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Bacteriophage-associated endoglycosidases selectively depolymerize capsular polysaccharides of *Klebsiella pneumoniae* belonging to the clonal groups 258 (clade II) and 307

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ABSTRACT

Klebsiella pneumoniae is a major opportunistic pathogen responsible for severe hospital-acquired infections, frequently sustained by multidrug-resistant high-risk clones. The capsular polysaccharide (CPS) represents a key virulence factor and a primary target of bacteriophage-associated polysaccharide depolymerases. In this study, we investigated the activity and substrate specificity of two phage-associated endoglycosidases from the lytic bacteriophages ΦBO1E and vB_KpS_GP-1 (ΦGP1), infecting *K. pneumoniae* strains belonging to clonal group 258 clade II and clonal group 307, respectively.

Purified CPS from strains KPB-1 and KP-395 were subjected to enzymatic depolymerization using intact phage particles. Enzymatic activity was qualitatively monitored by reducing sugar assays, while degradation products were isolated by size exclusion chromatography and characterized by electrospray ionization mass spectrometry and one- and two-dimensional NMR spectroscopy.

The ΦBO1E-associated endoglycosidase selectively cleaves the α-L-Rha*p*-(1→ 3)-β-D-Gal*p* linkage within the KPB-1 CPS, generating oligosaccharides corresponding to one and two repeating units. Similarly, the ΦGP1-associated enzyme depolymerizes the KP-395 CPS by specifically hydrolyzing the β-D-Gal*p*-(1 → 4)-α-D-Gal*p* linkage, yielding oligosaccharides corresponding to one and two repeating units. In both cases, the enzymes display strict specificity toward a single glycosidic bond within the CPS repeating unit.

These findings provide detailed structural insights into the mode of action of phage-associated endoglycosidases targeting clinically relevant *K. pneumoniae* CPS types. The high substrate specificity and efficient CPS depolymerization support the potential of these enzymes as enzybiotics or anti-virulence agents for the development of alternative strategies to fight multidrug-resistant *K. pneumoniae* infections.

1. Introduction

Bacteriophages are viruses that infect and replicate in bacterial hosts. They consist of single or double-stranded DNA or RNA enclosed in a protein case named capsid, whose shape has been a component of the first proposed classification scheme [1]. These prokaryotic viruses can undergo either a lytic cycle, leading to rapid bacterial cells lysis and release of newly formed phage particles in the surrounding environment, or a lysogenic cycle, in which the phage genome integrates into the host genome and is replicated along with it. In order to penetrate the bacterial cells, bacteriophages possess a set of enzymes specific for the different molecules which surround microbial cells. In Gram-negative species, bacteriophages are often equipped with polysaccharide depolymerases (PSDs) able to degrade specific components of the bacterial envelope, *i.e.* capsular polysaccharides (CPS), exopolysaccharides (EPS), and lipopolysaccharides (LPSs). These viruses are then able to recognize the receptors located on the cell-envelope surfaces, and, with the assistance of virion-associated peptidoglycan hydrolases, they penetrate

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the cell wall and start their lytic or lysogenic cycle [2]. In the case of bacteriophages specific for *Klebsiella pneumoniae*, PSDs very often target the extremely heterogeneous CPS [3] resulting in an exceptionally high strain specificity. Such enzymes cleave only one glycosidic linkage within the polymer repeating unit, thus generating oligosaccharides corresponding to one or multiple repeating units. PSDs are often phage-associated proteins, located on the virion tail, fibers, baseplate, or neck. Upon phage-induced bacterial lysis, free PSDs not incorporated into the virion during phage assembly, can be released resulting in the production of both soluble and phage-associated enzymes [2]. When testing for the presence of lytic bacteriophages, the free PSD forms can diffuse freely in the agar, causing only polysaccharide degradation of live bacteria, visualized as phage plaque-surrounding halo zones.

Bacteriophages are characterized by their extremely high host specificity which often reaches the strain level. Their specificity and ability to kill bacteria, make phages valuable antimicrobial weapons, especially against multi-drug resistant (MDR) bacterial strains. Indeed, the appearance and fast dissemination of antibiotic resistant bacteria in clinical settings has reached alarming levels [4], causing high-excesses of morbidity and mortality rates of hospitalized patients, who often die following bacterial infections sustained by MDR strains that cannot be effectively targeted by any available antibiotic [5].

A possible alternative to tackle bacterial infections could be the use of bacteriophages, or selected components thereof. This option has been already explored by Twort and d'Hérelle soon after their discovery, but then quickly abandoned in Western countries mostly given the huge success of "conventional" antibiotics. Phages are indeed specific "killers" targeting a single bacterial species or even single clonal lineages, thus leaving the beneficial microbiota largely undisturbed. In addition, phages are believed to be the most abundant biological entities on our planet, and the isolation of new candidates is relatively easy, fast and cheap. Moreover, their ability to often target and degrade major bacterial virulence factors such as CPS and LPS makes phages very promising tools against MDR bacteria. A further advantage to consider

