Carbapenem-resistance in *Klebsiella pneumoniae* (KP) sequence type ST258 is mediated by carbapenemases (e.g. KPC-2) and loss or modification of the major non-selective porins OmpK35 and OmpK36. However, the mechanism underpinning OmpK36-mediated resistance and consequences of these changes on pathogenicity remain unknown. By solving the crystal structure of a clinical ST258 OmpK36 variant we provide direct structural evidence of pore constriction, mediated by a di-amino acid (Gly115-Asp116) insertion into loop 3, restricting diffusion of both nutrients (e.g. lactose) and Carbapenems. In the presence of KPC-2 this results in a 16-fold increase in MIC to Meropenem. Additionally, the Gly-Asp insertion impairs bacterial growth in lactose-containing medium and confers a significant in vivo fitness cost in a murine model of ventilator-associated pneumonia. Our data suggests that the continuous selective pressure imposed by widespread Carbapenem utilisation in hospital settings drives the expansion of KP expressing Gly-Asp insertion mutants, despite an associated fitness cost.
The acquired resistance to Carbapenems by *Klebsiella pneumoniae* (KP) and other Gram-negative organisms is an increasing global problem that potentially jeopardises the future utility of a fundamentally important antibiotic class used for the treatment of life-threatening infections. KP infection is usually hospital-acquired where it accounts for around 30% of Gram-negative infections. In a US study covering long-term facilities between 2014 and 2015, almost 25% of KP isolates were Carbapenem-resistant (CRKP). CRKP is now endemic in some regions and are classified as ‘critical’ WHO Priority 1 organisms. Hospital-acquired CRKP infection mortality is high and there may be an increased risk of death when infection is caused by resistant versus sensitive KP strains (42 vs 21%). Importantly, while this may be due to the potential inferiority and known toxicity of the few alternative antibiotics, it illustrates the success of KP as a key antibiotic resistant pathogen and our reliance on Carbapenems.

The expression of carbapenemase enzymes, usually encoded on large resistance plasmids, represents the first genetic source of resistance. These enzymes inactivate Carbapenems by hydrolysis. The plasmids are transferred vertically from parental to daughter cells during cell division or horizontally by conjugal transfer. Chromosomally, modification of the major outer membrane porins, OmpK35 and OmpK36, limit antibiotic influx across the outer membrane in CRKP. These changes act in concert to effectively lower active Carbapenem concentrations at the site of their transpeptidase targets, the periplasm, abrogating their bactericidal effect.

One multiclonal sequence type (MLST), ST258, has been internationally successful in driving CRKP dissemination. ST258 strains have closely expanded with the KPC family of carbapenemases, which in tandem with modifications in OmpK35 and OmpK36, afford clinically relevant (high) minimum inhibitory concentrations (MICs) to Carbapenems. However, porins play important physiological roles and facilitate both the influx of small hydrophilic solutes, including nutrients, and the efflux of toxic products across the otherwise impermeable Gram-negative outer membrane. In keeping with this, deletion of both porin genes results in attenuation during in vivo infection. The overall structures of OmpK35 and OmpK36 display similar features to other general porins, a trimeric architecture composed of 16-stranded β-barrels. Two important structural components in porins are extracellular loops 3 and 4 (L3 and L4). L3 is not exposed at the cell surface but folds back into the barrel, forming a constriction zone half way inside the channel that contributes to the permeability properties, such as size exclusion limit and ion selectivity of the pore. L4 lies away from the pore and is involved in monomer trimerisation and subsequent stability.

OmpK35 is ubiquitously truncated in ST258 strain collections, where a common mutation (Genbank FJ577672) encodes a frame-shift that results in a premature stop codon (TGA) and large truncation (Fig. 1a, Supplementary Fig. 1a). This mutation results in an unstructured and non-functional pore due to the encoding of only a small conserved 19 amino acid N-terminal fragment following signal peptide cleavage. There is more heterogeneity in OmpK36 sequences. We chose to study an OmpK36ST258 variant from an ST258 strain (KPST258, Supplementary Table 1) that exhibits high Carbapenem MICs, which is associated with increased mortality. The OmpK36ST258 protein sequence of KPST258 is 100% identical to the consensus accession WP_00415112 present in 1132 NCBI assemblies. In comparison with the reference laboratory strain ATCC43816, the OmpK36 variant includes a Gly115-Asp116 (GD) insertion after the conserved Pro109-Glu-Phe-Gly-Gly-Asp114 motif in L3 and a Leu165-Ser-Pro167 (LSP) insertion in L4 (Fig. 1b, Supplementary Fig. 1b). The GD insertion and other sequence variations in L3 of OmpK36ST258 are correlated with increased resistance in clinical isolates from international KP collections.

