Molecular Microbiology (2006) **59**(4), 1083–1096

doi:10.1111/j.1365-2958.2005.05002.x
First published online 12 December 2005

The *Pseudomonas aeruginosa* type IV pilin receptor binding domain functions as an adhesin for both biotic and abiotic surfaces

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Summary

Pseudomonas aeruginosa **readily binds to stainless steel and other abiotic surfaces, causing major problems in both the medical and food industries. In this study, we show that *P. aeruginosa* binds to abiotic surfaces in a concentration-dependent, saturable manner during the initial stages of biofilm formation. *P. aeruginosa* type IV pili mediate binding to stainless steel as a pilus-deficient strain does not bind to steel, purified type IV pili bound in a concentration-dependent, saturable manner, and purified pili competitively inhibited whole cell binding. PAK pili can also bind polystyrene and polyvinylchloride in a concentration-dependant and saturable manner. As an antibody specific for the C-terminal pilin receptor binding domain inhibited adherence to abiotic surfaces, the role of the C-terminal receptor binding domain in mediating binding to steel surfaces was examined. A synthetic peptide of the PAK pilin epithelial cell receptor binding domain [PAK(128–144)ox] bound directly to steel with high affinity. The interaction of pili with steel was specifically inhibited by this peptide with an apparent K_i of ~0.2 nM and effectively inhibited the binding of viable homologous and heterologous *P. aeruginosa* strains to steel with an apparent K_i of ~4 nM. A single point mutation (K130I) in the PAO receptor binding domain was observed to abolish binding to stainless steel while binding to human buccal epithelial cells was enhanced. Therefore, the C-terminal receptor binding domain appears to have evolved for binding a variety of surfaces.**

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Introduction

Pseudomonas aeruginosa is an effective and common opportunistic pathogen of humans, causing serious infections in cystic fibrosis, intensive care, burn and immunocompromised patients (Bodey *et al*., 1983; Pier, 1985; Costerton, 2001). Initial binding of the bacterium to an abiotic or a cellular substratum is considered by many to be the initial stage of colonization for both biofilm formation (Watnick and Kolter, 2000) and initiation of an infection (Beachey, 1981). Recently, *P. aeruginosa* biofilms have been implicated during chronic infection of cystic fibrosis patients (O'Toole *et al*., 2000; Singh *et al*., 2000). In addition to chronic infection, *P. aeruginosa* biofilms contribute to morbidity of patients with medical implants including catheters (Kumon *et al*., 1997; Khaled *et al*., 2001), prosthetics (McNeil *et al*., 2001) and stainless steel implants (Traverso *et al*., 2005).

Stainless steel is widely used, particularly in the food sector, and commonly used in hospitals and in medical devices (Hood and Zottola, 1997). *P. aeruginosa* readily binds to stainless steel (Stanley, 1983; VanHaecke *et al*., 1990) to form highly recalcitrant, organized communities known as biofilms (Leake *et al*., 1982; Blenkinsopp *et al*., 1992; Johansen *et al*., 1997). *P. aeruginosa* biofilms on stainless steel surfaces can serve as a significant hospital reservoir for infection of susceptible patients that include the immunocompromised and burn patients (Tredget *et al*., 1992). Type IV pili are essential for the normal development of *P. aeruginosa* biofilms as mutants lacking functional pili are not able to develop past the microcolony stage in static or flow biofilm systems (O'Toole and Kolter, 1998; Klausen *et al*., 2003).

Type IV pili are composed of a single pilin subunit, PilA, and are assembled into long polar surface appendages (Folkhard *et al*., 1981). This assembly process ensures that the receptor binding domain is only located at the tip

of the pilus (Lee *et al*., 1994). The pilus-associated epithelial cell receptor binding domain is located within residues 128–144 of the C-terminal region of PilA, the pilin structural protein (Irvin *et al*., 1989). This terminal binding domain specifically recognizes GalNAc-β-D-(1,4)-Gal moieties of asialo-GM_1 as a minimal receptor (Sheth *et al*., 1994). Adherence to this receptor is specific and can be inhibited by a synthetic C-terminal receptor binding domain, PAK(128–144)ox, or by synthetic GalNAc-β-D-(1,4)-Gal (Sheth *et al*., 1994; Wong *et al*., 1995; Schweizer *et al*., 1998).

In this study, we examine the initial event of *P. aeruginosa* biofilm formation on stainless steel, namely the binding of the organism to the surface. We demonstrate that type IV pili specifically mediate binding to stainless steel and that the interaction with steel involves the previously identified pilin C-terminal epithelial cell receptor binding domain (Irvin *et al*., 1989). This synthetic peptide receptor binding domain was observed to bind to steel with high affinity and prevent binding of a variety of *P. aeruginosa* strains to steel. In addition, the C-terminal receptor binding domain also mediated binding to other abiotic surfaces including polyvinylchloride and polystyrene. Therefore, the C-terminal receptor binding domain has evolved to retain a structure for binding a variety of surfaces including both biotic and abiotic surfaces.

Results

Initial colonization of stainless steel is dependent upon type IV pili in P. aeruginosa

While the involvement of type IV pili in biofilm formation on abiotic surfaces has been well documented (O'Toole and Kolter, 1998; Klausen *et al*., 2003), the molecular basis for that involvement has not been firmly established. Therefore, we investigated the ability of *P. aeruginosa* wild-type strains PAK, PAO, K122-4 and KB7, which display considerable differences in their pilin sequences, to bind to stainless steel (Fig. 1A and B). *P. aeruginosa* pilins are characterized by a highly conserved N-terminal α-helix and a semi-conserved C-terminal disulphide loop region but display minimal sequence similarity through the bulk of the protein (Fig. 1A). However, structural studies indicate that *P. aeruginosa* pilins are strikingly similar (Fig. 1B) (Hazes *et al*., 2000; Craig *et al*., 2003; Audette *et al*., 2004). *P. aeruginosa* strains PAK, PAO, K122-4 and KB7 were observed to rapidly bind to stainless steel surfaces (Fig. 2A, D, G and J), in agreement with previous results (Stanley, 1983; VanHaecke *et al*., 1990). Strains PAO and PAK bound more significantly to stainless steel than either K122-4 or KB7 (Fig. 2A, D, G and J). *P. aeruginosa* strain MS591 (Starnbach and Lory, 1992), a *fliC*$^{-}$ non-flagellated mutant of PAK, was observed to bind at considerably reduced levels compared with the parental PAK strain (Fig. 2A and P). *P. aeruginosa* strain PAKNP, a non-piliated *pilA* deficient mutant of PAK (Saiman *et al*., 1990), did not bind to steel surfaces (Fig. 2M), suggesting that *P. aeruginosa* adherence to stainless steel may be mediated by type IV pili. To further investigate the initial colonization of stainless steel, the binding kinetics of PAK cells and pili to steel was examined in a quantitative manner employing viable biotinylated cells and purified biotinylated PAK pili. PAK cells (Figs 3A and 4) and purified PAK pili (Fig. 3B) were observed to bind to steel surfaces in a saturable, concentration-dependent manner while PAKNP biotinylated cells (Figs 3A and 4) did not bind appreciably to the steel surface. Biotinylation had no effect on the ability of the purified pili to bind stainless steel as identical binding kinetics were observed with native pili when anti-PAK pili antibodies were utilized to quantify binding (data not shown). Furthermore, addition of low concentrations of purified PAK pili competitively inhibited the binding of biotinylated PAK cells to steel (Fig. 3C). As the length of PAK pili is not known, the molarity of the pili cannot be determined (van Schaik *et al*., 2005).

We established that *P. aeruginosa* stained with acridine orange were readily visualized by epifluorescence microscopy following binding to stainless steel (Fig. 1D). *P. aeruginosa* cells were visualized as fluorescent orange rods bound to areas of the steel that fluoresced green either through non-specific interaction of the fluorochrome with grain boundary regions or due to non-specific interaction of the fluorochrome with organic material that interacted with the grain boundaries in the steel. Green fluorescent material primarily associated with grain boundaries in the steel was observed in both strains PAK and PAKNP (Fig. 1D and E). In confirmation of these results, *Pseudomonas* sp. has recently been shown to colonize preferentially with grain boundaries (Sreekumari *et al*., 2001).

