



Kingella kingae Surface Polysaccharides Promote Resistance to Human Serum and Virulence in a Juvenile Rat Model

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ABSTRACT *Kingella kingae* is a Gram-negative coccobacillus that is increasingly being recognized as an important cause of invasive disease in young children. The pathogenesis of *K. kingae* disease begins with colonization of the oropharynx, followed by invasion of the bloodstream, survival in the intravascular space, and dissemination to distant sites. Recent studies have revealed that *K. kingae* produces a number of surface factors that may contribute to the pathogenic process, including a polysaccharide capsule and an exopolysaccharide. In this study, we observed that *K. kingae* was highly resistant to the bactericidal effects of human serum complement. Using mutant strains deficient in expression of capsule, exopolysaccharide, or both in assays with human serum, we found that elimination of both capsule and exopolysaccharide was required for efficient binding of IgG, IgM, C4b, and C3b to the bacterial surface and for complement-mediated killing. Abrogation of the classical complement pathway using EGTA-treated human serum restored survival to wild-type levels by the mutant lacking both capsule and exopolysaccharide, demonstrating that capsule and exopolysaccharide promote resistance to the classical complement pathway. Consistent with these results, loss of both capsule and exopolysaccharide eliminated invasive disease in juvenile rats with an intact complement system but not in rats lacking complement. Based on these observations, we conclude that the capsule and the exopolysaccharide have important redundant roles in promoting survival of *K. kingae* in human serum. Each of these surface factors is sufficient by itself to fully prevent serum opsonin deposition and complement-mediated killing of *K. kingae*, ultimately facilitating intravascular survival and promoting *K. kingae* invasive disease.

KEYWORDS *Kingella kingae*, capsule, exopolysaccharide, serum resistance

The encapsulated Gram-negative coccobacillus *Kingella kingae* is a member of the commensal flora in the oropharynx in young children and is emerging as an important pathogen in the pediatric population (1). Recent epidemiological studies using sensitive PCR-based diagnostics have revealed that *K. kingae* is a leading cause of osteoarticular infections in young children between 6 and 36 months of age (2–4). In addition, *K. kingae* is a known cause of bacteremia and endocarditis in this population (2, 3). Following asymptomatic colonization of the upper respiratory tract, *K. kingae* can breach the epithelium, enter the bloodstream, and spread to distant sites to produce disease (1, 5–8). The mechanism by which *K. kingae* evades host innate immune responses during oropharyngeal colonization, in the bloodstream, and at sites of invasive disease is currently poorly understood.

Survival of bacteria in the bloodstream involves a complex interplay between the organism and the innate and adaptive immune systems. The innate immune system provides a rapid and immediate response to infection and plays an especially important

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role in children, who have a relatively naive adaptive immune system. A key component of innate immunity in the bloodstream is the complement system, a highly regulated and multifunctional group of circulating proteins that promote recognition of pathogens by immune cells through chemotaxis and opsonization and that are capable of direct killing of bacteria (9, 10). Complement is activated via the classical, the alternative, and the lectin pathways; all three of these pathways converge on the deposition of the protein fragment C3b on the bacterial surface. C3b promotes opsonization and formation of the membrane attack complex (MAC), which mediates direct lysis of Gram-negative bacteria (9, 10). Invasive bacterial pathogens express a variety of extracellular factors that mediate resistance to complement-mediated opsonin deposition and bacterial lysis.

Bacterial pathogens commonly express surface polysaccharides, which serve a multitude of functions and often allow the organism to tolerate environmental stressors, evade host immune mechanisms, and, ultimately, survive within the host. Capsular polysaccharides are lipidated, surface-anchored carbohydrate chains that have been widely shown to protect bacteria against mucosal and intravascular inflammatory responses by preventing phagocytosis and complement-mediated lysis (11–14). The polysaccharide capsules of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and a variety of other organisms have been extensively studied due to their importance as virulence factors and their effective use as vaccine antigens (15–18). In *K. kingae*, four distinct polysaccharide capsules, designated types a, b, c, and d, have been identified (19). Capsule types a and b account for greater than 95% of invasive disease isolates (19, 20). Previous work has demonstrated that the capsule is required for full *K. kingae* virulence in a juvenile rat model of invasive disease (21, 22).

Bacteria can also express additional or alternative surface polysaccharides, known as exopolysaccharides, which are secreted carbohydrate polymers that are not covalently anchored to the bacterial membrane and, hence, are different from polysaccharide capsules (23, 24). To date, exopolysaccharides have been studied largely in the context of bacterial biofilm formation and dispersal. In addition to expressing a capsular polysaccharide, *K. kingae* produces a galactofuranose homopolymer exopolysaccharide called the PAM galactan, which has been previously shown to have antibiofilm properties (21, 25). While a number of bacterial polysaccharide capsules have been studied for their ability to promote evasion of complement-mediated and neutrophil-mediated killing, understanding of the role of exopolysaccharides in these functions is limited (26–29).

In this study, we found that *K. kingae* is highly resistant to serum killing, resulting from the overlapping ability of the polysaccharide capsule and the exopolysaccharide to prevent opsonin deposition and complement-mediated lysis via the classical pathway. Elimination of both the capsular polysaccharide and the exopolysaccharide resulted in a complete loss of virulence in juvenile rats, highlighting the critical roles of these surface polysaccharides in *K. kingae* virulence.

RESULTS

***K. kingae* is highly resistant to complement-mediated lysis.** The ability of *K. kingae* to survive in the bloodstream in young children suggests that the organism is capable of evading the host innate immune response. To test the capacity of *K. kingae* to evade complement-mediated lysis, we performed serum bactericidal assays using normal human serum (NHS) as a source of active complement or heat-inactivated NHS (HI-NHS) as a source of inactive complement. *K. kingae* strain KK01 at an inoculum of 1.0×10^3 CFU was incubated for 1 h with serum concentrations ranging from 2% to 50% (Fig. 1A), using *Escherichia coli* strain DH5 α as a serum-sensitive control. To assess serum sensitivity, a survival ratio was calculated by dividing the number of CFU recovered from NHS by the number of CFU recovered from HI-NHS. The limit of detection for plating was 20 CFU, which enabled detection of up to a 98% reduction in bacterial survival. At low NHS concentrations, there was no killing of strain KK01 but complete killing of strain DH5 α (Fig. 1A). At an NHS concentration of 50%, strain KK01

