

DNA Binding: a Novel Function of *Pseudomonas aeruginosa* Type IV Pili

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The opportunistic pathogen *Pseudomonas aeruginosa* produces multifunctional, polar, filamentous appendages termed type IV pili. Type IV pili are involved in colonization during infection, twitching motility, biofilm formation, bacteriophage infection, and natural transformation. Electrostatic surface analysis of modeled pilus fibers generated from *P. aeruginosa* strain PAK, K122-4, and KB-7 pilin monomers suggested that a solvent-exposed band of positive charge may be a common feature of all type IV pili. Several functions of type IV pili, including natural transformation and biofilm formation, involve DNA. We investigated the ability of *P. aeruginosa* type IV pili to bind DNA. Purified PAK, K122-4, and KB-7 pili were observed to bind both bacterial plasmid and salmon sperm DNA in a concentration-dependent and saturable manner. PAK pili had the highest affinity for DNA, followed by K122-4 and KB-7 pili. DNA binding involved backbone interactions and preferential binding to pyrimidine residues even though there was no evidence of sequence-specific binding. Pilus-mediated DNA binding was a function of the intact pilus and thus required elements present in the quaternary structure. However, binding also involved the pilus tip as tip-specific, but not base-specific, antibodies inhibited DNA binding. The conservation of a Thr residue in all type IV pilin monomers examined to date, along with the electrostatic data, implies that DNA binding is a conserved function of type IV pili. Pilus-mediated DNA binding could be important for biofilm formation both in vivo during an infection and ex vivo on abiotic surfaces.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is a major cause of nosocomial infections and a leading cause of hospital-acquired pneumonia (10). *P. aeruginosa* is also a major cause of severe infections in immunocompromised individuals, including human immunodeficiency virus (32) and neutropenic cancer patients (33). Furthermore, *P. aeruginosa* can cause bacteremia in burn victims (43) and ulcerative keratitis of the cornea in users of extended-wear soft contact lenses (14). Individuals suffering from cystic fibrosis (CF) are normally colonized with *P. aeruginosa* by adolescence, which causes a severe persistent respiratory infection that results in extensive lung damage (6). Treatment of *P. aeruginosa* infections requires antimicrobial therapy, which is challenging due in part to a high intrinsic resistance to many antibiotics and disinfectants (37). Therefore, *P. aeruginosa* infections result in considerable morbidity and mortality of immunocompromised individuals.

After aspiration or inhalation, the initial step in the establishment of a *P. aeruginosa* infection is the adherence to susceptible host cells via type IV pili (59). *P. aeruginosa* type IV pili are assembled from 15-kDa pilin monomers, and adherence of *P. aeruginosa* to host cells through the type IV pilus is a tip-associated event that is mediated by the C-terminal re-

gion of the pilin structural subunit (28). Type IV pili are also important colonization factors for several other gram-negative human pathogens, including *Neisseria gonorrhoeae*, *Neisseria meningitidis* (36), enteropathogenic *Escherichia coli* (17), enterotoxigenic *E. coli* (18), *Vibrio cholerae* (55), and *Legionella pneumophila* (52). Although type IV pili have a central role during infection, these fibrous structures are multifunctional and therefore involved in other bacterial processes. In addition to their role as colonization factors, the type IV pili of many species also play a role in twitching motility (35), biofilm formation (39), natural transformation (13), and bacteriophage infection (4, 5).

Natural transformation is a process unique to bacteria and involves active DNA uptake from the environment that can result in the lateral (horizontal) acquisition of new genetic information in a heritable form (30). The involvement of type IV pili in natural transformation has been noted for several gram-negative bacteria, including *Thermus thermophilus* (15), *L. pneumophila* (53), *N. gonorrhoeae* (50), and *Pseudomonas stutzeri* (21). Although *P. aeruginosa* produces type IV pili, no evidence of natural transformation has been observed to date (8). However, the *pilA* gene of *P. aeruginosa* can restore natural transformation in a *P. stutzeri pilA* mutant (21) and can complement a *pilE* mutant in *N. gonorrhoeae* (1). Therefore, while *P. aeruginosa* itself is not transformation competent, the type IV pili of *P. aeruginosa* can function during the process of DNA uptake. Intriguingly, there is a link between the expression of type IV pili and the ability to bind DNA to the bacterial surface. Mutations that affect the expression of type IV pili in *N. gonorrhoeae* reduce the amount of nonspecific DNA binding

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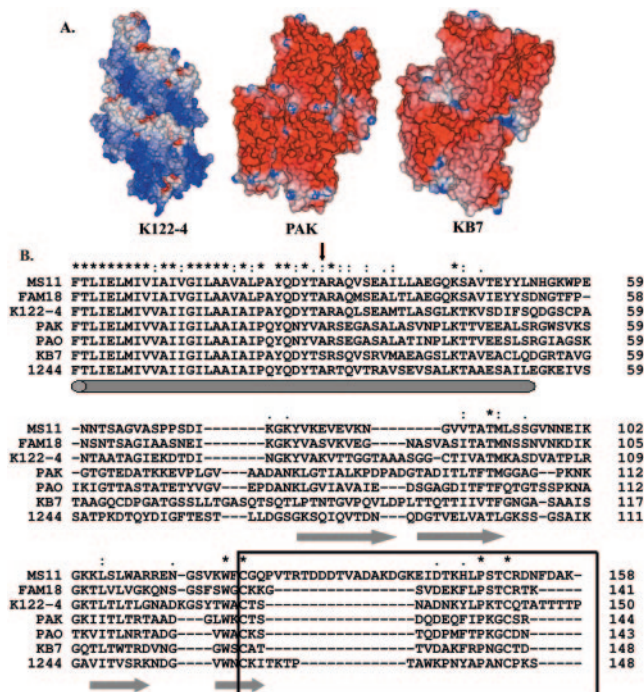


FIG. 1. Amino acid sequence and structural aspects of *P. aeruginosa* type IV pilins. (A) Electrostatic surface representation generated by using Delphi in InsightII of modeled pilins assembled into a pilus fiber (as described in reference 27) from *P. aeruginosa* strains K122-4, PAK, and KB-7. Blue represents a positive charge, red represents a negative charge, and white represents a neutral charge. (B) Multiple-sequence alignment of type IV pilins. Strictly, highly, and moderately conserved positions are indicated by asterisks, colons, and periods, respectively, above the sequences, and conserved secondary structural elements, based on an average for solved pilin structures, are indicated below the sequences. The N-terminal α -helix is indicated by a cylinder, and β -sheets are indicated by arrows. Production of soluble pilin monomers for structural studies (2, 24, 27) is achieved by truncation of the 28 N-terminal residues, indicated by a vertical arrow. The disulfide-bound receptor binding loop is enclosed in a box. The sequences are the sequences of *N. gonorrhoeae* strain MS11 (gi 3212472), *N. meningitidis* strain FAM18 (gi 2228578), and *P. aeruginosa* strains K122-4 (gi 77636), PAK (gi 120438), PAO (gi 120440), KB-7 (gi 3219798), and 1244 (gi 77632). The alignment was prepared with CLUSTALW (56) with minor manual editing.

at the cell surface (1). Furthermore, pilus-retraction-deficient mutants of *P. stutzeri* bind more DNA than wild-type bacteria, although the ability to take up DNA is greatly reduced (22).

