Genomic profiling reveals distinct routes to complement resistance in *Klebsiella pneumoniae*

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ABSTRACT

The serum complement (C') system is a first line of defense against bacterial invaders. Resistance to killing by serum enhances the capacity of Klebsiella pneumoniae to cause infection, but is an incompletely understood virulence trait. Identifying and characterising the factors responsible for preventing activation of, and killing by, serum C' could inform new approaches to treatment of K. pneumoniae infections. We have used functional genomic profiling to define the genetic basis of C' resistance in four diverse serum-resistant K. pneumoniae strains (NTUH-K2044, B5055, ATCC43816 and RH201207), and explored their recognition by key complement components. Over 90 genes contributed to resistance in one or more strains, but only three, rfah, lpp and arnD, were common to all four. Deletion of the anti-terminator rfah, controlling expression of capsule and O-side chains, resulted in dramatic C' resistance reductions in all strains. The murein lipoprotein gene lpp promoted capsule retention through a mechanism dependent on its C-terminal lysine residue; its deletion led to modest reductions in C' resistance. Binding experiments with the C' components C3b and C5b-9 showed that the underlying mechanism of evasion varied in the four strains: B5055 and NTUH-K2044 appeared to bypass recognition by C' entirely, while ATCC43816 and RH201207 were able to resist killing despite being associated with substantial levels of C5b-9. All rfah and lpp mutants bound C3b and C5b-9 in large quantities. Our findings show that, even amongst this small selection of isolates, K. pneumoniae adopts differing mechanisms and utilises distinct gene sets to avoid C' attack.
The opportunistic pathogen *Klebsiella pneumoniae* is a major public health threat due to its propensity to become extensively drug resistant (1, 2), the emergence of hypervirulent strains (3–5), and the recent evolution and increasing prevalence of strains displaying both hypervirulence and extensive drug resistance (6, 7). Virulence in *K. pneumoniae* is multifactorial and depends on both core-encoded and horizontally-acquired factors (8, 9). Capsule is a critical *K. pneumoniae* virulence determinant present in all clinical strains; mutants lacking capsule are avirulent, whilst overproduction of capsule is associated with hypervirulent strains and more severe disease in animal models (10, 11). Over 130 capsule locus types have been described in *K. pneumoniae* (12), and hypervirulent strains usually produce capsule type K1 or, less frequently, K2. Nine lipopolysaccharide (LPS) O side chain groups have been identified and characterized in *K. pneumoniae* (13); these moieties modulate innate immune signalling and may contribute towards serum resistance. Horizontally-acquired virulence genes include siderophores and capsule up-regulators (9, 14). In general, understanding *K. pneumoniae* pathogenesis is confounded by the phylogenetic breadth of infectious lineages, and by the diversity of the virulence factors themselves.

The complement (C') system, comprising more than twenty proteins in serum and tissue fluids, is a first line of defense against bacterial invaders that have breached the host’s epithelial barriers. Resistance to C’ is strongly correlated with the capacity for systemic survival, multiplication and spread of a wide range of Gram-negative pathogens (15), and is a major virulence trait enabling *K. pneumoniae* to elicit invasive infections (16, 17). The C’ cascade can be activated via the classical, alternative and lectin pathways, which each act in a precise sequence of reactions to facilitate C3b deposition on to the target bacterial surface. The classical pathway is initiated following recognition of antigen-antibody complexes on the bacterial cell surface by hexameric C1q, whereas the lectin pathway begins with detection of bacterial surfaces by pattern recognition molecules such as mannose-binding lectin or the ficolins (15, 18, 19). All pathways converge at C3 cleavage with the larger cleavage product C3b covalently bound to the target surface. Accumulation of anchored C3b...
by amplification leads to the assembly of C5 convertases that generate the C5b cleavage product which spontaneously associates with one molecule each of C6, C7, C8 and multiple copies of C9 to form the C5b-9 membrane attack complex. In C' susceptible bacteria, C5b-9 complexes intercalate into the outer membrane (OM) bilayer and perturb the cytoplasmic membrane through an incompletely defined process (20–22).

Gram-negative bacterial resistance to C' can be due to failure of activation of any of the C' pathways, degradation of activated C' proteins, arrest of activated pathways by C' inhibitors such as C1-inhibitor protein (C1-Inh), factor H (fH) and C4 binding protein (C4bp), or the inability of C5b-9 complexes to assemble and insert into the OM (which can be a result of impedance by bacterial surface structures) (15). The basis of the C' resistance of *K. pneumoniae* is still poorly understood.

Though it has been reported that limiting C' activation and C3b accumulation is the primary mode of resistance, both C' resistant and susceptible clinical isolates and mutants may activate C' cascades after exposure to human serum (23–26). Multiple different factors can influence serum resistance in *K. pneumoniae* including capsule type and amount, O-antigen type, and various surface proteins; capsules and O-antigens have each been invoked as the main determinant of C' resistance (9, 27). However, a recent study of >150 *K. pneumoniae* clinical isolates from Thailand of varying C' susceptibility concluded that susceptibility did not correlate with the presence of specific genes, particular capsule types, or even with the hyper-capsulation phenotype of the isolates (28). This study highlighted the main limitation of collective studies on C' resistance in *K. pneumoniae* to date - that although many resistance factors are individually well-characterised, there is very limited understanding of how their activities play out in different combinations, or across diverse isolates of *K. pneumoniae*.

Untangling the mechanisms behind C' resistance in *K. pneumoniae* will lead to better understanding of the virulence of this bacterium and will provide avenues to target C' resistance in the clinic, particularly in view of growing interest in the targeting of capsule and other virulence factors as an anti-infective strategy for *K. pneumoniae* (29–31). In particular, developing generally applicable
(rather than K type-specific) therapeutics that promote C' killing requires deeper knowledge of the activity of different C' resistance factors in diverse strains. In this study we used functional genomic profiling by transposon-directed insertion site sequencing (TraDIS) to define the genetic basis of serum survival in four diverse strains of *K. pneumoniae*. We show that C' resistance is multifactorial and strain-specific, and identify RfaH and Lpp as shared *K. pneumoniae* resistance determinants. Two of the strains evaded C' evasion by preventing C3b and C5b-9 accumulation at the cell surface, which was disrupted in ΔRfaH and Δlpp mutants, whilst the remaining two strains were resistant to serum despite substantial C5b-9 deposition. Our results present a picture of at least two distinct modes of C' resistance in *K. pneumoniae* and point to RfaH and Lpp as potential targets for C'-sensitizing therapeutics.

