Modulation of *Kingella kingae* Adherence to Human Epithelial Cells by Type IV Pili, Capsule, and a Novel Trimeric Autotransporter

Eric A. Porsch,[a,b] Thomas E. Kehl-Fie,[a*] and Joseph W. St. Geme III[a,b]

Department of Pediatrics[a] and Department of Molecular Genetics and Microbiology,[b] Duke University Medical Center, Durham, North Carolina, USA

* Present address: Thomas E. Kehl-Fie, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

ABSTRACT *Kingella kingae* is an emerging bacterial pathogen that is being recognized increasingly as an important etiology of septic arthritis, osteomyelitis, and bacteremia, especially in young children. Colonization of the posterior pharynx is a key step in the pathogenesis of *K. kingae* disease. Previous work established that type IV pili are necessary for *K. kingae* adherence to the respiratory epithelium. In this study, we set out to identify additional factors that influence *K. kingae* interactions with human epithelial cells. We found that genetic disruption of the gene encoding a predicted trimeric autotransporter protein called Knh (*Kingella* Nhha homolog) resulted in reduced adherence to human epithelial cells. In addition, we established that *K. kingae* elaborates a surface-associated polysaccharide capsule that requires a predicted ABC-type transporter export operon called *ctrA-BCD* for surface presentation. Furthermore, we discovered that the presence of a surface capsule interferes with Knh-mediated adherence to human epithelial cells by nonpiliated organisms and that maximal adherence in the presence of a capsule requires the predicted type IV pilus retraction machinery, PilT/PilU. On the basis of the data presented here, we propose a novel adherence mechanism that allows *K. kingae* to adhere efficiently to human epithelial cells while remaining encapsulated and more resistant to immune clearance.

IMPORTANCE *Kingella kingae* is a Gram-negative bacterium that is being recognized increasingly as a cause of joint and bone infections in young children. The pathogenesis of disease due to *K. kingae* begins with bacterial colonization of the upper respiratory tract, and previous work established that surface hair-like fibers called type IV pili are necessary for *K. kingae* adherence to respiratory epithelial cells. In this study, we set out to identify additional factors that influence *K. kingae* interactions with respiratory epithelial cells. We discovered a novel surface protein called Knh that mediates *K. kingae* adherence and found that a surface-associated carbohydrate capsule interferes with the Knh-mediated adherence of organisms lacking pili. Further analysis revealed that pilus retraction is necessary for maximal Knh-mediated adherence in the presence of the capsule. Our results may lead to new strategies to prevent disease due to *K. kingae* and potentially other pathogenic bacteria.

Address correspondence to Joseph W. St. Geme III, j.stgeme@duke.edu.

*K*ingella kingae is a Gram-negative bacterium in the family *Neisseriaceae* and was originally considered a rare cause of human disease. However, improvements in culture-based and molecular-analysis-based diagnostics over the past 2 decades have led to increased recognition of this organism as an emerging pathogen (1–8). *K. kingae* is the causative agent of a number of pediatric diseases, including septic arthritis, osteomyelitis, bacteremia, and endocarditis (6, 8). A recent report indicates that *K. kingae* is the leading cause of osteoarticular infections in children 6 to 36 months of age (1).

K. kingae initiates infection by colonizing the posterior pharynx and is a common commensal organism in young children (9, 10). The organism then enters the bloodstream and disseminates to distant sites, including joints, bones, and the endocardium (8, 11, 12). A key step in colonization of the respiratory tract is adherence to the respiratory epithelium. Previous studies have demonstrated that *K. kingae* type IV pili are essential for adherence to

human epithelial cells (13). In particular, genetic disruption of the gene encoding the major pilin subunit called PilA1 results in loss of piliation and complete loss of adherence (13, 14).

Expression of type IV pili is phase variable, allowing for evasion of the immune system. Our prototypic parent *K. kingae* strain, 269-492, grows on solid agar as two phenotypically stable colony types, referred to as nonspreading/noncorroding colonies and spreading/corroding colonies. The nonspreading/noncorroding colony variant (designated KK01) expresses low levels of type IV pili, while the spreading/corroding colony variant (designated KK03) expresses high levels of type IV pili. In a collection of clinical isolates from diverse anatomic sites, we have also identified a domed colony type associated with no pili. Further analysis of this collection revealed that the majority of respiratory and nonendocarditis blood isolates were piliated, while the majority of joint fluid, bone, and endocarditis blood isolates were nonpiliated, suggesting a role for pilus phase variation *in vivo* (15).
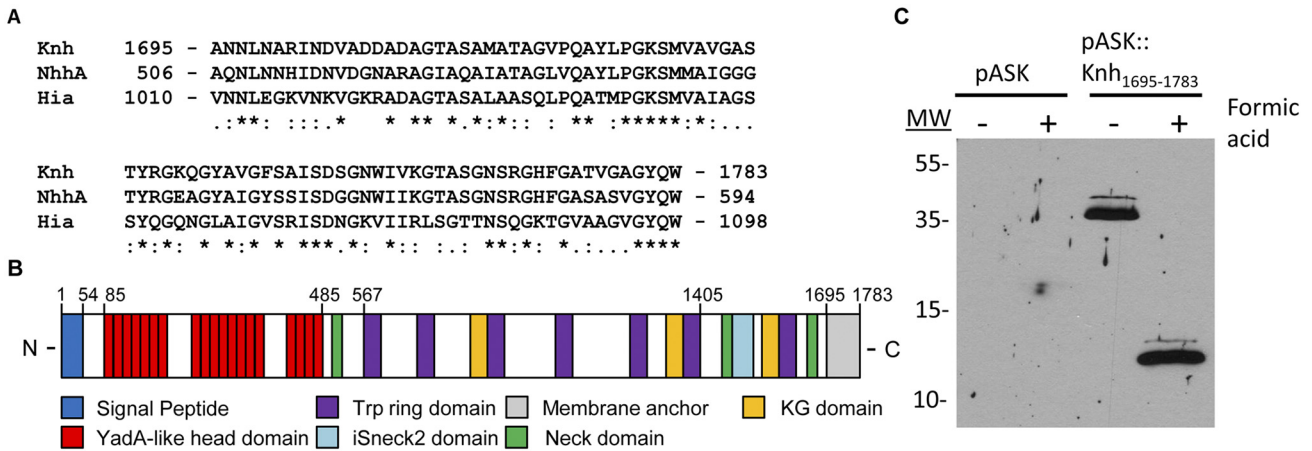
FIG 1 *K. kingae* Knh is a trimeric autotransporter. (A) Knh C-terminal membrane anchor amino acid sequence alignment with *N. meningitidis* NhhA and *H. influenzae* Hia. Asterisks indicate identical residues, colons indicate conserved substitutions, and periods indicate semi-conserved substitutions. (B) Annotation of Knh, highlighting the presence of numerous protein domains, including a signal peptide, a membrane anchor, YadA-like head domains, Trp ring domains, and an ISneck2 domain. (C) Strep-tag Western blot analysis of outer membrane preparations and formic acid-denatured outer membrane preparations following the induction of vector control pASK-IBA12 or pASK::Knh$_{1695-1783}$, which expresses the Knh beta-barrel domain fused to a Strep-tag. Molecular sizes (MW) are expressed in kilodaltons.

