Experimental Methods for Studying *Kingella kingae*

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Growth Conditions

Kingella kingae is a fastidious facultative anaerobe that grows optimally in a 37 °C humidified atmosphere supplemented with 5–10 % CO_2. The organism can be readily cultured on trypticase soy agar supplemented with 5 % lyophilized hemoglobin or 5 % sheep blood as a hemoglobin source (e.g., sheep blood agar) [1]. The organism also grows well on Columbia base or GC base agar supplemented with hemoglobin (e.g., chocolate agar). *K. kingae* does not grow well in liquid culture conditions using standard media formulations used for other fastidious gram-negative bacteria. However, it has been shown to grow in BD BACTEC™ Standard/10 Aerobic/F Culture Vials (BD, Franklin Lakes, NJ) [1, 2]. Using the same media formulation as the BACTEC vial media, *K. kingae* has been shown to grow in a 5 % CO_2-supplemented atmosphere at 37 °C without shaking, albeit at a slow rate.

Genetic Manipulation

Kingella kingae is naturally competent, resulting in the efficient uptake of extracellular DNA from the environment via natural transformation. If the imported DNA has high sequence homology to a segment of DNA of the recipient strain, the homologous recombination machinery can incorporate the exogenous DNA into the

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genome of the recipient strain, a process known as allelic exchange. This aspect of *K. kingae* biology has been exploited in the laboratory setting to introduce antibiotic resistance cassette-marked gene deletions, mutations, and complements. To transform *K. kingae*, bacteria are cultured overnight on chocolate agar and are then resuspended in brain–heart infusion (BHI) broth to an OD_{600} of ~0.8. Subsequently, the transforming DNA (typically 100–500 ng, but optimization may be necessary) is added to 250 μl of the bacterial suspension in a well of a 24-well plate, and the suspension is allowed to stand at room temperature for 30 min. Next, 250 μl of BHI supplemented with 5 % horse plasma is added to the transformation mixture, and the mixture is incubated at 37 °C in a 5 % CO_2-supplemented atmosphere for 2 h. Diluted and undiluted samples of the transformation reaction are then spread on chocolate agar plates containing the appropriate antibiotic to select for transformants, and plates are incubated overnight. Individual colonies are picked, and genomic DNA from these colonies is analyzed by PCR to confirm the presence and location of the integrated transforming DNA.

Beyond delivery by natural competence, exogenous DNA can be introduced into *K. kingae* by electroporation. Electroporation may be the only option for strains that are recalcitrant to transformation by natural competence, including strains that lack functional type IV pili, which appear to be necessary for transformation by natural competence. Briefly, bacteria are suspended from a chocolate agar plate into 25 ml of BD BACTEC™ Standard/10 Aerobic/F Culture Vial medium to an OD_{600} of ~0.1. After static growth overnight at 37 °C in 5 % CO_2, the bacteria are washed twice with 0.3 M sucrose at room temperature and then resuspended in 0.3 M sucrose. Subsequently, 100 ng of transforming DNA is electroporated into the electrocompetent bacteria at 12.5 kV/cm in a 2-mm cuvette. The electroporated bacteria are recovered in 500 μl of BHI supplemented with 5 % horse plasma and are incubated at 37 °C in a 5 % CO_2-supplemented atmosphere for 2 h prior to plating on selective media.

Plasmids

Attempts to introduce a variety of cloning plasmids with different origins of replication into *K. kingae* have not been successful. Recently, some *K. kingae* clinical isolates have been shown to carry up to two plasmids [3, 4]. More work is necessary to determine whether either of these plasmids will facilitate stable introduction of cloned DNA into *K. kingae*.

