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Surface Polysaccharides Promote Innate Immune Evasion By The Pediatric Pathogen Kingella Kingae

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Surface Polysaccharides Promote Innate Immune Evasion By The Pediatric Pathogen Kingella Kingae

Abstract
The gram-negative coccobacillus Kingella kingae is an emerging pediatric pathogen and is increasingly recognized as a common etiological agent of osteoarticular infections and bacteremia in young children. The pathogenesis of K. kingae disease involves colonization of the posterior pharynx, invasion into the bloodstream, and dissemination to distant sites of infection. Previous 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. The purpose of this work was to determine the role of the K. kingae polysaccharide capsule and exopolysaccharide in promoting resistance to complement-mediated and neutrophil-mediated killing. We determined that both the K. kingae capsule and exopolysaccharide prevented efficient binding of IgG, IgM, C4b, and C3b to the bacterial surface and inhibited complement-mediated killing. Abrogation of the classical complement pathway using EGTA-treated human serum restored survival of capsule-deficient, exopolysaccharide-deficient K. kingae to wild-type levels, demonstrating that capsule and exopolysaccharide promote resistance to the classical complement pathway. Consistent with these results, the capsule and exopolysaccharide enhanced K. kingae pathogenicity in juvenile rats with an intact complement system. Loss of the capsule and the exopolysaccharide resulted in avirulence, however not in rats lacking complement. Experiments using primary human neutrophils and a series of isogenic K. kingae mutants demonstrated the critical role of the capsule and the conditional role of the exopolysaccharide in preventing the neutrophil oxidative burst response and neutrophil-mediated killing of K. kingae. In the absence of capsule, the exopolysaccharide prevented K. kingae opsonization and subsequently, reduced ROS production. The loss of capsule promoted neutrophil binding of K. kingae but had no effect on neutrophil phagocytosis and bacterial internalization of K. kingae. In contrast, the exopolysaccharide efficiently blocked neutrophil phagocytosis of K. kingae and resisted the bactericidal effects of antimicrobial peptides. This work establishes that the K. kingae surface polysaccharides are multi-functional and play a critical role in K. kingae pathogenicity, facilitating evasion of host immunity. Our studies suggest that the K. kingae capsule and exopolysaccharide have potential as targets for vaccine development.

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Joseph W. St. Geme, III

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SURFACE POLYSACCHARIDES PROMOTE INNATE IMMUNE EVASION BY

THE PEDIATRIC PATHOGEN KINGELLA KINGAE

Vanessa Lynne Muñoz

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SURFACE POLYSACCHARIDES PROMOTE INNATE IMMUNE EVASION BY THE
PEDIATRIC PATHOGEN *KINGELLA KINGAE*

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Vanessa Lynne Muñoz
To my loving parents, Maritza and Sergio, thank you for your endless support throughout my scientific career. To my siblings, Sergio Jr., Alondra, Sarah, and Joanna, thank you for being my inspiration.
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ABSTRACT

SURFACE POLYSACCHARIDES PROMOTE INNATE IMMUNE EVASION BY THE PEDIATRIC PATHOGEN KINGELLA KINGAE

Vanessa Lynne Muñoz
Joseph W. St. Geme, III, M.D.

The gram-negative coccobacillus *Kingella kingae* is an emerging pediatric pathogen and is increasingly recognized as a common etiological agent of osteoarticular infections and bacteremia in young children. The pathogenesis of *K. kingae* disease involves colonization of the posterior pharynx, invasion into the bloodstream, and dissemination to distant sites of infection. Previous 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. The purpose of this work was to determine the role of the *K. kingae* polysaccharide capsule and exopolysaccharide in promoting resistance to complement-mediated and neutrophil-mediated killing.

We determined that both the *K. kingae* capsule and exopolysaccharide prevented efficient binding of IgG, IgM, C4b, and C3b to the bacterial surface and inhibited complement-mediated killing. Abrogation of the classical complement pathway using EGTA-treated human serum restored survival of capsule-deficient, exopolysaccharide-deficient *K. kingae* to wild-type levels, demonstrating that capsule and exopolysaccharide promote resistance to the classical complement pathway. Consistent with these results, the
capsule and exopolysaccharide enhanced *K. kingae* pathogenicity in juvenile rats with an intact complement system. Loss of the capsule and the exopolysaccharide resulted in avirulence, however not in rats lacking complement.

Experiments using primary human neutrophils and a series of isogenic *K. kingae* mutants demonstrated the critical role of the capsule and the conditional role of the exopolysaccharide in preventing the neutrophil oxidative burst response and neutrophil-mediated killing of *K. kingae*. In the absence of capsule, the exopolysaccharide prevented *K. kingae* opsonization and subsequently, reduced ROS production. The loss of capsule promoted neutrophil binding of *K. kingae* but had no effect on neutrophil phagocytosis and bacterial internalization of *K. kingae*. In contrast, the exopolysaccharide efficiently blocked neutrophil phagocytosis of *K. kingae* and resisted the bactericidal effects of antimicrobial peptides.

This works establishes that the *K. kingae* surface polysaccharides are multi-functional and play a critical role in *K. kingae* pathogenicity, facilitating evasion of host immunity. Our studies suggest that the *K. kingae* capsule and exopolysaccharide have potential as targets for vaccine development.
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1. Introduction

1.1 Kingella kingae history and pathogenicity

Kingella kingae was first isolated in the 1960’s by Elizabeth O. King at the Centers for Disease Control in Atlanta, GA (Bovre, 1976; Henriksen & Bovre, 1968). The species belongs to the Neisseriaceae family under the Kingella genus, along with K. denitrificans, K. oralis, K. negevensis, and K. potus (Bovre, 1976; C. Chen, 1996; El Houmami et al., 2017; Lawson et al., 2005; Maccato, McLean, Riddle, & Faro, 1991). K. kingae is a beta-hemolytic gram-negative coccobacillus and is most often isolated from human respiratory secretions and from systemic sites in patients with bacteremia, endocarditis, osteomyelitis, or septic arthritis (Henriksen & Bovre, 1968). The organism can be recovered from the human oropharynx as a part of the oral commensal flora and primarily colonizes children between the ages of 6 and 36 months (Amit, Dagan, & Yagupsky, 2013; Yagupsky, Porat, & Pinco, 2009).

As an emerging pediatric pathogen, K. kingae is increasingly recognized as the primary etiology of osteoarticular infections, a common etiology of bacteremia, and an occasional etiology of endocarditis in young children (Chometon et al., 2007b; Yagupsky, 2014; Yagupsky, Erlich, Ariela, Trefler, & Porat, 2006; Yagupsky, Porsch, & St Geme III, 2011). Invasive K. kingae disease is frequently associated with a concurrent viral respiratory infection in patients (J Amir & Yagupsky, 1998; Kiang et al., 2005; Seña, Seed, Nicholson, Joyce, & Cunningham, 2010; Yagupsky, 2004; Yagupsky & Dagan, 1994). Typically, patients under four years of age with invasive K. kingae disease are healthy, whereas older patients often have underlying health issues, including immunosuppression
or cardiac valve pathology (Dubnov-Raz et al., 2008, 2010; Yagupsky, 2004). The rate of
*K. kingae* colonization in the oropharynx peaks between 12- to 24-months of age and steadily decreases as the child ages (Yagupsky, Dagan, Prajgrod, & Merires, 1995; Yagupsky, Peled, & Katz, 2002). This age-related pattern of colonization correlates with the development of the adaptive immune response against *K. kingae* (Yagupsky et al., 1995, 2002). Asymptomatic colonization of the oropharynx is considered an immunizing event due to the increase in *K. kingae* cross-reactive antibody levels in older children and adults regardless of whether the child presents with invasive *K. kingae* disease (Slonim, Steiner, & Yagupsky, 2003; Yagupsky et al., 2011).

Oropharyngeal colonization by *K. kingae* presumably begins with adherence to respiratory epithelial cells. *In vitro* studies demonstrate that adherence to human epithelial cells is mediated by two identified adhesins in *K. kingae*, type IV pili and the autotransporter protein Knh (Kehl-Fie et al., 2010; Kehl-Fie, Miller, & St. Geme, 2008; Kehl-Fie, Porsch, Miller, & St. Geme III, 2009; Porsch, Kehl-Fie, & St. Geme III, 2012). *K. kingae* interaction with epithelial cells is initiated by initial low-affinity adherence through type IV pili engagement with host receptors (Kern, Porsch, & St. Geme, 2017; Porsch et al., 2012). Subsequently, pili binding and retraction results in polysaccharide capsule displacement and Knh-mediated high-affinity adherence (Kern et al., 2017; Porsch et al., 2012).

*K. kingae* type IV pili are comprised of the major pilin subunit, PilA1, and two minor pilus-associated proteins, PilC1 and PilC2 (Kehl-Fie et al., 2008; Porsch et al., 2013). The PilA1 protein in *K. kingae* is homologous to other major type IV pilins found
in pathogens such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Deletion of *pilA1* in *K. kingae* renders the bacterium nonpiliated and unable to adhere to epithelial cells or undergo twitching motility (pilus-dependent extension and retraction) *in vitro* (Kehl-Fie et al., 2008; Porsch et al., 2012). PilA1 expression is regulated by the PilS and PilR two-component system and sigma factor σ^54; elimination of PilR and σ^54 results in a drastic loss of piliation whereas elimination of PilS only slightly affects piliation (Kehl-Fie et al., 2009). Further examination of the pilin-encoding region of *K. kingae* determined that *pilA1* is transcribed upstream of two pilin-like genes, *pilA2* and *fimB* (Kehl-Fie et al., 2008). However, transcriptional analysis and transmission electron micrographs demonstrated that in-frame deletions of *pilA2* and *fimB* did not alter *K. kingae* piliation or adherence (the function of these two pilin-like genes remains unknown) (Kehl-Fie et al., 2008). The two minor pilus-associated proteins, PilC1 and PilC2, are encoded elsewhere in the genome and are homologous to the *N. meningitidis* and *N. gonorrhoeae* PilC proteins and the *Pseudomonas aeruginosa* PilY protein (Kehl-Fie et al., 2008; Porsch et al., 2013). Expression of either PilC1 or PilC2 promotes piliation, and deletion of both *pilC1* and *pilC2* results in a decrease of surface pili and loss of the adherence and twitching motility phenotype (Kehl-Fie et al., 2008; Porsch et al., 2013). Unlike the situation with mutations in PilR and σ^54, levels of PilA1 are not affected in the *pilC1* and *pilC2* double mutant, suggesting that the PilC proteins are either required or regulate pili assembly (Kehl-Fie et al., 2009; Porsch et al., 2013). The variability of *K. kingae* piliation across strains can be associated with the clinical site of isolation with most pharyngeal and bacteremia isolates,
expressing pili, while bone, joint, or endocarditis isolates generally being nonpiliated (Kehl-Fie et al., 2010, 2008).

Knh (Kingella NhhA homolog) is a predicted 540 kDa trimeric autotransporter adhesin that forms short fibers on the K. kingae bacterial surface (Kern et al., 2017; Porsch et al., 2012). Elimination of Knh reduces, but does not completely abrogate K. kingae adherence to human epithelial cells (Porsch et al., 2012). Knh-mediated adherence is dependent on the retraction of type IV pili to displace the polysaccharide capsule and unmask Knh (Kern et al., 2017; Porsch et al., 2012). The inhibition of Knh-mediated adherence by capsule is suspected to be caused by the stark differences in Knh length and capsule depth, ~110 nm and ~700 nm, respectively (Kern et al., 2017). Similar to the HMW1/HMW2 adhesins of Haemophilus influenzae, Knh is N-glycosylated by an HMWC-like glycosyltransferase enzyme (Rempe et al., 2015). In the absence of glycosylation, Knh levels in the outer membrane are reduced and adherence to epithelial cells and autoaggregation is lost (Rempe et al., 2015). Annotation and analysis of the Knh amino acid sequence unveiled the presence of two predicted domains associated with adhesive activity, an ISneck2 domain located near the membrane anchor and multiple YadA-like head domains located near the N-terminus (Porsch et al., 2012). Regulation of Knh expression and the mechanism of presentation on the bacterial surface remains largely uncharacterized. However, preliminary data suggest that two genes immediately downstream of knh designated knhB and knhC may play a role in the presentation of Knh on the bacterial surface, as elimination of KnhB and KnhC slightly alters Knh-mediated adherence and autoaggregation of K. kingae (unpublished data).
Biofilm formation facilitates bacterial adherence and establishment of colonization in microbial communities. *K. kingae* cell-free extracts from biofilms exhibit broad-spectrum anti-biofilm activity (Bendaoud et al., 2011). Examination of the *K. kingae* extracts determined the presence of two surface polysaccharides, the polysaccharide capsule and an exopolysaccharide (Bendaoud et al., 2011). The exopolysaccharide locus contains five genes (*pamA, pamB, pamC, pamD*, and *pamE*) and expression of *pamABCDE* or *pamABC* produces a galactan homopolymer (Bendaoud et al., 2011). Two unique exopolysaccharide structures have been identified so far (Bendaoud et al., 2011; Starr et al., 2013). Purified exopolysaccharide material, which is structurally distinct from the polysaccharide capsule, prevents the biofilm formation of numerous microbes through surface physiochemical modifications (Bendaoud et al., 2011). Release of exopolysaccharide by *K. kingae* is speculated to enhance colonization by inhibiting biofilm formation of other commensal organisms and establishing its niche in the oropharynx. In an infant rat infection model, elimination of the exopolysaccharide attenuated *K. kingae*-induced morbidity and mortality, suggesting that the exopolysaccharide plays a significant role in *K. kingae* virulence (Muñoz, Porsch, & St. Geme, 2018).

Following asymptomatic colonization in the respiratory tract, *K. kingae* must breach the respiratory epithelium, enter the bloodstream, and spread to distant sites for disease progression (Amit et al., 2013; Bidet et al., 2013; Yagupsky et al., 2006, 2009). It is hypothesized that bloodstream invasion of *K. kingae* is facilitated by disruption of the respiratory epithelium due to viral co-infection and secretion of the *K. kingae* RtxA cytotoxin (Amit et al., 2012; Kehl-Fie & St. Geme III, 2007; Yagupsky et al., 2011). *K.
*kingae* displays a wide range of cellular toxicity, and generation of a transposon mutant library identified the rtx locus as essential for cytotoxicity against epithelial, synovial, and macrophage-like cells *in vitro* (Kehl-Fie & St. Geme III, 2007). Furthermore, an RtxA-deficient *K. kingae* mutant was associated with higher survival percentages and increased white blood cell counts when compared to wild type *K. kingae* in an infant rat infection model (Chang, Nudell, Lau, Zakharian, & Balashova, 2014). The rtx locus contains five genes (*rtxB, rtxD, rtxA, rtxC*, and *tolC*) necessary for the production and secretion of the RtxA toxin (Kehl-Fie & St. Geme III, 2007). Genetic analysis of the rtx locus and flanking regions suggests that the locus was acquired by *K. kingae* via horizontal transfer given the high homology to *Moraxella bovis* insertional elements and the *M. bovis* rtx locus (Kehl-Fie & St. Geme III, 2007). RtxA is secreted by *K. kingae* through the type I secretion system (RtxB, RtxD, and TolC) and is present in cytotoxic and hemolytic outer membrane vesicles (Kehl-Fie & St. Geme III, 2007; Maldonado, Wei, Kachlany, Kazi, & Balashova, 2011). RtxA induces cytotoxicity through pore formation across planar lipid membranes; cation-selectivity of the RtxA channel induces a cation flux and programmed cell death (Bárceña-Uribarri, Benz, Winterhalter, Zakharian, & Balashova, 2015). Post-translational acylation of RtxA lysine residues and cholesterol binding are critical for the cytotoxic activity of RtxA (Osickova et al., 2018).

Bacterial encapsulation provides a variety of benefits to microbes by allowing the organism to tolerate environmental stressors and evade host immunity during infection. *K. kingae* produces a polysaccharide capsule that enhances virulence in the infant rat infection model (Muñoz et al., 2018; Starr, Porsch, Seed, & St. Geme III, 2016). The polysaccharide
capsule in conjunction with the exopolysaccharide can protect *K. kingae* from complement-mediated lysis by preventing opsonin deposition and complement activation (Muñoz et al., 2018). In *K. kingae*, four distinct polysaccharide capsules (types a, b, c, and d) have been identified, with capsule types a and b accounting for greater than 95% of invasive disease isolates (Porsch, Starr, Yagupsky, & St. Geme III, 2017; Starr, Porsch, Seed, Heiss, et al., 2016). Genetic analysis of *K. kingae* genomes revealed that clinical isolates contain one of four capsule-specific biosynthesis loci (*csa*, *csb*, *csc*, and *csd*), as well as a capsule export operon (*ctrABCD*) and capsule assembly genes (*lipA* and *lipB*) (Starr, Porsch, Seed, Heiss, et al., 2016; Starr, Porsch, Seed, & St. Geme III, 2016). Unlike the exopolysaccharide, the four *K. kingae* capsule types stain with cationic ferritin, which is consistent with anionic properties (Starr et al., 2013).

As outlined, *K. kingae* expresses a number of surface or secreted factors that have been characterized as potential virulence factors necessary for *K. kingae* colonization and dissemination during pathogenesis (Table 1, Fig. 1).

<table>
<thead>
<tr>
<th>Table 1: <em>K. kingae</em> surface or secreted factors</th>
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<td><strong>Factor</strong></td>
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| Capsule   | Protects against complement-mediated lysis, neutrophil activation and association, and antimicrobial peptides  
|           | Promotes virulence *in vivo*                      |
| Exopolysaccharide | Protects against complement-mediated lysis, neutrophil phagocytosis, and antimicrobial peptides  
|           | Inhibits biofilm production of other microbes  
|           | Promotes virulence *in vivo*                      |
| Knh       | Promotes adherence to epithelial cells           |
| OMVs      | Wide-spectrum cytoxicity                          |
|           | Contains RtxA, PilC2, and polysaccharide capsule |
| RtxA | - Wide-spectrum cytoxicity  
|      | - Promotes virulence *in vivo*
| Type IV pili | - Promote adherence to epithelial cells, twitching motility, and natural competence  
|      | - Contain PilC1 and PilC2

*OMVs, outer membrane vesicles

**Figure 1: Diagram of *K. kingae* virulence factors.**

*K. kingae* surface or secreted factors that are hypothesized to influence colonization (bottom) or dissemination (top).
1.2 Bacterial surface polysaccharides

1.2.1 Lipopolysaccharides

Gram-negative bacterial cell walls are multilayered barriers comprised of an inner membrane, an outer membrane, and a thin peptidoglycan layer within the periplasmic space between the two membranes. The bacterial outer membrane consists of phospholipids, lipoproteins, surface proteins, and lipopolysaccharides (LPS). For gram-negative bacteria, the outer membrane is an asymmetric bilayer, with LPS localizing to the outer leaflet (Funahara & Nikaido, 1980; Kamio & Nikaido, 1976; Mühlradt & Golecki, 1975). The LPS (also known as endotoxin) composition is characterized by three separate structural components: lipid A, a core oligosaccharide, and an O-antigen repeating polysaccharide.

Lipid A has a N-acetylglucosamine backbone that is highly acylated. As a phospholipid, lipid A anchors LPS to the hydrophobic outer membrane (Raetz, 1990; Raetz & Whitfield, 2002; Rietschel et al., 1994). For the majority of gram-negative bacteria, lipid A is required for growth, stability, and maintenance of the outer membrane barrier (Galloway & Raetz, 1990; Onishi et al., 1996; M. Vaara, 1993). Lipid A is highly conserved across bacterial species, and due to this conservation, fragmented or released LPS can be detected at the picomolar level by the host immune system (Aderem & Ulevitch, 2000; Medzhitov & Janeway, 2000). Innate immune cells, such as macrophages and neutrophils, along with endothelial cells, express Toll-like receptor 4 (TLR4), which binds lipid A moieties to trigger downstream immune cell activation and the release of inflammatory mediators (Beutler & Cerami, 1988; Dinarello, 1991; Drake, Cheng, Chang, & Taylor, 1993; Hoshino et al., 1999; Poltorak et al., 1998). While productive immune
responses to LPS promote bacterial clearance, lipid A is considered responsible for the
toxicity of LPS, and overproduction of inflammatory mediators systemically can lead to
cellular damage and septic shock in the host (Parrillo, 1993; van Deuren, Brandtzaeg, &
vander Meer, 2000).

The core oligosaccharide in LPS can be segmented further into the inner core,
which is proximal to lipid A, and the outer core, which attaches the O antigen (Raetz &
Whitfield, 2002). The inner core of most LPS structures contains keto-deoxyoctulosonate
(Kdo) residues and can be modified with phosphates, phosphorylcholine, or
phosphorylethanolamine residues (Brade, Opal, Vogel, & Morrison, 1999; Raetz &
Whitfield, 2002). Diversity in modifications of the inner core provides heterogeneity of the
LPS inner core; however, the outer core displays increased structural diversity (Raetz &
Whitfield, 2002). The structural diversity of the LPS outer core is thought to be shaped by
environmental pressures. Despite variation, the structural composition of the core
oligosaccharide remains largely conserved among species within the same genus or family
(Raetz & Whitfield, 2002). Conservation of the LPS core suggests that the structure is
essential for bacterial membrane stability and integrity (Raetz & Whitfield, 2002).

Unlike lipid A and the core oligosaccharide, O-antigen polymers are vastly diverse
even within a single species. Differences in the O-antigen structural composition can be
due to changes in the glycosidic linkages, monomers, branching patterns, and inclusion of
noncarbohydrate components (Brade et al., 1999; Knirel’ & Kochetkov, 1994).
Heterogeneity is also observed in the O-antigen chain length, and LPS visualization on a
gel exhibits a laddering effect due to variations in extension and addition of O units (Raetz
The O-antigen polysaccharide provides protection against a host of immune responses, and recognition of the O-antigen by immune components depends on the structure, abundance, and length of the O-antigen (Burns & Hull, 1998; Joiner, 1988; Weiss, Hutzler, & Kao, 1986). Moreover, a loss or shortening of the O-antigen can hinder bacterial colonization and/or virulence (Nesper et al., 2001; R. C. Sandlin et al., 1995; Robin C. Sandlin, Goldberg, & Maurelli, 1996; Van den Bosch, Manning, & Morona, 1997). The extent of protection and virulence provided by the O-antigen varies drastically across strains due to the wide range of structural diversity.