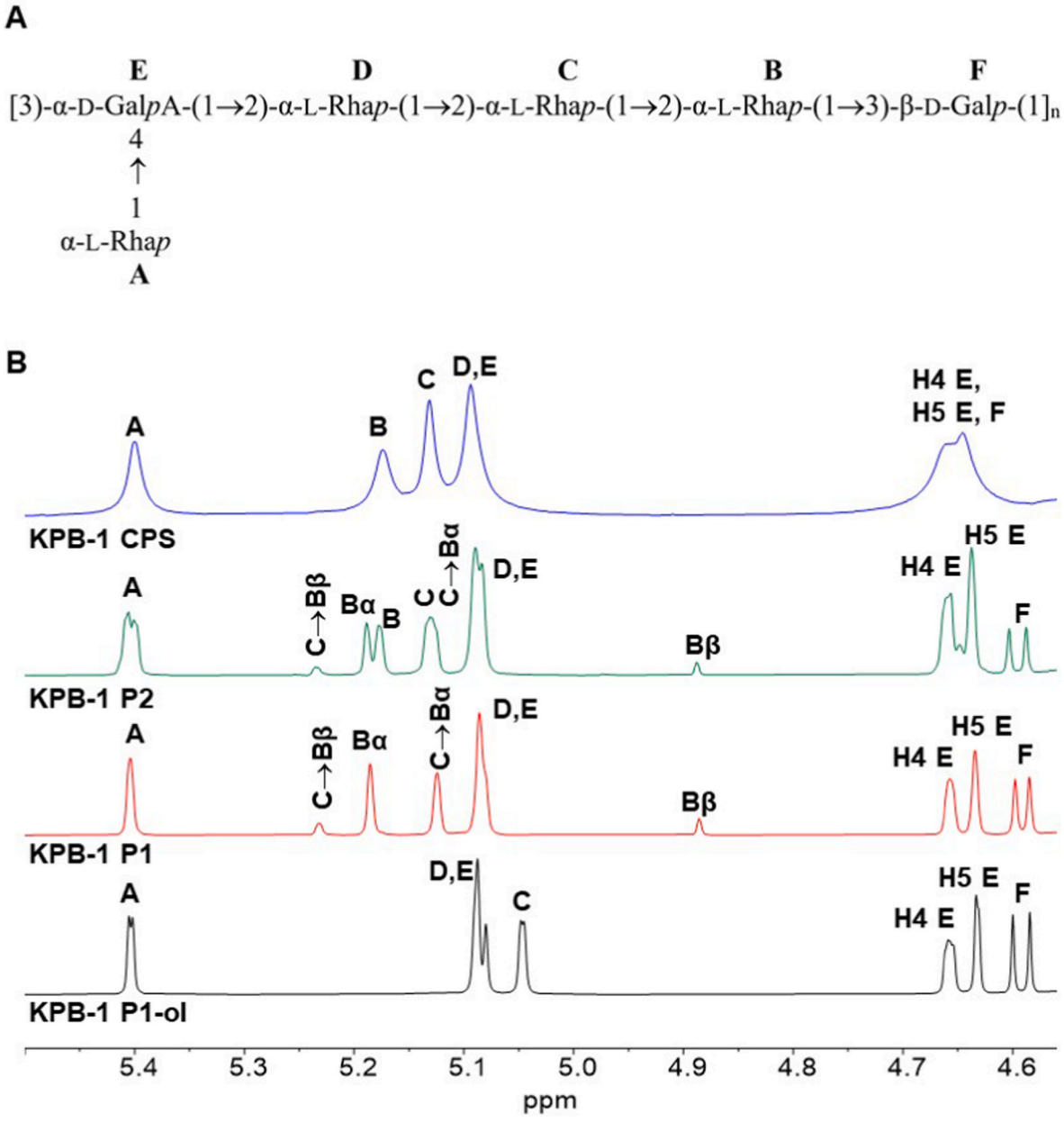

Fig. 1. Comparison of the ^{1}H NMR spectra anomeric regions of native and phage ΦBO1E degraded KPB-1 CPS. A) structure of KPB-1 CPS repeating unit. B) Anomeric regions of KPB-1 CPS, KPB-1 P2, KpB1 P1 and KPB-1 P1-ol recorded in D_2O at 50 °C. A more detailed assignment of KPB-1 P2 resonances is reported in Fig. S6.

phages or their components as promising anti-infective weapons, is that MDR infections are often sustained by a limited number of clones, named "high-risk clones". It is therefore envisaged that a restricted number of PSDs could be effective against a wide number of bacterial infections.

Here we describe the action of two PSDs associated with two different bacteriophages, ΦBO1E and vB_KpS_GP-1 (ΦGP1), specific for *K. pneumoniae* strains belonging to the Clonal Group (CG) 258 clade II and CG307, respectively, as potential enzybiotics candidates [2] to treat *K. pneumoniae* infections.

2. Results

2.1. Capsular polysaccharides

The capsular polysaccharides (CPS) produced by *K. pneumoniae* strains KPB-1 and KP-395, obtained from bacteria grown on solid medium, were subjected to ^{1}H NMR spectroscopy to check their structure and purity (Figs. 1 and 2, respectively). The CPS structure of *K. pneumoniae* KPB-1 has been already published and it is identical to that of two representatives of an outbreak clone of ST258 clade II KPC-Kp from USA [6] and of the epidemiologically unrelated carbapenem-resistant *K. pneumoniae* ST258 clade II strains KKBO-1, KKBO-4 and KMn7 isolated from inpatients in Italian hospitals [7,8] (Fig. 1).

Since *K. pneumoniae* KP-395 is a newly isolated strain, its CPS was subjected to an array of ^{1}H–^{1}H homonuclear experiments (COSY, TOCSY, NOESY, data not shown), and ^{1}H–^{13}C heteronuclear correlation experiment (HSQC, HMBC) (Fig. S1) in order to determine its structure. Complete assignment of the chemical shifts (Table S1) showed that KP-395 CPS is identical to K102 type CPS [9] (Fig. 2), consistently with the previously reported CPS genotyping result (KL102) [10].