Targeted Mutations

The first step to introduce targeted mutations or gene deletions into *K. kingae* involves generating a recombinant targeting plasmid using standard molecular

biology approaches. An example targeting plasmid for deletion of hypothetical gene A (*hypA*) is diagrammed in Fig. 1. Briefly, approximately 1 kb of upstream DNA and 1 kb of downstream DNA flanking the *hypA* gene are cloned into the multiple cloning site (MCS) of a standard cloning plasmid such as pUC19, leaving some restriction sites intact between the two cloned fragments. An antibiotic resistance cassette is then inserted between the upstream and downstream targeting fragments, generating a targeting deletion plasmid. To date, antibiotic resistance cassettes conferring resistance to kanamycin, erythromycin, tetracycline, or chloramphenicol have been used to generate marked *K. kingae* mutants and are detailed in Table 1.

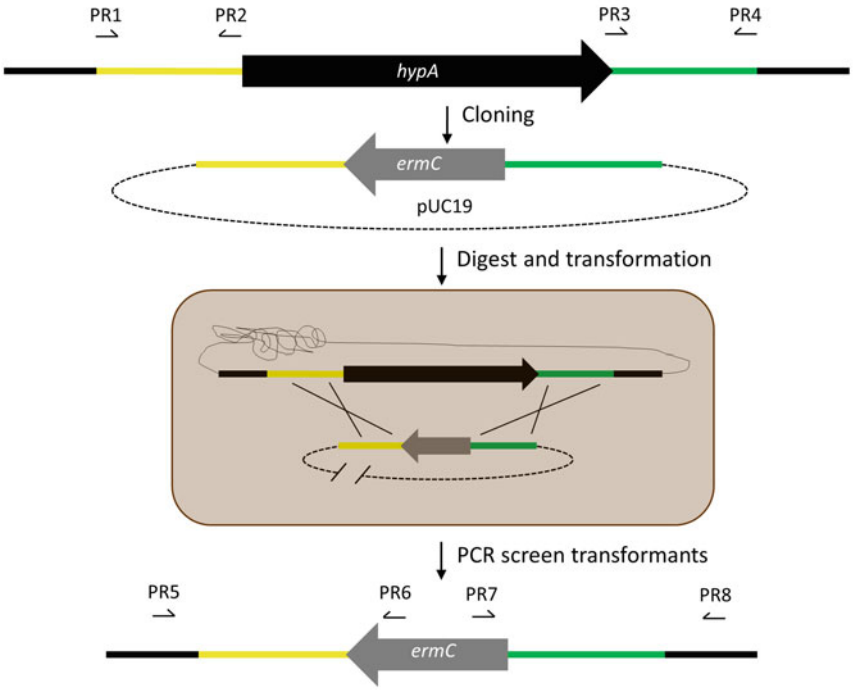

Fig. 1 Strategy for targeted gene deletion in *K. kingae*. Upstream (*yellow*) and downstream (*green*) homologous recombination targeting fragments (~1 kb each) are amplified from genomic DNA of the parental wild-type strain using primer pairs PR1/PR2 and PR3/PR4, respectively. Both fragments are cloned into pUC19, and the *ermC* erythromycin resistance cassette is cloned between the targeting fragments, generating a targeting deletion plasmid. The plasmid is then linearized by restriction digestion with an enzyme that cuts the vector backbone and is transformed into the wild-type parental strain via natural transformation. Individual transformants that grow on selective chocolate agar supplemented with 1 μg/ml erythromycin are single colony purified on the same selective media, and genomic DNA is prepared for use as the template for PCR screening. Two primer sets (PR5/PR6 and PR7/PR8) are used to confirm deletion of the target gene. Each primer set contains one primer that anneals outside of the cloned homologous recombination targeting regions (PR5 and PR8) and another primer that anneals to the *ermC* resistance cassette (PR6 and PR7)

Table 1 Antibiotic resistance cassettes for use in *K. kingae*

Antibiotic	Cassette	Source	Selection (μg/ml)	Reference
Kanamycin	*aphA3*	pFalcon2	50	[7]
Erythromycin	*ermC*	pIDN4	1	[22]
Tetracycline	*tetM*	pHSX*tetM*4	2	[23]
Chloramphenicol	*cat*	pACYC184	1	[24]

To eliminate the possibility of single-crossover homologous recombination and incorporation of the entire targeting plasmid, it is advisable to linearize the targeting plasmid prior to transformation by digesting the plasmid with an enzyme that only cuts in the vector backbone. Using this approach, only double-crossover homologous recombination events in the upstream and downstream targeting regions will result in incorporation of the resistance cassette and thus viable antibiotic resistant transformants.

