AEM Accepted Manuscript Posted Online 21 June 2019
Appl. Environ. Microbiol. doi:10.1128/AEM.00934-19

Phage-encoded K2 capsule depolymerase is highly stable, refractory to resistance and protects larvae and mice from *Acinetobacter baumannii* sepsis

Running title: K2 capsule-degrading enzyme

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Abstract

Acinetobacter baumannii is emerging as a major nosocomial pathogen in intensive care units. The bacterial capsules are considered major virulence factors and the particular *A. baumannii* capsular type K2 has been associated with high antibiotic resistance. In this study, we identified a K2 capsule-specific depolymerase in a bacteriophage tail spike C-terminus, a fragment that was heterologously expressed and its anti-virulence properties assessed by *in vivo* experiments. The K2 depolymerase is active in a broad range of environmental conditions and is highly thermostable, melting (T_m) at 67 ºC. In the caterpillar larvae model, the K2 depolymerase protects larvae from bacterial infections, using either pre-treatements or with single enzyme injection after bacterial challenge, in a dose dependent manner. In a mouse sepsis model, a single K2 depolymerase intraperitoneal injection of 50 µg is able to protect 60% of mice from an otherwise deadly infection, with a significant reduction in the pro-inflammatory cytokine profile. We showed that the enzyme makes bacterial cells fully susceptible to the host complement system killing effect. Moreover, the K2 depolymerase is highly refractory to resistance development, which make these bacteriophage-derived capsular depolymerases useful anti-virulence agents against multidrug resistant *A. baumannii* infections.

KEYWORDS: *A. baumannii*, bacteriophage, capsular depolymerase, anti-virulence

Importance

Acinetobacter baumannii is an important nosocomial pathogen resistant to many and sometimes all antibiotics. The *A. baumannii* K2 capsular type has been associated with elevated antibiotic resistance. The capsular depolymerase characterized here fits the new trend of alternative antibacterial agents needed against multidrug resistant pathogens. They are highly specific, stable and refractory to resistance as they do not kill bacteria per se, instead they remove bacterial surface polysaccharides, which diminish the bacterial virulence and expose them to the host immune system.

Introduction

Acinetobacter baumannii is an important nosocomial pathogen able to cause wound, skin, lung and bloodstream infections, especially problematic in intensive care units (1). Current treatments are becoming less effective, as many isolates are resistant to most and sometimes all available antibiotics (2, 3). International surveillance Healthcare Safety Network and Eurofins programs have already demonstrated that >50% of *A. baumannii* isolates in patients admitted to intensive care units have acquired resistance to carbapenem and that *A. baumannii* is by far the leading antibiotic-resistant pathogen surveyed (4, 5). Reports show that case-fatality rates associated with bloodstream infections range between 50 to 60% (6, 7).

The presence of capsule polysaccharide-based structures (K types) at the *A. baumannii* surface represents an important virulence factor. These polysaccharide coats modulate the growth within soft tissue infection sites (8), confer intrinsic resistance to peptide antibiotics (9) and protect bacteria from host immune defenses (8, 10). Currently, there are more than 125 capsule synthesis loci in *A. baumannii* found, being 40 different structures determined (11, 12). Nevertheless, the lack of serotyping or genotyping schemes have limited the information available about the virulence and prevalence of *A. baumannii* K types.

Capsular depolymerases are emerging as a new line of anti-virulence agents. These enzymes are typically displayed at the tips of bacteriophage (phage) tails or baseplates to “shave” bacterial capsules exposing their receptors for phage binding and further cell infection (12). Phage-derived capsular depolymerases have been shown to reduce the virulence of gram-negative bacteria, namely *Escherichia coli* (K1, K2, K5 and K30) (13, 14), *Klebsiella pneumoniae* (K1, K5, K64 and KN2) (15-18) and *A. baumannii* (undefined capsule type) (19) in murine models of sepsis, by enhancing killing by complement system, neutrophils, and macrophages. Our research group has also recently shown that capsular depolymerases are widespread in *Acinetobacter* phage genomes, being responsible for the activity and binding to specific *Acinetobacter* bacterial capsules (20), and that they can be heterologously expressed and digest *A. baumannii* capsules, making bacterial susceptible to serum