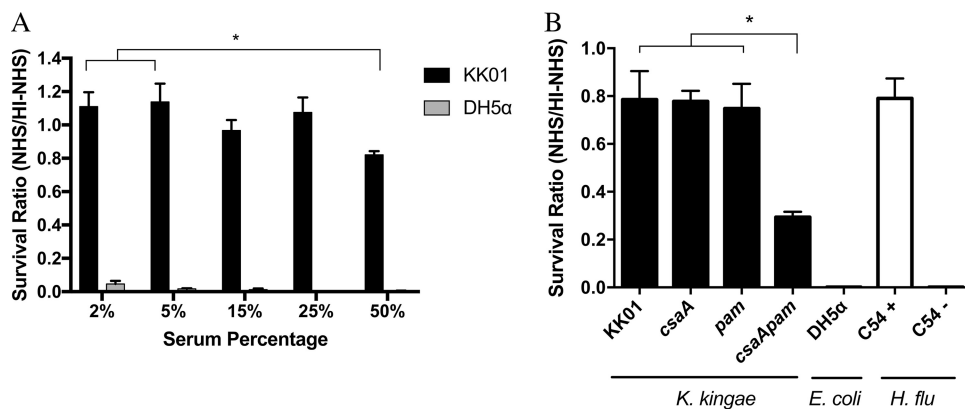


FIG 1 *K. kingae* is highly resistant to the bactericidal effects of complement present in pooled normal human serum, and elimination of both surface polysaccharides decreases serum resistance in *K. kingae*. (A) *K. kingae* strain KK01 and *E. coli* strain DH5α ($\sim 10^3$ CFU) were incubated with 2%, 5%, 15%, 25%, or 50% NHS or HI-NHS for 1 h. (B) *K. kingae* strains KK01, KK01 *csaA*, KK01 *pam*, and KK01 *csaA pam*, *E. coli* strain DH5α, and *H. influenzae* (*H. flu*) strains C54+ (encapsulated) and C54- (nonencapsulated) were incubated with 50% NHS or 50% HI-NHS for 1 h. The survival ratio was calculated by dividing the NHS CFU counts by the HI-NHS CFU counts. Abbreviations: *csaA*, KK01 *csaA*; *pam*, KK01 *pam*; *csaA pam*, KK01 *csaA pam*. A total of three biological replicates were performed ($n = 3$). Statistical significance was determined with an unpaired Student's *t* test, and the error bars represent the standard error of the mean. *, $P < 0.05$.

had a survival ratio of approximately 0.80 (Fig. 1A). As expected, HI-NHS had no effect on the survival of strain KK01 or strain DH5α at any serum concentration. These results demonstrate that *K. kingae* exhibits high-level resistance to complement-mediated lysis.

Surface polysaccharides prevent complement-mediated lysis of *K. kingae*. To determine the role of the *K. kingae* surface polysaccharides (the polysaccharide capsule and the exopolysaccharide) in serum resistance, we performed serum bactericidal assays using strain KK01 mutants deficient in expression of the polysaccharide capsule, the exopolysaccharide, or both, using strain KK01 *csaA* (a capsule-deficient mutant lacking the *csaA* capsule synthesis gene) (22), strain KK01 *pam* (an exopolysaccharide-deficient mutant lacking the *pamABCDE* galactan exopolysaccharide synthesis operon) (21), and strain KK01 *csaA pam* (a double mutant lacking both the capsule and the exopolysaccharide synthesis genes). Serum bactericidal assays were performed as described above using 50% serum for 1 h and using *E. coli* strain DH5α as a serum-sensitive control. In addition, isogenic encapsulated and nonencapsulated derivatives of *H. influenzae* strain C54 (designated + for encapsulated strain and - for nonencapsulated strain) were included as controls to demonstrate the importance of the polysaccharide capsule in serum resistance for another Gram-negative bacterium. Deletion of *csaA*, *pamABCDE*, or both *csaA* and *pamABCDE* resulted in no growth defects on solid agar and had no effect on survival in the presence of 50% HI-NHS. KK01 and the mutant derivatives maintained stable bacterial counts over the course of the 1-h assay in HI-NHS (data not shown). Survival in NHS by the capsule mutant (strain KK01 *csaA*) or the exopolysaccharide mutant (strain KK01 *pam*) was not affected and was similar to the survival of wild-type strain KK01 (Fig. 1B). In contrast, survival in NHS by the double mutant lacking both capsule and exopolysaccharide (strain KK01 *csaA pam*) was markedly reduced, with a survival ratio of less than 0.35 ($P < 0.05$) (Fig. 1B). While the absence of both the capsule and the exopolysaccharide resulted in reduced resistance to serum, survival of strain KK01 *csaA pam* was not reduced to undetectable levels, as observed with the serum-sensitive controls *E. coli* DH5α and *H. influenzae* C54 b-.

These results establish that elimination of both capsule and exopolysaccharide is critical for increased complement-mediated lysis and demonstrate the potent ability of *K. kingae* to evade complement-mediated lysis.