RESULTS

Serum resistant isolates of *K. pneumoniae*. Three well-studied hypervirulent *K. pneumoniae* strains and one recently-isolated classical strain were tested for survival in human serum (Table S1, Fig. 1A). B5055 (sequence type ST66) produces a type K2 capsule, and was originally isolated from a sputum sample in the 1920s. NTUH-K2044 is a hypervirulent strain (sequence type 23) producing a K1 capsule, and was the first characterized liver abscess-causing *K. pneumoniae* isolate (32). ATCC43816 is another K2 strain commonly used in mouse virulence studies (33). *K. pneumoniae* RH201207 is a colistin-resistant ST258 strain obtained from Public Health England in 2012 (34). These strains differ in their capsule production as determined by uronic acid assay, with B5055 and NTUH-K2044 producing copious capsule and ATCC43816 and RH201207 producing less (Fig. 1B). All four strains survived exposure to 66% normal human serum (a potent source of C') over a 3 h incubation period at 37 °C when an inoculum of 1 x 10^6 was employed (Fig. 1C); strains B5055, NTUH-K2044 and ATCC43816 proliferated in serum whereas viable counts for RH201207 did not change between 0-2 h but showed a slight reduction at 3 h. A sensitive control strain, *Escherichia coli* DH5α, showed no
viability after 30 min incubation and killing of all strains was completely abrogated by heat inactivation (56 °C, 30 min; data not shown).

TraDIS analysis of C’ resistance in K. pneumoniae isolates. We performed transposon-insertion sequencing of saturated mutant libraries exposed to serum to define the genes contributing to serum survival in each of the four K. pneumoniae strains. The K. pneumoniae B5055 library was constructed for this study by conjugative delivery of pDS1028 and contained 225,000 unique transposon insertions (see Materials and Methods; Table S2), while NTUH-K2044, ATCC43816 and RH201207 mutant library construction has been previously described (34, 35). Our experimental strategy was similar to that used in previous work with E. coli ST131 (36), with libraries treated with either normal human serum or heat-inactivated serum for 90 min, outgrown for 2 hours, and sequenced and mapped using the BioTraDIS pipeline (37). Putative serum resistance genes were defined as those with altered mutant abundance in the serum-treated libraries in comparison to the control libraries treated with heat-inactivated serum (log2-fold change of < -1 or > 1 NS vs HI-S, q-value of <0.005; Table S3). Comparing to the heat-inactivated serum control, rather than the input library, minimises the chance of spurious hits to mutants with general growth defects.

A total of 93 genes were identified that altered serum survival in one or more K. pneumoniae strains (Fig. 2; Table S4), with the number of hits in each strain ranging from 22 (for B5055) to 54 (for RH201207). These genes included 43 core or soft-core K. pneumoniae genes (present in >99% or 95-99% of K. pneumoniae strains), 24 shell genes (15-95% strains) and 26 cloud genes (<15% strains). Despite the high proportion of shell and cloud genes, 60 of the serum survival-related genes were present in all four of the strains examined. Putative serum resistance genes came from multiple functional categories including synthesis of surface polysaccharides, metabolism, cell surface or membrane structure and function, and transcriptional regulation (Fig. 3; Table S4). Overall, there was an unexpected strain specificity in the exact genes identified: even among the 60 hit genes present in all four strains, the majority (35 genes) influenced serum survival in only one strain, 22
genes were hits in two or three strains, and only three genes affected serum survival in all four K. pneumoniae (Fig. 2A; Fig. 3).

**Contribution of capsule to serum resistance.** Capsule biosynthetic genes (*cps*) were among the putative serum survival factors in the four strains investigated. The proportion of *cps* locus genes contributing to C' resistance and the magnitude of the fitness changes involved varied between isolates (Fig. 2B, Fig. 3). Note that mutagenesis of genes of the capsule locus can cause secondary cell envelope defects (shown for *wzo* and *wzi* (38)), and not all *cps* locus mutations entirely eliminate capsule production (35, 39); therefore complete consistency of selection across all genes of the *cps* locus is not expected. With *K. pneumoniae* B5055, which produces copious amounts of K2 capsule, 11/18 genes of the *cps* locus were called as hits, accounting for half of the serum survival determinants of this strain. They included the exporter *wzi*, the sugar precursor genes *manBC, galF* and the majority of K-type specific genes in the central operon of the K2 locus (Fig. 3; Fig. S1). The majority of these genes were also required in the K2 strain ATCC43816 (Fig. 3; Fig. S1), with the exceptions of *wzi*, and the sugar precursor genes *manB* and *ugd* (which had too few reads in this strain for serum-specific effects to be measured). In NTUH-K2044, 8/20 *cps* genes were called as hits and in RH201207 this proportion was 9/19 (Fig. 3; Fig. S1). Because the pDS1028 transposon has transcriptional read-out from one end, transposon insertions are not predicted to dramatically disrupt downstream gene expression in the NTUH-K2044, B5055 and ATCC43816 libraries. This effect is clear in NTUH-K2044, where transposon insertions in several genes of the *cps* locus (*magA/KP1_3714, wzc, wzb, wza/KP1_3718:KP1_2730*, none of which were defined as serum-related) are counter-selected by serum on one strand but unaffected on the other (Fig. S1). The RH201207 library was constructed using a different transposon, and transcriptional read-through is not expected in this library. Our TraDIS results indicate that all *K. pneumoniae* strains require capsule to some extent to withstand serum challenge. Known regulators of capsule biosynthesis also influenced serum survival, including the anti-terminator gene *rfaH* in all four isolates (40, 41), *rmpC* (BN49_pII0025) in B5055 only (42), and *rcsB* in RH201207 (43). We hypothesise that *rcsB* mutant...
showed a serum survival defect only in the RH201207 background because this strain produces less capsule than the other three strains, making it more sensitive to mutations that further reduce capsule expression. Mutation of the *rmpC* gene had no effect in NTUH-K2044; however, this strain encodes both chromosomal and plasmid copies of *rmpC*.