KP ST258 is evidently flourishing and a reduced permeability barrier is clearly beneficial in the face of prevailing antibiotic selection. This prompted us to explore the precise molecular

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**Fig. 1** OmpK35ST258 and OmpK36ST258 porin variants do not impact on growth in vitro. a The OmpK35ST258 frame-shift mutation results in a premature TGA stop codon. Sequence conservation with OmpK35WT is lost within the first β-strand resulting in a truncated protein. b OmpK36 sequences share 95% identity, with two insertions (GD in loop 3 and LSP in loop 4) in OmpK36ST258 (highlighted in green). The underlined PEGGD is a conserved loop 3 motif in OmpK36 porins. c Outer membrane preparations demonstrate a loss of OmpK35 in ICC8003 and ICC8004. OmpK36WT (ICC8001 and ICC8003) and OmpK36ST258 (ICC8002 and ICC8004) are present in similar abundance in isogenic strains. d, e Growth, measured by OD600, is not affected by the introduction of ST258 porins into ICC8001 in rich (Luria Bertani) media (d) or minimal (M9) media (e) with glucose as the sole carbon source (n = 3 repeats, error bars = s.d.)
resistance mechanism underlying changes in the outer membrane and to determine if these come at a fitness cost. Here we show that the GD insertion in OmpK36ST258 constricts the pore and restricts diffusion of Carbapenems and the disaccharide lactose across membranes. Pore constriction attenuates KP growth in medium containing lactose as a sole carbon source in vitro and infection in a murine model of ventilator-associated pneumonia in vivo. These results suggest that the selective pressure posed by the extensive usage of Carbapenems is a major driver in the continuous spread of CRKP, a key pathogen with a significant attributable morbidity, mortality and socioeconomic cost.

Results and Discussion

ST258 outer membrane porins enhance Carbapenem resistance. We investigated the impact of the sequence variations found in OmpK35 and OmpK36 of KPCST258 on Carbapenem resistance. To this end, we substituted the endogenous porin genes in a RifR derivative of ATCC43816, we named ICC8001 (Supplementary Table 1), with OmpK36ST258 (ICC8002), OmpK35ST258 (ICC8003) or both (ICC8004) (Fig. 1c and Table 1). The absence of OmpK35 in ICC8003 and ICC8004, resulting from the introduction of the ST258 truncation mutant (66aa vs 337aa WT sequence), was confirmed by analysis of outer membrane preparations (Fig. 1c). Replacement of the OmpK36 coding sequence alone, between the endogenous chromosomal promoter and terminator regions at the wild-type (WT) locus of ICC8001, resulted in a similar abundance of OmpK36WT and OmpK36ST258 in all four strains (Fig. 1c). No growth defects were detected when the different strains were grown in vitro in either rich (Luria Bertani, LB) or minimal (M9) media containing glucose (0.4% w/v) as the sole carbon source (Fig.1d, e). This suggested that the ST258 porin variants do not adversely affect KP’s ability to grow in extremes of extracellular osmotic pressure or when glucose is provided as the sole carbohydrate available for metabolism.

We next assessed the impact of sequential ST258 porin gene substitution on antimicrobial resistance in the absence or presence of the carbapenemase genes KPC-2 and OXA-48, encoded on epidemic pKpQIL-like and pOXA-48a-like circulating plasmids. Utilising a reference laboratory broth MIC panel, designed to evaluate resistant Gram-negative organisms, revealed that in the absence of carbapenemases all the strains remain Carbapenem sensitive (Supplementary Fig. 2a). Moreover, the isogenic strains remain sensitive to aminoglycosides and Tigecycline where porin loss or mutation do not influence susceptibility (Fig. 2e).

