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Campylobacter Bacteriophage Infection at Refrigeration Temperatures

Yang Hu, Bader Al Shaaer, Lu Liang and Ian F. Connerton *

Division of Microbiology, Brewing and Biotechnology, School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough LE12 5RD, UK; tony.yang.hu@outlook.com (Y.H.); alyba8@nottingham.ac.uk (B.A.S.)
* Correspondence: ian.connerton@nottingham.ac.uk

Abstract: The application of bacteriophages to control foodborne bacterial pathogens in foods has gained traction in recent years. Poultry meat is a major source of *Campylobacter jejuni*, and a target for the application of bacteriophages. To offer the prospect of a post-harvest control measure, the bacteriophage must function at refrigeration temperatures, where *C. jejuni* does not grow but can survive. Here, we report actions of three classes of *Campylobacter* bacteriophage at 4 °C. The pre-incubation of broth cultures at 4 °C before a shift to 42 °C under conditions that support the growth of the host bacteria revealed differences in the time to lysis compared with cultures incubated at 42 °C. The pre-adsorption of the bacteriophage to a sub-population of bacteria is consistent with the observation of asynchronous infection. To ascertain whether the bacteriophages adsorb and infect (the commitment to replicate), we investigated bacteriophage transcription at 4 °C. RNA transcripts for all the bacteriophage host combinations were detected after 15 min, indicating that the interaction is not merely passive. Bacteriophages can infect *C. jejuni* at refrigeration temperatures, but the infection does not proceed to lysis in the absence of host cell division.

Keywords: bacteriophage; *Campylobacter*; phage; biosanitisation; biocontrol; food safety; chicken

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1. Introduction

Campylobacter species are believed to be one of the most common causes of bacterial gastroenteritis worldwide [1]. *Campylobacter* diarrheal disease was the most frequently reported zoonotic disease in the European Union in 2021 [2]. Similarly, in the USA, the Centers for Disease Control and Prevention have reported *Campylobacter* as the most common form of bacterial foodborne infection since 2013 [3]. Most cases are associated with *C. jejuni* (88.4%) infection, followed by *C. coli* (10.1%), which accounts for most of the remaining case load [2]. *C. jejuni* and *C. coli* are thermophilic species that grow at 42 °C—the body temperature of avian species—and as a consequence, readily colonise the intestinal tracts of poultry [4]. Poultry are a major source of *Campylobacter*, with estimates suggesting that 80% of human illnesses emanate from poultry sources [5]. Foodborne campylobacteriosis is frequently associated with the consumption of undercooked poultry or ready-to-eat foods that have been cross-contaminated with raw poultry. One source attribution study referenced at the point of exposure indicated that 65–69% of human cases were derived from chicken meat [6]. Poultry meat is frequently contaminated with intestinal content during slaughter and carcass processing, and birds carrying the highest levels of *Campylobacter* represent a disproportionally higher risk to public health [7]. To reduce the risk, the EU has specified a carcass limit of 1000 CFU/g on broiler chicken [8].

On-farm biosecurity measures to reduce the exposure and transmission of campylobacters that colonise poultry are an important baseline for control, but in themselves they do not eliminate campylobacters from poultry [9]. Intervention strategies have been developed that can reduce *Campylobacter* intestinal loads to mitigate the impact of highly contaminated broiler chicken flocks and the processed carcasses from these flocks [9,10]. Modelling studies have suggested interventions that can produce a one- to two-log reduction in intestinal

Campylobacter concentrations can reduce the relative risk by between 44% and 95% [11]. One such intervention is the application of lytic *Campylobacter*-specific bacteriophages to reduce the intestinal carriage of broiler chickens [12–16], and post-processing to selectively decontaminate the surface of chicken skin [17–19].

The selective application of lytic bacteriophages (referred to as phage) for the biocontrol of foodborne bacterial pathogens in food production is an attractive premise since they are naturally present in foods and will reduce pathogens without harming beneficial bacteria or the product [20]. Based on these benefits, phage applications have been approved for use in the USA by the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA), where commercial products are targeting *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes* are available [21]. Consistent with the ubiquitous nature of bacteriophages and their host bacteria in the environment [22], *Campylobacter* bacteriophages are frequently present in poultry production units [23–25] and can be recovered from retail poultry meat [26]. It is evident that bacteriophage intervention will not introduce any agent that is not already encountered in poultry sources [26,27]. The application of bacteriophages against a wider array of bacteria that contribute to economic losses and concerns for public health in poultry production are now under consideration [28].

Phage applications often use multiple phage types that target different host receptors in the form of phage cocktails, which could concurrently expand the range of host bacteria affected within the target species and limit the potential to develop resistance to all components of the cocktail [29]. *Campylobacter*-specific bacteriophages with contractile tails and ds-DNA genomes have been classified in three classes based on their genome size and morphology [30]. Class I bacteriophages have large genomes reported to be in excess of 320 kb, class II genome sizes in the range of 172–182 kb and class III genome sizes in the range of 132–135 kb [31]. Bacteriophage classes II and III have been taxonomically classified as members of the *Myoviridae* family and placed within the subfamily *Eucampyvirinae* as, respectively, Firehammervirus and Fletchervirus [31]. Phage classes I and II differ from class III in the host surface structures they interact, with classes I and II showing dependence on a functional flagellar [32–35] and class III showing dependence on the composition of the capsular polysaccharide [35,36]. Differences in host specificity contribute to the selection of class II and III phages as suitable components of phage cocktails, which have been used to treat *Campylobacter*-colonised chickens [37–39].