The observations described above, combined with the surface electrostatic profiles and structural data for the type IV pili of *P. aeruginosa* (Fig. 1), led to the hypothesis that type IV pili may bind DNA nonspecifically. Although this phenomenon may not be important for productive natural transformation, it may facilitate other pilus-associated functions. Thus, we investigated the ability of type IV pili from *P. aeruginosa* to bind DNA. We demonstrated that PAK, K122-4, and KB-7 pili bind to DNA obtained from several sources and that DNA binding is specific, concentration dependent, and saturable. Binding is dependent on the intact pilus structure and is also a tip-associated event that occurs through dominant interactions with the backbone and a preference for pyrimidine bases.

MATERIALS AND METHODS

Bacterial strains, DNA sources, and antibodies. PAK pili were produced from *P. aeruginosa* strain PAK/2Pfs, a multipiliated mutant of *P. aeruginosa* PAK (4). K122-4 and KB-7 pili were expressed and purified from *P. aeruginosa* strain DB2, a pilus-deficient and retraction-defective strain (46). *P. aeruginosa* cells were cultured in Luria-Bertani broth or tryptic soy broth (Difco) at 37°C. *E. coli* DH5 α cells were used as the host cells for the purification of pUCP19 (49). *E. coli* DH5 α harboring pUCP19 was cultured in Luria-Bertani broth supplemented with 100 μ g of carbenicillin (Sigma) per ml, and pUCP19 was purified by using a QIAfilter Giga kit (QIAGEN). Lyophilized salmon sperm DNA (Roche), polyphosphate (Sigma), and pyrophosphate (Fisher) solutions were prepared in 0.01 M phosphate buffer (PB) (pH 7.4). Generation of the following antibodies used in this study has been described previously: polyclonal PAK (48), PK3B, and PK9H (11).

Pilus purification, biotinylation, and confirmation of activity. Purification of PAK pili, K122-4 pili, and KB-7 pili was performed as described previously by Paranchych et al. (40). Purification of pili was confirmed by sodium dodecyl sulfate (SDS)–15% (wt/vol) polyacrylamide gel electrophoresis (PAGE) by using standard procedures (47) and by electron microscopy (EM) after staining with 1% aqueous molybdate (pH 7.0) by using an Hitachi H-700i electron microscope operating at an accelerating voltage of 75 kV (data not shown). His-Tagged K122-4 and PAK pilin monomers were constructed by inserting a five-His tag at the N terminus of Δ (1-28) truncated pilins expressed off a PET expression vector in *E. coli* BL21. His-tagged pilins were purified from *E. coli* BL21 after induction with isopropyl- β -D-thiogalactopyranoside (IPTG) as follows. Cells were harvested by centrifugation and lysed with a French press. Filtered lysate was applied to a HiTrap chelating HP (Amersham) nickel column, and an imidazole-eluted fraction was then applied to an SP10/16 ion-exchange column (Amersham) with the Unicorn version 3.00 software (Amersham). The authenticity of the His-tagged truncated pilins was confirmed by N-terminal protein sequencing, mass spectroscopy, SDS-PAGE, and Western blotting with appropriate antibodies. The procedure used for biotinylation of the purified pili has been described previously (60). The ability of the biotinylated pili to bind to asialo-GM₁ and GM₁ was determined in order to confirm the functional binding activity of the pili as previously described (28).

Direct DNA binding assays. Poly-L-lysine (Sigma) was used to coat polystyrene microtiter plates (Costar) at a concentration of 20 μ g/ml in 0.01 M sodium carbonate buffer (pH 9.5) (100 μ l per well) overnight at 4°C, and this was followed by three washes with phosphate-buffered saline (PBS) (0.01 M phosphate, 0.15 M NaCl; pH 7.4) containing 0.05% (wt/vol) bovine serum albumin (BSA) (buffer A). The wells were then blocked with 5% (wt/vol) BSA in PBS (pH 7.4) overnight at 4°C and washed three times with buffer A. Salmon sperm DNA or pUCP19 was bound to the microtiter plates at a concentration of 60 μ g/ml in 0.01 M PB (pH 7.4) (100 μ l per well) overnight at 4°C and washed three times with buffer A. Biotinylated pili or His-tagged pilin was diluted in PB (pH 7.4) containing 5 mM MgCl₂, and aliquots were transferred (100 μ l/well) into the microtiter plates. The microtiter plates were then incubated for 1.5 h at room temperature (RT) and washed five times with buffer A. Streptavidin-peroxidase (Sigma) and poly-His antibody (Sigma) were diluted 1:30,000 and 1:4,000, respectively, into buffer A, and 100 μ l was added to each well in the plates. The plates were incubated for 1.5 h at RT and then washed five times with buffer A. Secondary rabbit anti-mouse horseradish peroxidase (HRP) (Bio-Rad) diluted 1:3,000 was added to plates containing His-tagged pilin, incubated for 1.5 h, and washed five times with buffer A. Substrate buffer (0.01 M sodium citrate buffer [pH 4.2] containing 1 mM 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt [ABTS] and 0.03% [vol/vol] hydrogen peroxide) was added (125 μ l per well) to each plate. The microtiter plates were incubated at RT for 10 to 45 min with shaking at 150 rpm. The absorbance at 405 nm was determined by using a Multiskan Plus version 2.01 plate reader.

Competitive DNA binding assays. The microtiter plates were prepared as described above. A fixed concentration of biotinylated PAK (0.3 μ g/ml), K122-4 (1.4 μ g/ml), or KB-7 (3.5 μ g/ml) pili were mixed with various concentrations of pUCP19, salmon sperm, polyphosphate, or pyrophosphate. The reaction mixtures were incubated at RT for 30 min. Aliquots of each reaction mixture were transferred (100 μ l per well) into microtiter plates containing immobilized pUCP19 or salmon sperm DNA. The incubation times, washing procedures, and procedures used for development of the plates were identical to those used in the direct DNA binding assays.