Many mucosal pathogens such as *H. influenzae* and *N. meningitidis* express solely the lipid A moiety and the core oligosaccharide, producing a lipooligosaccharide (LOS) that lacks the O-antigen (Raetz & Whitfield, 2002). LOS is also found in the outer leaflet of the outer membrane and has been demonstrated to mediate adherence and invasion of host cells in some species (Song, Ma, Chen, & Stein, 2000; van Vliet et al., 2009; Wetzler, Barry, Blake, & Gotschlich, 1992). Despite lacking the hypervariable O-antigen polymer, mucosal pathogens can adapt to immune and environmental pressures through phase variation prompting the expression of various forms of the LOS structure (Brade et al., 1999; Giardina, Weiss, Gibson, & Apicella, 2001; Hood et al., 1999). The LOS core is comprised of an inner core that is often decorated with either mono- or oligosaccharide branches (considered the outer core), which are the determinants of antigenicity (Brade et al., 1999; Giardina et al., 2001; Hood et al., 1999). Sialylation and host-antigen mimicry of LOS structures promotes colonization and immune evasion by mucosal pathogens (R.

1.2.2 Polysaccharide capsules

Capsular polysaccharides are lipidated, surface-anchored carbohydrate chains that contain multiple repeating saccharide units. Similar to the O-antigen present in LPS, capsules can consist of variations in sugar polymers, cyclic and isomer forms, and glycosidic linkages to generate unique antigenic epitopes within and across bacterial species. Polysaccharide capsules are generally considered anionic and highly hydrated, generating a mucoid-like phenotype (Gotschlich, Fraser, Nishimura, Robbins, & Liu, 1981). While the capsule biosynthesis genes necessary for polysaccharide production tend to be genetically diverse, the export and assembly machinery remains highly conserved across encapsulated gram-negative bacteria (Whitfield, 2006; Whitfield & Roberts, 1999).

For *N. meningitidis*, *H. influenzae*, and *E. coli*, a set of six conserved genes allow for polysaccharide capsule presentation (M. Frosch, Edwards, Bousset, Krausse, & Weisgerber, 1991). Four of six conserved gene products form an ATP-binding cassette transporter system that facilitates proper export through ATP-hydrolysis and insertion of the polysaccharide capsule into the outer membrane (M. Frosch, Müller, Bousset, & Müller, 1992; Larue, Ford, Willis, & Whitfield, 2011; Pazzani et al., 1993). As the result of the highly selective pressure exerted by the immune system, polysaccharide diversity within a given species can be relatively high (e.g., *E. coli*, ~80 serotypes; *N. meningitidis*, 13 serotypes; *H. influenzae*, 6 serotypes) (Cartwright, 1995; Moxon & Vaughn, 1981; Orskov, Orskov, Jann, & Jann, 1977; Whitfield, 2006). Interestingly, the pathogenicity of
a given pathogen can be dependent on the capsular type expressed, as certain capsules highly correlate with invasive disease, most notably the *H. influenzae* serotype b capsule (Cartwright, 1995; Moxon & Kroll, 1988; Moxon & Vaughn, 1981).

Polysaccharide capsules are critical virulence factors for many microorganisms and have been widely shown to protect bacteria against mucosal and intravascular inflammatory immune responses (Campos et al., 2004; Lambris, Ricklin, & Geisbrecht, 2008; Moxon & Vaughn, 1981; Nelson et al., 2007; Zwahlen, Kroll, Rubin, & Moxon, 1989). The presence of capsule masks bacterial surface antigens recognized by antibodies or complement fragments. By preventing detection of these antigenic epitopes, capsules impede antibody and complement opsonization and thus efficiently protect the bacterium from complement-mediated lysis and neutrophil phagocytosis. Neutrophils have negatively charged surfaces, and therefore, expression of polysaccharide capsules increases electrostatic repulsion and reduces or prevents phagocytosis by enhancing the anionic properties of the bacterial surface (Jarvis & Vedros, 1987). Beyond physical hinderances, polysaccharide capsules can incorporate sialic acids into the repeating unit mimicking host moieties and effectively averting detection by the host immune system (Matthias Frosch & Maiden, 2006). Furthermore, sialic acid incorporation into the polysaccharide structure can recruit the complement regulatory protein, factor H (Meri & Pangburn, 1990; Schneider et al., 2006). Factor H acts on the complement alternative pathway by binding factor I, which cleaves complement fragment C3b into an inactive form, iC3b, and promotes decay of the C3 convertase, inhibiting the feedback loop of the alternative pathway.
Immunogenic studies performed in mice revealed a T-cell independent response to polysaccharide capsules during immunization (Barrett, 1985; A. Coutinho, Möller, Anderson, & Bullock, 1973; Antonio Coutinho & Möller, 1973). Due to the nature of the response, a lack of IgM to IgG class-switching during initial infection leads to inefficient antibody production and B-cell memory response upon re-challenge. To overcome the hallmark characteristics of a T-cell independent response, chemical conjugation of polysaccharide capsules to carrier proteins redirects the capsule to a T-cell dependent response and boosts capsule-specific memory B-cells, providing lasting immunity (Kelly et al., 2006; MacLennan et al., 2001). The polysaccharide capsules of *S. pneumoniae*, *H. influenzae*, and *N. meningitidis* have been extensively studied due to their importance as virulence factors and their effective use as polysaccharide-conjugate vaccines (Goldblatt, 2000).

1.2.3 Exopolysaccharides

Exopolysaccharides are high molecular weight homo- or hetero-sugar polymers synthesized by bacteria and secreted into the extracellular milieu. Distinct characterization and clustering of exopolysaccharides can be difficult due to the heterogeneity across species, but polymers most commonly incorporate glucans, fructans, polygalactans, glucose, galactose, and rhamnose into the exopolysaccharide repeating unit (Gherardini & Helmer-Citterich, 2008; Ruas-Madiedo, Hugenholtz, & Zoon, 2002). The functionality of a given exopolysaccharide depends on the polymer structure, composition, and abundance.

Bacterial exopolysaccharides have been studied primarily in relation to biofilms, specifically attributing to adherence, bacterial cell aggregation, water retention, nutrient
scavenging, and various other processes during biofilm formation and dispersal (Cerning, 1991; De Vuyst & Degeest, 1999; Flemming, Neu, & Wozniak, 2007; Watnick & Kolter, 2000). As a major contributor to the formation of biofilms, exopolysaccharides can provide scaffolding for the biofilm matrix and are necessary for adherence and cohesiveness during initial attachment to surfaces and microcolony establishment (Flemming & Wingender, 2010; Rehm, 2010). Furthermore, differences in hydrophobicity of diverse exopolysaccharides can alter the biofilm architecture, stability, and storage of nutrients and enzymes (Nwodo, Green, & Okoh, 2012). While exopolysaccharide production is species-specific, environmental factors such as temperature, pH, nutrient availability, and growth conditions can influence exopolysaccharide yield and biofilm formation (Looijesteijn, van Casteren, Tuinier, Doeswijk-Voragen, & Hugenholtz, 2000).

Exopolysaccharides produced by pathogens such as Pseudomonas aeruginosa, extraintestinal pathogenic E. coli, and Bordetella pertussis promote pathogenesis beyond biofilm formation through evasion of cellular and humoral innate immune components (Ganguly, Johnson, Kock, Parks, & Deora, 2014; Jones & Wozniak, 2017; Miajlovic, Cooke, Moran, Rogers, & Smith, 2014; Mishra et al., 2012). Despite lacking a membrane-anchor, these exopolysaccharides exhibit similar properties to bacterial polysaccharide capsules by providing protection against neutrophil and complement activation and decreasing sensitivity to antimicrobial peptides and reactive oxygen species. Moreover, exopolysaccharides have also been demonstrated to interact with immune cells and can stimulate or dampen inflammatory responses during infection (Jones & Wozniak, 2017; Totté et al., 2015).
Various bacterial exopolysaccharides exhibit advantageous properties such as high viscosity, gelling capacity, tensile strength, stability, and low solubility (Nwodo et al., 2012). Synthetically polymerized exopolysaccharides, which can be valuable biomaterials, are frequently used for commercial and medical purposes. Common exopolysaccharides used in the food, petroleum, and pharmaceutical industries are alginate, cellulose, dextran, and xanthan (Nwodo et al., 2012). Several distinct forms of alginate are effective in the treatment of thromboembolic disorders due to the therapeutic characteristics, including anti-angiogenesis and anticoagulation (Alban, 2002). Xanthan gum has been utilized for a broad spectrum of diverse applications not limited to oil drilling, pesticides, and aqueous systems (Nwodo et al., 2012; Rosalam & England, 2006).

1.2.4 \textit{K. kingae} surface polysaccharides

Analysis of an Israeli collection containing over 400 \textit{K. kingae} clinical isolates from invasive disease and asymptomatic carriers revealed that greater than 99% of isolates screened were encapsulated (Starr, Porsch, Seed, Heiss, et al., 2016). PCR amplification revealed four distinct amplicons in the capsule synthesis locus (Starr, Porsch, Seed, Heiss, et al., 2016). Nucleotide sequencing revealed four unique loci, with one locus containing a single gene and three of the loci containing three genes (\textit{csaA}, \textit{csbABC}, \textit{cscABC}, and \textit{csdABC}) (Starr, Porsch, Seed, Heiss, et al., 2016). The capsule loci nomenclature was designated based on the type of capsule produced (e.g., \textit{csaA}, capsule synthesis type A) (Starr, Porsch, Seed, Heiss, et al., 2016; Starr, Porsch, Seed, & St. Geme III, 2016). Structural analyses were performed for purified capsule material from 12 \textit{K. kingae} strains expressing one of the four capsule loci. The analysis revealed the distinct
polymer composition and linkages of the four capsule types (type a, [3]-β-GalpNAc-(1→5)-β-Kdop-(2→]; type b, [6]-α-D-GlcNAc-(1→5)-β-(8-OAc)Kdop-(2→]; type c, [3]-β-D-Ribf-(1→2)-β-D-Ribf-(1→2)-β-D-Ribf-(1→4)-β-Kdop-(2→]; and type d, [P-(O→3)[β-D-Galp-(1→4)]-β-D-GlcNAc-(1→3)-α-D-GlcNAc-1-) (Fig. 2) (Starr, Porsch, Seed, Heiss, et al., 2016). Within the Israeli K. kingae collection, 239 strains were isolated from healthy carriers and 178 strains were isolated from K. kingae infections (Starr, Porsch, Seed, Heiss, et al., 2016). Capsule typing revealed that greater than 95% of invasive isolates expressed either a type a or type b capsule; in comparison, 68% of carrier isolates expressed either a type a or type b capsule (Starr, Porsch, Seed, Heiss, et al., 2016). Types c and d represented 12% and 20% of carrier isolates, respectively; these percentages dropped to a combined 4% representation in the invasive isolates (Starr, Porsch, Seed, Heiss, et al., 2016). The distribution of capsule types in invasive versus carrier isolates was further validated by applying a multiplex PCR approach to an international collection of K. kingae clinical isolates (Porsch et al., 2017).
Figure 2: Structure of the polysaccharide capsules and exopolysaccharide types present in *K. kingae* clinical isolates.

The polysaccharide capsule repeating unit structures (top panel) and the exopolysaccharide repeating unit structures (bottom panel). Top panel: capsule type a (A, GalNAc-Kdo), capsule type b (B, GlcNAc-Kdo), capsule type c (C, ribose-Kdo), and capsule type d (D, galacose-GlcNAc). Bottom panel: exopolysaccharide type 1 (1, galactan monosaccharide) and exopolysaccharide type 2 (2, galactan disaccharide). Adapted from (Starr et al., 2013) and (Starr, Porsch, Seed, Heiss, et al., 2016).

Studies performed with *K. kingae* strain KK01, which expresses a type a capsule, demonstrated that capsule expression was necessary for full virulence in an infant rat infection model (Muñoz et al., 2018; Starr, Porsch, Seed, & St. Geme III, 2016). Furthermore, due to the depth of the polysaccharide capsule, surface antigens, including the autotransporter adhesin, Knh, present on the bacterial surface are masked. Masking of
these surface epitopes enhances survival of *K. kingae* in the presence of serum and modulates *K. kingae* adherence to epithelial cells (Kern et al., 2017; Muñoz et al., 2018; Porsch et al., 2012).

Gas chromatography and nuclear magnetic resonance determined the glycosyl structure of surface polysaccharides from invasive *K. kingae* strains, KK01 and PYKK181 (Bendaoud et al., 2011; Starr et al., 2013). Analysis determined that in addition to the polysaccharide capsule, strain KK01 produces a →5)-β-Gal-(1→ exopolysaccharide and strain PYKK181 produces a →3)-β-Gal-(1→6)-β-Gal-(1→ exopolysaccharide (Fig. 2) (Bendaoud et al., 2011; Starr et al., 2013). Both exopolysaccharides are linear galactan homopolymer exopolysaccharides with distinct glycosyl linkages (Bendaoud et al., 2011; Starr et al., 2013). The KK01 and PYKK181 exopolysaccharides were designated as type 1 and type 2, respectively (Fig. 2). As previously mentioned in Ch. 1.1, expression of the type 2 exopolysaccharide from *K. kingae* or from an inducible plasmid in *E. coli* demonstrated that the exopolysaccharide exerted potent anti-biofilm properties inhibiting biofilm formation by gram-negative and gram-positive bacteria, as well as the fungal pathogen, *Candida albicans* (Bendaoud et al., 2011). *In vivo* juvenile rat studies using derivatives of strain KK01 demonstrated that the type 1 exopolysaccharide was necessary for full *K. kingae* virulence (Muñoz et al., 2018).

So far, there are no current publications identifying LPS genes or loci present in the *K. kingae* genome. Likewise, chemical or structural composition analyses of the *K. kingae* LPS, or more likely LOS, in clinical isolates has not been performed. Current ongoing
studies aim to address these critical gaps in knowledge in the attempt to broaden the understanding of *K. kingae* surface polysaccharides and involvement in pathogenesis.

### 1.3 Innate immunity

The immune system functions to protect the host against a variety of disease states, including invasion by microbial pathogens. Broadly, immunity can be divided into two main branches: innate immunity and adaptive immunity. Innate immunity is considered a non-specific, general response to foreign bodies, whereas adaptive immunity is considered a specialized, targeted response to specific antigens. Children rely heavily on the protective advantages of the innate immune system, as the adaptive immunity fully develops during childhood (Janeway, Travers, Walport, & Shlomchik, 2001). While innate immunity is composed of multiple cellular and humoral components, two key players are neutrophils and the complement system.

#### 1.3.1 Neutrophils

Neutrophils are the most abundant leukocyte (50-70%) present in human blood (Döhrmann, Cole, & Nizet, 2016). Human neutrophils are characterized by their segmented nucleus and the presence of multiple cytoplasmic vesicles (Borregaard, 2010). Generated in the bone marrow, terminally-differentiated neutrophils circulate in the bloodstream and are considered the first line of cellular defense during infection. Macrophages, mast cells, and complement recruit neutrophils to the site of inflammation by releasing chemoattractants (e.g., C5a, interleukin 8 (IL-8), and monocyte chemotactic factor), promoting expression of endothelial-leukocyte adhesive molecules (e.g., P- and E-selectins
and integrin CD11b/CD18) and increasing cell permeability (Kolaczkowska & Kubes, 2013; Phillipson & Kubes, 2011). Upon recruitment and extravasation, activated neutrophils contain an arsenal of anti-microbial mechanisms to effectively combat infection.

As phagocytic cells, neutrophils bind and engulf microbes through the expression of surface receptors that recognize opsonins and foreign surfaces (Borregaard, 2010; Phillipson & Kubes, 2011). After phagocytosis, phagocytic vacuoles fuse with lysosomes to form mature phagolysosomes, and neutrophils utilize both oxidative and non-oxidative methods for optimal killing of engulfed bacteria (Borregaard, 2010; Falloon & Gallin, 1986). Membrane-associated NADPH oxidases generate reactive oxygen species that are either released into the extracellular milieu or into phagolysosomes to target both intracellular and extracellular bacteria for oxidative killing (Borregaard, 2010; Nauseef, 2007). Moreover, phagolysosomes fuse with granules for the release of antimicrobial peptides and proteases, including elastase, myeloperoxidase, defensins, and cathepsin G, to enhance bacterial killing inside the vacuoles (Falloon & Gallin, 1986). The release of cytotoxic and antimicrobial proteins into the extracellular milieu can also occur through a cellular process termed degranulation. During neutrophil maturation, three distinct types of granules are formed (azurophilic, specific, and tertiary) and are ultimately exocytosed as a consequence of neutrophil activation by inflammatory stimuli during infection (Borregaard, 2010; Falloon & Gallin, 1986). Degranulation also promotes recruitment of adaptive immune cells by enhancing lymphocytic infiltration through chemokine release (Nathan, 2006; Tecchio, Micheletti, & Cassatella, 2014).
Lastly, neutrophils can undergo the release of neutrophil extracellular traps (NETs), which are primarily composed of DNA bound to histones, enzymes, and antimicrobial peptides (Brinkmann et al., 2004). NETs form a ‘sticky’ matrix immobilizing extracellular bacteria from further dissemination and aiding in phagocytosis (Papayannopoulos & Zychlinsky, 2009; Phillipson & Kubes, 2011). Trapped pathogens can also be directly killed by the densely concentrated, chromatin-associated antimicrobial proteins and peptides (Brinkmann et al., 2004).

1.3.2 Complement

Complement, a multifunctional group of proteins present in the blood and on cell surfaces, is a highly regulated component of the innate immune system. Complement activation promotes the recruitment of immune cells and recognition of pathogens through chemotaxis and opsonization (Gros, Milder, & Janssen, 2008). Complement is activated via three separate pathways: classical, lectin, and alternative. While activation of a distinct complement pathway is target-specific (antigen-antibody complexes—classical pathway, carbohydrate-lectin complexes—lectin pathway, and foreign surfaces—alternative pathway), all three pathways converge on the cleavage of C3 and the deposition of the protein fragment C3b on the target surface (Walport, 2001a, 2001b). C3b deposition promotes opsonization and formation of the membrane attack complex (MAC). MAC assembly, also known as the terminal pathway, is the sequential association of complement proteins, C5b, C6, C7, C8, and C9, which insert into the bacterial membrane to form a pore and mediate lysis of gram-negative bacteria (Bhakdi & Tranum-Jensen, 1988; Müller-Eberhard, 1986). The cleavage of C3 and C5 leads to the release of anaphylatoxins, C3a.
and C5a, which induce inflammatory responses that promote recruitment of phagocytes to the site of infection (Lambris et al., 2008). Phagocytes, including neutrophils and macrophages, recognize opsonized pathogens through membrane-associated receptors (e.g., CR1-4 and FcR); recognition by receptors prompts phagocytosis and bacterial clearance (Zipfel & Skerka, 2009).

Over 20 soluble and surface-associated regulators control aberrant complement activation and allow complement to discriminate between foreign and non-foreign cell surfaces. To protect host cells from complement activation during inflammation, complement regulators inhibit C3b deposition on ‘self’ cells or cleave deposited C3b to its inactive form to prevent further complement activation and deposition (Zipfel & Skerka, 2009). Complement regulators can also target anaphylatoxins C3a and C5a for degradation to dampen release of inflammatory mediators and prevent unnecessary recruitment of immune cells (Mueller-Ortiz et al., 2009; Skidgel & Erdös, 2007). Complement regulation functions beyond immune surveillance and facilitates neuroprotection, cellular homeostasis, and removal of cellular debris (Ricklin, Hajishengallis, Yang, & Lambris, 2010; Walport, 2001a, 2001b). Soluble regulators are generally categorized by the complement pathway targeted: classical and lectin pathways (C1 inhibitor and C4b-binding protein (C4BP)), alternative pathway (factor H and properdin), and terminal pathway (vitronectin and clusterin) (Blom, Villoutreix, & Dahlbäck, 2004a; Davis, Mejia, & Lu, 2008; Hourcade, 2008; Józsi & Zipfel, 2008; Preissner & Seiffert, 1998; Schwarz et al., 2008). Membrane-associated regulators, including CR1, CR2, DAF, CD46, and CD59, can
act on all three pathways of complement activation by inactivating C3 and C4 (Zipfel & Skerka, 2009).

1.4 Bacterial immune evasion mechanisms

Bacterial pathogens have developed a multitude of parallel mechanisms that serve to withstand environmental stressors, evade host immune defenses, and promote survival within a host. As the first line of host defense during infection, neutrophils and complement are common targets of bacterial evasion mechanisms (Fig. 3 and Fig. 4).

1.4.1 Neutrophil evasion

Recruitment

Chemoattractants recruit neutrophils to the site of inflammation and promote expression of surface adhesins to allow for neutrophil extravasation across the endothelial layer. The family of staphylococcal superantigen-like proteins (SSLs) promotes bacterial survival by interfering with recruitment of neutrophils during infection (Thammavongsa, Kim, Missiakas, & Schneewind, 2015). SSL5 and SSL11 interact with P-selectin glycoprotein ligand 1 (PSGL1) on neutrophil surfaces to prevent neutrophil adherence and extravasation through the endothelium (Bestebroer et al., 2007; Chung et al., 2007). Furthermore, SSL5 binds G protein-coupled receptors to inhibit anaphylatoxin-mediated activation of neutrophils and proinflammatory chemokine release (Bestebroer et al., 2007, 2009). Secretion of SSL3 and SSL10 avert neutrophil chemoattraction through association with lymphocyte receptors, TLR2 and CXCR4, respectively (Spaan, Surewaard, Nijland, & van Strijp, 2013; Yokoyama et al., 2012). Group A Streptococcus dampens neutrophil
recruitment through the expression of a membrane-anchored serine protease SpyCEP that degrades the chemokine IL-8 (Zinkernagel et al., 2008). Streptococcal secreted esterase (Sse) and C5a peptidase A (SCPA) are secreted by the organism and inactivate C5a and chemotactic platelet-activating factor to further suppress neutrophil recruitment (M. Liu et al., 2012; Terao, Yamaguchi, Hamada, & Kawabata, 2006).