Retail fresh chicken meat and processed products require refrigerated storage temperatures of 4 °C or below to prevent spoilage and to control pathogen growth before purchase. Low temperatures coupled with exposure to higher oxygen levels make retail chicken an inhospitable environment for the growth of campylobacters, although the bacteria are reported to preferentially survive in chicken carcass exudates [40] and to be able to form protective biofilms [41]. Phage applications to control bacterial pathogens on refrigerated products can bring about decontamination by binding and overwhelming the target bacteria (passive therapy) and have the potential to disrupt biofilms to make the bacteria accessible [42]. However, this form of therapy requires high concentrations of phages to saturate the space available to the host bacteria, including the surfaces and films associated with skin folds and feather follicles in the case of chicken carcasses. Empirically, phage applications of *Campylobacter*-contaminated chicken skins have produced reductions in viable counts between 0.7 and 1.5 $\log_{10}$ CFU [17,18,43,44], but phage application titres < $\log_{10}$ 5 PFU/cm^2 were observed to be ineffective and titres > $\log_{10}$ 8 PFU/cm^2 were markedly inefficient [19]. However, experiments designed to assess the ability of the phage to reduce the viable *Campylobacter* count will necessitate a rise in temperature that can elicit host infection and the commitment to lysis. The consumption of phage-treated products either directly, as undercooked meat, or via cross contamination will also lead to an increase in temperature. We therefore aimed to use culture models to understand the nature of the interaction of various phages with their host bacteria at low temperature, and the role of the inevitable temperature upshift.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Campylobacter jejuni strains used for this study as host bacteria to propagate bacteriophages included HPC5 isolated from the caecal content of a commercial broiler chicken in the UK ([12]; NCBI accession CP032316), PT14, the universal bacteriophage host strain isolated from human faeces ([45]; NCBI accession CP003871), and NCTC 12662 ([46]; NCBI accession CP019965) and NCTC11168 ([47]; NCBI accession AL111168) are reference strains. Blood agar (BA) base No. 2 plates (Oxoid, Basingstoke, UK) supplemented with 5% (*v*/*v*) defibrinated horse blood (TCS biosciences, Buckingham, UK) was used for routine bacterial culture. The bacteria were grown under microaerobic conditions (5% *v*/*v* oxygen, 2% *v*/*v* hydrogen, 5% *v*/*v* carbon dioxide and 88% *v*/*v* nitrogen) in a Whitley M35 Variable Atmosphere Workstation (Don Whitley Scientific, Bingley, UK) at 42 °C for 18 h.

Campylobacters were enumerated from liquid cultures by serial dilution in maximum recovery diluent (MRD; Oxoid) using a modification of the Miles Misra method [48] on blood-free Cefoperazone Charcoal Deoxycholate Agar (CCDA; Oxoid) plates containing 2% (*w*/*v*) additional agar to reduce swarming [49]. Five replicate 10 µL droplets from up to six dilutions were plated and incubated under microaerobic conditions at 42 °C for 36–48 h. Discreet colonies were counted and multiplied by 20 and then by the inverse of the dilution factor to determine the colony forming unit (CFU) per mL.

2.2. Bacteriophages, Propagation and Titration

Class I *Campylobacter* bacteriophage phi12 (NCTC12677) is a component of the UK typing scheme [50]. Class II bacteriophage (*Eucampyvirinae* Firehammervirus) CP20 (NCBI nucleotide accession MK408758) was isolated from poultry excreta [12]. Class III bacteriophages (*Eucampyvirinae* Fletchervirus) CP8 (NCBI nucleotide accession KF148616) and CP30A (NCBI nucleotide accession JX569801) were isolated from poultry excreta [12]. Bacteriophages were propagated using a whole plate lysis method whereby overnight bacterial growth on BA plates was harvested in 10 mL of 10 mM $MgSO_4$ solution. Five hundred microlitre aliquots of bacterial suspension were mixed with 100 µL of phage at a titre of approximately 6 $\log_{10}$ PFU. The *Campylobacter*/phage mix was then added to 5 mL of molten NZCYM top agar and poured onto an NZCYM plate (Oxoid). The plates were left to dry and incubated at 42 °C under microaerobic conditions for 18 h [50]. To harvest the phage, 5 mL of reverse osmosis water was transferred onto the plate, and incubated at 4 °C with shaking overnight. After incubation, the recovered phage lysate was filtered through a 0.22 µm filter (Minisart, Sartorius, Goettingen, Germany) to remove any remaining bacteria.

To titrate the propagated phage, a bacterial lawn was prepared by harvesting overnight bacterial growth on BA plates in 10 mL of 10 mM magnesium sulphate solution, followed by mixing 500 µL of bacterial suspension aliquot with 5 mL of molten NZCYM top agar and pouring onto a NZCYM plate. The plates were left to dry and 10 µL of 10-fold serial diluted phage samples were then spotted onto the bacterial lawn in triplicate. Once the spots had dried, the plates were incubated at 42 °C under microaerobic conditions for 18 h [50]. The resulting plaques were counted, and the titre determined as the plaque forming unit (PFU) per millilitre. As required, phages were pelleted by centrifugation at 37,500× *g* for 2 h before suspension in 1 mM $MgSO_4$ at a volume required to achieve titres of $\log_{10}$ 8 to 9 PFU/mL.