**Contribution of LPS O-side chains to serum resistance.** Enterobacteriaceae lacking LPS O-side chains are generally susceptible to CSb-9-mediated killing (44) and introduction of genes determining O-side chains into a highly C'-sensitive rough *E. coli* strain elicited a large increase in C' resistance (45). With our four *K. pneumoniae*, the majority of O-antigen genes showed a serum fitness defect when mutated (Fig. 2B; Fig. 3), with the exception of those of *K. pneumoniae* B5055. This is surprising because B5055 encodes the same O-antigen type (O1v1) as NTUH-K2044, in which O-antigen mutants showed a drastic fitness defect (Fig. 2B; Fig. 3). We suggest that the B5055 strain is almost completely protected from serum bactericidal activity by its thick K2 capsule, masking the additional protective activity of the O-antigen. *K. pneumoniae* ATCC43816 produces K2 capsule, albeit in lower amounts than B5055, but still required O-antigen for serum survival. These findings suggest that the K2 capsule is sufficient to completely protect from C'-mediated killing when produced in copious amounts, whilst K1 capsule is not, at least in these isolates.

LPS core biosynthetic genes contributed to serum fitness in isolates ATCC43816 and NTUH-K2044 (Table S4), although the same genes were either essential or had no effect on resistance in B5055, and were also not identified as statistically significant hits in RH201207. Note that mutation of many LPS core genes causes a severe general fitness defect, so their specific contributions to serum resistance are not always easy to define. A small subset of the genes (*arnD-arnF*) in the *arn/pmr* operon responsible for LPS lipid A modification showed C' resistance defects in one or more strains when mutated (Fig. 2B). This was unexpected as the L-Ara4N lipid A modification is rarely made in *vitro*, and is not produced in rich media conditions as used in this study (46). Loss of any of the *arnDEF* genes was previously shown to reduce *K. pneumoniae* mucoviscosity in a genome-wide
density-based screen (35), and we suggest that reduced capsule production underpins the serum survival defects seen here.

Other genes implicated in serum survival. Mutation of several genes involved in cell membrane or cell wall structure and function resulted in fitness defects in serum. These genes included dacA (RH201207 only) involved in cell wall biosynthesis, the inner membrane protein dedA (in isolates BS055, NTUH-K2044 and ATCC43816) which has a role in membrane integrity, and components of the tol-pal outer membrane transporter (NTUH-K2044 and ATCC43816). Finally, the outer membrane lipoprotein Lpp was required for full serum resistance in all four strains. A number of metabolic genes were also implicated in serum survival, primarily those involved in pyrimidine metabolism, and metabolism of carbohydrates (Fig. 3; Table S4). Some of these genes (pgi, pgm) are involved in precursor molecule biosynthesis for capsule and LPS.

Increased serum survival genes in K. pneumoniae RH201207. Five genes of K. pneumoniae RH201207, csrD, fabR, wecB, wecC and cyoA, led to increased serum fitness when mutated. CsrD promotes degradation of the capsule-regulating small RNA CsrB; mutation of csrD can promote capsule production (as measured by density) (35), which may explain the enhanced serum survival of this mutant. FabR, WecB and WecC are not predicted to affect capsule, but all three genes have roles relating to the cell envelope: FabR is a transcriptional regulator which controls the ratio of saturated to unsaturated fatty acids in the cell membrane, and WecB and WecC produce the second component of enterobacterial common antigen (N-acetyl-D-mannosaminuronic acid) and attach this to UDP-GlcNac. We speculate that loss of wecB and wecC increases the pool of UDP-GlcNac in the cell, which is then diverted into O-antigen biosynthesis (which also utilise UDP-GlcNac) (47). The cytochrome ubiquinol oxidase component CyoA also resulted in increased serum survival when mutated through an unknown mechanism. The identification of mutants with increased serum fitness in RH201207, but not the other K. pneumoniae strains, is consistent with the observation that serum survival of K. pneumoniae RH201207 is less dependent on capsule.
Confirmation of RfaH and Lpp as shared serum resistance factors in *K. pneumoniae* isolates. Only three genes affected serum survival in all four strains tested: the LPS modification gene *arnD*, the outer membrane lipoprotein *lpp*, and the transcription anti-terminator *rfaH* (Fig. 2; Fig. 3). We selected Lpp and RfaH for further characterisation as potential core serum resistance factors of *K. pneumoniae*. ArnD was not selected for follow-up because we failed to detect the relevant LPS modification in vitro (which is consistent with previous reports that the modification is made in vitro only under very specific conditions (46)) and therefore presumed its activity was indirect, though the potential role of lipid A modifications in *K. pneumoniae* C′ resistance may be of interest for a future study. Deletion mutants of *rfaH* and *lpp* were constructed in *K. pneumoniae* NTUH-K2044, B5055 and ATCC43816 by allelic exchange. In isolate RH201207, an insertion mutant in *rfaH* was obtained but a ∆*lpp* mutant could not be generated despite multiple attempts. Serum survival assays were conducted with an inoculum of 10⁶ cells in 66% normal human serum and bacterial counts monitored for 3 h (Fig. 4). Loss of *rfaH* caused a large reduction in serum survival in all four strains, and complementation of with plasmid-encoded *rfaH* expressed from its native promoter restored wild-type survival, confirming the importance of RfaH in C′ resistance (Fig 4). Loss of *lpp* caused a modest change in C′ sensitivity (Fig 4); these mutants lost the ability to proliferate in serum (note that *lpp* disruption does not cause a general growth defect, Fig S2B and Table S3), and with NTUH-K2044 ∆*lpp* delayed C′ killing was observed. The ∆*lpp* mutations could not be complemented by expression of *lpp* from its native promoter due to unexpected toxicity during cloning. Expression of *lpp* from an arabinose-inducible promoter also failed to complement the serum proliferation defect of the ∆*lpp* mutants. We suspect that this was due to insufficient expression. In addition, proliferation of the vector-only control strains was impaired by addition of arabinose (data not shown). Though we were unable to find an appropriate system for complementation of the ∆*lpp* mutants, their phenotypes align with the results of the genome-scale screens (Fig 2, Fig 3), as well as published work on Lpp in *K. pneumoniae* NTUH-K2044 (48).
We were intrigued by the variable requirement for different genes of the *cps* locus seen in TraDIS, and several randomly-isolated capsule locus mutants of ATCC43816 and RH201207 were also examined for serum survival in order to further validate the genome-scale screens (Fig S2). Each of these mutants showed the phenotype predicted based on TraDIS screening: ATCC43816 i-wcaJ, which was not identified as a serum resistance gene, multiplied to the same extent as wild type, ATCC43816 i-wza did not proliferate in serum, and RH201207 i-wzc was rapidly susceptible. RH201207 i-wcaJ, which was not a hit, was viable after 90 minutes (our TraDIS timepoint), but showed a delayed susceptibility to serum. Note that wcaJ deletion in *K. pneumoniae* does not completely eliminate K2 capsule production, and can also have pleiotrophic effects including rounded cell morphology and increased fitness under nutrient limitation (38, 39) – therefore, the full resistance of ATCC43816 i-wcaJ does not preclude a role for capsule in the C’ resistance of this strain. Taken together, the results of serum survival assays with defined mutants show perfect agreement with the phenotypes predicted from TraDIS screens (for 11/11 mutants), and establish RfaH and Lpp as shared serum resistance factors in *K. pneumoniae*. These experiments also revealed additional subtleties in the serum resistance phenotypes of the mutants, with survival patterns roughly following the underlying resistance of the parent strain (for example, ATCC43816 ΔrfaH and RH201207 ΔrfaH), and some differences only revealed at later stages of incubation (eg. RH201207 i-wcaJ).