Both OmpK35ST258 truncation (ICC8003) and OmpK36ST258 substitution (ICC8002) increased resistance to Carbapenems in the presence of KPC-2 and OXA-48 (Fig. 2a, b, c, d), although the absolute MIC values for these agents were found to be dependent on the enzymatic activity of each carbapenemase. The lower levels of resistance achieved by OXA-48 (Ambler class D) expressing strains is attributable to a weaker hydrolytic activity towards Carbapenems than that mediated by KPC-2 (Ambler class A). Of note, the contribution of OmpK36ST258 to Carbapenem resistance was greater than OmpK35ST258. For example, the MIC to Meropenem (Fig. 2a, d) is 1 mg/L in ICC8001, 8 mg/L in ICC8003 (encoding OmpK35ST258) and 16 mg/L in ICC8002 (encoding OmpK36ST258) in strains expressing KPC-2. This demonstrates that the WT OmpK35 and OmpK36 porins in ICC8001 allow sufficient periplasmic diffusion and bactericidal activity for this strain to remain below the Meropenem sensitivity breakpoint (2 mg/L), despite KPC-2 and OXA-48 mediated hydrolysis. This pattern extends to other classes of drugs with similar mechanisms of action, such as the third and fourth generation Cephalosporins, Cefotaxime and Cefepime (Fig. 2e).

The sensitivity to Ceftazidime is restored by the novel dizabicyclooctanone (DBO) non-β-lactam β-lactamase inhibitor, Avibactam (Fig. 2e), whereas other OmpK36 variants have been shown to contribute to resistance against this new agent. Importantly, the MIC to Meropenem of ICC8004 (encoding OmpK35ST258 and OmpK36ST258) was 32 mg/L, which reproduced the Carbapenem antibiogram (together with resistance to Imipenem and Ertapenem) of the KPCST258 strain harbouring the pKPQIL-like plasmid (Fig. 2d). ICC8004 far exceeded the Carbapenem resistance breakpoints of the agents tested. Indeed, the antibiotic levels required for treating KP, expressing this ST258 porin configuration, would be unachievable even in the context of attempting to optimise dosing by continuous infusion in current trials (NCT03213990).

OmpK36 pore constriction underpins Carbapenem resistance. The robust increase in Carbapenem resistance conferred by the expression of OmpK36ST258 compared with OmpK36WT, despite both proteins sharing 95% identity, prompted us to elucidate the underlying molecular resistance mechanism. As the OmpK36 used in previous structural work contains a Q235R mutation (PDB ID: 5O79), which is found behind L3 and therefore could have affected its conformation, we started by solving the structure of the WT OmpK36 (PDB ID: 6RD3, Table 2). This revealed that the two structures can be superimposed with an rmsd of 0.18 Å over 340 Cα atoms, suggesting that the Q235R substitution had no effect on the conformation of L3. We next solved the structure of OmpK36ST258 at 3.23 Å resolution by molecular replacement, using both OmpK36WT and the OmpK36ST258 as search models (Fig. 3a, Table 2). The overall architecture of the OmpK36ST258 porin is preserved despite multiple sequence variations, including L3 and L4 insertions. The OmpK36ST258 structure can be superimposed on OmpK36WT, with an rmsd of 0.43 Å over 340 Cα atoms. The structure revealed that the GD insertion in L3 of OmpK36ST258 resulted in an extended loop conformation that intrudes into the pore, at the constriction zone, which reduced the pore diameter by 26% (3.2 Å WT and 2.37 Å ST258 diameter) (Fig. 3b, Supplementary Fig. 3a). The structure suggests that L3 is further stabilised by the formation of a salt-bridge between D114 and R127 at the barrel face of the pore.

Table 1 Isogenic strains used in this study with their corresponding OmpK35 and OmpK36 composition

| Strain      | OmpK35         | OmpK36         |
|-------------|----------------|----------------|
| ICC8001     | OmpK35WT       | OmpK36WT       |
| ICC8002     | OmpK35ST258    | OmpK36ST258    |
| ICC8003     | OmpK35WT       | OmpK36ST258    |
| ICC8004     | OmpK35ST258    | OmpK36ST258    |
| WT + GD     | OmpK35ST258    | OmpK36ST258    |
| ST258 & GD  | OmpK35ST258    | OmpK36ST258    |
| ST258 ΔLSP  | OmpK35ST258    | OmpK36ST258    |
| ST258R127A  | OmpK35ST258    | OmpK36ST258    |
| OmpK35ST258 |                |                |
| OmpK36ST258 |                |                |
| OmpK35WT    |                |                |
| OmpK36WT    |                |                |
| OmpK35ST258 |                |                |
| OmpK36ST258 |                |                |
| OmpK35WT    |                |                |
| OmpK36WT    |                |                |
| OmpK35ST258 |                |                |
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| OmpK36ST258 |                |                |

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(Fig. 3a). In order to evaluate whether the L3 GD motif is the sole molecular mechanism contributing to the resistant phenotype we constructed two further mutants for evaluation in subsequent structural and functional experiments. We created an OmpK36\textsubscript{WT} chimera in which we inserted a GD in L3 (OmpK36\textsubscript{WT}GD) and a GD deletion mutant where the two amino acid motif was removed from L3 of the resistant OmpK36\textsubscript{ST258} (OmpK36\textsubscript{ST258GD}). Solving the OmpK36\textsubscript{WT+GD} structure at 2.03 Å resolution revealed a similar conformation to OmpK36\textsubscript{ST258} with formation of a D114/R127 salt-bridge and pore constriction to 2.87 Å (Fig. 3b, Table 2).