2.3. Bacteriophage Infection at 4 and 42 °C

Campylobacters were swabbed from whole BA plates and suspended in 20 mL of sterile Mueller–Hinton broth (Oxoid CM0405). The OD values at A600 were adjusted to 0.1 in 100 mL of Mueller–Hinton broth and pre-incubated with shaking at 100 rpm in 250 mL Erlenmeyer flasks with foam bungs under microaerobic conditions created by gas replacement in a jar (5.6% *v*/*v* oxygen, 3.6% *v*/*v* hydrogen, 7.3% *v*/*v* carbon dioxide and 83% *v*/*v* nitrogen) for 2 h at 42 °C [51]. After 2 h, the culture OD values at A600 were re-adjusted to 0.1 in 100 mL of Mueller–Hinton broth before the addition of 2 mL of SM buffer for

control or 2 mL of phage suspension (approximately 10^9 PFU/mL in SM buffer) to achieve a multiplicity of infection (MOI) of 10. Replicate flasks of 20 mL Mueller–Hinton broth in 100 mL Erlenmeyer flasks were prepared to enable single-use harvesting at timepoints over 24 h at 4 °C or 42 °C. For incubation at 42 °C, the cultures were assigned to gas replacement jars for microaerobic incubation and harvesting, as indicated. For incubation at 4 °C, the cultures were allowed to temperature acclimate for 1 h before the addition of buffer or phage suspension, and then assigned to gas replacement jars for microaerobic incubation with shaking before harvesting at 1 h and 2 hourly time points thereafter. For the temperature upshift experiments, the cultures were incubated at 4 °C for 2 h before transfer to a 42 °C shaking incubator with the flasks harvested at 0 h, 1 h and 2 h pre-upshift, and 1 h to 24 h post upshift. The culture samples were aliquoted accordingly: 50 µL for use in fluorescent cell staining, 1 mL for serial dilution for *Campylobacter* enumeration and 1 mL to be centrifuged at 13,000× *g* for 2 min to pellet the bacteria (flash frozen and stored at −80 °C) and to produce a cell-free supernatant that was passed through a 0.22 µm filter in preparation for phage titration. All experiments were performed in triplicate.

2.4. Fluorescent Cell Staining (Syto9/Propidium Iodide)

Freshly harvested 0.5 to 1 µL samples of culture were mixed with equal volumes of each stain from the *Bac*Light live–dead kit containing 3.34 mM SYTO 9 nucleic acid stain and 20 mM Propidium iodide solutions in DMSO (ThermoFisher Scientific, Loughborough, UK). The mixed cells and stains were incubated at room temperature for 15 min in a dark box before observation over 10 min at a magnification of 1250 (100×, plan Apo) with an epifluorescence microscope (Labophot; Nikon, Tokyo, Japan). The excitation/emission wavelengths for these dyes were 485/498 nm for SYTO 9 stain and 535/617 nm for propidium iodide. Photographic images were captured from 10 independent fields of triplicate slides using a vertical mounted digital camera. The percentage of red stained cells was calculated from the total number of red and green fluorescing cells for each time point. The red cell percentage of the non-infected cultures was subtracted from the phage-infected cultures for each time point to give the increase in red cells associated with phage treatment.

2.5. Campylobacter Genomic DNA Extraction

Genomic DNAs were prepared from BA plate swabs of *Campylobacter* cultures using GenElute Bacterial Genomic DNA Kit following the manufacturer's instructions (Sigma Aldridge, Gillingham, UK). DNA concentrations and quality were determined using a Nanodrop ND-1000 (Labtech International Ltd., Uckfield, UK) and Qubit (Thermo Fisher Scientific, Loughborough, UK).

2.6. Bacteriophage Genomic DNA Extraction

Bacteriophage DNAs were prepared from 1 mL phage suspensions (approximately $\log_{10}$ 9 PFU). To remove any host nucleic acids associated with the phage suspensions, 1 µL of 10 mg/mL DNase and 1 µL of 10 mg/mL RNase were added and the mixture incubated at 37 °C for 30 min. To this, 10 µL of 0.5M EDTA and 5 µL of 10 mg/mL Proteinase K was added, and the solution was mixed and incubated at 37 °C for a further 30 min. Wizard® DNA Clean-Up Resin (Promega, Southampton, UK) was mixed well before use and any crystals or aggregates were dissolve by warming the resin to 37 °C for 10 min. To each sample, 1 mL of Wizard® DNA Clean-Up Resin was added and mixed by inverting several times and packed into a 3 mL syringe. A Wizard mini-column was placed into a reaction tube and the syringe barrel attached to the column and the resin/lysate mix was pushed into the column until all liquid was forced through the resin. The column was washed with 2 mL of 80% isopropanol and centrifuged at 13,000× *g* for 2 min to dry the resin. The column was placed in a 1.5 mL microcentrifuge tube and 100 µL of sterile water at 80 °C was pipetted into the column. The column was centrifuged at 10,000× *g* for 1 min to elute the DNA. Residual guanidine contaminants were removed by ethanol precipitation of the DNA.

2.7. Polymerase Chain Reaction (PCR)

DreamTaq Green PCR Master Mix (ThermoFisher Scientific), nuclease-free water, forward primers and reverse primers were placed on ice for thawing. After defrosting, a brief vortex and centrifuge were applied to the Master Mix and primers to ensure the contents were well mixed and consolidated. As described by the manufacturer, each 25 µL reaction contained 1 µL template DNA, 1 µL forward primer, 1 µL reverse primer, 9.5 µL nuclease-free water and 12.5 µL DreamTaq Green PCR Master Mix. The reactions were loaded into a XP Thermal Cycler (Bioer Technology Co. Ltd., Hangzhou, China) programmed with an initial denaturation step of 95 °C for 2 min, then 40 cycles of denaturation 95 °C for 30 s, annealing at Tm −5 °C for 30 s and extension at 72 °C for 1 min, before a final extension at 72 °C for 15 min.

