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Examination of Type IV Pilus Expression and Pilus-Associated Phenotypes in *Kingella kingae* Clinical Isolates▽

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Kingella kingae **is a gram-negative bacterium that is being recognized increasingly as a cause of septic arthritis and osteomyelitis in young children. Previous work established that *K. kingae* expresses type IV pili that mediate adherence to respiratory epithelial and synovial cells. PilA1 is the major pilus subunit in *K. kingae* type IV pili and is essential for pilus assembly. To develop a better understanding of the role of *K. kingae* type IV pili during colonization and invasive disease, we examined a collection of clinical isolates for pilus expression and *in vitro* adherence. In addition, in a subset of isolates we performed nucleotide sequencing to assess the level of conservation of PilA1. The majority of respiratory and nonendocarditis blood isolates were piliated, while the majority of joint fluid, bone, and endocarditis blood isolates were nonpiliated. The piliated isolates formed either spreading/corroding or nonspreading/noncorroding colonies and were uniformly adherent, while the nonpiliated isolates formed domed colonies and were nonadherent. PilA1 sequence varied significantly from strain to strain, resulting in substantial variability in antibody reactivity. These results suggest that type IV pili may confer a selective advantage on *K. kingae* early in infection and a selective disadvantage on *K. kingae* at later stages in the pathogenic process. We speculate that PilA1 is immunogenic during natural infection and undergoes antigenic variation to escape the immune response.**

Kingella kingae is a gram-negative bacterium that is a member of the *Neisseriaceae* family and is being recognized increasingly as a cause of pediatric diseases, including septic arthritis, osteomyelitis, and endocarditis. *K. kingae* was originally identified by Henriksen and Bovre in 1968 (10) but was dismissed early on as an important pathogen due to its infrequent recovery from infected sites. Recent improvements in cultivation techniques and the application of PCR-based assays have led to increased detection of *K. kingae* in association with invasive disease (3, 6, 17, 25, 27, 28, 31). A recent study identified *K. kingae* as a major cause of pediatric joint and bone infections and the leading etiology of these infections in children under 36 months of age (3).

Invasive disease due to *K. kingae* is believed to begin with colonization of the upper respiratory tract (32). A sizeable percentage of children are colonized with *K. kingae* at least once per year during the first 2 years of life and appear to acquire the organism by person-to-person transmission (1, 14, 22, 27, 29–31). Following colonization, the organism must breach the respiratory epithelium, enter the bloodstream, and then disseminate to deeper tissues. An essential step in both colonization of the respiratory tract and seeding of remote sites is adherence to host tissues. Recent work demonstrated that *K. kingae* expresses type IV pili that are necessary for *in vitro* adherence to both respiratory epithelial and synovial cells (11). The major pilin subunit in *K. kingae* type IV pili is called PilA1 and is essential for pilus assembly (11, 12).

Type IV pili have been shown to be necessary for adherence and colonization in a variety of organisms, including the pathogenic *Neisseria* species (2, 4, 15, 16, 19, 20, 23, 24, 26). In this work, we examined a collection of clinical isolates of *K. kingae* for pilus expression, adherence, and antigenic diversity of PilA1. Our results revealed that *K. kingae* has three naturally occurring colony types that correlate with density of piliation, including high-density piliation, low-density piliation, and nonpiliation. Further analysis demonstrated that respiratory isolates and nonendocarditis blood isolates were generally piliated and that joint fluid, bone, and endocarditis blood isolates were usually nonpiliated. Only piliated isolates were capable of adherence to cultured respiratory epithelial and synovial cells *in vitro*. The PilA1 subunit in piliated isolates exhibited significant strain-to-strain variation in sequence and antibody reactivity.

MATERIALS AND METHODS

Bacterial strains, culture methods, and storage. Table 1 lists the clinical isolates that were examined in this study. *K. kingae* strain 269-492 is the prototype strain that we have examined in earlier studies, and *K. kingae* strain 269-492 *pilA1*::*aphA3* is a nonpiliated mutant that contains a kanamycin cassette in the *pilA1* gene (11). *K. kingae* strains were routinely grown on TSA II chocolate agar plates (Becton-Dickinson, Franklin Lakes, NJ) at 37°C with 5% CO_2 supplemented with 50 μg/ml kanamycin, as appropriate. *Escherichia coli* was routinely grown on Luria-Bertani (LB) agar or in LB broth supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin, as appropriate. To disrupt *pilA1* and eliminate piliation, strains were transformed by natural transformation with pUC19/

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TABLE 1. Analysis of the clinical isolates used in this study