Similar approaches with various modifications can be used to introduce other types of mutations into the *K. kingae* chromosome, as long as the mutation does not affect viability. Two example mutations other than gene deletions that have been generated in *K. kingae* are shown in Fig. 2. Figure 2a illustrates how a mutated *pilA1* promoter element with a scrambled sequence was generated. A targeting plasmid was constructed containing the entire *pilA1* locus and surrounding sequence in pUC19, with a kanamycin resistance cassette inserted into *recJ*, the gene upstream of *pilA1*. Next, PCR was performed with primers containing overhangs with the desired scrambled sequence and a SpeI restriction site that annealed to the opposite strands of the region surrounding the promoter element of interest. After treatment with DpnI to digest the template plasmid, the resulting PCR product with overhanging SpeI sites was gel-extracted and digested with SpeI. The SpeI-digested PCR product was gel-extracted and then self-ligated, generating a targeting plasmid with a scrambled *pilA1* promoter element with an internal SpeI site. Following sequencing to confirm that the promoter sequence was scrambled as desired, the mutated plasmid was transformed into *K. kingae*. Kanamycin-resistant transformants were picked and subjected to sequencing of the *pilA1* locus. Sequencing was necessary to confirm the presence of the desired mutation in the transformants because selection was solely based on the antibiotic resistance marker, which could potentially be kilobases away from the desired mutation, enabling selection of transformants with a homologous recombination event between the marker and the mutation of interest.

As illustrated in Fig. 2b, in order to introduce a calcium-binding site mutation in the pilus-associated protein PilC1, the entire *pilC1* gene with 1 kb of upstream and 1 kb of downstream targeting sequence was cloned into pUC19, with a kanamycin resistance cassette inserted into the *abcA* gene upstream of *pilC1*. The resulting plasmid was subjected to a standard site-directed mutagenesis procedure using the QuikChange XLII Site-Directed Mutagenesis Kit (Agilent Technologies, Wilmington, DE) to generate the calcium-binding site mutation. After sequencing