2.8. RNA Extraction

Following the manufacturer's instructions (NucleoSpin RNA plus, ThermoFisher Scientific), flash-frozen bacterial cell pellets were de-thawed and re-suspended into 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing 1 mg/mL lysozyme and vortexed vigorously. To this, 350 µL LBP buffer (containing guanidinium thiocyanate) was added and mixed by pipetting up and down. The suspension was incubated at 42 °C for 10 min. A NucleoSpin^{TM} gDNA Removal Column (ThermoFisher Scientific) was placed in the 2 mL collection tube provided. The mixed suspension was transferred to the column and centrifuged at 11,000× *g* for 30 s. The column was discarded and the flow-through was mixed with 100 µL binding solution (BS) to bind RNA in solution. After mixing by pipetting up and down, the mix was transferred to the NucleoSpin RNA plus column, which had been assembled with a collection tube. The sample was centrifuged at 11,000× *g* for 15 s and 200 µL buffer WB1 was added on the membrane and centrifuged at 11,000× *g* for another 15 s as the first wash. To this, 600 µL of Buffer WB2 was added to the columns and the assembly was centrifuged for 15 s at 11,000× *g* as a second wash. A further 250 µL Buffer WB2 was added to the column and centrifuged for 2 min at 11,000× *g* to remove the liquid. The RNA products were eluted twice by adding 30 µL RNase-free water and centrifuging at 11,000× *g* for 1 min.

2.9. Quantitative PCR

All pipettes, tips, microtitre plates and other materials used in qPCR preparation were exposed under UV for at least 2 h to inactivate free DNA and minimize environmental contamination [52]. Work was conducted in a class II microbiological cabinet. The cabinet was exposed to UV when not in use to prevent DNA contamination. An RT^2 First Strand Kit (Qiagen, Manchester, UK) was used for cDNA synthesis from 2 µL of the extracted RNAs. To this, 10 µL of genomic DNA elimination mix was added and mixed by pipetting up and down, and then centrifuged briefly to spin down any insoluble material. Next, the mixtures were incubated for 5 min at 42 °C, and then they were immediately placed on ice for 5 min. During this time, the reverse-transcription mix was prepared. For 10 µL aliquots of reverse-transcription mix, the following were mixed: 4 µL 5xBuffer BC3, 1 µL control P2, 2 µL RE3 reverse transcription mix and 3 µL nuclease-free water. The reverse-transcription mix was added to 10 µL genomic DNA elimination mix and mixed by pipetting up and down. The mix was incubated at 42 °C for another 15 min and then was immediately stopped by incubating at 95 °C for 5 min. To each sample, 91 µL RNase-free water was added and mixed by pipetting up and down. The cDNA products were stored at −20 °C ready for qPCR.

A 384-well qPCR plate was employed in a Real-Time PCR LightCycler® 480 System (Roche, Pleasanton, CA, USA) with settings of pre-incubation at 95 °C for 6 min, followed by 40 cycles of amplification. Every well was filled with 5 µL cyber green (PowerUp SYBR Green Master Mix from ThermoFisher Scientific), 2 µL nuclease-free water, 1 µL cDNA template, 1 µL forward primer (10 pmol) and 1 µL reverse primer (10 pmol). Melting curves were assessed for each RT-qPCR reaction using LightCycler® 480 System software (v 1.5.1.62;

Roche). Melting curves were generated by heating the samples from 65 °C to 97 °C at a ramp rate of 0.11 °C per second. Cycle threshold (CT) values were used to calculate the copy numbers of the template by plotting the data against a standard curve generated with five-fold serial dilutions of the target gDNAs until amplification was not detectable, and these corresponded to a range of 20 pg to 20 ng DNA per 10 µL reaction, depending on the target [53]. The genome copy number of the target bacterial DNA in each dilution was calculated based on its genome length and applied to the DNA quantity with the assumption of the mean molecular mass of one base pair as 650 Daltons. The methods were validated with DNA extracts from pure preparations of known CFU or PFU. The primer sequences were designed using the National Centre for Biotechnology Information (NCBI) primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed 7 May 2018) and purchased from Eurofins Genomics (Ebersberg, Germany). The primer sequences used in this study, with descriptions of the target genes and melting temperatures (Tm), are presented in Table 1.

Table 1. Primers used for the amplification of *C. jejuni* and bacteriophage cDNAs and gDNAs.

Oligo. Name	Sequence (5′-3′)	Tm (°C)	Product Size (bp)	Gene Product	Accession
PT14 RNApol F	AACATCTGCTTATACGCC (18)	49.5	69	DNA-directed RNA polymerase alpha	CP003871.4
PT14 RNApol R	GCACTGATTTTAGCCACA (18)	50.3			
HPC5 F	CGCTCTTGGTTGCCGATT (18)	56.1	179	RNA polymerase sigma-54	CP032316.1
HPC5 R	TTACTATCCACAAAAGCCGA (20)	51.3			
11168 F	ACCAGGAACGCAGCTAACTC (20)	57.2	176	RNA polymerase sigma-54	AL111168.1
11168 R	AAAACCGCCGCATTTCTACG (20)	56.5			
Phage 12 F	CTGGACGTGCAGGGAATAAA (20)	55	159	DNA-directed RNA polymerase beta	-
Phage 12 R	CCAAACCTAACCAAGCATCAT (21)	53.2			
CP30A sigma F	GTTCCTGCTCAGCTTTTC (18)	51.2	92	Phage sigma factor	JX569801.1
CP30A sigma R	CCAAAGAACCAGTTAAAGC (19)	49			
CP8 sigma F	TTGGTGAAATGTGTTTGCTTC (21)	52.1	245	Phage sigma factor	KF148616.1
CP8 sigma R	TCCTGCTCAGCTTTTCTTTC (20)	52.9			
CP20 16sMe F	GTGGTGCTGGTGCGATAG (18)	56.1	171	16s rRNA G1207 methylase	MK408758.1
CP20 16sMe R	TTGCTAAACTAAATGGTGGATT (22)	50.3			

2.10. Statistical Analysis

Statistical differences between control and treatment groups were assessed by using the Student's *t*-test with significance set at $p < 0.05$ using $\log_{10}$-transformed *Campylobacter* counts or phage titres. The data were collated, the figures constructed and statistical analysis performed using Microsoft Excel (2016).