2.2. Depolymerization of KPB-1 CPS with bacteriophages ΦBO1E and characterization of the products

ΦBO1E propagation yielded a titer of 10^{9} PFU/mL. Before proceeding with the KPB-1 CPS depolymerization, phages were spotted on the

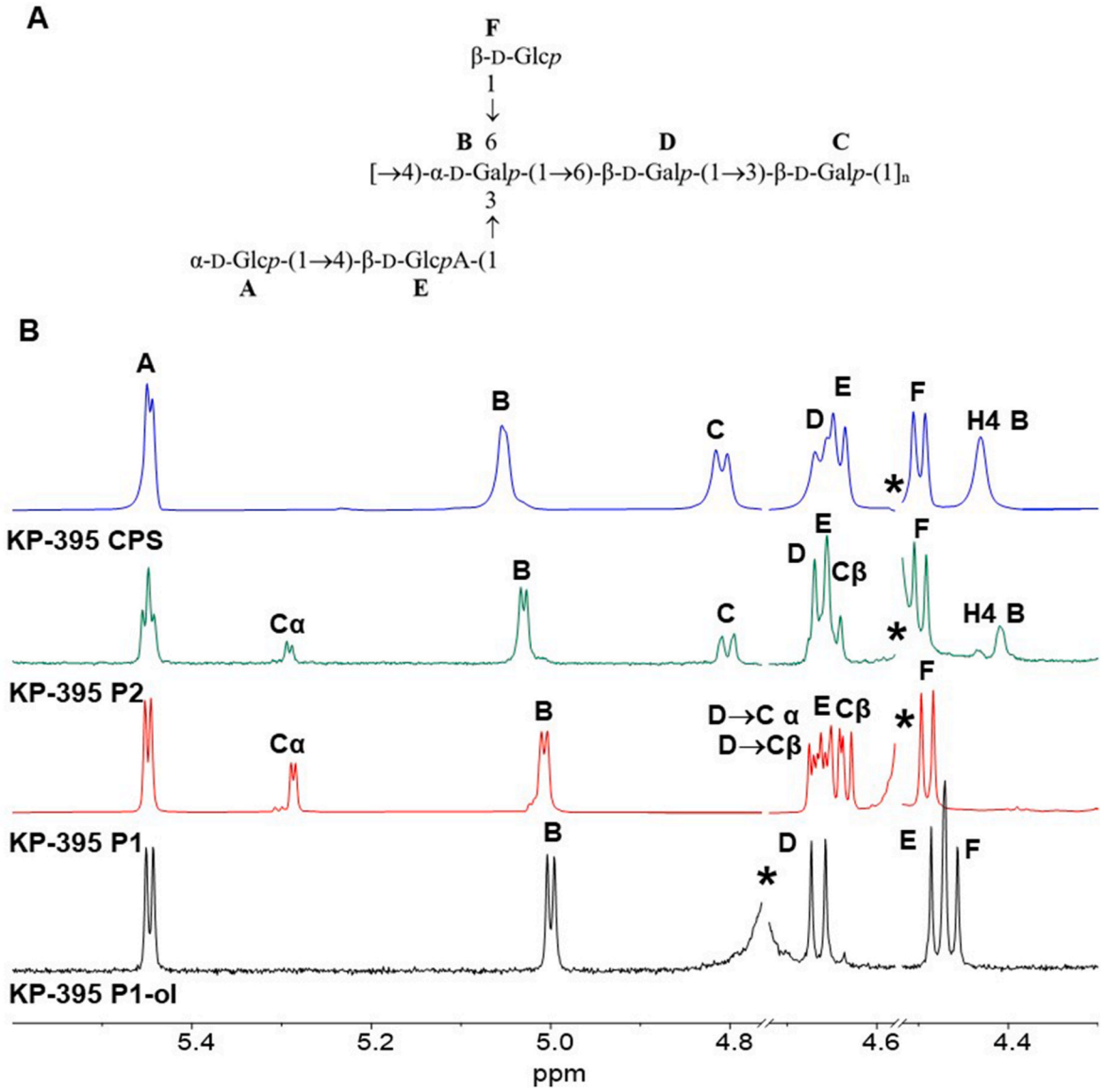

Fig. 2. Comparison of the ^{1}H NMR spectra anomeric regions of native and phage ΦGP1 degraded KP-395 CPS. A) structure of KP-395 CPS repeating unit. B) Anomeric regions of KP-395 CPS (DOSY), KP-395 P2, KP-395 P1, recorded at 45 °C, and KP-395 P1-ol recorded at 25 °C. The residual HOD signals (*) were cut from the spectra to simplify the Figure. A more detailed assignment of KP-395 P2 resonances is reported in Fig. S9.

host bacterial lawn to check the enzyme activity. As shown in Fig. S2, ΦBO1E produced lysis plaques surrounded by a halo; the latter is indicative of degradation of the capsule on the alive bacteria, caused by diffusion of unassembled phage components, including endoglycosidases, released by the lysed bacterial cells.

The phage-associated endoglycosidase activity was assessed by monitoring the formation of reducing sugars using the 3,5-dinitrosalicylic acid (DNS) colorimetric assay [11]. This method has the disadvantage that it may produce a "peeling" effect on the products [12]; furthermore, phage-associated endoglycosidase loses its activity with time and the generated fragments may act as inhibitors of the endoglycosidase, as previously reported [13]. Therefore, the DNS assay was used only qualitatively to obtain the time course of the phage-associated endoglycosidase CPS depolymerization. The graph showing the progression of the enzymatic reaction, as increasing concentration of the newly formed reducing ends as a function of time, is reported in Fig. 3A. Since the absorbance values depend on the oligosaccharide size (Fig. S3), maltohexaose was used to generate the calibration curve because the end degradation product of the endoglycosidase is the KPB-1 hexasaccharide repeating unit.