Lpp influences capsule retention but not capsule production and requires lysine-78. The antiterminator RfaH and the murein lipoprotein Lpp contributed to C’ resistance in all four *K. pneumoniae* strains. Lpp is an extremely abundant protein which contributes to cell envelope integrity by connecting peptidoglycan to the cell outer membrane (48, 49). We observed that the Δlpp mutant colonies were flat and unstructured in comparison to wildtype, although their opacity suggested they still produced capsule. To examine the effect of the *lpp* mutation further we measured total and cell-attached capsule using the uronic acid assay. All three *K. pneumoniae* Δlpp mutants produced capsule at wildtype levels, but showed moderate decreases in amounts of cell-
associated capsule (Fig 5A). Mutants of rfaH showed dramatically reduced capsule amounts (Fig 5B).

We then tested whether Lpp activity requires covalent linkage to peptidoglycan, mediated through the ε-amino group of the C-terminal lysine residue in Lpp and the meso-diaminopimelic acid residue on the peptidoglycan peptide stem (50). Expression of Lpp from an arabinose-inducible vector partially complemented the hypermucoid phenotype of NTUH-K2044 and B5055 (Fig 5C). Partial complementation was not seen with an Lpp-ΔK78 construct, confirming that the C-terminal lysine is required in order for Lpp to promote capsule retention in both K1 and K2 strains. The shared serum survival factors Lpp and RfaH therefore both appear to function at least partly through effects on capsule.

**Deposition of C3b and C5b-9 complexes.** Genome-scale screening revealed a very high degree of strain specificity in the serum resistance determinants across four *K. pneumoniae* strains. We decided to explore complement activation by these strains, and how this is affected by loss of rfaH or lpp. Activation of any or all C’ pathways will lead to C3b generation and binding to the target bacterial surface; subsequent formation of C5 convertase complexes may lead to deposition of membrane attack complexes and cell death (15, 19). Surface C3b deposition and C5b-9 formation on *K. pneumoniae* strains and mutants during incubation with human serum are reported in Figs 6, 7, S3 and S4. Unlike the three hypervirulent strains that showed little to no C3b binding, serum exposure of RH201207 led to a considerable increase in levels of C3b and C5b-9 over time (Fig 6A, 7A, S3, S4A). ATCC43816 also showed C5b-9 binding at later time points, while B5055 and NTUH-K2044 did not. In all backgrounds, the deletion of rfaH led to significant levels of C3b and C5b-9 binding compared to wild type, with a peak after 2-3 hours of serum exposure (Fig 6A and 7A), confirming that the mechanism of serum killing observed (Fig 4) is through formation of the membrane attack complex.

With B5055ΔrfaH, cells could not be examined beyond the 30 min time point due to cell lysis as determined by the release of cytoplasmic GFP from strain B5055 ΔrfaH pFLS21 (Fig S4B). Imaging of the ΔrfaH mutants showed that C3b binding is evenly distributed over the cell surface and occurs within 5 min of serum exposure (Fig 6B), whilst C5-9 deposition is minimal at 5 min (except for...
ATCC43816) and uniformly detected at 15 min (Fig 7B). Similarly, Δlpp mutants were also found to significantly bind C3b and C5b-9 compared to wild type, though to a lesser extent than the Δrfah mutants (Fig 6A, 7A, S3 and S4). Most Δlpp mutant cells maintained their rod-shape following 15 min of serum exposure (Fig 6C, 7C) which correlates with increased serum susceptibility only after longer exposure times (Fig 4). By examining cell population dynamics we observed that Δlpp mutants showed a similar distribution to wild type cells (Fig S3 and S4A, third columns). In contrast, Δrfah mutants displayed a more compact distribution in Q2 quadrant, suggesting that not only do more Δrfah cells bind C3b and C5b-9 over time, but that the level of binding to individual cells increases. These findings indicate that B5055, NTUH-K2044, ATCC43816 and RH201207 activate the complement system to different extents, and that loss of lpp or rfah increases the recruitment of complement components.