*Phagocytosis*

Neutrophils recognize antibodies, complement fragments, and pathogen associated molecular patterns through the expression of various surface receptors that promote neutrophil association and phagocytic uptake of bacterial invaders. Bacterial cytotoxins play an integral role in promoting resistance to neutrophil phagocytosis and enhance bacterial invasion. *Staphylococcus aureus* can escape neutrophil phagocytic vacuoles through the expression of leukocidin LukAB (DuMont et al., 2013). Group A *Streptococcus* produces streptolysin S (SLS) and streptolysin O (SLO) that initiate neutrophil apoptosis and hinder phagocytosis (Datta et al., 2005; Timmer et al., 2009). During pathogenesis, mutations in the two-component system, CovRS, up-regulate streptolysin O production and boost innate immune evasion by streptococci (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; M. J. Walker et al., 2007).
Figure 3: Neutrophil antimicrobial functions and bacterial evasion factors.

Evasion factors expressed by pathogen bacteria that target neutrophil recruitment (A), phagocytosis (B), the oxidative burst response/degranulation (C), and neutrophil extracellular traps (NETs) (D). Adapted from (Serruto, Rappuoli, Scarselli, Gros, & Van Strijp, 2010) and (Criss & Seifert, 2012).

Encapsulated pathogenic bacteria physically avoid phagocytosis by preventing opsonization and neutrophil recognition of surface antigens. Moreover, bacterial capsules expressed by pathogens, such as *N. meningitidis*, prevent phagocytosis by enhancing the anionic properties of the bacterial surface (Jarvis & Vedros, 1987). Group A *Streptococcus* produces a membrane-anchored M protein that binds complement regulators (factor H and C4BP) and blocks opsonization to remain undetected by neutrophils (Oehmcke, Shannon, Mörgelin, & Herwald, 2010).

Oxidative burst and degranulation
Neutrophils contain oxidative and non-oxidative methods to effectively target and kill intracellular and extracellular bacteria. *N. gonorrhoeae* porin and group A *Streptococcus* streptolysin O impair stimulation of the neutrophil oxidative burst response and suppress reactive oxygen species (ROS) generation (Uchiyama et al., 2015; Wetzler, Blake, Barry, & Gotschlich, 1992). Streptococcal and staphylococcal superoxide dismutases enzymatically detoxify neutrophil superoxides to prevent bacterial killing via ROS production (Henningham, Döhrmann, Nizet, & Cole, 2015; Karavolos, Horsburgh, Ingham, & Foster, 2003). *S. aureus* also synthesizes enzymes that protect the bacterium from nitrosative stress, hydrogen peroxide, and hydroxy radicals (Cosgrove et al., 2007; G. Y. Liu et al., 2005; Richardson, Libby, & Fang, 2008).

Bacterial resistance to neutrophil degranulation and the influx of antimicrobial peptides is mediated by the expression of proteinases and efflux pumps that target granular contents (Criss, Katz, & Seifert, 2009; Hagman et al., 1995; Stapels et al., 2014; Stohl, Criss, & Seifert, 2005). Enzymatic modifications of the *S. aureus* peptidoglycan layer and teichoic acids impede bactericidal activity of neutrophil antimicrobial peptides and secreted proteases (Bera, Herbert, Jakob, Vollmer, & Götz, 2005; Ernst & Peschel, 2011; Peschel et al., 1999).

**Neutrophil extracellular traps**

NET formation is considered a last effort attempt to kill extracellular bacteria by entangling microbes in DNA meshwork, increasing proximity to antimicrobial peptides, and facilitating phagocytosis. DNase production by streptococcal and staphylococcal strains is a significant effector for NET dispersal and evasion (Berends et al., 2010;
Buchanan et al., 2006). Degradation of NETs prevents entrapment and bacterial killing via antimicrobial peptides or neutrophil phagocytosis. Beyond the release of nucleases, the *S. pneumoniae* polysaccharide capsule protects against the antimicrobial effects of NET expulsion by reducing DNA interaction (Wartha et al., 2007). As previously mentioned, the group A *Streptococcus* SpyCEP peptidase targets IL-8 for degradation (Zinkernagel et al., 2008). IL-8 chemokine secretion promotes NET production and bacterial clearance.

### 1.4.2 Complement evasion

Complement activation relies on a cascade of events to deposit opsonins on the bacterial surface and promote formation of the membrane attack complex and/or recognition by phagocytes. Microbes express a host of proteins that interrupt or impede proper activation of complement to escape detection by the immune system (Fig. 4).

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**Figure 4:** The complement system and evasion strategies by pathogenic bacteria.

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The three pathways of complement activation and selected bacterial evasion factors listed near their relevant complement targets. Adapted from (Serruto et al., 2010).

Complement regulators

Acquisition of complement regulators is a common strategy for complement evasion by bacterial pathogens, such as *E. coli*, *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* (Hallström & Riesbeck, 2010; Kugelberg, Gollan, & Tang, 2008; Lambris et al., 2008; Thammavongsa et al., 2015). Soluble complement regulators (factor H, factor H-like protein 1, and C4BP) are present in high concentrations in human serum, making them readily accessible for acquisition by invading microbes. Neisserial species express multiple and often redundant surface proteins or structures that interact with complement regulators to promote survival in the host. *N. meningitidis* produces factor H-binding proteins, NspA and fHbp, as well as the C4BP-binding protein PorA (Jarva, Ram, Vogel, Blom, & Meri, 2005; Lewis et al., 2010; Lewis, Carter, & Ram, 2012; Madico et al., 2006); *N. gonorrhoeae* produces the factor H-binding protein PorB1A, which also binds C4BP along with PorB1B (Ram et al., 2001; Ram, McQuillen, et al., 1998). The sialylated LOS produced by *N. gonorrhoeae* has also been observed to interact with human factor H (Ram, Sharma, et al., 1998).

Antigen masking

Despite the vast diversity of polysaccharide capsule structures, bacterial encapsulation provides protection by masking surface antigens from antibody recognition and physically preventing complement deposition on the bacterial surfaces (Achtman, 1995; Horwitz & Silverstein, 1980; Kugelberg et al., 2008; Peterson, Wilkinson, Kim,
Schmeling, & Quie, 1978). Similarly, *N. meningitidis* can evade antibody detection by mimicking host-cell surfaces with sialylated LOS and masking bacterial epitopes (Schneider, Exley, Ram, Sim, & Tang, 2007; van Vliet et al., 2009).

*Proteolytic degradation*

The release of bacterial proteases or acquisition of host enzymes for antibody or complement degradation has been readily exploited by bacteria to prevent complement activation. *Pseudomonas* species prevent activation of the classical complement pathway by inactivating or degrading immunoglobulins, C1q, and C3 to non-functional fragments by the elastase PaE and alkaline protease PaAP (Lambris et al., 2008; Rooijakkers & van Strijp, 2007). Group A and B streptococci express C5a peptidases that degrade C5a and halt chemotactic signaling and proinflammatory responses (Chmouryguina, Suvorov, Ferrieri, & Cleary, 1996). Furthermore, exotoxin B from *S. pyrogenes* targets complement regulator properdin for degradation, destabilizing C3 convertases deposited on bacterial surfaces (Tsao et al., 2006).

*Professional complement evader – Staphylococcus aureus*

Gram-positive bacteria passively inhibit MAC formation due to their thick peptidoglycan cell wall; however, gram-positive bacteria can still be opsonized by antibodies or complement fragments and targeted by phagocytic cells. Complement evasion by *S. aureus* has been extensively studied due to its vast array of surface factors that inhibit multiple complement pathways and regulators. In addition to many other complement-targeted staphylococcal proteins, *S. aureus* produces staphylococcal complement inhibitor (SCIN), which inhibits the alternative pathway C3 convertase to
prevent C3b opsonization and release of C3a and C5a (Rooijakkers, Ruyken, et al., 2005). Secreted proteins SSL7 and Sbi-I/Sbi-II bind to human immunoglobulins to interfere with antibody recognition and block activation of the classical complement pathway (H. K. Kim et al., 2012; Thammavongsa et al., 2015; Zhang, Jacobsson, Vasi, Lindberg, & Frykberg, 1998). *S. aureus* expresses plasminogen kinase that binds and converts endogenous plasminogen into plasmin; on the bacterial surface, plasmin can degrade both IgG and C3 (Lähteenmäki, Kuusela, & Korhonen, 2001; Rooijakkers, van Wamel, Ruyken, van Kessel, & van Strijp, 2005). Efb and Ecb are multi-functional staphylococcal proteins that disrupt the C3 and C5 convertases and bind C3d (a C3b cleavage product) and factor H to prevent complement activation and proinflammatory responses (Amdahl et al., 2013; H. Chen et al., 2010; Jongerius et al., 2007).

1.5 Dissertation overview

Chapter 2 describes how the *K. kingae* polysaccharide capsule and exopolysaccharide promote survival in the presence of human serum and active complement by preventing antibody and opsonin deposition. Additionally, Chapter 2 demonstrates that both surface polysaccharides are necessary for full virulence of *K. kingae* in an infant rat infection model. Chapter 3 examines the distinct roles of the polysaccharide capsule and exopolysaccharide in protecting *K. kingae* against neutrophil-mediated killing. Chapter 4 delves into the characterization of the exopolysaccharide locus and determines the diversity of the exopolysaccharide in clinical isolates. Lastly, Chapter 5 summarizes the future directions and implications of this work.
2. *Kingella kingae* surface polysaccharides promote resistance to human serum and virulence in a juvenile rat model

2.1 Introduction

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 (Yagupsky et al., 2009). 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 (Chometon et al., 2007a; Yagupsky, 2014; Yagupsky et al., 2011). In addition, *K. kingae* is a known cause of bacteremia and endocarditis in this population (Yagupsky, 2014; Yagupsky et al., 2011). 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 (Amit et al., 2013; Bidet et al., 2013; Kehl-Fie & St. Geme III, 2007; Yagupsky et al., 2006, 2009). 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 role in children, who have a relatively naïve 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 (Ricklin et al., 2010; Sarma & Ward, 2011). 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 (Ricklin et al., 2010; Sarma & Ward, 2011). 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 (Geoffroy, Floquet, Métais, Nassif, & Pelicic, 2003; Horwitz & Silverstein, 1980; Noel, Hoiseth, & Edelson, 1992; Peterson et al., 1978). 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 (Goldblatt, 2000; Hyams, Camberlein, Cohen, Bax, & Brown, 2010; Moxon & Kroll, 1988; Swartley et al., 1997). In K. kingae, four distinct polysaccharide capsules have been identified, designated types a, b, c, and d (Starr, Porsch, Seed, Heiss, et al., 2016). Capsule types a and b account for greater than 95% of invasive disease isolates.
(Porsch et al., 2017; Starr, Porsch, Seed, Heiss, et al., 2016). Previous work has demonstrated that the capsule is required for full *K. kingae* virulence in a juvenile rat model of invasive disease (Starr et al., 2013; Starr, Porsch, Seed, & St. Geme III, 2016).

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 (Nwodo et al., 2012; Sutherland, 1982). 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 anti-biofilm properties (Bendaoud et al., 2011; Starr et al., 2013). 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 (Ganguly et al., 2014; Jones & Wozniak, 2017; Miajlovic et al., 2014; Mishra et al., 2012).

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.
2.2 Methods

2.2.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 2. The parent *K. kingae* strain 269-492 (Kehl-Fie & St. Geme III, 2007) 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 µg/ml kanamycin or 1 µg/ml erythromycin, as appropriate. *E. coli* strains were grown at 37°C on LB agar or in LB broth supplemented with 100 µg/ml ampicillin, as appropriate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK01</td>
<td>Nonspreading/noncorroding derivative of 269-492</td>
<td>(Kehl-Fie &amp; St. Geme III, 2007)</td>
</tr>
<tr>
<td>PYKK98</td>
<td>Clonal group B isolate from case of bacteremia, capsule type a</td>
<td>Yagupsky, P.</td>
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<tr>
<td>PYKK93</td>
<td>Clonal group P isolate from case of bacteremia, capsule type a</td>
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<tr>
<td>PYKK58</td>
<td>Clonal group N isolate from case of septic arthritis, capsule type b</td>
<td>Yagupsky, P.</td>
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<tr>
<td>KK146</td>
<td>Clonal group N isolate from case of bacteremia, capsule type b</td>
<td>Yagupsky, P.</td>
</tr>
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<td>PYKK060</td>
<td>Clonal group D isolate from case of endocarditis, capsule type c</td>
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</tr>
<tr>
<td>ATCC23330</td>
<td>Clonal group D isolate from a healthy carrier, capsule type c</td>
<td>ATCC</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>E3339</td>
<td>Clonal group F isolate from a healthy carrier, capsule type d</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>BB270</td>
<td>Clonal group U isolate from a healthy carrier, capsule type d</td>
<td>Yagupsky, P.</td>
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<td>KK01csaA</td>
<td>Contains csaA deletion</td>
<td>(Starr, Porsch, Seed, &amp; St. Geme III, 2016)</td>
</tr>
<tr>
<td>KK01pam</td>
<td>Contains pamABCDE deletion</td>
<td>(Starr et al., 2013)</td>
</tr>
<tr>
<td>KK01csApam</td>
<td>Contains csaA deletion and pamABCDE deletion</td>
<td>This study</td>
</tr>
<tr>
<td>KK01SwapEmpty</td>
<td>Contains the capsule synthesis locus flanking genes and a deletion of the csaA region</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
</tr>
<tr>
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<td>KK01SwapEmpty with csa locus recombined</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
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<td>KK01SwapEmpty with csb locus recombined</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
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<td>KK01SwapEmpty with csc locus recombined</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
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<tr>
<td>KK01Swapcsd</td>
<td>KK01SwapEmpty with csd locus recombined</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
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<td>KK01SwapEmpty_pam</td>
<td>KK01SwapEmpty with pamABCDE deletion</td>
<td>This study</td>
</tr>
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<td>KK01Swapcsapam</td>
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<td>This study</td>
</tr>
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<td>KK01Swapcsb with pamABCDE deletion</td>
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<td>KK01Swapcsc with pamABCDE deletion</td>
<td>This study</td>
</tr>
<tr>
<td>KK01Swapcsdpam</td>
<td>KK01Swapcsd with pamABCDE deletion</td>
<td>This study</td>
</tr>
</tbody>
</table>

**E. coli**

DH5α: *E. coli* F<sup>−</sup> φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r<sup>K</sup> m<sup>K</sup> <sup>+</sup> ) phoA supE441 thi-1 gyrA96 relA1 (Sambrook, Fritsch, & Maniatis, 1989)

**H. influenzae**
C54  |  *Haemophilus influenzae* serotype b isolate  | (Pichichero, Loeb, Anderson, & Smith, 1982)
C54 b- | Spontaneous capsule deficient derivative of strain C54 | (St. Geme & Falkow, 1991)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> pUC19pam::ermC</td>
<td>pUC19 with <em>ermC</em> erythromycin cassette flanked by surrounding 5’ and 3’ regions of the <em>pamABCDE</em> locus</td>
<td>(Starr et al., 2013)</td>
</tr>
</tbody>
</table>

### 2.2.2 Strain construction

The *csaA* mutant, the *pam* mutant, and the capsule swap strains were generated as previously described (Starr et al., 2013; Starr, Porsch, Seed, Heiss, et al., 2016; Starr, Porsch, Seed, & St. Geme III, 2016). To generate the KK01*csaA*pam double mutant and the capsule swap strains containing the *pam* locus deletion, strains KK01*csaA*, KK01Swapcsa, KK01Swapcsb, KK01Swabcsc, KK01Swapcsd, and KK01SwapEmpty were transformed with linearized plasmid pUC19pam::ermC. Briefly, the plasmid was purified from *E. coli*, linearized with NdeI, and introduced into the parental strains (*csaA* mutant or swap strains) via natural transformation. Transformants were recovered on chocolate agar plates containing 1.0 µg/ml erythromycin. Correct insertion of the gene disruption construct was confirmed via PCR and sequencing of the deletion site. The absence of capsule and exopolysaccharide was confirmed in strain KK01*csaA*pam and the capsule swap *pam* deletion strains using Alcian blue and silver staining as previously described (Porsch et al., 2012; Starr et al., 2013), with the parental strains and strain KK01 as controls.
2.2.3 Polysaccharide extraction and staining

For capsule extractions, bacteria were washed and resuspended in Tris acetate pH 5 for one hour. Cells were removed by centrifugation, and extracts were treated with proteinase K for one hour, and then concentrated as previously described (Porsch et al., 2012). For exopolysaccharide extractions, bacteria were resuspended in 5 ml PBS and vortexed. Cells were removed by centrifugation, and bacterial supernatants were treated with proteinase K for one hour, and then concentrated as previously described (Porsch et al., 2012).

Aliquots of supernatant and purified capsule from *K. kingae* derivatives were separated on 7.5% or 10% SDS-PAGE gels, respectively. For supernatant samples, SDS-PAGE gels were treated first with silver staining and subsequently with Alcian blue staining. For purified capsule samples, SDS-PAGE gels were treated with Alcian blue staining. For silver staining, gels were treated as previously described (J. S. Kim, Reuhs, Rahman, Ridley, & Carlson, 1996). For Alcian blue staining, gels were stained with 0.125% Alcian blue as previously described (Porsch et al., 2012).

2.2.4 Serum bactericidal assays

*K. kingae* strains and *E. coli* strain DH5α 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 µg/ml NAD and 0.1% lysed horse blood to an OD₆₀₀ of 0.2, and were grown to an OD₆₀₀ between 0.6-0.8 shaking at 250 rpm. Samples were diluted in PBS-G to obtain a final inoculum of
approximately 4.0 x 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 time points at 37°C with 5% CO₂. Complement activity was assessed and confirmed using a total hemolytic assay as previously described (Costabile, 2010). 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 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.

2.2.5 Flow cytometry analysis

*K. kingae* strains were grown on chocolate agar plates, resuspended to an OD₆₀₀ 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 opsoninization (Agarwal, Vasudhev, DeOliveira, & Ram, 2014; Mishra et al., 2012). 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
hr 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 hr 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 (mu 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).

2.2.6 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-PAGE 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.

2.2.7 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 hrs, and the bacteria were swabbed from plates and resuspended in PBS to a final density of 1 x 10^8 cfu/0.1 ml. While the in vitro serum bactericidal assays used 1 x 10^3 cfu to conserve human serum, 1 x 10^8 cfu was the necessary infectious dose in vivo to consistently generate invasive disease in rat pups with strain KK01. Three hours post-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 six hours for the first 30 hrs and then twice daily for a total of five days. Animals found to be moribund were euthanized by using CO2 inhalation followed by secondary decapitation.
2.2.8 Ethics statement

All animal experiments described within were conducted in accordance with the Animal Welfare Act, the Public Health Service Policy on 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. 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.

2.2.9 Statistical analysis

Statistical analyses were performed with GraphPad Prism version 7.0a for Mac (GraphPad Software, San Diego, CA) where \( P < 0.05 \) 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.

2.3 Results

2.3.1 \( K. \) 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 (HI)-NHS as a source of inactive complement. *K. kingae* strain KK01 at an inoculum of $1.0 \times 10^3$ cfu (colony forming units) was incubated for 1 hr with serum concentrations ranging from 2% to 50% (Fig. 5A), using *E. coli* strain DH5α as a serum-sensitive control. To assess serum sensitivity, a survival ratio was calculated, 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 DH5α (Fig. 5A). At a NHS concentration of 50%, strain KK01 had a survival ratio of approximately 0.80 (cfu in NHS divided by cfu in HI-NHS) (Fig. 5A). As expected, HI-NHS had no effect on 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.

### 2.3.2 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 KK01csaA (a capsule-deficient mutant lacking the *csaA* capsule synthesis gene) (Starr, Porsch, Seed, & St. Geme III, 2016), strain KK01pam (an exopolysaccharide-deficient mutant lacking the *pamABCDE* galactan exopolysaccharide synthesis operon) (Starr et al., 2013), and strain KK01csaApam (a double mutant lacking both capsule and exopolysaccharide synthesis genes). Serum
bactericidal assays were performed as described above using 50% serum for 1 hr and using
*E. coli* strain DH5α as a serum-sensitive control. In addition, isogenic encapsulated and
non-encapsulated derivatives of *H. influenzae* strain C54 (designated [+] for encapsulated
and [−] for non-encapsulated strains) 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 hr 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. 5B). In contrast, survival in NHS by
the double mutant lacking both capsule and exopolysaccharide (strain KK01*csaApam*) was
markedly reduced, with a survival ratio of less than 0.35 (*P* < 0.05, Fig. 5B). While the
absence of both capsule and exopolysaccharide resulted in reduced resistance to serum,
survival of strain KK01*csaApam* was not reduced to undetectable levels, as observed with
the serum sensitive controls *E. coli* DH5α and *H. influenzae* C54 b-.
Figure 5: *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*.

*K. kingae* strain KK01 and *E. coli* strain DH5α (~10^3 CFU) were incubated with 2%, 5%, 15%, 25%, or 50% NHS or HI-NHS for 1 h (A). *K. kingae* strains KK01, KK01csaA, KK01pam, and KK01csApm, *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 (B). The survival ratio was calculated by dividing the NHS CFU counts by the HI-NHS CFU counts. Abbreviations: csaA, KK01csaA; pam, KK01pam; csaApm, KK01csApm. 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.

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.

### 2.3.3 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 (Domenico, Tomas, Merino, Rubires, & Cunha, 1999; Howard & Glynn, 1971; Tomás, Camprubi, Merino, Davey, & Williams, 1991). 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 (Fig. 6) To further investigate whether the capsule and/or the exopolysaccharide prevents antibody binding, we performed flow cytometry assays to determine 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. 7A and 7B; gray bars), thus demonstrating the absence of non-specific binding of the secondary antibody. No significant difference in deposition was observed between strain KK01 and the single mutant strains KK01csaA and KK01pam after incubation with HI-NHS (Fig. 7A and 7B). In contrast, there was a statistically significant increase in deposition of both IgG and IgM on the surface of strain KK01csaApam, consistent with our observations regarding serum sensitivity (Fig. 7A and 7B).
Figure 6: Human IgG reactivity to *K. kingae* whole cell and outer membrane fractions.

Whole-cell (WC) and outer membrane (OM) fractions were purified from *K. kingae* strains KK01, KK01csaA, KK01pam, and KK01csaApam. Samples were run on a 7.5% SDS-PAGE gel, probed with 2% HI-NHS, and followed by an anti-human Ig–HRP-conjugated secondary antibody. Molecular sizes in kDa.