Effect of flagella and type IV pili mutations on binding to steel

Previous studies using *P. aeruginosa* FilC^{-} and PilB^{-} strains have established the importance of flagella and type IV pili during the initiation and development of biofilms on abiotic surfaces in static cultures (O'Toole and Kolter, 1998). To confirm our qualitative microscopic examination, a quantitative analysis of adherence to steel was performed. The binding of PAK-BΩ (PilB^{-} mutant), a strain that does not assemble pili but does express PilA (Koga *et al*., 1993), to steel was compromised relative to wild-type and equivalent to that observed for PAKNP and PAKMS591 (Fig. 4). ELISA evidence indicates that

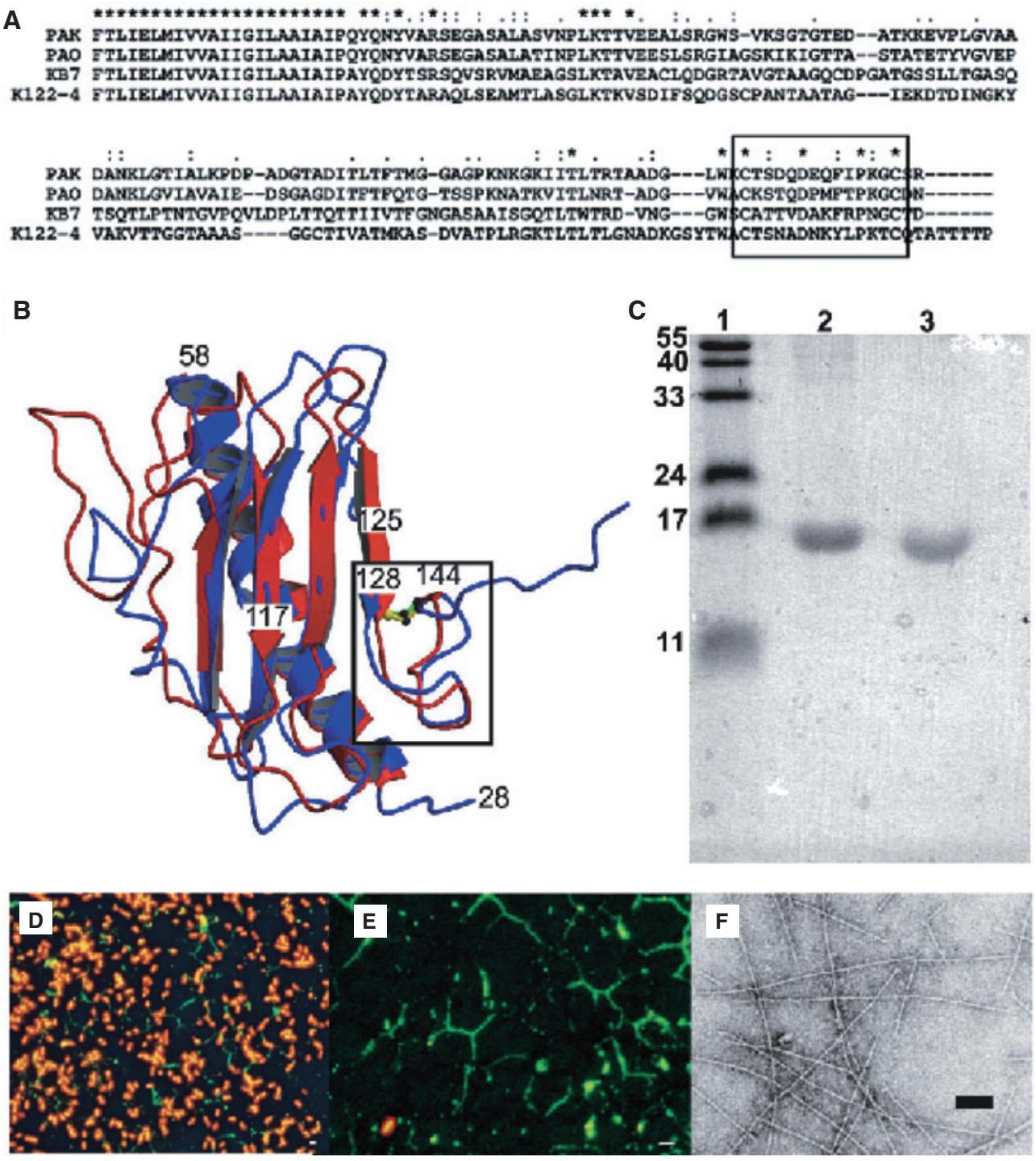

Fig. 1. A. Full-length pilin sequences of *P. aeruginosa* strains PAK, PAO, KB7 and K122-4. Boxed area represents disulphide loop region of residues 128–144 of the PAK sequence. The disulphide loop region contains an epithelial cell binding domain and displays a conserved antigenic epitope despite extensive sequence variation.
B. Structural overlay of *P. aeruginosa* strains PAK (red) and K122-4 (blue) truncated monomeric pilins. Disulphide loop region is highlighted by boxed area, cysteine residues are shown in black and the disulphide bonds are shown in green and yellow for PAK and K122-4 strains respectively.
C. Fifteen per cent SDS-PAGE gel (Sambrook *et al.*, 1989), lane 2 shows pili preparation before final cesium chloride gradient and lane 3 shows a single non-contaminated band of pili after density ultacentrifugation. Lane 1 represents a pre-stained protein ladder (Fermentas). Epifluorescence micrograph of acridine orange stained PAK wild-type cells (D), and PAKNP (PilA^{-} strain) (E) bound to stainless steel. Bacterial cells fluoresce orange, while grain boundaries fluoresce green due to non-specific staining. The bar represents 5 μM.
F. Electron micrograph picture of PAK pili. The bar represents 100 nM.

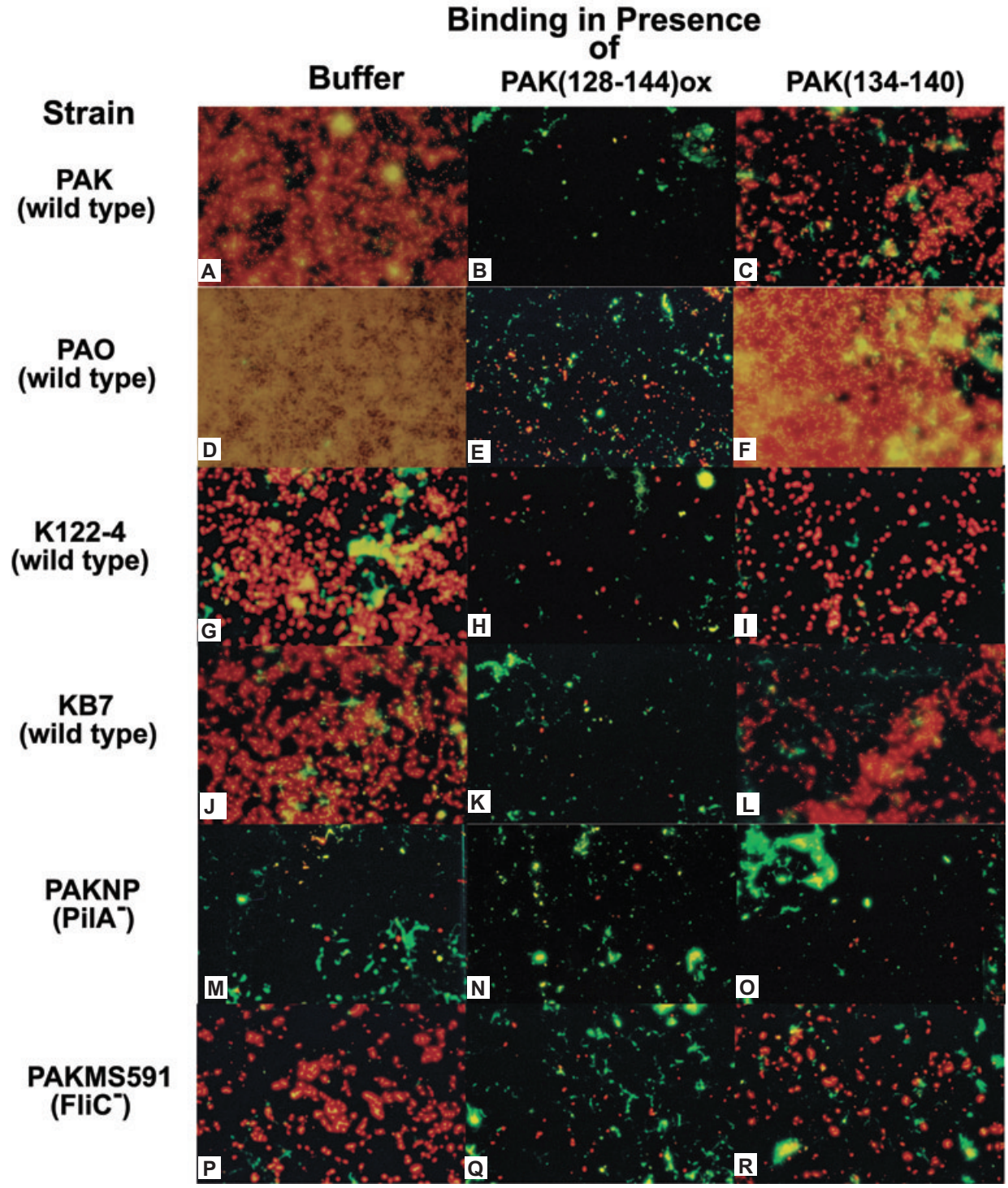

Fig. 2. Epifluorescence microscopy of stainless steel after binding of *P. aeruginosa* strains PAK (panels A, B and C), PAO (panels D, E and F), K122-4 (panels G, H and I), KB7 (panels J, K and L), PAKNP (panels M, N and O) and PAKMS591 (panels P, Q and R). Viable cells were allowed to incubate directly (panels A, D G, J, M and P) with a stainless steel grade 304 surface for 60 min at 37°C and washed five times with 10 mM PBS pH 7.4 containing 0.05% BSA. The cells were then stained using acrydine orange and a 40× objective field was photographed with a Leitz Laborlux microscope equipped with epifluorescent illumination and a Wild automatic exposure 35 mm camera system. Note that bound *Pseudomonas* cells are stained orange while green fluorescence indicates non-specific interaction of the fluorochrome with the surface. *P. aeruginosa* strains PAK, PAO, K122-4, KB7, PAKNP and PAKMS591 were incubated with the synthetic peptide PAO(128–144)ox (panels B, E, H, K, N and Q respectively) or with synthetic peptide PAK(134–140) (panels C, F, I, L, O and R respectively). Note that PAK(128–144)ox reduced the binding to steel of strains PAK, PAO, K122-4, KB7 and PAKMS591 but did not reduce the binding of strain PAKNP. PAK(134–140) did not inhibit the binding of any strain to the steel surface.