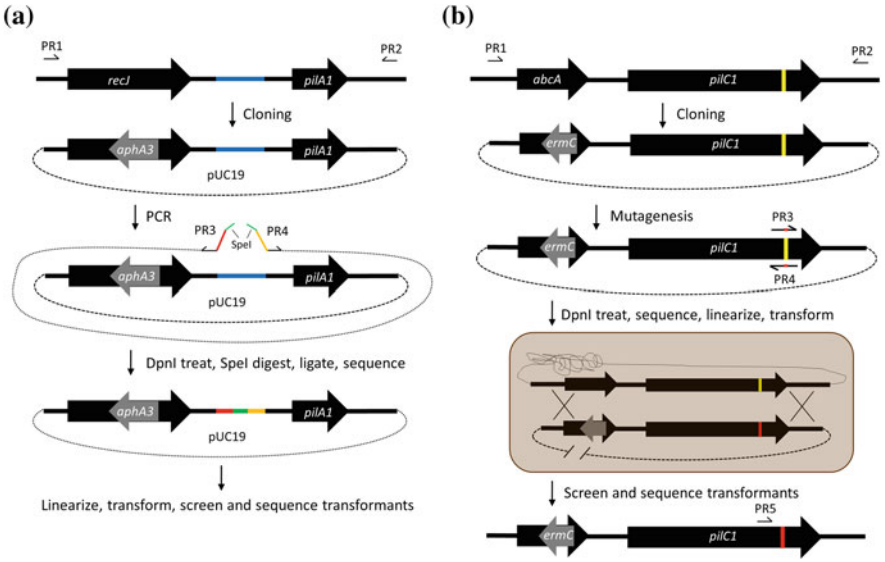

Fig. 2 Mutagenesis strategies in *K. kingae*. The strategy to generate a scrambled *pilA1* promoter element mutation in *K. kingae* is shown in (**a**) [10]. The native promoter element (not to scale) is shown in *blue*. The *pilA1* region is amplified with PR1/PR2 and is cloned into pUC19. The *aphA3* kanamycin resistance cassette is then cloned into the *recJ* ORF (a mutation that was previously shown to have no impact on type IV pilus expression or function). The plasmid is then used as the template for PCR with PR3, which has an overhang with half of the desired promoter scramble sequence (*red*) and a SpeI site (*green*), and PR4, which has an overhang with the other half of the desired promoter scramble sequence (*orange*) and a SpeI site (*green*). After cycling, the reaction is treated with DpnI to digest the template plasmid. The amplicon is then digested with SpeI, ligated, and transformed into laboratory *E. coli* strain DH5α for propagation. After sequencing of the entire *pilA1* locus to confirm the presence of the promoter scramble and absence of unwanted PCR-introduced mutations, the promoter scramble plasmid is linearized and transformed into *K. kingae*. Genomic DNA is isolated from the transformants and is subjected to PCR and sequencing to confirm the presence of the desired promoter scramble. A similar strategy is used to generate coding sequence mutations, including the procedure outlined in (**b**) used to generate a single amino acid substitution in the PilC1 calcium-binding site [12]. The primers PR3 and PR4 are complementary sense and antisense oligos with the necessary nucleotide changes (*red*) to change the native codon (*yellow*). The Agilent QuikChange XLII Site-Directed Mutagenesis kit is used to generate the mutation, and the resulting plasmid is sequenced and transformed as described above. The greater the distance from the selectable marker that the desired mutation is located, the greater the number of transformants will need to be screened by sequencing. This relationship is due to the fact that there is a greater chance of homologous recombination between the marker and the desired mutation as the distance from the selectable marker increases

to determine that only the desired mutation was generated, the plasmid was transformed into *K. kingae*. Introduction of the desired mutation was confirmed as described above.

Genetic Complementation

Given that there are no known plasmids for the introduction of episomal DNA into *K. kingae*, a strategy for chromosomal complementation of mutations was developed [5]. Briefly, a targeting plasmid (complementation plasmid) was created to direct the insertion of individual genes or clusters of genes into the *K. kingae* chromosome. The chromosomal region that the complementation plasmid was designed to target lacks predicted genetic elements (e.g., open reading frames, tRNAs, or regulatory elements) to minimize the chance that the introduction of cloned DNA into this region will affect *K. kingae* biology at the genetic level. The complementation plasmid contains an antibiotic marker and a fragment of the pUC19 MCS, including the restriction sites KpnI, BamHI, XbaI, PstI, and SphI, between the upstream and downstream targeting regions [5]. For complementation analysis, the gene to be complemented (with its native promoter) can be cloned into the MCS, introduced into the mutant strain via natural transformation, and screened for targeted recombination into the complementation locus. A modified version of this complementation plasmid with the *lacI* gene and *trc* promoter has been developed to enable isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced expression of cloned genes without their native promoter [6].