3. Results

3.1. Campylobacter Phage Infection at 4 °C

Campylobacter phages of classes I, II and III were mixed with host campylobacters (MOI 10) at 4 °C and incubated in Mueller–Hinton broth culture under microaerobic conditions. Replica flasks were harvested to enumerate the *Campylobacter* and titre the phage over 24 h (Supplementary Figure S1). The non-infected control cultures showed no evidence of growth at 4 °C, but remained viable over the period. The infected cultures showed a fall in phage titre and reductions in viable count over the first hour compared to the control cultures, and thereafter remained static. All phage classes interacted with their hosts, with 76–94% reductions in free phage recorded over 1 h. Table 2 summarises these data with respect to initial phage adsorption and the fall in viable count measurable after 1 h. After

the initial decline in viable count at 4 °C, the infected host cells did not vary significantly ($p > 0.05$) or show any evidence of productive phage replication ($p > 0.05$). The effects of the phages observed on the cell counts may have arisen due to the passive inundation of the host cells or phage adsorption that results in active infection and cell lysis once the cells are removed for enumeration at the permissive growth temperature (42 °C).

Table 2. Phage adsorption and *C. jejuni* host reduction at 4 °C.

Phage/Host	Phage Adsorption (% Free Phage Reduction)	Viable Count Reduction (log_{10} CFU/mL)	(%)
Class I (flagellar dependence)			
Phage 12/NCTC 11168	80.1 ± 9.2	0.4 ± 0.1	60.2 ± 0.1
Class II (flagellar dependence)			
CP20/HPC5	76.4 ± 6.2	0.24 ± 0.4	43.2 ± 0.4
Class III (capsular polysaccharide)			
CP8/PT14	88.2 ± 4	0.28 ± 0.4	42.5 ± 0.4
CP30A/NCTC 12662	94.7 ± 15	0.21 ± 0.2	39.8 ± 0.2

Means and SD ($n = 3$) data are recorded after 1 h of phage infection at MOI 10.

3.2. Microscopic Assessment of Host Cell Integrity and Permeability in the Presence of Bacteriophages at 4 °C

As anticipated for exponentially growing campylobacters in Mueller–Hinton broth at 42 °C, the cells appeared motile on microscopic examination and Gram stains revealed typical short spiral rods (1.5–2.5 μm). Upon transfer of these cultures to 4 °C, the cells retained their motility and short spiral morphology over the phage infection period in the first hour. The addition of the phage at the MOI of 10 did not result in a change in the cells' appearance, and specifically no change was observed over the initial 1 h period that produced the reduction in cell viability.

Vital staining using the Baclight reagents is frequently used for the microscopic monitoring of the viability of cells within bacterial populations. The method uses a combination of Syto 9 that stains intact cells green, and propidium iodide (PI) that relies upon the presence of damaged cells to affect entry and stain them red. The stains are differential indicators of membrane integrity, but in *Campylobacter* species the demarcation between live and dead is less clear as red cells can be fully motile, observable in exponential growth and can retain the ability to divide [54]. In this study, we therefore sought to use the Baclight stains solely as an indicator of differential permeability to the dye for cells from the control and phage-infected cultures at 4 °C. Culture samples were stained, applied to glass microscope slides and fluorescence microscope images recorded at time points post mixing of the phage with the host bacteria at MOI 10 (see Supplementary Figure S2 for representative images). Bacteriophage-infected cultures incubated at 4 °C showed modest increases of 3 to 5% in the frequency of PI-stained cells over that observed for non-infected cultures in the first 2 h after mixing, and remained at similar levels thereafter for the 24 h period of incubation (Table 3). At 4 °C, the phages bound the host efficiently to bring about a reduction in cell viability; however, the proportions of the surviving PI permeable cells appear invariant thereafter.

Table 3. Phage-induced increase in propidium iodide permeability of *C. jejuni* hosts at 4 °C.

Phage/Host	Differential PI Cell Permeability (PI Infected–PI Control)	
	2 h	24 h
Class I (flagellar dependence)		
Phage 12/NCTC 11168	4.76 ± 0.25	5.36 ± 0.72
Class II (flagellar dependence)		
CP20/HPC5	3.63 ± 0.34	4.62 ± 0.59
Class III (capsular polysaccharide)		
CP8/PT14	3.16 ± 0.49	4.14± 0.44
CP30A/NCTC 12662	3.05 ± 0.23	3.62 ± 0.41

PI = (propidium iodide-stained cells/total stained cells) × 100; means and SD (n = 3) data are reported after 2 h and 24 h of phage infection at MOI 10.

3.3. Bacteriophage Infection upon Transition from 4 °C to 42 °C

To attempt to distinguish whether the passive inundation of the host cells brings about reductions in viable count at 4 °C, or if the phages infect but cannot complete the replication cycle to achieve cell lysis without transfer to a permissive growth temperature, we examined the kinetics of the process when the phages and their host bacteria were pre-incubated in broth cultures for 2 h at 4 °C before upshift to the growth temperature of the host at 42 °C for 12 h. If the first contention was true, then the expected outcome would be to show little or no difference between the kinetics of the host growth and phage replication obtained at 42 °C and the same experiments performed with pre-incubation at 4 °C. If the alternative was true, the latent period would show variability if infection and replication proceeded during the preincubation period at 4 °C.