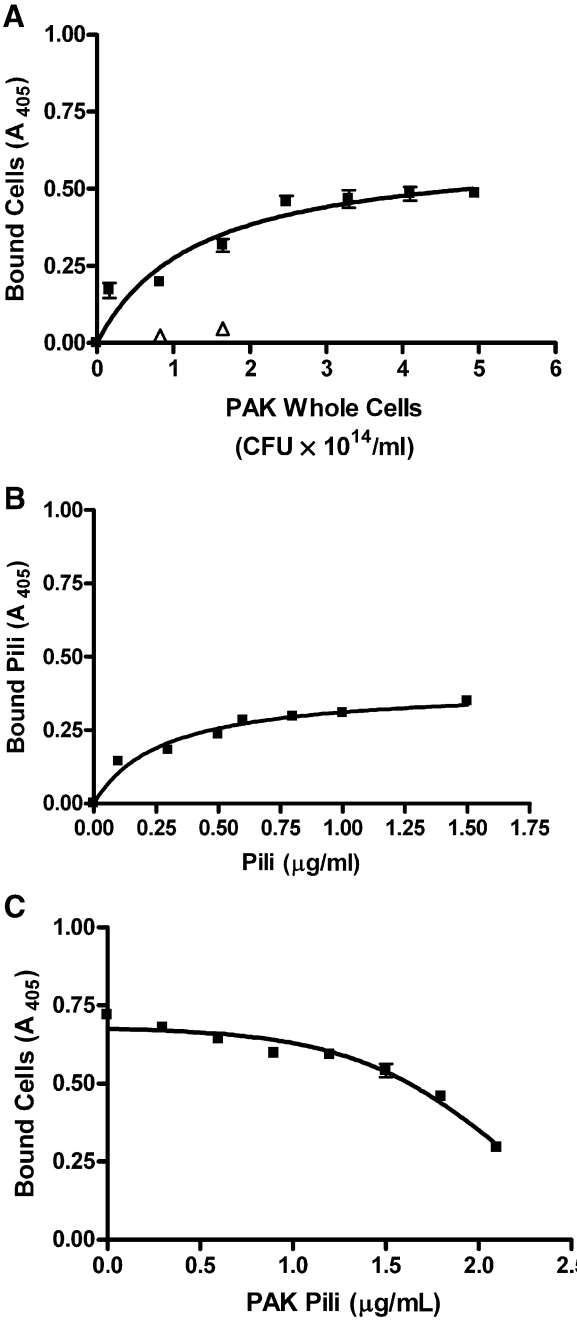

Fig. 3. A. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) and a pilin-deficient strain PAKNP (△) to stainless steel at cell densities of 10^{14} cfu ml^{-1}. The quantity of PAK cells bound to the stainless steel surface area was determined by measuring the amount of biotin bound to the stainless steel surface employing a modified ELISA with streptavidin-HRP and utilizing ABTS as a substrate. The symbols and bars in this and subsequent figures report the mean ± SEM (experiments were duplicated with individual studies employing at least six replicates).
B. Binding of biotinylated PAK pili to stainless steel. Biotinylated pili were washed and suspended in 10 mM PBS pH 7.4, and allowed to bind to stainless steel for 60 min at 37°C.
C. Competitive inhibition of viable biotinylated-PAK whole cell binding to stainless steel surfaces by purified homologous unlabelled pili. Pili and bacteria were mixed and then directly added to the stainless steel surface.

PAKMS591 has less surface-exposed pili than does the PAK wild-type strain (data not shown). Strain PAK-DΩ (*pilD*-mutant) which lacks the prepilin peptidase and therefore does not express surface-exposed PilA (Koga *et al.*, 1993) or functional type IV pili, bound roughly equivalent to mutant strains (PAKNP, PAKMS591 and PAK-BΩ) (Fig. 4). Although the binding curves differ slightly between mutant strains, all pili-deficient strains (PAKNP, PAK-BΩ and PAK-DΩ), and PAKMS591, bound significantly less than wild-type (Fig. 4). These results indicate that any mutation which abolishes the production of functional pili also reduces the ability to bind stainless steel.

Antibody inhibition studies

Addition of rabbit polyclonal anti-PAK pili antibodies (Lee *et al.*, 1989) but not rabbit pre-immune serum strongly inhibited the binding of biotinylated PAK cells and pili (Fig. 5) to steel in a dose-dependent manner. These data indicate that the type IV pili mediates binding. Murine monoclonal antibody PK99H, which recognizes PAK PilA residues 134–140 exposed at the tip of the pilus (Lee *et al.*, 1994; Wong *et al.*, 1992), significantly inhibited the binding of PAK cells and pili to steel (Fig. 5). The inhibition of PAK binding to stainless steel by PK99H suggests that the C-terminal disulphide loop region of pilin, which contains an epithelial cell binding domain, may also function in mediating attachment to steel surfaces.

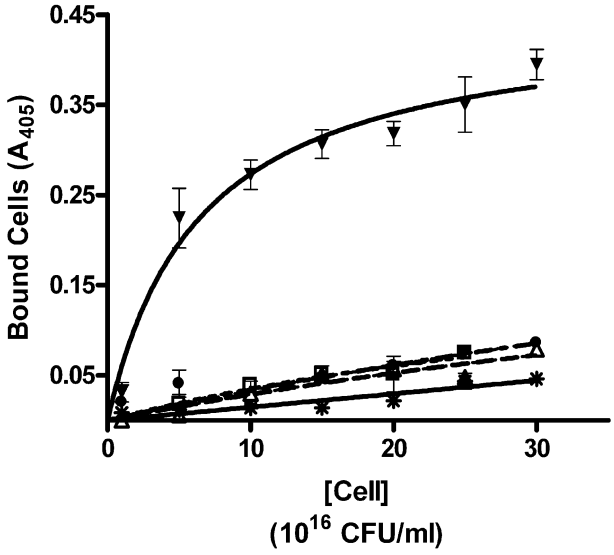

Fig. 4. Binding of biotinylated viable cells of *P. aeruginosa* strains PAKwt (▼), PAKMS591 a FliC⁻ strain (△), PAK-BΩ a PilB⁻ strain (●), PAK-DΩ a PilD⁻ strain (□), and PAKNP a PilA⁻ strain (✱) binding to stainless steel. The quantity of PAK cells bound to the stainless steel surface area was determined by measuring the amount of biotin bound to the stainless steel surface employing a modified ELISA with streptavidin-HRP and utilizing ABTS as a substrate.

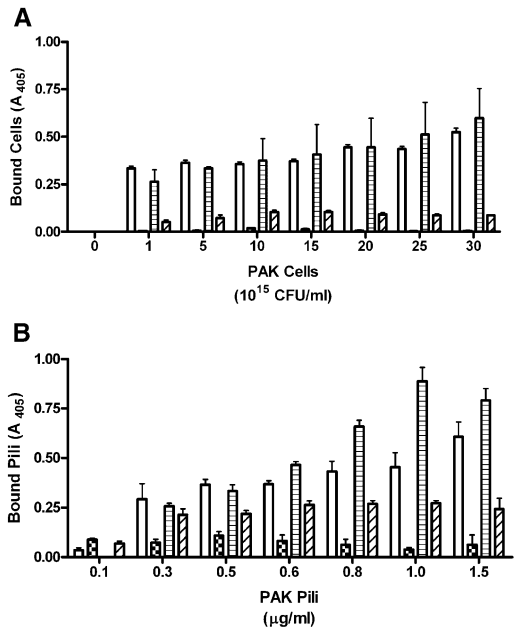

Fig. 5. A. Antibody inhibition of the binding of viable biotinylated PAKwt cells to stainless steel relative to PAKwt cells in buffer (□) or exposed to rabbit pre-immune antisera (▩). Antibodies utilized include rabbit polyclonal anti-PAK pili antisera (▤), and murine monoclonal antibody PK99H (▨) all of which are specific for PAK pili.
B. Antibody inhibition of the binding of biotinylated PAK pili to stainless steel relative to PAK pili in buffer (□) or exposed to rabbit pre-immune antisera (▩). Antibodies utilized include rabbit polyclonal anti-PAK pili antisera (▤), and murine monoclonal antibody PK99H (▨), all of which are specific for PAK pili.

