Pathogenesis of *Kingella kingae* Disease

Eric A. Porsch and Katherine A. Rempe

Colonization

The pathogenesis of *Kingella kingae* disease is believed to begin with colonization of the oropharynx. In order to colonize this site, the organism must adhere to respiratory epithelial cells. Early studies demonstrated that *K. kingae* expresses hair-like surface fibers and suggested that these fibers may be type IV pili, in particular given that they react with antibodies raised against *Kingella denitrificans* type IV pili. Kehl-fie et al. [1] confirmed that the *K. kingae* fibers are type IV pili and established that *K. kingae* type IV pili mediate adherence to respiratory epithelial cells [2]. The density of piliation correlates with three different colony morphologies when *K. kingae* is grown on solid medium: a spreading/corroding colony type associated with expression of abundant phase variable pili, a non-spreading/noncorroding colony type associated with sparse pili, and a domed colony type associated with no pili [3, 4].

Type IV pili are found in a wide variety of gram-negative species, with examples including enteropathogenic *Escherichia coli*, *Salmonella enterica serovar typhi*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Vibrio cholerae*. These polymeric surface fibers can be up to several μm in length and are composed primarily of a major pilus subunit. Type IV pili major pilus subunits share a number of characteristics, including a short hydrophobic signal peptide that is cleaved during pilus biogenesis, a methylated N-terminal residue, and two cysteine residues near the C-terminus [5]. Structurally, type IV pili major pilus subunits are predicted to have an N-terminal alpha-helical domain and a C-terminal globular domain [6–8].

E.A. Porsch (✉) · K.A. Rempe
Department of Pediatrics, Division of Infectious Diseases, The Children's Hospital of Philadelphia, 3615 Civic Center Blvd, ARC Lab 1205D, Philadelphia, PA 19104, USA
e-mail: PorschE@email.chop.edu

Type IV pili are unique in their ability to be retracted by the organism [9]. Retraction is responsible for a form of surface motility called twitching motility, allowing the organism to pull itself across a surface as a consequence of sequential rounds of extension, anchoring, and then retraction of pilus fibers [10]. Type IV pilus retraction is also involved in DNA uptake, an essential step in *K. kingae* natural competence.

Functional expression of type IV can require over 20 gene products, depending on the particular bacterial species. The main steps in type IV pilus biogenesis are as follows: The major pilus subunit (PilA1 in *K. kingae*) is translated as a pre-protein, which undergoes cleavage of the signal sequence by a dedicated inner membrane peptidase called PilD [11]. After processing, the mature subunit can be incorporated into the growing type IV pilus fiber by the pilus assembly complex, which contains the cytoplasmic protein PilM, the periplasmic proteins PilN, PilO, and PilP, the inner membrane spanning protein PilG, and the oligomeric outer membrane secretin PilQ [12–15]. Pilus extension is powered by ATP hydrolysis by the PilF (PilB in the *Pseudomonas* type IV pilus nomenclature) cytoplasmic assembly ATPase [16, 17]. Retraction of the pilus through the PilQ secretion is mediated by the PilT retraction ATPase [18, 19].

Gene deletion studies have revealed that *pilA1* and *pilF* are essential for type IV pilus expression in *K. kingae* [2]. The *pilT* gene is essential for pilus retraction, and deletion of *pilT* results in accumulation of dense nonretractile fibers [20]. Representative transmission electron micrographs of negatively stained wild type, *pilF* mutant, and *pilT* mutant derivatives of *K. kingae* strain 269–492 are shown in Fig. 1. Abundant pili are observed emanating from the surface of the wild-type strain (Fig. 1a) and the retraction-deficient *pilT* mutant (Fig. 1c) but are absent in the *pilF* assembly mutant (Fig. 1b). *K. kingae* contains minor pilins called PilA2 and FimB, which are likely incorporated into the pilus fiber but have unclear functions, as elimination of these proteins via deletion of the relevant gene has no effect on type IV pilus phenotypes [2].
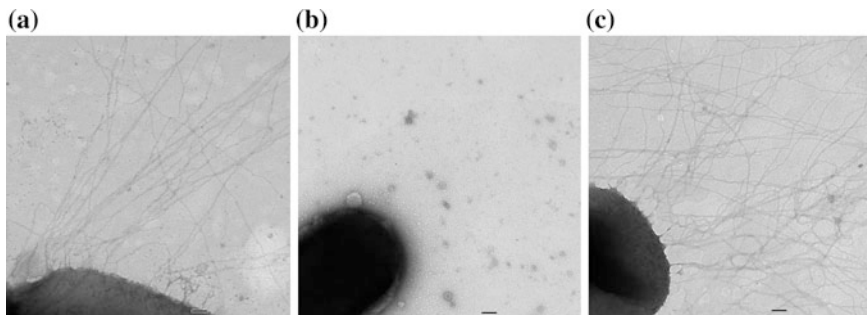