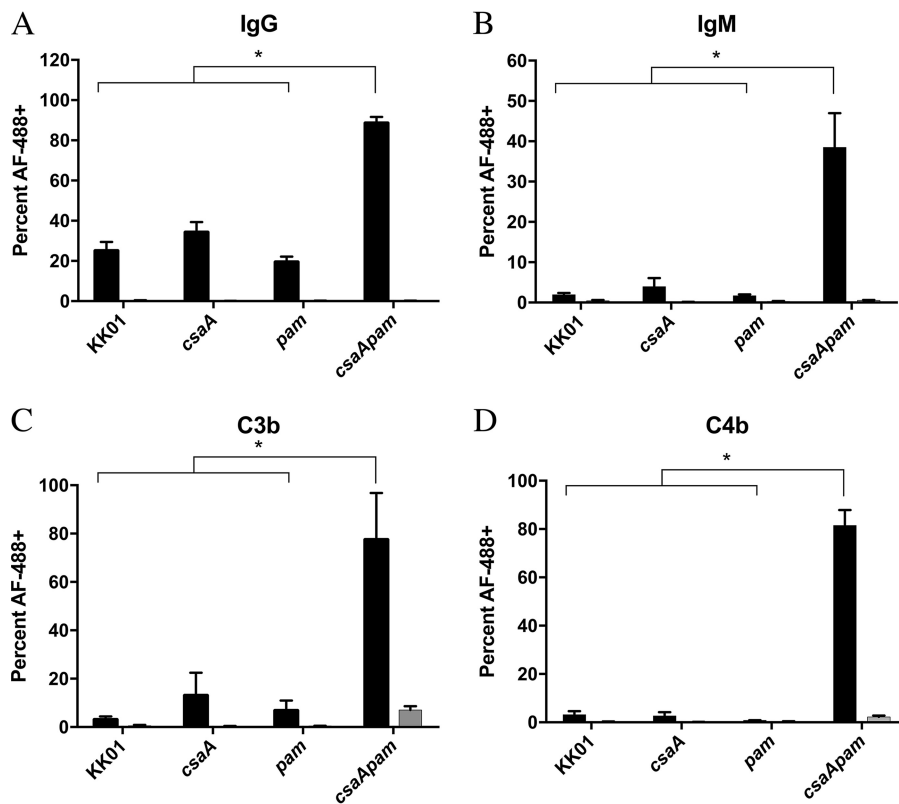


FIG 2 Opsonin deposition increases in the surface polysaccharide-deficient mutant. Binding of IgG (A), IgM (B), C3b (C), or C4b (D) to the bacterial surface was determined using flow cytometry. Cells were stained with propidium iodide (PI) prior to analysis; 30,000 propidium iodide-positive events per biological replicate were analyzed, and a total of three biological replicates were performed ($n = 3$). The percentages represent events that registered as Alexa Fluor 488 positive (AF-488+). Black bars, HI-NHS (A and B) or NHS (C and D); gray bars, secondary antibody-only controls (these bars are negligible in size due to the low signal). Abbreviations: *csaA*, KK01 *csaA*; *pam*, KK01 *pam*; *csaA pam*, KK01 *csaA pam*. Statistical significance was determined with an unpaired Student's *t* test, and the error bars represent the standard error of the mean. *, $P < 0.05$.

Opsonin deposition is increased in the absence of surface polysaccharides.

Elimination of the polysaccharide capsule in other organisms has been shown to expose bacterial surface factors and promote antibody recognition and, thus, activation of complement via the classical pathway (30–32). To determine whether *K. kingae*-specific antibodies were present in HI-NHS, Western blot analyses of whole-cell lysates and outer membranes were performed using HI-NHS as the primary antibody. Human serum immunoglobulins reacted with proteins present in *K. kingae* whole-cell and outer membrane fractions (data not shown). To further investigate whether the capsule and/or the exopolysaccharide prevents antibody binding, we performed flow cytometry assays to determine the relative levels of IgG and IgM deposition on the *K. kingae* surface after incubation with HI-NHS as the source of antibodies or with secondary antibody alone as a control. There was negligible fluorescence after the incubation with the secondary antibody alone (Fig. 2A and B, gray bars), thus demonstrating the absence of nonspecific binding of the secondary antibody. No significant difference in deposition was observed between strain KK01 and the single mutant strains KK01 *csaA* and KK01 *pam* after incubation with HI-NHS (Fig. 2A and B). In contrast, there was a statistically significant increase in the deposition of both IgG and IgM on the surface of strain KK01 *csaA pam*, consistent with our observations regarding serum sensitivity (Fig. 2A and B).

The constant region of IgG and IgM provides a platform for the initiation of the complement cascade (33–36). To determine whether antibody deposition activates the

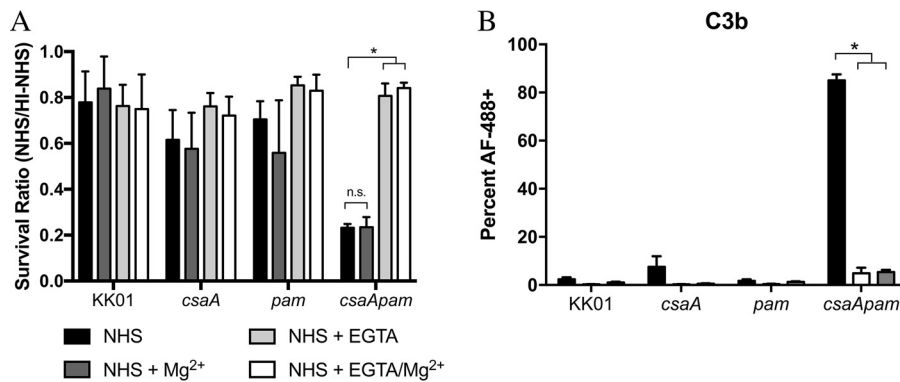


FIG 3 Inhibition of the classical pathway restores survival and reduces C3b deposition in the surface polysaccharide-deficient mutant. (A) Serum survival was assessed with either 50% NHS alone, 50% NHS plus 9 mM Mg²⁺, 50% NHS plus 5 mM EGTA, 50% NHS plus 9 mM Mg²⁺ and 5 mM EGTA, or 50% HI-NHS for 1 h. The survival ratio was calculated by dividing the NHS CFU counts by the HI-NHS CFU counts. (B) *K. kingae* strains were fixed and incubated for 15 min in medium alone, in medium containing 20% NHS, or in medium containing 20% NHS plus 9 mM Mg²⁺ and 5 mM EGTA. Binding of C3b (*n* = 3) to the bacterial surface was determined using flow cytometry, and 30,000 propidium iodine-positive events per experiment were analyzed. The percentages represent events that registered as Alexa Fluor 488 positive. Black bars, NHS; white bars, NHS plus 9 mM Mg²⁺ and 5 mM EGTA; gray bars, secondary antibody-only control. Abbreviations: *csaA*, KK01 *csaA*; *pam*, KK01 *pam*; *csaApam*, KK01 *csaA pam*. Statistical significance was determined with an unpaired Student's *t* test, and the error bars represent the standard error of the mean. n.s., not significant; *, *P* < 0.05.

classical pathway and leads to activation of the terminal pathway, we performed flow cytometry assays to measure the deposition of C4b and C3b, two well-documented opsonins and products of complement activation, on the bacterial surface. As shown in Fig. 2C and D, there was no significant difference in the binding of C4b and C3b to strain KK01 and the single mutant strains KK01 *csaA* and KK01 *pam*. In contrast, there was a statistically significant increase in C4b and C3b deposition on strain KK01 *csaA pam*, consistent with the increase in antibody deposition and serum sensitivity observed with this strain (Fig. 2C and D). These data suggest that the survival of strains KK01, KK01 *csaA*, and KK01 *pam* in NHS is due in part to reduced antibody deposition and the resultant absence of activating complement fragments on the bacterial surface. Elimination of both the polysaccharide capsule and the exopolysaccharide on the bacterial surface results in increased opsonin deposition, promoting complement activation.

