RESEARCH ARTICLE

Fine capsule variation affects bacteriophage susceptibility in Klebsiella pneumoniae ST258

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Abstract
Multidrug resistant (MDR) carbapenemase-producing (CP) Klebsiella pneumoniae, belonging to clonal group CG258, is capable of causing severe disease in humans and is classified as an urgent threat by health agencies worldwide. Bacteriophages are being actively explored as therapeutic alternatives to antibiotics. In an effort to define a robust experimental approach for effective selection of lytic viruses for therapy, we have fully characterized the genomes of 18 K pneumoniae target strains and tested them against novel lytic bacteriophages (n = 65). The genomes of K pneumoniae carrying blaNDM and blaKPC were sequenced and CG258 isolates selected for bacteriophage susceptibility testing. The local K pneumoniae CG258 population was dominated by sequence type ST258 clade 1 (86%) with variations in capsular locus (cps) and prophage content. CG258-specific bacteriophages primarily targeted the capsule, but successful infection is also likely blocked in some by immunity conferred by existing prophages. Five tailed bacteriophages against K pneumoniae ST258 clade 1 were selected for further characterization. Our findings show that effective control of K pneumoniae CG258 with bacteriophage will require mixes of diverse lytic viruses targeting relevant cps variants and allowing for variable prophage

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Abbreviations: CG, clonal group; CP, carbapenemase-producing; cps, capsular locus; ESBL, extended-spectrum-β-lactamase producing; EOP, efficiency of plating; ICE, integrative and conjugative element; LB, lysogeny broth; LPS, lipopolysaccharide; MDR, multidrug resistant; MLST, multilocus sequence typing; PFGE, pulse field gel electrophoresis; ROD, region of difference; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ST, sequence type; TEM, transmission electron microscopy; WGS, whole genome sequencing.

Carola Venturini and Nouri L. Ben Zakour have contributed equally to this work.

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content. These insights will facilitate identification and selection of therapeutic bacteriophage candidates against this serious pathogen.

KEYWORDS
bacteriophages, capsular locus, Klebsiella pneumoniae ST258, multidrug resistance

1 | INTRODUCTION

Klebsiella pneumoniae is an important ubiquitous Gram-negative species capable of causing disease in both humans and animals.\(^1\) The rise in recent decades of K pneumoniae that are multidrug resistant (MDR), including to last-line antibiotics such as carbapenems, has resulted in the classification of this species as an urgent threat by health agencies worldwide and its recognition as an important antimicrobial resistance reservoir.\(^2,3\) Carbapenemase-producing (CP) MDR strains, carrying the \(\text{bla}_{\text{KPC}}\) and \(\text{bla}_{\text{NDM}}\) genes, can be asymptomatic residents of the human gut and a major cause of serious nosocomial infections associated with high morbidity and mortality worldwide.\(^4-7\)

The genetically diverse clonal group CG258, comprising sequence types ST258, ST11, ST512, and a few rarer single locus variants, is largely responsible for the global dissemination of MDR CP-K pneumoniae.\(^6,7\) Population studies looking at the genomes of CG258 have shown that diversification within this CG is linked to a series of large-scale genomic rearrangements and high frequency of recombination, some of which result in switching or variation in the capsule polysaccharide-encoding (\(\text{cps}\)) locus.\(^6,8\) On the basis of this variation, the ST258 group can be subdivided into two separate lineages clade 1 and clade 2. ST258 clade 2 strains have been the main cause of disease outbreaks worldwide,\(^7,9\) while clade 1 uniquely predominated a recent Australian outbreak.\(^9\)

Alternative or adjuvant therapies to antibiotics against MDR pathogens are urgently needed.\(^2\) Naturally occurring lytic bacterial viruses (bacteriophages, phages) were recognized as effective therapeutic agents early in the 20th century, but were little valued by Western medicine after the success of antibiotics.\(^10\) The rise in multidrug resistance, however, has renewed the interest in their potential for both decontamination and eradication of pathogens refractory to antibiotics. Lytic bacteriophages against problematic bacterial species can be readily isolated, but medical applicability is hindered by limited understanding of key issues such as optimal clinical protocols, penetration, and resistance development, as well as disappointing outcomes of phage therapy in humans associated with inconsistent protocols or poor targeting.\(^10-12\)

Bacteriophages capable of lysing K pneumoniae, including MDR strains have been described,\(^13-15\) with complete genomes for more than 80 full double-stranded (ds) DNA bacteriophages available in NCBI databases to date. However, no effective therapeutic product has yet reached the bedside.

We are currently exploring bacteriophage therapy against extended-spectrum-\(\beta\)-lactamase producing (ESBL) Enterobacteriaceae isolated in Australia from humans with the aim of defining a robust experimental protocol for the rapid design of effective targeted phage preparations. Here, we characterize five K pneumoniae ST258-specific lytic bacteriophages (AmPh_EK29, AmPh_EK52, AmPh_EK80, JIPh_Kp122, JIPh_Kp127), isolated from wastewater in Australia, and correlate bacteriophage susceptibility profiles with genomic variation within K pneumoniae ST258 isolates.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates

In this study, we have fully characterized the bacterial genomes of ESBL K pneumoniae CG258 strains from Australia (n = 18, with n = 16 CP-K pneumoniae) and tested the infectivity of novel bacteriophages (n = 65) selected from our existing libraries or isolated de novo from local environmental sources. All MDR K pneumoniae isolates containing the carbapenem resistance genes commonly associated with CG258, \(\text{bla}_{\text{NDM}}\) or \(\text{bla}_{\text{KPC}}\),\(^16\) in our extensive clinical collection were selected as potential target isolates for this study (Table 1).\(^17\)

