Isolation of a Klebsiella bacterium-bacteriophage combination from human caecum effluent

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Introduction

The human gastrointestinal (GI) tract harbours a diverse range of bacteria, viruses and eukaryotes, with this microbial consortium referred to as the GI microbiota. A very large body of work exists in relation to the microbiota associated with the human colon, but little is known about the microbiota associated with the caecum.

The caecum is a pouch that connects the ileum to the proximal colon, and is considered to be the beginning of the large intestine. The mucosal surface of the human caecum is heavily populated with bacteria, with substantially more biofilm formation in this region of the GI tract than the proximal or transverse colon on the basis of the examination of the colon contents of one deceased organ donor (Bollinger *et al.*, 2007, *J Theor Biol* **249**, 826–831). Colonoscopic examination of the caecum reveals the presence of highly mucoid material lining its mucosal surface (Fig. 1), from which aerobic and anaerobic bacteria can be easily isolated (unpublished data). Therefore, the biofilm associated with the caecum represents "*adherent colonies of microbes growing within an extracellular matrix*" (Bollinger *et al.*, 2007).

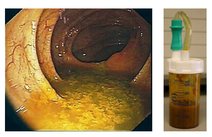

Fig. 1. Appearance of the caecum as viewed from the proximal colon (left panel). The yellow mucoid material lining the surface of the caecum was collected by suction during a routine colonoscopy procedure, and transported under anaerobic condtions to the University of Reading in a trap container (right panel). Ethical approval for the collection of caecum effluent from patients was obtained from St Thomas' Hospital Research Ethics Committee (06/Q0702/74) covering Guy's and St Thomas' Hospitals and transferred by agreement to London Bridge Hospital.

During a study of the microbiota associated with the caecum of patients with irritable bowel syndrome (IBS), attempts were made to isolate bacterium–bacteriophage combinations from samples of caecum effluent. Herein, we report the isolation of the first such combination from the caecum effluent of one patient.

Methods

Isolation of strain L4-FAA5 and bacteriophage ΦKLPN1 from caecum effluent. The sample was processed within 3 h of collection. Strain L4-FAA5 was isolated from diluted caecum effluent at 37 °C on fastidious anaerobe agar under anaerobic conditions. The remaining neat caecum effluent was diluted with sterile 0.5 % Lablemco/6 % NaCl, and homogenized in a stomacher for 2 min at 'high' speed, and placed on ice for 1 h. The homogenate was centrifuged at 4500 ***g*** for 20 min at 4 °C, and the supernatant passed through sterile 0.45 μm cellulose acetate filters; the sterile filtrate contained the virus-like particles isolated from the caecum sample. Bacteria isolated were identified by determining their 16S rRNA gene sequences. *Klebsiella* strains were grown in tryptone soya medium, and used in spot-overlay assays with the filtered caecum sample and double-agar-overlay-plaque assays with the purified bacteriophage. Various characteristics of the bacteriophage were determined (Fig. 2), as was its ability to infect a panel of 20 *Klebsiella pneumoniae* subsp. *pneumoniae* clinical isolates representing various capsule types (K2, K5, K20, K54 and K57). Transmission electron microscopy was used to examine the morphology of ΦKLPN1 (Fig. 3).

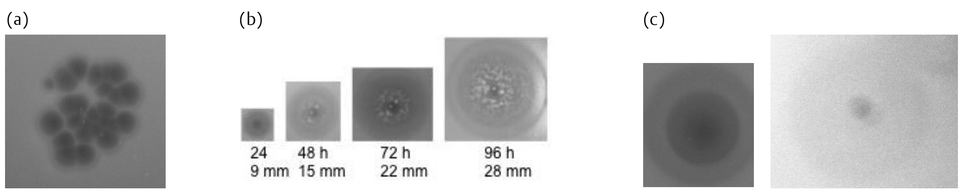

Fig. 2. Characteristics of plaques of bacteriophage ΦKLPN1 when infecting strain L4-FAA5. (a) Appearance of plaques upon initial isolation from caecum effluent. (b) Increase in the size of halo surrounding plaques over the course of 4 days when cultures were incubated aerobically at 37 °C, although the central clear area of the plaques remained 2 mm in diameter. (c) Halo morphology was dependent on atmospheric conditions under which cultures were incubated. After 24 h, aerobically grown L4-FAA5 infected with ΦKLPN1 produced haloes with concentric circles (left panel), whereas anaerobically grown infected cultures produced less distinct, but uniform haloes that were larger than those produced aerobically (11 mm vs 9 mm) (right panel). The two images have been manipulated to allow the halo around the plaque in the anaerobically grown infected culture to be seen.