Concentrated ΦBO1E particles (~1 × 10^{11} plaque-forming units, PFU), were incubated with 30 mg of CPS and the products were separated by size exclusion chromatography on a Bio-Gel P-4 column giving three main peaks (Fig. S4): the first eluting one was shown by NMR to contain low amounts of non-CPS carbohydrates likely produced by the bacteria (Fig. S5), while the following two were named KPB-1 P2 and KPB-1 P1 and eluted in the separation range of the column, indicative of CPS extensive degradation. The oligosaccharide KPB-1 P1 was reduced with $NaBH_4$ and subjected to Electrospray Ionization Mass Spectrometry (ESI-MS) in the negative ion mode. In the mass spectrum obtained (Fig. 4A in Fig. S6 full spectrum) a major ion $[M - H]^-$ was detected at 941.6 *m/z*, corresponding to an oligosaccharide of MM 942 Da, in agreement with one repeating unit of the KPB-1 CPS which is composed of one GalA, one Gal and four Rha residues, with one of these sugars present as an alditol. The oligosaccharide KPB-1 P1 was then subjected to fragmentation in the positive ion mode after reduction with $NaBD_4$. The fragment ions obtained (Fig. S6), detected as Na^+ adduct and dehydrated Na^+ adducts, identified the following sequence of the residues in the oligosaccharide KPB-1 P1:

Gal—GalA—Rha—Rha—Rha-ol
|
Rha

The oligosaccharides KPB-1 P1 and the $NaBH_4$ reduced KPB-1 P1 (KPB-1 P1-ol) derived from ΦBO1E hydrolysis of KPB-1 CPS were subjected to 1D NMR spectroscopy. The anomeric regions of the ^1H NMR spectra obtained are shown in Fig. 1 in comparison with that of the native KPB-1 CPS. Peaks are labeled as in the repeating unit structure (Fig. 1A). The anomeric region of KPB-1 P1 (Fig. 1B) is similar to that of the native KPB-1 CPS except for two small peaks at 5.23 and 4.88 ppm. The latter was attributed to the β-anomer of the newly formed reducing end, and the former to the twinning effect on the sugar next to the reducing end. After reduction of the reducing end, the resonance at 5.20 ppm (H1 **B**) disappeared from the anomeric spectral region, as well as the two signals at 5.23 and 4.88 ppm, thus identifying residue **B** with the rhamnitol reducing end and, the linkage α-L-Rha*p*-(1 → 3)-β-D-Gal*p* with the site of cleavage of the endoglycosidase. At the same time, H1 of **C** moved from 5.13 ppm to 5.06 ppm indicating that it is the residue directly linked to the reducing end **B**, in agreement with the structure of the repeating unit. As expected, the H1 chemical shifts of the other residues, **A**, **D**, **E**, **F**, did not change much, except for the complete separation of the resonances H1 **F**, H4 **E**, and H5 **E**.

The data obtained indicates that KPB-1 P1 has the structure reported in Fig. 5.

The sample KPB-1 P2 was also subjected to 1D and 2D NMR spectroscopy. The ^1H NMR spectrum anomeric region was compared with that of KPB-1 P1 (Fig. 1, Fig. S7) for chemical shifts and peaks integration values. Moreover, the chemical shifts assignments thus obtained were confirmed by inspection of the HSQC plot anomeric region (Fig. S7) and established that the oligosaccharide KPB-1 P2 is composed of two repeating units with the structure reported in Fig. S7.

In conclusion, ESI-MS and NMR spectroscopy data showed that ΦBO1E associated endoglycosidase cleaves only one glycosidic linkage of the KPB-1 CPS repeating unit, specifically α-L-Rha*p*-(1→ 3)-β-D-Gal*p*, generating oligosaccharides corresponding to one (KPB-1 P1) and two (KPB-1 P2) repeating units.

2.3. *Depolymerization of KP-395 CPS with bacteriophages ΦGP1 and characterization of the products*

ΦGP1 propagation led to the obtainment of 10^9 PFU/mL. Before proceeding with the KP-395 CPS depolymerization, phages were spotted on the host bacterial lawn to check the enzyme activity. ΦGP1 produced lysis plaques surrounded by a halo, as previously observed for ΦBO1E (Fig. S2).

KP-395 CPS was degraded with ΦGP1 associated endoglycanase and the reaction was monitored with the DNS test, as previously described. Maltohexaose was used again as standard for the colorimetric test, since the KP-395 CPS repeating unit is also an hexasaccharide. The graph showing the progression of the enzymatic reaction, as increasing concentration of the newly formed reducing ends as a function of time, is reported in Fig. 3B. The fact that the red line in Fig. 3B is not close to zero value might be due to the different reducing power possessed by different sugars. Concentrated ΦGP1 particles (~1 × 10^{11} PFU), were incubated with 30 mg of KP-395 CPS and the products were separated by size exclusion chromatography on a Bio-Gel P-4 column giving three