81 killing (21). The *in vivo* therapeutic potential of phage depolymerases remains however poorly
82 explored against *A. baumannii* infections.
83 In this study, we tested the anti-virulent properties of a new phage-derived capsular depolymerase
84 against a prevalent bacterial capsular type (K2), which has been associated with high antibiotic
85 resistance (e.g. encoding oxacillinase and cephalosporinases genes) (22, 23) and reported to be one
86 of the most common K type in patients admitted in hospitals of Portugal (24). The enzyme was
87 tested in *Galleria mellonella*, murine models and human serum.
88
89 **Methods**
90 **Capsular depolymerase cloning and expression**
91 The depolymerase coding sequence was amplified (primer forward: 5' -
92 GGATCCGATCCGAATATTGATATGACTGG, primer reverse: 5' - CTCGAGTTAACTCGTTGCTGTAAATGC,
93 with restriction sites for BamHI and XhoI) from the C-terminal region of the *B3_42* open reading
94 frame (genetic region 567 bp to 2,100 bp) of the previously isolated *A. baumannii* phage
95 vB_AbaP_B3 (GenBank accession number MF033348), further mentioned as phage B3 (20). This
96 fragment, named K2 depolymerase, was cloned into a pTSL vector (GenBank accession KU314761)
97 containing a N-terminal SlyD leader protein, as well as solubility enhancer, with a His tag as a leader
98 (25). The K2 depolymerae was expressed and purified exactly as previously described (21). Briefly,
99 recombinant hybrid protein (SlyD-k2 depolymerase) was expressed at in BL21 cells at 21ºC for 16 h
100 with agitation, pelleted, suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl), disrupted
101 by sonication and purified in immobilized metal affinity chromatography using nickel resins. The
102 hybrid protein (SlyDK2 depolymerase) was digested with TEV protease (protease:protein ratio of
103 1:100 (wt/wt)) to separate the His–SlyD expression tag from the K2 depolymerase and re-purified.
104 The K2 depolymerase eluted fraction was dialyzed in 20 mM PBS and quantified by the Pierce™
105 Bicinchoninic Acid Protein Assay Kit (Thermo Scientific).
106

Capsular depolymerase functional analysis

K2 depolymerase was characterized *in vitro* in terms of (i) activity spectrum, (ii) degradation of extracted exopolysaccharides, (iii) structural thermostability and (iv) ability to remove the phage B3 receptor. First, the enzyme activity spectrum was determined using drop tests. Enzyme serial dilutions drops between 0.0005 to 5 µg (equivalent to 10 µL drops of 0.001 to 10 µM solutions) were spotted onto 28 *A. baumannii* strains (having 22 different capsular types, namely K1-K3, K9, K11, K15, K30, K33, K35, K37, K40, K43-K49, K57, K73 and K83, referenced in (21) and listed in **Table 1**) overlaid in tryptic soy both-agar plates (TSB with 0.6% (w/v) agar). The presence of a hazy spot indicates capsular-degrading activity. Second, the ability of the K2 depolymerase to hydrolyse extracted exopolysaccharides on different environmental conditions was performed exactly as described elsewhere (21). Briefly, 5 mg/mL of extracted exopolysaccharides were dissolved into different buffer systems to simulate different pH (5 to 9), ionic strength (0 to 500 mM) and temperatures (20 to 80°C) conditions and incubated with the K2 depolymerase or with PBS at 37 °C for 1 h. A final concentration 0.05 µg/µL was used (equivalent to 1 µM). The hydrolytic activity was assessed with the 3,5-dinitrosalicylic acid (DNS) method. Third, Circular Dichroism (CD) spectroscopy was employed to measure the melting temperature (T_m) exactly as described here (21). Briefly, thermal denaturation was monitored through changes in the ellipticity recorded at 222 nm from 25 °C to 90 °C with a heating rate of 1ºC/min. Measurements were performed using the K2 depolymerase at 10 µM in 10 mM potassium phosphate buffer (pH 7). The melting curves were plotted as a function of temperature and fitted to the Boltzmann sigmoidal curve. Finally, we further enlighten the role of K2 depolymerase in phage adsorption onto the host bacterium as described here (21). Briefly, NIPH 2061 strain was incubated with PBS or K2 depolymerase for 2 hours, spun down, washed twice and added to the phage B3 at a multiplicity of infection of 0.001. After 5 min incubation, phage B3 was quantified in the supernatants and the adsorptions were calculated in percentage by the difference between total phage titre and the phage that did not adsorb.

Resistance development

To evaluate possible resistance development, the frequency of bacterial variants insensitive to K2 depolymerase under selective pressure was determined as follows. An overnight culture of NIPH 2061 strain was 100x diluted in TSB and incubated with PBS or K2 depolymerase for 24 h with agitation, pelleted, washed twice with TSB and sub-cultured two more times in presence of PBS or K2 depolymerase. Three different k2 depolymerase concentrations of 0.005 µg/µL, 0.05 µg/µL and 0.25 µg/µL (equivalent to 0.1, 1 and 5 µM) were used. The cultures were plated to isolate colonies and 20 were sub-cultured three times in TSA plates before testing the sensitivity towards K2 depolymerase using drop tests.

