EPIDEMIOLOGICAL TYPING

Characterisation of 16 *Campylobacter jejuni* and *C. coli* typing bacteriophages

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Taxonomic classification of bacteriophages specific for *Campylobacter jejuni* and *C. coli* has not been reported previously. A set of 16 virulent phages, distinguishable by their lytic spectra, has been used extensively for epidemiological typing of *C. jejuni* and *C. coli* at Preston Public Health Laboratory. These phages were investigated by electron microscopy, pulsed-field gel electrophoresis and restriction endonuclease analysis. All phages had icosahedral heads and long contractile tails. Accordingly, they were classified as members of the Myoviridae family. These phages could be subdivided into three groups according to genome size and head diameter: group I, two phages with head diameters of 140.6 and 143.8 nm and genome sizes of 320 kb; group II, five phages with average head diameters of 99 nm and average genome sizes of 184 kb; and group III, nine phages with average head sizes of 100 nm and average genome sizes of 138 kb. Phages NCTC12676 and NCTC12677 of group I had unusually large genomes of c. 320 kb which are two of the largest phage genomes to be described. Restriction endonuclease analysis demonstrated that DNA from the 16 phages was refractory to digestion by a number of restriction enzymes.

Introduction

More than 170 bacteriophages of *Campylobacter* spp. have been reported. A few temperate bacteriophages have been isolated from strains of *C. jejuni* [1–3], *C. fetus* subsp. *fetus* [4–7] and *C. fetus* subsp. *venerealis* [5]. The majority of *C. jejuni* and *C. coli* phages are virulent and were isolated from poultry manure, pig manure, abattoir effluents, human faeces and sewage [8–11]. Most of these studies describe either the methods of isolation of phages, or the application of phages to the bacteriophage typing of *C. jejuni* and *C. coli* strains for epidemiological purposes [1, 2, 9, 10, 12, 13] but there are few reports of the characterisation of these phages.

The most important criterion for phage taxonomy is ultrastructure [14]. However, not all of these campylobacter phages have been investigated by electron microscopy and exact particle dimensions are not available. Ten phages have been classified in the Siphoviridae family (Bradley's morphotype B1: non-contractile tail) [4, 5, 7] and another 71 phages in the Myoviridae family (Bradley's morphotype A1: tail

with contractile sheath) [2, 8, 12]. Molecular characterisation of campylobacter phages has not been reported and a taxonomic classification has not been described.

Sixteen virulent campylobacter bacteriophages are used at Preston Public Health Laboratory for the epidemiological typing of *C. jejuni* and *C. coli* strains by the scheme described by Salama *et al.* [13]. Six of these phages originated from Chicago, USA, and formed part of the phage typing scheme of Grajewski and colleagues [9]; the remaining 10 phages were isolated by Salama [12]. The objective of this study was to characterise these phages by electron microscopy, pulsed-field gel electrophoresis (PFGE) and restriction endonuclease analysis (REA).

Materials and methods

Bacterial strains, phages and media

The 16 typing phages and their propagating strains are listed in Table 1. All bacterial strains were stored at –70°C in Brain Heart Infusion (BHI) Broth (Difco) containing glycerol 20% w/v. Prior to use, host strains were subcultured on charcoal, ferrous sulphate, pyruvate (CFP) agar [15] with New Zealand Agar (Davis

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Table 1. *Campylobacter* phages and corresponding propagating strains

Bacteriophages		Propagating strains	
Reference number			
NCTC	ATCC	Species	NCTC no.
12669		<i>C. jejuni</i>	12658
12670		<i>C. jejuni</i>	12659
12671		<i>C. jejuni</i>	12660
12672		<i>C. jejuni</i>	12660
12673	35925-B2	<i>C. jejuni</i>	12661
12674	35922-B2	<i>C. jejuni</i>	12661
12675		<i>C. jejuni</i>	12622
12676	35920-B1	<i>C. jejuni</i>	12663
12677	35920-B2	<i>C. jejuni</i>	12663
12678		<i>C. jejuni</i>	12664
12679	35922-B3	<i>C. jejuni</i>	12664
12680	35924-B1	<i>C. jejuni</i>	12665
12681		<i>C. coli</i>	12666
12682		<i>C. coli</i>	12667
12683		<i>C. coli</i>	12668
12684		<i>C. coli</i>	12668

NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection.