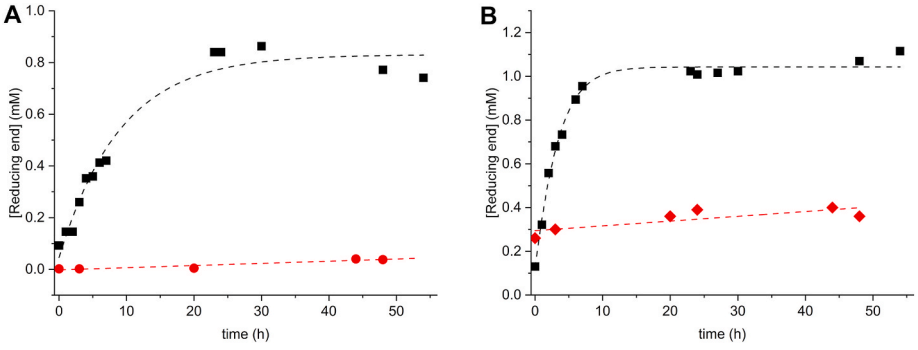

Fig. 3. Graphs showing the progression of the enzymatic reactions as increase in reducing ends concentration upon incubation of KPB-1 CPS with ΦBO1E (A) and KP-395 CPS with ΦGP1 particles (B). (A): ■ KPB-1 CPS + ΦBO1E; ● KPB-1 CPS alone. (B) ■ KP-395 CPS + ΦGP1 ◆ KP-395 CPS alone. Graphs were obtained with OriginPro2021 software.

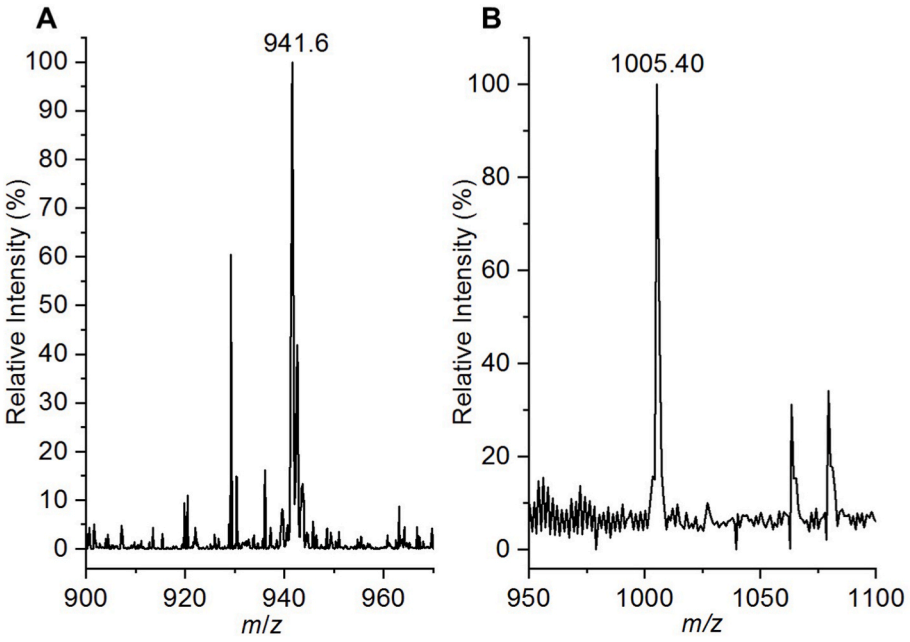

Fig. 4. ESI-MS mass spectra of KPB-1 P1-ol (A) and KP-395 P1-ol (B) recorded in the negative ion mode.

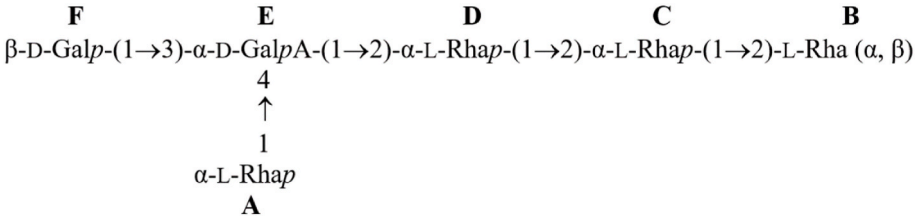

Fig. 5. Structure of the oligosaccharide KPB-1 P1, corresponding to one repeating unit, obtained after phage-mediated hydrolysis of KPB-1 CPS.

peaks (Fig. S8): the first eluting one was shown by ^{1}H NMR to contain low amounts of non-CPS carbohydrates likely produced by the bacteria (Fig. S9), while the following two were named KP-395 P2 and KP-395 P1 and eluted in the separation range of the column, indicative of CPS extensive degradation.

As reported in the previous paragraphs, the products were characterized by ESI-MS and NMR spectroscopy. The ESI-MS of $NaBH_4$ reduced KP-395 P1 (KP-395 P1-ol) was recorded in the negative ion mode (Fig. 4B, in Fig. S10 full spectrum) and showed a major ion $[M - H]^-$ at 1005.4 *m/z*, corresponding to an oligosaccharide of MM 1006 Da, composed of 1 HexA, 4 Hex, and 1 Hex-ol (alditol at the reducing end), in agreement with the composition of one CPS repeating unit. The oligosaccharide KP-395 P1-ol was then subjected to fragmentation in the positive ion mode (Fig. S10). The molecular ion at 1067.33 *m/z*, corresponding to the Na^+ adduct of KP-395 P1-ol with GlcA in the carboxylate K^+ form, was subjected to MS^2: the ion at 905.30 *m/z* formed by loss of one Hex was then fragmented and the MS^3 spectrum is shown in Fig. S10. Fragmentation arose from both ends of the molecule but the sequence of the residues in the oligosaccharide could not be established, due to its composition containing four hexoses with identical mass.