***Galleria mellonella* model**

Wax moth larvae *G. mellonella* were reared in our insectarium at 25 °C in the darkness, from egg to last instar larvae on natural diet (beeswax and pollen grains). Worms of the final instar larval stage, weighing 250±25 mg, were selected to be used in the experiments. The *G. mellonella* survival experiment was adapted from previous studies with small changes (26, 27). Briefly, NIPH 2061 strain overnight cultures were grown in fresh TSB at 37 °C and 200 rpm to exponential phase, cells harvested, washed and suspended in 20 mM HEPES (pH 7) to 10^6 CFU per volume of injection (5.5 µL). Using a hypodermic microsyringe, the larvae were injected with 10^6 CFU suspensions via the hindmost left proleg, previously surface-sanitized with alcohol at 70% (v/v). To assess the anti-virulence effect of the K2 depolymerase, the wax moth larvae were injected either with bacteria pre-treated with K2 depolymerase for 2 h at 37 °C prior to inoculation or with K2 depolymerase administered 30 min after bacterial infection, via the penultimate right proleg. Three different enzyme doses (0.25 µg, 0.5 µg and 3 µg/larvae) were used. Two group controls were performed: larvae injected with HEPES to monitor the killing due to injection trauma and larvae injected with K2 depolymerase to evaluate the toxicity of the enzyme. After inoculation, larvae were kept in petri

dishes and maintained in the dark at 37 °C for 72 h. The larval survival was assessed daily during that period and caterpillars were considered dead based on the lack of mobility in response to touch.

Mouse model

Animal experimentation was performed at the Life and Health Sciences Research Institute at the University of Minho, where a license in accordance with European Guidelines for the Care and Use of Animals for Research Purposes is granted. Animals were handled in accordance with the Directive 2010/63/EU of the European Parliament and of the Council, on the protection of animals used for scientific purposes (transposed to Portuguese law – Decreto-Lei 2013/113, 7th of august). The study was approved by the Portuguese national authority for animal experimentation Direção Geral de Alimentação e Veterinária (DGAV 8421 from 2018).

In vivo experimentation was carried out with 6-8 week old BALB/C mice (n=20). To avoid gender bias, both sexes were used, and mice were caged separately. After a period of acclimatization, mice were immunosuppressed with two intraperitoneal injections of cyclophosphamide at a dose of 100 mg/kg, 4 days and 1 day before infection. Mice were challenged with $1x10^7$ CFU suspension of NIPH 2061 strain via intraperitoneal injections. One hour after challenge, mice were treated with PBS (n = 10) or K2 depolymerase (n = 10) with a dose of 50 µg/mice, via intraperitoneal injections. Mock- and enzyme-treated groups were monitored up to 30 days to assess survival rate.

Pathological examinations

In an independent experiment, cyclophosphamide pre-treated mice (n = 24) were challenged with $1x10^7$ CFU with NIPH 2061 strain and treated 1 hour later with PBS (n = 12) or K2 depolymerase (n = 12) at 50 µg/mice via intraperitoneal injections. Before organ harvesting, mice were euthanized via CO_2 inhalation, starting with a CO_2 flow rate that displaced 10-30% of the cage volume per minute. Spleen and livers were aseptically removed from mice 12 h-post infection and homogenates were

10-fold serial diluted in sterile saline and cultured on LB agar plates, at 37ºC for 18 h. Quantification of viable bacteria was assessed by CFU counts and expressed as Log_{10}CFU per organ.

Blood biochemical assays

Serum was collected from whole blood by centrifugation at 3,000 x g for 15 min. Tumor necrosis factor-α (TNFα) and IL-6 were measured in the mocked and enzyme-treated groups by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (mouse TNFα max standard set, mouse IL-6 max standard set Biolegend), according to manufacturer's instructions.

Human serum assay

The serum killing assay was adapted from a previous protocol (28). The NIPH 2061 strain was grown overnight in TBS, diluted in TBS to ≈$5x10^4$ CFU/mL and mixed with fresh human serum in a 1:3 (serum/cells) ratio. The serum was isolated from donated by healthy volunteers. The samples were mixed with (i) PBS, (ii) K2 depolymerase or (iii) heat-inactivated (100 °C at 15 min) enzyme for 1 h at 37 °C. A final concentration of 0.05 µg/µL of enzyme was used (equivalent to 1 µM). Percent survival of enzyme-treated was determined based on viable counts relative to controls without enzyme. A similar experimental was also performed using ten isolated colonies from NIPH 2061 challenge cultures with the K2 depolymerase to evaluate their susceptibility towards to serum killing effect.

Statistical analysis

Differences between two experimental groups were analysed with the two-tailed Student t test. For survival curves, they were plotted using the Kaplan–Meier method and the differences in survival were calculated by using the log-rank Mantel-Cox statistical test, all performed with GraphPad Prism6 (GraphPad Software, Inc., La Jolla, USA). $P < 0.05$ was considered statistically significant.