Synthetic peptide inhibition of binding to steel

Antibody inhibition assays suggested that the cellular receptor binding domain of the pilus may also mediate binding to stainless steel. Therefore, competitive binding assays were utilized to test the ability of the C-terminal receptor binding domain to inhibit adherence of *P. aeruginosa* to steel. Previous studies demonstrated that the native C-terminal receptor binding domain (PAK128–144ox) mediates binding to GalNAc-β-D-Gal containing glycoconjugates (Sheth *et al*., 1994). The PAK pilin receptor binding domain (PAK128–144ox) inhibited the binding of both PAK wild-type cells and PAK pili to steel surfaces with apparent K_i's of ~4 nM and ~0.2 nM respectively (Fig. 6A and C). PAK(128–144)ox also inhibited the binding of PAKMS591 (compare Fig. 2P with Fig. 2Q) but had no effect on the binding of PAKNP (compare Fig. 2M and N). The peptide PAK(134–140), which constitutes a portion of the receptor binding domain, and has been demonstrated to bind with low affinity to respiratory epithelial cells (Yu *et al*., 1996), did not inhibit binding of PAK wild-type cells or PAK pili to steel surfaces, even at the exceptionally high peptide concentration of 100 μg ml^{-1} (Fig. 6B and D), nor did it inhibit the binding of strains PAO, K122-4 or KB7, in contrast to peptide PAK(128–144)ox (Fig. 2). In addition, neither PAK(22–52), a peptide derived from the N-terminal α-helix (residues 1–58) which should be buried in the native pilus fibre, nor PAK(117–125), a peptide consisting of a portion of β-strands 3 and 4 which models of the pilus fibre suggest will be displayed on the fibre surface (Hazes *et al*., 2000) (see Table 1 for peptide sequences and Fig. 1B), had any effect, even at high concentrations, on the binding of PAK wild-type cells or PAK pili to steel surfaces (Fig. 6B and D), indicating that these regions do not participate in pilus-mediated binding.

To determine whether the ability to interact with steel surfaces was a general attribute of the C-terminal receptor binding domain or a specific property of the PAK receptor binding domain, the ability of the PAO receptor binding domain to inhibit binding was examined. The synthetic PAO receptor binding domain, PAO(128–144)ox was observed to inhibit pilus-mediated binding to stainless steel in a similar manner to the native PAK peptide (Fig. 6E). To further confirm the specific nature of the receptor binding domain's interaction with steel surfaces, two additional control peptides, a scrambled PAO receptor binding domain PAO(128-144)ox_Scrambled and a linear variant of that sequence where the two cysteine residues have been replaced by alanine residues to eliminate the disulphide bridge, PAO(128–144)C129A/C142A_Scrambled, were utilized

Table 1. Synthetic peptides and peptide sequences employed or referred to in this study.

Peptide	Sequence
PAK(128–144)ox	Ac-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
PAK(134–140)	Ac-D-E-Q-F-I-P-K-amide
PAK(117–125)	Ac-T-L-T-R-T-A-A-D-G-OH
PAK(22–52)	Ac-P-Q-Y-Q-N-Y-V-A-R-S-E-G-A-S-A-L-A-S-V-N-P-L-K-T-T-V-E-E-A-D-P-OH
$PAO(128–144)_{ox}$_Scrambled	Ac-N-C-P-D-F-D-P-T-K-K-G-M-Q-A-C-T-S-OH
PAO(128–144)C129A/C142A_Scrambled	Ac-N-**A**-P-D-F-D-P-T-K-K-G-M-Q-A-**A**-T-S-OH
PAK(128–144)ox	Ac-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
PAO(128–144)ox	Ac-A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH
PAO(128–144)oxK130I	Ac-A-C-**I**-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH

Peptides were synthesized by solid phase and are N-α-acetylated with a free carboxyl except for PAK(134–140) which was synthesized as the N-α-acetylated amide peptide due to its short length. Peptides with a formed disulphide bridge between cysteine 129 and 142 are identified by an ox.

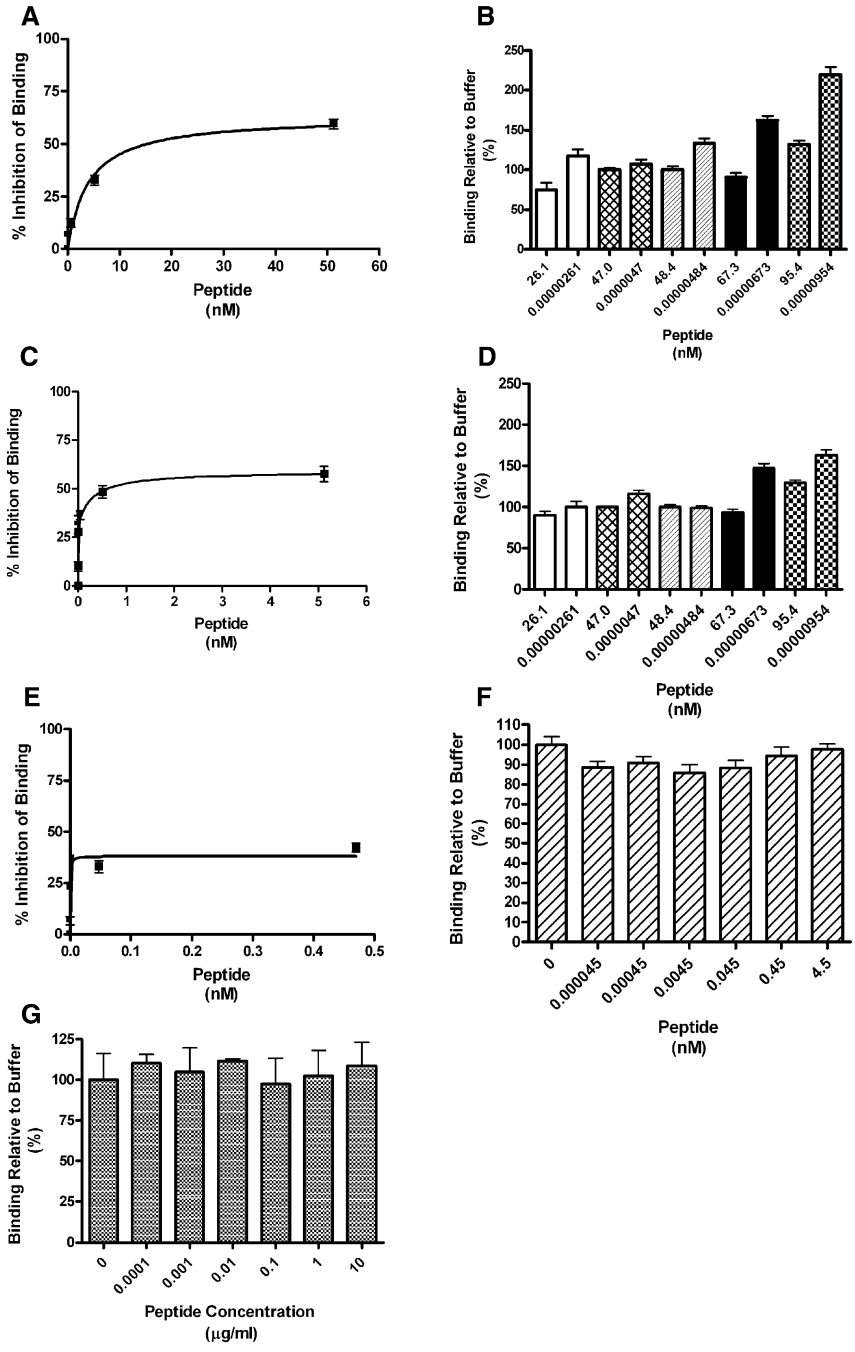

Fig. 6. A. Competitive inhibition of biotinylated PAKwt cell binding to stainless steel by the synthetic peptide PAK(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells. The apparent K_i of the peptide inhibition of PAKwt binding to steel is ~4 nM as determined by Prism 4 curve fitting.
B. Bar graph of the effect of various synthetic peptides on the binding of biotinylated PAKwt cells to stainless steel. Synthetic peptides consisting of the PAK PilA sequences PAK(22–52) (□) a portion of the N-terminal α-helix which is buried in the pilus fibre, PAK(117–125) (⊠) a solvent exposed sequence of PilA located N-terminally to the receptor binding domain, and PAK(134–140) (▨) a sequence from the PilA receptor binding domain that has low affinity for mucosal cell surface receptors. Two scrambled peptide sequences were utilized as further controls, PAO(128–144)ox_Scrambled (■), a PAO PilA receptor binding domain scrambled sequence that retains the intrachain disulphide bond and PAO(128–144)C129A/C142A_Scrambled (⊠) a linear variant of the initial scrambled sequence where the two cysteine residues have been replaced by alanine residues. See Table 1 for a list of sequences. Note that the control peptides do not inhibit cell binding to steel even at high concentrations. Indeed, PAK(117–125) appears to enhance cell binding to steel rather than inhibiting the binding function.
C. Competitive inhibition of biotinylated PAK pili binding to stainless steel by the synthetic peptide PAK(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells. The apparent K_i of the peptide inhibition of PAK pilus binding to steel is ~0.2 nM as determined by Prism 4 curve fitting.
D. Bar graph of the effect of various synthetic peptides on the binding of biotinylated PAK pili to stainless steel. Synthetic peptides and symbols are as for Fig. 5B. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations. Indeed, PAK(117–125) appears to enhance the binding of pili to steel rather than inhibiting binding.
E. Competitive inhibition of biotinylated PAKwt cell binding to stainless steel by the synthetic peptide PAO(128–144)ox, the PilA receptor binding domain that binds to human respiratory epithelial cells.
F. Bar graph of the effect of PAO(128–144)oxK130I (▨) on the binding of biotinylated PAK pili to stainless steel. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations.
G. Bar graph of the effect of trypsinized peptide (⊠) on the binding of biotinylated PAK pili to stainless steel. Note that the control peptides do not inhibit the binding of pili to steel even at high concentrations.