Fig. 1 Transmission electron micrographs of *K. kingae* strain 269–492 type IV pili surface fibers. **a** Abundant pili are seen emanating from the surface of wild-type strain 269–492. **b** Pili are absent from the surface of an isogenic *pilF* assembly ATPase mutant. **c** Mutation of the *pilT* retraction ATPase results in a hyper-piliated phenotype. Bar, 100 nm

The adhesive components of the *K. kingae* type IV pilus are likely the PilC1 and PilC2 pilus-associated proteins, which also play a role in pilus biogenesis [2, 21]. Both of these proteins have similarity to the well-characterized PilC1 and PilC2 pilus adhesins of the pathogenic *Neisseria* sp. and the PilY1 adhesin in *P. aeruginosa* type IV pili. However, unlike the PilC proteins in the pathogenic *Neisseria* sp., the *K. kingae* PilC proteins share limited amino acid sequence homology, with overall identity of 7 % and similarity of 16 % [2]. Elimination of either PilC1 or PilC2 results in a decrease in *K. kingae* adherence to cultured human epithelial cells, and elimination of both PilC1 and PilC2 results in a non-piliated phenotype [2, 21]. While there is not yet any direct evidence showing that the *K. kingae* PilC1 and/or PilC2 proteins mediate direct interaction with host cells, recent studies demonstrating that a *K. kingae* mutant that is able to express pilus fibers lacking PilC1 and PilC2 is non-adherent suggest that these proteins are the adhesive components of the fiber (Porsch and St. Geme, unpublished data). The host cell receptor for *K. kingae* type IV pili is currently unknown.

Similar to *P. aeruginosa* PilY1 and the pathogenic *Neisseria* sp. PilC proteins, both PilC1 and PilC2 in *K. kingae* contain calcium-biding sites [21–23]. Structural analysis of the PilY1 C-terminal domain showed a β-propeller fold containing a bound calcium ion, and mutational studies of the 9 amino acid calcium-binding pocket revealed that calcium binding is essential for the ability of PilY1 to potentiate PilT-mediated pilus retraction and twitching motility [23]. In *K. kingae*, both PilC1 and PilC2 contain calcium-binding pockets, with PilC1 containing a 9 amino acid PilY1-like calcium-binding site and PilC2 containing a 12 amino acid calmodulin-like calcium-binding site [21]. Calcium binding is necessary for PilC1 to facilitate twitching motility and adherence but has little to no effect on PilC2 function [21]. Although both PilC1 and PilC2 facilitate twitching motility and adherence, they appear to do so by different mechanisms.

The current model for *K. kingae* adherence to epithelial cells involves initial adherence by type IV pili, pilus retraction, and subsequent tight adherence mediated by the non-pilus trimeric autotransporter adhesin Knh (*Kingella* Nhh A homolog). Trimeric autotransporters are members of the Type V Secretion family and contain an N-terminal signal sequence, an internal passenger domain, and a C-terminal β-barrel outer membrane anchoring domain [24]. The N-terminal signal sequence directs the protein through the Sec secretion system into the periplasm. The protein then integrates into the outer membrane as a trimer, with the C-terminal outer membrane domains trimerizing to form a pore. The internal passenger domain is translocated through this pore and is displayed on the bacterial surface. Trimerization of the passenger domain is facilitated by a coiled-coil domain adjacent to the outer membrane pore [25].