Results

Depolymerase functional characterization

We have previously isolated a phage vB_AbaP_B3 (B3 for short) that infects *A. baumannii* K2 capsule type (named NIPH 2061 strain) (12). This virus makes clear plaques with large sorrowing haloes, which is indicative of capsule depolymerisation activity (29). The analysis of the phage B3 genome confirmed the existence of a tail spike gene (B3gp42) with a high level of identity to other five *Acinetobacter* phage tail-associated proteins (<97 % overall amino acid identity). This gene harbours a conserved N-terminal phage_T7 domain (PF03906.14) and a less conserved C-terminal pectate lyase domain (PF12708.7), which has been previously linked to enzymes able to degrade bacterial capsules (20) (**Figure 1a**). We therefore further cloned, recombinantly expressed and purified the C-terminal fragment, named K2 depolymerase, which contained the pectate lyase domain (**Figure 1a**). The heterologous production yielded a protein with >95% purity and with a molecular mass of 55.2 kDa matching the theoretical value (**Figure 1b**)

To assess the K2 depolymerase ability to degrade bacterial capsules we performed drop tests. The enzyme was only active on K2 out of 22 different K types of *A. baumannii* (**Table 1**). It remained active from a 0.0005 to 5 µg range (**Figure 1c**). To determine the protein hydrolytic activity, we digested extracted capsular polysaccharides and quantified the sugar reducing ends with the DNS method. K2 depolymerase exhibited high and similar hydrolytic activity in all ranges of pH values (pH 5 to 9) and ionic strengths (0 to 500 mM) (**Figure 2a-b**). The enzyme was also highly active between 20 and 60 ºC, retained 50% of activity at 70 ºC and was inactivated at higher temperatures (**Figure 1b)**. These results are in agreement with the heat-induced changes in the K2 depolymerase secondary structure, where thermal unfolding curves were achieved by following the CD signal at 222 nm as a function of temperature. Thermal unfolding curves showed a sigmoidal transition with melting temperature (T_m) = 67 ºC (**Figure 2d**), temperatures after which no enzymatic activity was observed.

233 To further enlighten the role of the K2 depolymerase in phage B3 adsorption onto the host
234 bacterium, experiments were conducted using hosts with and without enzymatic treatments. Results
235 showed that phage adsorbs 97% vs 20% for PBS- and enzyme-treated cells ($P < 0.01$), respectively
236 (**Figure 2e**). Therefore, by stripping the NIPH 2061 cells from their capsules, the K2 depolymerase is
237 removing the receptors that are no longer available for phage adsorption. This demonstrates an
238 important role of the K2 depolymerase, which is located at the tail spike of the virion particle, in the
239 initial step of the phage B3 infection.
240
241 **Resistance development**
242 The emergence of resistant isolates towards K2 depolymerase under selective pressure was also
243 analysed, by challenging NIPH 2061 strain with PBS or with K2 depolymerase at 3 different enzyme
244 concentrations (at 0.1, 1 or 5 µM) and three sub-cultures (of 24 h each) as described in the material
245 and methods. There were no differences in the growth rate of NIPH 2061 strain in presence of either
246 PBS or K2 depolymerase at 0.1, 1 or 5 µM, which presented a steady increase reaching an OD from
247 0.1 to 1.0 in a few hours. As expected, all 20 isolated colonies from PBS-challenged cultures
248 remained sensitive to enzyme drops. Interestingly, similar observations were made with all 20
249 isolated colonies from enzyme-challenged cultures and for all three different enzyme concentrations
250 used. This demonstrates that resistance development of *A. baumannii* cells towards K2
251 depolymerase under selective pressure is not easily observed.
252
253 **Capsular depolymerase treatment in *A. baumannii*-infected larvae**
254 To validate the K2 depolymerase anti-virulence properties *in vivo*, we used the *G. mellonella*
255 infection model (**Figure 3**). Two types of experiments were performed. Larvae survival rates were
256 measured by either (i) injecting K2 depolymerase pre-treated NIPH 2061 cells for 2 h or (ii) injecting
257 the K2 depolymerase 30 min after NIPH 2061 inoculation, without pre-treatment. In all experiments,

controls groups with either HEPES or enzyme alone resulted in 100% of survival rate, seems to demonstrate absence of toxicity (data not shown).