Direct and competitive oligonucleotide binding assays. The oligonucleotides used in this study are listed in Table 1. Double-stranded oligonucleotides were prepared by heating preparations to 65°C and cooling them to room tempera-

TABLE 1. Oligonucleotides used for direct and competitive inhibition assays

Oligonucleotide	Sequence ^a
ss-NG	*ACTCGCCGTCTGAACCTA
ds-NG.....	*ACTCGCCGTCTGAACCTA
	TGAGCGGCAGACTTGGAT
BLAD.....	TGAGTATTCAACATTTCCGTGTCG
Poly(A).....	AAAAAAAAAAAAAAAAAAAA
Poly(T).....	TTTTTTTTTTTTTTTTTT
Poly(G).....	GGGGGGGGGGGGGGGGGG
Poly(C).....	CCCCCCCCCCCCCCCC

^a An asterisk indicates the location of biotinylation.

ture. PAK, K122-4, and KB-7 pili were used to coat polystyrene microtiter plates (Costar) at a concentration of 100 µg/ml in 0.01 M sodium carbonate buffer (pH 9.5) (100 µl per well) overnight at 4°C, and this was followed by three washes with buffer A. The wells were then blocked with 5% (wt/vol) BSA in PBS (pH 7.4) overnight at 4°C, and this was followed by three washes with buffer A. The double-stranded biotinylated oligonucleotide or mixtures of single-stranded biotinylated oligonucleotides and single-stranded unlabeled oligonucleotides were diluted in PB (pH 7.4) containing 5 mM MgCl₂, and 100-µl aliquots were added to the wells of the microtiter plates and incubated at room temperature for 1.5 h. The incubation times, washing procedures, and procedures used for development of the plates were identical to those used in the direct DNA binding assays.

Antibody inhibition assays. Microtiter plates were prepared as described above. Biotinylated PAK pili were incubated with saturating amounts of antibody (PK3B, PK99H, or polyclonal PAK) for 30 min at RT. Aliquots of the reaction mixtures were then transferred (100 µl per well) into the microtiter plates. The incubation times, washing procedures, and procedures used for development of the plates were identical to those used in the direct DNA binding assays. Enzyme-linked immunosorbent assays (ELISA) were performed by coating microtiter plates with biotinylated or unlabeled PAK pili (100 µl per well) at a concentration of 10 µg/ml in 0.01 M sodium carbonate buffer (pH 9.5) overnight at 4°C. The plates were then washed three times with buffer A and blocked overnight with 5% (wt/vol) BSA in PBS (pH 7.4). Antibodies and streptavidin-HRP were diluted in buffer A, and aliquots (100 µl per well) were incubated in the microtiter plates for 1 h at 37°C. The plates were washed three times with buffer A, and the secondary antibodies (either goat anti-rabbit HRP or rabbit anti-mouse HRP [Bio-Rad]) were added and incubated for 1 h at 37°C. The plates were washed three times with buffer A, and 125 µl of substrate buffer per well was added; this was followed by development and determination of the absorbance at 405 nm with a Multiskan Plus version 2.01 plate reader.

RESULTS

Pilin electrostatics and structure-based hypothesis for a pilus-associated DNA binding function. An analysis of the pilus surface electrostatics of the left-handed modeled K122-4 pilus revealed a definite helical band with a positive charge along the length of the fiber (27). Electrostatic surface analysis of right-handed pili modeled from the structure of PAK pilin (24) and the predicted structure of KB-7 pilin indicated that while the pilus surface electrostatics varied substantially among strains, a subtle helical pattern of solvent-exposed positive charge was a feature of the three pili (Fig. 1A). Although these models are speculative, the electrostatic surface charges are similar in other models of PAK pili (9) and K122-4 pili (2, 3). The pattern of positive charge occurs even in the absence of sequence conservation among the various pili (Fig. 1B). K122-4 pili have a more pronounced band of solvent-exposed positive charge; this band is less distinct on the surface of PAK and KB-7 pili. Based on the electrostatics alone, K122-4 pili should have the highest affinity for DNA and PAK and KB-7 should have lower but similar affinities for DNA.

Prediction of DNA binding through electrostatics is justified, since in general DNA binding sites of proteins contain patches with large positive electrostatic scores (26). Furthermore, examination of the β-sheet structure, which should produce the helical band of positive charge in the assembled pilus in the structures of the solved *P. aeruginosa* pilin monomers of PAK (24) and K122-4 (2, 3, 27), revealed that this domain is dominated by solvent-exposed Thr residues (Fig. 1B), which contribute to DNA binding in other proteins through interactions with the DNA backbone (31). Therefore, we hypothesized that type IV pili of *P. aeruginosa* could mediate DNA binding through interactions with the DNA backbone and that such binding should not display sequence specificity.

Pilus-mediated DNA binding. Pili were purified as previously described (40) from *P. aeruginosa* strain PAK/2Pfs and from *P. aeruginosa* DB2 expressing K122-4 or KB-7 pilin expressed from full-length *pilA* from the respective strains cloned into pUCP19, and the purity of these pili was confirmed by SDS-PAGE and EM (data not shown). Purified pili were biotinylated as previously described in order to probe pilus-DNA interactions (60). As *P. aeruginosa* pili bind specifically to asialo-GM₁ (28), binding of the biotinylated pili to asialo-GM₁ and GM₁ was examined to confirm that the biotinylated pili were functional (data not shown). A novel assay was used to quantify DNA binding, as methods used to assay specific DNA binding, like electrophoretic mobility shift assays, were not appropriate for the hypothesized nonspecific DNA binding activity of *P. aeruginosa* pili. Therefore, pUCP19 or salmon sperm DNA was immobilized in the wells of microtiter plates through electrostatic interactions with immobilized poly-L-Lysine.

Pili from all three strains of *P. aeruginosa* examined bound to immobilized DNA in a concentration-dependent and saturable manner, albeit with different apparent affinities (Fig. 2). As purified pili are variable in length, it was not possible to calculate molarity. However, estimates of the binding affinity based on binding isotherms could be calculated. PAK exhibited the highest apparent affinity for DNA, with a *K_d* of ~0.5 µg/ml, followed by K122-4 (*K_d*, ~1.3 µg/ml); KB-7 had the lowest affinity (*K_d*, ~4.5 µg/ml) (Fig. 2). These data correspond to a 2.6-fold difference in apparent affinity between PAK and K122-4, a 9-fold difference between PAK and KB-7, and a 3.4-fold difference between K122-4 and KB-7. Due to the low binding affinity, KB-7 binding to immobilized DNA did not saturate under the experimental conditions described, but saturation of KB-7 binding to DNA was observed when considerably higher KB-7 pilus concentrations were employed (data not shown). These results did not support the original hypothesis based solely on electrostatics that K122-4 should have the highest apparent affinity for DNA (Fig. 1A). Furthermore, as pili bound to both the pUCP19 plasmid (Fig. 2A) and salmon sperm DNA (Fig. 2B), pili can bind both eukaryotic and prokaryotic DNA. Again, it was not possible to determine if there were major differences between affinities for each type of DNA as the amount of DNA immobilized on a plate could not be calculated and also it was not possible to calculate the molarity of pili. The binding isotherm for K122-4 pili is distinctly sigmoidal and may indicate that there were multiple interacting binding interactions. His-tagged monomers from K122-4 and PAK were used to evaluate whether binding is a function of the