Knh is a 1783 amino acid protein with a predicted molecular mass of approximately 179 kDa. The N-terminal signal sequence consists of amino acids 1–54, and the C-terminal membrane domain consists of amino acids 1695–1783 [20]. Amino acids 55–1694 constitute the internal passenger domain, which is constructed in a modular manner and contains multiple YadA-like head domains, Trp domains, KG domains, Neck domains, and a single iSneck2 domain, as
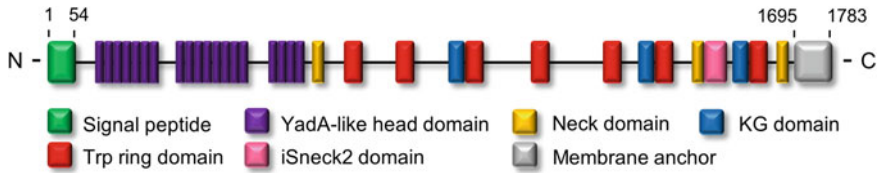
Fig. 2 Domain annotation of the trimeric autotransporter adhesin Knh expressed by *K. kingae* strain 269–492. The structural domain annotation was carried out using the Domain Annotation of Trimeric Autotransporter Adhesin (daTAA) program (toolkit.tuebingen.mpg.de/dataa) [26]

diagrammed in Fig. 2 using the Domain Annotation of Trimeric Autotransporter Adhesin program (daTAA) [26]. Mutation of *knh* results in an approximately 50 % reduction in adherence to epithelial cells, and full Knh-mediated adhesive activity is dependent on Knh glycosylation by the cytoplasmic N-linking glycosyltransferase called HMW1C$_{Kk}$ (the name HMW1C$_{Kk}$ reflects the homology to the well-characterized HMW1C glycosyltransferase in nontypable *Haemophilus influenzae*) [27]. HMW1C$_{Kk}$ modifies a least 32 asparagines throughout the Knh passenger domain [27].

Invasion

The pathogenic model of invasive *K. kingae* disease postulates that the source of invading bacteria is the colonizing population in the upper respiratory tract. According to this model, it should be possible to isolate genotypically identical bacteria from the oropharynx and the site of invasive disease (i.e., blood, joint fluid, and bone) in patients with invasive *K. kingae* disease. In support of this model, multiple studies have shown invading organisms to be genetically identical to colonizing isolates in individual patients [28, 29]. For the bacterium to transition from colonizer to invasive pathogen, it must breach the respiratory epithelial barrier in the oropharynx and enter the bloodstream.

Multiple epidemiological studies of patients with invasive *K. kingae* disease have revealed an association with antecedent or coincident viral infection. Yagupsky et al. [30] found that approximately 50 % of children with invasive *K. kingae* disease displayed symptoms of upper respiratory tract infection, 15 to 20 % had stomatitis, and 15 to 20 % had diarrhea. In agreement with these findings, a study in France found that 19/21 patients with proven *K. kingae* osteoarticular infection and only 3/8 patients with non-*K. kingae* osteoarticular infection had at least one respiratory virus present in the oropharynx, including human rhinovirus (12/21), coronavirus OC43 (4/21), parainfluenza virus 1, 2, 3, or 4 (3/21), enterovirus (2/21), or adenovirus (2/21) [31]. Of note, human rhinovirus infection is known to enhance bacterial adherence to respiratory cells and is associated with invasive bacterial infection [32, 33]. Recently, Houmami et al. [34] described a

series of five epidemic and four sporadic *K. kingae* osteoarticular infections between April and October of 2013 in a French hospital. Of the seven patients in this report who were younger than 24 months of age, five had antecedent hand, foot, and mouth disease and two had antecedent stomatitis. Both patients older than 2 years of age did not have evidence of antecedent or concurrent upper respiratory tract viral infection. Further analysis of an 11-month-old with septic arthritis and antecedent hand, foot, and mouth disease revealed the presence of *K. kingae* DNA in stored joint fluid and coxsackievirus-A6 in a stored stool specimen [34]. These studies strongly support the hypothesis that upper respiratory tract viral infection contributes to the development of *K. kingae* invasive disease, possibly related to virus-induced damage to the respiratory epithelium.

