Klebsiella pneumoniae subsp. *pneumoniae*-bacteriophage combination from the caecal effluent of a healthy woman

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Klebsiella pneumoniae subsp. *pneumoniae*– bacteriophage combination from the caecal effluent of a healthy woman

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Introduction

Given their staggering abundance and diversity, coupled to their perceived crucial role in the functioning of ecosystems, it is surprising that virus-like particles (VLPs) (and, by extension, bacteriophages) remain the most poorly characterized biological entities on Earth. Metagenomic studies on samples from a range of environments and the potential of bacteriophage therapy to treat antibiotic-resistant, clinically relevant bacteria have renewed interest bacteriophages. Virome and classical studies examining VLPs in the faeces of adults and infants have demonstrated that there is a vast diversity and abundance of bacteriophages associated with the human gut microbiota, particularly faeces and the caecum [1–4].

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 gastrointestinal tract than the proximal or transverse colon [5]. The biofilm associated with the caecum represents "*adherent colonies of microbes growing within an extracellular matrix*" [5]. During a study of the microbiota associated with the caecum of patients with irritable bowel syndrome and healthy controls, we isolated and characterized a *Klebsiella pneumoniae* subsp. *pneumoniae*–bacteriophage combination from the caecal effluent of a healthy woman.

Methods

Ethical approval to collect caecal 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. The caecal effluent was collected as described previously [4] from a 31-year-old female who showed no evidence of colonic abnormalities or disease as based on a routine colonoscopic examination.

A 1-ml aliquot of effluent was diluted (1-in-10) in sterile, anaerobic half-strength peptone water. A dilution series (10^{-1} to 10^{-6}) was prepared from the homogenate, and aliquots (20 µl) were plated in triplicate onto fastidious anaerobic agar (BIOTEC laboratories, Ipswich, UK) containing 5 % laked horse blood. Bacteria were incubated anaerobically for 5 days at 37 °C, and then enumerated. Ten colonies were selected randomly and streaked to purity, and identified using 16S rRNA gene sequence analysis. One of the five *Klebsiella pneumoniae* isolates (L4-FAA5) recovered was typed using capsular-type-specific, variable number tandem repeat and virulence gene targets [6].

The remaining neat caecal effluent was processed as described previously [4], and used in a spot assay on tryptone soya agar seeded with L4-FAA5. One plaque was selected, and propagated to purity. The ability of the isolated bacteriophage (named KLPN1) to infect clinical *K. pneumoniae* subsp. *pneumoniae* strains was tested (Table 1). Morphology of phage KLPN1 was determined by transmission electron microscopy [4]. The whole-genome sequence of phage KLPN1 was determined using pyrosequencing technology on a 454 FLX instrument (Macrogen Inc., Korea). GeneMark (http://exon.gatech.edu/GeneMark/gm.cgi) was used to predict ORFs. ORF boundaries were verified and, where required, adjusted by manual inspection of Shine–Delgarno sequences. BLASTP, InterPro (http://www.ebi.ac.uk/interpro/) and HHpred (http://toolkit.tuebingen.mpg.de/hhpred) were used to assign functionality to genes. The genome sequence of KLPN1 was compared with available genomes of *Klebsiella* phages (BLASTP), and public virome datasets (BLASTN) available from METAVIR (http://metavir-meb.univ-bpclermont.fr; *n* = 70; 51,992,208 sequence reads associated with 70 projects from a range of habitats, including human faeces [1–3,7,8]).

Results and Discussion

K. pneumoniae subsp. *pneumoniae* is a member of the gut microbiota and an important nosocomial and community-acquired opportunistic pathogen, causing pneumonia, and wound, burn, urinary tract and blood infections. There are 79 recognized capsular types of *K. pneumoniae* subsp. *pneumoniae*, with K2 strains among those most frequently associated with pyogenic liver abscesses. It has been suggested that the majority of *K. pneumoniae*-associated liver infections are preceded by colonization of the gastrointestinal tract, and infections arise from bacteria originating in the faecal microbiota [9].

Using caecal effluent recovered from a healthy woman, we have isolated a capsular type K2 *rmpA*$^+$ strain (L4-FAA5) of *K. pneumoniae* subsp. *pneumoniae*. Our isolation of a *K. pneumoniae* strain with virulence traits from the human caecum supports the assertion that the human gut microbiota is a source of potentially infectious *K. pneumoniae*.

Table 1. *K. pneumoniae* subsp. *pneumoniae* clinical isolates against which bacteriophage KLPN1 was screened

Strain*	Capsular type (K PCR result)†	*rmpA*	*wcaG*	VNTR type	Source	Infected by phage KLPN1
L4-FAA5	K2	+	-	nd	Human caecal effluent	Yes
K/5216	K1 (K1 cluster of CC23)	+	+	nd	Liver abscess (Taiwan)	No
NCTC 5055	K2 (reference strain)	+	-	nd	Human	Yes
NCTC 9660	K5 (reference strain)	-	-	nd	Cloacae of horse	No
PHE1	-	-	-	6,4,6,-,2,2,4,4,1	Rectal swab	No
PHE2	-	-	-	nd	Human clinical	No
PHE3	-	-	-	nd	Sputum, transplant patient	No
PHE4	-	-	-	nd	Urine, spinal injury patient	No
PHE5	-	-	-	nd	Human blood	No
PHE6	-	-	+	nd	Urine, incontinent patient	No
PHE7	-	-	-	nd	Human clinical	No
PHE8	-	-	-	nd	Human blood	No
PHE9	-	-	-	nd	Human clinical	No
PHE10	-	-	-	-,3,3,0,1,1,4,1,1	Human blood	No
PHE11	K2	-	-	6,3,4,0,1,1,4,1,1‡	Blood, patient with urinary tract infection	Yes
PHE12	K2	-	-	6,3,4,0,1,1,4,1,1‡	Urine	Yes
PHE13	K2	-	-	8,3,5,1,-,1,2,3,1	Blood and sputum, patient with bacteraemia and pneumonia	Yes
PHE14	K2	+	-	5,-,6,1,1,2,3,2,1	Sputum, patient with bacteraemia	Yes
PHE15	K2	-	-	nd	Urine, cardiac patient	Yes
PHE16	K20	+	-	nd	Sputum, transplant patient	No
PHE17	K54	-	+	nd	Intensive care unit	No
PHE18	K57	-	-	nd	Sputum, transplant patient	No