Therefore, in order to establish the glycosidic bond cleaved by the phage endoglycosidase, a ^{1}H NMR spectrum of KP-395 P1 was recorded and compared with the spectrum of the polysaccharide (Fig. 2). The resonance at 4.81 ppm of β-Gal*p* (**C**) H1 is absent in the oligosaccharide KP-395 P1, but 2 additional peaks at 5.29 and 4.63 ppm were detected and assigned to the α- and β-forms of the reducing end, respectively. Inspection of the anomeric region of the HSQC spectrum (Fig. S11) confirmed the shifts of the reducing end and allowed assignments of the other signals of the anomeric region. Furthermore, the cross-peak of H4/C4 of α-Gal (**B**) at 4.43/77.5 ppm in the spectrum of the CPS, site of a glycosidic linkage in the polysaccharide, was shifted to 4.23/69.9 ppm (data not shown), indicating that C4 was not a linkage site in the oligosaccharide KP-395 P1. Together these data indicated that the cleavage site of the ΦGP1 endoglycosidase is the (1-4) linkage between the residues β-Gal*p* (**C**) and α-Gal*p* (**B**), and that P1 has the primary structure reported in Fig. 6.

The ^{1}H NMR spectrum anomeric region of KP-395 P2 was compared with that of KP-395 P1 (Fig. 2, Fig. S12) for chemical shifts and peaks integration values. While the chemical shifts of the alfa reducing end (Cα) at 5.29 ppm has the same value for both samples, the peak at about 5.45 ppm (**A**) attributed to t-Glc-α is now composed of two doublets and its integration value has also doubled, indicating that there are two of such residues per reducing end. At the same time the signals at 4.80 ppm (H1 of **C**) and at 4.44 ppm (H4 of α-Gal (**B**)), with integral values close to 1, are diagnostic for establishing that KP-395 P2 is composed of two repeating units. Assignments were confirmed by the HSQC experiment of KP-395 P2 (Fig. S12).

In conclusion, ESI-MS and NMR spectroscopy data showed that ΦGP1 associated endoglycosidase cleaves only one glycosidic linkage within KP-395 CPS repeating unit, specifically β-D-Gal*p*-(1→ 4)-α-D-Gal*p*, generating oligosaccharides corresponding to one (KP-395 P1) and two (KP-395 P2) repeating units.

3. Discussion

The extensive global establishment of multidrug-resistant *Klebsiella pneumoniae* high-risk clones in clinical settings has prompted the search

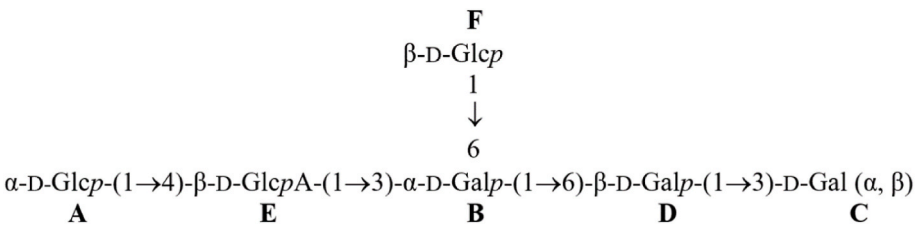

Fig. 6. Structure of the oligosaccharide KP-395 P1 obtained after phage-mediated hydrolysis of KP-395 CPS.

for alternative strategies beyond conventional antibiotics. Among these, bacteriophages and phage-derived enzymes have attracted increasing attention due to their high specificity and ability to target key bacterial virulence determinants, such as CPS [14]. In this study, we provide a detailed structural characterization of CPS depolymerization mediated by two bacteriophage-associated endoglycosidases specific for two clinically relevant *K. pneumoniae* clones, CG258 clade II and CG307. Both CPS types are composed of hexasaccharide repeating units but differ in monosaccharide composition and linkage patterns, reflecting the remarkable structural diversity of *K. pneumoniae* capsules. This diversity underlies the extreme host specificity of bacteriophages and their associated polysaccharide depolymerases.

Depolymerization assays using intact phage particles on bacterial layers demonstrated that both ΦBO1E and ΦGP1 produce plaques surrounded by halos, consistent with the diffusion of soluble depolymerases, suggesting the phage-mediated CPS depolymerization. These observations indicate that CPS components may act as the primary receptors for both phages. Consistent with this hypothesis, resistance to ΦBO1E has been previously associated with loss of CPS production [15], and two of the three KL102 strains resistant to ΦGP1 reported by Ponsecchi et al. [16] carried mutations within *cps* gene cluster genes. Moreover, bioinformatics analysis of the ΦBO1E and ΦGP1 phage genomes revealed the presence of at least two endoglycosidase encoding genes *i. e.* HOQ99_gp59 and vBKpSGP1_0058, respectively.

Structural analysis of the degradation products revealed that, in both cases, the enzymatic activity results in highly specific cleavage of a single glycosidic linkage within the CPS repeating unit. The ΦBO1E-associated endoglycosidase cleaves the α-L-Rha*p*-(1→ 3)-β-D-Gal*p* linkage in the KPB-1 CPS, yielding oligosaccharides corresponding to one and two repeating units. Similarly, the ΦGP1-associated endoglycosidase hydrolyzes the β-D-Gal*p*-(1→ 4)-α-D-Gal*p* linkage in the KP-395 CPS. It would be of interest to determine whether phages from distinct genera, such as the ΦKP-C01 phage infecting K102 strains [17], display the same CPS cleavage specificity as ΦGP1, considering that their endoglycosidases share 55.3% amino acid identity.

This strict bond specificity is a hallmark of phage-associated CPS depolymerases and contrasts with the broader activity of other bacterial polysaccharide-degrading enzymes produced by the gut microbiota [18,19]. From a biological perspective, such precision ensures efficient capsular degradation, thereby exposing the bacterial cell surface and facilitating phage adsorption and infection. From an applied standpoint, the generation of well-defined oligosaccharides highlights the potential of these enzymes as precise molecular tools for CPS structural analysis and as candidates for targeted anti-virulence therapies.