Strain[e]	Site/yr of isolation	Disease state	Colony type[a]	Pili[b]	Adherence to Chang/Hig-82[c]	PilA1 expression[d]
PYIDO	Blood/2007	Tenosynovitis	SC	+	+/+	+
PYKH-B	Blood/2007	Septic arthritis	NS/NC	+	+/+	++++
PYHER	Blood/2005	Osteomyelitis	D	−	−/−	NA[g]
PYITK-B	Blood/2007	Croup	SC	+	+/+	++
PYKK096	Blood/1992	Croup	NS/NC	+	+/+	−
PYKK060	Blood/1994	Endocarditis	NS/NC	+	+/+	+++
PYKK190	Blood/2002	Endocarditis	D	−	−/−	NA
PYKK197	Blood/2003	Endocarditis	D	−	−/−	NA
PYKK199	Blood//2004	Endocarditis	D	−	−/−	NA
PYKK061	Blood/1995	Bacteremia	SC	+	+/+	−
PYKK092	Blood/1996	Bacteremia	D	−	−/−	NA
PYKK093	Blood/1995	Bacteremia	NS/NC	+	+/+	−
PYKK097	Blood/1992	Bacteremia	NS/NC	+	+/+	−
PYKK128	Blood/1997	Bacteremia	D	−	−/−	NA
PYKK129	Blood/1997	Bacteremia	NS/NC	+	+/+	++++
PYKK181	Blood/2002	Bacteremia	NS/NC	+	+/+	+
PYKK243	Blood/2005	Bacteremia	SC	+	+/+	++
PYKK079	Blood/1994	Osteomyelitis	NS/NC	+	+/+	−
PYIDO PH-1	Respiratory/2007	Tenosynovitis	SC	+	+/+	+
PYKH PH-1	Respiratory/2007	Septic arthritis	NS/NC	+	+/+	++++
PYITK PH-1	Respiratory/2007	Croup	SC	+	+/+	++
PYITK PH-2	Respiratory/2007	Croup	SC	+	+/+	++
PYAA026	Respiratory/2006	Carrier	SC	+	+/+	++++
PYAA417	Respiratory/2006	Carrier	NS/NC	+	+/+	++
PYBB285	Respiratory/2006	Carrier	SC	+	+/+	++
PYC1639	Respiratory/2006	Carrier	SC	+	+/+	+
PYKK002	Respiratory/1994	Carrier	NS/NC	+	+/+	++
PYKK003	Respiratory/1994	Carrier	D	–[f]	−/−	−
PYKK012	Respiratory/1994	Carrier	SC	+	+/+	++++
PYKK019	Respiratory/1993	Carrier	D	−	−/−	NA
PYKK021	Respiratory/1993	Carrier	D	−	−/−	NA
PYKK029	Respiratory/1994	Carrier	NS/NC	+	+/+	−
PYKK048	Respiratory/1994	Carrier	SC	+	+/+	++
PYKK063	Respiratory/1994	Carrier	D	−	−/−	NA
PYKK065	Respiratory/1994	Carrier	D	−	−/−	NA
PYKK068	Respiratory/1994	Carrier	NS/NC	+	+/+	−
PYKK069	Respiratory/1994	Carrier	NS/NC	+	+/+	−
PYKK104	Respiratory/1996	Carrier	SC	+	+/+	+
PYKK113	Respiratory/1996	Carrier	SC	+	+/+	−
PYKK114	Respiratory/1996	Carrier	NS/NC	+	+/−	−
PYKK119	Respiratory/1996	Carrier	SC	+	+/+	−
PYKK120	Respiratory/1996	Carrier	SC	+	+/+	−
PYKK125	Respiratory1997	Carrier	SC	+	+/+	−
PYKK200	Respiratory/2004	Carrier	SC	+	+/+	+
PYO4a	Respiratory/2005	Carrier	NS/NC	+	+/+	−
PYP8	Respiratory/2005	Carrier	D	−	−/−	NA
PYKK070	Skeletal/1993	Osteomyelitis	D	−	−/−	NA
PYKK101	Skeletal/1992	Osteomyelitis	D	−	−/−	NA
PYKK141	Skeletal/1996	Osteomyelitis	NS/NC	+	+/+	−
PYKK088	Skeletal/1996	Osteomyelitis	D	−	−/−	−
PYKK057	Synovial/1995	Septic arthritis	NS/NC	+	+/+	−
PYKK058	Synovial/1993	Septic arthritis	D	−	−/−	NA
PYKK064	Synovial/1994	Septic arthritis	D	−	−/−	NA
PYKK077	Synovial/1991	Septic arthritis	D	−	−/−	NA
PYKK080	Synovial/1996	Septic arthritis	D	−	−/−	NA
PYKK081	Synovial/1991	Septic arthritis	NS/NC	+	+/+	−
PYKK082	Synovial/1990	Septic arthritis	SC	+	+/+	++++
PYKK083	Synovial/1991	Septic arthritis	D	−	−/−	NA
PYKK094	Synovial/1995	Septic arthritis	D	−	−/−	NA
PYKK100	Synovial/1996	Septic arthritis	SC	+	+/+	+
PYKK102	Synovial/1992	Septic arthritis	D	−	−/−	NA
PYKK103	Synovial/1992	Septic arthritis	D	−	−/−	NA
PYKK123	Synovial/1997	Septic arthritis	NS/NC	+	+/+	−
PYKK56	Synovial/1994	Septic arthritis	NS/NC	+	+/+	−