While it is clear that type IV pili are important for adherence to human epithelial cells, the fact that a significant percentage of pharyngeal isolates are nonpiliated suggests that *K. kingae* expresses additional surface factors that modulate interactions with host cells. Accordingly, to gain a more thorough understanding of the bacterial factors that potentially influence colonization of the pharynx, we set out to identify additional determinants of adherence to human epithelial cells. This search revealed a novel trimeric autotransporter called Knh (*Kingella* NhhA homolog), which is necessary for full-level *K. kingae* adherence to human epithelial cells. Additional studies demonstrated that *K. kingae* elaborates a surface-associated polysaccharide capsule that interferes with Knh-mediated adherence. Analysis of a *K. kingae* mutant incapable of pilus retraction suggested that capsule-mediated interference is overcome by the retraction of type IV pili, presumably displacing polysaccharide and facilitating access of Knh to the host cell surface. This adherence mechanism allows *K. kingae* to adhere efficiently to host cells while remaining encapsulated and resistant to host immune clearance strategies.

RESULTS

***K. kingae* encodes a novel trimeric autotransporter.** *K. kingae* type IV pili are essential for adherence to human epithelial cells (13). To identify other factors that might contribute to *K. kingae* adherence, we examined the draft genome sequence of strain 269-492 for putative adhesive factors by searching for homologs of genes encoding adhesins in other *Neisseriaceae* family members, including the *Neisseria meningitidis* Opa, Opc, NadA, and NhhA proteins. This analysis revealed a 5,352-bp open reading frame (ORF) encoding a protein that has C-terminal sequence homology to the *N. meningitidis* NhhA trimeric autotransporter (accession no. AAK09243.1) and was named Knh (16, 17). Trimeric autotransporters are a family of adhesive outer membrane proteins classified largely on the basis of the amino acid sequence in the conserved C-terminal beta-barrel membrane anchor domain. Sequence alignment revealed strong membrane anchor domain homology between Knh and NhhA, as well as the *Haemophilus*

influenzae Hia protein (accession no. AAC43721.2) (Fig. 1A). Additional analysis with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) revealed a predicted N-terminal signal peptide corresponding to amino acids 1 to 54 and a predicted signal peptidase cleavage site between amino acids 54 and 55, consistent with the possibility that Knh is secreted and surface localized. Domain annotation of Knh was accomplished with the Domain Annotation of Trimeric Autotransporters program (http://toolkit.tuebingen.mpg.de/dataa) and demonstrated an ISneck2 domain with architecture similar to that of the ISneck1 domain that has been associated with adhesive activity in the *H. influenzae* Hia and Hsf proteins (18), a series of 19 YadA-like head domains predicted to form a beta-roll on the basis of the *Yersinia enterocolitica* YadA head domain crystal structure (19, 20), and a series of eight Trp ring domains spaced between the YadA-like head domain region and the membrane anchor (Fig. 1B).

To confirm that Knh is a trimeric autotransporter, the ability of the C-terminal beta-barrel domain to trimerize in the outer membrane was examined. A 264-nucleotide fragment of *knh* encoding amino acids 1695 to 1783, corresponding to the predicted beta-barrel membrane anchor, was cloned in frame into pASK-IBA12 (IBA BioTAGnology), which contains an N-terminal *Escherichia coli* OmpA signal sequence fused to a Strep-tag. The resulting construct, designated pASK::Knh$_{1695-1783}$, was transformed into *E. coli* BL21 omp8 for induction. After secretion through the inner membrane and cleavage of the OmpA signal sequence, the recombinant Strep-tagged Knh$_{1695-1783}$ protein has a predicted monomeric molecular mass of 12 kDa. As is shown in Fig. 1C, induction of pASK::Knh$_{1695-1783}$ resulted in the outer membrane localization of a 36-kDa band detected with an anti-Strep monoclonal antibody. As has previously been reported with trimeric autotransporter proteins (21), the trimer was extremely stable under standard boiling and SDS-PAGE denaturing conditions and required an additional formic acid denaturation step to dissociate into monomers (Fig. 1C). These findings establish that the Knh beta-barrel is sufficient for trimerization in the outer membrane
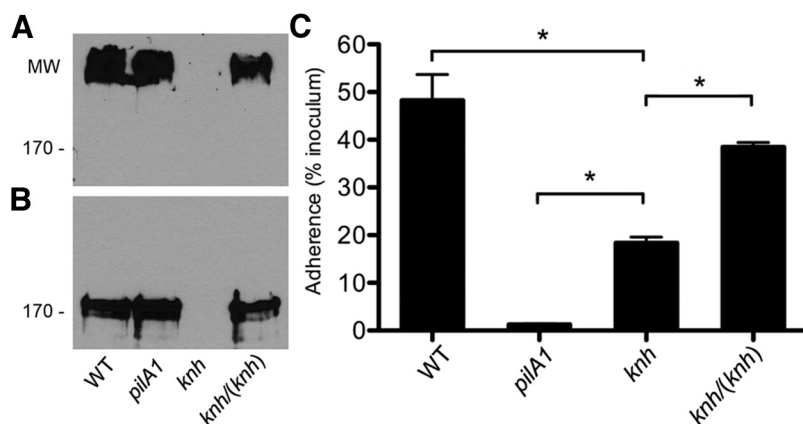
FIG 2 Knh is an outer membrane protein required for full-level adherence to Chang human epithelial cells. (A) Western blot analysis of outer membrane preparations from *K. kingae* strains KK03 (WT), KK03*pilA1*, KK03*knh*, and KK03*knh*/(*knh*) under standard SDS-PAGE denaturing conditions. (B) Western blot analysis of formic acid-treated outer membrane preparations from *K. kingae* strains KK03 (WT), KK03*pilA1*, KK03*knh*, and KK03*knh*/(*knh*) under standard SDS-PAGE denaturing conditions. Molecular sizes (MW) are expressed in kilodaltons. (C) Adherence of *K. kingae* strains KK03 (WT), KK03*pilA1*, KK03*knh*, and KK03*knh*/(*knh*) to Chang human epithelial cells. Adherence assays were conducted in triplicate. Error bars indicate standard errors of the means. Statistical analyses were performed by using the unpaired *t* test to compare the adherence levels of two strains as follows: $P < 0.05$ (*) for KK03 versus KK03*knh*, KK03*pilA1* versus KK03*knh*, and KK03*knh* versus KK03*knh*/(*knh*).

and confirm that Knh is a member of the growing class of trimeric autotransporter proteins.