Inhibition of the classical pathway when surface polysaccharides are absent restores serum survival and decreases opsonin deposition. The deposition of IgG and IgM on the bacterial surface prompts complement activation via the classical pathway. Ca²⁺ is considered necessary to maintain the integrity of the C1 complex, which associates with the Fc region of antigen-bound IgG or IgM and initiates the complement cascade (36, 37). The presence of EGTA in serum resistance assays chelates Ca²⁺ and thereby inhibits classical pathway activation, and the concomitant addition of Mg²⁺ preserves the alternative pathway (38).

To elucidate the importance of classical pathway activation in the serum killing of strain KK01 *csaA pam*, we performed serum bactericidal assays in the presence of EGTA plus Mg²⁺. As expected, the survival of strains KK01, KK01 *csaA*, and KK01 *pam* was not influenced by the introduction of 9 mM Mg²⁺ alone, 5 mM EGTA alone, or 9 mM Mg²⁺ plus 5 mM EGTA (Fig. 3A). Conversely, the survival ratio of strain KK01 *csaA pam* in the presence of either EGTA alone or EGTA plus Mg²⁺ was restored to levels similar to the survival ratios of strains KK01, KK01 *csaA*, and KK01 *pam* (Fig. 3A). As anticipated, the presence of Mg²⁺ alone had no effect on the survival ratio of strain KK01 *csaA pam* (Fig. 3A).

In order to confirm that chemical inhibition of the classical pathway via EGTA prevents complement activation, flow cytometry assays were performed to determine

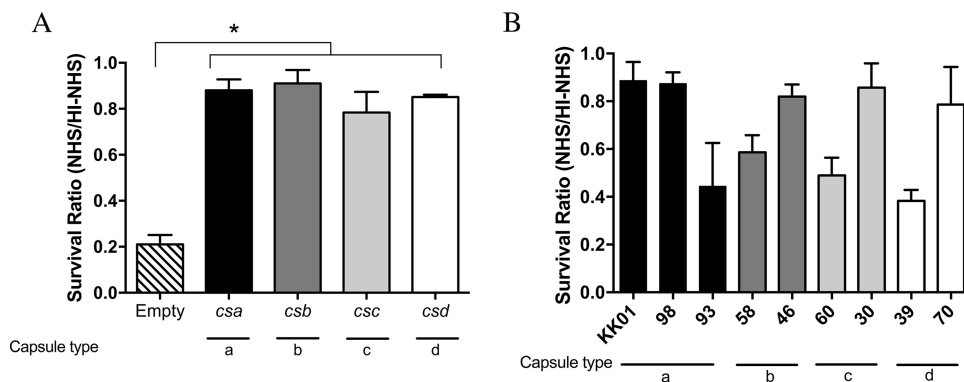


FIG 4 Specific capsule types do not dictate serum sensitivity in *K. kingae* clinical isolates. *K. kingae* strains ($\sim 10^3$ CFU) were incubated with either 50% NHS or 50% HI-NHS for 1 h. The survival ratio was calculated by dividing the CFU counts in NHS by the CFU counts in HI-NHS for isogenic derivatives of strain KK01 *pam* (A) or clinical isolates of *K. kingae* (B). Abbreviations: Empty, nonencapsulated strain KK01SwapEmpty *pam*; *csa*, capsule type a KK01Swap *csa pam*; *csb*, type b KK01Swap *csb pam*; *csc*, type c KK01Swap *csc pam*; *csd*, type d KK01Swap *csd pam*; 98, *K. kingae* clinical isolate PYKK98; 93, *K. kingae* clinical isolate PYKK93; 58, *K. kingae* clinical isolate PYKK58; 46, *K. kingae* clinical isolate KK146; 60, *K. kingae* clinical isolate PYKK60; 30, *K. kingae* clinical isolate ATCC 23330; 39, *K. kingae* clinical isolate E3339; 70, *K. kingae* clinical isolate BB270. A total of three biological replicates were performed ($n = 3$). Statistical significance was determined with an unpaired Student's *t* test, and the error bars represent the standard error of the mean. *, $P < 0.05$.

the percentage of C3b deposition on the surface of *K. kingae* in the presence of EGTA plus Mg^{2+} . As shown in Fig. 3B, no significant differences in C3b deposition were observed between strains KK01, KK01 *csaA*, and KK01 *pam* in the presence or absence of EGTA plus Mg^{2+} . In contrast, there was a significant decrease in C3b deposition on strain KK01 *csaA pam* when this strain was incubated in NHS containing EGTA plus Mg^{2+} (Fig. 3B). The C3b deposition on strain KK01 *csaA pam* in NHS containing EGTA plus Mg^{2+} mirrored the C3b deposition on strain KK01 *csaA pam* in medium alone (Fig. 3B). Taken together, these data provide further support that the classical complement pathway is activated in the absence of *K. kingae* surface polysaccharides.

The four distinct capsule types of *K. kingae* promote serum survival in isogenic strains. To date, a total of four distinct capsule types have been identified in diverse collections of *K. kingae* clinical isolates, including type a ([3]- β -GalpNAc-(1 \rightarrow 5)- β -Kdop-(2 \rightarrow)), type b ([6]- α -D-GlcpNAc-(1 \rightarrow 5)- β -(8-OAc)Kdop-(2 \rightarrow)), type c ([3]- β -D-Ribf-(1 \rightarrow 2)- β -D-Ribf-(1 \rightarrow 2)- β -D-Ribf-(1 \rightarrow 4)- β -Kdop-(2 \rightarrow)), and type d ([P-(O \rightarrow 3)] β -D-Galp-(1 \rightarrow 4)]- β -D-GlcpNAc-(1 \rightarrow 3)- α -D-GlcpNAc-1-)] (19–22). The prototype strain KK01 used in this study expresses the type a capsule. To assess whether the four capsule types have different abilities to protect the organism against complement-mediated lysis, we performed bactericidal assays on isogenic derivatives of strain KK01 *pam* expressing either capsule type a (strain KK01Swap *csa*), capsule type b (strain KK01Swap *csb*), capsule type c (strain KK01Swap *csc*), or capsule type d (strain KK01Swap *csd*) (19). As shown in Fig. 4A, all four capsule types were associated with a survival ratio of 0.80, similar to the survival ratio of strain KK01. These data suggest that the presence of any of the four *K. kingae* capsule types is protective and is adequate for promoting serum resistance in the absence of exopolysaccharide *in vitro*.