The constant region of IgG and IgM provides a platform for the initiation of the complement cascade (Duncan & Winter, 1988; Gadjeva et al., 2008; Perkins, Nealis, Sutton, & Feinstein, 1991; Zlatarova et al., 2006). To determine whether antibody deposition activates the 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. 7C and 7D, there was no significant difference in binding of C4b and C3b to strain KK01 and the single mutant strains KK01csaA and KK01pam. In contrast, there was a statistically significant increase in C4b and C3b deposition on strain KK01csaApam,
consistent with the increase in antibody deposition and serum sensitivity observed with this strain (Fig. 7C and 7D).

These data suggest that survival of strains KK01, KK01csaA, and KK01pam 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.

Figure 7: 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 iodine (PI) prior to analysis; 30,000 propidium iodine-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, KK01csaA; pam, KK01pam; csaApa, KK01csa4pam. 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.

2.3.4 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. Ca\(^{2+}\) 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 (Bryant & Jenkins, Jr., 1968; Gadjeva et al., 2008). The presence of EGTA in serum resistance assays chelates Ca\(^{2+}\) and thereby inhibits classical pathway activation, and the concomitant addition of Mg\(^{2+}\) preserves the alternative pathway (Des Prez, Bryan, Hawiger, & Colley, 1975).

To elucidate the importance of classical pathway activation in serum killing of strain KK01csaA, we performed serum bactericidal assays in the presence of EGTA + Mg\(^{2+}\). As expected, survival of strains KK01, KK01csaA, and KK01pam was not influenced by the introduction of 9 mM Mg\(^{2+}\) alone, 5 mM EGTA alone, or 9 mM Mg\(^{2+}\) + 5 mM EGTA (Fig. 8A). Conversely, the survival ratio of strain KK01csaA in the presence of either EGTA alone or EGTA + Mg\(^{2+}\) were restored to levels similar to the survival ratio of strains KK01, KK01csaA, and KK01pam (Fig. 8A). As anticipated, the
presence of Mg\(^{2+}\) alone had no effect on the survival ratio of strain KK01\textit{csaApam} (Fig. 8A).

Figure 8: Inhibition of the classical pathway restores survival and reduces C3b deposition in the surface polysaccharide-deficient mutant.

Serum survival was assessed with either 50% NHS alone, 50% NHS plus 9 mM Mg\(^{2+}\), 50% NHS plus 5 mM EGTA, 50% NHS plus 9 mM Mg\(^{2+}\) and 5 mM EGTA, or 50% HI-NHS for 1 h (A). The survival ratio was calculated by dividing the NHS CFU counts by the HI-NHS CFU counts. \textit{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 Mg\(^{2+}\) and 5 mM EGTA (B). 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 Mg\(^{2+}\) and 5 mM EGTA; gray bars, secondary antibody-only control. Abbreviations: \textit{csaA}, KK01\textit{csaA}; \textit{pam}, KK01\textit{pam}; \textit{csaApam}, KK01\textit{csaApam}. 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; *, \(P < 0.05\).

In order to confirm that chemical inhibition of the classical pathway via EGTA prevents complement activation, flow cytometry assays were performed to determine the percentage of C3b deposition on the surface of \textit{K. kingae} in the presence of EGTA + Mg\(^{2+}\). As shown in Fig. 8B, no significant differences in C3b deposition were observed between strains KK01, KK01\textit{csaA}, and KK01\textit{pam} in the presence or absence of EGTA + Mg\(^{2+}\). In
contrast, there was a significant decrease in C3b deposition on strain KK01csaApam when this strain was incubated in NHS containing EGTA + Mg\(^{2+}\) (Fig. 8B). C3b deposition on strain KK01csaApam in NHS containing EGTA + Mg\(^{2+}\) mirrored the C3b deposition on strain KK01csaApam in media alone (Fig. 8B). Taken together, these data provide further support that the classical complement pathway is activated in the absence of \textit{K. kingae} surface polysaccharides.

### 2.3.5 The four distinct capsule types of \textit{K. kingae} promote serum survival in isogenic strains.

To date, a total of four distinct capsule types have been identified in diverse collections of \textit{K. kingae} clinical isolates, including type a ([3]-\(\beta\)-GalpNAc-(1\(\rightarrow\)5)-\(\beta\)-Kdo-\(\rightarrow\))\), type b ([6]-\(\alpha\)-D-GlcNAc-(1\(\rightarrow\)5)-\(\beta\)-(8-OAc)Kdo-\(\rightarrow\)), type c ([3]-\(\beta\)-D-Ribf-(1\(\rightarrow\)2)-\(\beta\)-D-Ribf-(1\(\rightarrow\)2)-\(\beta\)-D-Ribf-(1\(\rightarrow\)4)-\(\beta\)-Kdo-\(\rightarrow\)), and type d ([P-(O\(\rightarrow\)3)[\(\beta\)-D-Galp-(1\(\rightarrow\)4)]-\(\beta\)-D-GlcpNAc-(1\(\rightarrow\)3)-\(\alpha\)-D-GlcpNAc-1-] (Porsch et al., 2017; Starr et al., 2013; Starr, Porsch, Seed, Heiss, et al., 2016; Starr, Porsch, Seed, & St. Geme III, 2016). 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 KK01pam expressing either capsule type a (strain KK01Swapcsa), capsule type b (strain KK01Swapcsb), capsule type c (strain KK01Swapcsc), or capsule type d (strain KK01Swapcsd) (Starr, Porsch, Seed, Heiss, et al., 2016). As shown in Fig. 9A, 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 \textit{K. kingae} capsule
types is protective and is adequate for promoting serum resistance in the absence of exopolysaccharide in vitro.

Figure 9: Specific capsule types do not dictate serum sensitivity in *K. kingae* clinical isolates.

*K. kingae* strains (~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 KK01pam (A) or clinical isolates of *K. kingae* (B). Abbreviations: Empty, nonencapsulated strain KK01SwapEmptypam; csa, capsule type a KK01Swapcsapam; csb, type b KK01Swapcsbpam; csc, type c KK01Swapcscpam; csd, type d KK01Swapcsdpam; 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.

2.3.6 *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. 9B, Table 2). Additionally, the eight clinical isolates contain the *pam* locus and produce exopolysaccharide. *K. kingae* strains PYKK98, KK146, ATCC23330, and BB270 showed high levels of serum resistance, with a survival ratio of approximately 0.80 (Fig. 9B), while strains PYKK93, PYKK58, PYKK60, and E3339 showed moderate levels of serum resistance, with survival ratios between 0.40 and 0.60 (Fig. 9B). The results demonstrate that the clinical isolates tested were resistant to human serum; however, there was no consistent distinction in level of susceptibility in relation to capsule type or clinical site of isolation.

### 2.3.7 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 (Starr, Porsch, Seed, & St. Geme III, 2016). 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 ng 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 (Bauman, 1978; Van den Berg, Aerts, & Van Dijk, 1991). CVF has been used previously in different animal models, including juvenile rats, to
successfully deplete complement *in vivo* (Kauppi-Korkeila, van Alphen, Madore, Saarinen, & Käyhty, 1996; Till, Morganroth, Kunkel, & Ward, 1987; Van den Berg et al., 1991). Three hours post injection with PBS alone or PBS with CVF, the rat pups were injected i.p. with $1 \times 10^8$ cfu of strain KK01, strain KK01csA, strain KK01pam, or strain KK01csApaA, using PBS as a control (Fig. 10A and 10B).

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 hrs (Fig. 10A). At five days post-infection, survival among pups infected with strain KK01csA was 29% ($p < 0.0001$, Fig. 10A), and survival among pups infected with strain KK01pam was 53% ($p < 0.0001$, Fig. 10A). In contrast, all pups injected with strain KK01csApaA were alive and healthy at five days post-infection, similar to pups injected with PBS alone ($p < 0.0001$, Fig. 10A). These results suggest that elimination of either capsule or exopolysaccharide results in reduced virulence of *K. kingae* and that elimination of both capsule and exopolysaccharide results in an avirulent strain.
Figure 10: 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 µg CVF in 0.1 ml PBS (B). The graphs plot Kaplan-Meyer survival curves for rats subsequently i.p. injected with 0.1 ml PBS or $1 \times 10^8$ 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, KK01csaA; pam, KK01pam; csaApam, KK01csaApam.

Among pups that received CVF pretreatment, all infected with strain KK01, strain KK01csaA, strain KK01pam, or strain KK01csaApam succumbed to infection within 12 hrs (Fig. 10B). All pups injected with PBS alone after CVF pretreatment survived five days post-infection (Fig. 10B). These results suggest that resistance to complement via the polysaccharide capsule and the exopolysaccharide is critical for *K. kingae* virulence in juvenile rats.

2.4 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 (Chometon et al., 2007a; Yagupsky, 2014; Yagupsky et al., 2011). This organism causes disease by breaching the respiratory epithelium, invading the bloodstream, and disseminating to distant sites (Amit et al., 2013; Bidet et al., 2013; Kehl-Fie & St. Geme III, 2007; Yagupsky et al., 2006, 2009). 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 KK01csaApam 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 KK01csaApam.

In recent years, a number of reports have described bacterial factors associated with evasion of complement activity in non-encapsulated organisms (Del Tordello, Vacca, Ram, Rappuoli, & Serruto, 2014; Griffiths et al., 2011; Hallström, Blom, Zipfel, & Riesbeck, 2009; Ho, Ram, Nelson, Bonthuis, & Smith, 2007; Murphy, Kirkham, & Lesse, 2006). 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 (Geoffroy et al., 2003). 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 30-70% of carrier isolates are non-encapsulated (Ala’Aldeen et al., 2000; Kugelberg et al., 2008; van Deuren et al., 2000). 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 (Noel, Brittingham, Granato, & Mosser, 1996; Noel et al., 1992; Swift, Moxon, Zwahlen, & Winkelstein, 1991). In this study, we observed that non-encapsulated 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 non-covalent mechanism of association (Starr et al., 2013). 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 (Cerning, 1991; De Vuyst & Degeest, 1999; Flemming et al., 2007; Nwodo et al., 2012). Exopolysaccharide function
depends on the polymer structure and composition and has been studied related to adherence, bacterial cell aggregation, water retention, and nutrition (Flemming et al., 2007; Flemming & Wingender, 2002; Nwodo et al., 2012). Few studies have characterized exopolysaccharides in the context of inhibiting complement deposition and subsequent complement and/or leukocyte evasion (Ganguly et al., 2014; Jones & Wozniak, 2017; Miajlovic et al., 2014; Mishra et al., 2012). 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 our studies of *K. kingae* (Mishra et al., 2012). 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 colonic acid (Miajlovic et al., 2014). 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 *K. kingae* (Miajlovic et al., 2014). In *K. kingae*, the level of protection against complement activation and complement-mediated killing provided by the exopolysaccharide mimics the protection provided by the polysaccharide capsule. Thus far, two exopolysaccharide structures have been described in *K. kingae*: \( \rightarrow 3 \)-\( \beta \)-Galf\-(1→5)-\( \beta \)-Galf\-(1→5)-\( \beta \)-Galf\-(1→) (Bendaoud et al., 2011; Starr et al., 2013). It has been reported that biofilm formation and dispersal of *K. kingae* clinical strain PYKK181
is dependent on the PAM galactan exopolysaccharide (Bendaoud et al., 2011). 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 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 that was reduced to ~40% of the level in the parent strain (Fig 5B). 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 capsule and/or exopolysaccharide play 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 promoting a balanced Th1/Th2 response in mice (Surana & Kasper, 2012). Interestingly, Totté et al. have reported that a galactan homopolysaccharide in Mycoplasma mycoides subsp. mycoides similar to the K. kingae exopolysaccharide binds TLR2 and promotes the production of the anti-inflammatory cytokine IL-10 (Totté et al., 2015). 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*.

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3. *Kingella kingae* surface polysaccharides promote resistance to neutrophil phagocytosis and killing

3.1 Introduction

*Kingella kingae*, a gram-negative coccobacillus, is a common commensal organism in the oropharynx of young children (Yagupsky et al., 2009). Despite being an oral commensal, *K. kingae* is a primary cause of osteoarticular infections and a common etiology of bacteremia in children between 6 and 36 months of age (Chometon et al., 2007b; Yagupsky, 2014; Yagupsky et al., 2011). Recent studies have elucidated *K. kingae* surface and secreted factors that promote virulence via adherence to epithelial cells, cytotoxicity, and immune evasion (Chang et al., 2014; Kehl-Fie et al., 2008; Kehl-Fie & St. Geme III, 2007; Maldonado et al., 2011; Muñoz et al., 2018; Porsch et al., 2012).

To colonize the oropharynx and survive in the hostile intravascular environment, *K. kingae* must evade innate immunity. *K. kingae* produces a polysaccharide capsule and a galactan homopolymer exopolysaccharide, both of which have been demonstrated to contribute to virulence in an infant rat infection model (Muñoz et al., 2018; Starr et al., 2013; Starr, Porsch, Seed, & St. Geme III, 2016). Recent work has determined that the *K. kingae* polysaccharide capsule and exopolysaccharide confer high-level resistance to human serum (Muñoz et al., 2018). Elimination of both surface polysaccharides is detrimental to the organism in the presence of human serum, resulting in increased deposition of antibodies and complement fragments, and ultimately, complement activation and bacterial lysis (Muñoz et al., 2018).
Neutrophils are the most abundant leukocyte type in the blood and the predominant infiltrating leukocyte type during acute inflammation (D. M. Walker, 2004). These cells mobilize to clear pathogenic bacteria through various extracellular and intracellular mechanisms and are primed and activated by a variety of inflammatory stimulants, including conserved bacterial ligands known as pathogen associated molecular patterns (PAMPs). PAMPs are recognized by membrane-associated Toll-like receptors (TLRs), and subsequent TLR activation primes neutrophils and promotes phagocytosis, degranulation, and production of reactive oxygen species (ROS).

Given the role of neutrophils in combating microbial invaders, bacteria have evolved multiple mechanisms to evade neutrophil-mediating killing. Encapsulation by invasive pathogens such as *Streptococcus pneumoniae* and *Neisseria meningitidis* has been demonstrated to promote bacterial survival by inhibiting neutrophil recognition and activation (Beiter et al., 2008; Campos et al., 2004; Hyams et al., 2010). Encapsulation has also been demonstrated to prevent antibody recognition of surface antigens present on the bacterial surface and to inhibit complement deposition and activation (Geoffroy et al., 2003; Horwitz & Silverstein, 1980; Hyams et al., 2010; Peterson et al., 1978). Opsonization by immunoglobulins and complement components augments neutrophil recognition and enhances neutrophil antimicrobial activity, including phagocytosis of opsonized bacteria.

In this study, we found that the *K. kingae* polysaccharide capsule promotes neutrophil evasion by preventing neutrophil activation, dampening ROS production, and inhibiting initial neutrophil binding of *K. kingae*. Interestingly, we observed a distinctive role for the exopolysaccharide in promoting *K. kingae* survival in the presence of
neutrophil antimicrobial peptides and blocking neutrophil phagocytosis of bound bacteria. The absence of both the polysaccharide capsule and the exopolysaccharide increased neutrophil opsonophagocytosis of \textit{K. kingae}. This study demonstrates the importance of the \textit{K. kingae} polysaccharide capsule and exopolysaccharide in neutrophil evasion, presumably promoting hematogenous dissemination of \textit{K. kingae}.

3.2 Methods

3.2.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 3. \textit{K. kingae} strains were stored at 
-80°C in brain heart infusion (BHI) broth with 20% glycerol. \textit{E. coli} strains were stored at 
-80°C in Luria-Bertani (LB) broth with 15% glycerol. \textit{K. kingae} strains were grown at 37°C with 5% CO$_2$ on chocolate agar plates supplemented with 50 µg/ml kanamycin or 1 µg/ml erythromycin, as appropriate. \textit{E. coli} strains were grown at 37°C on LB agar or shaking at 250 rpm in LB broth supplemented with 100 µg/ml ampicillin or 50 µg/ml kanamycin, as appropriate.

<table>
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<th>Description</th>
<th>Reference</th>
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<td>Nonscaping/noncorroding derivative of 269-492</td>
<td>(Kehl-Fie &amp; St. Geme III, 2007)</td>
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<td>KK01</td>
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<td>-------------------------</td>
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<td>--------------------------------</td>
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<td>E. coli pUC19rtxACDB::kan</td>
<td>pUC19 with aphA3 kanamycin cassette flanked by surrounding 5' and 3' regions of the rtxACDB locus</td>
<td>This study</td>
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### 3.2.2 Strain construction

Strains KK01 *csaA*, KK01 *pam*, and KK01 *csaApam* were generated as previously described (Muñoz et al., 2018; Starr et al., 2013; Starr, Porsch, Seed, & St. Geme III, 2016). Briefly, plasmid-based gene disruption constructs were created in *E. coli*, linearized, and introduced into *K. kingae* using natural transformation. Transformants were recovered by selectively plating on chocolate agar plates with the appropriate antibiotic. Gene disruptions were confirmed by PCR.

To generate pUC19rtxBDCA::kan, DNA fragments of the homologous recombination targeting sequence corresponding to the ~1 kb upstream of *rtxB* and the ~1 kb downstream of *rtxA* were PCR amplified from strain KK01 genomic DNA using primers *rtx 5’ For/ rtx 5’ Rev* and *rtx 3’ For/ rtx 3’ Rev*, respectively, and were cloned into pUC19 (Table 4). A kanamycin resistance cassette was then ligated into the BamHI site located...
between the cloned upstream and downstream homologous recombination targeting sequences. The plasmid was linearized with NdeI and was transformed into strain KK01, KK01 csaA, KK01 pam, and KK01 csaApam via natural transformation. Transformants were recovered on chocolate agar plates containing 50 µg/ml kanamycin. Correct insertion of the gene disruption construct was confirmed via PCR and sequencing of the deletion site.

<table>
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<td>This study</td>
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<tr>
<td>rtx 3’ Rev</td>
<td>GCAGAAGCTTAGAGCTGCGTAAACCTATG</td>
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3.2.3 Neutrophil purification

Human neutrophils were obtained from healthy adult donors using approved IRB protocol IRB 16-012812 at the Children’s Hospital of Philadelphia. Whole blood was collected in a Vacutainer K2E 18mg (BD, Franklin Lakes, NJ). Neutrophils were isolated as previously described (Ermert, Zychlinsky, & Urban, 2009). Briefly, neutrophils were purified using 3% dextran sedimentation for 30 min at room temperature (RT) to separate red blood cells (RBCs) from the leukocyte-rich supernatant. The supernatant was transferred into a new conical tube, and 1 volume of Hypaque Ficoll (Sigma-Aldrich, St. Louis, MO) was added underneath the supernatant. The sample was centrifuged at 300 g for 30 min to pellet the neutrophils. The remaining RBCs were subjected to hypotonic lysis with cold double-distilled water, and the isotonicity was restored by the addition of RPMI-
HEPES (RPMI (Lonza, Walkersville, MD) without phenol red, substituted with 10 mM HEPES (Sigma-Aldrich, St. Louis, MO)). Neutrophils were pelleted and resuspended in RPMI-HEPES for experimental use.

### 3.2.4 Neutrophil killing assay

Killing assays with neutrophils were performed as previously described (Ermert et al., 2009). Briefly, neutrophils were diluted to 2.0 x 10^6 cells/ml, and 500 µl was seeded onto 24-well plates in RPMI-HEPES with 1% human serum albumin (HSA) (Alfa Aesar, Ward Hill, MA) or 1% normal human serum (NHS) (Immucor, Norcross, GA). Bacteria were resuspended to an OD₆₀₀ of ~0.8 in phosphate-buffered saline (PBS) and added at both a high and low multiplicity of infection (MOI), 10:1 or 1:10, respectively, and centrifuged at 1000 rpm for 2 min. Samples were incubated for various time points at 37°C with 5% CO₂. After incubation, the supernatant was removed and placed in 1.5-mL reaction tubes. Neutrophils were lysed using cold double-distilled water, wells were scraped, and the lysate was added to the corresponding collected supernatants. 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 CFU counts (limit of detection for plating: 20 CFU). To perform inhibition assays, neutrophils were incubated with 10 µg/ml cytochalasin D (Life Technologies Corp., Frederick, MD), 10 mM N-acetylcysteine (MilliporeSigma, Burlington, MA), 4 µM diphenyleneiodonium (XXX), or 1x protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) for 30 min prior to infection with bacteria.
3.2.5 Oxidative burst studies

To detect intracellular and extracellular reactive oxidative species (ROS) generated by neutrophils, the neutrophils were diluted to $2.0 \times 10^5$ cells/ml and seeded onto a white tissue culture-treated 96-well microtiter plate with 1% HSA or 1% NHS as previously described (Engels, Endert, Kamps, & Van Boven, 1985; Mishra et al., 2012). The bacteria were resuspended to an $OD_{600}$ of ~0.8 in phosphate-buffered saline (PBS) and added at a MOI of 10:1 to wells in the presence of 50 µM luminol. The microtiter plate was centrifuged at 100 g for 2 min and the relative amount of ROS generated by neutrophils was detected at 5 min intervals over 60 min by luminescence. Phorbol 12-myristate 13-acetate (PMA) (Alfa Aesar, Wardhill, MA) at a concentration of 40 ng/ml was used as a positive control for the generation of ROS by neutrophils.

3.2.6 Microscopy

*Bacterial ingestion by neutrophils*

To detect binding and phagocytosis of bacteria by neutrophils, neutrophils were diluted to $2.0 \times 10^6$ cells/ml and seeded onto tissue culture-treated glass coverslips with 1% HSA or 1% NHS (Mishra et al., 2012; Tuominen-Gustafsson, Penttinen, Hytönen, & Viljanen, 2006). Bacteria were resuspended to an $OD_{600}$ of ~0.8 in PBS, stained with 5 µg/ml CFSE (Tonbo Biosciences, San Diego, CA) for 20 min at 37°C, and washed twice with PBS. CFSE-stained bacteria were added at an MOI of 10:1 and centrifuged at 1000 rpm for 2 min. Samples were incubated for 30 min at 37°C with 5% CO₂. Cells were washed twice with PBS to remove non-adherent bacteria and were fixed at RT for 15 min using 4% paraformaldehyde in PBS. Samples were incubated with anti-*K. kingae* antibody
overnight at 4°C. The anti-K. kingae antibody was generated against an acetone powder of K. kingae strain KK01 whole bacteria. Cells were washed twice with PBS and incubated with a 1:500 dilution of goat anti-guinea pig IgG DyLight 649-conjugated antibody (Rockland, Limerick, PA).