Random Transposon Mutagenesis

Given the natural transformability of *K. kingae*, the organism has been shown to be amenable to random in vitro transposon mutagenesis. Genomic DNA is isolated using the Promega Wizard Genomic DNA Isolation Kit (Promega, Madison, WI) according to the manufacturer's instructions. The isolated DNA is mutagenized using a purified maltose-binding protein (MBP)-*Himar*1 transposase fusion (purified from *E. coli* lysates after expression from the pMAL-*Himar* plasmid) and the plasmid pFalcon2 as the source of a kanamycin-marked mini Solo transposon, as described by Hendrixson et al. [7]. Briefly, 1 μg of genomic DNA is incubated with 500 ng of pFalcon2 and 500 ng of purified Himar1 transposase in a final volume of 80 μl of a solution containing 25 mM HEPES pH 7.9, 250 μg/ml BSA, 1 mM dithiothreitol (DTT), 100 mM NaCl, and 5 mM $MgCl_2$ for 4 h at 30 °C. The mutagenized chromosomal DNA is extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1) and is then ethanol-precipitated. To repair transposon/chromosome junctions, the purified mutagenized DNA is first treated with 1.5 units of T4 DNA polymerase for 20 min at 11 °C. The enzyme is heat-inactivated by incubating the reaction for 15 min at 75 °C. To complete the repair, the DNA is then treated with 600 units of T4 DNA ligase for 1 h at 22 °C. The repaired DNA is then transformed into *K. kingae* using the natural transformation protocol described above. After overnight growth on

chocolate agar containing kanamycin, the recovered colonies can be pooled to generate a transposon mutant library.

To confirm that individual transposon mutants only have one transposon insertion, purified genomic DNA is digested with BspHI and examined by Southern hybridization using a ~500-bp biotinylated fragment of the *aphA3* cassette from pFalcon2 as the probe. Following complete digestion, the presence of one hybridizing band indicates a single insertion. To determine the location of the transposon insertion, arbitrary PCR and sequencing are performed. The first round of a nested PCR is carried out using arbitrary primers ARB1 (5′ GGCCAC GCGTCGACTAGTACNNNNNNNNNNGATAT 3′) or ARB6 (5′ GGCCACGCG TCGACTAGTACNNNNNNNNNNACGCC 3′), where N represents a random nucleotide and specific primer Solo5 Arb#1 (5′ GCCCGGGAATCATTT GAAGGTTG 3′) or Solo3 Arb#1 (5′ CGCGTCGCGACGCGTCAATTC GAGG 3′). Solo5 Arb#1 anneals at the 5′ end of the Solo transposon, and Solo3 Arb#1 anneals at the 3′ end of the Solo transposon. A second round of amplification, using the first PCR product as the template, uses ARB2 (5′ GGCCACGCGTCGA CTAGTAC 3′), which anneals to the 5′ end of ARB 1 and ARB 6 with Solo5 outN (5′ AATATGCATTTAATACTAGCGACGCC 3′) or Solo3 outN (5′ CGCTCTTGAAGGGAACTATGTTG 3′), which are external to Solo5 Arb#1 and Solo3 Arb#1, respectively. The PCR products from the second round of amplification are gel-purified and sequenced using either Solo5 outN or Solo3 outN, as appropriate, to sequence across the chromosome/transposon junction. The utility of random transposon mutagenesis is best exemplified by Kehl-Fie et al., who used this approach to identify the RTX toxin locus responsible for the broad cell-type cytotoxicity of *K. kingae* [8].

Surface Factors

Given the importance of bacterial surface factors in mediating interactions with the host and their potential roles as colonization and virulence factors, much of the *K. kingae* molecular pathogenesis research has been focused in this area. *K. kingae* is generally amenable to a variety of standard techniques previously developed to study surface factors in other organisms. For example, the type IV pili of *K. kingae* are readily visualized by negative-staining transmission electron microscopy (TEM) [8–11], pilus retraction can be assessed using a modified agar plate stab assay [5, 12], outer membrane fractions can be isolated based on sarkosyl insolubility [5], and the presence of the polysaccharide capsule can be visualized by cationic ferritin staining and thin section TEM [5, 6]. The following sections describe experimental methods that have been optimized for the study of *K. kingae* surface factors.