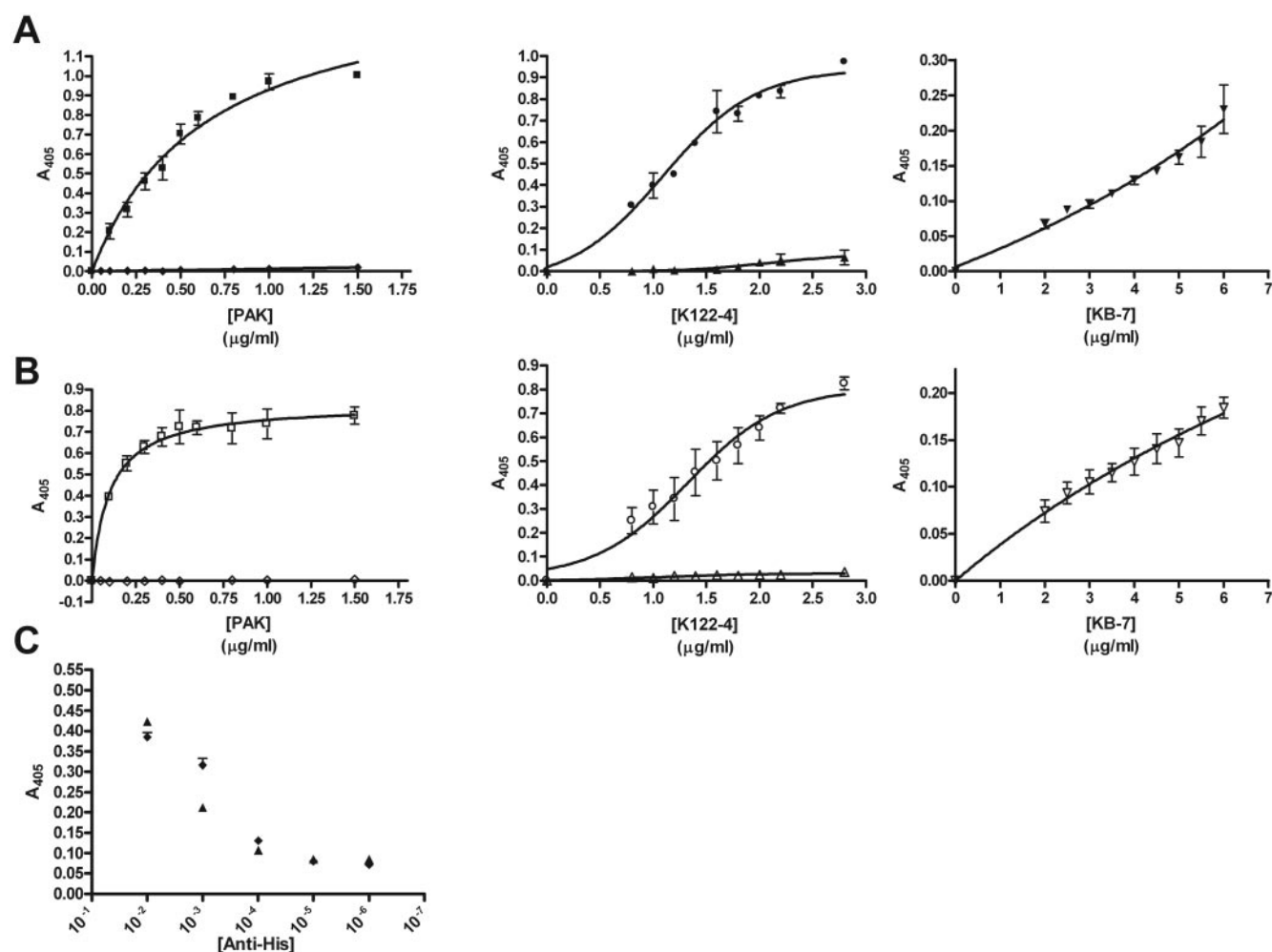


FIG. 2. Concentration dependence of DNA binding by purified biotinylated pili from *P. aeruginosa* strains PAK, K122-4, and KB-7. (A and B) The pUCP19 plasmid (A) (solid symbols) or salmon sperm DNA (B) (open symbols) was immobilized in the wells of microtiter plates by using poly-L-lysine. Various concentrations of biotinylated PAK (■ and □), K122-4 (● and ○), or KB-7 (▼ and ▽) pili or His-tagged PAK (◆) or K122-4 (▲) pilin were added to the plates and incubated for 1.5 h at room temperature. Binding was quantified spectrophotometrically by using streptavidin-HRP or primary mouse His antibodies and secondary HRP conjugate. The data are means and standard deviations of at least three replicates from two independent experiments. (C) ELISA of His-tagged PAK (◆) or K122-4 (▲) monomers. His-tagged monomers were immobilized in the wells of microtiter plates at a concentration of 10 $\mu\text{g/ml}$. Antibody dilutions were added to the wells containing the immobilized His-tagged monomers in the presence of 5 mM MgCl_2 and incubated for 1 h at 37°C. Binding was quantified spectrophotometrically by using anti-mouse HRP. The data are the means and standard deviations of three replicates.

intact pilus. Using an ELISA, we determined that the polyhistidine antibody recognized our constructs (Fig. 2C) under the conditions used in the binding assays. Monomers from PAK and K122-4 lacked the ability to bind DNA from both sources (Fig. 2), indicating that DNA binding is a function of the intact pilus.

Pilus-mediated DNA binding is a specific event that involves interactions with the DNA backbone. To demonstrate that poly-L-lysine and biotinylation did not interfere with the pilus-mediated DNA binding, competitive binding assays were performed. First, to demonstrate that poly-L-lysine has no effect on the ability of pili to bind DNA, purified biotinylated pili from the three strains were preincubated with either salmon sperm DNA or pUCP19 and then added to microtiter plates containing immobilized salmon sperm or pUCP19 DNA. Exogenous DNA from both sources reduced binding to immobilized DNA on the plates by more than 50%, and in the case

PAK pili over 90% inhibition was observed with both forms of DNA (Fig. 3). The apparent affinities of both eukaryotic and prokaryotic DNA for pili were high, as the apparent K_i was <5 nM (Fig. 3). These observations suggest that DNA binding is specific, that both prokaryotic and eukaryotic DNA are recognized, and that the use of poly-L-lysine to immobilize DNA on the microtiter plates did not compromise the assay or generate a high degree of nonspecific binding.