to assess the relative importance of sequence versus amino acid composition. Neither scrambled sequence was able to inhibit binding of PAK wild-type cells or PAK pili to steel surfaces, even at very high peptide concentrations (Fig. 6B and D). As a further control, peptides obtained through the trypsinization of bovine serum albumin (BSA) were utilized to confirm that the inhibition of binding was sequence-specific and not a common property of peptides. No inhibition of adherence was observed for either PAK whole cells (data not shown) or PAK pili even at high peptide concentrations (Fig. 6G). A PAO receptor binding domain mutant, PAO(128–

144)oxK130I which has high affinity for human buccal epithelial cells (BECs) (Fig. 7C) was unable to inhibit *P. aeruginosa* whole cells or pili adherence to steel (Fig. 6). To further determine whether the receptor binding domain inhibited binding to steel by a competitive mechanism or by interacting with *P. aeruginosa* cells or pili, the ability of PAK(128–144)ox to bind to stainless steel was determined using the monoclonal antibody PK99H as a probe of peptide binding to steel (PK99H has been demonstrated to bind to both PAK(128–144)ox and PAK(134–140) when these peptides are bound to a cell surface receptor) (Irvin *et al.*, 1989; Yu *et al.*, 1996). PAK(128–144)ox bound with high affinity to stainless steel while PAK(134–140) bound only marginally to the steel surface at very high concentrations (Fig. 7A and B).

As *P. aeruginosa* strains vary considerably in their ability to bind to steel surfaces (Fig. 2A, D, G and J), we sought to determine whether the PAK pilin receptor binding domain, PAK(128–144)ox, could inhibit the binding of other *P. aeruginosa* strains. Utilizing microscopy, we found that at very low concentrations (51 nM), PAK(128–144)ox substantially inhibits the binding of strains PAO, K122-4 and KB7 (compare Fig. 2D, G and J with Fig. 2E, H and K) while very high concentrations (100 μg ml^{-1}) of PAK(134–140) have a minimal effect on binding to steel (compare Fig. 2A, D, G and J with Fig. 2C, F, I and L). The pilin receptor binding domain sequences of strains PAK, PAO, K122-4 and KB7 vary substantially (Fig. 1A and B, and Table 1), but all these receptor binding domains display a conserved antigenic epitope and compete for epithelial cell surface receptors (Sheth *et al.*, 1995).

Binding to other abiotic surfaces

As type IV pili have been implicated in biofilm formation on polystyrene and polyvinylchloride surfaces, we sought to determine if the C-terminal receptor binding domain may function to mediate attachment to a variety of abiotic surfaces. PAK whole cells and pili were found to bind in a concentration-dependent and saturable manner to both polyvinylchloride and polystyrene plates (Fig. 8A–D). The murine monoclonal antibody PK99H significantly inhibited binding to both polyvinyl chloride and polystyrene surfaces (Fig. 8E and F). These data indicate that type IV pili mediate binding to these plastic surfaces which may be dependant on the C-terminal receptor binding domain.

Discussion

The aggressive colonization of stainless steel surfaces, apart from being of enormous industrial significance, is also of medical relevance; *P. aeruginosa* infections are prevalent in burn units where large stainless steel tubs,

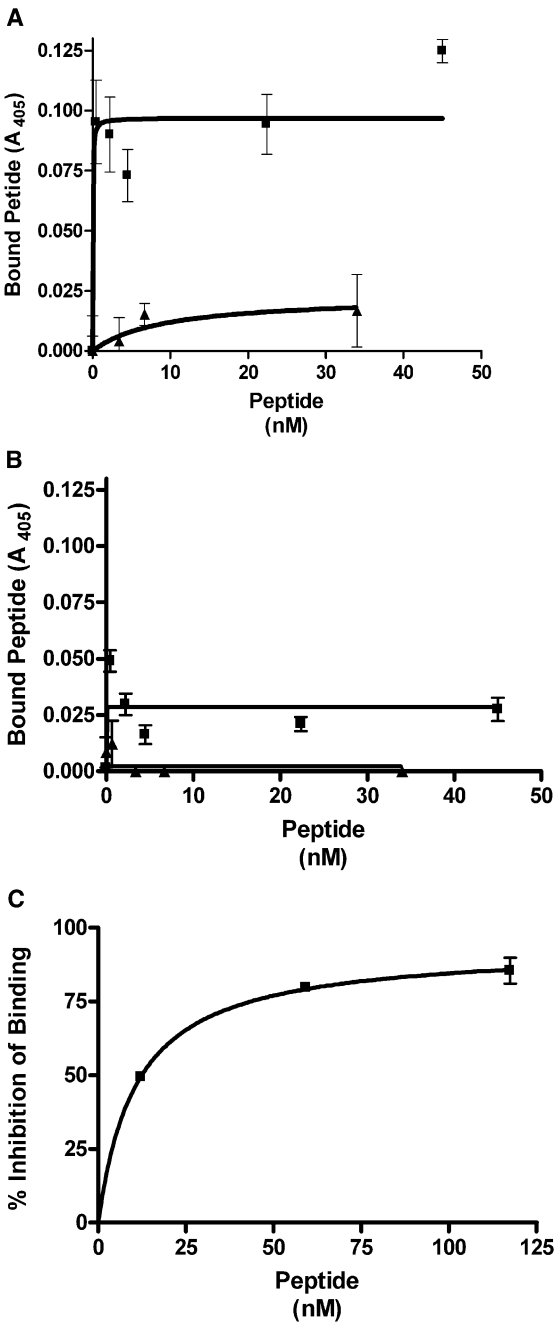

Fig. 7. A. Binding of PAK(128–144)ox (■) and PAK(134–140) (▲) to stainless steel as determined by a direct immunoassay employing murine monoclonal antibody PK99H. PK99H binds with high affinity to both of these peptides (Doig *et al.*, 1990; Wong *et al.*, 1992) even when the peptides are bound to receptors (Irvin *et al.*, 1989; Yu *et al.*, 1996). Note that PAK(128–144)ox binds with high affinity to steel while PAK(134–140) binds only slightly at very high concentrations.
B. Binding of PAK(128–144)ox (■) and PAK(134–140) (▲) to stainless steel as determined by a direct immunoassay employing biotinylated peptide. Note that PAK(128–144)ox binds with high affinity to steel while PAK(134–140) binds only slightly at very high concentrations.
C. Competition assay using BECs and wild-type PAO with PAO(128–144)oxK130I peptide in increasing concentrations.