In the first experiment (**Figure 3a**), bacterial-infected larvae pre-treated with HEPES resulted in a quick and abrupt death, with only 25%, 20% and 10% survival rate after 24, 48 and 72 h, respectively. In opposite, bacterial-infected larvae pre-treated with K2 depolymerase significantly prevented worm death in a time- and dose-dependent manner. After 72 h, 53%, 69% and 88% of larvae survived using 0.25 µg, 0.5 µg and 3 µg of K2 depolymerase pre-treatments, respectively (P <0.01, P <0.0001 and P <0.0001). We also confirmed that K2 depolymerase did not affect bacterial viability given that no differences were observed in the number of the NIPH 2061 CFU before and after of K2 depolymerase treatments.

In the second set of experiments using larvae with a bacterial established infection (**Figure 3b**), in the first 24 h, HEPES injections resulted in 35% survival of larvae, whereas increasing doses of the K2 depolymerase increased the survival rate from 73% up to 100% of larvae. At 48 h, only 22% of bacterial-infected and HEPES-treated larvae survived, being significantly increased between 40% to 76% when treated with increasing doses of the K2 depolymerase. At 72 h, bacterial-infected and untreated larvae displayed less than 15% survival rate. K2 depolymerase treatments with 0.25 µg did not show any improvement. Nevertheless, larvae injected with 0.5 µg and 3 µg of K2 depolymerase still exhibited 56% and 70% worm survival, respectively (P <0.0001 and P <0.0001). Thus, the NIPH 2061 bacterial capsule (K2 type) influences bacterial virulence and higher doses of K2 depolymerase improve the therapeutic effect.

Capsular depolymerase treatment in *A. baumannii*-infected mice.

To further validate the K2 depolymerase anti-virulence properties in vertebrate animals, we used mice infected with NIPH 2061 strain (10^7 CFU) and treated with a single 50 µg dose of K2 depolymerase 1 h-post infection (**Figure 4a**). In the control group, infection in all PBS-treated mice quickly progressed 20 h-post infection. Mice exhibited severe signs of septicaemia, with abnormal

posture behaviour and more than 10% weight loss, being therefore sacrificed for ethical reasons. In contrast, at 20 h post-infection, 90% of enzyme-treated mice had survived, decreasing to 60% at 42 h post-infection, after which mice remained healthy until the end of the experimental period. Statistical analysis of survival rates corroborated that the K2 depolymerase therapy was successful against *A. baumannii* infection (P = 0.004). The fact that in treated mice the CFU counts were lower in the spleen, but not in the liver, comparatively to the control group, also attest for the successful depolymerase treatment (**Figure 4b**).

Capsular depolymerase treatment biochemical effect

Levels of TNF-α and IL-6 were analysed from serum of mock and enzyme-treated mice and the results showed the induction of these pro-inflammatory cytokine production upon infection. As shown in **Figure 5**, the amount of both TNF-α and IL-6 were significantly higher in non-treated mice than in enzyme-treated mice.

Serum sensitivity of depolymerase-treated *A. baumannii*

To further validate the anti-virulence effect of K2 depolymerase, we used human serum mixed with NIPH 2061 strain (**Figure 6**). After adding K2 depolymerase to the contaminated serum, NIPH 2061 CFU were reduced below detection limit (<10 CFU/mL). As expected, addition of heat-inactivated K2 depolymerase did not sensitize NIPH 2061 strain to the serum killing. Thus, enzymatic capsule removal via K2 depolymerase is crucial to help the host complement system to control the infection. As an additional study, we also tested the susceptibility to serum killing of the previously isolated bacterial colonies (n = 20) plated after being challenged with K2 depolymerase. These strains survived to serum complement system, but after being in contact with K2 depolymerase, were reduced below detection limit (< 10 CFU/mL) (data not shown).

310

311 **Discussion**

312 Capsular polysaccharides, also termed as K types, are major virulence factors of bacteria, involved in
313 protecting cells from a range of environmental pressures, mostly against host immunity (30, 31). As
314 documented for *E. coli* (32) and *K. pneumoniae* (33), capsules may also be the primary virulence
315 determinant of *A. baumannii*. However, so far, only few K types of *A. baumannii* were proved to
316 invade or overwhelm mammalian defences (K1 and K45) (9, 21, 34). The existence of at least 125 K
317 types in *A. baumannii* might be related to different degrees of clinical manifestation of infections
318 and antibiotic resistance (21). However, this knowledge is limited due to the lack of robust typing
319 schemes available to determine the most predominant and virulent K types.

320 To the best of our knowledge, the first epidemically capsule typing study demonstrated that the *A.*
321 *baumannii* bacterial capsule type K2 has been frequently associated with infections in Portugal (24).
322 Strains of this capsular type, such as NIPH 2061 used in this study, also have been linked to high
323 antibiotic resistance (encoding oxacillinase and cephalosporinases genes), thereby demanding the
324 development of new therapeutic options (22, 23). Therefore, the present study aimed at isolating a
325 new phage-derived capsular depolymerase that degrades the *A. baumannii* K2 and evaluating its
326 anti-virulence potential and efficacy in controlling *A. baumannii* systemic infections, using *in vivo*
327 models.