[a] Colony types were designated spreading/corroding (SC), nonspreading/noncorroding (NS/NC), and domed (D).
[b] Piliation was assessed by negative-staining transmission electron microscopy.
[c] Adherence to Chang and Hig-82 cells was assessed by qualitative adherence assay.
[d] Expression of PilA1 was assessed by Western blot analysis using antiserum GP65. The level of expression was compared to expression by strain 269-492, which was defined as ++++.
[e] Strains PYHER, PYKK197, PYKK199, PY04a, PYP8, PYKK070, and PYKK057 were recovered from patients in central Israel, strains PYKK092, PYKK128, PYKK141, PYKK088, and PYKK064 were recovered from patients in northern Israel, and all other strains were recovered from patients in southern Israel. Strains PYKK060 and PYKK197 were recovered from adults, and all other strains were recovered from children between the ages of 6 months and 6 years.
[f] Atypical short fibers were observed.
[g] NA, not applicable.

pilA1::*aphA3* (11). *K. kingae* strains were stored at −80°C in brain heart infusion (BHI) broth with 30% glycerol, and *E. coli* strains were stored at −80°C in LB broth with 30% glycerol.

Adherence assays. Bacterial adherence was assessed in assays with Chang (human conjunctiva) cells and Hig-82 (rabbit synovium) cells, which were obtained originally from the American Tissue Culture Collection and were maintained as previously described (13). Adherence assays were performed as previously described (11). Briefly, bacteria were grown for 17 to 18 h on chocolate agar and then resuspended in BHI broth to an optical density at 600 nm (OD_{600}) of 0.8. The bacteria were inoculated onto a fixed confluent monolayer of cells in 24-well plates, and the plates were centrifuged for 5 min at 1,000 rpm and then incubated for 25 min at 37°C. Monolayers were rinsed with phosphate-buffered saline (PBS) to remove nonadherent bacteria and were then stained with Giemsa for examination by light microscopy. *K. kingae* isolates were classified as adherent if light microscopy at ×400 magnification revealed more than 50 bacteria/field (about five times more bacteria than observed with 269-492 *pilA1*::*aphA3*).

Analysis of pilus expression. *K. kingae* isolates were examined for the presence of pili by negative-staining transmission electron microscopy as previously described, and a minimum of 20 organisms per strain were examined for the presence of pili (11, 13).

Colony morphology. To determine colony morphology, isolates were grown for 17 to 18 h on chocolate agar and assessed by two independent observers with the aid of a hand lens. Colonies were classified as spreading/corroding, nonspreading/noncorroding, or domed.

Western analysis. To assess PilA1 antigenic variability, isolates were grown for 17 to 18 h on chocolate agar, resuspended in 1 ml of PBS to an OD_{600} of 0.8, and then centrifuged at 21,130 × *g* for 2 min. The bacterial pellets were resuspended in 200 μl PBS and mixed with 3× protein running buffer to produce whole-cell lysates. The resulting lysates were examined by Western analysis using guinea pig antiserum GP65 raised against PilA1 from *K. kingae* strain 269-492 and an anti-guinea pig horseradish peroxidase-conjugated secondary antibody (12).

DNA sequencing and analysis. Chromosomal DNA was prepared using the Wizard Genomic Purification kit (Promega, Madison, WI). The *pilA1* gene was amplified by nested PCR. The first round of amplification was performed with primers Pilin Region Rev#2 (ACGTGTCGACCCAGCAACACCGTC CAATCCAG) and Pilin Region Fwd#1 (ACGTGAATTCAAGCGCGTAT GCCGTGCGAC), and the second round of amplification was performed with primers PilA1seq#2Fwd (GCATGCACTCTGCTACCAAGTAAGGC) and PilA2seqRev#2 (AAACCAAACACCAAAGCCGCC). Comparison of predicted amino acid sequences was performed as previously described by Obert et al. (18).

Statistical analysis. Statistical analysis was performed using chi-square testing. *P* values were two sided, and *P* values of <0.05 were considered significant.

Nucleotide sequence accession numbers. The *pilA1* DNA sequences determined in this study were deposited in GenBank and assigned accession numbers as follows for the *K. kingae* strains listed: GU581047 for strain PYO4a, GU581048 for strain PYKK113, GU581049 for strain PYKK114, GU581050 for strain PYKK123, GU581051 for strain PYKK125, GU581052 for strain PYC1639, GU581053 for strain PYKK200, GU581054 for strain PYKK243, GU581055 for strain PYKK56, GU581056 for strain PTKH-B, GU581057 for strain PYKHPH-1, GU581058 for strain PYKK061, GU581059 for strain PYKK068, GU581060 for strain PYKK069, GU581061 for strain PYKK096, GU581062 for strain PYKK129, and GU581063 for strain PYKK181.