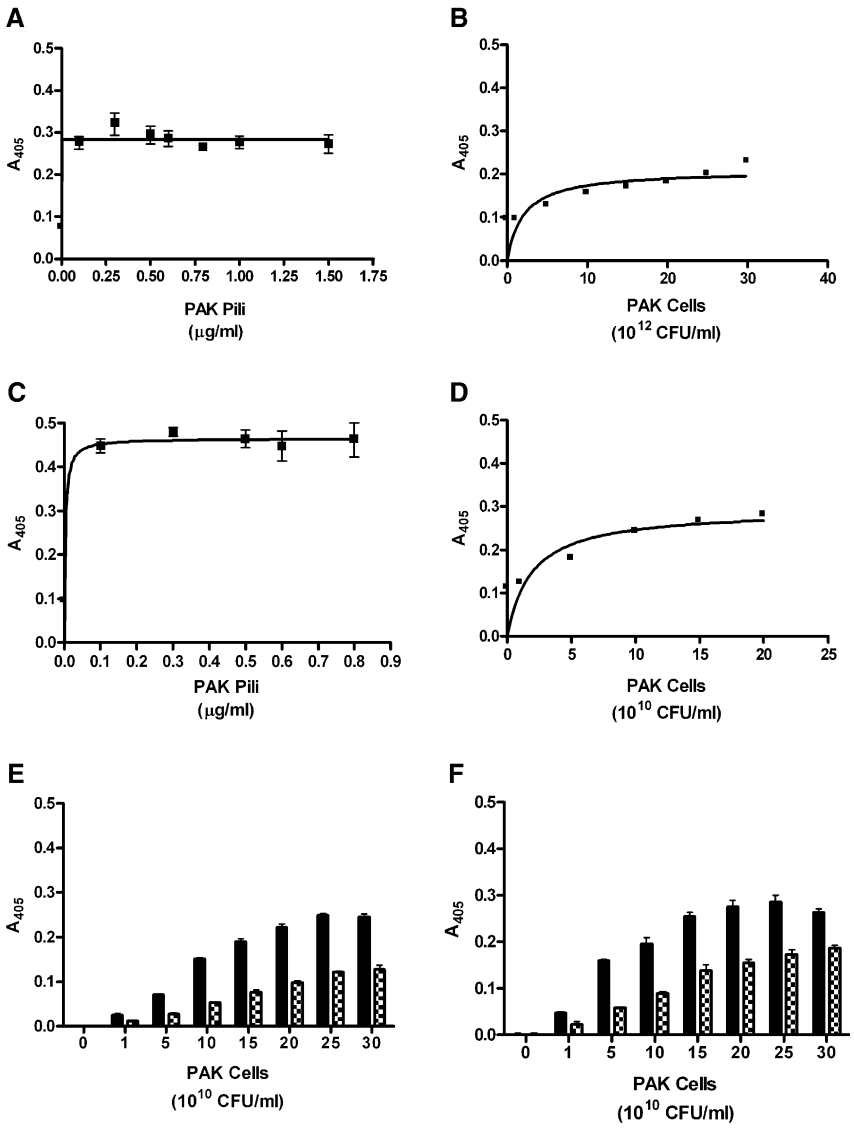

Fig. 8. A. Binding of biotinylated PAK pili (■) to polystyrene plastic. Biotinylated pili were washed and suspended in 10 mM PBS pH 7.4, and allowed to bind to polystyrene for 60 min at 37°C.
B. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) to polystyrene at cell densities of 10^{12} cfu ml^{-1}. The quantity of PAK cells bound to the polystyrene surface area was determined by measuring the amount of biotin bound to the polystyrene surface employing a modified ELISA with streptavidin-HRP and utilizing ABTS as a substrate.
C. Binding of biotinylated PAK pili (■) to polyvinylchloride plastic.
D. Binding of biotinylated viable whole cells of *P. aeruginosa* strain K (PAK) (■) to polyvinylchloride at cell densities of 10^{10} cfu ml^{-1}.
E and F. Antibody inhibition of the binding of viable biotinylated PAKwt cells to polyvinylchloride (E) or polystyrene (F) relative to PAKwt cells in buffer (■) or exposed to murine monoclonal antibody PK99H (▩).

known as hydrotherapy units, are often used to treat patients with severe burns (Tredget *et al*., 1992). Tredget *et al*. (1992) have demonstrated a significant decrease in *P. aeruginosa* infection rates in burn units where stainless steel hydrotherapy units were removed. Biofilm formation on stainless steel and other substrata, as a function of physical and chemical modifications, has been widely investigated (Arnold *et al*., 2004; Balazs *et al*., 2004; Groessner-Schreiber *et al*., 2004; Lomander *et al*., 2004). Rough stainless steel surfaces more readily develop biofilms compared with smooth, or electropolished, steel (Characklis *et al*., 1990; VanHaecke *et al*., 1990; Bagge *et al*., 2001; Balazs *et al*., 2004; Lomander *et al*., 2004). We sought to clarify the role of the *P. aeruginosa* type IV pilus in the initial colonization of abiotic surfaces, particularly with stainless steel given the classic genetic evidence that flagella are more likely responsible for the initial stages of *P. aeruginosa* biofilm formation (O'Toole and Kolter, 1998).

We demonstrated that the initial binding of *P. aeruginosa* to stainless steel is concentration-dependent and exhibits classical saturation kinetics (Fig. 3). *P. aeruginosa* pili clearly play a major role in mediating whole cell binding to stainless steel as (i) pili-deficient strains are unable to adhere, (ii) pili bind to steel in a concentration-dependent, saturable manner, and (iii) pili competitively inhibit whole cell binding in a direct competition assay (Fig. 3).

Although Wozniak *et al*. (2003) demonstrated that alginate, the primary exopolysaccharide of *P. aeruginosa*, did not have a significant effect on the initial binding of *Pseudomonas* to abiotic surfaces, we have demonstrated that type IV pili are involved in the initial adherence. Therefore, while extracellular polysaccharide has long been pro-

posed to play a major role in mature biofilms (Stoodley *et al*., 2002), type IV pili protein subunits may provide the initial attachment to abiotic surfaces (Figs 2 and 3). Evidence of an initial involvement of protein in biofilm formation on copper surfaces has been reported previously (Bremer and Geesey, 1991). Interestingly, a variety of proteins have the ability to mediate tight interactions with abiotic surfaces, including the *Mytilus edulis* foot proteins which are capable of mediating interactions with a variety of abiotic surfaces including metals and plastics (Suci and Geesey, 2001). A direct role for protein in mediating interactions with an abiotic surface, particularly a metal surface, is thus not without precedent.

While O'Toole and Kolter (1998) found that flagella play a significant role in biofilm formation, we found that *P. aeruginosa* lacking flagella yet expressing pili were able to bind to steel (Figs 2 and 4). In addition, any strain lacking the ability to assemble functional pili were unable to bind (Figs 2 and 4). The difference between our findings and those of O'Toole and Kolter (1998) may reflect the ability of bound cells to remain surface attached and differentiate into a microcolony, and subsequently a biofilm, as our data only report on events that occur within 1 h of a potential bacterial interaction with the surface.

To determine whether the C-terminal receptor binding domain of the pilus was responsible for adherence to stainless steel, as for immobilized BECs (Irvin *et al*., 1990; Schweizer *et al*., 1998), we employed monoclonal antibodies specific for residues in the C-terminal binding domain in a competitive inhibition assay. Monoclonal antibody PK99H recognizes residues 134–140 of PAK PilA (Wong *et al*., 1992), inhibits pilus-mediated binding to respiratory epithelial cells (Irvin *et al*., 1989), and confers protection from challenge with strain PAK in a mouse infection model (Sheth *et al*., 1995). PK99H inhibited pilus-mediated binding to stainless steel, indicating that the pili C-terminal receptor binding domain, residues 128–144 of PAK PilA, may specifically mediate the interaction with steel (Fig. 5).

To confirm that residues 128–144 were mediating the interaction between pili and steel, a variety of synthetic peptides were used in competitive inhibition assays. Strikingly, PAK(128–144)ox effectively inhibits the adherence of heterologous *P. aeruginosa* strains to stainless steel even at low concentrations (Fig. 2B, E, H and K), indicating that the ability to bind to steel through the C-terminal binding domain is conserved in all *P. aeruginosa* strains. The ability of the synthetic receptor binding domain, PAO(128–144)ox, to inhibit the binding of both PAK cells and pili to steel further supports our hypothesis that the C-terminal pilin receptor binding domain of the various pilins mediates binding to steel in addition to mediating binding to human respiratory epithelial cells.

Peptides with limited affinity for steel have been identified by phage display methodology and their affinity for steel has been correlated to their amino acid composition (Zuo *et al*., 2005). The interaction of the receptor binding domain with steel is not simply a function of the peptide amino acid composition as two scrambled sequences of the PAO receptor binding domain (one retaining the disulphide bridge and the other a linear variant where the two cysteine residues are replaced with alanine residues) failed to inhibit binding to steel (Fig. 6B and D). The PilA receptor binding domain binds to steel with high affinity in a sequence-specific manner, indicating that binding is likely dependent upon both the sequence and three dimensional structure of the peptide rather than upon the amino acid composition. The steel binding function is sequence-specific as PAO(128–144)oxK130I failed to inhibit binding to steel even though the affinity of this peptide for human BECs was enhanced (compare Fig. 6F with Fig. 7C). This observation suggests that the ability to bind to steel and human epithelial cells can be differentiated, indeed, the short synthetic peptide PAK(134–140) which has previously been demonstrated to have a low affinity for human BECs (Yu *et al*., 1996) did not inhibit binding to steel (Fig. 2). These results indicate that the two binding functions are readily differentiated by a single point mutation, suggesting that the molecular basis of the interaction with steel and epithelial cells is quite distinct, although both are dependent on the C-terminal receptor binding domain.

As type IV pili have also been implicated in biofilm formation on plastic surfaces, the ability of the PilA receptor binding domain to mediate adherence to two widely used plastics, polyvinylchloride and polystyrene, was examined. Direct binding assays demonstrate that PAK whole cells and pili adhere to plastics in a concentration-dependent and saturable manner (Fig. 8). As well, the monoclonal antibody PK99H was able to inhibit *P. aeruginosa* binding to both polyvinylchloride and polystyrene plates (Fig. 8). These data suggest that the C-terminal receptor binding domain is involved not only in adherence to stainless steel and BECs, but also to other abiotic substrates including plastics.

