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Original article

Host adaption to the bacteriophage carrier state of *Campylobacter jejuni*

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Abstract

The carrier state of the foodborne pathogen *Campylobacter jejuni* represents an alternative life cycle whereby virulent bacteriophages can persist in association with host bacteria without commitment to lysogeny. Host bacteria exhibit significant phenotypic changes that improve their ability to survive extra-intestinal environments, but exhibit growth-phase-dependent impairment in motility. We demonstrate that early exponential phase cultures become synchronised with respect to the non-motile phenotype, which corresponds with a reduction in their ability to adhere to and invade intestinal epithelial cells. Comparative transcriptome analyses (RNA-seq) identify changes in gene expression that account for the observed phenotypes: downregulation of stress response genes *hrcA*, *hspR* and per and downregulation of the major flagellin *flaA* with the chemotactic response signalling genes *cheV*, *cheA* and *cheW*. These changes present mechanisms by which the host and bacteriophage can remain associated without lysis, and the cultures survive extra-intestinal transit. These data provide a basis for understanding a critical link in the ecology of the *Campylobacter* bacteriophage.

Keywords: Campylobacter; Bacteriophage; Carrier state life cycle; RNA-seq

1. Introduction

The bacterial pathogen *Campylobacter jejuni* is a common cause of human diarrhoeal disease worldwide. Infection can arise from food and water-borne sources, but is notably associated with the consumption of contaminated poultry products [1]. The intestines of poultry are often colonised by campylobacters without compromising the health of the birds, but their presence represents a foodborne hazard to humans when transferred to poultry meat during processing. Bacteriophages have the potential to control bacterial pathogens, and the application of bacteriophages that predate campylobacters can reduce the intestinal carriage of poultry [2,3] and the contamination of poultry meat [4]. *Campylobacter*-specific bacteriophages can be recovered from the intestines of poultry where their host bacteria proliferate [5], and readily infect and replicate within campylobacters in the laboratory [6]. Under these circumstances the bacteriophages are exclusively virulent in that they propagate by infection and lysis of host bacteria. Consistent with a virulent life style, these bacteriophages have contractile tails and icosahedral head morphologies that would place them in the family *Myoviridae* as part of the T4-like phage superfamily [5]. More recently, a new subfamily, the *Eucampyvirinae*, has been proposed that consists of two genera: "Cp220likevirus" and "Cp8unalikevirus", which characteristically possess genome sizes in the range of 130–140 and 170–190 kb respectively [7].

Treatments of *Campylobacter* biofilms with Cp8unalikevirus bacteriophages result in a reduction of the viable bacteria and dispersal of the matrix [8]. Within mature biofilms, bacterial growth is severely restricted, and it is from these restrictive conditions that we have reported the recovery of campylobacters that remain bacteriophage-associated in a relationship that has been referred to as the carrier state life cycle (CSLC) [9]. The CSLC has been observed with strictly lytic bacteriophages infecting various bacterial genera [10–17], and describes mixtures of bacteria and

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bacteriophages that persist in a more or less stable equilibrium [18]. In the *C. jejuni* CSLC cultures, the phage titres remained equivalent to the numbers of viable bacteria following repeated subculture, implying that phage replication was continuing within a subpopulation of sensitive cells, whilst the remaining bacteria were capable of evading phage infection despite their close proximity. The recovery of infectious bacteriophages following treatments of CSLC cultures with either chloroform or bacteriophage-neutralising antibodies established that the phage particles are pre-assembled and intimately associated with the bacteria in that they are sheltered from the antibody [9]. However, the association does not prevent the release of bacteriophages, which allows for free phage particles to explore the environment for new host bacteria. *C. jejuni* CSLC cultures could not efficiently colonise chickens, but when administered to *Campylobacter* pre-colonised chickens, the CSLC phage readily replicated to bring about a reduction in the intestinal counts of the resident population [9]. The dissemination of free phage particles, whilst maintaining host association of a subpopulation phage to ensure against the low probability of encountering permissive hosts upon entering extra-intestinal environments, constitutes a hedge betting strategy that would ensure survival of the phage. CSCL bacteria also have notable phenotypic changes compared to wild type bacteria. These include improved aerotolerance under nutrient-limited conditions, which would confer a survival advantage in extra-intestinal environments, and a lack of motility, which would account for their inability to colonise chickens [9].