Figure 1 shows the viable counts and phage titres of phage-infected cultures over 12 h at 42 °C with and without preincubation at 4 °C. The class I phage 12 did not exhibit productive replication in broth culture at 42 °C, and therefore was not amenable to the analysis. After initial adsorption, the class II phage CP20 exhibited a rise in phage titre over 4 h from 2 h post infection at 42 °C (Figure 1A). In contrast, preincubation at 4 °C resulted in a greater adsorption and a more familiar single-step rise in phage titre from 2 to 4 h after the temperature upshift (Figure 1B). Under these conditions, CP20 also showed evidence for a secondary adsorption event followed by an increase in phage titre. In either case, the maximum fall in the host viable count after 6 h at 42 °C was $\leq$1 Log_{10} CFU/mL compared to the non-infected control culture. The class III phages CP8 (Figure 1C,D) and CP30A (Figure 1E,F) produced greater falls in host counts of 2.2 to 3.1 Log_{10} CFU/mL compared to the non-infected control cultures after 6 h at 42 °C. The reductions in host count coincided with a rise in phage titre as a single burst between the 2 and 4 h timepoints for the cultures maintained at 42 °C, which marks 2 h as the end of the latent period for CP8 with the host *C. jejuni* PT14 and CP30A with the host *C. jejuni* NCTC 12662. Preincubation at 4 °C elongated the rise in phage titre from 2 to 6 h for CP8 and from 1 to 4 h for CP30A. The extension in the time elapsed for the rise in the phage titre implies the cultures are exhibiting multiple burst events, and therefore demonstrates asynchronicity in the timing of the infection and the latent periods observed.

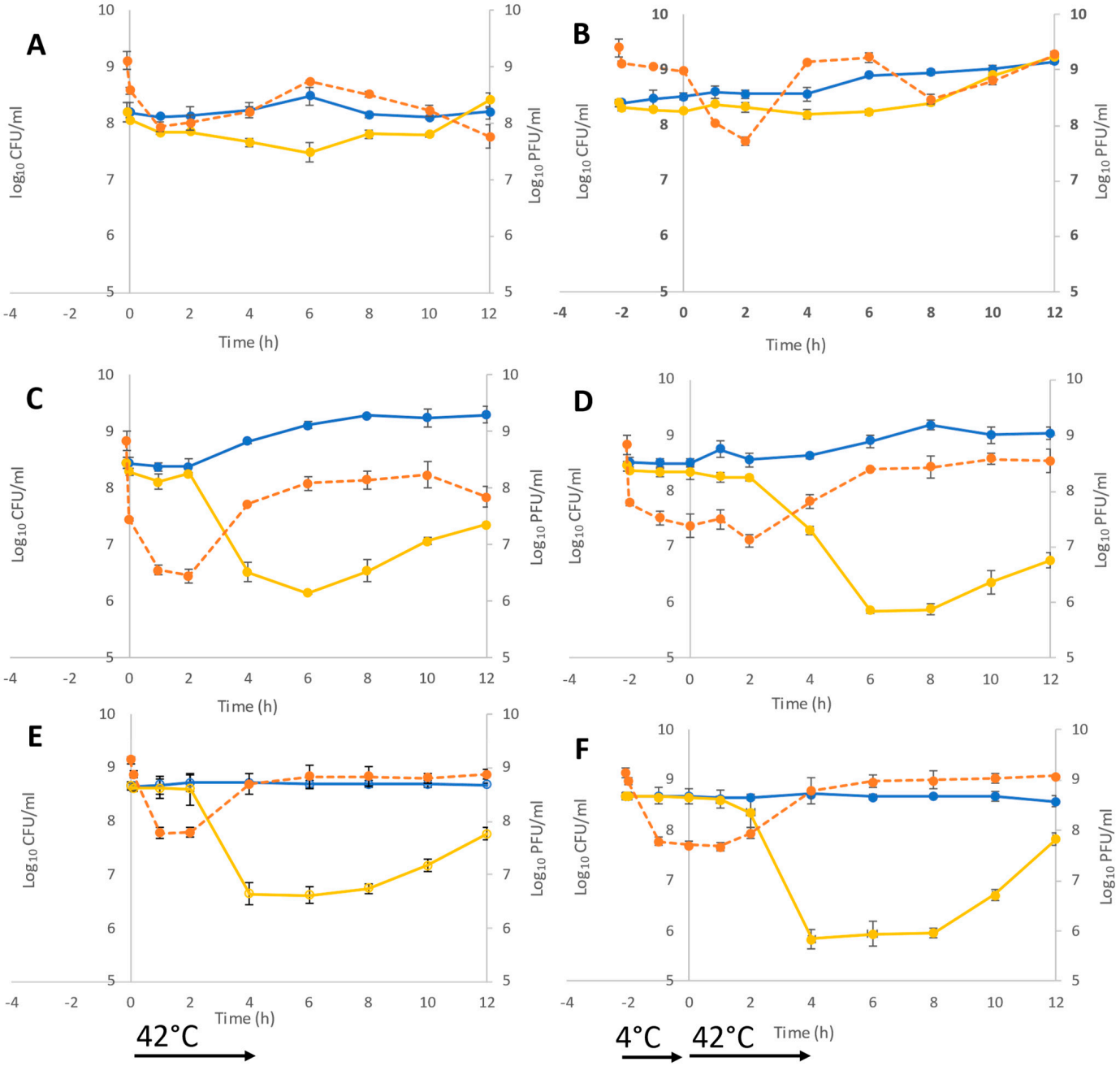

Figure 1. Kinetics of *C. jejuni* growth and bacteriophage replication at 42 °C and after pre-incubation at 4 °C. The solid blue lines represent bacterial counts of control non-infected *C. jejuni* cultures, the solid yellow lines represent the bacterial counts of phage-infected cultures (left axis Log_{10} CFU/mL) and the dashed orange lines represent the corresponding phage titres (right axis Log_{10} PFU/mL). Panels (**A**,**C**,**E**) show cultures incubated at 42 °C. Panels (**B**,**D**,**F**) show cultures pre-incubated for 2 h at 4 °C before an upshift in the temperature to 42 °C. Panels (**A**,**B**) show the host *C. jejuni* HPC5 and with infection by phage CP20. Panels (**C**,**D**) show the host *C. jejuni* PT14 and with infection by phage CP8. Panels (**E**,**F**) show the host *C. jejuni* NCTC 12662 and with infection by phage CP30A. The data are presented as the mean bacterial counts or phage titres with the error bars ± the standard deviations (n = 3).