Importantly, CPS degradation does not directly kill bacterial cells but removes a major virulence factor, potentially rendering bacteria more susceptible to host immune defenses or to antibiotics [15,20,21]. This action does not provide a selective pressure for resistance, contrary to bactericidal agents, and thus it is particularly attractive in the context of infections caused by high-risk clones, which are often responsible for outbreaks and express a limited number of CPS types. The targeting of CG258 clade II and CG307 is especially relevant, as these lineages are among the most prevalent and clinically problematic *K. pneumoniae* clones worldwide.

The release of soluble depolymerases during phage infection, as suggested by halo formation, further supports the feasibility of using purified enzymes independently of whole phage particles. Indeed, despite its therapeutic promise, phage therapy still faces major challenges, including rapid emergence of phage-resistant mutants, limited clinical standardization, pharmacokinetic constraints, and unresolved regulatory hurdles. Such "enzybiotic" approaches could circumvent some limitations associated with phage therapy, including regulatory concerns and potential immune responses to whole viral particles.

In conclusion, this study expands the molecular understanding of bacteriophage-associated endoglycosidases targeting *K. pneumoniae* CPS and highlights their high specificity toward clinically important capsular types. The detailed structural insights presented here lay the groundwork for future investigations aimed at assessing the *in vivo* efficacy, stability, and immunological properties of these enzymes, as well as their potential integration into combinatorial strategies to combat multidrug-resistant *K. pneumoniae* infections.

4. Materials and methods

4.1. Bacterial strains and bacteriophages

K. pneumoniae KPB-1 [22] is a clinical strain isolated from the pleural fluid of an inpatient admitted to an Italian hospital. This strain belongs to the ST512, a derivative of CG258 clade II [23,24], responsible for the early pandemic dissemination of the KPC-3 carbapenemase. *K. pneumoniae* KP-395 (originally referred to as EuSCAPE_IT395) [10] is a clinical isolate of ST307, a high-risk clone recently emerged in many countries, including Italy. Lytic bacteriophages, ΦBO1E [25] and ΦGP1 [16], were used to degrade the capsules of KPB-1 and KP-395, respectively.

4.2. Bacterial growth and capsular polysaccharides purification

An overnight liquid culture of KPB-1 or KP-395 was diluted 50 times in Wolfer-Ferguson (W–F) medium. An aliquot of 100 μL of the cell suspension was spread on W–F agar plates and incubated at 30 °C for 4 days. Then, the cells were harvested with sterile 0.9% NaCl, and the suspension was incubated at 10 °C with shaking for 3 h. The cell suspension was then centrifuged at 40,000 RCF at 4 °C for 30 min; the supernatant containing the soluble polysaccharide was precipitated in 4 vol of cold ethanol and let sit at −20 °C for 16 h. The precipitated CPS was recovered by centrifugation at 40,000 RCF for 30 min at 4 °C, dissolved in water, then dialyzed (Servapor 12–14 kDa molecular weight cut-off) against water until the conductivity reached 1 μS. The solution was brought to neutral pH, filtered through a 0.45 μm pore size membrane filter (MF™ membrane filters, Merck Millipore), and lyophilized. The CPS samples were checked by ^{1}H NMR spectroscopy for purity and chemical structure.

4.3. Bacteriophages propagation and titration

For bacteriophages propagation KPB-1 and KP-395 bacterial strains were grown in 10 mL of Lysogeny Broth (LB) overnight at 37 °C with shaking. Cells were harvested by centrifugation and resuspended in 1 mL of Saline Magnesium (SM) buffer (Tris-HCl 50 mM, NaCl 100 mM,

$MgSO_4$ 8 mM, pH 7.5). Aliquots of 5 mL of Molten soft agar (LB supplemented with 0.7% agar) maintained at 50 °C were transferred into a sterile test tube. Subsequently, 10 μL of phage suspension (≈10^8 PFU/mL; ΦBO1E or ΦGP1) and 400 μL of the concentrated bacterial culture were added. The mixture was gently mixed and poured on the LB agar plate and allowed to solidify, before overnight incubation at 37 °C. The number of LB agar plates prepared depended on the amount of phage needed. The subsequent day, 3-5 mL of SM buffer were added to each plate to recover the soft agar with a spatula. The suspension was subjected to orbital shaking for about 1 h, followed by centrifugation at 5000 RCF for 30 min. The supernatant was recovered, centrifuged again to eliminate the remaining soft agar particles and filtered with 0.22 μm syringe filters (KX Sterile Syringe Filter, Kinesis). In order to recover as much phages as possible, the soft agar recovered from the plates was resuspended in SM buffer a second time for at least 1 more hour with orbital shaking and all the subsequent steps of purification were repeated. This operation allowed the recovery of almost the same amount of phage particles obtained in the first purification steps.

For phage titration, the phage suspension was serially diluted in SM buffer to get 1 mL aliquots. To each dilution, 1 mL of 1.5•10^8 CFU/mL of an overnight host bacterial culture was added, followed by incubation at 30 °C for 30 min. The phage and bacteria suspension were mixed with 5 mL of soft agar at 50 °C and poured on LB plates, which, after cooling, were incubated at 30 °C for 4 h or overnight at room temperature. By counting the lytic plaques formed and multiplying them for the dilution factor the phage concentration was obtained. The concentration of phage particles obtained was usually around 1•10^9 PFU/mL, with some variation depending on the quantity of buffer that was added during the recovery. The phage concentration was increased up to 10^{11}-10^{12} PFU/mL using centrifugal concentrators (Amicon® Ultra centrifugal filters 10 kDa MWCO, Merck Millipore).