Visualization of bacteria–neutrophil association was performed by microscopy using a Nikon Eclipse Ni-E (Nikon Instruments Inc., Melville, NY) with either a 20x objective or 60x oil objective. For quantification, 100 neutrophils per n=1 were chosen at random for analysis; the cell-associated versus extracellular bacteria were counted by a blinded reader.

3.2.7 Antibacterial peptide bactericidal assays

K. kingae strains were grown on chocolate agar plates and then resuspended in PBS. Samples were diluted in PBS to obtain a final inoculum of approximately $4.0 \times 10^3$ CFU/0.1 ml. The respective inocula were mixed with polymyxin B (Alfa Aesar, Ward Hill, MA), HNP 1-3 (MilliporeSigma, Burlington, MA), or LL-37 (Bachem, Torrance, CA) at various concentrations and incubated at 37°C with 5% CO$_2$ for 30 min. After incubation, 9 mM of MgCl$_2$ was added to the reaction prior to plating. Serial dilutions of the inoculum and reaction samples were plated on chocolate agar plates and incubated overnight at 37°C with 5% CO$_2$ to determine the CFU counts (limit of detection for plating, 20 CFU).

3.2.8 Ethics statement

Blood samples for neutrophil isolation were obtained from healthy adult donors that provided oral informed consent under approved Children’s Hospital of Philadelphia
Institutional Review Board (CHOP IRB) protocol IRB 16-012812 in compliance with all institutional and federal guidelines. Due to the nature of the study, the CHOP IRB indicated that oral consent was appropriate and approved its use. Oral informed consent from each study participant was recorded on a copy of the Verbal Consent Form, which were maintained in the study records.

3.2.9 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. The specific statistical tests used for each experiment are specified in the figure legends.

3.3 Results

3.3.1 The *K. kingae* capsule and exopolysaccharide prevent neutrophil-mediated killing of *K. kingae*.

In previous work we demonstrated that the polysaccharide capsule and exopolysaccharide expressed by *K. kingae* protect the organism from complement-mediated lysis and promote virulence in an infant rat model of infection (Muñoz et al., 2018; Starr et al., 2013; Starr, Porsch, Seed, & St. Geme III, 2016). To further characterize the role of these surface polysaccharides in innate immune evasion, we performed neutrophil-killing assays using *K. kingae* strain KK01, the capsule-deficient mutant KK01 csaA (Starr, Porsch, Seed, & St. Geme III, 2016), the exopolysaccharide-deficient mutant KK01 pam (Starr et al., 2013), and the capsule-deficient and exopolysaccharide-deficient mutant KK01 csaApam (Muñoz et al., 2018). Using an MOI of 0.1 or 10, the *K. kingae*
strains were incubated with or without purified human neutrophils for 1 hr in the presence of 1% normal human serum (NHS) as a source of serum opsonins or 1% human serum albumin (HSA) as a control. To assess extracellular and intracellular survival of *K. kingae* in the presence of neutrophils, supernatant and neutrophil lysates were diluted and plated to determine colony forming units (CFU). Survival percentage was calculated by dividing the recovered CFU by the inoculated CFU.

Figure 11: Elimination of the *K. kingae* capsule results in *K. kingae* killing by neutrophils.

*K. kingae* strains KK01 (A), KK01 csaA (B), KK01 pam (C), and KK01 csaApam (D) were incubated with human neutrophils at an MOI of 10 or 0.1 for 1 hr in the presence of 1% HSA or 1% NHS. Black bars indicate survival in the absence of neutrophils, and white bars represent survival in the presence of neutrophils. Survival was determined by dividing the recovered CFU by the inoculum CFU. Statistical
significance was determined using two-way ANOVA and the Tukey post hoc test. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.

Elimination of the capsule, the exopolysaccharide, or both the capsule and the exopolysaccharide resulted in no growth defects on solid agar (Muñoz et al., 2018) and had no effect on survival in the absence of neutrophils or 1% NHS alone (Fig. 11, black bars and Fig. 12). Survival of strains KK01 and KK01 pam was only slightly affected at an MOI of 0.1 in the presence of neutrophils with 1% NHS (Fig. 11A, C). Survival of strain KK01 csaA was reduced in the presence of neutrophils with 1% NHS at both MOIs and in the presence of neutrophils with 1% HSA at an MOI of 0.1 with ~60% survival (Fig. 11B). Interestingly, survival of strain KK01 csaApam was markedly reduced in the presence of neutrophils at both MOIs (Fig. 11D). In contrast to the other strains tested, strain KK01 csaApam was more susceptible to neutrophil-mediated killing when opsonins were present, with ~30% survival with opsonins compared to ~50% survival without opsonins (Fig. 11D). These results establish that the capsule is critical for K. kingae neutrophil evasion and that the exopolysaccharide plays a conditional protective role when opsonins are present and the capsule is absent.
Figure 12: Survival of *K. kingae* in the presence of 1% normal human serum.

*K. kingae* strains KK01, KK01 *csaA*, KK01 *pam*, and KK01 *csaApam* were incubated with 1% normal human serum at an MOI equivalent of 0.1 (white bars) or 10 (black bars) for 1 hr in the absence of neutrophils. Survival was determined by dividing the recovered CFU by the inoculum CFU.

3.3.2 The *K. kingae* capsule inhibits the neutrophil oxidative burst response.

The oxidative burst response generated by human neutrophils is a critical innate immune defense mechanism against bacterial pathogens. *K. kingae* expresses a cytotoxin called RtxA that is capable of rapidly killing a variety of human cell types, including leukocytes (Chang et al., 2014; Kehl-Fie & St. Geme III, 2007; Maldonado et al., 2011). *In vitro*, strains KK01 and KK01 *rtx* (a mutant with the *rtx* locus deleted) demonstrate similar survival in the presence of neutrophils at an MOI of 10 (Fig. 13). However, to eliminate the influence of the RtxA toxin as a confounding variable on neutrophil activation and ROS production in the presence of *K. kingae*, we deleted the *rtx* locus in strains KK01, KK01 *csaA*, KK01 *pam*, and KK01 *csaApam*. To determine the oxidative burst response, we incubated the *K. kingae rtx* mutant strains at an MOI of 10 with human neutrophils and
either 1% HSA or 1% NHS in the presence of luminol (Fig. 14A-C). Based on measurement of chemiluminescence (relative light units, RLU) at 5 min intervals over a 1 hr incubation period, incubation of strains KK01 _rtx_ and KK01 _pamrtx_ with neutrophils resulted in a moderate neutrophil response when compared to the uninfected control (Fig. 14A, B). In contrast, incubation of strains KK01 _csaArtx_ and KK01 _csaApamrtx_ resulted in a rapid and robust neutrophil response, peaking at the 25 min time point (Fig. 14A, B).

![Figure 13: Elimination of RtxA toxin has no effect on K. kingae survival in the presence of neutrophils.](image)

*K. kingae* strains KK01 (A), KK01 _rtx_ (A), KK01 _csaA_ (B), KK01 _csaArtx_ (B), KK01 _pam_ (C), KK01 _pamrtx_ (C), KK01 _csaApam_ (D), and KK01 _csaApamrtx_ (D) were incubated with human neutrophils at an MOI of 10 for 1 hr in the presence of 1% HSA (white bars) or 1% NHS (black bars). Survival was determined by dividing the recovered CFU by the inoculum CFU.
Focusing on the 25 min time point, the neutrophil response was compared across the KK01 \( rtx \) mutant strains in either 1% HSA or 1% NHS. In both 1% HSA and 1% NHS, we observed a statistically significant increase in the neutrophil response to strains KK01 \( csaArtx \) and KK01 \( csaApamrtx \) when compared to strain KK01 \( rtx \) (Fig. 14C). Comparison of strains KK01 \( rtx \) and KK01 \( pamrtx \) in 1% HSA or 1% NHS revealed no statistically significant difference in neutrophil response (Fig. 14C). Similarly, comparison of strains KK01 \( csaArtx \) and KK01 \( csaApamrtx \) in 1% HSA revealed no statistically significant difference in neutrophil response (Fig. 14C). In contrast, there was a statistically significant increase in neutrophil response to strain KK01 \( csaApamrtx \) compared to strain KK01 \( csaArtx \) in 1% NHS (Fig. 14C). These data suggest that \( K. kingae \) encapsulation prevents neutrophil activation and ROS production and release. Moreover, the neutrophil response is most robust when both surface polysaccharides are absent and opsonins are present.
Figure 14: The absence of the *K. kingae* capsule results in an enhanced neutrophil oxidative burst response.

*K. kingae* rtx mutant strains were incubated with human neutrophils at an MOI of 10 in the presence of 1% HSA (A) or 1% NHS (B). Chemiluminescence was measured at 5-min intervals for 60 min for kinetic analysis (A, B) or at the 25-min time point (C). Statistical significance was determined using two-way ANOVA and the Tukey post hoc test. RLU, relative light unit; n.s., not significant; **, *P* < 0.01; ****, *P* < 0.0001.

3.3.3 Neutrophil-mediated killing of capsule-deficient *K. kingae* is dependent on phagocytosis and ROS production.

Neutrophils are recruited early during infection and can eliminate pathogens through a variety of mechanisms, including phagocytosis and the oxidative burst (Nathan, 2006). To determine whether phagocytosis and/or ROS production facilitates killing of non-encapsulated *K. kingae* strains, we performed neutrophil-killing assays in the presence of cytochalasin D (Cyto D), the antioxidant N-acetylcysteine (NAC), or the NADPH oxidase inhibitor, diphenyleneiodonium (DPI). Using an MOI of 0.1, strains KK01 *csaA*
and KK01 csaApam were incubated with Cyto D, NAC, or DPI pre-treated human neutrophils for 30 min. Pretreatment of neutrophils with Cyto D restored survival of strains KK01 csaA and KK01 csaApam in the presence of neutrophils (Fig. 15A, B). Pretreatment of neutrophils with NAC led to a roughly two-fold increase in the percent survival for strains KK01 csaA and KK01 csaApam in 1% HSA and 1% NHS, but did not completely restore survival to the level observed with vehicle-only pretreated neutrophils (Fig. 15C, D). Similarly, pretreatment of neutrophils with DPI led to a significant increase in the percent survival for strain KK01 csaA in 1% HSA (Fig. 15E). Pretreatment of neutrophils with DPI led to a roughly two-fold increase in the percent survival for strain KK01 csaApam but did not completely restore survival (Fig. 15F). These data suggest that phagocytosis and ROS are key mechanisms contributing to neutrophil-mediated killing of non-encapsulated K. kingae.
Figure 15: Inhibition of neutrophil phagocytosis and ROS production enhances survival of non-encapsulated *K. kingae*.

*K. kingae* strains KK01 csaA and KK01 csaApam were incubated with human neutrophils at an MOI of 0.1 for 30 min. Neutrophils were preincubated with 10 µg/ml cytochalasin D, Cyto D (A, B), 10 mM N-acetylcysteine, NAC (C, D), or 4 µM diphenyleneiodonium (E, F) in the presence of 1% HSA or 1% NHS. Black bars indicate survival in the absence of neutrophils, white bars represent survival in the presence of neutrophils, and gray bars represent survival in the presence of neutrophils pretreated with Cyto D, NAC, or DPI. Survival was determined by calculating the recovered CFU by the inoculum CFU. Statistical significance was determined using two-way ANOVA and the Tukey post hoc test. n.s., not significant, *, P < 0.05; ***, P < 0.001; ****, P < 0.0001.

3.3.4 The *K. kingae* exopolysaccharide protects against neutrophil antimicrobial peptides.

Neutrophil granules are cytoplasmic vesicles that can fuse with phagosomes or the plasma membrane to release degradative enzymes (e.g. myeloperoxidase, elastase, and cathepsin G), antimicrobial peptides (e.g. defensins and the cathelicidin LL37), and reactive radicals to kill intracellular and extracellular microorganisms (Nathan, 2006). To determine whether proteases released during degranulation facilitate killing of *K. kingae*, we performed neutrophil-killing assays in the presence of a protease inhibitor cocktail (PI). Using an MOI of 0.1, strains KK01 csaA and KK01 csaApam were incubated with PI-pretreated human neutrophils for 30 min. Pretreatment of neutrophils with PI had no statistically significant effect on levels of survival for strains KK01 csaA and KK01 csaApam (Fig. 16A, B). To determine the sensitivity of *K. kingae* in the presence of antimicrobial peptides, we performed bactericidal assays with a bacterial peptide, polymyxin B, and human peptides, LL-37 and HNP 1-3, at a range of physiologic concentrations. Survival of strain KK01 csaA compared to strain KK01 was not affected.
in the presence of polymyxin B and was slightly decreased in the presence of LL-37 (Fig. 16C, D). Interestingly, survival of strain KK01 pam compared to strain KK01 was significantly decreased in the presence of polymyxin B or LL-37 (Fig. 16C, D). Strain KK01 csaApam was most sensitive in the presence of polymyxin B and LL-37 compared to strain KK01 (Fig. 16C, D). We observed no change in survival of the mutant strains compared to strain KK01 in the presence of HNP 1-3 (Fig. 16E). These results establish that the exopolysaccharide is critical for survival of *K. kingae* in the presence of antimicrobial peptides and demonstrate a moderate role for the capsule.
Figure 16: The surface polysaccharides protect *K. kingae* against antimicrobial peptides.

*K. kingae* strains KK01 *csaA* and KK01 *csaApam* were incubated with human neutrophils preincubated with a protease inhibitor cocktail (PI) at an MOI of 0.1 for 30 min in the presence of 1% HSA or 1% NHS (A, B). Black bars indicate survival in the absence of neutrophils, white bars represent survival in the presence of neutrophils, and gray bars represent survival in the presence of neutrophils.
pretreated with PI. *K. kingae* strains and *E. coli* strain DH5α were incubated with various concentrations of antimicrobial peptides, polymyxin B (C), cathelicidin LL-37 (D), or HNP1-3 (E) for 30 min. Survival was determined by calculating the recovered CFU by the inoculum CFU.

### 3.3.5 The *K. kingae* surface polysaccharides inhibit neutrophil binding and phagocytosis of bacteria.

To further delineate the mechanism of neutrophil-mediated killing of *K. kingae* strains, we performed immunofluorescence microscopy, examining the number of *K. kingae* associated with neutrophils and the nature of association. *K. kingae* rtx mutant strains were used to prevent cell lifting during infection. *K. kingae* strains were stained with carboxyfluorescein succinimidyl ester (CFSE) prior to infection and were incubated at an MOI of 10 with human neutrophils for 30 min or 60 min in presence of 1% HSA or 1% NHS. The wells were washed vigorously to remove any unassociated bacteria, and the samples were incubated with an anti-*K. kingae* whole organism guinea pig antiserum to detect extracellular bacteria associated with the neutrophils. For the 30 min time point, the total number of associated bacteria (intracellular and extracellular) was 2.8-fold higher for strain KK01 csaArtx compared to strain KK01 rtx in 1% HSA and 1.6-fold higher for strain KK01 csaArtx compared to strain KK01 rtx in 1% NHS (Fig. 17A, Table 5). The total number of associated bacteria was 4.0-fold higher for strain KK01 csaApamrtx compared to strain KK01 rtx in 1% HSA and 3.1-fold higher for strain KK01 csaApamrtx compared to strain KK01 rtx in 1% NHS (Fig. 17A, Table 5). While we observed an increase in total bacterial association with strain KK01 csaArtx compared to strain KK01 rtx, there was no significant difference between the number of intracellular bacteria (Fig. 17B, C, Table 5). In contrast, we observed a significant increase in the number of intracellular KK01
csaApamrtx compared to strains KK01 rtx, KK01 csaArtx, and KK01 pamrtx in 1% HSA and 1% NHS (Fig. 17B-E). We also observed a statistically significant increase in the total number of associated bacteria for strain KK01 csaApamrtx in 1% NHS compared to 1% HSA (Table 5).

At the 60 min time point, we observed an increase in total number of associated bacteria for strains KK01 rtx, KK01 csaArtx, and KK01 pamrtx; however, the number of intracellular bacteria remained similar across the 30 min and 60 min time points (Table 5, Fig. 17B-D, F-H). We observed a slight increase in total number of associated bacteria for strain KK01 csaApamrtx at 60 min (Table 5). Comparing the 60 min time point to the 30 min time point, there was a significant decrease in the number of extracellular bacteria and a slight increase in the number of intracellular bacteria for strain KK01 csaApamrtx (Fig. 17E, I).

Taken together, these results demonstrate that the absence of capsule results in increased bacterial association with neutrophils and that only the elimination of both the capsule and the exopolysaccharide results in a significant increase of K. kingae phagocytosis by neutrophils, regardless of opsonin deposition.
Figure 17: The presence of the *K. kingae* surface polysaccharides results in reduced neutrophil association and phagocytosis of *K. kingae*.

Immunofluorescence images (A) and quantitative analysis (B-I) of *K. kingae rtx* mutant strains incubated with human neutrophils at an MOI of 10 for 30 min (A-I) or 60 min (B-I) in the presence of 1% HSA or 1% NHS. *K. kingae* strains were stained with CFSE (green) prior to infection. Neutrophils were fixed and incubated with a guinea pig anti-*K. kingae* antiserum and subsequently with anti-guinea pig AF-649 to detect extracellular bacteria (red). DAPI was used to stain the nuclei of the neutrophils (blue). Images were acquired using a 20x objective. Arrows indicate extracellular bacteria, and arrowheads indicate intracellular bacteria. Scale bar is equal to 5 µm. Graphs depict the total number of CFSE+/AF649+ (intracellular, B-E) events and the total number of CFSE+/AF649− (extracellular, F-I) for strains KK01.
rtx (B, F), KK01 csaArtx (C, G), KK01 pamrtx (D, H), and KK01 csaApamrtx (E, I). The data represents total number of CFSE\(^+\) events per 100 neutrophils chosen randomly per n=1. Abbreviations: H, HSA; N, NHS; KK, K. kingae; ecKK, extracellular K. kingae.

Table 5: Total number of CFSE\(^-\) bacteria associated with human neutrophils

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<th>1% NHS</th>
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<tr>
<td></td>
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<tr>
<td>KK01 rtx</td>
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<td>(59±9.2)</td>
</tr>
<tr>
<td>KK01 csaArtx</td>
<td>(59 ± 9.5)</td>
<td>(79±7.1)</td>
</tr>
<tr>
<td>KK01 pamrtx</td>
<td>(36 ± 5.5)</td>
<td>(58±5.9)</td>
</tr>
<tr>
<td>KK01 csaApamrtx</td>
<td>(85 ± 9.0)</td>
<td>(96±9.0)</td>
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*The number (mean ± SD) of associated bacteria is indicated in parentheses. The data represents total number of CFSE\(^+\) events per 100 neutrophils chosen randomly per n=1.

3.4 Discussion

The pathogenesis of K. kingae disease involves bacterial invasion of the bloodstream, evasion of complement- and neutrophil-mediated killing, and dissemination to distant sites (Amit et al., 2013; Bidet et al., 2013; Kehl-Fie & St. Geme III, 2007; Yagupsky et al., 2006, 2009). The K. kingae surface polysaccharides have proven to be critical for complement evasion, and expression of both the capsule and the exopolysaccharide is essential for full virulence in the infant rat model of infection (Muñoz et al., 2018). In this study, we observed that in the absence of the polysaccharide capsule, K. kingae was sensitive to neutrophil-mediated killing. This phenotype was enhanced when
the exopolysaccharide was also absent. Further analysis demonstrated that the polysaccharide capsule prevented neutrophil ROS production and interfered with neutrophil binding of *K. kingae* but had a limited effect on resistance to neutrophil antimicrobial peptides and no direct effect on phagocytosis. In contrast, the exopolysaccharide conferred resistance to neutrophil antimicrobial peptides and blocked neutrophil phagocytosis but had a limited effect on neutrophil binding of *K. kingae*. Overall, we have established a novel interplay between the *K. kingae* polysaccharide capsule and exopolysaccharide, which have complementary but distinct roles in mediating resistance to human neutrophils.

Polysaccharide capsules expressed by invasive pathogens such as *N. meningitidis* and *S. pneumoniae* promote bacterial survival during infection by interfering with neutrophil recognition, activation, and phagocytosis (Hyams et al., 2010; Spinosa et al., 2007; Wartha et al., 2007). In this study, we demonstrated that the presence of the *K. kingae* polysaccharide capsule significantly reduced the oxidative burst response by neutrophils. Many encapsulated pathogens prevent ROS production by inhibiting serum opsonization (Horwitz & Silverstein, 1980; Hyams et al., 2010; Peterson et al., 1978). In the case of *K. kingae*, elimination of the polysaccharide capsule increased ROS production regardless of the presence of human serum. Interestingly, only elimination of both the polysaccharide capsule and the exopolysaccharide led to a significant increase in ROS production when opsonins were present. These data are consistent with our previous study demonstrating that the presence of either the polysaccharide capsule or the exopolysaccharide was sufficient to prevent opsonization of *K. kingae* (Muñoz et al., 2018). Our results suggest
that encapsulation prevents ROS production through mechanisms beyond hindering opsonin deposition. We hypothesize that when the *K. kingae* polysaccharide capsule is absent, increased neutrophil recognition of bacterial lipopolysaccharide (LPS) prompts TLR4 activation. TLR4 is activated by LPS and upon activation recruits the NADPH-oxidase complex, Nox4, leading to the generation of ROS (Kaisho & Akira, 2006; Ngkelo, Meja, Yeadon, Adcock, & Kirkham, 2012; Park et al., 2004; Qureshi et al., 1999). Future studies will assess the stimulatory potential of *K. kingae* LPS.