The PilA C-terminal receptor binding domain, displayed at the tip of the type IV pilus, mediates direct binding to both biotic and abiotic surfaces. Although the pilus is able to bind both substrates, the affinities differ by several orders of magnitude. However, as the single point mutation in the C-terminal receptor binding domain increased the affinity for BECs, it abolished the affinity for stainless steel. This supports our hypothesis that the receptor binding domain has retained sequence and structural elements required for adherence to a variety of surfaces. Therefore, we have determined that the C-terminal receptor binding domain is responsible for adherence to stain-

less steel and although the sequence varies widely between strains, attachment via this C-terminal receptor binding domain is a conserved function of the type IV pilus.

Experimental procedures

Bacterial strains, DNA and antibody sources

The *P. aeruginosa* strains used in this study were PAK, PAK 2Pfs (Bradley and Pitt, 1974), PAK-BΩ, a 2 kBΩ fragment containing a transcriptional terminator from pHP45 was inserted into the pilB gene, PAK-DΩ, the same transcriptional terminator was inserted into the pilD gene (Koga *et al*., 1993), PAKMS591, the gentamycin cassette from a pPC110 was inserted into the fliC gene (Starnbach and Lory, 1992), PAKNP the tetracycline cassette from pB322 was inserted into the pilA gene (Saiman *et al*., 1990), K122-4, a clinical isolate from a cystic fibrosis patient in Toronto which possesses both pili and flagella (Pasloske *et al*., 1988) and KB7, an isolate containing both pili and flagella (Wong *et al*., 1995). Several of these strains were generously provided by Dr Jessica Boyd (NRC Institute for Marine Biosciences, Halifax, Nova Scotia). The strain PAK 2Pfs, a multipiliated retraction-deficient strain, was used for the purification of pili only and not used in experimental conditions. The phenotypes of the *P. aeruginosa* strains with respect to expression of pili was experimentally verified by Western blotting with anti-PAK pilus-specific antisera and by direct ELISA with whole cells and heat inactivated whole cells (to determine the presence of surface-exposed pili), by the sensitivity to type IV pilus-specific phage, and by monitoring the twitching motility of the strains. *P. aeruginosa* was routinely grown at 37°C in Luria–Bertani broth (LB) or LB supplemented with 50 μg ml^{-1} tetracycline (Sigma) for PAKNP, 100 μg ml^{-1} of gentamycin for PAKMS591, or 50 μg ml^{-1} of streptomycin for strains PAK-BΩ and PAK-DΩ. The polyclonal antibodies generated against the PAK pili and associated pre-immune antisera used in this study have been reported previously (Paranchych *et al*., 1979).

Biotinylation of P. aeruginosa *cells and purified pili*

Biotinylation of bacteria was performed as previously described by Yu *et al*. (1996) with the following modifications. Harvested cells were suspended in 5 ml of phosphate buffered saline (PBS) (pH 6.8) with 75 μl of 20 mg ml^{-1} biotinamidocaproate N-α-hydroxysuccinimidyl ester dissolved in dimethylsulfoxide and incubated at 22°C with agitation (200 rpm) in a water bath shaker for 1 h. Cells were harvested by centrifugation (10 000 *g* for 10 min at 4°C) and washed four times before resuspension in 1.0 ml PBS, pH 6.8. Viable counts were performed before and after biotinylation. PAK pili were purified from PAK 2Pfs as described previously (Paranchych *et al*., 1979). The purity and integrity of the pili were assessed by 15% SDS-PAGE (Sambrook *et al*., 1989) and electron microscopy (Fig. 1C and F). The procedure used for the biotinylation of the purified pili has been previously described (Yu *et al*., 1996). The ability of the biotinylated pili to bind to asialo-GM_1 and GM_1 was determined as previously described (Lee *et al*., 1994) and the binding specificity for asialo-GM_1 was confirmed to establish the functional binding activity of the pili following biotinylation.

Stainless steel binding assay

Grade 304 stainless steel 2B finish plates (20 gauge – 1 mm thick and 7.6 × 11.5 cm) were washed in 95% ethanol for 10 min, and rinsed with distilled water. Immediately before the binding studies, coupons were washed with 20 ml of acetone for 1 min with gentle agitation and rinsed with distilled water. Coupons were then assembled into a Schleicher and Schuell MinifoldTM System (Mandel Scientific). Biotinylated viable PAK cells or purified PAK pili (biotinylated or unbiotinylated) were added (100 μl per well in replicates of six) to the stainless steel manifold and incubated at 37°C for 1 h with gentle agitation. The manifold was subsequently washed five times with 250 μl per well Buffer A (PBS pH 7.4 containing 0.05% BSA). Binding was assessed using either streptavidin-horseradish peroxidase (HRP) or polyclonal PAK antibodies and secondary goat-anti-rabbit HRP (Bio-Rad). Substrate buffer (0.01 M sodium citrate buffer pH 4.2 containing 1 mM 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma) and 0.03% (v/v) hydrogen peroxide) was added (125 μl per well) and the manifolds were incubated at room temperature (RT) for 10 min with shaking at 150 rpm. The absorbance was determined at 405 nm using a Multiskan Plus version 2.01 plate reader following transfer of the reaction solution to 96 well flat-bottomed micro titer plates (Corning).

Buccal epithelial cell assay

Buccal epithelial cell assay was completed as described by McEachran and Irvin (1985), with the following modifications. Corning 24 well tissue culture treated ELISA plates were incubated with 500 μl of a 1 μg ml^{-1} poly L-lysine solution at 75°C overnight before washing for 15 min with PBS three times. Gluteraldehyde (25%, 125 μl per well) was incubated for 1 h at 37°C. Wells were washed as previously stated. BECs, harvested from 10 healthy volunteers, were filtered through a fine (70 μl) nylon mesh (Spectrum) and added to the prepared ELISA plate for overnight fixation at 37°C. Binding studies were performed as noted above.

Antibody inhibition studies

Biotinylated viable PAK whole cells or biotinylated purified PAK pili were mixed with 50 μl of a 10^{-2} dilution of pilus-specific antibody (note that all antibodies were initially set to the same titer via an ELISA employing purified pili as an antigen) or pre-immune rabbit sera in PBS buffer, pH 7.4, with a vortex mixer and incubated for 1 h at 37°C. The cell or pili mixture was utilized for binding assays as previously described (Yu *et al*., 1996). Concentrations of biotinylated PAK cells or biotinylated PAK pili ranged from 0 to 3.0×10^{16} cfu ml^{-1} and 0 to 1.5 μg ml^{-1} respectively. The steel surface was washed five times with Buffer A, incubated with 100 μl of either a rabbit anti-IgG HRP, for the polyclonal antibody or mouse anti-IgG HRP for the monoclonal antibodies. After an 1 h incubation at 37°C, the steel surface was

washed as described, and ABTS substrate solution (125 μl per well) was added for 15 min. The two pilus-specific antibodies used in this study, a polyclonal anti-PAK pili antibody (Lee *et al*., 1989) and monoclonal antibody PK99H (Doig *et al*., 1990), have been previously described. Rabbit preimmune serum, which had previously been determined to be free of anti-*P. aeruginosa* antibodies by ELISA, was utilized as a control.

Peptide synthesis and competitive peptide inhibition assays

The peptides described in Table 1 were synthesized as the N-α-acetylated free carboxyl form, except for PAK(134–140) which was synthesized as the N-α-acetylated and C-terminal amide form, by solid-phase peptide synthesis and purified by reversed-phase high-performance liquid chromatography (HPLC) as previously reported (Wong *et al*., 1992; Wong *et al*., 1995). Peptides containing two cysteine residues were air-oxidized to generate the disulphide bridged form of the peptide with disulphide formation being experimentally confirmed (Campbell *et al*., 1995). Synthetic peptides PAK(128–144)ox, PAK(117–125), PAK(134–140), PAO(128–144)ox, PAK(128–144)oxK130l, PAO(128–144)ox_Scrambled and PAO(128–144)C129A/C142A_Scrambled were dissolved in Buffer A and incubated with either 10^{15} cfu ml^{-1} biotinylated viable PAKwt cells or 0.75 μg ml^{-1} of biotinylated purified PAK pili such that the final peptide concentration ranged from 51 nM to 51 μM. The samples were then utilized directly in a steel surface binding assay as described above.

Trypsinized BSA peptides

Bovine serum albumin (BSA) (Biotech grade Fisher) was dissolved in PBS pH 7.4, and heat denatured by boiling in a water bath for 1 h. Trypsin (50 μl of a 1 mg ml^{-1} solution) was then added to a 10 mg ml^{-1} heat denatured BSA solution and incubated at RT overnight with gentle agitation. Trypsin was heat inactivated via boiling water bath for 1 h before use in competitive peptide inhibition assays as listed above.