Gelatin, Leamington Spa) 1.2%. Phage stocks were stored in SM buffer [16] at -70°C . Phages were propagated on NZY agar comprising NZY Broth (Gibco BRL) and Select Agar (Gibco BRL) at a concentration of 1.2% for the basal agar and 0.7% for soft agar overlays. BHI broth modified by the addition of 0.01 M MgSO_4 , and 0.001 M CaCl_2 was used to prepare suspensions of *campylobacter* host strains prior to propagation.

Phage propagation and concentration

Propagating strains were subcultured on to CFP agar plates which were incubated micro-aerobically at 37°C for 24 h and harvested into BHI broth to give suspensions equivalent to 10^8 cfu/ml. Phages were propagated by the soft agar overlay method of Adams [17] and harvested with SM buffer overlays [16]. Phage suspensions were centrifuged at 3000 *g* for 25 min at 4°C (IEC-Centra-8R) and the supernatant fluid was filtered through 0.2- μm pore size disposable filters (Minisart NML, Sartorius, Germany) to remove any remaining bacterial debris. The suspensions were transferred into 12-ml centrifuge tubes, and the phage particles were pelleted at 207 000 *g* for 60 min at 4°C (Damon/IEC 628 rotor). The phage pellets were resuspended in SM buffer (1 ml) and stored at -20°C . These suspensions were used for the determination of phage morphology by electron microscopy, sizing of the phage genomes by PFGE and the extraction of phage DNA for REA.

Electron microscopy

Purified phage particles were adsorbed to pyroliform-coated 400-mesh copper grids, negatively stained with phosphotungstic acid (pH 6.5) 3% w/v, and examined with an AEI EM801 electron microscope at an acceleration voltage of 80 kV. Photographs were taken

at a magnification of 51 000. For calibration purposes, astroviruses were photographed at the same magnification (a mean figure of 27.5 nm being used for the diameter of the astroviruses).

Pulsed-field gel electrophoresis

For PFGE, 200 μl of GTG Seaplaque agarose (FMC Bioproducts, USA) 1.5% dissolved in sterile de-ionised water was added to 100 μl of purified phage suspension. The suspension was mixed and pipetted into a plug mould (CHEF, BioRad, USA). The plug was allowed to solidify at room temperature for 30 min. The plug was expelled into a sterile plastic 30-ml screw-capped container and 2 ml of EET buffer (0.1 M EDTA, 0.01 M EGTA, Tris-HCl, pH 8.0), containing lysozyme (Amresco, USA) 1 mg/ml and RNAase (Amresco) 20 $\mu\text{g}/\text{ml}$ were added. The suspension was incubated at 37°C overnight. After incubation, the plugs were washed three times, for 30 min each, with 5 ml of TE buffer [16] at room temperature, on a rocking platform. After washing, plugs were incubated overnight at 50°C in 1 ml of ESP buffer (EET containing sodium dodecyl sulphate 1% w/v). After incubation, plugs were washed twice in TE buffer containing 0.001 M phenylmethanesulphonyl fluoride (Sigma) and then given three further washes with TE buffer.

For electrophoresis, one-third of each plug was loaded into one well of a Fastlane agarose running gel (FMC Bioproducts, USA) 1%. The gel was placed in a CHEF-DR II electrophoresis cell (BioRad) and submerged in $0.5 \times \text{TBE}$ buffer [16] equilibrated to 14°C . Bacteriophage lambda DNA concatemers (48.5–1018.5 kb; BioRad) 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, 5 s; final pulse, 20 s; voltage 200 V (6 V/cm); time, 16 h; and temperature, 15°C . After electrophoresis, the gels were stained with ethidium bromide 0.5 mg/ml solution for 60 min and then destained for 120 min in de-ionised water. Gels were then visualised under UV transillumination and photographed.