Aside from preventing ROS production, the *K. kingae* polysaccharide capsule decreased the total number of neutrophil-associated bacteria. It is well-documented that bacterial polysaccharide capsules prevent neutrophil association by blocking opsonization (Horwitz & Silverstein, 1980; Hyams et al., 2010; Peterson et al., 1978). Interestingly, in our studies neutrophil binding of *K. kingae* occurred regardless of opsonization, similar to ROS production. Studies have shown nonopsonic neutrophil interactions by *N. gonorrhoeae* and *H. pylori* through the expression of adhesins on the bacterial surface (Craig, Lackie, Parton, & Freer, 1988; Fischer & Rest, 1988; Rautelin, Blomberg, Järnerot, & Danielsson, 1994; Rest & Frangipane, 1992). In our previous work, we established that the *K. kingae* polysaccharide capsule can interfere with adherence to epithelial cells (Porsch et al., 2012). In the absence of type IV pili, the polysaccharide capsule masks the trimeric autotransporter, Knh, and deletion of the capsule locus restores Knh-mediated adherence to wild-type levels (Porsch et al., 2012). Neutrophil-killing assays have not been performed using Knh-deficient or pili-deficient *K. kingae* strains to determine whether association with neutrophils is affected by these adhesins.
It is noteworthy that despite an increase in the total number of bound bacteria, the elimination of capsule did not increase phagocytosis regardless of opsonization. This observation suggests that the *K. kingae* exopolysaccharide inhibits or delays neutrophil phagocytosis. Exopolysaccharide secretion in other bacterial pathogens is commonly associated with biofilm formation, promoting survival in the host by inhibiting opsonization and phagocytosis (Jesaitis et al., 2003; Jones & Wozniak, 2017; Yamanaka et al., 2011). Based on previous studies, we presume that the *K. kingae* exopolysaccharide remains loosely tethered to the *K. kingae* cell surface, at least in part (Muñoz et al., 2018; Starr et al., 2013). Surface analysis using scanning electron microscopy may be beneficial for identifying structural or physical changes in the *K. kingae* cell surface that alter interactions with human neutrophils to inhibit or reduce neutrophil phagocytosis. Alternatively, the presence of the exopolysaccharide on the *K. kingae* bacterial surface may mask surface antigens that are recognized by neutrophil receptors and trigger phagocytosis.

Cationic antimicrobial peptides function through electrostatic interactions with anionic bacterial surfaces (Brogden, 2005; Hancock & Chapple, 1999; Nicolas & Mor, 1995; Martti Vaara, 1992). While bacterial polysaccharide capsules vary in their polymer composition, most capsules are anionic and have been shown to bind antimicrobial peptides to prevent or reduce undesirable interactions with the bacterial surface (Campos et al., 2004; Spinosa et al., 2007; Wartha et al., 2007). Surprisingly, there are cases where the lack or loss of the polysaccharide capsule increases resistance to antimicrobial peptides, with examples including *S. pneumoniae* and *Campylobacter jejuni* (Beiter et al., 2008; Zilbauer et al., 2005). Given that the *K. kingae* polysaccharide capsule blocks antibody
recognition of surface antigens (Muñoz et al., 2018), we hypothesized that the capsule would be necessary for complete protection against antimicrobial peptides. Interestingly, loss of the polysaccharide capsule had no effect on *K. kingae* survival in the presence of polymyxin B and only slightly affected survival in the presence of cathelicidin LL-37. In contrast, elimination of the exopolysaccharide resulted in a marked decrease in *K. kingae* survival in the presence of increasing concentrations of either polymyxin B or LL-37 when compared to the wild-type strain, suggesting that the exopolysaccharide is essential for antimicrobial peptide resistance. Llobet et al. demonstrated that free capsular material shed or purified from *K. pneumoniae*, *S. pneumoniae*, or *Pseudomonas aeruginosa* acted as a decoy by sequestering and neutralizing polymyxin B and HNP-1 (Llobet, Tomás, & Bengoechea, 2008). The secretion and release of the high molecular weight exopolysaccharide by *K. kingae* may provide a physical barrier and also actively scavenge antimicrobial peptides to protect *K. kingae* more efficiently than the polysaccharide capsule. Similar to *K. kingae* survival in the presence of neutrophils, elimination of both the capsule and the exopolysaccharide led to the most sensitive phenotype in assays with antimicrobial peptides, suggesting that the capsule plays a minor role in protection when the exopolysaccharide is absent.

Given the robust oxidative burst response and phagocytic activities of neutrophils in assays with surface polysaccharide-deficient *K. kingae*, pretreatment of neutrophils with ROS inhibitors, DPI and NAC, or an actin polymerase inhibitor, Cyto D, restored survival of *K. kingae* mutants to wild-type levels. Together, our data suggest that killing of the capsule-deficient *K. kingae* strains is predominately due to ROS production, whereas the
enhanced killing of the capsule-deficient and exopolysaccharide-deficient *K. kingae* is meditated through both ROS production and phagocytosis. Furthermore, degranulation and release of antimicrobial peptides by neutrophils may augment killing of exopolysaccharide-deficient *K. kingae*.

In our earlier work, we observed redundancy of the the *K. kingae* polysaccharide capsule and exopolysaccharide in preventing antibody and complement deposition on the bacterial surface (Muñoz et al., 2018). In this study, we observed largely distinct but interdependent roles for the polysaccharide capsule and the exopolysaccharide in protecting *K. kingae* from neutrophil-mediated killing and antimicrobial peptides. This study demonstrates the protective functions of the *K. kingae* polysaccharide capsule and exopolysaccharide against human neutrophils and highlights the robust and multifaceted mechanisms employed by *K. kingae* to evade innate immunity.

**Acknowledgements**

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Muñoz V. L., Porsch E. A., St. Gme J. W., III. *Kingella kingae* surface polysaccharides promote resistance to neutrophil phagocytosis and killing.
4. Diversity and genetic characterization of the *Kingella kingae* exopolysaccharide among clinical isolates

4.1 Introduction

*Kingella kingae* is a fastidious gram-negative oropharyngeal commensal bacterium in humans and has been described as an emerging pediatric pathogen (Yagupsky et al., 2009, 2011). In previous work, we described the production of two surface polysaccharides, the polysaccharide capsule and the exopolysaccharide, by the *K. kingae* strain KK01 (a septic arthritis clinical isolate). Genetic and structural analyses of the capsule synthesis loci from a diverse collection of *K. kingae* clinical isolates established the presence of four distinct capsule types (types a, b, c, and d) (Starr, Porsch, Seed, Heiss, et al., 2016). Interestingly, capsule types a and b were predominant among invasive disease isolates suggesting an association between capsule type and clinical presentation (Porsch et al., 2017; Starr, Porsch, Seed, Heiss, et al., 2016). While the genetic determinants and diversity of the *K. kingae* polysaccharide capsule have been extensively studied, little is known about the exopolysaccharide synthesis locus, *pamABCDE*, and diversity among carrier and invasive clinical isolates.

Exopolysaccharide function depends on the polymer structure and composition and in other bacterial species has been studied primarily as a major contributor to the formation and dispersal of biofilms (Cerning, 1991; De Vuyst & Degeest, 1999; Flemming et al., 2007; Nwodo et al., 2012). Thus far, two galactan homopolymer exopolysaccharide structures with distinct linkages have been described in *K. kingae*: \( \rightarrow 5\)-\(\beta\)-Galf-(1\( \rightarrow \)) and \( \rightarrow 3\)-\(\beta\)-Galf-(1\( \rightarrow 5\))-\(\beta\)-Galf-(1\( \rightarrow \)) (Bendaoud et al., 2011; Starr et al., 2013), which we refer to as the type 1 and type 2 exopolysaccharide, respectively. Based on previous studies of
K. kingae strain KK01, the type 1 exopolysaccharide by prevents opsonin deposition and complement activation, protecting the organism from human serum (Muñoz et al., 2018). In studies of K. kingae strain PYKK181, the type 2 exopolysaccharide has been demonstrated to contribute to biofilm dispersal (Bendaoud et al., 2011). The ability of the K. kingae exopolysaccharides to inhibit biofilm formation and promote serum resistance may benefit K. kingae during colonization and invasive disease. Further analysis of the biosynthesis genes and/or machinery will provide a better foundation to determine the presence and diversity of exopolysaccharides in K. kingae clinical isolates. Interestingly, the exopolysaccharide pam loci present in strains KK01 and PYKK181 are highly homologous.

In this study, we describe the genetic composition of the pam locus and found two distinct alleles that correlate with the exopolysaccharide types. Furthermore, using PCR-typing, we examine the diversity of the exopolysaccharide among carrier and invasive clinical isolates and established that over 99% of all isolates express the type 1 exopolysaccharide. Both the type 1 and type 2 exopolysaccharides promote serum resistance in K. kingae isolates. Notably, the lack of diversity of the exopolysaccharide among isolates makes the exopolysaccharide a preferential target for polysaccharide-conjugate vaccines.

4.2 Methods

4.2.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 6. 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 µg/ml kanamycin or 1 µg/ml erythromycin, as appropriate. *E. coli* strains were grown at 37°C on LB agar or shaking at 250 rpm in LB broth supplemented with 100 µg/ml ampicillin or 50 µg/ml kanamycin, as appropriate.

### 4.2.2 Strain construction

Gene disruptions were introduced into *K. kingae* by natural transformation (Kehl-Fie et al., 2008). Following recovery, transformants were plated on selective medium and correct insertion of the gene disruption construct was confirmed via PCR and sequencing of the insertion site. All of the plasmids used in this study are listed in Table 6, and the sequences of all of the primers used are listed in Table 7.

The strains KK01 *csaA*, KK01 *pam*, and KK01*csaApam* were generated as previously described (Muñoz et al., 2018; Starr et al., 2013; Starr, Porsch, Seed, & St. Geme III, 2016). To generate mutants with deletion of *pamABC* or *pamDE*, strains KK01 and KK01*csaA* were transformed with the linearized disruption plasmids pUC19*pamABC::kan* or pUC19*pamDE::kan*. To generate pUC19*pamABC::kan*, DNA fragments of the homologous recombination targeting sequence corresponding to ~1 kb upstream of *pamA* and ~1 kb downstream of *pamC* were PCR amplified from strain KK01 genomic DNA using primers *pam 5’ For/ pamABC 5’ Rev* and *pamABC 3’ For/ pamABC 3’ Rev*, respectively, and were ligated into pUC19. To generate pUC19*pamDE::kan*, DNA fragments of the homologous recombination targeting sequence corresponding to ~1 kb upstream of *pamD* and ~1 kb downstream of *pamE* were PCR amplified from strain KK01
genomic DNA using primers pamDE 5’ For/ pamDE 5’ Rev and pam 3’ For/ pam 3’ Rev, respectively, and were ligated into pUC19. For both plasmids, a kanamycin resistance cassette was then ligated into the BamHI site located between the cloned upstream and downstream homologous recombination targeting sequences.

To generate strain KK143csb, strain KK143 was transformed with the linearized disruption plasmid pSwapEmpty (Starr, Porsch, Seed, Heiss, et al., 2016). To generate pamABCDE deletion mutants, strains KK143 and KK143csb were transformed with the linearized disruption plasmid pUC19pam::ermC (Starr et al., 2013). The plasmids were purified from E. coli and linearized with NdeI.

To generate pUC19pamABC, DNA fragments were amplified from strain KK01 genomic DNA by PCR using primers pam Amp For/ pamABC Amp Rev and were ligated into pUC19. Plasmids pUC19EmptyVector and pUC19pamABC were transformed into E. coli strain DH5α by electroporation, and transformants were recovered by plating on agar supplemented with 100 µg/ml ampicillin.

### Table 6: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kingella kingae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KK01</td>
<td>Nonspreading/noncorroding derivative of 269-492</td>
<td>(Kehl-Fie &amp; St. Geme III, 2007)</td>
</tr>
<tr>
<td>PYKK181</td>
<td>Clonal group V isolate from patient with bacteremia</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>CC254</td>
<td>Clonal group V isolate from a healthy carrier</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK242</td>
<td>Clonal group V isolate from patient with bacteremia</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK430</td>
<td>Clonal group V isolate from patient with bacteremia</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK143</td>
<td>Clonal group V isolate patient with septic arthritis</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>Code</td>
<td>Isolate Type and Details</td>
<td>Source</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>KK405</td>
<td>Clonal group V isolate patient with septic arthritis</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>Sch1614</td>
<td>Clonal group B isolate from a healthy carrier</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>ATCC23330</td>
<td>Clonal group D isolate from a healthy carrier</td>
<td>ATCC</td>
</tr>
<tr>
<td>KK245</td>
<td>Clonal group A isolate from patient with bacteremia</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK247</td>
<td>Clonal group A isolate from patient with endocarditis</td>
<td>Yagupsky, P.</td>
</tr>
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<td>BB060</td>
<td>Clonal group D isolate from a healthy carrier</td>
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</tr>
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<td>KK113</td>
<td>Clonal group C isolate from a healthy carrier</td>
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<td>AA046</td>
<td>Clonal group F isolate from a healthy carrier</td>
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</tr>
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<td>KK86</td>
<td>Clonal group K isolate from a healthy carrier</td>
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<td>KK145</td>
<td>Clonal group K isolate from patient with septic arthritis</td>
<td>Yagupsky, P.</td>
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<td>KK12</td>
<td>Clonal group H isolate from a healthy carrier</td>
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<td>KK136</td>
<td>Clonal group H isolate from patient with bacteremia</td>
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<td>Clonal group P isolate from patient with endocarditis</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK141</td>
<td>Clonal group N isolate from patient with osteomyelitis</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>AA068</td>
<td>Clonal group N isolate from a healthy carrier</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>KK245</td>
<td>Clonal group A isolate from patient with bacteremia</td>
<td>Yagupsky, P.</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Reference</td>
</tr>
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<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
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<td>EE160</td>
<td>Clonal group A isolate from a healthy carrier</td>
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<td>BB114</td>
<td>Clonal group A isolate from a healthy carrier</td>
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<td>KK01csaA</td>
<td>Derivative of KK01 with deletion of csaA type a capsule synthesis gene</td>
<td>(Starr, Porsch, Seed, &amp; St. Geme III, 2016)</td>
</tr>
<tr>
<td>KK01pam</td>
<td>Derivative of KK01 with deletion of pamABCDE exopolysaccharide locus</td>
<td>(Starr et al., 2013)</td>
</tr>
<tr>
<td>KK01csaApam</td>
<td>Derivative of KK01 with deletion of csaA and pamABCDE locus</td>
<td>(Muñoz et al., 2018)</td>
</tr>
<tr>
<td>KK01pamABC</td>
<td>Derivative of KK01 with deletion of pamABC locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK01pamDE</td>
<td>Derivative of KK01 with deletion of pamDE locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK01csaApamABC</td>
<td>Derivative of KK01 with deletion of csaA and pamABC locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK01ScsaApamDE</td>
<td>Derivative of KK01 with deletion of csaA and pamDE locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK143csb</td>
<td>Derivative of KK143 with deletion of csbABC type b capsule synthesis locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK143pam</td>
<td>Derivative of KK143 with deletion of pamABCDE exopolysaccharide locus</td>
<td>This study</td>
</tr>
<tr>
<td>KK143csbpam</td>
<td>Derivative of KK143 with deletion of csbABC and pamABCDE loci</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td>lhs (Sambrook et al., 1989)</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>E. coli F− φ80dlacZΔM15 Δ(lacZYAargF)U169 deoR recA1 endA1 hsdR17(rK− mK+) phoA supE441 thi-1 gyrA96 relA1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19pam::ermC</td>
<td>pUC19 derivative that contains pamABCDE disruption construct marked with the ermC erythromycin cassette</td>
<td>(Starr et al., 2013)</td>
</tr>
<tr>
<td>pSwapEmpty</td>
<td>Contains the capsule synthesis locus flanking genes with the capsule synthesis variable region deleted</td>
<td>(Starr, Porsch, Seed, Heiss, et al., 2016)</td>
</tr>
<tr>
<td>pUC19pamABC::kan</td>
<td>pUC19 derivative that contains pamABC disruption construct marked with the aphA3 kanamycin cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19p&lt;sub&gt;pamDE:&lt;/sub&gt;kan</td>
<td>pUC19 derivative that contains &lt;em&gt;pamDE&lt;/em&gt; disruption construct marked with the &lt;em&gt;aphA3&lt;/em&gt; kanamycin cassette</td>
<td>This study</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pUC19p&lt;sub&gt;pamABC&lt;/sub&gt;</td>
<td>pUC19 derivative that contains the &lt;em&gt;pamABC&lt;/em&gt; locus and upstream promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

**4.2.3 Polysaccharide extraction and staining**

For capsule extractions, <em>K. kingae</em> strains were grown at 37°C with 5% CO<sub>2</sub> on chocolate agar plates. Bacteria were resuspended in PBS to an OD<sub>600</sub> ~1.2, and cells were pelleted, washed, and resuspended in Tris acetate pH 5.0 for 1 hr. Cells were removed by centrifugation, and extracts were treated with proteinase K for 1 hr and then concentrated as previously described (Porsch et al., 2012). For capsule and exopolysaccharide co-purifications, bacteria were resuspended in 5 ml PBS and vortexed. Cells were removed by centrifugation, and bacterial supernatants were treated with proteinase K for 1 hr and then concentrated as previously described (Porsch et al., 2012).

Aliquots of supernatant and purified capsule from <em>K. kingae</em> derivatives were separated on 7.5% and 10% SDS-PAGE gels, respectively. For supernatant samples, SDS-PAGE gels were treated first with silver staining and subsequently with Alcian blue staining. For purified capsule samples, SDS-PAGE gels were treated with 0.125% Alcian blue as previously described (Porsch et al., 2012). For silver staining, gels were treated as previously described (J. S. Kim et al., 1996).
4.2.4 RNA isolation and RT-PCR

Total RNA from *K. kingae* strain KK01 was extracted and purified as previously described (Kehl-Fie et al., 2009). Briefly, RNA was extracted using TRIreagent (Sigma, St. Louis, MO) and the RNeasy minikit by following the lipid-rich tissue protocol (Qiagen, Valencia, CA). Residual DNA was removed with RQ1 DNase (Fisher Scientific, Pittsburgh, PA). The concentration and purity of the RNA were determined by measuring the A260/A280 ratio with a Nanodrop 2000 (Thermo Fisher, Rockford, IL). The qScript cDNA SuperMix kit (Quanta Biosciences, Beverly, MA) was used according to the manufacturer’s instructions for RT-PCR. The primer sets used for RT-PCR are listed in Table 7.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pam</em> 5’ For</td>
<td>GC*GAAATTCGGCGTTGGTGGAAATATCCTG</td>
<td>(Starr, Porsch, Seed, &amp; St. Geme III, 2016)</td>
</tr>
<tr>
<td><em>pam</em> 3’ For</td>
<td>GCCGATCCTCAAAAGGCTGGTATAAACAC</td>
<td>(Starr, Porsch, Seed, &amp; St. Geme III, 2016)</td>
</tr>
<tr>
<td><em>pam</em> 3’ Rev</td>
<td>GCAAGCTTCCATATCGCTTTTGCTTTGC</td>
<td>(Starr, Porsch, Seed, &amp; St. Geme III, 2016)</td>
</tr>
<tr>
<td><em>pam</em>AB outside R</td>
<td>GCTTTCGGAATGCTTTGCGTACC</td>
<td>This study</td>
</tr>
<tr>
<td><em>pam</em>A locator Rev</td>
<td>GCAGGCTTGTACAGGTCTTTG</td>
<td>This study</td>
</tr>
<tr>
<td><em>pam</em> inside For</td>
<td>GCGACTGTTCCTCATTACGATAG</td>
<td>This study</td>
</tr>
</tbody>
</table>
4.2.5 Serum bactericidal assays

*K. kingae* strains were grown at 37°C with 5% CO₂ on chocolate agar plates and then resuspended in PBS containing 0.1% gelatin (PBS-G). *E. coli* strains were grown on LB agar or shaking at 250 rpm in LB broth supplemented with 100 µg/ml ampicillin at 37°C to OD₆₀₀ 0.8. Samples were diluted in PBS-G to obtain a final inoculum of approximately 4.0 x 10³ 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 time points at 37°C with 5%
CO₂. 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 cfu counts (limit of detection for plating: 20 cfu).

4.2.6 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. The specific statistical tests used for each experiment are specified in the figure legends.

4.3 Results

4.3.1 Exopolysaccharide purification and visualization

In order to extract and co-purify the polysaccharide capsule and exopolysaccharide, we collected bacterial supernatants from K. kingae strains KK01, KK01csaA, KK01pam, and KK01csaApam in 5 ml PBS. After concentration and proteinase K treatment, supernatants were separated on 7.5% SDS-PAGE gels (Fig. 18). The polysaccharide capsule from the surface of strains KK01 and KK01pam stained prominently with silver stain and Alcian blue reagents (Fig. 18), as demonstrated previously (Starr et al., 2013). The exopolysaccharide capsule from the surface of strains KK01 and KK01csaA stained prominently with silver stain but did not stain with Alcian blue (Fig. 18, data not shown). The supernatant from strain KK01csaApam failed to stain with either silver stain or Alcian blue reagents, as expected (Fig. 18).
Figure 18: Silver and Alcian blue staining of *K. kingae* surface polysaccharides present in bacterial supernatants.

Samples were proteinase K treated and resolved on a 7.5% SDS-PAGE gel. Lane 1, protein ladder, molecular sizes in kDa; lane 2, strain KK01; lane 3, strain KK01*csaA*; lane 4, strain KK01*pam*; lane 5, strain KK01*csaApam*.

**4.3.2 KK01 *pamABC* is sufficient for serum resistance.**

Previous work determined that expression of *pamABC* from *K. kingae* clinical isolate PYKK181 in *E. coli* was sufficient for inhibiting biofilm formation (Bendaoud et al., 2011). To determine whether *pamD* and *pamE* were necessary for exopolysaccharide-mediated serum resistance, we performed serum bactericidal assays using human serum. We deleted the entire *pam* locus (*pamABCDE*), or portions of the *pam* locus (*pamABC* or *pamDE*) in either strain KK01 or strain KK01*csaA*. Inocula of $1.0 \times 10^3$ cfu were incubated for 1 hr with 50% human serum (Fig. 19), using strain KK01*csaApam* (containing a deletion of *pamABCDE*) as a serum-sensitive control. To assess serum sensitivity, a survival ratio was calculated, dividing the number of cfu recovered from NHS by the number of cfu recovered from HI-NHS.
Deletion of *pamD* and *pamE* does not affect *K. kingae* serum resistance in the absence of capsule.