328 We found that phage vB_AbaP_B3 genome (B3 for short) contains a tail spike (B3gp42) with a
329 pectate lyase domain at the C-terminal fragment. We further showed that this C-terminal fragment
330 (named K2 depolymerase) has a specific depolymerase activity on K2 out of 22 different capsular
331 types tested, with activity between micro to millimolar range. A similar extremely narrow host range
332 has been found in capsular depolymerases from phages infecting *A. baumannii* (21), *E. coli* (13) and
333 *K. pneumoniae* (16, 18, 26), often restricted to one or two K types. We also showed that the K2
334 depolymerase binds to the host receptor as NIPH 2061 cells pre-treated with the enzyme heavily
335 affected phage B3 adsorption. As phage tail spikes function as host recognition elements (35), such

results suggest that phage tail spike proteins have high diversity, evolved to encode several depolymerase domains to recognize a high range of bacterial K antigens possibilities.

K2 depolymerase also demonstrated to be a versatile and thermostable enzyme as it is active across a range of different environmental conditions of pH (5 to 9), ionic strength (0 to 500 mM) and temperatures (20 to 70 °C). We noted that the loss of enzymatic activity at temperatures ≥70 °C related well with the loss of protein structure observed during CD spectroscopy measurements (T_m = 67 °C). These impressive characteristics have been shown for the few capsular depolymerases also tested from *K. pneumoniae*-infecting phages (26, 36). They are likely a reflection of their structural nature i.e., being part of the virion particle, all structural proteins are evolved to endure broad and harsh conditions to maintain phage infectivity and survival.

To assess if *A. baumannii* capsule type K2 is an important virulence factor, we first used the *G. mellonella* larvae model for its simplicity. These insects are easy to use and have host mechanisms of resistance similar of those of vertebrates against *A. baumannii* infections (37). We demonstrated that K2 capsule could overcome the immune system of larvae causing significant death. We also showed that the K2 depolymerase could significantly rescue larvae either by pre-treating K2 cells with K2 depolymerase before inoculation or by injecting the enzyme 2 h post infection. As expected, these results were dose dependent. Overall, since capsule removal by the K2 depolymerase attenuated the pathogenicity of the cells and prolonged the larvae lifespan, it was demonstrated that the capsule K2 type is a major virulence factor of *A. baumannii*. Generally, other researchers have also shown success in preventing and treating the lethal effects of encapsulated *K. pneumoniae* (K3, K21 and K36) (26, 38) and *A. baumannii* (undefined capsule type) (19) with single injections of capsular-specific depolymerases. However, these models have administrated enzymes immediately after infection (5 min or less), which may raise the question if they are only providing a protective effect rather than a therapeutic one.

To further investigate the anti-virulence efficacy in a model that better mimics the human immune systems, we tested the K2 depolymerase in a murine model of sepsis. Our results indicated that a

362 single administration of the K2 depolymerase (50 μg/mice) had a significant therapeutic effect, by
363 rescuing 60% of mice, controlling bacterial proliferation and reducing local inflammation. Other
364 studies have also demonstrated that a single administration of capsular depolymerases could
365 significantly protect mice infected with *E. coli* (K1, K2, K5 and K30) (13, 14) and *K. pneumoniae* (K1,
366 K5, K64 and KN2) (15-18). A recent study also showed that a capsule depolymerase administered
367 intraperitoneally could rescue 100% of mice with *A. baumannii* systemic infections (19). In that
368 study, the *A. baumannii* strain used has an undefined capsule type, which unable us to correlate the
369 activity with our K2 depolymerase. Furthermore, the authors used the whole tail spike protein (N-
370 terminal tail domain plus the C-terminal pectate lyase 3 domain), while we cloned and used only the
371 pectate lyase fragment that confers capsule depolymerase activity, which further difficults a direct
372 comparison. Still, all these works prove that these phage-derived proteins are able to reproductively
373 reduce bacterial virulence *in vivo*. Nevertheless, particular attention must be given to the time and
374 route of administration, as well as the serotype strains used, which have been shown to influence
375 the therapeutic outcomes (39). Overall, the *in vivo* efficacy of capsular depolymerases suggests that
376 a therapeutic approach based on capsular depolymerases represents a promising alternative to treat
377 bacterial infections and in particular, those of *A. baumannii* associated with extended drug
378 resistance.
379 Aiming to elucidate the mechanism of cooperation between the capsular depolymerase and host
380 immune system to control bacterial infections, we performed additional tests. We hypothesised that
381 a complement system-dependent mechanism should be responsible for the clearance of enzyme-
382 treated bacteria and protection against infection, since cyclophosphamide used to
383 immunocompromise mice reduces the level of leukocytes but not those associated with the host
384 complement system (40). To prove this, we isolated human serum and mixed with the NIPH 2061
385 strain (K2 type) cells in presence and absence of the K2 depolymerase. The experiments indicated
386 that the enzyme could efficiently sensitize the bacteria in serum. The fact that the bacteria could not

be sensitized to the host complement system in the presence of heat-inactivated enzyme, showed that the killing effect was solely attributed to the lytic effect of the complement system.