Restriction endonuclease analysis

DNA was extracted and purified from pelleted phage particles by the standard method of Sambrook *et al.* [16] (caesium chloride centrifugation and subsequent dialysis steps were omitted). Purified phage DNA was resuspended in sterile de-ionised water, and stored at -20°C .

DNA samples (*c.* 1 μg) from phages NCTC12673 and NCTC12674 were tested with the following restriction endonucleases: *Ava*II, *Cla*I, *Eco*RV, *Hae*III, *Hha*I, *Hinf*I, *Pst*I, *Pvu*I, *Rsa*I, *Sca*I *Taq*I (Appligene, France) and *Bam*HI, *Eco*RI, *Hind*III,

*Hpa*II (Boehringer Mannheim, Germany) according to the suppliers' recommendations. DNA samples from the remaining 14 phages were tested with *Taq*I, *Eco*RV, *Hha*I and *Sca*I. DNA fragments were separated by electrophoresis in agarose 0.8% gels containing ethidium bromide 0.5 µg/ml in TBE buffer [16]. Fragment sizes were determined from co-migrating *Hind*III and *Pst*I digests of Lambda DNA (Appligene).

Results

Electron microscopy

The morphological characteristics of the 16 phages are summarised in Table 2. Electron micrographs of phages

NCTC12676 and NCTC12673 are shown in Fig. 1a and b, respectively. All 16 phages had icosahedral heads and possessed long contractile, non-flexible tails. Fourteen phages were morphologically similar and could not be distinguished by electron microscopy. Two phages, NCTC12676 and NCTC12677, had significantly larger head dimensions (head diameters 140.6 and 143.8 nm, respectively) and these two phages also possessed collars attached at the tail-head interface which have annular thickenings and may be beaded. In Fig. 1a, the collar has collapsed against the head. These collars were not observed on the other 14 phages. Seven of the phage preparations examined contained only phages which appeared to have undergone tail contraction and, therefore, it was not possible to determine tail length and tail diameter.

Table 2. Morphological characteristics and genome sizes of 16 campylobacter bacteriophages

Phage	Genome size (kb)	Head diameter, nm (SD)	Tail		Other ultrastructures	n
			Diameter, nm (SD)	Length, nm (SD)		
12669	135	97 (1.34)	12 (0.00)	103 (1.34)	—	2
12670	140	97 (1.34)	13 (1.34)	98 (5.59)	—	2
12671	135	107 (1.10)	12 (0.00)	108 (3.41)	—	3
12672	140	97 (1.10)	12 (1.10)	99 (4.93)	—	3
12673	140	101 (4.80)	13 (1.02)	101 (2.96)	—	7
12674	140	99 (2.25)	13 (0.98)	99 (1.51)	—	7
12675	180	98 (0.95)	ND	ND	—	4
12676	320	141 (5.66)	ND	ND	Collar	3
12677	320	144 (4.90)	ND	ND	Collar	9
12678	140	98 (1.38)	15 (1.10)	103 (2.23)	—	5
12679	130	102 (0.00)	11 (1.41)	107 (1.34)	—	2
12680	140	101 (3.79)	12 (0.95)	94 (5.15)	—	4
12681	180	105 (1.16)	ND	ND	—	3
12682	190	96 (2.78)	ND	ND	—	4
12683	190	99 (1.16)	ND	ND	—	3
12684	180	97 (2.25)	ND	ND	—	3

n, number of particles measured; ND, not determined.

Fig. 1. Electron micrographs of: **a**, phage NCTC12676 (×163 000); **b**, phage NCTC12673 (×204 000). Bar, 100 nm.

Pulsed-field gel electrophoresis

The genome size of all 16 phages was determined by PFGE and the results are shown in Table 2. The 16 phages could be separated into three groups according to genome size. Bacteriophages NCTC12676 and NCTC12677 had the largest genomes of *c.* 320 kb; five phages formed a group with genome sizes of 180–190 kb; and the remaining phages were in a group with genomes of 130–140 kb.