RESULTS

Colony morphology and piliation among *K. kingae* clinical isolates. Earlier reports described two *K. kingae* colony types called spreading/corroding and nonspreading/noncorroding colonies (5, 9). The spreading/corroding colony type is characterized by a uniform small raised central colony surrounded by a large fringe and correlates with high-density piliation (5, 9, 12). In contrast, the nonspreading/noncorroding colony type is characterized by a large, flat colony with a smaller fringe and correlates with low-density piliation (5, 9, 12). Upon examining our collection of 64 clinical isolates of *K. kingae*, we observed an additional colony type that was similar in size to nonspreading/noncorroding colonies but was more domed and lacked a fringe, resembling the colonies formed by the nonpiliated *K. kingae* strain 269-492 *pilA1*::*aphA3* (Fig. 1). We refer to this colony type as domed.

To assess the relationship more generally between expression of type IV pili and colony morphology, we examined our collection of clinical isolates by negative-staining transmission electron microscopy. As summarized in Table 1, 64% (41/64) of the isolates had surface fibers that resembled the type IV pili present on *K. kingae* strain 269-492. All of the piliated isolates formed either spreading/corroding or nonspreading/noncorroding colonies, and all of the nonpiliated isolates formed domed colonies. Strain PYKK003 expressed atypical short fibers and formed domed colonies and was considered nonpiliated. To confirm that the type IV pilus-like fibers on the 41 piliated isolates were truly type IV pili, we insertionally inactivated *pilA1* in five fiber-expressing clinical isolates, namely, PYKK012, PYKK060, PYKK061, PYKK081, and PYKK082. Examination of the resulting mutants by negative-staining transmission electron microscopy revealed an absence of fibers in all cases (data not shown), confirming that the fibers in the parent strains are type IV pili.

Considered together, these results demonstrate that there are three colony morphologies among clinical isolates of *K. kingae*, including spreading/corroding, nonspreading/noncorroding, and domed colony types. Spreading/corroding colonies are associated with high-density piliation, nonspreading/noncorroding colonies are associated with low-density piliation, and domed colonies are associated with a lack of pili (12).

Relationship between piliation and colony morphology and site of isolation. Given that only some of the isolates in our collection were piliated, we examined whether pilus expression correlated with the anatomic site of isolation. As shown in Tables 1 and 2, a high percentage of respiratory isolates (79%; 22/28) and nonendocarditis blood isolates (79%; 11/14) were piliated and a relatively low percentage (36%; 8/22) of joint fluid, bone, and endocarditis blood isolates were piliated. Among the piliated respiratory isolates, 64% (14/22) formed spreading/corroding colonies (5, 9). Among the piliated nonendocarditis blood isolates, 36% (4/11) formed spreading/corroding colonies (5, 9). Among the piliated joint fluid, bone, and endocarditis blood isolates (referred to as invasive isolates), 25% (2/8) formed spreading/corroding colonies.

Analysis using chi-square testing revealed that piliation was more common among respiratory and nonendocarditis blood isolates than among joint fluid, bone, and endocarditis blood isolates ($P = 0.02$ for respiratory and nonendocarditis blood isolates compared to joint fluid, bone, and endocarditis blood isolates) (Table 2). Additional analysis demonstrated that high-density piliation was more common among respiratory tract isolates than among nonendocarditis blood and focal invasive isolates (chi-square test, $P = 0.008$ for respiratory isolates compared to nonendocarditis blood and joint fluid, bone, and endocarditis blood isolates) (Table 2).

Correlation between piliation and adherence. In previous work, we demonstrated that *K. kingae* strain 269-492 requires type IV pili for adherence to respiratory epithelial and synovial cells (11). To assess whether type IV pili are required in general for *K. kingae* adherence, we assessed our collection of clinical isolates for the ability to adhere to Chang respiratory epithelial cells and Hig-82 synovial cells. Overall, 64% (41/64) of the isolates adhered to Chang cells and 62% (40/64) of the isolates adhered to