3.4. Bacteriophage Transcription at 4 °C

To further assess whether the phages interacting with host bacteria become committed to replication but are unable to complete the life cycle at low temperatures, we sought evidence of phage transcription at 4 °C using quantitative PCR. Specific primers targeting

the host and phage gene sequences are listed in Table 1. The primers were demonstrated as specific for the phage or the host bacterial strain by PCR amplification of genomic DNAs. Bacterial cells were incubated with the phage at an MOI of 10 over 24 h at 4 °C, harvested by centrifugation and flash frozen. RNAs were extracted from the flash-frozen cells, treated to ensure the removal of DNA and demonstrated to be dependent on reverse transcription to support the amplification of phage nucleic acid sequences. Phage RNAs could not be detected from non-infected control cells. RNA transcript levels were determined by qPCR and calibrated against Ct values of genomic DNAs to determine the copy number. Figure 2 shows the copy numbers of the phage RNAs per cell determined from infected cultures at 4 °C. RNA transcripts were detected for all the phage–host combinations after 15 min incubation at 4 °C. The transcripts produced maximum levels of 2 to 3.6 copies per cell 1 to 2 h post infection, and thereafter plateaued over 24 h. This evidence indicates that the bacteriophages can infect *C. jejuni* host cells at low temperatures, but the infection does not appear to progress with static mRNA levels that are consistent with the absence of lysis if the culture does not encounter a permissive growth temperature for the host.

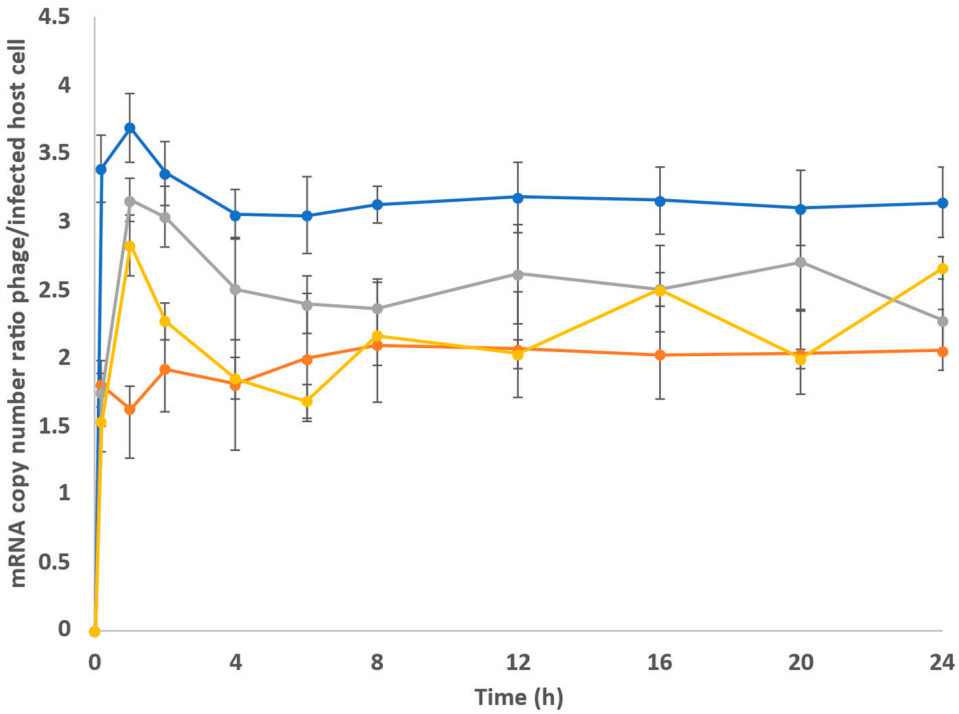

Figure 2. Phage transcript copy numbers per host cell determined over 24 h at 4 °C. The blue line represents class I phage 12 infection of *C. jejuni* NCTC 11168, the orange line represents class II phage CP20 infection of *C. jejuni* HPC5, the yellow line represents class III phage CP8 infection of *C. jejuni* PT14 and the grey line class III phage CP30A infection of *C. jejuni* NCTC 12662. The graph shows the mean copy numbers of the target transcripts per host cell determined from three independent cultures with the error bars ± the standard deviations.

4. Discussion

At the outset of these studies, we sought to understand the nature of the interaction of *Campylobacter* bacteriophages with their host bacteria at low temperatures in the absence of growth, and the consequence of an increase in temperature that would support bacterial metabolism and growth. These studies were founded on the potential use of bacteriophages to control *Campylobacter* foodborne diseases by decontaminating chicken meat and carcasses stored at refrigeration temperatures [55] that pose a high risk to the consumer, either by undercooking or through cross-contamination from the raw product [19]. However, from an ecological standpoint, there are parallels with the ability of phages to interact with target

bacteria in the environment at temperatures that do not enable the growth of the host, at which point they must either delay replication or undergo inefficient replication and lysis under conditions where the availability of further prey bacteria will be limited. In this context, it is interesting to note that bacteriophages that can infect mesophilic *Escherichia coli* at low temperatures have been isolated from environmental sources [56,57], and have been investigated for their potential to control harmful shiga-toxin-producing *E. coli* in foods [58].