Type IV Pili

Initial studies of *K. kingae* type IV pili examined pilus density and morphology using negative-staining TEM of whole bacteria [8, 9]. A protocol for large-scale purification of pili was developed based on a method for purifying type IV pili from *Eikenella corrodens* [13]. Briefly, this method involves resuspension of bacterial growth from 20 chocolate agar plates in 150 mM ethanolamine pH 10.5, shearing the fibers with a handheld homogenizer, and completion of two rounds of 10 % ammonium sulfate precipitation and dialysis [10, 13], yielding highly pure type IV pilus fibers as assessed by negative-staining TEM and SDS-PAGE separation and Coomassie blue staining of the ~14-kDa major pilus subunit. To complement this method, a small-scale procedure to more rapidly examine the relative piliation levels across multiple *K. kingae* strains or isogenic mutants was developed. The small-scale pilus preparation procedure is delineated in Table 2 and allows quantitative assessment of pilus levels in less than a day.

Type IV pilus-mediated twitching motility can be assessed using a modified agar plate stab assay originally developed for *Pseudomonas aeruginosa* [14]. For *K. kingae*, chocolate agar is poured as a thin layer into tissue culture-treated 100-mm Petri dishes. Following solidification and cooling of the agar, 1 µl of a 0.8 OD_{600} bacterial suspension is stab-inoculated at the plate/agar interface. After incubation under standard *K. kingae* growth conditions for 2–3 days, the agar is carefully peeled away from the plate to expose the twitching zone surrounding the inoculation site. The plate is air-dried, and a 0.1 % crystal violet solution is then applied to stain the twitching zone. The diameter of the zone can be measured to quantitate the level of twitching motility [5, 12].

Table 2 *K. kingae* small-scale type IV pilus purification procedure

Step	Description
1	Grow *K. kingae* strains on chocolate agar for 17–18 h
2	Resuspend bacteria in PBS to an OD_{600} of 1.0
3	Add 1.5 ml of the bacterial suspension to a 1.7-ml microcentrifuge tube
4	Vortex at the maximum setting for 1 min
5	Centrifuge the vortexed suspension for 2 min at 21,000 × *g*
6	Transfer 1.25 ml of the supernatant to a new 1.7-ml microcentrifuge tube
7	Add 250 µl of saturated ammonium sulfate, vortex, and incubate on ice for 2 h
8	Centrifuge for 5 min at 21,000 × *g* to pellet the precipitated, sheared fibers
9	Resuspend the pellet in 40 µl 1 × SDS-PAGE loading buffer
10	Load different volumes of the resuspended pellet onto a 15 % SDS-PAGE gel
11	Stain the gel with Coomassie blue

Outer Membrane Proteins

Outer membrane fractions can be readily isolated from *K. kingae* whole-cell sonicates based on sarkosyl insolubility. Briefly, bacteria are resuspended in 10 mM HEPES, pH 8.0, and sonicated to create whole-cell lysates. Bacterial debris is removed from the lysates by centrifuging at 21,000 × *g* and 4 °C for 2 min. The supernatant is recovered and centrifuged at 100,000 × *g* and 4 °C for 1 h to pellet the total membrane fraction. The pellet is then resuspended in 10 mM HEPES supplemented with 1 % sarkosyl and incubated with gentle agitation for 30 min to solubilize the inner membrane fraction. The sample is centrifuged again at 100,000 × *g* and 4 °C for 1 h to pellet the outer membrane fraction. The outer membrane fraction is washed once with 10 mM HEPES and can be resolved on an SDS-PAGE gel. Prior to SDS-PAGE analysis, the outer membrane fraction can be treated with formic acid to denature extremely stable multimeric proteins, such as trimeric autotransporters [5, 15, 16].