To determine if the observed pore diameter reduction, mediated by L3 GD insertion, influenced permeability, we performed liposomal swelling assays and quantified carbohydrate and Carbapenem diffusion across the OmpK36 isoloms. While not diffusing into empty proteoliposomes, glucose (180 g/mol), the smallest carbohydrate tested, freely diffused across all OmpK36 isoloms, demonstrating formation of functional pores (Supplementary Fig. 4b). The L3 GD mediated pore constriction did not impact on glucose diffusion, in keeping with the in vitro growth curves in minimal media containing glucose as the sole carbon source (Fig. 1e). Stachyose, a tetrasaccharide, with the highest molar mass tested at 666 g/mol was unable to diffuse across either OmpK36\textsubscript{WT} or OmpK36\textsubscript{WT+GD} expressing strains compared with glucose and stachyose. Diffusion of lactose was impaired across OmpK36\textsubscript{WT+GD} expressing strains compared with OmpK36\textsubscript{WT} expressing strains (Supplementary Fig. 5f).

Consistent with this finding, an isogenic strain expressing OmpK36\textsubscript{WT+GD} in M9 containing lactose as the sole carbon source, demonstrated a growth defect compared with the OmpK36\textsubscript{WT} expressing strain (Supplementary Fig. 5f).

Meropenem (383 g/mol) diffusion was significantly reduced in OmpK36\textsubscript{ST258}-containing proteoliposomes compared with OmpK36\textsubscript{WT} (Fig. 3c, d). This diffusion barrier is reproduced by GD insertion in OmpK36\textsubscript{WT+GD}, and completely reversed by L3 GD deletion in OmpK36\textsubscript{ST258GD} (Fig. 3c, d). Expression of the molecular mechanism contributing to the resistant phenotype we defined by EUCAST breakpoints. Antibiotic key: IPM Imipenem, MEM Meropenem, ETP Ertapenem. e Resistance to other antibiotics in different classes tested. Individual values are colour coded (green—sensitive, yellow—intermediate and red—resistant) according to their antibiotic resistance defined by EUCAST breakpoints. Antibiotic key: AMP Ampicillin, AMC Amoxicillin/Clavulanate (2:1), TAZ Piperacillin/Tazobactam, CTX Cefotaxime, CZA Cefazidime/Avibactam, CAZ Cefazidime, CEP Cefepime, C_T Cefotolozone/Tazobactam, ATM Aztreonam, CIP Ciprofloxacin, TOB Tobramycin, AMK Amikacin, GEN Gentamicin, TGC Tigecycline. 

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**Fig. 2** The impact of OmpK35\textsubscript{ST258} and OmpK36\textsubscript{ST258} substitution on resistance to antibiotics used in Gram-negative infections. a Meropenem, b Imipenem and c Ertapenem broth minimum inhibitory concentrations presented graphically in isogenic strains expressing the KPC-2 carbapenemase. Dotted lines represent sensitive and resistant EUCAST breakpoints. d Broth MIC of the isogenic KP strains expressing the KPC-2 or OXA-48 carbapenemase. Individual values are colour coded (green—sensitive, yellow—intermediate and red—resistant) according to their antibiotic resistance defined by EUCAST breakpoints. Antibiotic key: IPM Imipenem, MEM Meropenem, ETP Ertapenem. e Resistance to other antibiotics in different classes tested. Individual values are colour coded (green—sensitive, yellow—intermediate and red—resistant) according to their antibiotic resistance defined by EUCAST breakpoints. Antibiotic key: AMP Ampicillin, AMC Amoxicillin/Clavulanate (2:1), TAZ Piperacillin/Tazobactam, CTX Cefotaxime, CZA Cefazidime/Avibactam, CAZ Cefazidime, CEP Cefepime, C_T Cefotolozone/Tazobactam, ATM Aztreonam, CIP Ciprofloxacin, TOB Tobramycin, AMK Amikacin, GEN Gentamicin, TGC Tigecycline.
ST258 porins impact on fitness in a severe pneumonia model. Our results show that the pore constriction directly contributes to Carbapenem resistance and implies that the combined ST258 OmpK35 and OmpK36 configuration would be advantageous in the face of Carbapenem exposure. We next aimed to investigate if the ST258 OmpK35 and OmpK36 configuration is advantageous, neutral or costly during infection. For this, we developed an acute murine infection model, in which KP is inoculated directly to the trachea and lung parenchyma via placement of an oral endotracheal tube. This mimics the inoculation route in ventilator-associated pneumonia (VAP) encountered in hospital settings. We validated the accuracy and reproducibility of intubation and inoculum delivery using both standard microbiological techniques and in vivo imaging. First, we harvested the lungs immediately after inoculation and enumerated colony forming units (CFU) in the lung parenchyma (Supplementary Fig. 8a). The number of KP reaching the site of infection is reproducible across animals and accurately reflected the inoculation dose (e.g. 1000 CFU). Furthermore, we generated a bioluminescent tagged strain of ICC8001, where the Photorhabdus luminescens bacterial luciferase operon was introduced downstream of the glmS gene. (see details in the Method section). Following intubation of the tagged strain the total flux from the lung region was measured, confirming accurate administration across animals (Supplementary Fig. 8b, c). 3D-diffuse light imaging of this bioluminescent source immediately following inoculation demonstrates distribution deep into the dependent lung zones reproducing the common nidus of infection in the human host (Fig. 4a).