Knh is expressed in the *K. kingae* outer membrane and is required for full-level adherence to human epithelial cells. To determine if Knh is expressed and present in the outer membrane of *K. kingae* strain KK03, sarkosyl-insoluble membrane fractions were isolated and examined by Western blotting with anti-Knh serum. Strain KK03 is a naturally occurring derivative of our prototype *K. kingae* strain, 269-492, which expresses stable levels of type IV pili and was used as the wild-type (WT) strain throughout this study. As a negative control, a Knh mutant was created by insertionally inactivating *knh* in strain KK03. Under standard SDS-PAGE denaturing conditions, a high-molecular-mass band was evident in outer membrane preparations of strain KK03 and the nonpiliated, nonadherent *pilA1* mutant but not in those of the *knh* mutant (Fig. 2A). While the high molecular mass of this reactive band precludes accurate size estimation, it is consistent with the trimer form of Knh, which has a predicted molecular mass of 540 kDa. Formic acid treatment of outer membranes resulted in elimination of the high-molecular-mass band and detection of a band corresponding to the predicted monomeric molecular mass (180 kDa) of Knh in strains KK03 and KK03*pilA1* but not in strain KK03*knh* (Fig. 2B). To complement the *knh* mutation, the *knh* ORF with the native promoter region was recombined into a separate locus in the genome in the KK03*knh* strain background (see Fig. S1 in the supplemental material for the complementation strategy used). (To date, plasmids that replicate in *K. kingae* are unavailable.) As shown in Fig. 2A and B, outer membrane localization of Knh was restored in complemented strain KK03*knh*/(*knh*). Semiquantitative extracellular pilus preparation analysis using strain KK03*pilA1* as a negative control revealed no difference in piliation levels among strains KK03, KK03*knh*, and KK03*knh*/(*knh*) (data not shown).

To determine if Knh is involved in mediating *K. kingae* adher-

ence to host cells, we examined the adherence of strains KK03, KK03*pilA1*, KK03*knh*, and KK03*knh*/(*knh*) in assays with Chang human epithelial cells. As shown in Fig. 2C, elimination of Knh resulted in a significant reduction in the level of adherence, compared to that of strain KK03 ($P < 0.05$), and complementation of the *knh* mutation almost completely restored adherence. Unlike the situation when type IV pili were eliminated by disruption of the *pilA1* gene encoding the major pilin subunit, the disruption of *knh* did not completely abrogate adherence ($P < 0.05$ for KK03*pilA1* versus KK03*knh*), suggesting that *K. kingae* type IV pili mediate low-level adherence and require Knh for full-level adherence.

Considered together, these data demonstrate that Knh is required for full-level adherence of *K. kingae* to Chang human epithelial cells and that Knh cannot mediate appreciable adherence in the absence of type IV pili.

***K. kingae* produces a surface-associated polysaccharide capsule.** The observation that type IV pili are required for Knh-mediated adherence suggested that *K. kingae* may express another surface factor that blocks Knh adhesive activity. Examination of *K. kingae* strain KK03 revealed a mucoid colony phenotype similar to that seen with other bacterial species that produce a polysaccharide capsule (Fig. 3A). To explore the possibility that *K. kingae* expresses a polysaccharide capsule, the draft genome sequence was searched for homologs to genes involved in capsule synthesis and export in *N. meningitidis*, a well-studied *Neisseriaceae* family member that contains the *siaABCD*, *ctrABCD*, *ctrE/lipA*, and *ctrF/lipB* capsule-associated genes. As summarized in Table S1 in the supplemental material, an intact locus with homology to the ABC-type capsule export operon *ctrABCD* of *N. meningitidis* and unlinked genes with homology to the *ctrE/lipA* and *ctrF/lipB* genes were identified in the *K. kingae* genome. To determine if the *ctrABCD* operon plays a role in *K. kingae* encapsulation, *ctrA* was insertionally inactivated. As shown in Fig. 3B, examination of KK03*ctrA* revealed nonmucoid colonies, in marked contrast to the colonies of parental strain KK03. To confirm that *K. kingae* elaborates a polysaccharide capsule, strains KK03 and KK03*ctrA* were stained with cationic ferritin and then examined by thin-section transmission electron microscopy (TEM). Because of the highly anionic nature of most bacterial polysaccharide capsules, cationic ferritin typically binds to the capsular material, producing an electron-dense rim on the bacterial surface when visualized by thin-section TEM. A thick layer of electron density was evident on the surface of WT strain KK03 (Fig. 3C) and was absent from mutant strain KK03*ctrA* (Fig. 3D). Attempts to complement the mutation in *ctrA* and the likely polar effect on the *ctrBCD* genes were unsuccessful. Sequencing of our chromosomal complementation construct containing *ctrABCD* generated in *E. coli* consistently revealed nonsense or frameshift mutations in *ctrA*, presumably reflecting the inability of *E. coli* to tolerate the expression of the outer membrane protein encoded by *ctrA*. However, quantitative reverse transcription (qRT)-PCR analysis revealed that transcription of the ORF downstream of the *ctrABCD* operon was unaffected by the *ctrA* insertion mutation
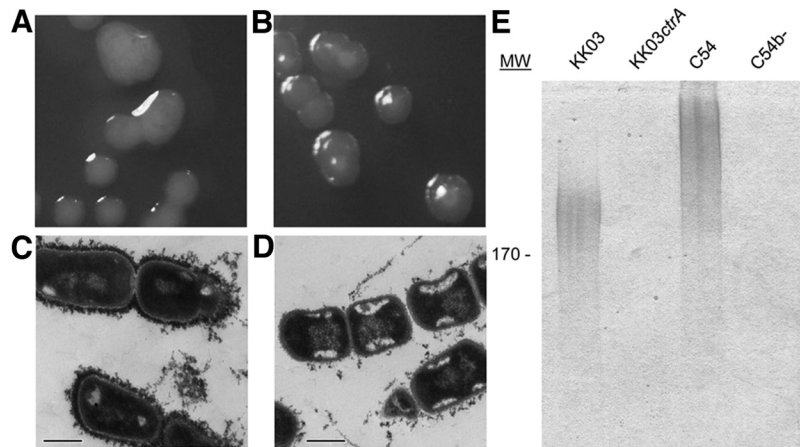
FIG 3 *K. kingae* has a surface capsule. (A, B) Colony phenotypes of *K. kingae* strains KK03 (A) and KK03*ctrA* (B). (C, D) Thin-section TEM analyses of cationic ferritin-stained *K. kingae* strains KK03 (C) and KK03*ctrA* (D). Scale bars, 500 nm. (E) Capsular material was extracted from strains KK03 and KK03*ctrA* with heat, separated by 7.5% SDS-PAGE, and then stained with the cationic dye alcian blue. Encapsulated *H. influenzae* type b strain C54 and an isogenic nonencapsulated mutant, C54b-, were included as controls. Molecular size (MW) is expressed in kilodaltons.