Like other gram-negative bacteria, *K. kingae* produces outer membrane vesicles (OMVs) that can be readily purified for analysis. Maldonado et al. [17] described a purification protocol that involves the scraping of bacterial growth from agar plates and then centrifugation at 150,000 × *g* and 4 °C for 15 min. Following centrifugation, the supernatant is collected, filtered through a 0.45-μm pore membrane, and centrifuged again at 150,000 × *g* and 4 °C for 2 h. The final pellet consists of the OMV fraction, which can be examined by TEM to visualize the OMVs and can be plated on growth media to confirm sterility.

Extracellular Polysaccharides

Kingella kingae expresses a polysaccharide capsule that can be purified by using a method that releases the capsule polymer from the surface of the organism and removes protein and other cell component contaminants [6, 18]. To prepare strains for extraction of capsule polysaccharide, bacterial lawns are grown on 40 chocolate agar plates overnight. Subsequently, the lawns are swabbed from the plates, resuspended in 50 ml of 1 % formaldehyde in PBS, and incubated at room temperature for 30 min. The bacteria are pelleted by centrifugation at 4000 × *g* for 10 min and are then resuspended in 40 ml of 50 mM Tris-acetate pH 5. Following vigorous shaking at room temperature for 1 h, the bacteria are pelleted by centrifugation at 12,000 × *g* for 20 min. The supernatant is filtered using a 0.22-μm filter and is adjusted to pH 7 with 1 M Tris pH 9. To remove contaminating DNA and RNA, a total of 10 units of DNase I and 0.1 mg of RNase A are added to the filtered material, and the mixture is incubated at 37 °C for 4 h. To remove contaminating proteins, 0.18 mg of proteinase K is added, and the sample is incubated at 55 °C overnight. To remove the proteinase K and any remaining protein contaminants, the sample is concentrated using a 100-kDa molecular weight cutoff

filter, extracted with Tris-saturated phenol pH 7.4, and then extracted with chloroform. The extracted material is dialyzed overnight in deionized water, and the purified polysaccharide can then be flash-frozen and lyophilized to allow for extended storage.

Kingella kingae also expresses a secreted exopolysaccharide galactan homopolymer (PAM galactan) that copurifies along with the polysaccharide capsule [18, 19]. To prevent contamination of capsule preparations with the PAM galactan, the *pamABCDE* locus can be deleted in *K. kingae* strains prior to capsule extraction [18]. To specifically purify the PAM galactan, the bacterial growth from a capsule synthesis-deficient or capsule export-deficient strain is directly resuspended in PBS, the suspension is agitated for 1 h, the bacteria are pelleted, and the supernatant is subjected to the same purification protocol as described for the capsule polysaccharide [18].

Interactions with Eukaryotic Cells

The *K. kingae* RTX toxin is present in all *K. kingae* strains examined to date and has broad-range cytotoxicity, including activity against human epithelial cells, synovial cells, macrophage-like cells, and red blood cells [8, 17]. This cytotoxicity precludes studies of in vitro interactions between wild-type *K. kingae* and cultured human cell lines. Early studies aiming to define *K. kingae* adherence factors found that fixation of cell monolayers with 2 % glutaraldehyde protected the monolayers against cytotoxicity and still allowed high-level in vitro adherence [9]. Many of the subsequent studies exploited this fixation protocol to identify and characterize *K. kingae* surface factors that influence adherence to epithelial and synovial cells, including type IV pili, the Knh trimeric autotransporter, and the capsular polysaccharide [5, 10–12, 18]. The protocol for assessing quantitative adherence levels of *K. kingae* to fixed cultured cell monolayers is detailed in Table 3. As an alternative to fixation to allow assessment of *K. kingae* interactions with host cells, the *rtx* locus can be deleted, as described thus far in two *K. kingae* strain backgrounds [8, 20].