***K. kingae* clinical isolates show moderate to high levels of serum resistance.** Serum resistance plays an important role in pathogenicity and has been shown to be variable from strain to strain in other bacterial species. To determine the variability of serum resistance across *K. kingae* clinical isolates, we performed serum bactericidal assays on eight clinical isolates that represented all four capsular groups and included both invasive and healthy carrier isolates (Fig. 4B; Table 1). Additionally, the eight clinical isolates contain the *pam* locus and produce exopolysaccharide. *K. kingae* strains PYKK98, KK146, ATCC 23330, and BB270 showed high levels of serum resistance, with a survival ratio of approximately 0.80 (Fig. 4B), while strains PYKK93, PYKK58, PYKK60, and E3339 showed moderate levels of serum resistance, with survival ratios of between

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>Kingella kingae</i>		
KK01	Nonspreading/noncorroding derivative of 269-492	8
PYKK98	Clonal group B isolate from a case of bacteremia, capsule type a	P. Yagupsky
PYKK93	Clonal group P isolate from a case of bacteremia, capsule type a	P. Yagupsky
PYKK58	Clonal group N isolate from a case of septic arthritis, capsule type b	P. Yagupsky
KK146	Clonal group N isolate from a case of bacteremia, capsule type b	P. Yagupsky
PYKK60	Clonal group D isolate from a case of endocarditis, capsule type c	P. Yagupsky
ATCC 23330	Clonal group D isolate from a healthy carrier, capsule type c	ATCC
E3339	Clonal group F isolate from a healthy carrier, capsule type d	P. Yagupsky
BB270	Clonal group U isolate from a healthy carrier, capsule type d	P. Yagupsky
KK01 <i>csaA</i>	Contains <i>csaA</i> deletion	22
KK01 <i>pam</i>	Contains <i>pamABCDE</i> deletion	21
KK01 <i>csaA pam</i>	Contains <i>csaA</i> deletion and <i>pamABCDE</i> deletion	This study
KK01SwapEmpty	Contains the capsule synthesis locus flanking genes and a deletion of the <i>csaA</i> region	19
KK01Swap <i>csa</i>	KK01SwapEmpty with <i>csa</i> locus recombined	19
KK01Swap <i>csb</i>	KK01SwapEmpty with <i>csb</i> locus recombined	19
KK01Swap <i>csc</i>	KK01SwapEmpty with <i>csc</i> locus recombined	19
KK01Swap <i>csd</i>	KK01SwapEmpty with <i>csd</i> locus recombined	19
KK01SwapEmpty <i>pam</i>	KK01SwapEmpty with <i>pamABCDE</i> deletion	This study
KK01Swap <i>csa pam</i>	KK01Swap <i>csa</i> with <i>pamABCDE</i> deletion	This study
KK01Swap <i>csb pam</i>	KK01Swap <i>csb</i> with <i>pamABCDE</i> deletion	This study
KK01Swap <i>csc pam</i>	KK01Swap <i>csc</i> with <i>pamABCDE</i> deletion	This study
KK01Swap <i>csd pam</i>	KK01Swap <i>csd</i> with <i>pamABCDE</i> deletion	This study
<i>E. coli</i> DH5 α	<i>E. coli</i> F $^-$ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r $_{K}^-$ m $_{K}^+$) <i>phoA supE441 thi-1 gyrA96 relA1</i>	64
<i>H. influenzae</i>		
C54	<i>Haemophilus influenzae</i> serotype b isolate	65
C54 b $^-$	Spontaneous capsule-deficient derivative of strain C54	66
<i>E. coli</i> plasmid pUC19 <i>pam::ermC</i>	pUC19 with <i>ermC</i> erythromycin cassette flanked by surrounding 5' and 3' regions of the <i>pamABCDE</i> locus	21

0.40 and 0.60 (Fig. 4B). The results demonstrate that the clinical isolates tested were resistant to human serum; however, there was no consistent distinction in the level of susceptibility in relation to capsule type or clinical site of isolation.

Capsule and exopolysaccharide are required for full *K. kingae* virulence in a juvenile rat infection model. Given the importance of the capsular polysaccharide and exopolysaccharide in protecting *K. kingae* from the bactericidal effects of human serum *in vitro*, we sought to elucidate the role of these factors *in vivo*. To examine the role of the *K. kingae* surface polysaccharides in virulence and to determine the influence of complement in limiting infection *in vivo*, we modified a previously described juvenile rat infection protocol (22). Briefly, we injected 5-day-old Sprague-Dawley rat pups via the intraperitoneal (i.p.) route with either phosphate-buffered saline (PBS) alone or PBS with 10 μ g of cobra venom factor (CVF), which forms a complex with complement components factor B and factor D and creates a convertase that depletes C3 and C5 to undetectable levels in the serum (39, 40). CVF has been used previously in different animal models, including juvenile rats, to successfully deplete complement *in vivo* (40–42). At 3 h postinjection with PBS alone or PBS with CVF, the rat pups were injected i.p. with 1×10^8 CFU of strain KK01, strain KK01 *csaA*, strain KK01 *pam*, or strain KK01 *csaA pam*, using PBS as a control (Fig. 5A and B).

Among the rat pups that did not receive pretreatment with CVF, all that were infected with strain KK01 succumbed to infection, with a median survival of 12 h (Fig. 5A). At 5 days postinfection, survival among pups infected with strain KK01 *csaA* was 29% ($P < 0.0001$) (Fig. 5A), and survival among pups infected with strain KK01 *pam* was 53% ($P < 0.0001$) (Fig. 5A). In contrast, all pups injected with strain KK01 *csaA pam* were alive and healthy at 5 days postinfection, similar to the results for pups injected with PBS alone ($P < 0.0001$) (Fig. 5A). These results suggest that elimination of either capsule or exopolysaccharide results in reduced virulence of *K. kingae* and that elimination of both the capsule and the exopolysaccharide results in an avirulent strain.