Restriction endonuclease analysis

The DNA extracts of the 16 phages were refractory to digestion by many of the restriction endonucleases. DNA from phages NCTC12673 and NCTC12674 was resistant to digestion with *Ava*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hinf*I, *Hpa*II, *Pst*I, *Pvu*I, *Rsa*I and *Sca*I. However, restriction digests were obtained with *Hha*I and *Taq*I. The enzymes *Sca*I and *Eco*RV failed to cleave DNA from any of the 16 phages investigated. *Taq*I cleaved DNA from 14 of the 16 phages and the multiple fragments generated were all < 2000 bp in size. The restriction endonuclease *Hha*I cleaved DNA from four of the 16 phages and produced multiple fragments of size range 8–23 kb. Although no identical fragment patterns were observed, the enzyme *Hha*I yielded similar patterns for phages NCTC12673 and NCTC12674.

Discussion

Taxonomic classification of campylobacter bacteriophages has not been described, although 81 phages have been characterised morphologically [2–5, 7, 8, 12]; all had icosahedral heads and the majority (68 phages) had contractile tails (A1 morphotype), whereas the remaining 13 phages possessed non-contractile tails (B1 morphotype). All 16 phages examined in the present study had icosahedral heads and contractile tails, corresponding with the A1 morphotype, and were accordingly classified as members of the family Myoviridae.

The 16 phages can be sub-divided into three groups according to genome size, head size and ultrastructure (Table 3). The two group I phages possessed collars not observed on any of the other 14 phages. Such

Table 3. Proposed taxonomic grouping of campylobacter phages based upon genome size and ultrastructural characteristics

Group	Average genome size, kb (SD)	Average head diameter, nm (SD)	Phages in group
I	320 (0.00)	143 (2.12)	12676, 12677.
II	184 (5.48)	99 (3.43)	12675, 12681, 12682, 12683, 12684.
III	138 (3.63)	100 (3.09)	12669, 12670, 12671, 12672, 12673, 12674, 12678, 12679, 12680.

structures have not been reported previously for other campylobacter bacteriophages and their relevance to phage classification is unknown [18, 19]. All phages from groups I and II had undergone tail contraction, but it is not clear whether this is an important ultrastructural difference or an artefact of the preparation protocol. Previous studies by Salama [12] have demonstrated that these 16 phages are specific for strains of *C. jejuni* and *C. coli* and do not lyse strains of *Escherichia coli*, *Pseudomonas* spp., *Bacillus* spp., *Streptococcus* spp., *Staphylococcus* spp. and *Salmonella* spp. Further studies at this laboratory have demonstrated that these phages do not lyse strains of *C. lari*, *C. upsaliensis* and *C. fetus* subsp. *fetus*.

The Preston phage-typing scheme uses 16 lytic phages arranged in four sets of four phages and the phage-type is determined by generating a hexadecimal score based on the reactions given by each of the phages in each of the sets. This produces a unique four-figure or alpha-numeric code for each phage-type [20]. Over a 5-year period, this scheme was used to phage-type > 9000 strains of *C. jejuni* and *C. coli* from human, animal and environmental sources in the UK [20]. There have been over 400 phage types observed, but only 35 phage types have a frequency of > 1% and most of these are defined by reactions with phages from only one of these three groups described. However, some phage types are defined by reaction with phages from one or more of these groups of phages. In this study, the three most common phage-type codes were 1010 (12%), 2146 (6%) and CEA9 (5%) [20]. Remarkably, these phage-type codes correlate with the taxonomic groupings proposed in Table 4. For example, code 1010 is attributable to lysis with the phages in group I, code 2146 is associated with lysis by phages of group II and code CEA9 is associated with lysis by phages of group III. These data demonstrate that this may represent the first report of a genetic basis for the specific lytic