Unlabeled pili were able to inhibit DNA binding by biotinylated pili, indicating that biotinylation does not affect the ability of pili to bind to DNA (data not shown). The structure-based hypothesis that DNA binding can be mediated by residues on the pilin exposed in the β -sheet with the phosphate backbone of the DNA was tested by employing polyphosphate and pyrophosphate as competitors in binding assays with immobilized pUCP19 and salmon sperm DNA. Polyphosphate

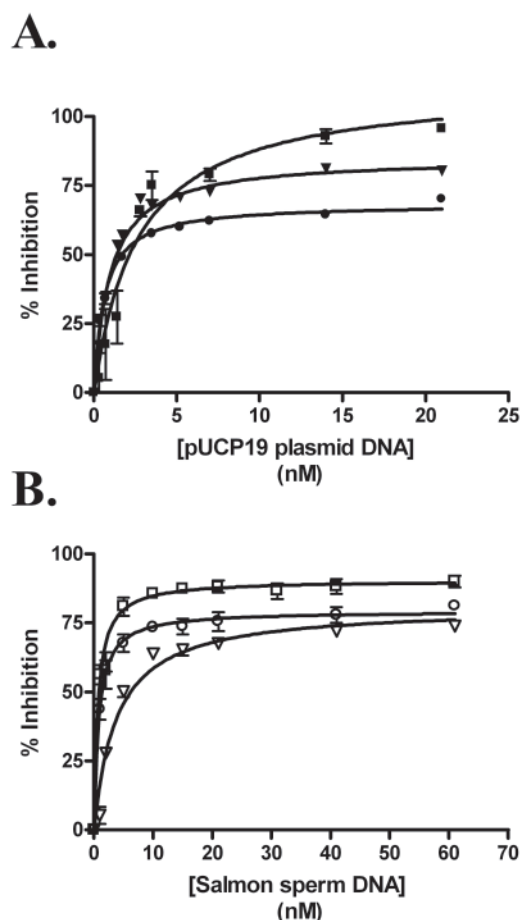


FIG. 3. Exogenous DNA inhibits binding of biotinylated pili to immobilized DNA. pUCP19 plasmid DNA (A) or salmon sperm DNA (B) was immobilized in the wells of microtiter plates by using poly-L-lysine. Biotinylated PAK (■ and □), K122-4 (● and ○), or KB-7 (▼ and ▽) pili were preincubated with various amounts of exogenous DNA for 30 min. The reaction mixtures were then added to microtiter plates containing immobilized DNA and incubated for 1.5 h at room temperature. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of at least three replicates from two independent experiments.

but not pyrophosphate was able to inhibit binding to immobilized DNA by more than 50% and, in the case of KB-7 pili, by more than 90% (Fig. 4). These data suggest that the interaction between KB-7 pili and DNA is almost completely dependent on electrostatics. However, there is also a hydrophobic component to binding as increasing the salt concentration enhanced binding to immobilized DNA (Fig. 4C). K122-4 and PAK pili showed similar binding patterns in the presence of different salt concentrations (Fig. 4C and data not shown). The ability of all types of pili to bind DNA at low salt concentrations suggested that binding is specific and not mediated by nonspecific hydrophobic interactions (Fig. 4C). These observations support the hypothesis that interactions with the DNA backbone are important for DNA binding by type IV pili but suggest that there is also a specific component to DNA binding.

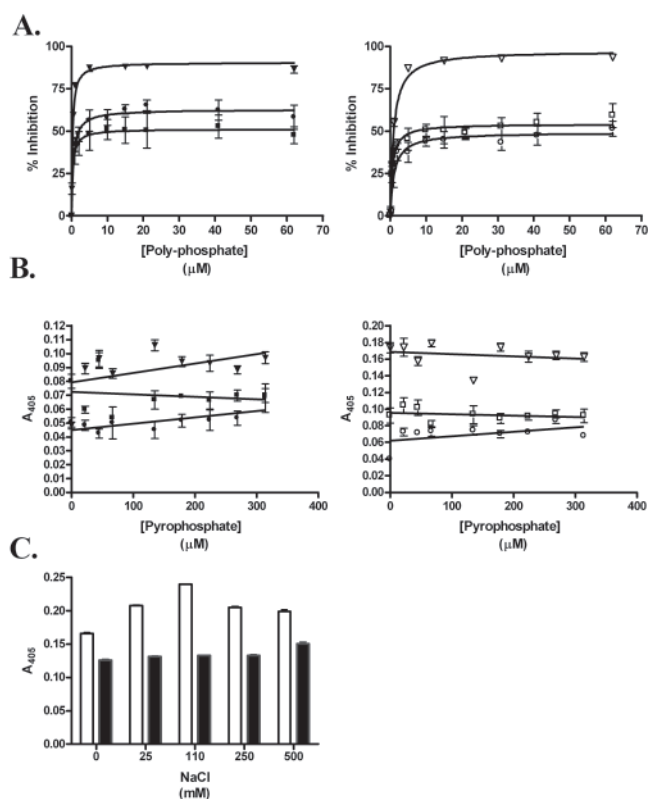


FIG. 4. (A and B) Polyphosphate but not pyrophosphate inhibits binding of biotinylated pili to immobilized DNA. pUCP19 plasmid DNA (open symbols) or salmon sperm DNA (solid symbols) was immobilized in the wells of microtiter plates by using poly-L-lysine. Biotinylated PAK (■ and □), K122-4 (● and ○), or KB-7 (▼ and ▽) pili were preincubated with various concentrations of polyphosphate (A) or pyrophosphate (B) for 30 min. The reaction mixtures were then added to microtiter plates containing immobilized DNA and incubated for 1.5 h at room temperature. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of at least three replicates from two independent experiments. (C) Salt dependence of pilus-mediated DNA binding. Biotinylated K122-4 pili (open bars) or KB-7 pili (solid bars) were added to microtiter plates containing immobilized pUCP19 at a concentration of 0.5 μ g/ml in 0.01 M PB (pH 7.4) containing various amounts of NaCl and incubated for 1.5 h at RT. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of three replicates.

Pilus-mediated DNA binding is not sequence specific, although binding to pyrimidine bases is preferred. To evaluate the possibility that DNA binding is also partially dependent on the nucleotide sequence or base composition, short oligonucleotides were employed. A preferred DNA binding sequence was not known; however, it has been demonstrated that *pilA* effectively complements a *pilE* mutant of *N. gonorrhoeae* (1), and as the pUCP19 plasmid contains the sequence GCGGTG TGAA (which is almost identical to the *N. gonorrhoeae* uptake sequence), a biotinylated synthetic oligonucleotide containing the *N. gonorrhoeae* 10-bp uptake sequence was employed.