In addition to virus-induced damage to the respiratory epithelial barrier, *K. kingae* may breach the epithelium by means of a potent RTX cytotoxin. The *K. kingae* RTX toxin is a member of the repeats-in-toxin family and has been shown to be cytotoxic to a wide range of cell types in vitro, including respiratory epithelial cells, synovial cells, macrophage-like cells, and red blood cells [35]. The *K. kingae* RTX toxin was discovered following the observation that introduction of *K. kingae* onto a cell monolayer resulted in cell rounding and lysis. A transposon library screen identified a locus with high homology to the Mbx locus in *Moraxella bovis* that is absent in the less pathogenic *Kingella* species *K. oralis* and *K. denitrificans* [35]. This locus also has high homology to RTX toxins in *H. influenzae* and *N. meningitidis*, suggesting that *K. kingae* acquired the *rtx* genes through horizontal gene transfer [35]. Testing on a wide range of cultured human cells showed that synovial cells and macrophages are more sensitive to RTX toxin effects than respiratory epithelial cells, although respiratory epithelial cells are still lysed [35]. Additional investigation revealed that the RTX toxin is abundant in *K. kingae* outer membrane vesicles, which induce cytotoxicity when directly delivered to host cells [36].

The *K. kingae* RTX toxin contains glycine-rich nonapeptide calcium-binding repeats and is secreted by the type I secretion system [37]. It is a pore forming toxin and forms 0.94 nm radius pores in the host cell membrane that display voltage-dependent gating, with a channel conductance similar to general diffusion [37]. Comparison of a wild type and an isogenic RTX mutant strain in an infant rat model of invasive *K. kingae* disease revealed decreased virulence by the RTX mutant [38]. The *rtx* genes are present in all *K. kingae* strains examined to date, and the *rtxA* and *rtxB* genes have proven to be reliable targets for PCR-based detection of *K. kingae* in clinical samples [39–41].

Immune Evasion

Once *K. kingae* breaches the respiratory epithelial layer and enters the bloodstream, the organism must be able to evade host immune mechanisms in order to persist and disseminate to sites of invasive disease. While there are only a few studies

addressing potential *K. kingae* immune evasion strategies, current evidence suggests that expression of a polysaccharide capsule and antigenic variation of selected surface-displayed proteins play a role in survival of the bacterium during invasive disease.

Examination of *K. kingae* strains after 48 h of growth on chocolate agar reveals a striking mucoid colony morphology, consistent with production of a polysaccharide capsule [20]. A number of pediatric pathogens (e.g. *N. meningitidis*, *H. influenzae*, and *Streptococcus pneumoniae*) express a polysaccharide capsule, a layer of surface-associated polysaccharide chains that surround and 'encapsulate' the bacterium. Polysaccharide capsules are considered classical virulence factors and have been shown to play many roles in host–pathogen interactions, including masking of antigenic surface epitopes, prevention of complement factor deposition, prevention of phagocytosis, and host mimicry [42]. Disruption of a predicted *ctrABCD* capsule export operon in *K. kingae* eliminates the mucoid colony morphology and ablates the polysaccharide capsule, as highlighted in Fig. 3 [20]. Structural analysis of the polysaccharide capsule purified from *K. kingae* strain 269–492 revealed a repeating unit of N-acetylgalactosamine (GalNAc) and 3-deoxy-D-manno-octulosonic acid (Kdo) with the structure of $[\rightarrow 3)\text{-}\beta\text{-Gal}p\text{NAc-}(1 \rightarrow 5)\text{-}\beta\text{-Kdo}p\text{-}(2 \rightarrow]_n$ [43]. A mutant lacking surface capsule was significantly less virulent in the juvenile rat infection model, demonstrating the likely importance of capsule as a *K. kingae* virulence factor.