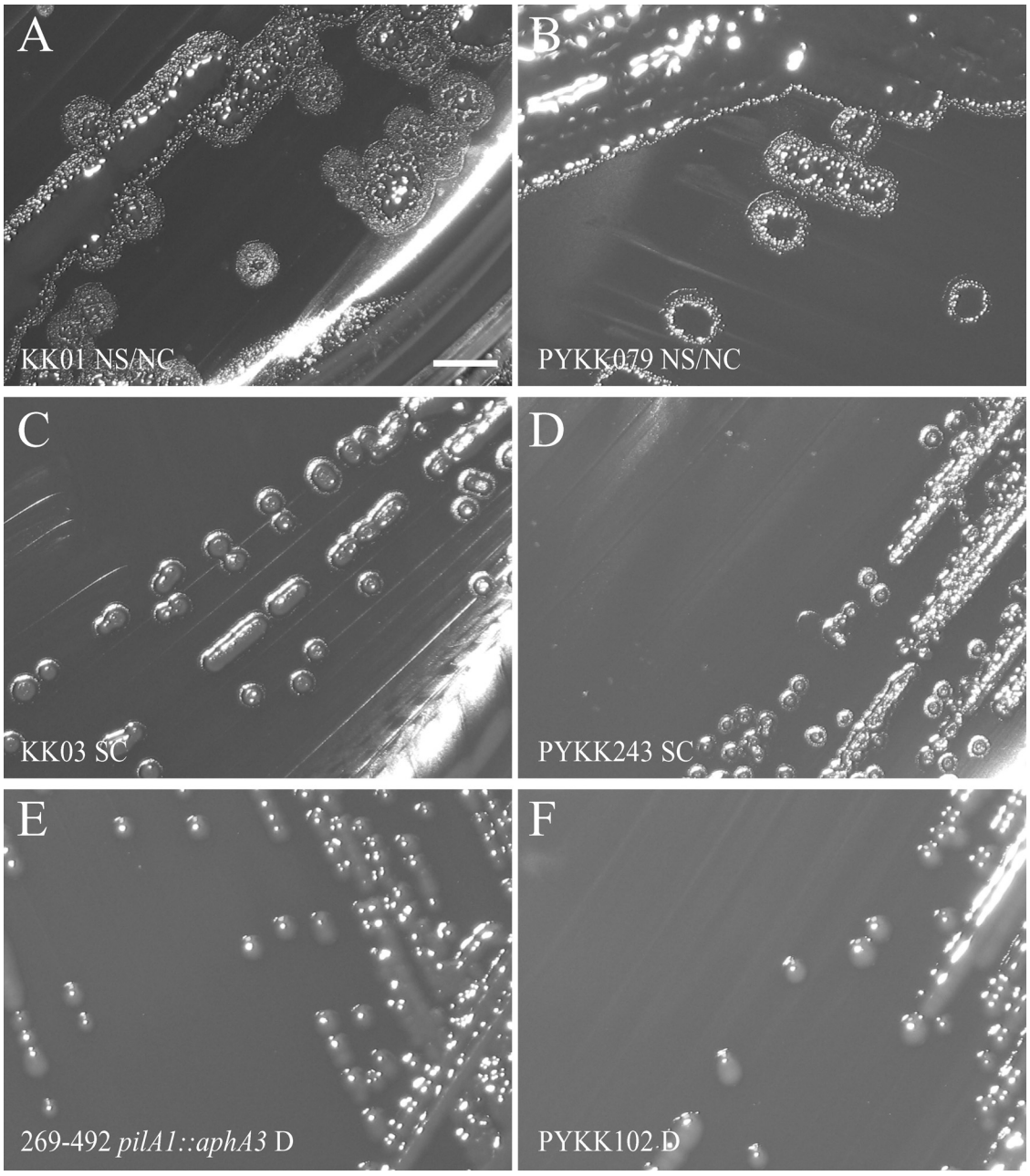

FIG. 1. Representation of the three different colony types formed by *K. kingae* clinical isolates and *K. kingae* strain 269-492 derivatives. (A) Strain KK01, nonspreading/noncorroding derivative of 269-492. (B) Strain PYKK79, nonspreading/noncorroding clinical isolate. (C) Strain KK03, spreading/corroding derivative of 269-492. (D) Strain PYKK243, spreading/corroding clinical isolate. (E) Strain 269-492 *pilA1*::*aphA3*, nonpiliated derivative of 269-492 with domed morphology. (F) Strain PYKK102, nonpiliated clinical isolate with domed morphology. NS/NC stands for nonspreading/noncorroding, SC stands for spreading corroding, and D stands for domed. The bar in panel A equals 3 mm.

Hig-82 cells (Table 1 and Fig. 2). Consistent with our earlier observations with *K. kingae* strain 269-492, only the piliated isolates were adherent (11). Furthermore, the *pilA1* mutants of strains PYKK012, PYKK060, PYKK061, PYKK081, and PYKK082 were nonadherent (data not shown). Strain PYKK114 was sparsely piliated and was adherent to Chang cells and nonadherent to Hig-82 cells.

Variability in PilA1 sequence among strains. Earlier analysis demonstrated that *K. kingae* surface proteins exhibit antigenic variability among strains (33). To assess the level of strain-to-strain variability in PilA1, we began by examining the piliated isolates by Western analysis using an antiserum raised against PilA1 from *K. kingae* strain 269-492. Overall, only 53% (22/41) of the piliated isolates reacted with our antiserum against PilA1. As summarized in Table 1 and highlighted with a representative sampling of piliated isolates in Fig. 3, of the 22 isolates with detectable levels of PilA1 by Western analysis, only 27% (6/22) reacted as well as strain 269-492.

TABLE 2. Summary of pilus expression and colony morphology by site of isolation

Site of isolation	% Piliated[a]	% Spreading/corroding colony type[b]
Total	64 (41/64)[c]	49 (20/41)
Respiratory	79 (22/28)	50 (14/28)
Blood nonendocarditis	79 (11/14)	29 (4/14)
Focal invasive	36 (8/22)	9 (2/22)
Joint fluid	43 (6/14)	14 (2/14)
Bone	25 (1/4)	0 (0/4)
Blood endocarditis	25 (1/4)	0 (0/4)

[a] Percent piliation is lower in focal invasive isolates than in respiratory and blood nonendocarditis isolates (chi-square test, $P = 0.02$).
[b] Percent spreading/corroding colony type is lower in blood endocarditis and focal invasive isolates than in respiratory isolates (chi-square test, $P = 0.008$).
[c] The values in parentheses are the number of isolates with that morphology/total number of isolates.