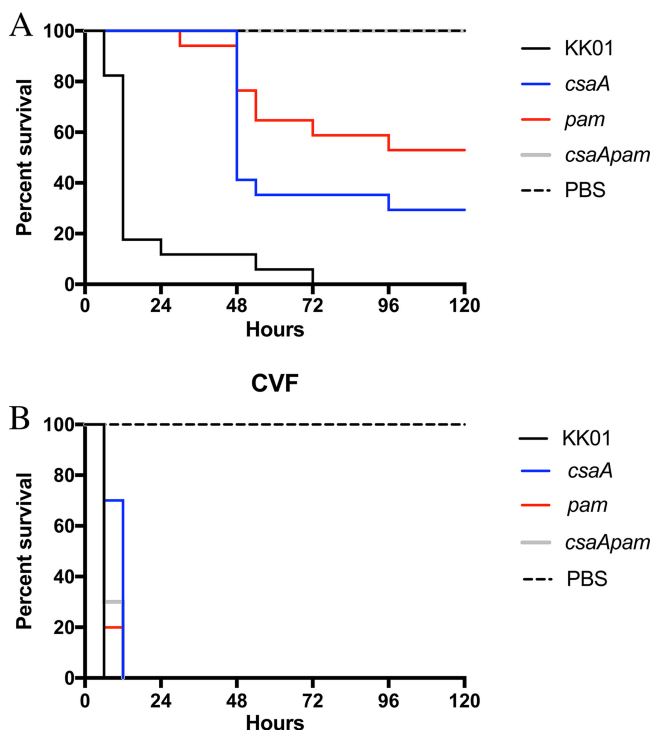


FIG 5 Surface polysaccharides are required for full *K. kingae* virulence in a rat infection model. Five-day-old Sprague-Dawley rats were injected via the intraperitoneal (i.p.) route with either 0.1 ml PBS (A) or 10 μ g CVF in 0.1 ml PBS (B). The graphs plot Kaplan-Meier survival curves for rats subsequently i.p. injected with 0.1 ml PBS or 1×10^8 CFU of the KK01 strains in PBS. Data are for 17 (A) and 10 (B) animals infected with each strain and 15 (A) and 9 (B) animals injected with PBS. Abbreviations: *csaA*, KK01 *csaA*; *pam*, KK01 *pam*; *csaA**pam*, KK01 *csaA pam*.

Among pups that received CVF pretreatment, all infected with strain KK01, strain KK01 *csaA*, strain KK01 *pam*, or strain KK01 *csaA pam* succumbed to infection within 12 h (Fig. 5B). All pups injected with PBS alone after CVF pretreatment survived at 5 days postinfection (Fig. 5B). These results suggest that resistance to complement via the polysaccharide capsule and the exopolysaccharide is critical for *K. kingae* virulence in juvenile rats.

DISCUSSION

Kingella kingae is a common commensal organism in the oropharynx and is an emerging pediatric pathogen that represents a leading etiology of joint and bone infections in young children (2–4). This organism causes disease by breaching the respiratory epithelium, invading the bloodstream, and disseminating to distant sites (1, 5–8). In order to survive in the intravascular environment and then disseminate, the organism must evade innate immune mechanisms.

Our initial findings revealed that *K. kingae* is highly resistant to the bactericidal effects of complement present in human serum. Further examination revealed that the presence of either the capsule or the exopolysaccharide alone was sufficient to fully prevent opsonin deposition and completely protect *K. kingae* against complement-mediated killing, a novel observation. Elimination of both the capsule and the exopolysaccharide was required for efficient binding of opsonins (IgG, IgM, C4b, and C3b) to the bacterial surface and for significant complement-mediated killing *in vitro*. Abrogation of the classical complement pathway *in vitro* restored survival of the capsule/exopolysaccharide double mutant, clarifying the mechanism by which these surface polysaccharides promote survival in human serum. In agreement with our *in vitro* data, elimination of both the capsule and the exopolysaccharide rendered *K. kingae* completely avirulent, abolishing morbidity and mortality in the juvenile rat infection model.

To confirm that the attenuation of strain KK01 *csaA pam* *in vivo* was a consequence of efficient activation of complement, pups were injected with CVF to eliminate complement. As predicted, the resulting complement deficiency was associated with an increase in virulence of all *K. kingae* strains, including strain KK01 *csaA pam*.

In recent years, a number of reports have described bacterial factors associated with evasion of complement activity in nonencapsulated organisms (43–47). However, bacterial polysaccharide capsules generally predominate as the most potent mechanism of bacterial resistance to complement-mediated serum killing, as highlighted by studies of *H. influenzae*, *N. meningitidis*, and *E. coli*. In *N. meningitidis*, transposon mutagenesis and large-scale analysis of the genome searching for genes contributing to serum resistance identified only genes involved in polysaccharide capsule or lipooligosaccharide synthesis (14). Consistent with the conclusion that the polysaccharide capsule is important for *N. meningitidis* serum resistance, almost all meningococcal isolates recovered from blood are encapsulated, while 30 to 70% of carrier isolates are nonencapsulated (48–50). Similarly, in *H. influenzae*, loss of capsule reduces or eliminates serum resistance, and overexpression of the serotype b capsule synthesis genes due to duplication events promotes greater serum resistance (13, 51, 52). In this study, we observed that nonencapsulated *K. kingae* exhibits high levels of serum resistance *in vitro*, similar to the serum resistance of wild-type *K. kingae*, underscoring the importance of the exopolysaccharide.

In considering our observation that the *K. kingae* capsule and exopolysaccharide play crucial and apparently redundant roles in serving to prevent IgG, IgM, C4b, and C3b deposition on the bacterial surface and resultant bacterial lysis, it is noteworthy that the capsule is tethered to the bacterial surface via a lipid anchor and the exopolysaccharide is unanchored. We presume that the exopolysaccharide is loosely associated with the bacterial surface, blocking access of opsonins. In earlier work, we observed that the exopolysaccharide can be released from the bacterial surface by resuspending bacteria in PBS and vigorously shaking, consistent with a noncovalent mechanism of association (21). The nature of the association and the balance between surface-associated and released exopolysaccharide during natural infection are currently unknown.