As observed in previous studies, the treatment of *C. jejuni* with phages brought about modest reductions in the viable count of the bacteria in broth culture at refrigeration temperatures [44,59]. All phage classes were adsorbed by their host bacteria, with 76–94% reductions in the free phage evident within an hour at an MOI of 10. The greatest reduction in viable count was observed for class I phage 12 at 0.40 $\log_{10}$ CFU/mL (60.2%). *C. jejuni* infection by phage 12 is dependent on the presence of a functional flagella [32], the efficient expression of which requires growth temperatures of 42 °C [60]. However, the host bacteria in these studies were observed to be motile over the initial infection period at 4 °C. In these experiments, all the host bacteria were harvested from agar plates and incubated at 42 °C under microaerobic conditions that would ensure flagella synthesis before chill and acclimation at 4 °C for 1 h in advance of infection. Similarly, the class II phage show motility dependence [34], and accordingly the class II phage CP20 was able to bind the host and reduce the viable count by 0.24 $\log_{10}$ CFU/mL at 4 °C. Class II phages have been applied to chicken skins under atmospheric conditions and demonstrated to reduce *Campylobacter* counts [17], and it therefore likely that a proportion of the campylobacters present retain their flagella on refrigerated fresh chicken to enable flagellar-dependent phage adsorption.

Class III phages are dependent on the composition of the capsular polysaccharide to affect adsorption [36] and have been reported to demonstrate more efficient binding than class II phages [44]. The class III phages CP8 and CP30 examined here also exhibited more efficient adsorption at 4 °C (88 and 95% compared to 76%), but did not exhibit as great a difference as that reported previously (55%) [44]. The respective reductions in viable count of 0.28 and 0.21 $\log_{10}$ CFU/mL for CP8 (42.5%) and CP30 (39.8%) were comparable to the reduction of 0.24 $\log_{10}$ CFU/mL observed for the class II phage CP20 (43.2%).

Although the bacteriophages efficiently adsorbed to the host cells to produce measurable falls in the viable count at 4 °C compared to non-infected controls, this was not accompanied by proportional changes in the propidium iodide permeability of the cells as an indicator of impaired cell integrity. The phage-infected cultures were observed to show increases of 3 to 5% in the propidium iodide-stained cells in excess of the control cultures over 2 h and remained similar thereafter. The maintenance of cell integrity is consistent with the retention of cell viability at 4 °C after the initial fall in the counts upon the addition of the phage.

The application of phage therapy requires an understanding of the kinetics of the processes that relate to key thresholds that govern the outcomes: the proliferation threshold that is the concentration of the target bacterial population required to support an increase in the phage titre, and the inundation threshold that relates to the phage concentration, above which a decline will be brought about in the bacterial population [61]. Bacteriophages applied directly to foods or environmental surfaces in food production settings operate by exceeding the inundation threshold to overwhelm the target food-borne pathogenic bacteria. This is passive therapy, which does not rely on phage replication. In contrast, active therapy relies on phage replication under conditions where the target bacterial density exceeds the proliferation threshold to bring about a crash in the bacterial population. The two forms of phage therapy are not mutually exclusive. Cairns et al. have combined experimental and modelling approaches to establish the key thresholds for *C. jejuni* with a class III phage [62]. Using a target MOI of 10, the phage titres and bacterial concentrations were selected to allow observations of low temperature passive inundation and the bacterial population crashes when the culture temperatures were shifted up and the phages able to replicate.

Incubating the phage-infected cultures at 42 °C produced the anticipated population crashes in the host bacteria (120-fold reduction) with a defined latent period of 2 h for the class III phages CP8 and CP30A to increase the phage titre (18-fold). However, the class II phage CP20 produced a decline in the bacterial population (3-fold) and rise in phage titre over 4 h (5-fold), suggesting the initiation of replication in a component of the population, with the remaining members lagging. Preincubating these cultures at 4 °C appears to have allowed the adsorption to progress to completion and produce uniform lysis and achieve a 25-fold increase in phage titre. It is possible that CP20 uses more than one kinetically distinguishable receptor. Preincubating the class III cultures at 4 °C asynchronized the burst periods, with the rises in phage titre taking place over longer periods of 2 to 6 h for CP8 (18-fold) and from 1 to 4 h for CP30A (13-fold). We sought to distinguish between a passive inundation mode of action and limited infection at 4 °C where the phage cannot complete replication until transfer to a permissive growth temperature for the host bacteria. If the bacteria were killed immediately after adsorption via passive inundation, then they could not further influence the outcome of the infection kinetics upon transfer to 42 °C. If the alternative was true, the pre-adsorbed phage would lead to asynchronous infection. These experiments provide the first evidence for the pre-adsorption of a phage to a sub-population of bacteria. To investigate if the phage adsorbed at 4 °C can commit to replication, we sought evidence of phage transcription using quantitative-PCR. We were able to detect RNA transcripts for all the phage–host combinations. The transcripts reached maximal levels of 2 to 3.6 copies per cell 1 to 2 h post infection, but did not show any further increase over 24 h.

5. Conclusions

Campylobacter bacteriophages can infect their host cells at low temperatures, but do not progress to lysis unless the host can grow and provide the supporting metabolism.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3040094/s1, Figure S1: Campylobacter phages of classes I, II and III with host campylobacters in broth culture under microaerobic conditions at 4 °C; Figure S2: Fluorescent microscope images of Baclight stained *Campylobacter jejuni* PT14 cultures and cultures infected with bacteriophage CP8 at 4 °C.

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