Once the accuracy of the model was confirmed we assessed whether OmpK35ST258 and OmpK36ST258 affect fitness in vivo. First, we inoculated 500 CFU (±10%) of ICC8001, a dose that results in a primary pneumonic focus and secondary bacteraemia. Following intubation, the mice developed a septic phenotype displaying severe dyspnoea, weight loss and become unresponsive to external stimuli. The infected mice reached the severity endpoint at 36h post infection, when quantification revealed 2.0 × 10^9 CFU (mean, n = 10) in the lung tissue and 9.33 × 10^4 CFU/ml (mean, n = 10) in the blood.

We next compared the outcome of infection with each of the three isogenic strains (ICC8002-ICC8004) and with OmpK35ST258ΔOmpK36 (that functionally represents the ΔOmpK35/ΔOmpK36) to confirm the reported phenotype of the double porin mutant in our model (Fig. 4b, c). Inoculating these strains individually illustrated that the OmpK35ST258 truncation (ICC8003) or isofirm variation in OmpK36ST258 (ICC8002) alone or in combination (ICC8004) resulted in significant expansion in the lungs and dissemination to the blood. No significant differences from ICC8001 infection were observed in the total CFU from the lungs or dissemination to the blood. In contrast, the loss of both porins in OmpK35ST258/ΔOmpK36 caused significant attenuation and this strain failed to reach a high pulmonary burden (mean 3.93 × 10^5, n = 10) associated with a low level of bacteraemia detectable in only one mouse (n = 10).