2.2 | CP-K pneumoniae phenotypes

2.2.1 | Biofilm production

Biofilm formation by growing bacteria in polypropylene microtiter plates was estimated by crystal violet staining of adherent cells, following the protocol of O’Toole and Kolter\(^18\) with minor modifications. Briefly, overnight bacterial cultures in lysogeny broth (LB; Oxoid, Basingstoke, UK) adjusted to OD\(_{600}\) 0.4 were added (0.1 mL) to microtiter plate (Corning Life Sciences, Corning, NY, USA) wells and grown overnight in a static incubator at 37°C. Wells were carefully washed twice with RO water before addition of 0.1% crystal violet (Sigma-Aldrich, MO, USA) (225 μL) and incubation at room temperature. Plates were gently washed four times with RO water and dried at room temperature for at least 2 hours. For quantitation, 200 μL of ethanol (95%) was added to each well and left for 10-15 minutes. An aliquot (125 μL)
| Name (JIE)* | Patient identifier | State | Collection† | Carbapenemase encoding gene‡ | Antibiotic resistance phenotypes** | Reference |
|-------------|--------------------|-------|-------------|-----------------------------|-----------------------------------|-----------|
| 2487        | 1 Vic              | 2012  | None        | None                        | AMK AMC AMP ATM CAZ CRO CIP SXT GEN TZPi TIM TOB TMP | (17)      |
| 2709        | 2 Vic              | 2012 (June 06) | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP SXT MEM MXF TZP TIM TOB TMP | (16)      |
| 2713        | 3 Vic              | 2012  | *bla*<sub>NDM</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP SXT GEN MEMi TZP TIM TOB TMP | (17)      |
| 2733        | 4 NSW              | 2012  | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP SXT TZPi TIM TOB TMP | (16)      |
| 2740        | 2 Vic              | 2012 (June 21) | None | AMK AMC AMP ATM FEP CAZ CRO CIP SXT TZPi TIM TOB TMP | (16)      |
| 2771        | 5 NSW              | 2012  | *bla*<sub>KPC</sub> | AMK AMC AMP ATM CAZ CRO CIP MEM TZP TIM TOB TMPi | (16)      |
| 2783        | 6 NSW              | 2010  | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP CST SXT MEM TZP TIM TOB TMP | (16)      |
| 2793        | 7 WA               | 2012  | *bla*<sub>KPC</sub> | AMK AMC AMP ATM CAZ CRO CIP SXT MEM TZP TIM TOB TMP | (16)      |
| 3095        | 8 Vic              | 2012  | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP GENi MEM TZP TIM TOB TMPi | This work |
| 4005        | 9 Vic              | 2014 (Jan 09) | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP GENi MEM TZP TIM TOB | This work |
| 4019        | 9 Vic              | 2014 (Jan 31) | *bla*<sub>KPC</sub> | AMK AMC AMP ATM FEP CAZ CRO CIP SXT MEM TZP TIM TOB TMP | This work |

(Continues)
of the solubilized solution was then transferred to a new flat-bottom microtiter dish and absorbance at 540 nm was measured in a SpectraMax Vmax microplate reader (Biomolecular Devices, San Jose, CA, USA). Experiments were performed in triplicate.

### 2.2.2 Polysaccharide capsule production

Total capsule production in *K. pneumoniae* CG258 was quantified according to previously described methods. Briefly, overnight bacterial cultures in Mueller-Hinton broth (Oxoid, Basingstoke, UK) were mixed with 1% of Zwittergent 3-14 detergent (Millipore, Billerica, MA, USA) in 100 mM citric acid (pH 2.0) and incubated for 30 minutes at 50°C with occasional mixing. After pelleting the bacteria, 300 µL of supernatant was mixed with absolute ethanol to a final concentration of 80% and left on ice for 30 minutes to allow for capsule precipitation. After centrifugation, the precipitates were allowed to dry, and resuspended DNase-free water (Lonza, Rockland, ME, USA) and kept at 4°C overnight. Capsule quantitation was assayed by measuring uronic acid content on ethanol-precipitated culture supernatants by addition of 1.2 mL of 12.5 mM tetraborate (Sigma-Aldrich, St. Louis, MO, USA) in concentrated sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA), and detection (absorbance at 520 nm) using 0.15% of m-hydroxydiphenyl (Sigma-Aldrich, St. Louis, MO, USA) in 0.5% of NaOH (Amresco, Solon, OH, USA). Sodium hydroxide added to the tetraborate/sulfuric acid solution was used as the baseline for quantification. Capsule quantification was performed in triplicate for each bacterial strain.

### TABLE 1 (Continued)

| Name (JIE)* | Patient identifier | State | Collection† | Carbapenemase encoding gene‡ | Antibiotic resistance phenotypes** | Reference |
|-------------|--------------------|-------|-------------|----------------------------|-----------------------------------|-----------|
| 4020        | 10 Vic             | 2014  |             | *bla* KPC                  | AMK AMC AMP ATM FEP CAZ CRO CIP MEMi TZP TIM TOB | This work |
| 4046        | 11 Vic             | 2014  |             | *bla* KPC                  | AMKi AMC AMP ATM FEP CAZ CRO CIP MEMi TZP TIM TOB | This work |
| 4203        | 12 Vic             | 2014  |             | *bla* KPC                  | AMK AMC AMP ATM FEP CAZ CRO CIP MEMi TZP TIM TOB | This work |
| 4282        | 13 Vic             | 2014  |             | *bla* KPC                  | AMC AMP ATM FEP CAZ CRO CIP MEMi TZP TIM TOB | This work |
| 4455        | 14 NSW             | 2015  |             | *bla* KPC                  | AMK AMC AMP ATM FEP CAZ CRO CIP SXT GEN MEMi TZP TIM TOB | This work |
| 4626        | 16 NSW             | 2015  |             | *bla* KPC                  | AMK AMC AMP ATM FEP CAZ CRO CIP CST SXT GEN MEMi TZP TIM TOB | This work |
| 4660        | 17 NSW             | 2015  |             | *bla* KPC                  | AMKi AMC AMP ATM FEP CAZ CRO CIP CST SXT GEN MEMi TZP TIM TOB | This work |