Table 4. Three most common phage lysis patterns and associated phages

Phage no. (NCTC)	Phage type		
	1010	2146	CEA9
12676	+	–	–
12677	+	–	–
12682	–	+	–
12681	–	+	–
12683	–	+	–
12675	–	+	–
12684	–	+	–
12673	–	–	+
12674	–	–	+
12678	–	–	+
12680	–	–	+
12671	–	–	+
12669	–	–	+
12679	–	–	+
12672	–	–	+
12670	–	–	+

+, lysis; –, no lysis.

activity of phages used in a bacteriophage typing scheme. Further molecular characterisation of the phages and an extensive analysis of the phage typing codes produced by the Preston scheme is necessary to establish the significance of minor differences between the lytic reactions of closely related phage types. Scrutiny of these data should produce a more rational approach to the structure and interpretation of phage-typing schemes.

PFGE can separate large fragments of DNA with high resolution [21] and has been used to investigate the genomes of streptococcal bacteriophages [22]. Very few phages have been described with genomes > 100 kb: the T-even phages of *E. coli* (T2, T4 and T6) have a genome size of 160 kb [23]; two lactococcal phages, 1289 and ϕ 111, 134 kb [22]; phage A511 of *Listeria* spp., 116 kb [24]; two *B. subtilis* phages, SPO1 (150 kb) and PBS1 (280 kb) [25]. The genome sizes of the 16 campylobacter phages in the present study were determined by PFGE. The group I phages had genomes of c. 320 kb. The group II and III phages had average genome sizes of 184 kb and 138 kb, respectively. Therefore, the 16 phages in this study represent a group of phages with unusually large genomes.

REA has been applied to the characterisation of bacteriophages from a wide range of bacterial genera including phages of *Str. cremoris* [19, 26–28], *Enterobacter* spp. [29], *Rhodococcus rhodochrous* [30] and *Listeria* spp. [24]. Other workers have reported that bacteriophage DNA is refractile to cleavage by one or more restriction endonucleases [22, 27–29, 31] and, in the present study, DNA from all 16 campylobacter phages was refractile to cleavage by the enzymes *Sca*I and *Eco*RV. The enzyme *Taq*I cleaved DNA from 14 of the phage genomes although the fragments generated were all < 2000 bp in size; no identical fragment patterns were observed. In contrast, the two phages with the 320-kb genomes were not cleaved by *Taq*I. This enzyme has also been reported to be able to digest DNA from the *B. subtilis* phages PBS1 and SPO1, the bacillus phage SP15, the *Xanthomonas oryzae* phage XP12 and the coliphage T4. DNA from all of these phages was refractile to cleavage by a wide range of other restriction endonucleases [32]. Four of the campylobacter phage genomes were digested by the enzyme *Hha*I and the restriction profiles of strains NCTC12673 and NCTC12674 were similar; they differed only in the presence of one additional fragment.

Overall, the campylobacter bacteriophages studied were resistant to the majority of restriction endonucleases examined. A possible explanation for this observation is that there is a paucity of recognition sites for these enzymes in the genomes of these phages which is an evolutionary response of the phage to the presence of restriction modification systems in

host strains [31, 33]. Alternatively, the phage genomes may code for one or more methyltransferases that modify specific nucleotides at the recognition site of one or more of the restriction endonucleases. An identical mechanism has been described for two temperate phages of *B. subtilis*, SP β [34] and ϕ 3T [35]; both code for a methyltransferase (M. *Bsu* RI) that methylates the central cytosine in the recognition sequence of the restriction endonuclease *Bsu*RI of *B. subtilis*, thus preventing cleavage [36]. A search for altered recognition sites for known restriction endonucleases in the phage DNA might lead to a comparison of the sensitivity of different phages to possible campylobacter restriction enzymes. The proposed taxonomic grouping based upon genome size and ultrastructural characteristics is a novel approach to the classification of campylobacter phages. Additional molecular studies of campylobacter phages based on GC-ratios, PFGE profiles and DNA–DNA homology between the phages is necessary to confirm the taxonomic status of the groups.

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