In order to more stringently determine if OmpK35ST258 and OmpK36ST258 have a fitness cost, we tested ICC8002, ICC8003 and ICC8004 in competition with ICC8001 utilising a fluorescent based in vivo competition assay. We chromosomally tagged the strains at the 3rd glmS site with either green (sFGFP) or red fluorescent (mRFP1) protein coding genes and confirmed...
Gly-Asp insertion in L3 of OmpK36 reduces pore diameter, restricts Meropenem diffusion and mediates Meropenem resistance. a Cartoon representation of the OmpK36ST258 trimer. The OmpK36ST258 mutations have been mapped onto the structure. All mutations are shown as orange sticks. The majority of the mutations are found in L3/4. Inset: close-up view of the pore constriction zone. In order to accommodate the GD insertion, L3 has undergone a conformational change, stabilised by a salt-bridge, with subsequent constriction of the pore relative to the OmpK36WT (shown in grey cartoon). b Lateral view of OmpK36 monomer aligned to minimal pore diameter graph, calculated using the HOLE algorithm, demonstrating a reduction in minimal pore diameter in both OmpK36ST258 and OmpK36WT+GD compared with OmpK36WT. c OmpK36 isoforms were reconstituted into proteoliposomes and Meropenem diffusion was assessed by liposomal swelling assay. Meropenem influx is reduced in OmpK36ST258 compared with OmpK36WT. This effect is abolished by GD deletion (OmpK36ST258ΔGD) and reproduced by GD insertion (OmpK36WT+GD) (n = 3 repeats, error bars = ± s.d.). d Calculated Meropenem uptake rate over 20 seconds (ΔOD400nm/Δt(s)) from (c). ***p < 0.0001, error bars ± s.e.m. e GD insertion in OmpK36 isoforms mediates Meropenem resistance assayed by agar dilution in KP (OmpK35ST258 background with KPC-2). Substitution of OmpK36WT with OmpK36WT+GD increases the MIC to that of OmpK36ST258, a phenotypic pattern reversed by GD deletion in the OmpK36ST258ΔGD mutant expression by fluorescent microscopy (Fig. S9a). We confirmed that the cost of fluorescent protein expression was silent by growth in vitro (Supplementary Fig. 9b, c). Furthermore, we verified neither tag confounded competition by co-inoculating ICC8001GFP and ICC8001RFP (250 CFU (±10%) of each), resulting in a 95% confidence interval traversing 50% (mean 51.3%, 95% confidence interval 47–55.7% in the lung and mean 47.12%, 95% confidence interval 37.4–56.8% in the blood) (Fig. 4d and S9). This demonstrated that in vivo competition could be used to assess relative fitness of ICC8002 ICC8004. In contrast to the results of single strain inoculations (Fig. 4b, c), when assessed in competition with ICC8001, ICC8003 (OmpK35ST258) and ICC8002 (OmpK36ST258) showed a trend towards reduced fitness in the lung (Fig. 4e, f), which reached significance in dissemination to the blood (Supplementary Fig. 9e, f). Importantly, ICC8001 completely out-competition ICC8004 (expressing the dual ST258 combination of OmpK35/OmpK36 mutations), with no recoverable ICC8004 in either the lung (Fig. 4g) or blood (Fig. 4h).

In order to exclude the possibility that the out-competition of ICC8004 was due to pleiotropic effects of porin constriction on production of capsule, which is a key KP virulence factor, we quantified abundance of capsule between the different strains. This revealed no difference between ICC8001, ICC8002, ICC8003 and ICC8004 (Supplementary Fig. 10), suggesting that in the absence of antibiotics the ST258 porin composition has a significant fitness cost in KP. Finally, we tested if the GD insertion alone, which mediates increased Carbapenem resistance, is sufficient to reproduce the observed virulence disadvantage during in vivo competition. For this, we carried out a series of infections using L3 GD mutants (on an OmpK35ST258 background), competing OmpK36WT against OmpK36WT+GD and OmpK36ST258 against OmpK36ST258ΔGD. In both sets of isogenic pairs the presence of the GD insertion resulted in almost complete out-competition in the severe murine pneumonia model (Fig. 4i). These data provide direct evidence that the structural determinant conferring porin constriction and retarding antibiotic entry disadvantages KP during infection.

The rapid dissemination of a single global resistant sequence type has occurred in other pathogens, such as ST131 E. coli, another frequently hospital-acquired pathogen. However, the traits conferring a resistant phenotype are proposed to be neutral, i.e. there is no attributable fitness cost in vivo. The attenuation seen in the OmpK35ST258/ΔOmpK36 was evident during infection, resulting in marked failure to replicate efficiently in the lungs and disseminate to the blood. Our OmpK36ST258 variant occupies a middle ground; it is able to expand in vivo to high levels when strains containing this isoform are inoculated alone. However, it demonstrated a marked disadvantage in vivo when in competition with its WT counterpart. We infer from
Fig. 4 The Gly-Asp insertion in Asp L3 causes a fitness disadvantage in an in vivo model of Ventilator-associated pneumonia. a Delivery of bioluminescent KP to the lung parenchyma by intubation recorded by 3D-diffuse light imaging immediately post inoculation. Images were reconstructed into 3D using 3D living image. b Mice were intubated with 500 CFU of ICC8001-ICC8004 or OmpK35ST258/ΔOmpK36 (n = 10/strain, error bars ± s.d.). Enumeration of CFU in lungs and blood, collected at 36 h post infection, revealed no significant difference between ICC8001 and all strains tested except OmpK35ST258/ΔOmpK36. **p < 0.001, ***p < 0.0002. d Competition between ICC8001-GFP and ICC8001-RFP was tested by intubating 250 CFU of each competing strain. Enumeration of CFU of each strain in lungs and blood, identified by their colony fluorescence, was performed at 36 h post infection. Top bar represents mean value, points below represent individual mice (n = 10 per competition, error bars ± 95% confidence interval). No significant difference between ICC8001-GFP and ICC8001-RFP was detected in either the lungs or blood (Fig S9d). e Competition between ICC8001 and ICC8002 (OmpK35ST258) and f ICC8001 and ICC8003 (OmpK36ST258) result in non-significant fitness disadvantage in the lung, but significant attenuation in dissemination to the blood (Fig S9e, f) (n = 10 per competition, error bars ± 95% confidence interval). g Competition between OmpK36 isogenic pairs with or without Gly-Asp Loop 3 insertion demonstrates that in vivo disadvantage results from pore constriction. Top bar represents mean value (n = 20 mice, error bars ± 95% confidence interval), OmpK36WT in competition with OmpK36WT+GD (open circles, each point represents 1 mouse (n = 10)) or OmpK36ST258 in competition with OmpK36ST258GD (closed circles, each point represents 1 mouse (n = 10)).
these data, that in OmpK36ST258, pore diameter reduction is a trade-off between Carbapenem resistance and retaining partial WT functionality. Indeed, we show that diffusion of lactose is reduced in the presence of L3 GD insertion. Whilst this is probably not a substrate utilised by KP in the murine host, it provides a putative mechanism leading to fitness cost in vivo; i.e. the resistance mutations may impair the ability of the strain to compete for resources that are limited during infection.