Note: *from JIE_G1046886_Iredell collection; †in brackets, month and day specified for isolates collected from the same patient; ‡determined by diagnostic PCR screening at collection facility (ICPMR, Westmead Hospital, Westmead, NSW, Australia); **determined by BD Phoenix (Becton Dickinson, Wokingham, Berkshire, UK) screening at collection facility. Cutoff values in accordance with the EUCAST system. i, intermediate. For isolate 2793, FEP susceptibility was not determined.
2.2.3 Lipopolysaccharide profiles

Lipopolysaccharide (LPS) profiles of *K. pneumoniae* CG258 strains were obtained using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) subsequent to proteinase K digestion of whole cell lysates. Overnight bacterial cultures were pelleted and washed twice in saline; the final pellet resuspended in 35 µL sterile saline. Pellets were treated with 4X SDS reducing buffer (0.0625 M Tris-HCl, pH 8.8, 10% glycerol, 2% SDS, 5% 2-b-mercaptoethanol, 0.0125% bromophenol blue) at 100°C for 10 minutes. Proteins were digested by addition of 20 mg/mL proteinase K and incubation at 60°C for 1 hour. Samples were run under reducing conditions using Tris-glycine running buffer (mini-PROTEAN system, Bio-Rad Laboratories, Hercules, CA, USA). Silver staining using a reference-based core genome alignment as an input.

2.3 Sequencing and analysis of bacterial genomes

The genomes of selected MDR *K. pneumoniae* isolates were sequenced by Illumina NextSeq (paired-end; 2 x 150 bp). Bacterial DNA extraction was performed using the DNeasy Blood and Tissue DNA isolation kit (Qiagen, Hilden, Germany) to obtain high purity (OD260/280 1.8-2.0; OD260/230 1.8) preparations for sequencing. DNA libraries for whole genome sequencing (WGS) were prepared using the Nextera XT kit and sequencing was performed at the Australian Genome Research Facility (AGRF, Melbourne, Australia). De novo assembly of sequencing reads and simulated reads of NCBI reference genomes were performed as previously described, using our WGS analysis workflow based on publicly available tools, including SPAdes 3.9.0,24 Nullarbor 1.2.0,25 Kleborate 0.2.0,26 to confirm identity (in silico multilocus sequence typing [MLST]), virulence and antibiotic resistance genotypes. A maximum-likelihood recombination-free phylogenetic tree was computed using RAxML 8.2.427 and Gubbins 2.2.0,28 using a reference-based core genome alignment as an input. The publicly available genome sequences of five representative *K. pneumoniae* strains were also added for comparative purposes: AUSMDU00008079, used as mapping reference (CP022691); HS11286 (CP0033200); NJST258-1 (CP0066923); Kb140 (AQROD00000000); and VA360 (ANGI00000000). The pangenome, determined using Roary version 3.11.0,29 was used to classify regions of differences across the strain data set, based on their contiguity and functional categories of the genes encoded. Kleborate 0.2.0,26 was used to type capsule, O antigen (LPS) and siderophores. Plasmid replicon identification and typing was performed using PlasmidFinder and pMLST implemented in BAP.30 Prophage-associated contigs were annotated using PHASTER.31 Further *cps* locus comparative analysis was performed using Easyfig and Geneious v9.1 (https://www.geneious.com). Gap closure between separate contigs in the capsular locus (*cps*) was achieved by PCR amplification and Sanger sequencing of purified linkage amplicons (AGRF, Melbourne, Australia).

2.3.1 *bla*KPC genomic context

To confirm plasmid content and genomic context of the *bla*KPC gene in target ST258 (n = 16) isolates, we performed pulse field gel electrophoresis (PFGE) on S1 nuclease (Promega, Madison, WI, USA) digested DNA, as before, and Southern hybridization with *bla*KPC and *rep* IncFIIK DIG-labeled probes prepared using published primers and the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) following manufacturer’s instructions. Images were obtained on a ChemiDoc MP System (Bio-Rad Laboratories, Richmond, CA, USA).

2.4 De novo isolation of CP-*K. pneumoniae*-specific bacteriophages

Bacteriophages against target *K. pneumoniae* CG258 were isolated from sewage and wastewater samples collected in the Greater Sydney District (Sydney, NSW, Australia). Specimens were clarified by centrifugation and filtration through a 0.22 µm filter. Aliquots of environmental filtrates were then incubated overnight with a single target *K. pneumoniae* isolate (either JIE2709, JIE4282, JIE4455, or ATCC 13883). Bacteriophages were selected from single plaques in double-layer agar assays and purified through three rounds of plating. High-titer stocks were prepared by propagating bacteriophages over several double-layer plates washed in SM buffer (50 mM Tris-HCl, 8 mM MgSO₄, 100 mM NaCl, pH 7.4) and filtered through a 0.22 µm filter. The concentration as plaque forming units per mL (PFU/mL) was determined by spotting 10 µL of 10-fold serial dilutions onto a double-layer of the target bacteria. High-titer (>10⁹ PFU/mL) bacteriophage stocks were stored at 4°C.

2.5 Bacteriophage host range

The identified CG258 *K. pneumoniae* strains were tested against bacteriophages (n = 65) selected from our extensive library or isolated de novo against one of the target isolates. Bacteriophage lytic activity was determined by measuring the efficiency of plating (EOP) for each phage-bacteria combination using the spotting technique as above, and by co-incubation (18 hours) of bacteriophage and target bacteria (multiplicity of infection (MOI) = 100) in liquid microcosmos.
(Mueller Hinton broth) in 96-well plates, with spectrophotometric automated OD<sub>600</sub> measurement (SpectraMax iD5, Molecular Devices, San Jose, CA, USA) to assess bacterial growth at 10 minutes intervals.<sup>37</sup> Klebsiella oxytoca, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus, and Staphylococcus pseudintermedius were used as cross-species controls. In order to further test bacteriophage specificity, we also blind tested the host ranges of three bacteriophages with unique specificities (AmPh_EK52, AmPh_EK38, and JIPh_Kp122) on a set of CP-K pneumoniae isolates from Europe (n = 48) to determine their predictive diagnostic value linked to K pneumoniae sequence or capsule type. Bacteriophage activity against each strain was scored as: 1. “full activity” for presence of clear plaques at highest dilution; 2. “poor activity” for presence of turbid plaques, or isolated bacterial colonies within clearings, or EOP three or more log<sub>10</sub> lower than that of the original host; 3. “partial activity” for evidence of clearing in bacterial lawn, but absence of distinct plaques; 4. “negative” for very faint, difficult to observe, clearing or absence of any visible plaques or clearing zones in the bacterial lawn.