*K. kingae* strains KK01, KK01*pam*, KK01*csaApam*, KK01*pamABC*, KK01*csaApamABC*, KK01*pamDE*, and KK01*csaApamDE* 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. 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.

Deletion of *pamABC* or *pamDE* resulted in no growth defects on solid agar and had no effect on survival in the presence of 50% HI-NHS. Survival in 50% NHS by strains KK01*pam*, KK01*pamABC*, KK01*pamDE*, and KK01*csaApamDE* was not significantly different from the survival of wild-type strain KK01 (Fig. 19). In contrast, survival in 50% NHS by strains KK01*csaApam* and KK01*csaApamABC* was markedly reduced, with a survival ratio of ~0.40 (*P* < 0.05, Fig. 19).

To further confirm that the KK01 *pamABC* genes promote serum resistance, we transformed *E. coli* strain DH5α with either an empty vector pUC19 plasmid or the pUC19*pamABC* plasmid. Due to the sensitivity to serum of strain DH5α, the serum resistance assays were performed by incubating DH5α derivatives with 2% human serum.
for 30 min (Fig. 20). Five individual transformants harboring the pUC19*pamABC* plasmid were selected and assessed for changes in serum resistance, using DH5α/pUC19 as a serum-sensitive control. We observe in our preliminary data (n=2) that the survival ratio of DH5α/pUC19 in 2% NHS was ~0.18 (Fig. 20). In contrast, the survival ratio of DH5α/pUC19*pamABC* clones 1-3 in 2% NHS was much higher, between 0.55-0.70 (Fig. 20). The survival ratio of DH5α/pUC19*pamABC* clones 4 and 5 in 2% NHS was moderately increased, approximately ~0.40 (Fig. 20).

![Graph showing survival ratio](image)

**Figure 20:** Expression of the KK01 *pamABC* genes in serum-sensitive *E. coli* increases serum resistance.

*E. coli* strains DH5α/pUC19 (Vector) and DH5α/pUC19*pamABC* were incubated with 2% NHS or 2% HI-NHS for 30 min. The survival ratio was calculated by dividing the NHS CFU counts by the HI-NHS CFU counts. A total of three biological replicates were performed (n = 2).
So far, these results establish that the *pamABC* genes are sufficient to promote serum resistance and confirm that *pamD* and *pamE* are not critical for exopolysaccharide-mediated serum resistance in *K. kingae* and *E. coli*.

### 4.3.3 Transcriptional analysis of KK01 *pam* locus.

To determine expression of polycistronic transcripts in the exopolysaccharide biosynthesis locus, the transcription of *K. kingae* strain KK01 *pamA, pamB, pamC, pamD,* and *pamE* was assessed by RT-PCR. We performed RT-PCR from *K. kingae* KK01 cDNA using three forward primers that fall within the *pamA, pamC,* or *pamD* open reading frames (ORF) and four reverse primers within the *pamB, pamC, pamD,* or *pamE* ORF (Fig. 21A). Using Virtual Footprint analysis, predicted promoters were upstream of the *pamA* and *pamD* genes; no terminator sequences were detected within the *pamABCDE* locus (Münch et al., 2005). We detected amplicons of the expected size when using the specific primers for the contiguous genes *pamAB, pamABC, pamCDE, pamCD,* and *pamDE* (Fig. 21B-D). Conversely, amplicons were less abundant when using specific primers for the contiguous genes *pamABCD* and *pamABCDE* (Fig. 21B, C).
Figure 21: Transcription and polycistronic mRNA of *pamABCDE* locus in *K. kingae* strain KK01.

Genomic structure of *K. kingae pamABCDE* genes and surrounding genes (A). Specific primers for RT-PCR designed to amplify PCR products are shown in arrows (forward primers, red; reverse primers, black). Polycistronic mRNA PCR products for *pamAB*, *pamABC*, *pamABCDE*, (B) *pamABCDE*, *pamDE*, (C), *pamCD*, and *pamCDE* (D). Lane 1, genomic DNA; Lane 2, cDNA; Lane 3, no reverse transcriptase (RT).
4.3.4 The type 1 and type 2 exopolysaccharides are associated with two distinct PamC proteins.

Previous work determined that the septic arthritis clinical isolate KK01 expresses a \( \rightarrow 5 \)-\( \beta \)-Gal\(-(1\rightarrow\) galactan homopolymer designated as a type 1 exopolysaccharide, whereas the bacteremia clinical isolate PYKK181 expresses a \( \rightarrow 3 \)-\( \beta \)-Gal\(-(1\rightarrow 5 \)-\( \beta \)-Gal\-(1\rightarrow \) galactan homopolymer designated as a type 2 exopolysaccharide. To determine whether the differences in polymer linkages were due to genes present in the \( pam \) locus, we performed amino acid sequence alignments to compare the percent identity and similarity of the predicted PamA, PamB, PamC, PamD, and PamE proteins across the two \( K. kingae \) strains. This comparison revealed a lower percent identity and similarity between PamC alleles relative to the other proteins encoded by the \( pamABCDE \) locus, with an overall identity of 85% and a similarity of 91% (Fig. 22). The identity and similarity of PamA and PamB between strains was 99% (Fig. 22), the identity and similarity of PamD between strains was 100% (Fig. 22), and the identity and similarity of PamE between strains was 98% (Fig. 22).

![Figure 22: Comparison of the predicted protein products of the pam genes in K. kingae strains KK01 and PYKK181.](image)

Genomic organization of the \( pamABCDE \) loci in \( K. kingae \) strains KK01 (top) and PYKK181 (bottom). The light blue boxes highlight the identity and similarity based on amino acid sequence alignments of the predicted PamA, PamB, PamC, PamD, and PamE proteins (center); one number is denoted if the percent identity and similarity are the same.
Based on BLAST analyses, PamC is a predicted galactofuranosyl transferase, with potential to create linkages in polymer chains. Further analysis of the 592 amino acid PamC protein revealed a 146-amino acid stretch with relatively low identity (46%) and low similarity (67%) when comparing strain KK01 to strain PYKK181 (Fig. 23). Of the 55 non-synonymous amino acid changes between strain KK01 PamC and strain PYKK181 PamC, 48 were present in this 146-amino acid stretch of low homology (Fig. 23). These results suggest that PamC is the molecular determinant of exopolysaccharide diversity in *K. kingae* clinical isolates.

**Figure 23: Comparison of PamC across *K. kingae* strains KK01 and PYKK181.**

Schematic highlighting a region of low identity and similarity between KK01 PamC (blue) and PYKK181 PamC (red) (A). The light blue boxes highlight the identity and similarity between the full-length PamC proteins (A) and the 146-amino acid PamC fragments (B). Predicted amino acid sequence alignment of KK01 PamC (top) and PYKK181 PamC (bottom) highlighting the region with the lowest amount of homology (pink) (C).
4.3.5 *pamC1* is predominant in clinical isolates of *K. kingae*.

To determine the presence of the KK01 *pamC* allele (*pamC1*) or the PYKK181 *pamC* allele (*pamC2*) in *K. kingae* clinical isolates, we designed allele-specific forward primers that anneal within the variable region of the *pamC* gene and a reverse primer that anneals within a conserved region of the *pamC* gene, producing allele-specific amplicons (Fig. 24A). PCR amplification of genomic DNA from *K. kingae* strains KK01, PYKK181, and KK01 pam confirmed primer specificity to either the *pamC1* or *pamC2* allele (Fig. 24B).

![Figure 24: K. kingae pamC allele PCR-typing method.](image)

Illustration of *pamC* alleles of *K. kingae* strains KK01 and PYKK181 highlighting the variable region in *pamC1* (blue) and *pamC2* (red) (A). Specific primers designed are shown in arrows (allele-specific forward primer, blue or red; conserved reverse primer, black). PCR amplification of strains KK01 pam, KK01, and PYKK181 using the *pamC1*-specific forward primer (top) or the *pamC2*-specific forward primer (bottom). Lane 1, DNA ladder; Lane 2, KK01 pam; Lane 3, KK01; Lane 4, PYKK181.
Using the *pamC* allele PCR-typing method, we screened a collection of 24 clinical isolates for the presence of the *pamC1* or *pamC2* allele. The 24 clinical isolates represent 14 sequence types based on multilocus sequence typing; 12 of the 14 sequence types are clustered into seven sequence type complexes (two of the sequence types represented do not fall into a sequence type complex) (Basmaci et al., 2014). Additionally, based on previous PFGE analysis, the clinical isolates represent 11 PFGE clonal groups (A, B, C F, H, K, N, P, V, and 9), including the five major clonal groups that account for >72% of invasive disease isolates (B, H, K, N, and P) (Amit et al., 2012; Starr, Porsch, Seed, Heiss, et al., 2016). The cohort represents 12 strains from healthy carriers and 12 strains from patients with invasive disease, including bacteremia (5), septic arthritis (4), endocarditis (2), and osteomyelitis (1) (Basmaci et al., 2014).

Amplicons were detected in all of the clinical isolates screened for one of the two *pamC* alleles (Fig. 25). The *pamC1* allele was predominant among clinical isolates, present in 87.5% (21 of 24) of these strains (Fig. 25). The *pamC2* allele was present in the other three clinical isolates screened (3 of 24, 12.5%) (Fig. 25). All three clinical isolates containing the *pamC2* allele, *K. kingae* strains CC254, KK242, and Sch1614, fall within the same sequence type as strain PYKK181. These data suggest that the type 1 exopolysaccharide is the predominant exopolysaccharide present in *K. kingae* clinical isolates. No significant associations between exopolysaccharide type and clinical presentation were identified. Clinical isolates present in this study had been examined previously for capsule type (Starr, Porsch, Seed, Heiss, et al., 2016); however, no significant associations between exopolysaccharide type and capsule type were found.
Figure 25: PCR screening of a diverse cohort of *K. kingae* clinical isolates.

PCR amplification of genomic DNA from *K. kingae* strains using the *pamC1*-specific forward primer (top) or the *pamC2*-specific forward primer (bottom). *K. kingae* strains KK01, KK01, and PYKK181 were used as controls. Color blocks above image represent clinical isolates present in a similar sequence type (red or purple) or sequence type complex (pink, orange, yellow, blue, green, sky, or navy). Lane 1, ATCC 23330; Lane 2, BB060; Lane 3, AA046; Lane 4, KK86; Lane 5, KK145; Lane 6, KK12; Lane 7, KK136; Lane 8, KK83; Lane 9, BB012; Lane 10, KK142; Lane 11, KK98; Lane 12, KK164; Lane 13, KK144; Lane 14, KK190; Lane 15, CC254; Lane 16, KK242; Lane 17, Sch1614; Lane 18, KKBB114; Lane 19, KK245; Lane 20, EE160; Lane 21, KK247; Lane 22, KK141; Lane 23, AA068; Lane 24, KK113.

4.3.6 *pamC2* is present in PFGE V clinical isolates.

To determine whether clinical isolates within the same PFGE group as *K. kingae* strain PYKK181 possess the *pamC2* allele, we used the *pamC* allele PCR-typing method to screen a collection of six clinical isolates present within the PFGE V or 9 group. The cohort was comprised of two healthy carrier isolates and four invasive isolates, including isolated associated with bacteremia (2) and septic arthritis (2). The *pamC1* allele was
present in 0% (0 of 6) of all clinical isolates screened (Fig. 26). The pamC2 allele was present in 100% (6 of 6) of all clinical isolates screened (Fig. 26). These results suggest that the pamC2 allele may be restricted to a distinct set of clinical isolates. Interestingly, all clinical isolates within these two PFGE groups express a type b polysaccharide capsule; however, based on Fig. 25, clinical isolates that produce a type b capsule may contain either the pamC1 or pamC2 allele.

**Figure 26: PCR screening of PFGE V K. kingae clinical isolates.**

PCR amplification of K. kingae strains using the pamC1-specific forward primer (top) or the pamC2-specific forward primer (bottom). K. kingae strains KK01pam, KK01,
and PYKK181 were used as controls. Lane 1, CC254; Lane 2, KK242; Lane 3, KK430; Lane 4, KK143; Lane 5, KK405; Lane 6, Sch1614.

4.3.7 Both type 1 and type 2 exopolysaccharides confer serum resistance.

To determine whether the type 2 exopolysaccharide also confers serum resistance in the absence of the polysaccharide capsule, we performed serum bactericidal assays using derivatives of *K. kingae* strain KK143, a septic arthritis clinical isolate producing a type b capsule and possessing the *pamC2* allele. Unlike *K. kingae* strain PYKK181, *K. kingae* strain KK143 was amenable to genetic manipulation. Serum bactericidal assays were performed using strain KK143, strain KK143*csb* (a capsule-deficient mutant lacking the *csbABC* capsule synthesis locus), strain KK143*pam* (an exopolysaccharide-deficient mutant lacking the *pamABCDE* galactan exopolysaccharide synthesis operon), and strain KK143 *csbpam* (a double mutant lacking both capsule and exopolysaccharide synthesis genes). Assays were performed using 50% serum for 1 hr, and strains KK01, KK01*csaA*, KK01*pam*, and KK01*csaApam* were used as controls (Fig. 27A). Deletion of *csb*, *pam*, or both *csb* and *pam* resulted in no growth defects on solid agar and had no effect on survival in the presence of 50% HI-NHS. Survival in 50% NHS of strains KK143*csb* and KK143*pam* was not significantly different from the survival of wild-type strain KK143 (Fig. 27B). In contrast, survival in 50% NHS by strain KK143*csbpam* was markedly reduced, with a survival ratio of less than 0.35 (*P* < 0.05, Fig. 27B).
Figure 27: The type 2 exopolysaccharide promotes serum resistance for *K. kingae* strain KK143.

*K. kingae* strains KK01, KK01csaA, KK01pam, KK01csaApam, (B) KK143, KK143csb, KK143pam, and KK143csbpam (B) 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. 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.

These results establish that in the absence of the polysaccharide capsule, the type 2 exopolysaccharide is critical for survival in the presence of human serum, similar to observations with the type 1 exopolysaccharide.

4.4 Discussion

Polysaccharide-conjugate vaccines against *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* have provided long-lived protection,
reflecting stimulation of robust immune responses in infants (Goldblatt, 2000; Hyams et al., 2010; Moxon & Kroll, 1988; Swartley et al., 1997). Polysaccharide-conjugate vaccine design is typically guided by polysaccharide capsule diversity and correlation with invasive disease (Goldblatt, 2000). Analyses of a large collection of K. kingae clinical isolates revealed four distinct capsule structures and capsule biosynthesis loci, two of which account for >95% of invasive disease cases (Porsch et al., 2017; Starr, Porsch, Seed, Heiss, et al., 2016). Identification of the polysaccharide capsule led to the discovery of the exopolysaccharide, which has a distinct polymer structure separate from the polysaccharide capsule (Starr et al., 2013). In this study, we hoped to enhance understanding of the exopolysaccharides produced by K. kingae to better evaluate the potential of this factor as a vaccine candidate.

Previous work from Bendaoud et al. utilized an inducible plasmid containing the pamABC locus from K. kingae strain PYKK181 in E. coli and demonstrated exopolysaccharide production and anti-biofilm properties (Bendaoud et al., 2011). Using a high copy number plasmid, we were able to demonstrate that expression of the strain KK01 pamABC genes in E. coli promoted serum resistance, further confirming the importance of the exopolysaccharide in preventing complement-mediated lysis. It is interesting to note that expression of the pamABC biosynthesis locus alone was sufficient for biofilm dispersal and serum resistance in E. coli, unlike the K. kingae polysaccharide capsule biosynthesis locus, which requires additional genes necessary for lipidation and capsule export (Bendaoud et al., 2011; Starr, Porsch, Seed, & St. Geme III, 2016).
Based on previous work and data from this study, PamD and PamE are not essential for exopolysaccharide biosynthesis, serum resistance, or biofilm dispersal (Bendaoud et al., 2011). Based on BLAST analyses, PamD and PamE are predicted putative glycosyltransferases. There is a predicted promoter binding site upstream of pamD, suggesting that pamD and pamE are separate from the pam exopolysaccharide biosynthesis locus. Unpublished data suggest that pamD and pamE affect K. kingae lipopolysaccharide (LPS) biosynthesis; however, it is unclear to what extent. Deletion of the pamABC locus did not visibly alter the LPS migration pattern, providing further evidence for distinct roles for the pamABC and pamDE genes (unpublished data). In ongoing studies, LPS purification and analysis will clarify the role of the pamD and pamE genes.

Previous descriptions of the pam loci between K. kingae strains KK01 and PYKK181 noted high homology within the pamABCDE gene cluster; however, individual predicted amino acid sequence alignments of the five gene products revealed significant differences in the pamC products, suggesting that these gene products may be the molecular determinant of exopolysaccharide diversity. PCR-typing of the pamC alleles identified the pamC1 allele (type 1 exopolysaccharide) as predominant in the clinical isolates screened; although 11 PFGE clonal groups were represented, expanding our screening to a larger collection of isolates would provide a broader view of exopolysaccharide diversity. Interestingly, the pamC2 allele was predominant in the PFGE V clonal group. The PFGE V clonal group contained both carrier and invasive isolates with the pamC2 allele. Despite the lack of correlation between clinical presentation and exopolysaccharide type, use of the infant rat infection model may provide better insight to
differences in exopolysaccharide virulence properties in vivo. Further genetic manipulation is needed to confirm the function and properties of the two exopolysaccharide types and pamC alleles.

The lack of cross-protection against antigenically distinct, encapsulated strains is a limitation of capsule-conjugate vaccines. Recently through the use of genomic sequencing, outer membrane proteins have been targeted as vaccine antigens in a variety of pathogens due to the conservation across strains (Etz et al., 2002; Pizza et al., 2000; Tan, Carlone, & Borrow, 2010; Wizemann et al., 2001). We demonstrate the presence of only two exopolysaccharide loci in K. kingae strains, with a majority of clinical isolates containing the type 1 exopolysaccharide locus, thus providing further evidence of the exopolysaccharide as an attractive vaccine candidate.

The type 1 exopolysaccharide had been previously described as promoting K. kingae survival in human serum. Through the generation of surface polysaccharide mutants in K. kingae strain KK143, we were able to confirm that the type 2 exopolysaccharide exhibits similar properties. Exopolysaccharide pam swap constructs will be created to further confirm the galactofuranosyl transferase activity of pamC and to express the type 1 and type 2 exopolysaccharides in an isogenic background for functional analyses in future experiments.

To summarize, in this study we have established a SDS-PAGE staining protocol for visualization of co-purified surface polysaccharides and a pamC PCR-typing method. Analyses of the pam loci confirmed that pamABC is sufficient to promote serum resistance in both K. kingae as well as in E. coli. PCR-typing allowed us to determine the level of
diversity in the pamC allele across a collection of 27 K. kingae clinical isolates and confirm the presence of only two pamC alleles thus far. We were able to demonstrate that the type 2 exopolysaccharide also functions to protect K. kingae against complement-mediated lysis. Future studies will focus on exopolysaccharide antigenicity and in vivo infection studies.

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5. Future Directions

5.1 Conclusion

*K. kingae* displays high levels of resistance to innate immune components, specifically to complement- and neutrophil-mediated killing. The data presented in Chapters 2 and 3 demonstrate the significance of surface polysaccharides (the polysaccharide capsule and exopolysaccharide) in *K. kingae* immune evasion and virulence *in vivo*. Chapter 4 delves into the biosynthesis locus of the exopolysaccharide and explores the diversity of the exopolysaccharide present in *K. kingae* clinical isolates. The future directions and experimental approaches outlined below will identify and characterize other determinants of innate immune evasion in *K. kingae*. Moreover, we will investigate the multi-faceted functions of the *K. kingae* surface polysaccharides in immune evasion and begin to provide better insight into polysaccharide diversity and its contribution to *K. kingae* pathogenicity.

5.2 Evaluate the impact of polysaccharide capsule diversity on immune evasion

Capsule production by invasive pathogens promotes host survival by inhibiting complement activation and neutrophil recognition (Beiter et al., 2008; Campos et al., 2004; Hallström & Riesbeck, 2010; Hyams et al., 2010; Lambris et al., 2008; Wartha et al., 2007). Many species of bacteria express multiple different capsule serotypes, and in some cases a specific capsule type is associated with invasive disease potential, as is the case with *H. influenzae* serotype b capsule (Cochi, Broome, & Hightower, 1985) and *Klebsiella pneumoniae* capsule types K1 and K2 (Mizuta et al., 1983; Paczosa & Mecsas, 2016). *K.*
*K. kingae* clinical isolates can be classified into four distinct capsule types (types a, b, c, and d) (Starr, Porsch, Seed, Heiss, et al., 2016). Strains expressing the type a and type b capsule account for >95% of invasive disease isolates (Porsch et al., 2017; Starr, Porsch, Seed, Heiss, et al., 2016), suggesting that the distinct capsule composition may play a role in the pathogenicity of *K. kingae*.

As previously shown in Chapters 2 and 3, *K. kingae* strain KK01 expresses a type a polysaccharide capsule that protects *K. kingae* against complement- and neutrophil-mediated killing (Muñoz et al., 2018). Furthermore, isogenic derivatives of strain KK01pam expressing one of the four capsule types were associated with a serum survival ratio similar to the serum survival ratio for strain KK01 (the parental strain) (Muñoz et al., 2018). To assess whether the four capsule types differentially protect *K. kingae* against neutrophil-mediated killing, we performed neutrophil killing assays on isogenic derivatives of strain KK01pam expressing either capsule type a (strain KK01Swapcsapam), capsule type b (strain KK01Swapcsbpam), capsule type c (strain KK01Swapcscpam), or capsule type d (strain KK01Swapcsdpam). The survival of strains KK01pam, KK01pamSwapcsa, KK01pamSwapcsb, KK01pamSwapcsd was not affected at an MOI of 0.1 in the presence of neutrophils with 1% HSA or 1% NHS (Fig. 28A-C, E). The survival of strain KK01pamSwapcsc was slightly affected at an MOI of 0.1 in the presence of neutrophils with 1% NHS (Fig. 28D). As expected, survival of strain KK01pamSwapEmpty was significantly decreased at an MOI of 0.1 in the presence of neutrophils with 1% HSA and 1% NHS (Fig. 28F). These data suggest that the presence of any of the four naturally occurring *K. kingae* capsule type is adequate in serving a protective role for *K. kingae* strain.
KK01 under *in vitro* serum and neutrophil experiments. Future studies will investigate the slight changes in neutrophil sensitivity when the type c capsule is expressed in the KK01Swap background.