DISCUSSION

Resistance to killing by C′ is an important yet incompletely understood feature of K. pneumoniae pathogenesis (4, 8, 28). The prominent polysaccharide capsule has been invoked as a key determinant of resistance by virtue of its capacity to limit C3b deposition or assembly of the membrane attack complex (8, 27) but it is clear that other factors also contribute to the C′ resistant phenotype (28). Resistance to serum killing is associated with K. pneumoniae hypervirulence and we therefore selected three well-studied hypervirulent strains as well as a recently-isolated clinical strain for our analyses. To our knowledge, this study represents the first multi-strain functional genomics study of C′ resistance in any bacterial species.

TraDIS identified 93 genes that impacted serum survival in one or more strains but only three of these, rfah, lpp and arnD, were common to all four strains. All three genes influence the physical characteristics of the outer surface of K. pneumoniae. RfaH controls transcription of operons that direct synthesis, assembly and export of the lipopolysaccharide core and capsular polysaccharide in E. coli and other gram-negative bacteria (41), the abundant peptidoglycan-linked outer membrane
protein Lpp is involved in the maintenance of cell envelope integrity and retention of capsule at the cell surface (Fig 5A)(35, 50), and the arr operon encodes proteins that participate in the addition of 4-amino-4-deoxy-L-arabinose to lipid A (51) and may also affect capsule levels through an unknown mechanism (35). Deletion of rfaH markedly increased C’ susceptibility in all four strains, confirming the key contributions of capsule and LPS O-side chains to the resistant phenotype. However, there were strain differences in the rate of C’ killing; K. pneumoniae RH201207ΔrfaH was rapidly killed, while ATCC43816 and NTUH-K2044 displayed a delayed killing response typical of smooth (O-side chain-replete) C’ susceptible Gram-negative bacteria (52). RH201207 possesses LPS O-side chains but elaborates less capsule than the other three. These C’ susceptibility profiles emphasize the interdependence of the various surface structures that contribute to serum resistance. Deletion of lpp in the three hypervirulent isolates modified the serum responses but to a variable degree: the loss of proliferation in serum of strains ATCC43816 and B5055 was not sufficient to convert them to full C’ susceptibility whereas the degree of C’ killing of K. pneumoniae NTUH-K2044Δlpp was more pronounced. Although capsule retention is impaired in the lpp mutants, reducing the protective barrier against C’ binding, the presence of large amounts of unattached polysaccharide is likely to have caused off-target C’ activation (26) and depletion of C’ components in the serum, resulting in less pronounced killing than rfaH mutants.

Removal of the capsule by deletion of rfaH (Fig 5B) led to significant deposition of C3b on the outer surface in all four strain backgrounds. Deletion of rfaH presumably caused loss of O-side chains as well as capsule, as shown in E coli and other gram-negative bacteria including Salmonella enterica and Yersinia enterocolitica (41, 53, 54). The formation of C5b-9 complexes at the cell surface and subsequent changes in cell morphology point to a loss in integrity of both the outer membrane and peptidoglycan layer, eventually leading to cell lysis, though the exact mechanism by which the inner membrane is disrupted is not yet understood. With the Δlpp mutants, which have detached capsule (Fig 5) and increased membrane permeability but retain their O-antigen (48), sufficient deposition of C3b and perturbation of the cell envelope by C5b-9 complexes occurred to prevent proliferation in
serum as seen in Fig. 4. While B5055 and NTUH-K2044 did not show detectable C3b or C5b-9 levels by flow cytometry, the C' resistant ATCC43816 showed a limited increase in levels of C5b-9 complexes following serum exposure, despite these not functioning as bactericidal entities (Figs 6A & 7A; Fig. S4A). Finally, although the classical isolate RH201207 survived 2-3 h serum incubation, both C3b and C5b-9 levels rose dramatically following incubation with serum.

These differences in the interplay between surface factors and the C' system are unlikely to be due to differences in strain-to-strain gene content. Around half of the hit genes were present in all four strains but contributed to complement resistance in only one or two (46 of 93 total genes, 60 of which were present in all strains), and this trend held when the classical RH201207 strain was excluded (of 36 hits in B5055-NTUH-K2044-ATCC43816 shared genes, 12 were specific to one strain, 19 were hits in two strains and only 5 were hits in 3 strains). However, the degree of strain specificity we found is broadly comparable to that observed for daptomycin resistance genes in two strains of Streptococcus pneumoniae, which showed only 50% overlap despite the two strains sharing 85% of their genes (55). Furthermore, bacteria such as Salmonella spp, Mycobacterium tuberculosis and Pseudomonas aeruginosa have been shown to possess strain-specific essential gene sets by TraDIS/TnSeq methods (56–58).

Strain specific effects are likely to be due to a combination of imperfect hit identification, functional divergence of genes in different strains, and context-dependent fitness contributions of genes with the same activity, due to either redundancy with other factors or differences in the relative contribution of each gene to overall bacterial surface architecture. For example, the O1v2-type O-antigen produced by both NTUH-K2044 and B5055 contributed to serum resistance only in the former strain, presumably because in B5055 the protection from the capsule is so strong that other factors are not needed. We speculate that such context-dependent fitness effects may be a common feature in bacterial populations. Our finding that vastly different gene sets underpin serum survival in four strains support the notion that serum resistance is determined by the overall biophysical
properties of the cell surface, rather than any single factor, and also show that there are multiple routes by which a C'-resistant cell surface can be generated.