This study aims to investigate changes in gene expression and regulation associated with *C. jejuni* CSLC host phenotypes and to understand how the hedge betting strategy of the bacteriophage is implemented.

2. Materials and methods

2.1. *Campylobacter* strains and bacteriophages

C. jejuni PT14 [19,20] was routinely grown on horse blood agar (BA) at 42 °C under microaerobic conditions for 18 h as previously described [2]. *Campylobacter* cultures were resuspended in Mueller-Hinton (MH) broth (CM0337; Oxoid, Basingstoke, UK) using a sterile swab to use as inoculums to initiate broth cultures. Microaerophilic conditions were maintained using anaerobic jars employing gas replacement (85% N_2, 5% O_2 and 10% H_2).

The *Eucampyvirinae* Cp8unalikevirus bacteriophages CP8 and CP30A [8] were isolated in the UK from poultry excreta [2]. Cp220like virus bacteriophage CP220 [20,21] was isolated from poultry meat in the UK. These phages were propagated on the bacterial hosts to be embedded in soft agar overlay using NZCYM agar as previously described [6,22].

2.2. *Growth characteristics of* C. jejuni *CSLC strains*

To determine the primary characteristics C. *jejuni* PT14 and CSLC isolates were inoculated into 50 ml of sterile MH broth to final optical density (A_{600}) 0.01–0.02 (approximately 10^5 CFU/ml). For RNA extractions, the procedure was scaled-up to 500 ml MH broth cultures in 2 l flasks. Samples were incubated shaking at 42 °C under microaerobic conditions over 24 h. Aliquots of 100 μl were removed every 2 h and immediately tenfold serially diluted using maximal recovery diluent (MRD; Oxoid). Campylobacters were enumerated as described previously [8]. Briefly, serial tenfold dilutions were made in MRD (CM0733; Oxoid) and enumerated in triplicate on mCCDA (CM0739, Oxoid) agar with additional agar (L13; Oxoid) to a total of 2% (w/v) added to reduce swarming. Plates were incubated under microaerobic conditions at 42 °C for 48 h before typical *Campylobacter* colonies were counted. Bacteriophages were enumerated using the soft agar overlay method as previously described [22]. Briefly, serial tenfold dilutions of phage suspensions were applied as 10 μl droplets in triplicate to the surface of prepared host bacterial lawns and allowed to dry. Plates were then incubated under microaerobic conditions at 42 °C for 48 h before the plaques were counted.

2.3. *Carrier state and motility tests*

The carrier state phenotype was defined by plaque formation following microaerobic incubation of a soft agar overlay containing the test isolate alone using the method described above. Motility was assessed by inoculation of 0.4% MH agar followed by incubation for 24 h under microaerobic conditions. Motility was assessed as a function of the radius of the motility halo with a strain being defined as motile if the halo radius exceeded 20 mm.

2.4. *Cell culture, adherence and invasion*

HCA-7 colonic epithelial cells [23] were grown as monolayer cultures in 24-well plates in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with foetal calf serum (FCS) at 10% (v/v) (Invitrogen Ltd) at 37 °C in 5% (vol/vol) CO_2 [24]. Cell viability was monitored by performing microscopic examination of 0.4% Trypan-Blue-stained cells. Duplicate cell monolayers at 70% confluence were covered with bacterial cells at an approximate multiplicity of infection of 100 in D-MEM and incubated at 37 °C for 3 h in 5% (vol/vol) CO_2. One monolayer plate was then washed 3 times with sterile phosphate-buffered saline (PBS), monolayers were lysed from one of the plates by adding 0.1% (vol/vol) Triton X-100 and viable bacterial counts enumerated on mCCDA plates recorded as the total adherent and invaded cell numbers. The monolayers of the second plate were similarly washed with PBS and 1 ml of fresh D-MEM supplemented with 250 μg gentamicin ml^{-1} and incubated for a further 2 h to kill all extracellular bacteria. The monolayers were then washed three times with PBS and lysed with 0.1% (vol/vol) Triton X-100 in PBS to release the internalized bacteria, which were enumerated on CCDA plates. The adherent bacterial count was derived by subtracting the number of internalized bacteria from the total and expressed as the percentage of the inoculum. The invasion efficiency was expressed as the percentage