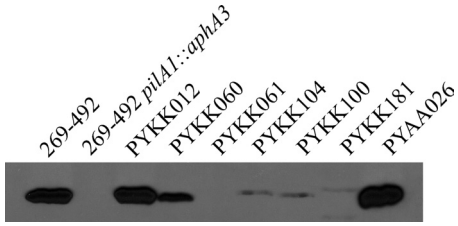

FIG. 3. Representative Western blot assay showing the range of signals detected when whole-cell lysates of piliated *K. kingae* clinical isolates were examined for PilA1 reactivity using antiserum GP65 raised against PilA1 from *K. kingae* strain 269-492. Strain 269-492 was used as a positive control, and strain 269-492 *pilA1*::*aphA3* was used as a negative control.

To assess whether the range of reactivity by Western analysis reflected variability in the PilA1 sequence or variability in the density of piliation, we determined the nucleotide sequence of the *pilA1* gene from 17 clinical isolates with various levels of detectable PilA1. As shown in Fig. 4, pairwise analysis of the predicted amino acid sequences revealed substantial variability among the 17 isolates, with sequence identity ranging between 66% and 100% and averaging 79%. Only 52% of the residues were identical across all isolates. The N-terminal one-third of the protein was highly conserved, and the sequence over residues 66 to 120 and 153 to 164 (the C terminus of the protein) was highly divergent (Fig. 5). Overall, the Western analysis and sequencing data indicate that PilA1 is antigenically diverse among strains of *K. kingae*.

DISCUSSION

K. kingae is being recognized increasingly as a leading cause of pediatric joint and bone infections. Previous work established that type IV pili are necessary for *K. kingae* strain 269-492 adherence to respiratory epithelial and synovial cell lines (11). To gain further insight into the importance of pili at different points in the pathogenic process, we examined a collection of clinical isolates for piliation. We found that a high percentage of respiratory and nonendocarditis blood isolates and a low percentage of joint fluid, bone, and endocarditis blood isolates expressed pili. Additionally, we observed that only piliated isolates were capable of adherence to respiratory epithelial and synovial cells. We also discovered that the major

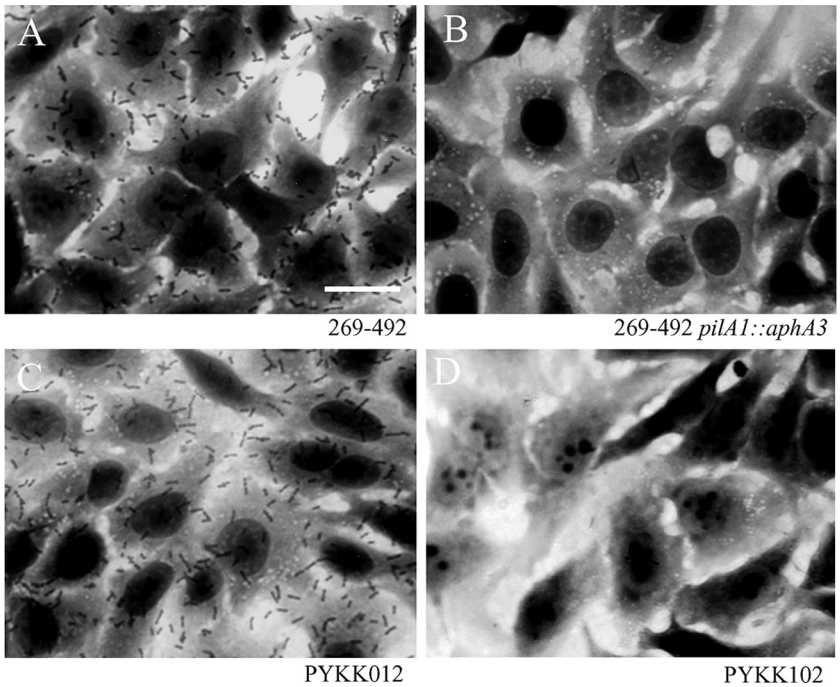

FIG. 2. Representative light micrographs of *K. kingae* adherence to Chang cells. (A) Strain 269-492, piliated. (B) Strain 269-492 *pilA1*::*aphA3*, nonpiliated. (C) Strain PYKK012, piliated. (D) Strain PYKK102, nonpiliated. Strain PYKK012 is representative of adherent clinical isolates, and strain PYKK102 is a representative nonadherent clinical isolates. The bar in panel A equals 15 μm.