Juvenile Rat Infection Model

Basmaci et al. reported the first animal model for examining virulence differences among *K. kingae* clinical isolates [21]. These investigators inoculated 5-day-old Sprague–Dawley rats via the intraperitoneal (i.p.) route with $\sim 10^7$ cfu of three *K. kingae* strains resuspended in PBS from overnight growth on chocolate agar. The animals were examined for 5 days for signs of infection, including skin discoloration, signs of peritonitis, and survival. Signs of infection included necrotic lesion formation around the injection site, abscess formation, lethargy, and death.

Table 3 *K. kingae* in vitro adherence assay procedure with Chang epithelial cells

	Description
Day 1	
1	Start with a T75 or T150 adherent tissue culture flask of Chang epithelial cells at ~80 % confluency cultured in Minimum Essential Medium (MEM) supplemented with 10 % fetal calf serum and 1 × MEM non-essential amino acid mix (complete media)
2	Release the cells with 0.05 % trypsin–EDTA and dilute to 1.8×10^5 cells/ml in complete media
3	Add 1 ml of the cell suspension to each well of a 24-well tissue culture plate (3 wells per *K. kingae* strain) and incubate at 37 °C in a humidified atmosphere supplemented with 5 % CO_2 for 17–18 h
4	Streak out each *K. kingae* strain on chocolate agar and incubate for 17–18 h
Day 2	
5	Remove the complete media from the 24-well tissue culture plate, add 300 µl of 2 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4 to each well, and gently rock at 4 °C for 2 h
6	Remove the glutaraldehyde solution, wash the wells 3 times with 500 µl of Tris-buffered saline (TBS), and add 300 µl of fresh complete media to each well
7	Suspend the bacteria in ~3 ml of BHI broth to an OD_{600} of 0.8 (~6.5×10^8 cfu/ml) and add 10 µl of each strain suspension to 3 wells
8	Gently swirl the plate and centrifuge for 5 min at 165 × *g*
9	Transfer the plate to a 37 °C incubator for 25 min
10	During the incubation, prepare a 1:100 dilution series in BHI in duplicate for each inoculating strain, plate on chocolate agar, and incubate overnight (inoculum plates)
11	Remove the media from the wells and wash 4 times with 500 µl of PBS
12	Add 100 µl of 0.05 % trypsin–EDTA to each well and incubate at 37 °C for 20 min
13	Add 900 µl BHI to each well, scrape the entire well surface with a pipette tip, and pipette up and down repeatedly to mix
14	Prepare a 1:100 dilution series in BHI for each well, plate on chocolate agar, and incubate overnight (adherence plates)
Day 4	
15	Determine the total cfu of the inoculum and adherence plates
16	Divide the total number of cfu on the adherence plates by the number of cfu on the inoculum plates to determine the adherence level expressed as % of inoculum

Significant differences in survival were observed among the strains. Chang et al. expanded on the model and tested the role of the *K. kingae* RTX toxin in virulence using both 7-day-old and 21-day-old Sprague–Dawley rats [20]. Significant differences in survival were observed in the 7-day-old pups injected i.p. with ~1.2×10^7 cfu of strain PYKK081 (a wild-type septic arthritis clinical isolate) versus an isogenic RTX-deficient mutant. In contrast, no differences in survival were observed in the 21-day-old animals. This report also found evidence of bacteremia and significant histopathology in the thymus, spleen, and bone marrow [20]. Starr et al. used 5-day-old Sprague–Dawley rats to investigate the role of the *K. kingae* polysaccharide capsule on virulence. Using an i.p. inoculum of 10^8 cfu of

strain KK01 (a stable derivative of septic arthritis isolate 269–492) and isogenic capsule-deficient mutants, the capsule-deficient mutants were significantly less virulent as assessed by survival through the 5-day experiment [6]. The development of the juvenile rat model was a significant first step for examining *K. kingae* virulence, but an animal model that more closely replicates *K. kingae* disease in humans, such as a septic arthritis model, will be critical for future studies. In addition, an animal upper respiratory tract colonization model will be necessary to test the in vivo relevance of potential colonization factors.

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