A limitation of our study is that *K. pneumoniae* is a highly genetically diverse species (59), and the four isolates that we studied do not cover the range of potential combinations of cell surface structures that may impact survival in serum. We did note that the classical strain RH201207 was markedly different from the three hypervirulent strains in terms of genes involved in C’ resistance and C’ binding patterns; it would be useful to explore the properties of other classical strains in future studies. Another limitation is that in order to maintain library diversity and provide enough material for sequencing we based our TraDIS strategy on that used by Phan and co-workers (36), employing a library inoculum of 10⁸ CFU with only a single 90 min time point, which may have missed delayed or subtle effects on C’ resistance. Though high-throughput mutagenesis studies such as ours are the only way to profile the contributions of all non-essential genes to serum survival, mutation or deletion of genes encoding major surface structures (such as capsule, LPS O-side chains and abundant membrane proteins) may force a major reconfiguration of the cell surface as a compensatory mechanism to deal with envelope stress (60); conversion of C'-resistant cells to C'-susceptible could be a consequence of this compensatory response as well as loss of the structure itself. In particular, capsule locus mutations can have a range of secondary effects including changes to cell envelope integrity, cell morphology and growth rate, and some do not fully abolish capsule production (38, 39). Such effects cannot be detected or avoided by employing different mutagenesis strategies (eg. gene deletion vs transposon insertion) or by complementation. While these findings fit with the range of serum resistance phenotypes we observed among different cps locus mutants (Fig S1 and Fig S2A), they also suggest that any data derived from capsule mutant strains should be interpreted with caution. More direct information comes from recent studies using phage-derived capsule depolymerases, where *K. pneumoniae* strains are stripped of capsule prior to treatment with serum. In this way, at least eight different capsular types of *K. pneumoniae* have been confirmed to protect from serum to date, including type K1 (of NTUH-K2044) (61–66).
change in serum sensitivity following enzymatic capsule removal varies depending on both the strain and the capsule type. We are currently examining the impact of capsule removal on complement susceptibility in systematic fashion using enzymes selective for the most frequently isolated K. pneumoniae capsular serotypes. Despite its inherent limitations, our genome-scale screening gives a picture consistent with recent phage depolymerase work and the collective molecular microbiological studies (9, 27)– K. pneumoniae capsule can protect from serum killing, and the strength of this protection depends on capsule type, capsule thickness and the strain background.

The data suggest that K. pneumoniae may adopt different strategies for evasion of C1- mediated attack. Isolates may fail to strongly activate C1 pathways (B5055; NTUH-K2044) or activate one or more pathways but avoid C5b-9-mediated lethality (ATCC43816). With either scenario the capsule is likely to be critical. Implicit in the design of bactericidal assays is the assumption that normal human serum contains IgM or IgG subclasses directed against exposed bacterial surface antigens with the capacity to efficiently activate the classical pathway (67); this is certainly the case with much-studied E. coli strains but less clear with K. pneumoniae. After activation, C5b-9 will engender lethal membrane damage only after disruption of lipid domains on the bacterial surface, resulting in a drastic change to membrane topology and architecture. C1 resistant bacteria may not only mask their cell surface from the initial recognition by the three C1 pathways, but also inhibit later stages of the C1 pathway by altering their surface configuration in response to envelope stress and preventing membrane insertion and MAC pore formation. Our findings that distinct K. pneumoniae strains can have distinct C1 evasion mechanisms, underpinned by dramatically different gene sets, highlights the complexity associated with predicting serum resistance based on genome sequence or single virulence factors – an undertaking which is not yet possible for K. pneumoniae (28). A comprehensive understanding of the basis of C1-resistance in Gram-negative bacteria will only be forthcoming when the behavior of such clinically relevant pathogens can be explained.

MATERIALS AND METHODS
Construction of the *K. pneumoniae* B5055 TraDIS library. The *K. pneumoniae* B5055 transposon mutant library was constructed by conjugation with *E. coli* β2163 pDS1028 as described (35), with selection of transposon-containing *K. pneumoniae* B5055 colonies performed at 25 °C on LB agar supplemented with 25 μg/ml chloramphenicol. Approximately 600,000 colonies were scraped, pooled and used as the final B5055 TraDIS library.

Serum challenge of TraDIS libraries. Experiments were performed in biological triplicate. TraDIS libraries were grown overnight in 10 ml LB with an inoculum of 10-20 μl, which was sufficient to ensure representation of the entire mutant library. Overnight cultures were diluted 1:25, subcultured in 25 ml LB in a 250 ml flask, and grown 37°C at 180 rpm on an orbital incubator to OD600 of 1. A 1ml aliquot of each culture was centrifuged for two min at 8000 g and resuspended in sterile PBS. 500 μl of bacterial suspension was added to 500 μl normal human serum (Sigma, S7023) and incubated at 37°C for 90 min. Control reactions were performed in the same way, except that serum was heat-inactivated at 56°C for 30 min prior to use. Following incubation, serum reactions were centrifuged, the pellets suspended in 10 ml LB and the surviving bacteria outgrown at 37°C for 2 h.

DNA extraction and next-generation sequencing. Genomic DNA (gDNA) was purified by phenol-chloroform extraction; 1-2 μg DNA was used for the construction of the TraDIS sequencing libraries as described previously (37). Amplification of transposon junctions was performed using primer FS108 (NTUH-K2044, B5055 and ATCC43816 libraries) or TnStetR_5PCR (RH201207 library). Libraries from the RH201207 strain were sequenced on the Illumina Miseq platform using primer TnStetR_5Seq. All other libraries were sequenced on the Illumina HiSeq platform using FS107. Sequencing was performed as described previously (37).

Analysis of TraDIS data. TraDIS sequencing reads were analyzed using the BioTraDIS pipeline as described previously (37, 68), with the following parameters passed to the bacteria_tradis script: "-v --smalt_y 0.96 --smalt_r -1 -t TAAGAGACAG -mm -1". Reads and insertion sites were assigned to each gene using a custom script (available at https://github.com/francescashort/tradis_scripts/tradis_insert_sites_FS.py), with reads mapping to the 3’ 10% of the gene
ignored. Output samples following treatment with serum or heat-inactivated serum were compared to the input sample, and to each other, using the tradis_comparison.R script without filtering. Hits were defined as those genes with log2FC < -1 or log2FC > 1, q-value < 0.005. Values reported in the manuscript are for serum compared to heat-inactivated serum. A previously generated pangenome(35) from a global collection of 265 *K. pneumoniae* strains(59) was used to identify orthologs between strains and to classify genes as belonging to the *K. pneumoniae* core or accessory genome. Where needed, pathway information on specific genes was extracted from Biocyc(47).

**Quantification of capsule.** Capsule production was measured using an assay for uronic acids as described previously (69). Overnight cultures of *K. pneumoniae* were grown in LB at 37 °C and 500 µl aliquots were used directly in the assay. To examine cell-attached capsule, the 500 µl culture samples were centrifuged at 8000 g for 2 min and cell pellets were then suspended in 500 µl fresh LB medium prior to uronic acid quantification. A standard curve of glucuronic acid (Sigma-Aldrich) was used to calculate uronic acid concentrations.