(see Fig. S2 in the supplemental material), supporting the conclusion that the phenotypes observed as a result of the *ctrA* mutation are due to disruption of the *ctrABCD* operon.

As a complementary approach to demonstrate the presence of a capsule, surface material was released from bacterial suspensions by heat extraction at 55°C, resolved by SDS-PAGE, and then stained with alcian blue (22). As shown in Fig. 3E, high-molecular-mass, alcian blue-reactive material was present in strain KK03 but absent from strain KK03*ctrA*. *H. influenzae* strain C54, which expresses a type b polysaccharide capsule, and *H. influenzae* strain C54b-, an isogenic mutant that is nonencapsulated, were used as controls. The alcian blue-reactive material did not stain with Coomassie blue and was unaffected by proteinase K treatment (data not shown), confirming that the extracted material is not proteinaceous.

Together, these data indicate that *K. kingae* strain KK03 expresses a surface-associated polysaccharide capsule that requires the predicted *ctrABCD* capsule export operon for surface localization.

Type IV pili, capsule, and Knh influence *K. kingae* adherence to human epithelial cells. To further explore the interrelationship between type IV pili, Knh, and the capsule in terms of their effects on bacterial adherence, we generated single, double, and triple mutations in *pilA1*, *knh*, and *ctrA*. Extracellular pilus preparations revealed no change in pilus expression levels in mutant strains with an intact *pilA1* gene (data not shown). As shown in Fig. 4, insertional inactivation of *ctrA* in strain KK03 resulted in a slight decrease in adherence that was not statistically significant. The adherence of the KK03*ctrA*/*knh* double mutant mimicked the levels seen in the *knh* single mutant, indicating that encapsulation does not influence type IV pilus-mediated adherence in the absence of Knh. Interestingly, while the *pilA1* mutant was nonadherent, simultaneous disruption of *pilA1* and *ctrA* in the KK03 background restored high-level adherence ($P < 0.05$ for the *pilA1/ctrA* mutant versus the *pilA1* mutant), indicating that encapsulation inhibits pilus-independent Knh-mediated adherence. The *pilA1*/

ctrA/knh triple mutant was nonadherent, providing strong evidence that Knh is an adhesin and is responsible for the *K. kingae* pilus-independent adherence observed in the *pilA1/ctrA* double mutant ($P < 0.05$ for the *pilA1/ctrA/knh* mutant versus the *pilA1/ctrA* mutant).

The type IV pilus retraction ATPase system is required for full-level adherence. The observation that the capsule interferes with Knh-mediated adherence in the absence of pili but not in the presence of pili raised the possibility that pilus-mediated adherence draws the organism closer to the host cell surface, perhaps via pilus retraction. In accordance with this possibility, *K. kingae* encodes a homolog of the PilT/PilU retraction ATPase machinery that has been shown to be essential for pilus retraction in several well-studied type IV pilus-expressing bacteria, including *N. meningitidis*, *N. gonorrhoeae*, *Pseudomonas aeruginosa*, and *Myxococcus xanthus* (23–26). To determine if pilus retraction is necessary for full-level *K. kingae* adherence, *pilT* was insertionally inactivated in strain KK03. As the insertion in *pilT* likely had a polar effect on *pilU*, the *pilTU* operon with the native promoter was recombined into the chromosome to achieve complementation. Examination of KK03*pilT* revealed increased levels of extracellular pili, as assessed by semiquantitative pilus preparations (Fig. 5A), but lacked twitching motility, as assessed by a modification of the *P. aeruginosa* agar stab assay (Fig. 5B) (27), consistent with the hypothesis that the PilT/PilU retraction ATPase machinery is necessary for *K. kingae* pilus retraction. As shown in Fig. 5C, elimination of PilT/PilU resulted in adherence that was decreased compared to
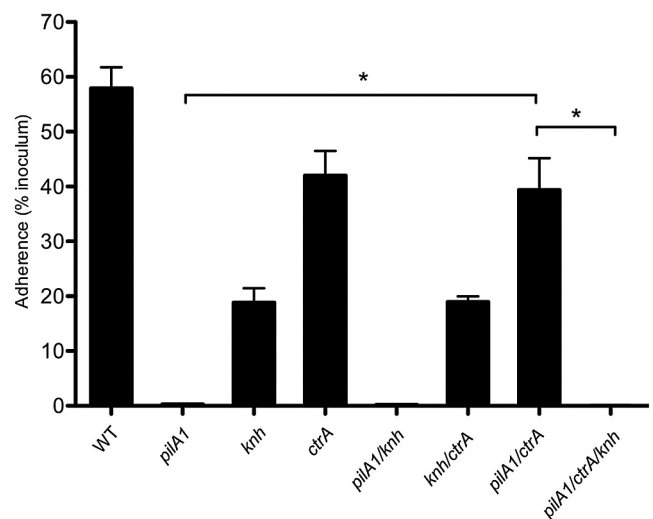
FIG 4 Influences of type IV pili, the capsule, and Knh on *K. kingae* adherence. The adherence of *K. kingae* KK03 (WT) and the *pilA1*, *knh*, *ctrA*, *pilA1/knh*, *knh/ctrA*, *pilA1/ctrA*, and *pilA1/ctrA/knh* mutant strains to Chang human epithelial cells was measured. Adherence assays were conducted in triplicate. Error bars indicate standard errors of the means. Statistical analyses were performed by using the unpaired *t* test to compare the adherence levels of two strains as follows: $P < 0.05$ (*) for *pilA1/ctrA* versus *pilA1* and for *pilA1/ctrA/knh* versus *pilA1/ctrA*.