Strain	269-492	PY04a	PYC1639	PYKK56	PYKK113	PYKK114	PYKK123	PYKK125	PYKK200	PYKK243	PYKK069	PYKK096	PYKK129	PYKK181	PYKH-B	PYKH-PH1	PYKK061	PYKK068	
Reactivity	++++	-	+	-	-	-	-	-	+	++	-	-	++++	+	++++	++++	-	-	
Colony	SC	NS/NC	SC	NS/NC	SC	NS/NC	NS/NC	SC	SC	SC	NS/NC	NS/NC	NS/NC	NS/NC	NS/NC	NS/NC	SC	NS/NC	
% Identity	***	67.8	78.3	78.3	80.6	76.3	81.9	80.6	78.3	78.3	100	77.1	100	79.5	100	100	68.5	81.9	269-492
		***	71.1	71.1	68.7	67.6	68	68.7	71.1	71.1	67.8	71.1	67.8	68.9	67.8	67.8	85.2	68	PY04a
			***	100	83.2	78.2	77.4	83.2	100	100	78.3	75.8	78.3	75.6	78.3	78.3	71.1	77.4	PYC1639
				***	83.2	78.2	77.4	83.2	100	100	78.3	75.8	78.3	75.6	78.3	78.3	71.1	77.4	PYKK56
					***	79.9	82.4	100	83.2	83.2	80.6	75.5	80.6	78.6	80.6	80.6	69.4	82.4	PYKK113
						***	80.5	79.9	78.2	78.2	76.3	75.6	76.3	75	76.3	76.3	68.2	80.5	PYKK114
							***	82.4	77.4	77.4	81.9	77.6	81.9	76.6	81.9	81.9	66	100	PYKK123
								***	83.2	83.2	80.6	75.5	80.6	78.6	80.6	80.6	69.4	82.4	PYKK125
									***	100	78.3	75.8	78.3	75.6	78.3	78.3	71.1	77.4	PYKK200
										***	78.3	75.8	78.3	75.6	78.3	78.3	71.1	77.4	PYKK243
											***	77.1	100	79.5	100	100	68.5	81.9	PYKK069
												***	77.1	81.6	77.1	77.1	71.8	77.6	PYKK096
													***	79.5	100	100	68.5	81.9	PYKK129
														***	79.5	79.5	69.6	76.6	PYKK181
															***	100	68.5	81.9	PYKH-B
																***	68.5	81.9	PYKH-PH1
																	***	66	PYKK061
																		***	PYKK068

FIG. 4. Pairwise analysis of PilA1 sequences. Reactivity indicates the level of reactivity with antiserum raised against PilA1 from *K. kingae* strain 269-492. Colony indicates the colony type; SC refers to spreading/corroding colonies, and NS/NC refers to nonspreading/noncorroding colonies.

pilus subunit PilA1 displays a relatively high degree of strain-to-strain variability in sequence.

Previous work described the presence of two *K. kingae* colony types called spreading/corroding and nonspreading/noncorroding, which correlate with high-density piliation and low-density piliation, respectively (5, 9, 12). In this study, we observed an additional spontaneously occurring colony type that correlated with an absence of pili and a lack of bacterial adherence. This nonpiliated colony type is similar in size to the nonspreading/noncorroding colony type but is slightly more domed and lacks any fringe, virtually identical to the colonies produced by *pilA1* mutants.