The following complementary oligonucleotides were used to investigate this possibility (the 10-bp uptake sequence from *N. gonorrhoeae* is underlined): biotin-ACTCGCCGTCTGAACCTA and TAGGTTTCAGACGCGAGT. Purified PAK, K122-4, and

KB-7 pili were immobilized into the wells of microtiter plates, and double-stranded biotinylated oligonucleotide was added at various concentrations. Binding to the double-stranded oligonucleotide was concentration dependent for PAK and K122-4, but no appreciable binding was observed for KB-7 pili (Fig. 5A). PAK and K122-4 pili bound the double-stranded oligonucleotide with roughly equivalent affinities, displaying K_d s of 57 and 50 μ M, respectively, and apparent K_a s of 1.8×10^4 and 2×10^4 M^{-1} , respectively (Fig. 5A). All three types of pili were also able to bind to a single-stranded oligonucleotide containing the *N. gonorrhoeae* uptake sequence with affinities that were slightly but not significantly higher than those for the double-stranded oligonucleotide (data not shown).

Competitive binding assays with immobilized PAK pili were then performed to determine if binding to the *N. gonorrhoeae* uptake sequence was specific. An unrelated single-stranded oligonucleotide that was approximately the same length (BLAD) (Table 1) was used to inhibit binding of the ss-NG oligonucleotide to the immobilized pili (Fig. 5B). The ability of this oligonucleotide to inhibit binding indicated that the pilus-mediated DNA binding was not specific for the *N. gonorrhoeae* uptake sequence. Again, binding of the single-stranded oligonucleotides to immobilized PAK pili was not influenced by the biotin tag as S1 nuclease-treated single-stranded biotin-tagged oligonucleotide was unable to bind (Fig. 5C). To evaluate the possibility that base composition influenced oligonucleotide binding, we employed unlabeled single-stranded poly(A), poly(T), poly(G), and poly(C) oligonucleotides (Table 1) as solution phase competitors in binding assays with the single-stranded biotinylated *N. gonorrhoeae* uptake sequence. The poly(C) and poly(T) oligonucleotides were able to inhibit binding of the PAK pili to the *N. gonorrhoeae* uptake sequence (Fig. 5B). However, the poly(A) and poly(G) oligonucleotides were unable to inhibit binding of the PAK pili to the *N. gonorrhoeae* uptake sequence (Fig. 5B). Therefore, pyrimidine base interactions may be favored over purine base interactions during pilus-mediated DNA binding in the absence of apparent sequence specificity.

DNA binding is a tip-associated event that is dependent on the quaternary structure of the pilus. In order to determine which attributes of the intact pilus are involved in mediating DNA binding, a series of antibody inhibition assays were performed. As we had established that DNA binding is dependent on the intact pilus structure as pilin monomers were unable to bind DNA (Fig. 2), the relative importance of the pilus base and the relative importance of the pilus tip in DNA binding were examined. Monoclonal antibodies, including PK3B and PK99H, which are specific for the base of the pilus and the C-terminal receptor binding domain involved in adherence to asialo-GM₁ (11), respectively, and polyclonal PAK antibodies (48) were employed in this study. PAK pili were preincubated with saturating amounts of antibodies and then added to microtiter plates containing immobilized plasmid pUCP19. Preincubation with PK3B produced only marginal inhibition compared to PAK pili, probably through steric interference (Fig. 6A). Preincubation with both PK99H and polyclonal PAK antibodies resulted in a large reduction in binding to the immobilized pUCP19 (Fig. 6A). These data indicate that DNA binding is a tip-associated event that requires other attributes of the intact pilus as polyclonal PAK antibodies cause further reduc-

tions in binding compared to PK99H. Biotinylation of PAK pili had no effect on antibody recognition, nor did streptavidin recognize unlabeled PAK pili (Fig. 6B). Since DNA binding is a tip-associated event but involves the intact pilus structure, the relative contribution of the C-terminal receptor binding domain to DNA binding was determined. A direct assay to examine competition of biotinylated PAK pili and the PAK(128-144)ox peptide (28) was performed. The PAK(128-144)ox peptide, which is an analog of the C-terminal receptor binding domain (28), was unable to inhibit pilus-mediated DNA binding even at concentrations that were 1,000-fold higher than those of the biotinylated PAK pili (data not shown). These observations again support the hypothesis that the intact pilus structure is required for DNA binding, and although DNA binding is tip associated, the binding domain did not appear to overlap the C-terminal binding domain involved in adherence to asialo-GM₁.

DISCUSSION

Electrostatic modeling of the solvent-exposed surface of K122-4, PAK, and KB-7 modeled pili suggested that a band of positive charge may be a common feature of type IV pili (Fig. 1A). This band is present in a groove formed by the β -sheet domain found in the pilin monomers (2, 24, 41). Examination of this domain in PAK (24) and K122-4 (2) revealed that in this region there are a large number of solvent-exposed Thr residues (Fig. 1B), which are residues that have been found to mediate DNA backbone interactions in other DNA binding proteins (31). Furthermore, a number of solvent-exposed Lys, Asp, and Gly residues were found in this area (Fig. 1B), and these side chains have also previously been ascribed roles in DNA binding events (31). The CATH protein structural classification database (38) indicates that the pilin structure may be classified as a two-layer sandwich of the α - β folding class. The closest structural match to pilin that contains only this architecture appears to be the major coat proteins of icosahedral RNA phages belonging to the *Leviviridae* family, which includes bacteriophages MS2, Ga, Q-beta, and PP7. These phages are simple viruses containing a single-stranded positive-sense RNA molecule, a maturation protein, and 180 identical coat protein molecules that form the shell of the virus and bind the phage RNA, forming an icosahedral lattice with a triangulation number of 3 (19). MS2 coat protein dimers bind RNA through direct interaction of solvent-exposed side chains of β -sheet residues and where Ser and Thr side chains contribute directly to purine binding specificity (25). Interestingly, there is a solvent-exposed Thr residue at position 98 in the K122-4 structure that is solvent exposed and conserved in a β -strand of all type IV pilus sequences examined to date (Fig. 1B) (2). This β -strand is also rich in solvent-exposed Thr residues (Fig. 1B); the additional Thr residues may contribute to a pyrimidine binding preference. Thr98 may be conserved to facilitate DNA binding, as all variants of pili tested in this study are able to bind DNA even though the surface electrostatics vary. The potential contributions of the conserved Thr and other Thr residues to DNA binding are being investigated.

We showed that PAK, K122-4, and KB-7 pili were all able to bind directly to bacterial plasmid DNA (Fig. 2A) and salmon sperm DNA (Fig. 2B) in a concentration-dependent fashion.