Further study by Starr et al. [44] established that the genetic requirements for encapsulation in *K. kingae* include the predicted *ctrABCD* export operon, *lipA*, *lipB*,
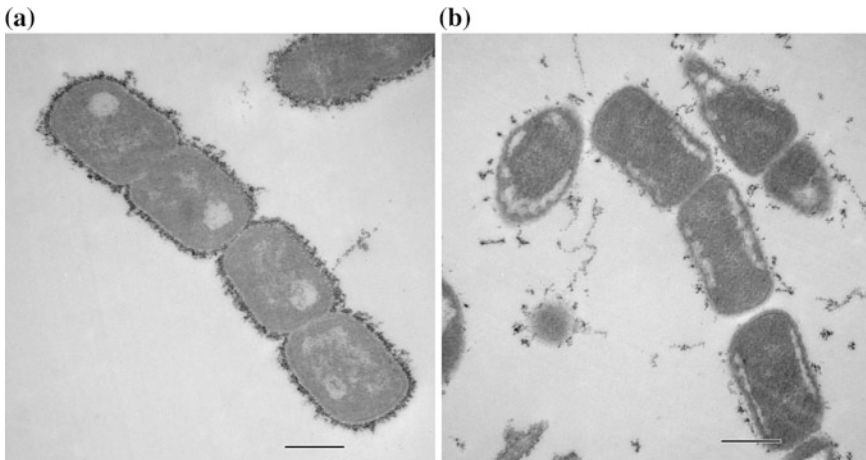
Fig. 3 Transmission electron micrographs of thin-sectioned cationic ferritin-stained wild-type *K. kingae* strain 269–492 (**a**) and an isogenic *ctrA* capsule export mutant (**b**). A layer of electron density due the cationic ferritin interactions with the surface capsular polysaccharide is visible on the surface of the wild-type strain (**a**) but is absent from the nonencapsulated ctrA mutant (**b**). Bar, 500 nm

and *csaA* (<u>c</u>apsule <u>s</u>ynthesis region <u>a</u> gene <u>A</u>). Based on homology to the *N. meningitidis lipA* and *lipB* gene products and the *Escherichia coli kpsC* and *kpsS* gene products, the *K. kingae lipA* and *lipB* gene products likely function as retaining βKdo transferases that create the poly-βKdo linker that connects the polysaccharide to the phosphatidylglycerol membrane anchor [44–46]. Mutational studies revealed that CsaA is a bifunctional glycosyltransferase responsible for synthesizing the GalNAc-Kdo capsule polymer [44].

Examination of a large collection of Israeli healthy carrier and invasive disease isolates revealed a total of four *K. kingae* capsule types, including the GalNAc-Kdo-containing capsule (designated type a), an N-acetylglucosamine-Kdo-containing capsule with a [→6)-α-GlcpNAc (1→5)-β-Kdop-(2→] repeat unit (designated type b), a ribose-Kdo-containing capsule with a [→3)-β-Ribf-(1→2)-β-Ribf-(1→2)-β-Ribf-(1→4)-β-Kdop-(2→] repeat unit (designated type c), and a GlcNAc-galactose-containing capsule with a [→3)(β-Gal-(1→4)]-β-GlcNAc-(1→3)-α-GlcNAc-1-P-(O→] repeat unit (designated type d) [47] (Starr, Porsch, and St. Geme, submitted). In the Israeli collection of isolates, approximately 96 % of invasive disease isolates expressed either the type a or type b capsule. In contrast, only 70 % of carrier isolates expressed the type a or type b capsule, suggesting that the type a and type b capsule structures may provide a selective advantage for the organism in the bloodstream and contribute to the development of *K. kingae* invasive disease (Starr, Porsch, and St. Geme, submitted). Given that these initial studies focused on Israeli clinical isolates, further research is necessary to determine the global distribution of *K. kingae* capsule types.

Porsch et al. [20] demonstrated that the polysaccharide capsule is able to mask the adhesive activity of Knh. When functional pili are present, retraction of these appendages following initial adherence to host cells is thought to displace the polysaccharide capsule, allowing Knh to interact with the host cell and mediate high affinity adherence. A mutant lacking type IV pili but still expressing Knh is non-adherent, presumably because Knh is masked by the polysaccharide capsule. Adherence by this mutant can be restored by eliminating capsule expression, uncovering Knh, and allowing Knh access to host cell receptors [20]. While not demonstrated experimentally, it is likely that the polysaccharide capsule is also able to mask other surface antigens, many of which are smaller than Knh.