Exopolysaccharides in other bacterial species have been studied primarily as major contributors to the formation and dispersal of biofilms (24, 53–55). Exopolysaccharide function depends on the polymer structure and composition and has been studied in relation to adherence, bacterial cell aggregation, water retention, and nutrition (24, 55, 56). Few studies have characterized exopolysaccharides in the context of inhibiting complement deposition and subsequent complement and/or leukocyte evasion (26–29). In *Pseudomonas aeruginosa*, expression of the Psl exopolysaccharide serves to protect the organism from neutrophil antimicrobial functions; however, Psl does not play a role in evasion of complement-mediated lysis *in vitro* and has been studied only in the context of capsule-deficient organisms, thus contrasting with the findings of our studies of *K. kingae* (26). Like *K. kingae*, extraintestinal pathogenic *Escherichia coli* (ExPEC) produces two surface polysaccharides that contribute to serum resistance, namely, a group 2 polysaccharide capsule and an exopolysaccharide called colanic acid (27). However, while colanic acid is important for evasion of complement-mediated killing, the absence of the polysaccharide capsule renders ExPEC sensitive to serum even in the presence of colanic acid, suggesting that the exopolysaccharide alone is not sufficient for mediating serum resistance and thus again contrasting with the findings of studies of *K. kingae* (27). In *K. kingae*, the level of protection against complement activation and complement-mediated killing provided by the exopolysaccharide mimics the level of protection provided by the polysaccharide capsule. Thus far, two exopolysaccharide structures have been described in *K. kingae*: $\rightarrow 3$ - β -GalF-(1 \rightarrow 5)- β -GalF-(1 \rightarrow) and $\rightarrow 5$ - β -GalF-(1 \rightarrow) (21, 25). It has been reported that biofilm formation and the dispersal of *K. kingae* clinical strain PYKK181 are dependent on the PAM galactan exopolysaccharide (25). In this study, we have shown that the PAM galactan produced

by *K. kingae* strain KK01 prevents opsonin deposition and complement activation, providing evidence for a novel function of the *K. kingae* exopolysaccharide.

In *K. kingae*, four distinct polysaccharide capsules have been identified, designated types a, b, c, and d. Using isogenic derivatives of strain KK01 *pam*, all four capsule types were associated with a survival ratio similar to the survival ratio for strain KK01. These data suggest that the presence of any naturally occurring *K. kingae* capsule type is adequate to protect KK01 *pam* against serum killing under our *in vitro* conditions. In addition, eight clinical isolates representing all four capsular groups and including both invasive and healthy carrier isolates were assessed for serum resistance. Interestingly, these isolates exhibited moderate to high levels of serum resistance, with no consistent distinctions in level of susceptibility in relation to capsule type or clinical site of isolation. The variability in the serum survival ratio among the different clinical isolates could be due to strain-to-strain variation in the quantity of capsule and/or exopolysaccharide. Despite the contributions of capsule and exopolysaccharide to serum resistance, the capsule and exopolysaccharide double mutant remained relatively resistant to human serum, with a survival ratio of 0.40 (Fig. 1B). This observation highlights the presence of additional factors deployed by *K. kingae* for innate immune evasion and may be another explanation for the variability of serum resistance observed in the clinical isolates.

While our *in vitro* serum resistance experiments suggest no differences in resistance between the wild-type strain and the single mutant strains lacking capsule or exopolysaccharide, our *in vivo* studies established that each surface polysaccharide plays a role in promoting virulence in juvenile rats. Elimination of either capsule or exopolysaccharide resulted in reduced virulence, including a lower mortality rate and delayed mortality. This finding suggests that perhaps the capsule and/or the exopolysaccharide plays a role in virulence beyond protecting against humoral immunity *in vivo*; alternatively, serum sensitivity *in vitro* may be unobservable in the absence of a single surface polysaccharide. Along these lines, polysaccharide capsules and exopolysaccharides have been implicated in both promoting and dampening host inflammatory responses. The zwitterionic capsular polysaccharide A from *Bacteroides fragilis*, the gut microfloral commensal, is implicated in mediating development of the host immune system through induction of CD4⁺ maturation and by promotion of a balanced Th1/Th2 response in mice (57). Interestingly, Totté et al. have reported that a galactan homopolysaccharide in *Mycoplasma mycoides* subsp. *mycoides* similar to the *K. kingae* exopolysaccharide binds Toll-like receptor 2 and promotes the production of the anti-inflammatory cytokine interleukin-10 (58). Further work is necessary to elucidate the immunoregulatory potential of the *K. kingae* surface polysaccharides both *in vitro* and *in vivo*.

In conclusion, both the polysaccharide capsule and the exopolysaccharide of *K. kingae* play a crucial role in preventing opsonin deposition and complement-mediated killing, presumably facilitating intravascular survival and resulting in enhanced virulence *in vivo*. These results highlight an underrecognized function of a bacterial exopolysaccharide and provide important information that may facilitate development of a polysaccharide-based vaccine against *K. kingae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. The parent *K. kingae* strain 269-492 (8) grows on solid agar as two phenotypically stable colony types, designated KK01 and KK03. As described in our earlier work, the KK01 and KK03 colony types differ in their density of piliation. *K. kingae* KK01 was used as the wild-type strain throughout this study. *K. kingae* strains were stored at -80°C in brain heart infusion (BHI) broth with 20% glycerol.

E. coli strains were stored at -80°C in Luria-Bertani (LB) broth with 15% glycerol. *K. kingae* strains were grown at 37°C with 5% CO₂ on chocolate agar plates supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin or 1 $\mu\text{g}/\text{ml}$ erythromycin, as appropriate. *E. coli* strains were grown at 37°C on LB agar or in LB broth supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin, as appropriate.

Strain construction. The *csaA* mutant, the *pam* mutant, and the capsule swap strains were generated as previously described (19, 21, 22). To generate the KK01 *csaA pam* double mutant and the capsule swap strains containing the *pam* locus deletion, strains KK01 *csaA*, KK01Swap *csa*, KK01Swap *csb*, KK01Swap *csc*, KK01Swap *csd*, and KK01SwapEmpty were transformed with linearized plasmid

pUC19*pam::ermC*. Briefly, the plasmid was purified from *E. coli*, linearized with NdeI, and introduced into the parental strains (the *csaA* mutant or swap strains) via natural transformation. Transformants were recovered on chocolate agar plates containing 1.0 $\mu\text{g/ml}$ erythromycin. Correct insertion of the gene disruption construct was confirmed via PCR and sequencing of the deletion site. The absence of the capsule and the exopolysaccharide in strain KK01 *csaA pam* and the capsule swap *pam* deletion strains was confirmed using alcian blue and silver staining as previously described (21, 59), with the parental strains and strain KK01 being used as controls.

Polysaccharide extraction and staining. For capsule extractions, bacteria were washed and resuspended in Tris acetate (pH 5) for 1 h. Cells were removed by centrifugation, and the extracts were treated with proteinase K for 1 h and then concentrated as previously described (59). For exopolysaccharide extractions, bacteria were resuspended in 5 ml PBS and vortexed. Cells were removed by centrifugation, and the bacterial supernatants were treated with proteinase K for 1 h and then concentrated as previously described (59).