A

B

C
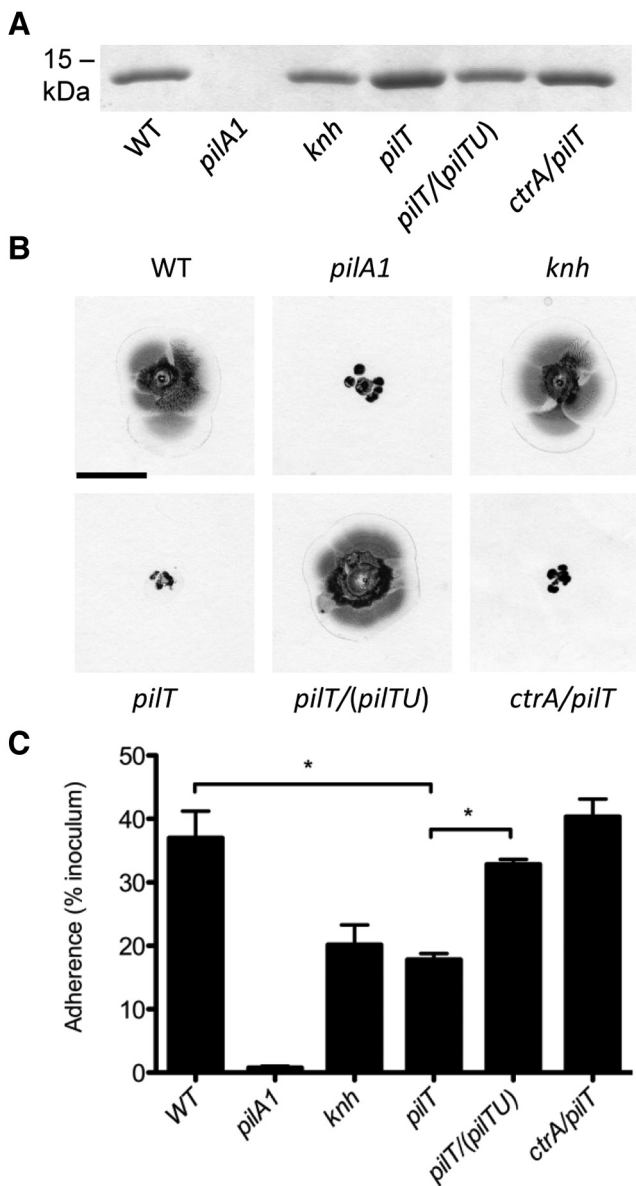
FIG 5 PilT/PilU retraction ATPase machinery influences *K. kingae* piliation, twitching motility, and adherence. *K. kingae* strains KK03, KK03*pilA1*, KK03*knh*, KK03*pilT*, KK03*pilT/(pilTU)*, and KK03*ctrA/pilT* were examined for extracellular pilus expression by semiquantitative pilus preparation (A), twitching motility by a modified agar stab assay (scale bar, 1 cm) (B), and adherence to Chang human epithelial cells (C). Adherence assays were conducted in triplicate. Error bars indicate standard errors of the means. Statistical analyses were performed by using the unpaired *t* test to compare the adherence levels of two strains as follows: $P < 0.05$ (*) for KK03 versus KK03*pilT* and for KK03*pilT* versus KK03*pilT/(pilTU)*.

that of parent strain KK03 ($P < 0.05$) but equal to that of the *knh* mutant. Complementation of the *pilTU* operon in the *pilT* mutant background resulted in reduction of extracellular pilus expression to the WT level and restoration of twitching motility and adherence (Fig. 5A to C). The observation that the *knh* and *pilT* mutants have similar adherence levels suggests that both retractable and nonretractable pili mediate a basal level of adherence and that the *pilT* mutant is unable to utilize Knh as an adhesin. We next inves-

tigated if full-level adherence could be restored in the *pilT* mutant by surface capsule elimination via disruption of *ctrA*. As expected, the *ctrA/pilT* double mutant adhered to epithelial cells at the WT level (Fig. 5C). These data support the hypothesis that retraction of type IV pili is necessary to overcome the inhibitory influence of the capsule on Knh-mediated adherence.

DISCUSSION

The pathogenesis of *K. kingae* disease is thought to begin with colonization of the upper respiratory tract. Previous work established that type IV pili promote *K. kingae* adherence to human epithelial cells (13). In this study, we identified a novel *K. kingae* trimeric autotransporter protein called Knh that also mediates adherence to human epithelial cells. Further analysis revealed that *K. kingae* expresses a polysaccharide capsule that interferes with Knh adhesive activity when type IV pili are absent. We observed that type IV pili are important to overcome the polysaccharide capsule and facilitate Knh-mediated adherence in the presence of the PilT/PilU ATPase retraction machinery, presumably as a result of pilus retraction.

Trimeric autotransporters are integral outer membrane proteins and appear to be universally associated with adhesive activity (28). In our study, *K. kingae* derivatives expressing the trimeric autotransporter Knh but lacking type IV pili and a capsule were adherent, while those lacking Knh, type IV pili, and a capsule were nonadherent, providing strong evidence that Knh is an adhesin. Analysis of the Knh amino acid sequence revealed the presence of conserved domains possibly involved in adhesive activity. The N-terminal region of Knh is predicted to encode three clusters of β-roll degenerate repeats termed YadA-like head domains based on characterization of the *Y. enterocolitica* YadA trimeric autotransporter adhesin. Studies of YadA have demonstrated that the head domain is responsible for YadA-mediated adhesive activity (20, 29), raising the possibility that this domain is involved in Knh adhesive function as well. In addition, Knh has an ISneck2 domain that is similar to the ISneck1 domain identified in the adhesive regions of the *H. influenzae* Hia and Hsf adhesins (18). It is interesting to speculate regarding the location of the adhesive domain(s) of Knh relative to the outer membrane and the presence of a capsule. We have clearly demonstrated that a nonpiliated *K. kingae* mutant that expresses Knh and a capsule is nonadherent. One possible explanation for this phenotype is that the adhesive domain of Knh is buried within the capsule and is thus not accessible to the host cell receptor. The ISneck2 domain is proximal to the membrane anchor, suggesting that it is likely to be located close to the bacterial surface and may be masked by surface structures, including the polysaccharide capsule. The YadA-like head domains are located distally and are also presumably masked by the polysaccharide capsule, perhaps unless the capsule is displaced.