We provide compelling evidence that the molecular mechanism conferring resistance is actually disadvantageous in vivo. Given KP ST258 success, we cannot rule out the existence of fitness costs in other resistance mutations in successful clades, the elucidation of which could help in our continual fight against the spread of multidrug-resistant pathogens.

Clinically, our data points towards a model where selection pressure imposed by utilisation of Carbapenem in hospital settings, drives the expansion of ST258 KP. Accordingly, we provide evidence to support that we should continue to Start Smart-Then Focus in order to reduce the selective pressure in healthcare, and implement restrictive prescribing policies aimed at minimising the inappropriate use of broad-spectrum agents such as Carbapenems.

Methods

Generation of isogenic strains and tagging. Strains (S), plasmids (V, vector) and primers (P) are listed in the supplementary material (Table 1, S1, S2 and S3, respectively). All genomic deletions and substitutions were made in ICC8001 (strain 1 in Table S2) using a two-step recombination methodology resulting in scarless and markerless mutants as previously described28. The isogenic strains generated are summarised in Table 1. Unless otherwise stated, all vectors were generated by Gibson Assembly (New England Biolabs).

We generated the OmpK35ST258 truncation coding sequence (FJ577672) derived by PCR for the presence of resistance genes (KPC-2 P39/40, OXA-48 P31/32 and meropenem, ertapenem or imipenem. Saturated overnight cultures of test strains were diluted in 0.8% saline and 20 µl (representing 10⁴ CFU) plated and incubated overnight at 37 °C.

Liposome swelling assays. Proteoliposomes were prepared as previously described34. The change in OD₄₀₀ of the mixture was measured for 90 s (1 reading intervals) with a SpectraMax M Series Multi-Mode Microplate Readers (Molecular Devices). A 5 s reading delay was imposed for all measurements in order to reduce initial reading spikes. The isotonic concentration of each substrate was empirically determined by measuring the change in OD₄₀₀ of control liposomes; isotonic solutions showed less than +0.001 units change over 90 s. The first 20 s of the measurements were used for analysis and plotting since they represent the linear decrease of OD₄₀₀. Each curve represents three separate liposome reconstitutions.

Crystallisation. OmpK36WT, OmpK36ST258 and OmpK36WT-R127A were exchanged into 10 mM HEPES pH 7, 150 mM LiCl, and 0.4% C₈E₄ using a PD-10 desalting column (GE Healthcare) and concentrated to 10 mg/ml. Plate-like crystals for OmpK36WT were grown from a solution containing 0.1 M NaCl, 0.1 LSO₄, 0.1 mM Tris HCl pH 8.5 and 30%PEG400 at 4 °C. Needle-like crystals for OmpK35ST258 and OmpK36WT-R127A were grown from a solution containing 0.1 M lithium sulphate, 0.1 M sodium citrate pH 5.6 and 12%PEG400 at 20 °C. The OmpK36WT crystals were cryoprotected by supplementing the crystallisation condition with 25% ethylene glycol and were frozen in liquid nitrogen. OmpK36WT and OmpK36WT-R127A crystals were directly frozen in liquid nitrogen. Diffraction data training and data collection were performed at Diamond Light Source synchrotron.

Data collection. OmpK36ST258 data to 3.23 Å were collected on I04 at Diamond Light Source and processed using autoPROC and STARANISO35. The space group was determined to be P2₁2₁2₁ with six copies of OmpK36WT and OmpK36WT-R127A crystals. The space group was determined to be R2₁ over the entire scale of resolution.