2.6 | Bacteriophage characterization

2.6.1 | Genome sequencing

Bacteriophages (n = 5) that specifically lysed K pneumoniae ST258 clade 1 with different host range profiles were selected for further characterization as potential therapeutic candidates. Bacteriophage DNA was extracted using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA) and used for WGS (Nextera XT Library Preparation kit and Nextera XT v2 Indexes; paired end 150 bp chemistry on the Illumina NextSeq 500 NCS v2.0; Illumina, San Diego, CA, USA). Error rates were calculated using PhiX Sequencing Control v3 for each run. De-multiplexing and FastQC generation was performed with default settings using BaseSpace (Illumina). Bacteriophage genomes were assembled using our in-house genomic pipeline and annotated using RAST-tk.<sup>38</sup> The absence of lysogeny modules, virulence, and resistance determinants was determined using our WGS analysis workflow (as for bacterial genomes) and PHASTER.<sup>31</sup> Genome comparisons with best database (GenBank, NCBI) matches were obtained using Easyfig.<sup>32</sup> PFGE of intact viral particles was performed to confirm relative size (Chef Mapper System, Bio-Rad Laboratories, Hercules, CA, USA).

2.6.2 | Imaging of bacteriophages

Bacteriophage preparations were dialyzed against 0.1 M of ammonium acetate in dialysis cassettes with a 10,000 membrane molecular weight cutoff (Pierce Biotechnology, Rockford, IL, USA), negatively stained with 2% of uranyl acetate and visualized using transmission electron microscopy (TEM).<sup>37</sup> TEM was conducted at the Westmead Electron Microscopy Facility (Westmead, Australia) on a Philips CM120 BioTWIN transmission electron microscope at 100 kV. Images were recorded with a SIS Morada digital camera using iTEM software (Olympus Soft Imaging Solutions GmbH, Munster, Germany). Bacteriophage morphology and related taxonomic assignment were confirmed following the guidelines set by the International Committee on Taxonomy of Viruses (http://www.ictvonline.org/).<sup>39</sup>

2.6.3 | Phage stability

Phage stability in SM buffer was determined by measuring the EOP after incubation at different temperatures (21°C for 24 hours, 21°C for 7 days, 37°C for 24 hours, 4°C for >1 month, and 4°C for >1 month with chloroform) and at different pH levels (pH 3, 6, 7, and 8; 4 hours at room temperature). Rate of adsorption and one-step growth curves (latent period and burst size) were calculated according to established protocols.<sup>37</sup>

2.7 | Data availability and accession numbers

The Illumina sequencing data sets of all K pneumoniae isolates obtained in this work were deposited in SRA (NCBI) database under Bioproject PRJNA529495. The cps locus variants were deposited in the GenBank database (NCBI) under accessions: MN443946 for KL106-D2 (JIE2783), MN443947 for KL106-D3 (JIE4005), and MN443948 for KL106-D4 (JIE4282). The complete annotated genomes of K pneumoniae bacteriophages AmPh_EK29, AmPh_EK52, AmPh_EK80, JIPh_Kp122, and JIPh_Kp127 were also deposited in the GenBank database (NCBI) under accession numbers MN434092, MN434093, MN434094, MN434095, and MN434096, respectively.

3 | RESULTS

3.1 | CP-K pneumoniae population

Of a total 21 clinical ESBL K pneumoniae isolates that carried a carbapenemase gene (by PCR), upon sequencing 18 were found to belong to CG258 (Figure 1; Table S1). This set contained two ST11 representatives, one ST512 (single locus variant of ST258 clade 2), one ST1199 (single SNP variant of ST258 clade 1), and 14 ST258 strains, segregated further into
two distinct lineages, known as clade 1 (n = 13) and clade 2 (n = 2) and distinguished by approximately 40 nonrecombinant core SNPs (Figure 1). Strains belonging to clade 1 dominated this population (86%), reflecting the epidemiology of a recent Australian outbreak. Inspection of the most common \textit{K pneumoniae} virulence-associated mobile genetic element, ICE\textsubscript{Kp}, revealed that all strains belonging to ST11 carried an ICE\textsubscript{Kp3} associated with a yersiniabactin operon \textit{ybt}, while all ST258 and the ST1199 strain carried an ICE\textsubscript{Kp2} associated with a yersiniabactin operon \textit{ybt}, a combination usually only found in clade 1 isolates (clade 2 isolates most commonly being associated with ICE\textsubscript{Kp10} with \textit{ybt}) (Figure 1).

### 3.2 | CP-\textit{K pneumoniae} CG258 bacteriophage susceptibility

Screening for bacteriophage susceptibility identified at least one bacteriophage with strong activity against each strain of CP-\textit{K pneumoniae} CG258, with potential therapeutic value (Figure 2C). A correlation pattern between specific regions of differences (RODs) and bacteriophage susceptibility was prominent in our data set (Figure 2D). Phage host range profiles largely grouped according to capsular types, KL15-1 (ST11), KL106-D1 (ST258 clade 1), and KL107 (ST258 clade 2 and ST512) each presenting unique patterns of phage susceptibility with few examples of cross-reactivity. Bacteriophage AmPh\textsubscript{EK29} was able to lyse both a subset of clade 1 isolates (KL106-D1 and D2) and JIE2793 (ST512; KL107), while bacteriophages JIPh\textsubscript{Kp122} and JIPh\textsubscript{Kp127} had preferential activity against few specific clade 1 isolates (Figure 2C).