Encapsulation of bacterial pathogens has been shown to confer phenotypes important for survival on the host, including antiphagocytic activity and serum resistance. In this study, we report that *K. kingae* expresses a surface-associated polysaccharide capsule. According to current understanding, the pathogenesis of *K. kingae* disease begins with colonization of the upper respiratory tract, followed by hematogenous dissemination to joints, bones, or the endocardium (8). This pathogenic sequence is supported by the finding that genotypically identical *K. kingae* strains can be isolated from the pharynx and bloodstream of patients with sys-

temic disease (12). We speculate that encapsulation is important for *K. kingae* to cause invasive disease by promoting intravascular survival, similar to observations with *N. meningitidis*, *H. influenzae*, and other encapsulated pathogens (30–34). Studies are under way to investigate the role of the *K. kingae* capsule in serum resistance and antiphagocytic activity. In addition to expressing a surface-associated polysaccharide capsule, *K. kingae* produces a secreted exopolysaccharide galactan that has broad antibiofilm activity (35). At this time, the relationship between the capsular polysaccharide and the galactan is unclear.

This study established that surface expression of the *K. kingae* capsule requires a predicted ABC-type transporter system with homology to CtrABCD in *N. meningitidis*. We also identified homologs of the *N. meningitidis* CtrE/LipA and CtrF/LipB proteins (36, 37). Both of these proteins also have homologs in the *E. coli* group 2 and 3 capsule biosynthesis systems (KpsC and KpsS) and in encapsulated *H. influenzae* (HcsA and HcsB) and appear to play a role in the export of capsule polymers to the bacterial surface (38, 39). Interestingly, the genetic organization of the polysaccharide synthesis machinery in *K. kingae* differs from that of *E. coli* groups 2 and 3, *H. influenzae*, and *N. meningitidis*. In *N. meningitidis*, a capsule synthesis operon (which differs in gene composition, depending on the specific capsule serotype) is located immediately upstream of the *ctrABCD* export operon and is divergently transcribed (40, 41). In *E. coli* group 2, the synthesis operon is located immediately between the export and assembly operons, and in *H. influenzae*, the synthesis operon is adjacent to the export operon (42–46). We have been unable to locate a putative capsule synthesis operon adjacent to *ctrABCD* or at any other locus in our draft *K. kingae* genome sequence, suggesting that *K. kingae* utilizes a unique genetic mechanism for the synthesis of capsular polysaccharide in a system that utilizes the ABC-type transporter system for export.

We first suspected that the capsule may be obstructing the ability of Knh to mediate adherence when we found that a mutant KK03 strain expressing a capsule and Knh but lacking pili was nonadherent. In considering this possibility, elaboration of a polysaccharide capsule has been shown to interfere with adhesive interactions with the host in several other pathogens. For example, the capsule interferes with adherence to human epithelial cells by *H. influenzae* type b (47), with adherence to gastrointestinal epithelium by enterotoxigenic *E. coli* (48), and with *N. meningitidis* Opa- and Opc-mediated adherence to epithelial and endothelial cells (49, 50). It is intriguing that the adherence of the *ctrA* mutant is slightly decreased compared that of parental strain KK03. While it is clear that the surface capsule blocks Knh-mediated adherence in the absence of type IV pili, we speculate that interaction of pili, Knh, and the capsule is necessary for full-level adherence of the WT organism. It is interesting to compare the *K. kingae* adherence data presented here with the adherence mechanism of *N. meningitidis*, a related encapsulated member of the *Neisseriaceae* family. *N. meningitidis* uses a two-step adherence process that starts with an initial type IV pilus-mediated interaction associated with microcolony formation on the host cell (51). Type IV pili are the key surface factor for adherence of encapsulated *N. meningitidis* to the epithelium and endothelium (52, 53). Following the initial adhesive interaction, type IV pili and the capsule are downregulated during the transition to the second stage of host cell interaction, which is termed intimate adherence (51, 54, 55). The shift from initial adherence to intimate adherence occurs over a course of

hours, leading to host cell cytoskeletal rearrangements and cortical plaque formation under the intimately adherent bacteria (51, 56). Interestingly, we were able to detect high-level adherence after an only 25-min incubation of WT *K. kingae* with human epithelial cells, indicating that type IV pili are able to rapidly overcome the inhibitory influence of the capsule on Knh-mediated adherence, apparently via PilT/PilU-mediated retraction. We have observed similar adherence after a 5-min incubation (data not shown), which argues against the downregulation of capsule expression. These results suggest that *K. kingae* has developed a unique mechanism to efficiently adhere to host cells without having to downregulate capsule expression and become more susceptible to immune clearance.

We propose a model for *K. kingae* adherence to human epithelial cells focusing on the three surface factors examined in this study: type IV pili, Knh, and the capsule (Fig. 6). This model involves a two-step process that begins with a low-affinity adhesive event mediated by type IV pili, as these fibers are able to extend beyond the polysaccharide capsule and engage their host cell receptor. PilT/PilU-mediated pilus retraction then brings the bacterium into close contact with the host cell, causing physical displacement of the surface polysaccharide capsule. Once the capsule has been displaced, Knh is able to engage its host cell receptor, leading to the high-level adherence we observe in adherence assays. It is important to note that this model relies on strong pilus retraction forces to displace the capsule. The force generated by a single *Myxococcus xanthus* type IV pilus has been measured at ~150 pN, making this the strongest linear molecular motor described to date (57). Similar measurements have been made for *N. gonorrhoeae* type IV pili, suggesting that the strong forces generated by these bacterial organelles may be conserved in the *Neisseriaceae* family (58).
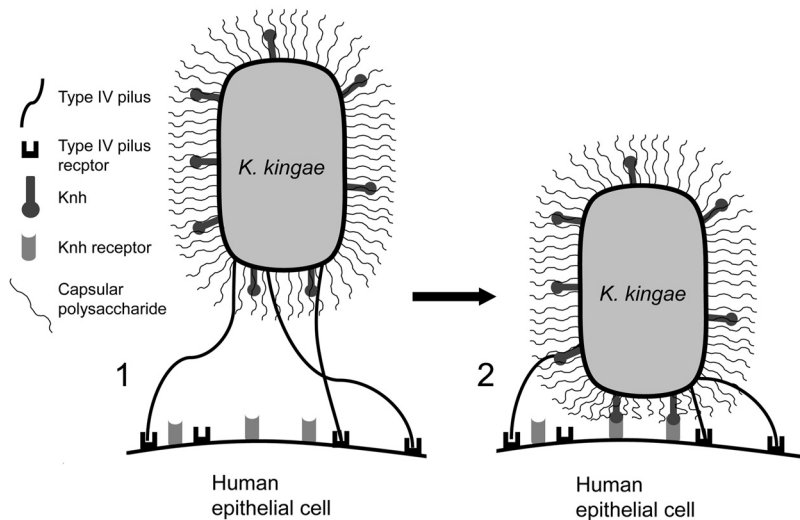
Our results establish a potential mechanism by which *K. kingae* adheres to the host respiratory epithelium and promotes colonization of the upper respiratory tract. However, the precise interrelationship and regulatory mechanisms of the three surface factors presented here have yet to be determined. Further investigation of the *K. kingae* adherence mechanism may lead to novel strategies for the prevention of disease due to *K. kingae* and to other encapsulated pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table S2 in the supplemental material. Strain 269-492 forms two distinct colony morphologies on solid agar that are referred to as KK01 and KK03. KK01 is associated with stable low-level piliation, and KK03 is associated with stable high-level piliation. KK03 was used as the WT strain throughout this study to control for the piliation level. *K. kingae* strains were stored at −80°C in brain heart infusion broth with 30% glycerol. *E. coli* strains were stored at −80°C in Luria-Bertani (LB) broth with 15% glycerol. *K. kingae* strains were routinely cultured at 37°C with 5% CO_2 on chocolate agar supplemented with 50 μg/ml kanamycin, 1 μg/ml erythromycin, or 2 μg/ml tetracycline, as appropriate. *E. coli* strains were routinely cultured at 37°C in LB broth or on LB agar supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 500 μg/ml erythromycin, or 12.5 μg/ml tetracycline, as appropriate.