Figure 28: The four distinct capsule types of *K. kingae* promote neutrophil survival in isogenic strain derivatives.

*K. kingae* strain KK01pam (A), or isogenic derivatives of strain KK01pam expressing either capsule type a (Swapcsapam) (B), capsule type b (Swapcsbpam) (C), capsule type c (Swapcsbcpam) (D), capsule type d (Swapcsdpam) (E), or an empty cassette (SwapEmpty pam) (F) were incubated with (white bars) or without (colored bars) neutrophils in the presence of 1% HSA or 1% NHS at an MOI of 0.1. The percent survival was calculated by dividing the cfu counts by the inoculum. Statistical
significance was determined using two-way analysis of variance and the Tukey post hoc test. The error bars represent the SEM of 3 independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Given the correlation between *K. kingae* capsule type and clinical presentation, we are interested in elucidating the virulence phenotype of capsule isogenic derivatives *in vivo*. To examine the virulence potential of the distinct *K. kingae* capsule types, we will use a previously described juvenile rat infection protocol (Starr, Porsch, Seed, & St. Geme III, 2016). Briefly, five-day-old Sprague-Dawley rat pups will be injected via the intraperitoneal (i.p.) route with KK01 and KK01pam isogenic capsule swap strains. Morbidity and mortality will be monitored over a five-day period. We hypothesize that the *K. kingae* isogenic derivatives expressing either capsule type a or capsule type b will be more virulent in an *in vivo* infant rat infection model than isogenic derivatives expressing either capsule type c or capsule type d.

Anti-capsular antibodies promote opsonization and lead to efficient complement and neutrophil activation as previously demonstrated in *H. influenzae* and *K. pneumoniae* (Jacob Amir, Liang, & Granoff, 1990; Anderson R. B., Jr.; Smith, D. H., 1972; Johnston, Anderson, Rosen, & Smith, 1973; Kobayashi et al., 2018). Currently, antisera against purified *K. kingae* capsular material conjugated to an immunogenic carrier protein is being generated in guinea pigs. ELISA analyses demonstrate the specificity of the *K. kingae* anti-capsule antibodies to their respective capsule type (data not shown). To determine whether *K. kingae* anti-capsule antibodies enhance killing of encapsulated *K. kingae*, we will perform serum resistance and neutrophil-killing assays using capsule isogenic derivative strains in the presence of anti-capsule antibodies generated against the type a, b, c, or d.
capsule. We predict that preincubation with anti-capsule antibodies will promote complement and neutrophil activation and enhance killing of encapsulated *K. kingae* strains. Additionally, *in vivo* administration of anti-capsule antibodies will be used in the juvenile rat infection model as a passive immunization strategy against *K. kingae* infection. We hypothesize that passive immunization will lead to attenuation of encapsulated *K. kingae* strains in our *in vivo* model.

In *H. influenzae* and *K. pneumoniae*, capsule overproduction is associated with disease and hypervirulent strains (Dorman, Feltwell, Goulding, Parkhill, & Short, 2018; Noel et al., 1996, 1992; Paczosa & Mecsas, 2016; Shon, Bajwa, & Russo, 2013; Swift et al., 1991). Earlier studies utilized atomic force microscopy to determine the average depth of the *K. kingae* type a capsule in strain KK01 (Kern et al., 2017). Evaluation of KK01 capsule isogenic derivative strains as well as clinical isolates from both invasive and carrier strains using atomic force microscopy may provide valuable insight into differences in capsule production and expression between the four capsule types and across a diverse cohort of *K. kingae* strains.

### 5.3 Binding of complement regulators and contribution to *K. kingae* serum resistance

Complement activation is highly inflammatory and can cause host tissue damage; therefore, the three complement pathways are highly regulated through a variety of effector proteins to prevent aberrant activation (Zipfel & Skerka, 2009). So far, over 20 soluble and surface-bound effector proteins have been identified, including C4b-binding protein (C4BP), factor H, and vitronectin. C4BP and factor H act as cofactors for factor I
recruitment; factor I mediates degradation of C4b and C3b (Blom et al., 2004a; Blom, Villoutreix, & Dahlbäck, 2004b; Józsi & Zipfel, 2008; Rodríguez de Córdoba, Esparza-Gordillo, Goicoechea de Jorge, Lopez-Trascasa, & Sánchez-Corral, 2004). Degradation of C4b and C3b accelerates the decay of C3 convertases, C4b2a and C3bBp, respectively. Vitronectin is present in plasma and considered an important component of the extracellular matrix; vitronectin functions to prevent insertion of the membrane attack complex into bacterial membranes (Preissner & Seiffert, 1998). Pathogenic bacteria can exploit complement regulation through the acquisition of these soluble effectors. *N. meningitidis* and *N. gonorrhoeae* (members of the Neisseriaceae family) bind factor H to prevent activation of the alternative pathway (Lewis et al., 2012; Ram, Sharma, et al., 1998). We hypothesized that *K. kingae*, a member of the Neisseriaceae family, binds factor H, promoting survival of the bacterium even in the absence of surface polysaccharides.

To investigate whether *K. kingae* binds complement regulator factor H, we performed flow cytometry assays to determine relative levels of factor H deposition on the *K. kingae* surface using human serum as a source for factor H. In comparison to *E. coli* strain DH5α, there was a significant increase in factor H deposition on the bacterial surfaces of K. kingae strains KK01, KK01csaA, KK01pam, and KK01csaApam when incubated with either 1% or 5% HI-NHS (Fig. 29). Interestingly, deletion of the exopolysaccharide biosynthesis locus, *pam*, led to a significant increase in factor H deposition, although it remains unknown as to why this occurs (Fig. 29). The data demonstrate that factor H is deposited on the surface of *K. kingae* and that presence of the
exopolysaccharide either hinders antibody recognition of surface-bound factor H or inhibits factor H binding.

Figure 29: Complement regulator, factor H, is present on the *K. kingae* surface after serum incubation.

Binding of factor H to the bacterial surface of *K. kingae* strains KK01, KK01csaA, KK01pam, KK01csaApam and *E. coli* strain DH5α was determined using flow cytometry. Cells were stained with propidium iodine (PI) prior to analysis; 30,000 propidium iodine-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+). Dark red bars, 5% HI-NHS; Red bars, 1% HI-NHS; gray bars, secondary antibody-only controls (these bars are negligible in size due to the low signal).

Using protein blast alignment against *K. kingae*, we uncovered glycoprotein 2 (GP2), which has predicted homology to the *N. meningitidis* factor H binding protein (FHBP). *K. kingae* GP2 is 21.2% identical and 34.2% similar to *N. meningitidis* FHBP and using Phyre2 structural analysis shows 18-27% identity to FHBP (Kelley, Mezulis, Yates,
Wass, & Sternberg, 2015). To investigate whether GP2 is necessary for factor H binding, we generated GP2-deletion strains in the *K. kingae* strains KK01, KK01csaA, KK01pam, and KK01csaApam. Using the KK01 GP2-deficient strains, we performed flow cytometry assays to determine relative levels of factor H deposition on the *K. kingae* bacterial surfaces. There was no change in factor H deposition when GP2 was present or absent (data not shown). This result suggests that either GP2 is not necessary for factor H-binding or that *K. kingae* expresses additional surface structures that promote factor H deposition.

To determine whether GP2 was necessary for *K. kingae* serum resistance, we performed serum bactericidal assays using GP2-deficient strains and KK01csaApam as a serum-sensitive control. No changes in the survival ratio were observed in the GP2-deficient strains when compared to KK01 (data not shown). No further analyses were performed using the GP2-deficient strains.

Unpublished data from the lab determined that the trimeric autotransporter, Knh, was necessary for adherence to vitronectin-coated plates. Vitronectin is present in human serum and is a regulator of the terminal complement pathway (Preissner & Seiffert, 1998). To determine the role of Knh in *K. kingae* serum resistance, we performed serum bactericidal assays using strains KK01, KK01csaApam, and Knh-deficient mutants. No changes in the survival ratio were observed in the Knh-deficient strains when compared to the parental strain (data not shown). To investigate whether Knh is necessary for factor H binding, we performed flow cytometry assays to determine relative levels of factor H deposition on the *K. kingae* surface in KK01 Knh-deficient strains. There was no change in factor H deposition when Knh was present or absent (Fig. 30). This result suggests that
either Knh is not necessary for factor H-binding or that \textit{K. kingae} expresses additional surface structures that promote factor H deposition. Knh and GP2 double mutants have not been generated.

**Figure 30:** Elimination of the trimeric autotransporter, Knh, does not affect factor H deposition.

Binding of factor H to the bacterial surface of \textit{K. kingae} strains KK01, KK01\textit{knh}, KK01\textit{csaApam}, and KK01\textit{csaApamknh} was determined using flow cytometry. Cells were stained with propidium iodine (PI) prior to analysis; 30,000 propidium iodine-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\(^+\)). Red bars, 1\% HI-NHS; gray bars, secondary antibody-only controls (these bars are negligible in size due to the low signal).

Future studies will determine whether vitronectin and C4BP are deposited on the bacterial surface of \textit{K. kingae} using the previously described flow cytometry assays. Additionally, we will perform serum resistance assays using depleted serum to determine the necessity of factor H, C4BP, and vitronectin deposition for \textit{K. kingae} survival in the
presence or absence of surface polysaccharides. While the loss of capsule and exopolysaccharide promotes activation of the classical pathway through antibody deposition, the deposition of factor H and vitronectin may inhibit the alternative and terminal pathways to promote a low level of survival as demonstrated in Ch. 2. We hypothesize that \textit{K. kingae} will be less resistant to human serum when complement regulators have been depleted from the serum.

5.4 Tn-seq analyses for additional \textit{K. kingae} immune evasion factors

5.4.1 Complement evasion

Our previous work demonstrates that the elimination of both the capsule and exopolysaccharide resulted in reduced resistance to complement-mediated lysis (Ch. 2) (Muñoz et al., 2018). Interestingly, elimination of these surface polysaccharides did not completely render the \textit{K. kingae} bacterium sensitive to human serum, suggesting that \textit{K. kingae} employs additional factors to prevent membrane attack complex formation and complement-mediated lysis.

To determine additional factors that mediate serum resistance in the absence of surface polysaccharides, we generated a \textit{K. kingae} transposon (Tn) mutant library in strain KK01\textit{csaApam} (Fig. 31A). The current mutant library contains \(~42,000\) random transposon mutants, an approximate 21x coverage of the \textit{K. kingae} genome. Serum survival assays were performed using 1 x 10^7 bacteria from the pooled mutant library in 20\% NHS or HI-NHS (Fig. 31B). Genomic DNA was recovered from four technical replicates in three separate experiments. In on-going experiments, we are processing purified genomic DNA for high-throughput sequencing, which will be used to identify transposon insertions
that are reduced or absent in the challenge condition (20% NHS). Candidate targets will be subjected to deletion and complementation to confirm the serum-sensitive phenotype.

Figure 31: Schematic representation of Tn-seq screen for *K. kingae* survival in human serum.

Generation of *K. kingae* mutant library by using the Himar transposase to introduce random transposon insertions into the *K. kingae* genome (A). Mutated DNA is then transformed back into *K. kingae*. Mutant library is challenged using 20% NHS or 20% HI-NHS and recovered DNA is processed and mapped using Illumina sequencing (B). Figure adapted from (van Opijnen, Bodi, & Camilli, 2009) and (Langereis, 2014).

5.4.2 Neutrophil evasion

The previously generated transposon mutant library described in Ch. 5 sub-section 5.4.1 may be utilized in neutrophil-killing assays to determine additional factors necessary for *K. kingae* neutrophil evasion in the absence of the polysaccharide capsule and exopolysaccharide. Tn-seq screens have been performed previously in nontypeable *H. influenzae* (NTHi) to elucidate LOS-modifying proteins that protect NTHi in the presence of neutrophils (Langereis & Weiser, 2014). Additionally, assays will be performed in the
absence or presence of human serum to identify candidate genes that prevent neutrophil-mediated killing in the presence of serum opsonins.

5.5 Determine mechanism of capsule inhibition of neutrophil ROS production

Elimination of the *K. kingae* capsule stimulates a rapid and robust release of reactive oxygen species (ROS) by human neutrophils during *K. kingae* infection (Ch. 3). In the absence of the *K. kingae* polysaccharide capsule, increased neutrophil recognition of bacterial LPS may prompt TLR4 activation and subsequent ROS production. TLR4 is activated by LPS and upon activation recruits the NADPH-oxidase complex, Nox4 (Kaisho & Akira, 2006; Park et al., 2004; Qureshi et al., 1999). Nox4 is then able to generate ROS and promote proinflammatory cytokine expression (Ngkelo et al., 2012; Park et al., 2004). To determine whether TLR4 was activated by *K. kingae*, we performed a chemiluminescence bio-assay using a neutralizing rat IgG monoclonal antibody to human TLR4 (anti-TLR4) to inhibit the biological activity of TLR4; an isotype control for rat IgG1 was included as a negative control (Fig. 32, 33). LPS from *Salmonella enterica* serotype enteritidis was included as a positive control for TLR4 activation and ROS production by neutrophils (Fig. 32A). Preliminary data (n=1) suggest a slight reduction in ROS production during neutrophil incubation with strain KK01csaA in the presence of the anti-TLR4 antibody (Fig. 32C, 33B). No changes in ROS production were observed with strains KK01, KK01pam, and KK01csaApam in either 1% HSA or 1% NHS in the presence of the anti-TLR4 antibody (Fig. 32, 33). As previously demonstrated, the preliminary data suggest a marked increase in ROS production when neutrophils are incubated with strain
KK01csaApam (Fig. 32E, 33D). Currently, ongoing experiments are focused on improving *K. kingae* LPS purification and structural analysis. Future studies will use purified *K. kingae* LPS and polysaccharide capsule to determine changes in neutrophil TLR4 activation.

Figure 32: Detection of neutrophil TLR4 activation during *K. kingae* infection.
Salmonella enterica serotype enteritidis LPS (A) and K. kingae rtx mutant strains, KK01 (B), KK01csaA (C), KK01pam (D), and KK01csaApam (E) were incubated with human neutrophils at an MOI of 10 in the presence of 1% HSA. Assays were performed in the presence of 10µg/ml rat anti-TLR4 antibodies (triangles), 10µg/ml rat IgG1 antibody control (squares), or media alone (circles). Chemiluminescence was measured at 5-min intervals for 75 min for kinetic analysis. Gray plot points represent neutrophil-only control (A-E). Data represents an n=1.

Figure 33: Detection of neutrophil TLR4 activation during K. kingae infection in the presence of serum opsonins.

K. kingae rtx mutant strains, KK01 (A), KK01csaA (B), KK01pam (C), and KK01csaApam (D) were incubated with human neutrophils at an MOI of 10 in the presence of 1% NHS. Assays were performed in the presence of 10µg/ml rat anti-TLR4 antibodies (triangles), 10µg/ml rat IgG1 antibody control (squares), or media alone (circles). Chemiluminescence was measured at 5-min intervals for 75 min for kinetic analysis. Gray plot points represent neutrophil-only control (A-E). Data represents an n=1.
5.6 Determine the mechanism by which exopolysaccharide inhibits neutrophil phagocytosis

K. kingae bacterial association to human neutrophils was significantly increased in the absence of the polysaccharide capsule (Ch. 3). Despite promoting neutrophil association, the loss of capsule alone did not significantly increase neutrophil uptake and phagocytosis (Ch. 3). Interestingly, elimination of both the capsule and exopolysaccharide led to an increase in intracellular bacteria, suggesting that the exopolysaccharide inhibited or delayed neutrophil uptake of associated bacteria (Ch. 3). We will perform live cell fluorescence microscopy to investigate interactions between neutrophils and various mutant strains of K. kingae KK01. To distinguish bacterial cells from neutrophils, we will stain each population prior to infection with the appropriate fluorescent cellular dye. We hypothesize that the exopolysaccharide may provide a physical barrier or blockage during bacterial uptake. Alternatively, the exopolysaccharide may play a direct role in phagocytic inhibition through interactions with neutrophil receptors. While the use of live cell fluorescence microscopy may not provide direct answers as to whether the exopolysaccharide is interacting with neutrophil receptors, visualization of neutrophil-bacterial association will provide a better understanding of cellular dynamics during K. kingae infection.

5.7 Effect of surface polysaccharides on cytokine expression and release

Beyond clearing pathogenic bacteria through intracellular and extracellular mechanisms, neutrophils can release of a large subset of cytokines and chemokines upon recruitment (Mayadas, Cullere, & Lowell, 2014; Mócsai, 2013). Various neutrophil
receptors, including pathogen recognition receptors (PRR) as mentioned previously, trigger cytokine and chemokine production (Scapini et al., 2000; Tecchio et al., 2014).

Given the differences in neutrophil activation upon infection with capsule-deficient *K. kingae*, ELISA assays will be performed to determine the secretion of IL-1α, IL-1β, IL-6, TNFα, and IFNγ following incubation of neutrophils with *K. kingae* in the presence or absence of serum opsonins. Additionally, we plan to determine proinflammatory and anti-inflammatory cytokine transcript levels in infected neutrophils using qRT-PCR. Assays will be performed using wild type and *K. kingae* mutants lacking the capsule, exopolysaccharide, or both to establish how these surface polysaccharides influence neutrophil cytokine expression and secretion. In Ch. 3, we demonstrated a lack of neutrophil activation and neutrophil-mediated killing when the capsule was present. We hypothesize that expression of the *K. kingae* polysaccharide capsule inhibits or reduces cytokine release. As speculated in Ch. 5.5, we assume elimination of capsule leads to LPS exposure and recognition by TLR4, thus triggering ROS production and perhaps changing the cytokine profile.

While the loss of exopolysaccharide alone did not render *K. kingae* sensitive to neutrophil-mediated killing, elimination of both surface polysaccharides promoted phagocytosis of *K. kingae*, suggesting that the exopolysaccharide may inhibit or prevent phagocytosis (Ch. 3). A reported galactan homopolysaccharide in *Mycoplasma mycoides* subsp. *mycoides* similar to the *K. kingae* exopolysaccharide binds TLR2 and promotes the production of anti-inflammatory cytokines (Totté et al., 2015). We hypothesize that the *K.
*kingae* exopolysaccharide may interact with neutrophil receptor(s) and prompt changes in cytokine expression and secretion.

5.8 Determine regulatory elements for polysaccharide capsule production

A mariner transposon mutagenesis strategy was implemented to determine essential genes or loci for encapsulation of *K. kingae* strain KK01 (Starr, Porsch, Seed, & St. Geme III, 2016). Around 10,000 transposon mutants were screened for a non-mucoid phenotype, which is expected of non-encapsulated organisms (Starr, Porsch, Seed, & St. Geme III, 2016). Through screening and bioinformatic approaches, capsule export, assembly, and biosynthesis genes were identified (*ctrABCD* operon, *lipA*, *lipB*, and *csaA*) (Starr, Porsch, Seed, & St. Geme III, 2016). Despite successfully identifying genes necessary for encapsulation, capsule regulatory components were absent from the transposon screen. Unfortunately, the non-mucoid phenotype was not suitable for identification of regulatory networks that alter the production and/or depth of the *K. kingae* capsule.

Recently, Dorman et al. employed a density-TraDISort strategy in *K. pneumoniae* to determine genes that influence capsule production (Dorman et al., 2018). Density-TraDISort combines density gradient centrifugation using varying percentages of Percoll with transposon insertion sequencing (Dorman et al., 2018). Our initial studies will determine migration patterns of wild-type KK01 and various KK01 capsule-mutant strains. We predict that optimization may be required to more accurately separate differentially encapsulated *K. kingae* bacteria. We will perform thiobarbituric acid reactivity assays as previously described to determine the amount of capsule present in each fraction and validate expected separation patterns (Starr, Porsch, Seed, & St. Geme III, 2016; Straus,
Lonon, Woods, & Garner, 1990; Warren, 1963). If *K. kingae* is amenable to density centrifugation, genomic DNA from strain KK01*pam* will be used for the generation of a transposon mutant library. Unlike *K. pneumoniae*, *K. kingae* strains express an exopolysaccharide that may alter the migration pattern of non-encapsulated *K. kingae*. The density-TraDISort strategy may be useful for identification of exopolysaccharide regulatory elements. However, the exopolysaccharide is secreted and not membrane-anchored like the polysaccharide capsule. Therefore, separation of bacteria based on levels of exopolysaccharide release through centrifugation may not be plausible.

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The methods for Ch. 5.4 have been published and are referenced below:

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**BIOGRAPHY**

Vanessa Lynne Muñoz was born June 28, 1991 in Elizabeth, NJ. She attended Brown University in Providence, RI and graduated with a B.A. in Business, Entrepreneurship, and Organization in 2013. She joined the University of Rochester Post-Baccalaureate Research Program in the fall of 2013 and spent a year studying *Streptococcus mutans* under Dr. Robert Quivey. In 2014, she entered graduate school in the Cell and Molecular Biology Program at the Perelman School of Medicine at the University of Pennsylvania. She joined the laboratory of Dr. Joseph St. Geme, III to study molecular determinants of innate immune evasion in the pediatric pathogen *Kingella kingae*. In 2016, she was awarded an NSF Graduate Research Fellowship (2016-2019) and was a Ford Foundation Predoctoral Fellowship honorable mention. In 2017, she won the Adelaide M. Delluva student travel award from the Philadelphia Chapter of the Association for Women in Science. Additionally, she was selected to speak at the Microbiology, Virology, and Parasitology Retreat and the Philadelphia Infection and Immunity Forum. In 2018, she was nominated and attended the St. Jude Future Fellow Research Conference. In 2019, she was selected to speak at the Mid-Atlantic Microbial Pathogenesis Meeting. In her free time, she enjoys playing recreational sports and travelling abroad.

**Publications and works-in-progress:**

**Muñoz V. L.,** Porsch E. A., St. Geme J. W., III. *Kingella kingae* surface polysaccharides promote resistance to neutrophil phagocytosis and killing. (Submitted)