Structure solution and refinement. All structures were determined by molecular replacement in Phaser37 using the OmpK36WTspace (PDB ID: 3O79)38 as search model. Initial refinement of OmpK36ST258 to 3.23 Å was carried out in REFMAC538 and at later stages in Phenix39 with non-crystallographic symmetry (NCS). After rigid body and restrained refinement electron density corresponding to the molecules and insertions were identified, built and refined. Additional density, possibly detergent molecules, that was observed on the surface of the protein was not modelled due to the low resolution. The OmpK36WT and OmpK36WT-R127A structures were refined to 1.92 and 2.0 Å, respectively, in Phenix39.
with NCS. All model building was performed in Coot[8]. Refinement statistics are summarised in Table 2.

**Pore size analysis.** Constriction of the pore diameter was determined by measurement of the channel path by HOLE[14].

**Animal studies.** Animal studies were performed on project license 78413 granted by the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986; ethical approval was granted by the Imperial College Animal Welfare and Ethical Review Body. All animal work complied with relevant ethical regulations for animal testing and research and results are reported in line with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Female BALB/c mice (8–10 week 18–20 g) (Charles River, UK) were housed under a 12 h light/12 h dark light cycle with access to food and water ad libitum. Anaesthesia was induced by i.p. administration of ketamine (80 mg/kg) and medetomidine (1 mg/kg), and procedural recovery took place at 35 °C following the administration of atipamezole (1 mg/kg) reversal. Intubation was achieved by placing a 24 G plastic cannula over a fibre-optic light illuminated cable, through the glottis under direct vision, of mice suspended according the kit protocol (Kent Scientific, CT, USA). In single strain infections inoculum was generated by the dilution of saturated overnight culture (LB) in 1xPBS (final volume 50 µl, final dose 500 CFU). This was pipetted into the hub of the cannula and instilled into the trachea under the inspiratory effort of spontaneously breathing animals. Two 400 µl (approximate to tidal volume) air flushes were used to evenly distribute the inoculum to the distal airways and CFU counts confirmed in the inoculum by plating onto LB Agar and overnight incubation at 37 °C.

3D bioluminescent imaging for the distribution post infection intubation was carried out using the IVIS SpectrumCT (Caliper Life Sciences, Massachusetts, USA). Inoculum (ICC8001_LUX) was prepared by sub-culture of saturated overnight culture (LB) in 1xPBS (final volume 50 µl, final dose 500 CFU). This was pipetted into the hub of the cannula and instilled into the trachea under the inspiratory effort of spontaneously breathing animals. Two 400 µl (approximate to tidal volume) air flushes were used to evenly distribute the inoculum to the distal airways and CFU counts confirmed in the inoculum by plating onto LB Agar and overnight incubation at 37 °C.

**Data availability**

The source data underlying Figs. 1a–e and 3b–d, 2b–l, Supplementary Fig. 4a, b, c, e, Supplementary Fig. 5b–e and Supplementary Fig. 7a–c and Supplementary Fig. 8a–c are available in the Source Data File. The coordinates and structure factors of PDB 6DR3 (OmpK36 WT, 6RPC (OmpK36 ST256)) and 6RCK (OmpK36 WT, +Glu) are available in the Protein Databank.

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Statistical analyses were performed in Prism (Graphpad Software, La Jolla, California, USA). Error bars are described in legends depicting either SD or SEM. Rates for meropenem influx were calculated over the first 20 s of data recording (5–25 s) by non-linear regression without imposed constraints. A multiple comparisons ANOVA, with replacement of ICC8001 (WT) as a control. 95% confidence intervals were calculated in Prism (Graphpad Software, La Jolla, California, USA).

Non-significance represents any p > 0.05, otherwise p values are presented in the legend of figures.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

J.L.C.W. designed the overall strategy and performed the molecular biology, biochemistry and in vivo experiments; he also analysed the data and wrote the paper. L.E.K. performed biochemical and in vivo experiments, she analysed data and wrote the paper. M.R. and H.S.K. performed the structural and proteoliposome assays. They analysed data. W.W.L. analysed the capsule. S.I.B provided clinical input and edited the paper. A.C. participated in supervision, data analysis and edited the paper. K.B. led the structural and proteoliposome aspects of the project, participated in data analysis and wrote the paper. G.F. led the in vivo aspects of the project, participated in data analysis and wrote the paper.

Additional information

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