Genetic disruptions. Gene disruptions were introduced into *K. kingae* by natural transformation (59). Following recovery of transformants by plating on selective medium, correct localization of the gene disruptions was confirmed by Southern blotting or PCR. The *pilA1* gene was disrupted as described previously (13). All of the plasmids used in this study are listed in Table S2 in the supplemental material, and the sequences of all of

Type IV pilus

Type IV pilus receptor

Knh

Knh receptor

Capsular polysaccharide

K. kingae

K. kingae

1

2

Human epithelial cell

Human epithelial cell

FIG 6 *K. kingae* adherence to human epithelial cells involves sequential adhesive events. Initial *K. kingae* interactions with the host respiratory epithelium are initiated by type IV pili (1). Once pili have engaged their host cell receptor, PilT-mediated pilus retraction pulls the bacterium into close contact with the host cell membrane. This action results in physical displacement of the polysaccharide capsule, exposing Knh to the host cell membrane. Unmasked Knh can then mediate high-affinity *K. kingae* interactions with human epithelial cells via the Knh host cell receptor without the need for genetic capsule downregulation (2).

the primers used are listed in Table S3. To disrupt the *knh* gene, fragments corresponding to the 5′ and 3′ regions of the gene were individually amplified from *K. kingae* strain 269-492 by using primers *knh*5′ F and *knh*5′ R and primers *knh*3′ F and *knh*3′ R, respectively. These fragments were ligated into BamHI/SalI-digested pTrc99A (introducing an internal deletion and a ClaI site within *knh*), generating pTrc99A/*knh*::ClaI. The *tetM* tetracycline resistance cassette was amplified with flanking ClaI sites from pHSX*tetM*4 using primers *tetM* F and *tetM* R and ligated into pTrc99A/*knh*::ClaI, generating pTrc99A/*knh*::*tetM*. To disrupt the *ctrA* gene, a fragment containing most of the *ctrA* ORF and the upstream flanking sequence was amplified from *K. kingae* strain 269-492 with primers *ctrA* F and *ctrA* R and ligated into BamHI/HindIII-digested pUC19, generating pUC19/*ctrA*. Subsequently, the QuikChange XL site-directed mutagenesis kit (Agilent Technologies) was used to insert an MluI site into the *ctrA* ORF by using primers *ctrA* MluI F and *ctrA* MluI R, generating pUC19/ *ctrA*::MluI. The erythromycin resistance cassette *ermC* was amplified with flanking MluI sites from pIDN4 by using primers *ermC* F and *ermC* R and ligated into pUC19/*ctrA*::MluI, generating pUC19/*ctrA*::*ermC*. To disrupt the *pilT* gene, a fragment containing most of the *pilT* ORF and the upstream flanking sequence was amplified from *K. kingae* strain 269-492 with primers *pilT* F and *pilT* R and ligated into BamHI/HindIII-digested pUC19, generating pUC19/*pilT*. Subsequently, site-direct mutagenesis was used to insert an MluI site into the *pilT* ORF by using primers *pilT* MluI F and *pilT* MluI R, generating pUC19/*pilT*::MluI. The kanamycin resistance cassette *aphA3* was excised from pFalcon2 by digestion with MluI and ligated into MluI-digested pUC19/*pilT*::MluI, generating pUC19/*pilT*::*aphA3*. To rule out PilA1 amino acid sequence variation as a source of phenotypic changes, the *pilA1* gene in all strains that did not contain the *pilA1* disruption was sequenced and confirmed to be 100% identical to the KK03 *pilA1* sequence. To exclude possible phase-variable events in the mutants generated in this study, all strains were examined for pilus production by using extracellular pilus preparations, Knh production using Western blotting, and capsule production using heat extraction and alcian blue staining to confirm the presence or absence of the three surface factors under study.

The genetic complementation strategy used is detailed in Fig. S1 and Text S1 in the supplemental material.

Generation of anti-Knh polyclonal antiserum. In order to generate an antiserum reactive with Knh, a 1.3-kb fragment corresponding to the 5′ coding region of *knh* (encoding amino acids 55 to 403, lacking the predicted signal sequence) was amplified with primers KnhAb F and KnhAb R and ligated into EcoRI/SalI-digested pGEX6p (GE Healthcare Life Sciences), creating pGEX::Knh$_{55-403}$, which encodes a glutathione *S*-transferase (GST) fusion protein. The GST-Knh$_{55-403}$ fusion protein was affinity purified on glutathione-agarose and treated with PreScission protease (GE Healthcare Life Sciences) to release Knh$_{55-403}$ from the GST fusion. The resulting 42-kDa protein was resolved by 10% SDS-PAGE, stained with Coomassie blue, excised, and then injected into a guinea pig, generating antiserum GP97.