The K2 depolymerase characterized here fit the new trend of alternative antibacterial agents needed against multidrug resistant *A. baumannii*. Besides being highly specific, stable and able to reduce the virulence of the *A. baumannii* in *in vivo* models, the enzyme also seem to be refractory to resistance development. This was shown by challenging the NIPH 2061 strain with the enzyme for 24 hours, where the isolated colonies remained sensitive to the K2 depolymerase in drop tests and to the serum complement only after striping the cells from their polymeric coats with the K2 depolymerase. This can be explained by the unique mode of action of the capsular depolymerases that do not kill bacteria, but instead degrade the extracellular polymers from the bacteria that protect them from the environment, which makes the development of bacterial resistant variants unlikely.

In conclusion, the anti-virulence properties together with the high stability, versatility of the K2 depolymerase towards extreme conditions of pH, ionic strength and temperatures, and the low probability of resistance development, make this enzyme a potential therapeutic agent against multidrug resistant *A. baumannii* infections. In an era where multidrug resistant infections are increasing, capsular depolymerases may play a vital role as surrogate antibiotics.

408 **Footnotes**

409 **Funding**

410 This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the
411 scope of the strategic funding of UID/BIO/04469/2019 unit and the Project PTDC/BBB-
412 BSS/6471/2014 and COMPETE 20202 (POCI-01-0145-FEDER-016678, POCI-01-0145-FEDER-006684).
413 This work was also supported by BioTecNorte operation (NORTE-01-0145-FEDER-000004, NORTE-01-
414 0145-FEDER-000013 and NORTE-01-0145-FEDER-000023) funded by the European Regional
415 Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. This
416 study was additionally supported by Infect-ERA grant Infect-ERA/0002/2015: BU_SPONT_HEAL. HO,
417 AGF and DMH acknowledge the FCT grants SFRH/BPD/111653/2015, FRH/BPD/112903/2015, and
418 SFRH/BDP/91831/2012. AM acknowledges the NORTE-08-5369-FSE-000041 project. Funding
419 received by iBB-Institute for Bioengineering and Biosciences from FCT (UID/BIO/04565/2013) and
420 from Programa Operacional Regional de Lisboa 2020 (Project N. 007317) is acknowledged.

421

422 **Acknowledgments**

423 We thank Filipa Grosso for providing the *A. baumannii* K2 isolates H466, H580, H603 and H678.

424

425 **Potential conflicts of interest**

426 Authors declare no conflict of interest.

427 **Figure Legends**

428 **Figure 1. K2 depolymerase identification**. A) *In silico* analysis of the *A. baumannii* phage
429 vB_AbaP_B3 tail spike (B3gp42). Protein data bank (PBD) database shows homology to phiAB6
430 tailspike (5JS4_A) and PFAM identifies a N-terminal tail domain and a C-terminal depolymerase
431 domain (pectate lyase 3, named K2 depolymerase) that was cloned (genetic region 567 bp to 2,100
432 bp, of the B3_42 open reading frame); B) SDS-PAGE gel demonstrating the expression and
433 purification of the hybrid protein expression (SlyD-K2 depolymerase) after TEV protease digestion, to
434 separate the K2 depolymerase from the SlyD fusion partner. C) Drops tests of the K2 depolymerase
435 onto NIPH 2061 strain.

436

437 **Figure 2. *In vitro* functional analysis**. Hydrolytic activity of the K2 depolymerase on extracted
438 exopolysaccharides from NIPH 2061 (K2 capsule type) host in different: A) ionic strengths (pH 6, 0-
439 500 mM NaCl, 37 °C), B) temperatures (pH 6, 0 mM NaCl and 37-80 °C) and C) pH values (5-9, 0 mM
440 NaCl, 37 °C). The results are expressed as relative activity, comparing with the best activity value
441 obtained, pH 6.0 mM, 37 °C. Significance was determined by Student test (*, $P < 0.05$). D) Circular
442 dichroism analysis of the K2 depolymerase. Melting curve was acquired measuring ellipticity at 222
443 nm from 25 to 90 °C. E) *A. baumannii* phage vB_AbaP_B3 adsorption onto NIPH 2061 cells pre-
444 treated with K2 depolymerase. Results are expressed as residual PFU percentages in comparison
445 with adsorption assays with NIPH 2061 cells pre-treated with PBS. * Statistically different ($P < 0.01$).