Within the clade 1 set, the capsule-specificity of the bacteriophages tested was further confirmed by the resistance to lysis of the capsular variant isolates JIE2783 and JIE4282 (see below) which were not lysed by the majority of the bacteriophages active against other clade 1 strains. \textit{K pneumoniae} bacteriophages AmPh\textsubscript{EK52} and JIPh\textsubscript{130} were effective in clearing most CP-\textit{K pneumoniae} ST258 clade 1 specifically, except for the ones carrying capsular variants. None of these clade-specific bacteriophages were cross-reactive against other \textit{K pneumoniae} sequence types (eg, ST14, ST25, ST208, ST278, ST938, ST1978) and any of the other control species tested.

### 3.3 | ESBL \textit{K pneumoniae} CG258 variable accessory genome

A comprehensive analysis of the pangenome using Roary showed that the CG258 accessory genome was composed of 1682 accessory genes, which could be further classified into discrete RODs based on their function and contiguity as follows: 33 capsule-associated genes, 334 phage-related genes, 622 plasmid-related genes, 328 RODs-associated genes, and 365 other genes that could not be assigned unambiguously to the aforementioned categories (RODs were numbered and are summarized in Table S1). Though the presence-absence profile of these RODs mainly reflected the ST of these isolates, a degree of intra-clade variation was also observed among the ST258 clade 1 strains in prophage and plasmid-related regions (Figure 2A,B,D; Table S1).
3.3.1 | Polysaccharide capsule synthesis locus

Bioinformatic analysis of the genomic region between the galF and ugd genes (cps locus) revealed distinct capsular types within the same ST, with KL106 and KL103 found in ST11 strains (JIE2713/JIE2487 and HS11286 reference strain, respectively), and KL106 and KL107 found in ST258 clade 1 and 2, respectively. This locus is a well-known recombination hot-spot in K pneumoniae and further inspection of our clade 1 isolates revealed the presence of three variants of the previously described KL106-D1 arrangement41,42 (Figures 2B, 3A). Variants were due to insertion of ISKpn26 (IS5 family) in two different locations: (i) within the glycosyltransferase-encoding gene wcaJ in two separate positions (KL106-D2 in JIE2783; KL106-D4 in JIE4282); and (ii) within an acyltransferase-encoding gene (KL106-D3 in JIE4005, JIE4019, and JIE4020) in the variable-content segment of the cps locus42 (Figure 3A). All the ISKpn26 insertions interrupt the genes coding sequence, producing characteristic 4 bp direct repeats (Figure 3A). Representative sequences for the three new cps locus variants were deposited in GenBank. Capsule production was significantly different among the CP-K pneumoniae strains (ANOVA, \( P < .001 \)) (Figure 3B). Low capsule content was observed in capsular variants JIE2783 and JIE4282 (ISKpn26-ΔwcaJ variants KL106-D2 and KL106-D4, respectively). In JIE3095, capsule production was significantly higher than in other clade 1 strains (except JIE2740, 4019, 4020, and 4203; Fisher's protected LSD, \( P < .05 \)), although no sequence variation in the JIE3095 cps locus was identified.

In our set of strains, a clear association was observed between bacteriophage susceptibility profiles and cps variants (Figure 2D) with very few of the tested bacteriophages (4/65; ~6%) showing cross-clade specificity. This correlation between capsule type and phage susceptibility held when a clade 1-specific bacteriophage (KL106) (AmPh_EK52), a clade 2-specific bacteriophage (KL107) (AmPh_EK38), and a bacteriophage with inter-clade range (JIPh_Kp122) were blind-tested on a larger panel (\( n = 48 \)) of CP-K pneumoniae isolates from Europe (Table S2).

3.3.2 | Other cell surface structures

In contrast to the variable cps locus structure, gene content and arrangement in the LPS encoding loci of the ST258 clade 1 isolates was conserved. Accordingly, LPS profiles from silver staining showed no significant differences in the O antigens of the short, long, or intermediate chains (Figure S1A).
The lipid A component of JIE4282 differed in size from all other K pneumoniae CG258 (Figure S1A). According to Kleborate results, JIE4282 is missing the \textit{wbbM} gene encoding a glycosyltransferase required for d-galactan I biosynthesis. Of note, JIE2793 (ST512) is also missing one of the hypothetical proteins (\textit{glmA}) in the O antigen operon (O2v2 type). Biofilm production levels also differed (unbalanced ANOVA, \( P < 0.001\)) and were found to be significantly higher in JIE2793, JIE3095, and JIE4282 compared to all others (Fisher's protected LSD, \( P < 0.05\)) (Figure S1B). Variable levels could be attributable to a number of factors, including variations associated with fimbrial genes.

As expected, conserved regions encoding for Type 1 (\textit{fim} operon) and Type 3 (\textit{mrk} operon) fimbriae as well as an identical \textit{ecp} operon were present in all clade 1 strains. Six additional putative chaperon/usher loci associated with fimbrial production (\textit{kpa}, \textit{kpb}, \textit{kpd}, \textit{kpe}, \textit{kpg}) and an additional \textit{ecp}-like region were also identified in all genomes, with the exception of JIE4046 and the KL106-D3 isolates (JIE4005, 4019, and 4020), where the additional \textit{ecp}-like operon is missing (ROD-6, Table S1). The Type 1 fimbrial locus \textit{kpg} (ROD-1, Table S1) linked to biofilm formation was also missing in JIE4046. Fimbriae are critical filamentous cell surface structures that allow the bacteria to adhere to host surfaces and form biofilms, mediating their virulence. The chaperone/usher-dependent pathway is the most abundant secretory pathway in Gram-negative \(\gamma\)-proteobacteria and up to eight usher-type loci have been identified in \textit{K pneumoniae}, most considered part of the accessory genome and likely able to modulate adherence properties. Limited capsule
production (eg, JIE4282) and expression of O-antigen variants (eg, JIE2793) could also affect adhesion necessary for biofilm establishment. In our study, none of these variations in cell surface structures could be directly correlated with bacteriophage susceptibility or host range.