nd, No data. *Strains with the prefix PHE were submitted for typing by healthcare providers to Public Health England – Colindale. †K PCR can pick up K1, K2, K5, K20, K54 and K57 capsular types. ‡Corresponds to multi-locus sequence type ST14. Often seen among multi-drug-resistant isolates producing carbapenemases.

Bacteriophages against *K. pneumoniae* subsp. *pneumoniae* L4-FAA5 were present at $2\times10^5 \pm 2.65\times10^3$ (n=3) pfu/ml caecal effluent (Fig. 1a). An isolated plaque was propagated to purity. Phage KLPN1 was chloroform-resistant and formed clear plaques of 2 mm in diameter within 3 h of spotting onto an agar overlay. After prolonged incubation, the area around its plaques developed opaque haloes caused by depolymerase activity, which increased in size over the course of 4 days (Fig. 1b, c). Phage KLPN1 displayed stability to prolonged storage in TSB at 4 °C: after 6 and 18 months' storage, titres for the bacteriophage were still 10^{10} pfu/ml, comparable with the original stock. The *Siphoviridae* bacteriophage presented a rosette-like tail tip (Fig. 1d).

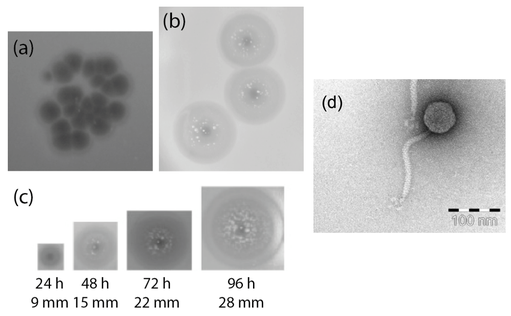

Fig. 1. Isolation of phage KLPN1. Appearance of plaques formed on *K. pneumoniae* subsp. *pneumoniae* L4-FAA5 by phage KLPN1. (a) Initial isolation of bacteriophages from filtered caecal effluent on TSA. (b) Appearance of plaques of pure phage stock after 24 h. (c) Growth of haloes surrounding plaques over the course of the course of 96 h. (d) Transmission electron micrograph of phage KLPN1.

When screened against a panel of clinical isolates of *K. pneumoniae* subsp. *pneumoniae*, phage KLPN1 was shown to infect and lyse capsular type K2 strains (Table 1), but did not exhibit depolymerase activity. Its virulence against K2 strains suggests phage KLPN1 has potential clincal applications, and that the gut microbiota is an untapped source of agents for phage therapy.

The genome of KLPN1 was determined to be 49,037 bp (50.5 GC %) in length, comprising 73 predicted ORFs, of which 23 encoded genes associated with structure, host recognition, packaging, DNA replication and cell lysis (Fig. 2). On the basis of sequence analyses, phages KLPN1 and 1513 (a *Siphoviridae* phage whose sequence was recently deposited in GenBank: KP658157) were found to be two new members of subfamily *Tunavirinae*, genus "Kp36likevirus". Based on HHpred data, ORF34 and/or ORF35 encode the depolymerase activity of phage KLPN1. No sequences with similarity to part of the genome of phage KLPN1 were found in publicly available virome sequences held by METAVIR. However, using InterProScan, the protein sequence encoded by ORF64 was found to belong to the family 'Protein of unknown function DUF3987', representing uncharacterized human-gut-microbiome-specific proteins [10]. ORF60 and ORF61 were predicted to encode holin (which destroys the cytoplasmic membrane) and endolysin (which degrades peptidoglycan), respectively. These gene products have antibacterial properties that can be used in phage-associated therapies; further characterization of the proteins encoded by these ORFs is required.

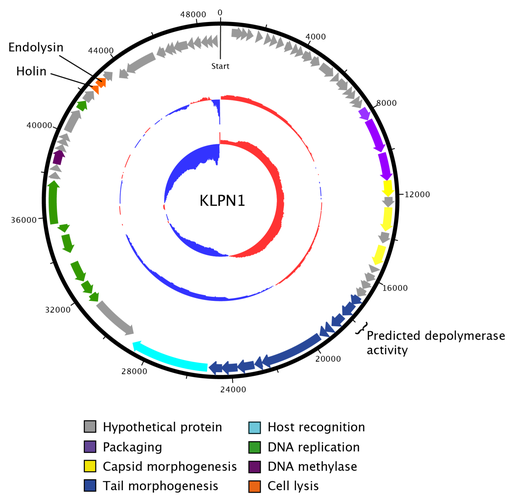

Fig. 2. Genome map of phage KLPN1. The linear genome of KLPN1 depicted in a circularized format. The three circular tracks describe (from inner to outer): GC skew [(G–C)/(G+C)], with blue peaks indicating a lower than average proportion of G; G+C content, with blue peaks indicating below-average G+C content; and predicted ORFs and their direction of transcription. The majority of the ORFs were transcribed on the positive strand.

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