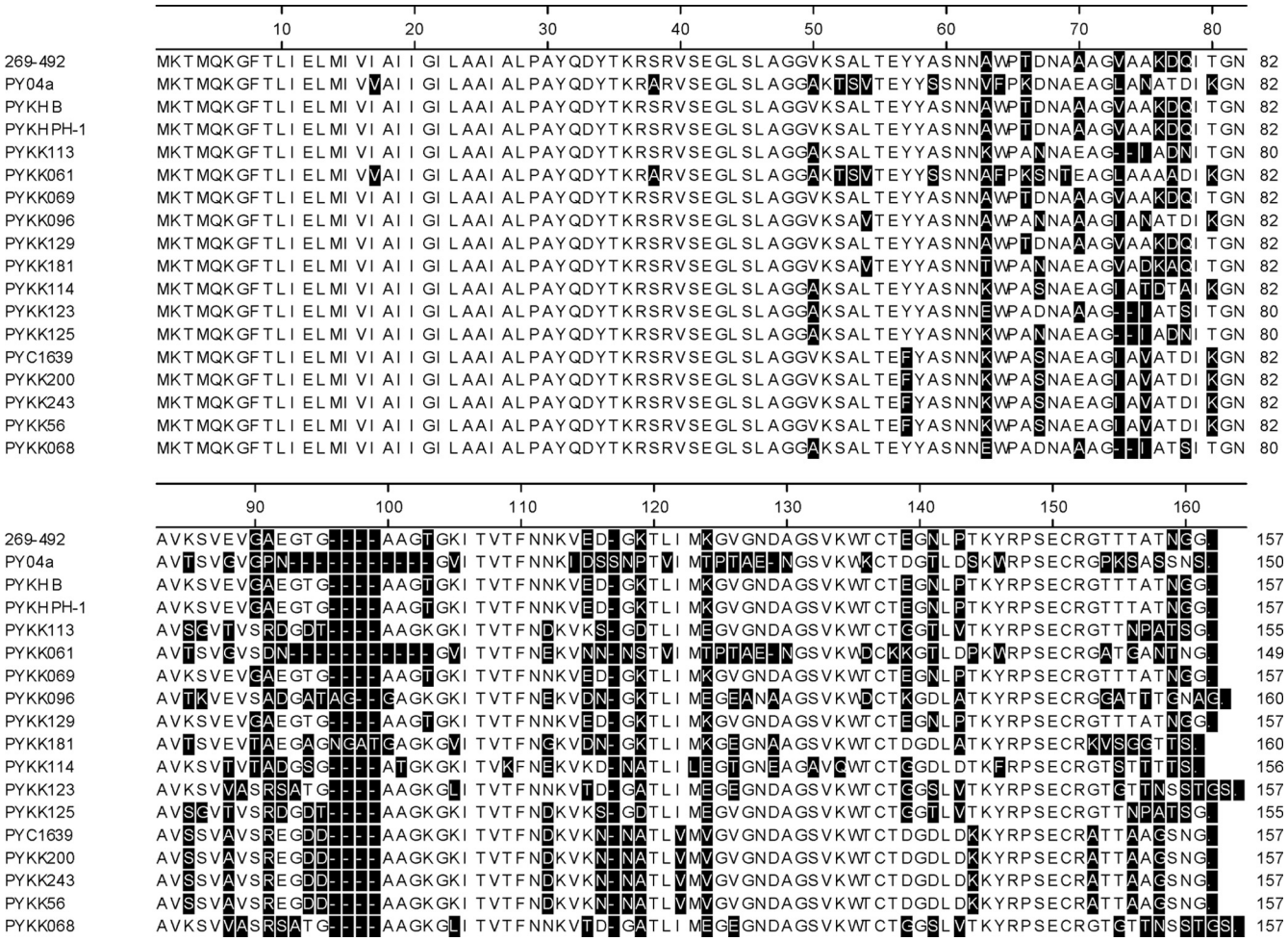

FIG. 5. Alignment of PilA1 predicted amino acid sequences from *K. kingae* strain 269-492 and 17 clinical isolates. Residues highlighted in black are nonidentical to the majority of the residues at that position.

Examination of our collection of clinical isolates for pilus expression revealed a high prevalence of piliation among respiratory isolates and nonendocarditis blood isolates and a low prevalence of piliation among invasive isolates. These results suggest that pili may provide a selective advantage early in infection and a selective disadvantage at later stages in the pathogenic process. In support of this conclusion, examination of the colony morphology and level of piliation revealed a progressive decrease in the density of pili on piliated isolates from the respiratory tract to those from the bloodstream to those from invasive sites. Interestingly, the selection against type IV pili during *K. kingae* invasive disease differs from observations with *Neisseria meningitidis* bacteremia and meningitis, which are characterized by persistence of bacterial piliation (7, 21).

The progressive loss of piliation during the development of *K. kingae* invasive disease highlights the importance of a process for controlling the level of pilus expression. In previous work, we found that mutation of the *pilS* gene, which encodes the PilS sensor histidine kinase, results in a shift from high-density piliation to low-density piliation (12), suggesting a mechanism for the change from spreading/corroding colonies to nonspreading/noncorroding colonies during infection. In addition, we discovered that insertional inactivation of the *rpoN* gene, which encodes σ54, or the *pilR* gene, which encodes the PilR response regulator, eliminated the expression of *pilA1* (12), raising the possibility that conversion from spreading/corroding or nonspreading/noncorroding colonies (piliated colonies) to domed colonies (nonpiliated colonies) as *K. kingae* transitions from the respiratory tract or the blood to invasive sites may be a consequence of mutations in *pilR* or *rpoN*. The observation that clinical isolates vary in the density of piliation also suggests that mutations in *pilS*, *pilR*, and *rpoN* are representative of the range of mutations that alter *K. kingae* pilus expression *in vivo*. More-detailed molecular studies are necessary to fully define the mechanisms used by *K. kingae* to alter pilus expression during natural infection.

Analysis of the predicted amino acid sequence of the *K. kingae* PilA1 protein demonstrated significant variation between strains. Similar to PilE in *Neisseria* species, the *K. kingae* PilA1 protein can be divided into three regions, namely, a highly conserved N-terminal region, a variable middle region, and a variable C-terminal region (8). However, the variable regions appears to be shorter in PilA1 from *K. kingae* than in PilE from *Neisseria* species. Interestingly in *Neisseria* species and *Pseudomonas aeruginosa*, the conserved N terminus of the pilin is located in the core of the pilus and the variable middle and C-terminal regions are surfaced exposed (2). If *K. kingae* type IV pili are structurally similar to *Neisseria* and *Pseudomonas* pili, the conserved regions of *K. kingae* type IV pili may be located in the pilus core and the variable middle and C-terminal regions may be present on the surface of the pilus. It is interesting to speculate that the PilA1 sequence variability observed may facilitate prolonged colonization by a particular strain or allow repeated colonization by different strains of *K. kingae*.

This work emphasizes the importance of type IV pili in *K. kingae* colonization and underscores the selection against type IV pili as *K. kingae* enters the bloodstream and disseminates to remote sites. In ongoing work, we are exploring the factors that control the density of pilus expression and that influence pilus antigenic variability in *K. kingae*. These studies will provide an improved understanding of the role of type IV pili and other factors in *K. kingae* pathogenesis.

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