3.3.3 Prophages

The prophage content in the clade 1 isolates also varied, with the subset roughly divided into three subgroups based on prophage profile (Figure 2D; Table S1), and this could impact bacteriophage susceptibility due to variation in the host of superinfection immunity or exclusion properties. For example, the lytic activity of bacteriophage AmPh_EK29 toward ST258 clade 1 isolates is specific to a subset of strains that differ from the rest specifically in their prophage content (ie, JIE3095, and JIE4005, 4019, 4020, and 4203) (Figure 2). In JIE3095, the prophage designated “phage_1” is missing head morphogenesis and tail assembly components. In all other clade 1 representatives in our set this is an intact (presumably inducible) temperate bacteriophage (~36 kb), closely related to bacteriophages P2 or 186 (Myoviridae; ~33.6 kb). The sequence modifications seen in JIE3095 likely inactivate this prophage with possible consequent modification of the phage susceptibility of the host. Another prophage found in clade 1 isolates, phage_4 (similar to members of the Siphoviridae; ~18 kb), is completely absent in JIE2783 and modified in JIE4005, 4019, 4020, and 4203. In the latter set of isolates, the prophage is missing several hypothetical proteins and the tail tape measure protein-encoding gene, which for some bacteriophages has been shown to play a role in superinfection exclusion mechanisms45 (Table S1). An additional intact prophage, phage_5 (~45 kb), was identified in the genome of JIE4005, 4019, and 4020 (Table S1). This prophage is syntenic with lambdoid Siphoviridae bacteriophages and is shared in its full-length only with one other genome in the GenBank database from an Australian K. pneumoniae ST258 [CP0250059].

3.3.4 Plasmids

All K. pneumoniae were MDR, and all ST258 isolates with the exception of JIE2740 carried the blaKPC gene (Table 2; Table S3). The overall plasmid signature for each ST258 clade was unique and remarkably uniform (Table 2; Figure S2). As expected,8,16 the blaKPC allele 2 (blaKPC-2) was exclusively associated with ST258 clade 1, while blaKPC-3 was associated with clade 2 isolates and the closely related ST512 strain (Table 2). These genes were found exclusively on large (>20 kb) plasmids and co-localized by Southern hybridization with the rep gene from IncFIK-type plasmids35 (Table 2; Figure S2). All isolates carried multiple genes conferring extended-spectrum β-lactam resistance other than blaKPC (Table S3). Antibiotic resistance genotypes determined by WGS analysis accounted for all resistance phenotypes determined by standard clinical screening (Table S3).

3.4 Bacteriophage characterization

Among those tested, we identified five unique ds DNA bacteriophages AmPh_EK29, AmPh_EK52, AmPh_EK80, JIPh_Kp122, and JIPh_Kp127 that selectively target CP-K pneumoniae ST258 clade 1 isolates, some of which may have therapeutic potential (Table 3 and 4). WGS of purified viral DNA produced 11,340 to 5,049,732 reads that de novo assembled into one contig in all instances (Table S4). Bacteriophage genomes size varied between 40.7 and 169.3 kb and GC content was lower than 50% (host genome) in all except for AmPh_EK52 (GC% 52.9) (Table 4; Figure 4). No lysogeny or virulence associated genes were identified, indicating suitability for therapeutic use (Figure 4). The high degree of sequence similarity (>95%) to characterized K pneumoniae-specific phage in the NCBI database and TEM imaging showed that the selected bacteriophages belonged to the order Caudovirales (Table 4; Figure 4).

AmPh_EK29 and JIPh_Kp122 (Myoviridae-like), presented a prolate head (approx. 80 by 100 nm) and long tail (100 nm) (Table 4; Figure 4A,D). AmPh_EK52 resembled Podoviridae bacteriophages, having a small thick tail (approx. 20 nm long) and close homology to members of this family including genome size of about 40 kb and absence of tRNAs in its genome (Table 4; Figure 4B). JIPh_Kp127 and AmPh_EK80 were both T5-like Siphoviridae viruses with long thin tails (Table 4; Figure 4C,E). Screening of entries in the NCBI database by BLASTn identified close relatives of these bacteriophages but no identical sequences (Table 4), and in genome comparisons with best matching GenBank entries, the modular structure and order were preserved in all cases, with the main regions of difference found in tail or tail-associated open reading frames (Figure 4).

All five bacteriophages efficiently lysed target bacteria in vitro at high titer (between 107 and 109 PFU/mL) and in combination captured the entire ST258 clade 1 subset (Table 3; Table S5). However, host ranges were unique for each bacteriophage. AmPh_EK80, JIPh_Kp122, and JIPh_Kp127 showed narrower activity toward ST258 strains, with preference toward specific targets. These bacteriophages produced confluent lysis zones on most of the tested isolates when titrated on solid media, indicating the possibility of non-productive lysis, that is, no exponential bacteriophage amplification, “lysis-from-without” or “abortive lysis”.48 All bacteriophages were highly stable in SM buffer maintaining high titer at a range of temperatures (4°C for > 1 month; 21°C
for 1 week; 37°C for 24 hours) and pH levels (pH 3, 6, 7, and 8 for 4 hours). Exposure to chloroform at 4°C decreased the stability of AmPh_EK29 by 2-3 orders of magnitude, but had no effect on the stability of the remaining four bacteriophages. One-step growth curves revealed latent periods of 80-250 minutes and burst sizes between 12 and 500 PFU/cell (Table 3; Figure S3). Growth curves for AmPh_EK80 and JIPh_Kp127 were comparable. Bacteriophage JIPh_Kp122 had the shortest latent time (80 minutes), while AmPh_EK52 had the shortest burst time (30 minutes).