446

447 **Figure 3. *In vivo Galleria mellonella* model.** The larvae survival rates infected with NIPH 2061 cells
448 (K2 capsule type) (10^6 CFU inoculum) were measured by injecting A) K2 depolymerase-pre-treated
449 NIPH 2061 cells for 2 h or B) K2 depolymerase 30 min after NIPH 2061 bacterial inoculation. Three
450 different enzyme doses of 0.25 µg, 0.5 µg and 3 µg were used. For clarity, control groups (HEPES-
451 treated and enzyme-treated larvae) resulted in 100% of survival rate and were omitted from the
452 figures. Significance was determined by Mantel-Cox test (*, $P < 0.05$).

Figure 4. *In vivo* murine model. A) The survival rates of mice infected with NIPH 2061 (K2 capsule type)cells (10^7 CFU inoculum) were measured after intraperitoneal injection of PBS or the K2 depolymerase (50 µg/mice) 1 h-after challenge. B) CFU counts of the spleen and liver of mice 12 h-after challenge. Significance was determined by Mantel-Cox test (*, $P < 0.05$).

Figure 5. Biochemical analysis. The biochemical levels of A) TNFα and B) IL-6 were measured in blood of PBS-treated and K2 depolymerase-treated mice with 50 µg. Significance was determined by two-tailed Student t test (*, $P < 0.05$).

Figure 6. Host serum complement system effect. Human serum was infected with NIPH 2061 (K2 capsule type) cells in a 3:1 ration and incubated with PBS, K2 depolymerase or heat-inactivated (15 min at 100 °C) enzyme, for 1 h at 37 °C. Enzyme final concentration of 1 µM was used. #, below detection limit (10 CFU/mL).

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Table 1. Activity spectrum of the K2 depolymerase. Drop test of the enzyme was made in bacterial lawns of a collection of *A. baumannii* with known capsular types. For all strains, the sequence types (ST) according the multilocus sequence analysis using the Pasteur scheme, capsular type (K) and respective accession numbers are given. K-type - determined capsule structure; N/A – not available

Strain	ST	K type	GenBank accession No.	K2 depolymerase spot test
Acinetobacter baumannii (n=28)				
NIPH 290 (= CIP 110431)	ST1	1	KB849940.1 (126651-104645 bp)	-
NIPH 2061 (= CIP 110467)	ST2	2	KB849309.1 (77375-101575 bp)	+
H466	ST2	2	N/A yet	+
H580	ST2	2	N/A yet	+
H603	ST2	2	N/A yet	+
H678	ST2	2	N/A yet	+
NIPH 501T (= ATCC 19606T)	ST52	3	KB849970.1 (174731-119233 bp)	-
NIPH 528 (= CIP 110436 = RUH 134)	ST2	9	KB849906.1 (78004-102899 bp)	-
NIPH 80 (= CIP 110427)	ST37	9	KB849944.1 (156383-131489 bp)	-
J9	ST49	11	KF002790	-
A85	ST1	15	KC118540 (8456-36738 bp)	-
RBH2	ST111	19	KU165787	-
NIPH 190 (= CIP 110429)	ST9	30	KB849477.1 (592918-572137 bp)	-
NIPH 67 (= CIP 110425)	ST35	33	KB849903.1 (1301423-1278652 bp)	-
LUH5535	N/A	35	KC526896	-
NIPH 146 (= CIP 110428)	ST25	37	KB849308.1 (572444-592959 bp)	-
ANC 4097 (= CIP 110499)	ST1	40	KB849962.1 (39134-62621 bp)	-
NIPH 60 (= CIP 110424)	ST34	43	KB849508.1 (140959-120981 bp)	-
NIPH 70 (= CIP 110426)	ST36	44	KB849923.1 (574942-546765 bp)	-
NIPH 201 (= CIP 110430)	ST38	45	KB849844.1 (365379-344305 bp)	-
NIPH 329 (= CIP 110432)	ST11	46	KB849871.1 (2591085-2567472 bp)	-
NIPH 601 (= CIP 110437)	ST40	47	KB849894.1 (3226845-3205785)	-
NIPH 615 (= CIP 110438)	ST12	48	KB849301.1 (114143-93442 bp)	-
NIPH 1734 (= CIP 110466)	ST15	49	KB849325.1 (2998512-2965610 bp)	-
NIPH 335 (= CIP 110433)	ST10	49	KB849886.1 (1318556-1286966 bp)	-
BAL_212	ST52	57	KY434631	-
SGH0703	ST2	73	MF362178	-
LUH5538	N/A	83	KC526898	-