4.4. Depolymerization of capsular polysaccharides with bacteriophages and purification of the products

An amount of 30 mg of pure polysaccharide was dissolved in 5 mL of SM buffer and autoclaved to sterilize. Then 1 mL of phage suspension (10^{11} PFU/mL) was added to the CPS solution and incubated at 37 °C with shaking. After 24 and 48 h, another aliquot of 1 mL of phage suspension was added. After 72 h of total incubation, the suspension was boiled for 30 min to inactivate the enzyme, centrifuged at 14500 RCF, and the supernatant was lyophilized. The products were subjected to size exclusion chromatography on a low-pressure chromatographic system constituted by a Bio-Gel P-4 (Bio-Rad, fractionation range: 800-4000 Da) column (1.6 cm i. d. × 90 cm) connected to a refractive index detector (Knauer, RI detector K-2301, Lab-Service Analitica) which was interfaced with a computer via PicoLog software. Samples were dissolved in 7.6 mL of 0.05 M $NaNO_3$, 1.9 mL were loaded each time on the column and eluted with 0.05 M $NaNO_3$ at 7.2 mL/h flow rate. Fractions were collected every 15 min, pooled according to the elution profile, dialyzed and freeze-dried.

The time course of the enzymatic degradation was obtained as follows. Aliquots of 60 mg of pure CPS were dissolved in 4 mL of SM buffer and autoclaved to sterilize. Then, 2 mL of phage suspension (10^{11} PFU/mL) was added to the CPS solution and incubated at 37 °C with stirring for 54 h. Every hour, an aliquot was withdrawn from the suspension and frozen immediately. Untreated CPS dissolved in the same buffer was used as control. The collected fractions were boiled for 15 min and centrifuged to remove the pellet. Supernatant aliquots of 100 μL were used for the DNS assay [R4] using maltohexaose to build the standard curve (Fig. S4). The graphs were obtained with OriginPro 2021, OriginLab corporation, Northampton, MA, USA.

4.5. Electrospray ionization mass spectrometry

The oligosaccharides KPB-1 P1 and KP-395 P1, obtained by phage-mediated degradation of the CPS, were reduced with $NaBD_4$ or $NaBH_4$, dialyzed against water, and freeze-dried. For ESI-MS analysis the oligosaccharides were dissolved in 50% aqueous methanol–11 mM NH_4OAc; spectra were recorded in the negative ion mode for molecular mass determination, while fragmentation was obtained in the positive ion mode. A Thermo Fisher LTQ XL™ Linear Ion Trap Mass Spectrometer was used for all analyses, except for fragmentation of KPB-1 P1-ol which was obtained with a Bruker Esquire™ 4000 Ion Trap Mass Spectrometer. Both instruments were connected to a syringe pump for the injection of the samples.

4.6. NMR spectroscopy

The polysaccharides and oligosaccharides were exchanged two times with 99.9% D_2O by lyophilization and subsequently dissolved in 0.6 mL of 99.96% D_2O and introduced into a 5 mm NMR tube for data acquisition. NMR spectra of KPB-1 CPS and its oligosaccharides were recorded on a 500 MHz VARIAN spectrometer operating at 50 °C. HSQC spectra of KPB-1 oligosaccharides were recorded using $J = 145$ Hz (for directly attached ^1H–^{13}C correlations). NMR spectra of KP-395 CPS were recorded on a Bruker Avance III 600 MHz NMR spectrometer equipped with a BBO Prodigy cryoprobe and processed using standard Bruker software (Topspin 3.2). The probe temperature was set at 45 °C. 1D (^1H and ^{13}C) and 2D, COSY, TOCSY, NOESY, HSQC and HMBC NMR experiments were performed. 2D COSY and NOESY experiments were recorded with pre-saturation of HOD, whereas the TOCSY experiments were performed using DOSY to remove signals from low molecular weight components (ledbpgpml2s2d). 2D TOCSY experiments were recorded using a mixing time of 150 ms and the 1D variants using 180–200 ms. 2D NOESY experiments and the 1D variants were recorded using a mixing time of 300 ms. The HSQC experiment was optimized for $J = 145$ Hz (for directly attached ^1H–^{13}C correlations), and the HMBC experiments optimized for a coupling constant of 6 Hz (for long-range ^1H–^{13}C correlations). To improve sensitivity by performing many scans, the 2D experiments were recorded using non-uniform sampling: 40 % for homonuclear and 20–30 % for heteronuclear experiments. ^1H and HSQC ($J = 145$ Hz) experiments of KP-395 oligosaccharides were recorded at 45 °C Bruker Avance NEO 600/54 Onebay NMR Spectrometer. Chemical shifts are expressed in ppm and referred to acetone (2.225 ppm for ^1H and 31.07 ppm for ^{13}C) in the CPS spectra; the oligosaccharides spectra were referred to the most downfield ^1H signal of the respective CPS. NMR spectra were processed using MestreNova 14.3 software.

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CRediT authorship contribution statement

Michela Zaro: Investigation, Validation, Writing – review & editing. **Dhanasri Jayamoorthi:** Investigation. **Barbara Bellich:** Investigation, Writing – review & editing. **Greta Ponsecchi:** Investigation, Writing – review & editing. **Lucia Henrici De Angelis:** Investigation, Writing – review & editing. **Neil Ravenscroft:** Writing – review & editing. **Marco Maria D'Andrea:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Paola Cescutti:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2026.109959.

Data availability

Data will be made available on request.

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