mutant derivatives of *C. rodentium*, with the exception of strain TJE4. This strain, a mutant of the wild-type DBS100, has a transposon insertion in a homologue of *rfaA*, encoding an enzyme involved in lipopolysaccharide (LPS) biosynthesis (Terry Evans, personal communication). This suggested that ϕ CR1 uses an LPS receptor.

During random transposon mutagenesis to generate mutant strains for transduction studies, four transposon insertion mutants affected in growth on Mac were also identified. Interestingly, all proved refractory to infection by ϕ CR1. Sequence analysis showed that the transposon had inserted into the following gene homologues: *rfaX* in NKP05 and NKP08, *rfaL* in NKP11, and *rfaJ* in NKP15. All these genes code for enzymes involved in LPS biosynthesis. The reason for these mutants' lack of growth on Mac is most likely due to the toxicity of the bile salts in the medium, affecting cells with a compromised cell surface. Reversion to growth on Mac was seen at a rate of around 1×10^{-3} revertants per c.f.u. for these LPS mutants, with varied colony morphology, suggesting random suppressor mutations to overcome the sensitivity to the medium. All these observations are consistent with the notion that ϕ CR1 uses LPS as its receptor.

Characterization and biological properties of ϕ CR1

The morphology of ϕ CR1 was determined by electron microscopy. The phage was found to have an icosahedral head of 116 nm diameter, a 9 nm long neck, and a 245 nm long contractile tail. The contracted tail length was 115 nm, and the diameters of the sheath and central tube were 43 nm and 9 nm respectively (all sizes are the means of measurements

from at least 50 individual virions). Evidence of a base plate and tail fibres was seen on a number of virions. The morphology observed (Fig. 1), which was consistent over different sample preparations and several different grids, allowed classification of ϕ CR1 into the order *Caudovirales* and family *Myoviridae* (Ackermann, 2003).

We were unable to identify any restriction enzyme capable of digesting the DNA of ϕ CR1, and we therefore presume that ϕ CR1 DNA is highly modified. This notion was supported by our inability to sequence its genome. Despite several different approaches to cloning, ϕ CR1 DNA could not be prepared for conventional capillary sequencing. The use of pyrosequencing methodology in an attempt to obtain genome sequence, using 454 Life Sciences' technology involving library preparation without cloning (Margulies *et al.*, 2005), was also unsuccessful. PFGE was performed to elucidate the size of the phage genome. From the PFGE results (Fig. 2), the genome of this phage is estimated to be approximately 300 kbp.

Adsorption assays, summarized in Fig. 3, showed that at 20 min post-infection, more than 80% of available phages were adsorbed to the host. The initial rapid adsorption rate slowed to a maximum adsorption of approximately 90% of ϕ CR1 within 30 min post-infection. Thirty minutes was chosen as the adsorption time for the transduction assay, to ensure that the maximum number of phage had adsorbed to the host, but keeping the total time of the assay within 70 min to reduce the consequences of phage burst (seen at 80 min post-infection, data not shown) and subsequent superinfection killing of transductants.

Transduction

Transduction was optimized for number of bacterial cells and m.o.i., to produce the highest rate of transduction. With 10^8 cells, transduction was not detectable with any m.o.i., and only with an m.o.i. of 1 were one or two transductants detected for 10^9 cells. Better results were seen for transductions using 10^{10} cells, and the highest transduction frequency, in the order of 10^{-8} transductants per p.f.u., was seen using 10^{10} cells with ϕ CR1 at an m.o.i. of 0.1 (see Table 1). Altering the temperature of transduction, temperature of incubation, or the introduction of a wash step had no effect on transduction efficiency.

Generalized transduction was shown by ϕ CR1-mediated transduction of Cm^R markers from five different mutant donor strains, and co-inheritance of the appropriate secondary phenotype, as shown in Table 2. Frequencies of transduction of 1×10^{-8} to 3×10^{-8} were observed for the different markers, which, although on the low side, are sufficient for the use of ϕ CR1 as a functional genomics tool. We were unable to detect transduction of plasmids (data not shown), but this is probably due to the low rates of transduction and the limits of detection of the assay.

Conclusions

ϕ CR1 is the first generalized transducing phage to be identified for *C. rodentium*. It has an icosahedral head and a long contractile tail, belonging to the order *Caudovirales* and family *Myoviridae*. Our data suggest that ϕ CR1 is a virulent phage, with highly modified DNA and a genome size of around 300 kbp, which infects *C. rodentium* via an LPS receptor. Its estimated large genome size indicates it should be able to transduce large amounts of DNA. ϕ CR1 is capable of generalized transduction at frequencies in the order of 10^{-8} transductants per p.f.u., which, although low, is more than sufficient for its use as a genetic tool. ϕ CR1 will be a very valuable tool for the molecular genetic analysis of pathogenesis in *C. rodentium*.

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Abbreviations

Cm	chloramphenicol
Nal	nalidixic acid

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Fig. 1. Electron micrographs of ϕ CR1 negatively stained with ammonium molybdate. (a) ϕ CR1 shown alongside phage lambda (the smaller of the two virions). (b) Intact virion with extended tail shown next to a virion with a contracted tail, exposed central tube and deformed head. (c) Intact virion showing evidence of base plate and tail fibres. Bars, 100 nm.

Fig. 2. PFGE of ϕ CR1 DNA. Phage DNA shown next to Low Range PFG Marker (New England Biolabs).

Fig. 3. Adsorption of ϕ CR1 to DBS100. The graph shows the average results of three separate experiments. An m.o.i. of 0.01 was used for the infection, and the supernatant titrated at various time points to determine the amount of phage remaining unadsorbed.

Table 1

Frequency of transduction of TnCm : : *croI* from IF232 to DBS100 using ϕ CR1, with varied m.o.i. and cell numbers

No. of DBS100 cells	Transduction frequency* at m.o.i. of:		
	0.1	1	10
1×10^8	$<1 \times 10^{-7}$	$<1 \times 10^{-8}$	$<1 \times 10^{-9}$
1×10^9	$<1 \times 10^{-8}$	2×10^{-9}	$<1 \times 10^{-10}$
1×10^{10}	1×10^{-8}	3×10^{-9}	2×10^{-11}

* Number of transductants per p.f.u.

Table 2 ϕ CR1-mediated transduction of different markers into DBS100

Donor strain	Relevant marker	Phenotype [*]	Mean frequency of transduction [†]	SD
NKP01	<i>purC</i> :: TnCm	Purine auxotroph	3×10^{-8}	8×10^{-9}
NKP04	<i>lacY</i> :: TnCm	Lactose ⁻	1×10^{-8}	6×10^{-9}
NKP07	<i>galE</i> :: TnCm	Galactose ⁻	2×10^{-8}	1×10^{-9}
NKP14	<i>thrA</i> :: TnCm	Aspartate-family amino acid auxotroph	2×10^{-8}	7×10^{-9}
IF232	<i>croI</i> :: TnCm	Lactone ⁻	2×10^{-8}	5×10^{-9}

^{*} The number of transductants was determined by the number of Cm^R colonies displaying co-inheritance of the listed phenotype.

[†] Transduction frequencies are expressed as the number of transductants per p.f.u. and are the means of at least 3 different experiments.