Aliquots of supernatant and purified capsule from *K. kingae* derivatives were separated on 7.5% or 10% SDS-polyacrylamide gels, respectively. For supernatant samples, the SDS-polyacrylamide gels were treated first with silver staining and subsequently with alcian blue staining. For purified capsule samples, the SDS-polyacrylamide gels were treated with alcian blue staining. For silver staining, the gels were treated as previously described (60). For alcian blue staining, the gels were stained with 0.125% alcian blue as previously described (59).

Serum bactericidal assays. *K. kingae* strains and *E. coli* strain DH5 α were grown on chocolate agar plates and then resuspended in PBS containing 0.1% gelatin (PBS-G). *H. influenzae* strains were grown on chocolate agar plates overnight, resuspended in BHI with 3.5 $\mu\text{g/ml}$ NAD and 0.1% lysed horse blood to an optical density at 600 nm (OD₆₀₀) of 0.2, and grown to an OD₆₀₀ of between 0.6 and 0.8 with shaking at 250 rpm. Samples were diluted in PBS-G to obtain a final inoculum of approximately 4.0×10^3 CFU/0.1 ml. The respective inocula were mixed with pooled NHS (Immucor, Norcross, GA) or HI-NHS (prepared by incubating NHS at 56°C for 20 min) diluted in PBS-G, as appropriate, and incubated for various times at 37°C with 5% CO₂. Complement activity was assessed and confirmed using a total hemolytic assay as previously described (61). Serial dilutions of the inoculum and reaction samples were plated on chocolate agar plates and incubated overnight at 37°C with 5% CO₂ to determine the CFU counts (limit of detection for plating, 20 CFU). To perform classical pathway inhibition assays, samples were incubated with EGTA at a final concentration of 5 mM and supplemental MgCl₂ at a final concentration of 9 mM prior to introduction of NHS.

Flow cytometry analysis. *K. kingae* strains were grown on chocolate agar plates, resuspended to an OD₆₀₀ of 0.8 in PBS, pelleted, and resuspended in 4% paraformaldehyde in PBS for fixation. After incubation for 20 min at room temperature (RT), bacteria were washed with Tris-buffered saline (TBS) and resuspended in PBS. We modified previously described protocols to detect bacterial opsonization (26, 62). Briefly, fixed bacteria were incubated with 20% NHS as the source of C3b or 1% NHS as the source of C4b. Samples were incubated with gentle agitation for 15 min at RT, washed with PBS, and resuspended in TBS containing 50 mM EDTA and 0.1% bovine serum albumin (BSA). Fixed bacteria were incubated with or without a 1:250 dilution of a mouse anti-human C3b monoclonal antibody (Thermo Fisher, Rockford, IL) or a 1:250 dilution of a rabbit anti-human C4b monoclonal antibody (Abcam, Cambridge, MA) for 1 h at RT. Samples were washed twice in PBS, resuspended in PBS containing 0.1% BSA, and incubated with a 1:200 dilution of a rabbit anti-mouse IgG DyLight 488-conjugated antibody (Rockland, Limerick, PA) or a 1:200 dilution of a goat anti-rabbit IgG DyLight 488-conjugated antibody (Rockland) for 45 min at RT, as appropriate. Bacteria were washed, resuspended in PBS, and stained with propidium iodide (Biotium, Fremont, CA) for flow cytometry analysis. For classical pathway inhibition assays, samples were incubated with EGTA at a final concentration of 5 mM and supplemental MgCl₂ at a final concentration of 9 mM prior to introduction of NHS.

For human IgG and human IgM surface deposition analysis, bacteria were fixed, resuspended in TBS containing 50 mM EDTA and 0.1% BSA, and then incubated with PBS as a control or 1% HI-NHS as a source of human IgG and human IgM. After gentle agitation for 1 h at RT, samples were washed twice with PBS, resuspended in PBS containing 0.1% BSA, and then incubated with a 1:200 dilution of a goat anti-human IgG (H&L) DyLight 488-conjugated antibody (Rockland) or a 1:200 dilution of a goat anti-human IgM (μ chain) DyLight 488-conjugated antibody (Rockland) for 45 min at RT. Bacteria were washed, resuspended in PBS, and stained with propidium iodide (Biotium) for flow cytometry analysis. Flow cytometry assays were performed using an Accuri C6 instrument (BD Biosciences, San Jose, CA).

K. kingae-specific serum antibody detection. Serum antibodies reactive to *K. kingae* whole-cell and outer membrane fractions were determined by Western blotting. Total membranes were recovered by centrifugation of cleared bacterial sonicates, and outer membrane fractions were isolated on the basis of Sarkosyl insolubility. The whole-cell and outer membrane fractions were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with 2% HI-NHS, followed by an anti-human Ig horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, MO) at a dilution of 1:5,000. The blot was developed by using a chemiluminescent substrate.

Juvenile rat infection model. Nursing 5-day-old Sprague-Dawley rat pups (Charles River Laboratories, Wilmington, MA) were injected via the intraperitoneal route with 0.1 ml PBS alone (control) or 10 μg of CVF (Complement Technology, Tyler, TX) in 0.1 ml PBS. *K. kingae* strains were grown on chocolate agar for 18 h, and the bacteria were swabbed from plates and resuspended in PBS to a final density of 1×10^8 CFU/0.1 ml. While the *in vitro* serum bactericidal assays used 1×10^3 CFU to conserve human serum, 1×10^8 CFU was the necessary infectious dose *in vivo* to consistently generate invasive disease in rat pups with strain KK01. At 3 h after CVF injection, rat pups were injected via the intraperitoneal route with

0.1 ml of the appropriate bacterial suspension or 0.1 ml of PBS alone (control). Rat pups were injected using a 27 1/2-gauge needle and then returned to their cage with a lactating dam. The experimental and control groups were housed separately with a lactating dam and were monitored for mortality and signs of illness every 6 h for the first 30 h and then twice daily for a total of 5 days. Animals found to be moribund were euthanized by using CO₂ inhalation followed by secondary decapitation.

Ethics statement. All animal experiments described within were conducted in accordance with the Animal Welfare Act, the Public Health Service policy on the humane care and use of laboratory animals from the U.S. Department of Health and Human Services, and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (63). The Children's Hospital of Philadelphia animal research facilities have full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The animal procedures were approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee (IACUC) under protocol IAC 16-001050.

Statistical analysis. Statistical analyses were performed with GraphPad Prism (version 7.0a) software for Mac (GraphPad Software, San Diego, CA), where a *P* value of <0.05 was considered statistically significant. Unpaired Student's *t* test was performed as appropriate. Mortality rates were calculated using the log-rank (Mantel-Cox) test for significance.

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