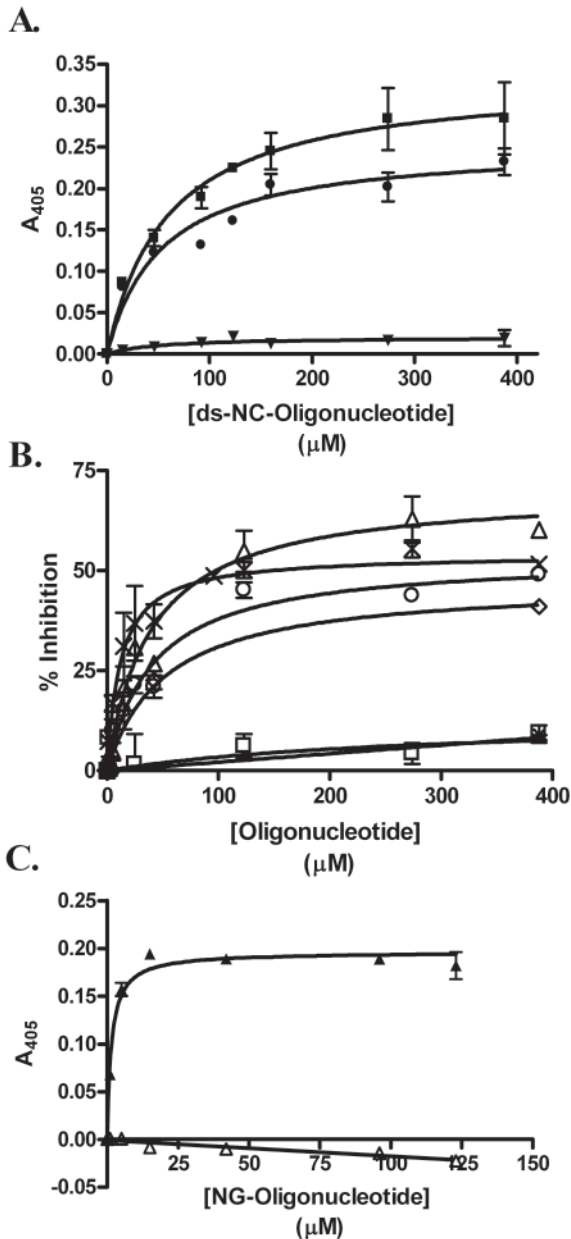


FIG. 5. Direct and competitive binding of biotinylated oligonucleotides to immobilized *P. aeruginosa* pili purified from strains PAK, K122-4, and KB-7. (A) Concentration dependence of binding of a biotinylated double-stranded oligonucleotide containing the *N. gonorrhoeae* uptake sequence (Table 1) to immobilized PAK pili (■), K122-4 pili (●), or KB-7 pili (▼). Various concentrations of the double-stranded NG oligonucleotide were added to the plates containing the immobilized pili and incubated for 1.5 h at room temperature. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of at least three replicates from two independent experiments. (B) Direct competitive binding of single-stranded oligonucleotides (Table 1) and biotinylated oligonucleotide ss-NG (15 μ M) was mixed with various concentrations of unlabeled NG (Δ), BLAD (×), poly(C) (○), poly(A) (□), poly(T) (◇), or poly(G) (*) added to the microtiter plates containing immobilized PAK pili and incubated for 1.5 h. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of at least three replicates from two independent experiments. (C) Direct binding of untreated ss-NG (Δ) or S1 nuclease-treated ss-NG (▲) to immobilized PAK pili.

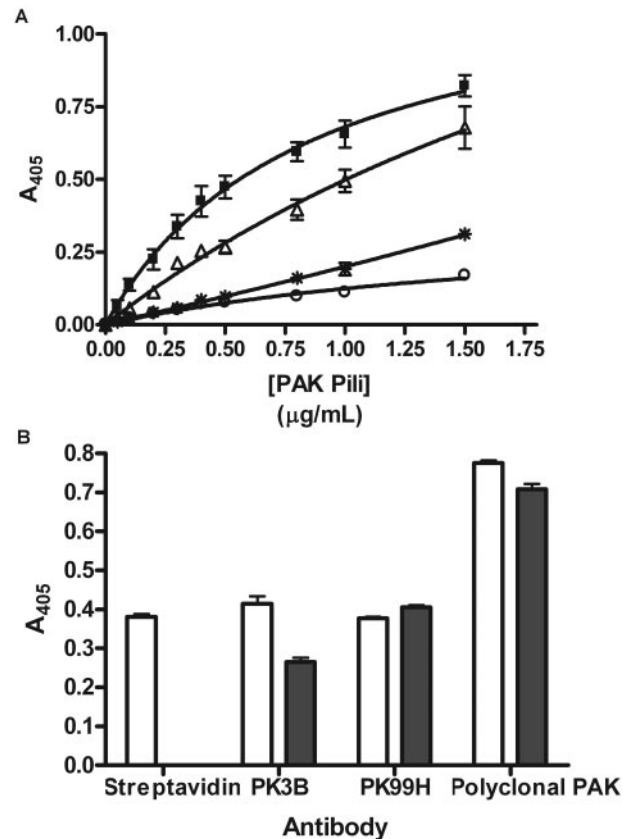


FIG. 6. Antibody inhibition of binding of biotinylated PAK pili to immobilized pUCP19. pUCP19 was immobilized in the wells of microtiter plates by using poly-L-lysine. (A) Biotinylated PAK pili were either not treated (■) or preincubated with PK99H (*), PK3B (Δ), or polyclonal PAK (○) antibodies for 45 min at room temperature. The reaction mixtures were then added to microtiter plates containing immobilized plasmid pUCP19 and incubated for 1.5 h at room temperature. Binding was quantified spectrophotometrically by using streptavidin-HRP. The data are the means and standard deviations of at least three replicates from two independent experiments. (B) ELISA performed with unlabeled or biotinylated PAK pili and streptavidin-HRP, PK99H, PK3B, and polyclonal PAK. The data are the means and standard deviation of three replicate wells.

This suggests that the polymorphism in the pilin proteins (23) does not affect the ability of the assembled pilus to bind to DNA. However, polymorphism in the pilin proteins does affect the affinity of the intact pilus for DNA as PAK had the highest apparent affinity, followed by K122-4 and KB-7 pili. In addition, DNA binding is a function of the intact pilus structure as pilin monomers are unable to bind to DNA from either source (Fig. 2). These observations indicate that the DNA binding site spans at least two pilin monomers or that the assembled monomer conformation in the pilus differs from the conformation of an unassembled monomer. Interestingly, Audette et al. (2) recently proposed that the K122-4 monomeric pilin may have a conformation in solution that differs from the pilin conformation observed in crystals that have a rather low solvent content. Although pilus-mediated DNA binding has been examined in *N. gonorrhoeae* and it has been concluded that pili do not bind DNA (12, 34), the studies were performed under conditions that would have disassembled the pilus structure

into monomeric units, and we demonstrated that pilin monomers are unable to bind DNA (Fig. 2).