**Serum survival assays.** Bacteria were grown overnight in LB, subcultured 1:100 in fresh LB medium, and grown to late exponential phase (OD\textsubscript{600} = 1). Cultures were then washed once in PBS and diluted 1:100 in sterile phosphate-buffered saline; 50 µl diluted culture was added to 100 µl pre-warmed human serum (Sigma) and incubated at 37 °C. Samples were taken at set time points, serially diluted and plated for enumeration of viable bacteria.

**Hypermucoidy assay.** Overnight cultures of the strains of interest carrying pBAD33-derived Lpp expression plasmids (Table S1) were grown in LB supplemented with 25 µg/ml chloramphenicol, and subcultured for 5 h in LB + 0.1% L-Ara to induce vector expression. Cultures were centrifuged at 1000 g for 5 min, and hypermucoidy expressed as a ratio of OD\textsubscript{600} of the supernatant / OD\textsubscript{600} of the original culture.

**Construction of mutants.** Clean single-gene deletion mutants in *K. pneumoniae* were constructed as described (35) using pKNG101Tc-derived allelic exchange vectors introduced by conjugation with *E. coli* β2163 as donor strain. Details of the plasmids and oligonucleotides used in mutant construction
are in Table S1. Defined transposon insertion mutants of ATCC43816 and RH201207 were isolated by subjecting the relevant TraDIS library to two rounds of density-gradient centrifugation (70). The non-capsulated fraction was grown as single colonies and mutant locations identified by random-primed PCR as described (71) using primers FS57-FS60 together with FS108-109 for ATCC43816 and FS346-347 for RH201207 (Table S1). Complementation plasmids were constructed using the primers listed in Table S1 and introduced by electroporation.

**Detection of surface-located C′ components.** Early mid-logarithmic-phase LB cultures (1ml) were washed in gelatin-veronal-buffered saline containing Mg^{2+} and Ca^{2+} (pH 7.35) (GVB^{2+}), and incubated in 66% prewarmed (37°C) pooled human serum (MP Biomedicals) for 15, 30, 60, 120 and 180 min (1). Prewarmed, heat-inactivated (56°C; 30 min) human pooled serum served to set T0. Human C3-deficient and C5-deficient serum (Sigma) were used as negative controls. For flow cytometric staining, after incubation the mixtures were washed in PBS and approximately 1x10^6 cells were stained. C3b binding was detected with a mouse monoclonal APC anti-C3b/iC3b antibody (Biolegend) (4 µl per 10^6 cells) and C5b-9 formation was detected by indirectly staining cells with 8µg/ml mouse anti-C5b-9 antibody [aE11] (abcam) as primary antibody and 2.5µg/ml Alexa Fluor®488 goat anti-mouse IgG H&L (abcam) as secondary antibody. After 20 min incubation at RT, mixtures were washed and suspended in 200 µl PBS. Samples were acquired using a MACSQuant® instrument (Miltenyi Biotec) within 60 min. Approximately 40,000 cell events were collected. Flow cytometry data analysis was carried out using FlowJo 10 Software. Graphpad 7.05 software was used for graph design and statistical analysis.

For microscopy, samples of early mid-logarithmic-phase LB cultures (equivalent to 1ml of OD_{600} of 0.5) were washed in GVB^{2+}, and incubated with serum for 0, 5 or 15 min. Cells were then washed with PBS, separated into two aliquots and stained with either 10 µg/ml mouse monoclonal APC anti-C3b/iC3b antibody (Biolegend) or 10 µg/ml mouse anti-C5b-9 antibody [aE11] (abcam) followed by 10µg/ml Alexa Fluor®488 goat anti-mouse IgG H&L (abcam) for 10min at RT. Cells were washed with PBS following each staining step, resuspended in PBS and mounted on 1% PBS agarose pads for.
imaging. Highly inclined and laminated optical sheet (HILO) microscopy was performed using the Nanoimager S MarkII from ONI (Oxford Nanoimaging) equipped with lasers 473nm/300mW (10%), 640nm/300mW (7%), dual emission channel split at 560nm, 100x oil-immersion objective (Olympus, numerical aperture (NA) 1.49) and an ORCA-Flash4.0 V3 CMOS camera (Hamamatsu). Images were acquired at an illumination angle of 51° with 100ms exposures for >40 frames and processed using FIJI software (72). In brief, transillumination images were generated as an average of 10 frames (total of 1sec exposure) while fluorescence images were processed by averaging 40 images (total of 4sec exposure). Brightness and contrast of images in Figs 6 & 7 are normalised.

**Measurement of cytoplasmic marker release by B5055 ΔrfaH**

Bacteria containing pFLS21 (Table S1), a pDiGc (73) derivative which expresses GFP from the constitutive rpsM promoter, were grown to early log-phase in LB medium, and washed once in PBS. 250 µl undiluted cell suspension was combined with 500 µl serum or heat-inactivated serum and incubated at 37 °C. The total GFP fluorescence of a 100 µl sample, and the fluorescence of 100 µl of supernatant following centrifugation at 8000g for 2 min, was measured in a Pherastar fluorimeter at set time points following incubation. Values were calculated as a percentage supernatant fluorescence intensity/total fluorescence intensity, with background signal from 66% human serum with PBS subtracted.

**Statistical analysis**

TraDIS comparisons were conducted using EdgeR as implemented in the BioTraDIS pipeline, for which the statistical approaches have been described in detail. The Benjamini-Hochberg correction for multiple testing was applied.

All quantitative experiments were performed in biological triplicate, with the exception of those shown in Fig S2B (n = 2, 7 technical replicates) and Fig S4B (n = 2). All graphs show mean ± 1SD, and statistical significance is indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 throughout. Serum survival data were compared between bacterial strains by two-factor repeated measures ANOVA on log_{10}-transformed bacterial viable counts with Huyhn and Feldt correction. Where the
ANOVA indicated a significant time*strain interaction, viability at \( t = 180 \) was compared by one-way ANOVA with Dunnett’s test for multiple comparisons. Uronic acid quantification and hypermucoidy data were compared between strains by one-way ANOVA on untransformed data followed by Dunnett’s post-hoc test to compare multiple strains to a single reference, or the Tukey-Kramer test for all-against-all comparisons. Complement binding time series data were tested for significance by two-factor repeated measures ANOVA on untransformed data, followed by Fisher’s protected LSD test to compare mutant to wild-type at individual time points.