In addition to the outer membrane-associated polysaccharide capsule, *K. kingae* has also been found to secrete a galactofuranose exopolysaccharide homopolymer called PAM galactan. Bendaoud et al. [47] reported a PAM galactan structure of [→3)-β-Galf-(1→6)-β-Galf-(1→]$_n$ in *K. kingae* strain PYKK181, and Starr et al. reported a PAM galactan structure of [→5)-β-Galf-(1→]$_n$ in strain 269–492 [43]. The PAM galactan in strain PYKK181 was reported to have broad spectrum biofilm inhibitory activity, which may play a role in dispersal of the organism from a biofilm community or may prevent biofilm formation by competing bacteria in the upper respiratory tract [47]. The potential role of the PAM galactan as a virulence factor has not been investigated, but exopolysaccharides from other gram-negative bacteria have been shown to have protective roles during pathogenesis [48, 49].

K. kingae can cause uncomplicated bacteremia or can disseminate from the bloodstream to joints, bones, or the endocardium [50, 51]. Analysis of a collection of *K. kingae* systemic isolates showed that strains recovered from the blood of patients with uncomplicated bacteremia were generally piliated but typically expressed relatively few pili [52]. In contrast, strains recovered from joint fluid samples, bone aspirates, or the blood of patients with endocarditis were generally non-piliated [52]. It is possible that low-density piliation facilitates a tropism for joints, bones, and the endocardium and potentiates an inflammatory response, which in turn selects against piliated organisms. Consistent with this possibility, pili promote efficient adherence to cultured synovial cells. Similar to type IV pili expressed by other pathogens such as *P. aeruginosa*, *K. kingae* pili are regulated by a transcription factor called σ^{54} and by the PilS/PilR two-component sensor/regulator system [53]. Mutations in the PilS sensor result in a reduced density of pili, similar to the relative reduction in density of pili observed in isolates recovered from the bloodstream compared to isolates from the posterior pharynx. Mutations in the PilR response regulator completely eliminate piliation, similar to the absence of pili observed in isolates from joints and bones [53]. It is unclear which environmental factors influence the activity of σ^{54}, PilS, and PilR and thereby control the density of piliation in *K. kingae*. The spreading/corroding colony morphology associated with dense piliation, the non-spreading/non-corroding colony morphology associated with sparse piliation, and the domed colony morphology associated with no pili may reflect the relative activity of PilS and PilR [52].

Immunity

To investigate the role of the host immune system in development of *K. kingae* disease in healthy individuals, Slonim et al. [54] examined serum IgA and IgG levels against outer membrane proteins in 19 children with invasive disease. As expected, there were significant increases in serum IgA and IgG levels in children convalescing from invasive disease. Further study revealed that the age incidence of disease is inversely correlated with serum IgA and IgG levels in healthy individuals. Infants younger than 6 months of age have undetectable levels of IgA but high levels of serum IgG, suggesting that protection from invasive disease in this age group is due to maternally derived IgG [54]. Children 6 to 18 months of age have the highest incidence of disease and the lowest serum IgG levels [55]. Serum IgG and IgA levels in children 2 years of age and older progressively increase while the incidence of disease progressively decreases, suggesting that *K. kingae* carriage or exposure in the first 2 years of life may be an immunizing event. However, *K. kingae* outer membrane protein epitopes have been shown to be polymorphic among diverse strains, raising the possibility of strain specific immune responses that may not prevent recolonization by an antigenically distinct strain [56]. In agreement with these studies, many of the *K. kingae* surface factors examined to date show strain-to-strain variation, suggesting that immune pressure potentially

drives antigenic diversity in this species. The PilA1 subunit of type IV pili is expressed by all piliated strains, yet only 52 % of PilA1 amino acid residues are identical across a collection of clinical isolates [52]. Recent evidence also suggests that there is strain-to-strain sequence diversity in Knh and the PilC proteins (Porsch and St. Geme, unpublished data).

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