Nonspecific DNA binding has previously been correlated to the expression of type IV pili composed of *P. aeruginosa* PAK pilin in *N. gonorrhoeae* (1) and in multipiliated retraction-deficient mutants of *P. stutzeri* (22). Although this nonspecific binding activity of the type IV pili may not be important for natural transformation, pilus-deficient strains of naturally transformable species have reduced DNA uptake potential (15, 21, 53). Furthermore, although *P. aeruginosa* is not naturally transformable, the type IV pili from this species can complement pilus mutants in natural transformation-competent species (1, 21), indicating that type IV pili have a conserved role during the process of natural transformation. Therefore, the nonspecific DNA binding activity of type IV pili may increase the DNA uptake potential in naturally transformable species, potentially through retraction of the pilus, bringing DNA to specific receptors on the cell surface (16). As functional type IV pili are not necessarily required for natural transformation (22, 29), the nonspecific DNA binding activity of the pili would only increase the amount of DNA available for uptake and therefore increase the uptake frequency. In this regard, it is interesting that *P. aeruginosa* has homologues of several of the proteins required for DNA uptake in *N. gonorrhoeae*, including ComE, ComA, and ComL (54). *P. aeruginosa* may therefore be able to accumulate DNA even though lateral gene transfer does not occur frequently; this possibility is under investigation.

The different pilus types displayed different apparent affinities for DNA, and the results of both direct and competitive binding assays suggest that PAK pili have the highest apparent affinity for DNA, followed by K122-4 and KB-7 pili (Fig. 2 and 3). Pilus-DNA backbone interactions contribute significantly to pilus-mediated DNA binding as polyphosphate is able to reduce the binding of K122-4 and PAK pili to both bacterial plasmid DNA (Fig. 4A) and salmon sperm DNA (Fig. 4B) by more than 50%. Electrostatics and backbone interactions are dominant features of KB-7 pilus-mediated DNA binding as polyphosphate could inhibit binding by more than 90% (Fig. 4). The type IV pili of *P. aeruginosa* potentially facilitate polyphosphate uptake, as the results of this study indicate that pili can bind directly to polyphosphate (Fig. 4) and polyphosphate active transport in *P. aeruginosa* is well documented (42). After binding, pilus retraction could bring the polyphosphate through the outer membrane to receptors on the inner membrane. Interestingly, polyphosphate kinase and/or polyphosphate appear to be required for twitching motility (44) and biofilm formation (45) in *P. aeruginosa*, two functions that also require type IV pili.

Although pilus-mediated DNA binding is dominated by electrostatics as DNA binding can be inhibited by polyphosphate (Fig. 4), other features of the DNA structure must also play a role in binding as the K122-4 pilus, which has the most positive β -sheet region (Fig. 1A), did not have the highest affinity for DNA, and binding studies performed in the presence of various salt concentrations have indicated that there is a specific component to DNA binding (Fig. 4C). Also, PAK and K122-4 pili, but not KB-7 pili, were able to bind a double-stranded oligonucleotide with approximately the same apparent affinities (Fig. 5A). Therefore, short sequences of DNA are

bound by PAK and K122-4 pili with similar energetics. Since KB-7 had a higher K_d for DNA (Fig. 2) and because electrostatics dominate the interaction as polyphosphate could inhibit DNA binding by more than 90% (Fig. 4), it is not surprising that a short oligonucleotide was unable to bind to any great extent (Fig. 5A). In addition, we concluded that DNA binding is not dependent on a specific sequence as several oligonucleotides were able to bind PAK pili with similar apparent affinities (Fig. 5B). However, single-stranded oligonucleotides containing only pyrimidine bases were able to inhibit binding of single-stranded *N. gonorrhoeae* oligonucleotide, indicating that pilus-mediated DNA binding displays base specificity or preference in the absence of sequence specificity (Fig. 5B). This apparent base composition preference may reflect the specificity of the DNA binding site on the pilus, or specific physicochemical features of the single-stranded oligonucleotides (e.g., the stiffness of purine oligonucleotides versus pyrimidine oligonucleotides) could influence binding.

DNA binding is a tip-associated event but not a base-associated event with PAK pili, as antibodies to the tip but not antibodies to the base of the pilus inhibited DNA binding (Fig. 6A). Additional features present in the quaternary structure of the pilus are also involved in DNA binding, as polyclonal PAK antibodies inhibited binding to a greater degree than the tip-specific antibodies inhibited binding (Fig. 6A). This suggests that binding occurs both at or near the pilus tip and along the pilus filament. Furthermore, an analog of the C-terminal receptor binding domain of PAK pili [synthetic peptide PAK(128-144ox)] and monomeric pilins were unable to inhibit pilus-mediated DNA binding (data not shown), providing further support for the hypothesis that DNA binding is a function of the intact pilus.

Pilus-mediated DNA binding likely plays a role during biofilm formation as both extracellular DNA and type IV pili are required for *P. aeruginosa* biofilm formation (39, 58). DNA increases *P. aeruginosa* and type IV pilus binding to stainless steel, which strongly suggests that the pilus DNA binding function plays a role in biofilm formation (R. T. Irvin, unpublished data). DNA may aid in the formation of microcolonies as type IV pili are involved in the formation of cell clusters that require both cell-cell and cell-surface interactions (39). Since pilus-mediated DNA binding is likely a multivalent interaction, aggregates of cells and DNA that are attached to a surface could potentially form. Interestingly, there are high concentrations of DNA in the lungs of patients with CF as a result of neutrophil necrosis (57) or specific secretion of DNA (7). This DNA may assist in the formation of a *P. aeruginosa* biofilm and thus contribute to persistent colonization. Biofilm formation is involved in chronic colonization, as *P. aeruginosa* isolates from the lungs of CF patients have a mucoid phenotype characterized by the production of alginate, an exopolysaccharide produced during biofilm formation (20). *P. aeruginosa* cells found in sputum obtained from CF patients are organized into clusters reminiscent of biofilms, produce quorum-sensing agents required for biofilm formation, and are not associated with the mucosal epithelial surface (51).

We demonstrated that the type IV pili from three strains of *P. aeruginosa* can bind directly to multiple forms of DNA. This is a function of the intact pilus and potentially is a tip-associated event involving other aspects of the quaternary structure.

This binding event is dominated through interaction with the DNA backbone; however, there is an apparent preferential interaction with the pyrimidine bases. The ability of the type IV pilus of *P. aeruginosa* to bind DNA could be involved in biofilm formation in the course of infection or during colonization of abiotic surfaces.

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