**DATA AVAILABILITY**

The TraDIS sequencing data generated for this study has been deposited in the European Nucleotide Archive (ENA) under project PRJEB20200. Sample-wise accession numbers are provided in Table S2.

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Figures

**Fig 1. Characteristics of *K. pneumoniae* strains used in this study** (A) Schematic of the four strains used in this study, with sequence type, O-antigen and capsule types indicated. (B) Quantification of capsular uronic acids in four *K. pneumoniae* strains (n = 3). Statistically significant differences between strains were determined by one-way ANOVA (overall p<0.0001) followed by Tukey’s HSD test, **p<0.01, ***p<0.001, ****p<0.0001. Overall significance is indicated above chart. (C) Resistance of the four *K. pneumoniae* strains to killing by pooled human serum (n = 3). Strains were compared by two-factor repeated measures ANOVA (overall p<0.0001), and Tukey’s HSD test at t=180 showed RH201207 to be significantly different from each of the other three strains, ***p<0.001.

**Fig 2. Genes contributing to serum resistance in four *K. pneumoniae* strains** (A) Venn diagram showing the overlap in genes involved in serum survival in each strain. Hit genes are defined as those with a log2FC < -1 or >1, q-value < 0.005. Full results are in Table S3 and TraDIS hits in Table S4. (B) Abundance of transposon mutants following serum treatment, relative to treatment with heat-inactivated serum. Volcano plots of log2-fold change and log10 p-value are shown for each strain. Genes with very low read counts in any condition are excluded. Key resistance factors (capsule, O-antigen, Lpp, RfaH and ArnDEF) are indicated by colour.

**Fig 3. Strain specificity of complement resistance in *K. pneumoniae*** Discontinuous heatmap of TraDIS hits for complement resistance. Homologues across different strains were determined by BLASTp (cutoff >90% amino acid identity) in the process of constructing the *K. pneumoniae* pangenome (see materials and methods). Capsule and O-antigen locus types was determined using Kaptive-Web and the corresponding gene names are used. The three genes outside of the capsule and LPS loci that were required for complement resistance in all four strains are indicated in red text. Genes marked “NA” are either absent from that strain, or have been excluded from the comparative analysis due to having very low read counts in any condition. Full details are in Table S3 and Table S4.
Fig 4. Validation of serum survival defects in ∆rfaH and ∆lpp mutants

Total bacterial viable count of 782 K. pneumoniae strains and key mutants following incubation with 66% pooled normal human serum (see Materials and Methods). The detection limit of the assay is 2x 10^3 viable cells per ml. Overall statistical significance was determined by two-factor repeated measures ANOVA (p<0.0001 for all strains), mutants were compared to wild-type at t=180 by single-factor ANOVA and Dunnett’s test at t=180 (**p<0.01). ATCC43816 WT and mutants n = 5, all other strains n = 3.

Fig 5. Effects of Lpp and RfaH on capsule production and retention

(A) Comparison of total and cell-associated capsule content of wild-type and ∆lpp mutants of K. pneumoniae ATCC43816, B5055 and NTUH-K2044 (n = 3). Uronic acids were either quantified directly from culture or following a single wash and resuspension in LB (see Materials and Methods), and ∆lpp values were normalised to the WT from the same strain and condition. All three strains showed a significant reduction in cell-associated capsule, while total capsule was unchanged or increased (one-way ANOVA relative to WT, *p<0.05 **p<0.01 ***p<0.001). (B) Comparison of capsule production in ∆rfaH mutant and complemented mutant strains (n = 3). Overall statistical significance for each strain was determined by one-way ANOVA, ∆rfaH mutant and complemented strains were compared to WT by Dunnett’s post-hoc test. RH201207 ∆rfaH was compared to WT by one-way ANOVA. *p<0.05, **p<0.01. (C) Partial complementation of K. pneumoniae ∆lpp mutants using an inducible vector (n = 3). The hypermucoidy assay for capsule was performed on stationary-phase, arabinose-induced cultures washed once in PBS. Induction of wild-type Lpp partially restored the hypermucoid phenotype of the NTUH-K2044 and B5055 ∆lpp mutants. This effect was not seen with the empty vector, or with an Lpp construct lacking its C-terminal lysine (∆K78). Overall significance for each strain was determined by one-way ANOVA, followed by Dunnett’s post-hoc test to compare each WT or complemented strain to ∆lpp + vector. *p<0.05, **p<0.01.

Fig 6. C3b binding to the bacterial cell surface

(A) Flow cytometry-based determination of C3b binding to ATCC43816, B5055, NTUH-K2044, RH201207 and their respective mutants were measured after 15, 30, 60, 120 and 180 min incubation in human pooled serum at 37°C (n = 3). Values were...
Fig 7. C5b-9 binding to the bacterial cell surface (A) Flow cytometry-based determination of C5b-9 formation on ATCC43816, B5055, NTUH-K2044, RH201207 and their respective mutants after 15, 30, 60, 120 and 180 min incubation in human pooled serum at 37°C (n = 3). Values were converted to A.U., setting T0 to 1. Two-way repeated measures ANOVA with uncorrected Fisher’s LSD test revealed: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001; ****, P ≤ 0.0001. For B5055 ΔrfaH, data was collected only at the first two time points due to cell lysis detected by release of cytoplasmic fluorescent marker from labelled cells. (B-C) Fluorescence microscopy of 5 min and 15 min serum exposed ΔrfaH and WT cells (B) or 15 min serum exposed Δlpp and WT cells (C) following incubation with APC conjugated anti-C3b antibody. Data are representative of three independent experiments. For comparison of binding patterns and intensity, transillumination (left) and fluorescence images (right) are normalised within each panel. Scale bar, 2μm.