Results and Discussion

Five of the ten colonies isolated from the caecum sample were identified as *Klebsiella pneumoniae*, representing ~11 % of the total microbiota (2.5×10^7 of 2.2×10^8 cfu/ml caecum effluent). Strain L4-FAA5 was selected for further characterization. Using the typing method of Turton *et al.* (2010, *J Med Microbiol* **59**, 541–547), strain L4-FAA5 was identified as *K. pneumoniae* subsp. *pneumoniae* capsule type K2, *rmpA*$^+$. When grown anaerobically for 24 h, strain L4-FAA5 was highly mucoid, forming amorphous colonies upon prolonged incubation. Colonies of aerobically grown cultures were 3 mm in diameter after 24 h, shiny and convex; although the colonies increased in size after prolonged incubation, they maintained their form.

Plaques identical to those shown in Fig. 2(a) were observed with all *K. pneumoniae* isolates in the spot-overlay assays with filtered caecum effluent. Strain L4-FAA5 was used as the host bacterium on which to purify and propagate the bacteriophage, ΦKLPN1. ΦKLPN1 formed clear plaques of 2 mm diameter within 3 h of inoculating an agar overlay, and was detected at $2\times10^5 \pm 2.65\times10^3$ pfu/ml caecum effluent (n=3). After prolonged incubation, the area around the plaques developed opaque haloes, suggesting ΦKLPN1 possesses an exopolysaccharide depolymerase (Fig. 2b). The concentric circles seen within the halo area of aerobically grown infected cultures were absent from anaerobically grown cultures (Fig. 2c). At an MOI of 0.008 and with aerobic incubation, 95 % of infectious particles adsorbed to strain L4-FAA5 (OD_{660} 0.4) within 5 min; burst size was ~2000 pfu. On the basis of its restriction profile (not shown), the genome of ΦKLPN1 was ~35 kb in size. SDS-PAGE analysis of structural proteins showed three major bands at 45, 30 and 25 kDa (not shown). Examination by transmission electron microscopy showed ΦKLPN1 to be a member of the *Siphoviridae* (Fig. 3).

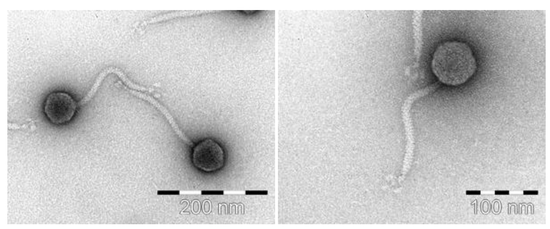

Fig. 3. Transmission electron micrographs of bacteriophage ΦKLPN1. The bacteriophage has a flexible, non-contractile tail, and belongs to the *Siphoviridae*. At the terminus of the striated tail are what appear to be short fibres. The capsid is ~64 nm at its widest point, tapering to ~54 nm; the tail is ~196 nm long.

When screened against 20 clinical isolates of *K. pneumoniae* subsp. *pneumoniae*, ΦKLPN1 infected 5/6 K2 strains, but none of the other strains tested (spot-overlay assays). The ability of ΦKLPN1 to infect K2 strains suggests the bacteriophage could have therapeutic potential in nosocomial infections. Consequently, the human GI tract should be viewed as a source of bacteriophages of medical interest.

The depolymerase activity of ΦKLPN1 on strain L4-FAA5 may be of ecological significance. We have found high concentrations of bacteria within caecum biofilm material. This material may be a preferred site for bacteriophage reproduction compared to the less accessible bacteria found in luminal contents of the colon. ΦKLPN1 may be a prophage of L4-FAA5 that has reverted to a lytic cycle of infection due to the cultivation of its host outside the human body. Within the caecum, the bacteriophage may regulate population numbers within the biofilm in response to environmental changes within this ecosystem.

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