| Isolate^ | ST258 clade | blaKPC allele | Plasmid replicons† | Plasmid sizes (kb)** |
|----------|-------------|---------------|--------------------|----------------------|
| 2487     | na (ST11)   | None          | IncFIK IncF-like, ColE-like | nd, nd              |
| 2713     | na (ST11)   | None          | IncFIK ColE-like    | nd, nd              |
| 2709     | 1           | 2             | IncFIK IncFIB-pKpQil-like, IncX3, ColE-like | none, 242.5; 104.5 |
| 2733     | 1           | 2             | IncFIK IncX3, ColE-like | 43, 194             |
| 2740     | 1           | None          | IncFIK IncX3, ColE-like | 41, 194             |
| 2771     | 1           | 2             | IncFIK IncFIB-pKpQil-like, IncX3, ColE-like | 41, 104.5           |
| 2783     | 1           | 2             | IncFIK IncFIB-pKpQil-like, IncX3, ColE-like | 43, 194; 104.5      |
| 3095     | 1           | 2             | IncFIK IncX3, ColE-like | 41, 165             |
| 4005     | 1           | 2             | IncFIK IncX3, ColE-like | 43, 160.5           |
| 4019     | 1           | 2             | IncFIK IncF IncX3, ColE-like | 43, 160.5, 150     |
| 4020     | 1           | 2             | IncFIK IncX3, ColE-like | 43, 160.5           |
| 4046     | 1           | 2             | IncFIK IncX3, ColE-like | 41, 160.5           |
| 4203     | 1           | 2             | IncFIK IncX3, ColE-like | 41, 194; 104.5      |
| 4282     | 1           | 2             | IncFIK IncFIB-pKpQil-like, IncX3, ColE-like | 41, 200; 110  |
| 4455     | 1           | 2             | IncFIK IncFIB-pKpQil-like, IncX3, ColE-like | 43, 160.5           |
| 2793     | na (ST512)  | 3             | IncFIK IncN, ColE-like | 58, 208             |
| 4626     | 2           | 3             | IncFIK IncR IncX3, ColE-like | 43, 208; 121     |
| 4660     | 2           | 3             | IncFIK IncR IncX3, ColE-like | 43, 208; 121     |

Note: *Data obtained from WGS analysis except where otherwise specified. ^, in bold isolates from same patient; †, based on PlasmidFinder scheme implemented in BAP10; **, approximate sizes determined by S1-PFGE; nd, not determined. In bold, plasmids co-localizing with the blaKPC gene by Southern blot hybridization. Underline, plasmids co-localizing with the IncFIK replicon by Southern blot hybridization.

**Table 2** Plasmid content in sequenced K pneumoniae CG258 isolates*
DISCUSSION

The increasing challenges posed by the rise of antibiotic resistance in human pathogens have revitalized interest in the use of bacteriophage for the treatment of bacterial infections. Among MDR pathogens, CP-K pneumoniae is a serious clinical concern, as both gut colonizer and agent of severe sepsis when invading sterile body sites. In this study, ST258 isolates were predominant in the local clinical CP-K pneumoniae population, with overrepresentation of ST258 clade 1 (KL106-D1), reflecting the epidemiology of a recent local outbreak. The incidence of blaKPC in Australia has been rather limited when compared to its dissemination in other countries, but tracking of these pathogens remains paramount due to the consistent association of multidrug resistance with mobilizable genetic elements facilitating its persistence and dissemination.

In the K pneumoniae genome, the cps locus, encoding the capsular polysaccharide outer layer, is a recognized recombination hotspot, responsible for the diversification of clonal lineages, particularly within the CG258 group, and over 100 capsular types have been identified in this species. The capsule is a complex structure of repeating sugar subunits that protects the cell from external threats (including phage attack) and enhances K pneumoniae virulence, being implicated in resistance to host defense mechanisms, immune evasion, adherence, and biofilm formation. In our study, we identified three novel

TABLE 3 Efficiency of plating of selected CP-Klebsiella pneumoniae ST258 clade 1 bacteriophages*

| Isolate (JIE) | ST  | Clade | AmPh_EK29 | AmPh_EK52 | AmPh_EK80 | JIPh_Kp122 | JIPh_Kp127 |
|--------------|-----|-------|-----------|-----------|-----------|------------|------------|
| 2713         | ST11| NA    | None      | None      | None      | 4          | **3**      | **3**      |
| 2487         | ST11| 1     | None      | None      | None      | **3**      | **3**      | **3**      |
| 2709         | ST258| 1    | **2.0 × 10^9**<br>(100) | **8.0 × 10^8**<br>(100) | **4.0 × 10^9**<br>(15) | **3**      | **3**      | **3**      |
| 2733         | ST258| 1    | **8.0 × 10^9**<br>(40) | **6.0 × 10^8**<br>(75) | **4.0 × 10^9**<br>(15) | **3**      | **3**      | **3**      |
| 2740         | ST258| 1    | **4.0 × 10^9**<br>(20) | **2.6 × 10^9**<br>(325) | None      | **3**      | **3**      | **3**      |
| 2771         | ST258| 1    | **1.8 × 10^9**<br>(90) | **4.0 × 10^8**<br>(50) | **3**      | **3**      | **3**      | **3**      |
| 2783         | ST258| 1    | **2.4 × 10^9**<br>(120) | None      | **6.0 × 10^9**<br>(100) | **3**      | **3**      | **3**      |
| 3095         | ST258| 1    | **4.6 × 10^9**<br>(23) | **8.0 × 10^8**<br>(100) | **3**      | **3**      | **3**      | **3**      |
| 4005         | ST258| 1    | None      | **1.6 × 10^9**<br>(200) | **3**      | **3**      | **3**      | **3**      |
| 4019         | ST258| 1    | None      | **2.6 × 10^9**<br>(325) | **3**      | **3**      | **3**      | **3**      |
| 4020         | ST258| 1    | None      | **4.0 × 10^9**<br>(500) | **3**      | **3**      | **3**      | **3**      |
| 4046         | ST258| 1    | **1.1 × 10^9**<br>(55) | **8.0 × 10^8**<br>(100) | **3**      | **3**      | **3**      | **3**      |
| 4203         | ST258| 1    | None      | **1.0 × 10^9**<br>(125) | None      | **3**      | **3**      | **3**      |
| 4282         | ST258| 1    | None      | **6.0 × 10^9**<br>(100) | **3**      | **3**      | **3**      | **3**      |
| 4455         | ST258| 1    | **2.0 × 10^9**<br>(100) | **2.5 × 10^8**<br>(31) | **3**      | **3**      | **3**      | **3**      |
| 2793         | ST512| NA   | **6.0 × 10^9**<br>(30) | None      | **4**      | **3**      | **3**      | **3**      |
| 4660         | ST258| 2    | None      | None      | **4**      | **3**      | **3**      | **3**      |
| 4626         | ST258| 2    | None      | None      | **4**      | **3**      | **3**      | **3**      |

Note: *in brackets percentage EOP when compared to amplification strain. ***, indicates clearing in bacterial lawn without single plaque formation (confluent lysis or abortive lysis). In bold, titer on original amplification host. Scores indicate: 1, high titer lysis; 3 and 4, poor or no lysis.
capsular variants in ST258 clade 1 due to ISKpn26 insertion in the cps locus, responsible for the intra-clade variation in our population which correlated remarkably well with reduced host range for many of the clade 1 infecting bacteriophages.