Protein analysis. A 264-bp fragment encoding Knh amino acids 1695 to 1783 encompassing the predicted beta-barrel membrane anchor was PCR amplified with primers KnhBB F and KnhBB R, digested with EcoRI and BamHI, and ligated into EcoRI/ BamHI-digested pASK-IBA12 (IBA BioTAGnologies). The resulting construct, designated pASK:: Knh$_{1695-1783}$, was transformed into *E. coli* BL21 omp8 and induced with 2 μg/ml anhydrous tetracycline for 3 h. Outer membranes were isolated on the basis of Sarkosyl insolubility, and an aliquot was treated with 95% formic acid overnight as previously described (21). Samples with or without formic acid treatment were separated by 15% SDS-PAGE and transferred to nitrocellulose. Western blot analysis of Strep-tagged recombinant KnhBB was performed by using the anti-Strep monoclonal antibody (IBA BioTAGnologies) according to the manufacturer's recommendations.

Western blot analysis of Knh in *K. kingae* was performed as described previously, with Knh-specific antiserum (60). Briefly, outer membrane fractions were isolated on the basis of sarkosyl insolubility and treated overnight with 95% formic acid. Samples were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with antiserum GP97 against Knh and a secondary goat anti-guinea pig horseradish peroxidase-conjugated antibody. Western blot assays were developed by using a chemiluminescent peroxidase substrate.

Pilus preparations. Derivatives of *K. kingae* strain KK03 were incubated on chocolate agar for 17 to 18 h, and growth was suspended in 1.5 ml 50 mM Tris–150 mM NaCl, pH 8.0, to an optical density at 600 nm (OD$_{600}$) of 1.0, vortexed at full speed for 1 min, and centrifuged at 21,000 $\times$ *g* for 2 min to pellet the bacteria. A 1.25-ml volume of the bacterium-free supernatant was subjected to 20% ammonium sulfate precipitation on ice for 2 h. Precipitated pili were collected via centrifugation at 21,000 $\times$ *g* for 5 min and resuspended in 1$\times$ SDS-PAGE loading buffer. Aliquots were separated by 15% SDS-PAGE and stained with Coomassie blue. The observed protein band was confirmed to be the major pilin subunit, PilA1, by Western blot analysis with antiserum GP65 as described previously (14).

Capsule extraction. Derivatives of *K. kingae* strain KK03 and *H. influenzae* type b strain C54 were incubated for 17 to 18 h on chocolate agar, suspended in 1 ml phosphate-buffered saline (PBS) to an OD$_{600}$ of 0.8, centrifuged for 2 min at 10,000 $\times$ *g*, and resuspended in 0.5 ml PBS. Suspensions were incubated at 55°C for 30 min to extract capsular material. Bacteria were pelleted, and supernatants were concentrated 10-fold in an Amicon Ultra centrifuge filter with a 10,000 molecular weight cutoff. Capsule extracts were separated by 7.5% SDS-PAGE and stained with the cationic dye alcian blue (0.125% alcian blue in 40% ethanol/5% acetic acid; Sigma) for 2 h and then destained overnight in 40% ethanol/5% acetic acid.

Cationic ferritin staining. Derivatives of *K. kingae* strain KK03 were incubated on chocolate agar for 17 to 18 h, and growth was suspended in 3 ml 0.1 M sodium cacodylate, pH 7.0 (cacodylate buffer), to an OD_{600} of 0.8. Glutaraldehyde was added to a final concentration of 5%, and samples were incubated for 20 min at ambient temperature. Following centrifugation at $750 \times g$ for 15 min, pellets were gently resuspended in 1 ml cacodylate buffer, polycationic ferritin (Sigma) was added to a final concentration of 1 mg/ml, and samples were incubated for 30 min at ambient temperature and then centrifuged at $750 \times g$ for 15 min. The pellets were washed once with 1 ml cacodylate buffer, resuspended in cacodylate buffer with 5% glutaraldehyde, incubated for 2 h at ambient temperature and then overnight at 4°C, and processed for TEM analysis.

TEM. To assess surface capsule expression, cationic-ferritin-stained bacteria were washed three times with cacodylate buffer, embedded in 2% low-melting-point agarose, and fixed in cacodylate buffer with 4% glutaraldehyde and 7.5% sucrose. The pellets were postfixed in 1% osmium tetroxide in cacodylate buffer, stained en bloc with 0.5% uranyl acetate, dehydrated with a graded series of ethanol, and embedded in epoxy resin. Blocks were cut into thin sections (60 to 90 nm), placed on copper-rhodium 200-mesh grids, and examined by using a Philips CM-12 electron microscope.

Twitching motility assays. Twitching motility was assessed by a modified agar plate stab assay originally developed for *P. aeruginosa* (27). Briefly, derivatives of *K. kingae* strain KK03 were incubated on chocolate agar for 17 to 18 h and growth was resuspended in $1\times$ PBS to an OD_{600} of 1.0. One microliter of the bacterial suspension was stab inoculated into the bottom of tissue culture-treated 100-mm plates containing chocolate agar and incubated at 37°C with 5% CO_2 for 2 days. Twitching motility-competent strains spread from the stab inoculation site at the plate-agar interface. The agar was carefully peeled away, and the plate was air dried and stained with crystal violet to visualize the twitching motility zones.

Eukaryotic cell lines and adherence. Chang cells (Wong-Kilbourne derivative [D] of Chang conjunctiva, HeLa origin; ATCC CCL-20.2) were cultivated at 37°C with 5% CO_2 in medium as described previously (13). Quantitative adherence assays were performed as described previously (13). Briefly, confluent and fixed cell monolayers in 24-well plates were inoculated with approximately 6.5×10^6 CFU of bacteria and the plates were centrifuged at $165 \times g$ for 5 min and then incubated for 25 min at 37°C in 5% CO_2. Monolayers were washed four times with PBS to remove nonadherent bacteria and then treated with $1\times$ trypsin-EDTA (Sigma) for 20 min at 37°C to release adherent bacteria. Percent adherence was calculated by dividing the number of adherent CFU by the number of inoculated CFU. All experiments were performed in triplicate. Statistical analysis was performed using the unpaired *t* test to compare the adherence levels of two strains as noted in the figure legends.

Nucleotide sequence accession numbers. The nucleotide sequences of the *ctrABCD*, *ctrE*, *ctrF*, and *knh* loci have been submitted to GenBank and assigned accession numbers JX944815 (*ctrABCD*), JX944816 (*ctrE*), JX944817 (*ctrF*), and JX944818 (*knh*).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00372-12/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3 , DOCX file, 0.1 MB.
Figure S1, TIF file, 0.3 MB.
Figure S2, TIF file, 0.1 MB.

ACKNOWLEDGMENTS

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