In two of our variants (KL106-D2, JIE2783; KL106-D4, JIE4282) with significantly reduced capsule production, the IS interrupts the wcaJ gene, encoding an enzyme that is involved in initiating the synthesis of capsular repeat units by catalyzing the transfer of glucose-1-phosphate and galactose-1-phosphate moieties onto undecaprenyl phosphate (an essential lipid for capsule biogenesis). WcaJ seems to be fairly conserved among K. pneumoniae of different capsular types. Variations in the wcaJ coding sequence have been linked to decreased capsule content and mucoidy in MDR K. pneumoniae, leading to altered response to antibiotics and to attack by the host immune system. In this study, we did not observe a significant reduction in capsule production in any of the three strains (JIE4005, 4019 and 4020) with ISKpn26 insertion into the acyltransferase-encoding gene (KL106-D3), though capsule content was slightly lower than in the isolates with unmodified cps locus. This gene is located in a segment of the ST258 clade 1 cps locus with variable gene content, between two essential genes, gnd and ugd, and is not present in all K. pneumoniae. Its product is responsible for acetylation of capsule components, and it was shown that mutations in its coding sequence are linked to both low capsule content and altered antigenicity, warranting further investigation of mutant phenotypes.

Matching of bacteriophage host range with bacterial genomic data allowed for the selection of a number of viruses with unique characteristics that could be further examined in combination for therapeutic applications (high lytic activity, poor resistance development) against K. pneumoniae ST258 clade 1. All the bacteriophages sequenced were highly homologous to previously characterized lytic viruses shown to be effective against specific K. pneumoniae types. However, none of these were reported to target ST258 as our bacteriophages do. Comparative analysis of bacteriophage genomes identified preferential loci of variability, mostly related with tail fibers or tail-associated genes, presumably responsible for phage target specificity. We also showed a clear association between the host range of most of the tested viruses with capsular types and subtypes (ISKpn26 variants).

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exception, lysed a subset of ST258 clade 1 strains (capsular type KL106 but different prophage profile) and the ST512 representative isolate (capsular type KL107), indicative of distinct receptor specificity for this virus. JIPh_Kp122 and
JIPh_Kp127 also showed spectra beyond capsular type, and overall lysed ST258 isolates less efficiently in solid media with plaque phenotypes suggestive of abortive or passive lysis, 48 though lysis kinetics improved in liquid assays. In this regard, further analysis is required to establish the true value of any of these bacteriophages for therapy either as single preparations (targeted therapy) or as mixes (cocktails; preferred for clinical applications). 59 Synergy between phage activity and antibiotics should be determined in all instances, and for cocktail preparation, in particular, synergy among bacteriophage candidates must also be defined as differences in lytic activity (infection kinetics) may affect overall efficacy of phages when in combination. 56

Capsule-targeting bacteriophages often act by production of depolymerases, enzymes that cleave glycosidic bonds disrupting capsule integrity 57, 58 with demonstrated specificity toward certain capsular types, 59, 60 indicating that differential enzymatic degradation may be responsible for the patterns of lytic activity observed in our study. Development of acapsular mutants with reduced virulence in response to depolymerase activity has been reported as a product of the adaptive interplay between Klebsiella and its bacteriophages, 50, 61 and may be an expected trade-off in their evolutionary arms race. The complex dynamic interactions of bacteriophages and their hosts and the coevolution mechanisms at play have complicated direct prediction of phage susceptibility from host genomics, even when dealing with clonal populations. Fine genomic diversity in our ST258 clade 1 strains was not only associated with the capsule, but also with variations in other elements such as prophages and plasmids. Prophage content is likely implicated in resistance to some of the tested bacteriophages (ie, AmPh_EK29) and should also be considered when selecting optimal therapeutic mixes. Other elements (lack of fimbrial locus in JIE4046, different plasmid content (JIE4005, 4019, and 4020), porin variants etc) seemed not to directly impact the susceptibility patterns to most of the tested bacteriophages, but have the potential to affect viral host range and synergy. 61 The tight relationship between K pneumoniae capsule and its bacteriophages, highlighted in this study, must therefore, be carefully considered for future progress in therapeutic applications specifically targeting ST258 isolates. Better bioinformatic tools and larger well characterized microbial collections may allow for the definition of predictive algorithms for a priori selection of optimal therapeutic candidates targeting these pathogens.

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CONFLICT OF INTEREST
None to declare.

AUTHOR CONTRIBUTIONS
C. Venturini planned project, performed experiments and data analyses, wrote the manuscript; N.L. Ben Zakour performed genomic analysis of viral and bacterial sequences, wrote the manuscript; B. Bowring performed bacteriophage isolation, microbiology experiments, and participated in writing the manuscript; S. Branston and Z. Kovach contributed to project planning and data analysis, planned initial phage screening, and provided bacteriophages (AmPh); R. Cole performed PFGE analysis of AmPh bacteriophages and purified their DNA for sequencing; S. Branston and Z. Kovach performed initial phage susceptibility testing; E. Kettle performed and supervised preparation of samples for TEM; N. Thomson participated in project planning and genomic analyses of bacterial isolates; J.R. Iredell planned project and wrote manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.