Direct binding of peptides to steel

To confirm that the pilin receptor binding domain was directly interacting with the steel surface rather than indirectly inhibiting cell or pilus binding to steel, direct binding of the synthetic receptor binding domain was assessed. The binding of the synthetic peptides to steel was determined by a modified immunoassay that has been previously described (Yu *et al*., 1996). Synthetic peptides PAK(128–144)ox and PAK(134–140) were prepared in Buffer A (0–51 μM). The peptides were then added directly to wells (100 μl per well in replicates of six) formed on the steel surface and incubated for 1 h at 37°C without agitation. Following five washes with Buffer A (250 μl per well), a 1:5000 dilution of PK99H was added to each well (100 μl per well) and incubated for 1 h, and washed as described. Secondary antibody Goat anti Rabbit IgG HRP (100 μl per well of a 1:3000 dilution) was added and again incubated for 1 h. ABTS substrate solution was added (125 μl per well) and allowed to incubate for 25 min before the absorbance at 405 nm was determined as described.

Acridine orange staining and microscopy

Stainless steel plates, prepared and utilized in bacterial binding studies as described above, were incubated in 1 mM Acridine Orange stain for 1 min, and thoroughly rinsed with distilled water. Coupons were visualized using a Leitz laborlux K microscope equipped with a MSP4 camera, and 40× Neoflour lens with epifluorescent illumination. Micrographs were recorded with Kodak Colormax 35 mm film, processed and digitally scanned immediately after film processing.

Acknowledgements

This work was supported by an operating grant from the Canadian Institutes for Health Research (MOP-38004) and an RGPIN-Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (to R.T.I) and by Public Health Service Grants grant from the National Institutes of Health (D.J.H., AI-40541, GM-69845, and RSH, AI-48717) and Cystic Fibrosis Foundation (HASSET03P0, D.J.H). The generosity of Jessica Boyd in the provision of some of the strains used in this study is gratefully acknowledged.

References

Arnold, J.W., Boothe, D.H., Suzuki, O., and Bailey, G.W. (2004) Multiple imaging techniques demonstrate the manipulation of surfaces to reduce bacterial contamination and corrosion. *J Microsc* **216:** 215–221.

Audette, G.F., Irvin, R.T., and Hazes, B. (2004) Crystallographic analysis of the *Pseudomonas aeruginosa* strain K122–4 monomeric pilin reveals a conserved receptor-binding architecture. *Biochemistry* **43:** 11427–11435.

Bagge, D., Hjelm, M., Johansen, C., Huber, I., and Gram, L. (2001) *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. *Appl Environ Microbiol* **67:** 2319–2325.

Balazs, D.J., Triandafillu, K., Wood, P., Chevolot, Y., van Delden, C., Harms, H., *et al.* (2004) Inhibition of bacterial adhesion on PVC endotracheal tubes by RF-oxygen glow discharge, sodium hydroxide and silver nitrate treatments. *Biomaterials* **25:** 2139–2151.

Beachey, E.H. (1981) Bacterial adherence: adhesion–receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J Infect Dis* **143:** 325–345.

Blenkinsopp, S.A., Khoury, A.E., and Costerton, J.W. (1992) Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **58:** 3770–3773.

Bodey, G.P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* **5:** 279–313.

Bradley, D.E., and Pitt, T.L. (1974) Pilus-dependence of four *Pseudomonas aeruginosa* bacteriophages with non-contractile tails. *J Virol* **24:** 1–15.

Bremer, P.J., and Geesey, G.G. (1991) Laboratory-based model of microbiologically induced corrosion of copper. *Appl Eviron Microbiol* **57:** 1956–1962.

Campbell, A.P., McInnes, C., Hodges, R.S., and Sykes, B.D. (1995) Comparison of NMR solution structures of the receptor binding domains of *Pseudomonas aeruginosa* pili strains PAO, KB7, and PAK: implications for receptor binding and synthetic vaccine design. *Biochemistry* **34:** 16255–16268.

Characklis, W.G., McFeters, G.A., and Marshall, K.C. (1990) Physiological ecology in biofilm systems. In *Biofilms.* Characklis, W.G., and Marshall, K.C. (eds). New York: John Wiley & Sons, pp. 341–394.

Costerton, J.W. (2001) Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trend Microbiol* **9:** 50–52.

Craig, L., Taylor, R.K., Pique, M.E., Adair, B.D., Arvai, A.S., Singh, M., *et al.* (2003) Type IV pilin structure and assembly: X-ray and EM analysis of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol Cell* **11:** 1139–1150.

Doig, P., Sastry, P.A., Hodges, R.S., Lee, K.K., Paranchych, W., and Irvin, R.T. (1990) Inhibition of pilus-mediated adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells by monoclonal antibodies directed against pili. *Infect Immun* **58:** 124–130.

Folkhard, W., Marvin, D.A., Watts, T.H., and Paranchych, W. (1981) Structure of polar pili from *Pseudomonas aeruginosa* strains K and O. *J Mol Biol* **149:** 79–93.

Groessner-Schreiber, B., Hannig, M., Duck, A., Griepentrog, M., and Wenderoth, D.F. (2004) Do different implant surfaces exposed in the oral cavity of humans show different biofilm compositions and activities? *Eur J Oral Sci* **112:** 516–522.

Hazes, B., Sastry, P.A., Hayakawa, K., Read, R.J., and Irvin, R.T. (2000) Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. *J Mol Biol* **299:** 1005–1017.

Hood, S.K., and Zottola, E.A. (1997) Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *Internat J Food Microbiol* **37:** 145–153.

Irvin, R.T., Doig, P.C., Lee, K.K., Sastry, P.A., Paranchych, W., Todd, T., and Hodges, R.S. (1989) Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. *Infect Immun* **57:** 3720–3726.

Irvin, R.T., Doig, P.C., Sastry, P.A., Heller, B., and Paranchych, W. (1990) Usefulness of equilibrium parameters of adhesion in predicting the outcome of competition for bacterial receptor sites on respiratory epithelial cells by *Pseudomonas aeruginosa* strains of heterologous pilus type. *Microbial Ecol Health Dis* **3:** 39–47.

Johansen, C., Falholt, P., and Gram, L. (1997) Enzymatic removal and disinfection of bacterial biofilms. *Appl Environ Microbiol* **63:** 3724–3728.

Khaled, G.H., Finkelstein, F.O., Carey, H.B., Warlaw, S.C., Kilger, A.S., and Edburg, S.C. (2001) Method for studying development of colonization and infection of dialysis catheters. *Adv Perit Dial* **17:** 163–171.

Klausen, M., Aaes-Jorgensen, A., Molin, S., and Tolker-Nielsen, T. (2003) Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Mirobiol* **50:** 61–68.

Koga, T., Ishimoto, K., and Lory, S. (1993) Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. *Infect Immun* **61:** 1371–1377.

Kumon, H., Watanabe, T., Vincent, P., and Nickel, J.C. (1997) Fully hydrated images of *Pseudomonas aeruginosa* biofilm on the surface of catheter material. *Can J Urol* **4:** 416–421.

Leake, E.S., Gristina, A.G., and Wright, M.J. (1982) Use of chemotaxis chambers for studying *in vitro* bacterial colonization of biomaterials. *J Clin Microbiol* **15:** 320–323.

Lee, K.K., Sastry, P.A., Paranchych, W., and Hodges, R.S. (1989) Immunological studies of the disulfide bridge region of *Pseudomonas aeruginosa* PAK and PAO pilins, using anti-PAK pilus and antipeptide antibodies. *Infect Immun* **57:** 520–526.

Lee, K.K., Sheth, H.B., Wong, W.Y., Sherburne, R., Paranchych, W., Hodges, R., *et al.* (1994) The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol Microbiol* **11:** 705–713.

Lomander, A., Schreuders, P., Russek-Cohen, E., and Ali, L. (2004) Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces. *Bioresour Technol* **94:** 275–283.

McEachran, D.W., and Irvin, R.T. (1985) A new method for the irreversible attachment of cells or proteins to polystyrene tissue culture plates for use in the study of bacterial adhesion. *J Microbiol Method* **5:** 99–111.

McNeil, S.A., Nordstrom-Lerner, L., Malani, P.N., Zervos, M., and Kauffman, C.A. (2001) Outbreak of sternal surgical site infections due to *Pseudomonas aeruginosa* traced to a scrub nurse with onychomycosis. *Clin Infect Dis* **33:** 317–323.

O'Toole, G.A., and Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30:** 295–304.

O'Toole, G.A., Kaplan, H.B., and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54:** 49–79.

Paranchych, W., Sastry, P.A., Frost, L.S., Carpenter, M., Armstrong, G.D., and Watts, T.H. (1979) Biochemical studies on pili isolated from *Pseudomonas aeruginosa* strain PAO. *Can J Microbiol* **25:** 1175–1181.

Pasloske, B.L., Sastry, P.A., Finlay, B.B., and Paranchych, W. (1988) Two unusual pilin sequences from different isolates of *Pseudomonas aeruginosa*. *J Bacteriol* **170:** 3738–3741.

Pier, G.B. (1985) Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host–bacterium interaction. *J Infect Dis* **151:** 575–580.

Saiman, L., Ishimoto, K., Lory, S., and Prince, A. (1990) The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. *J Infect Dis* **161:** 541–548.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

van Schaik, E.J., Giltner, C.L., Audette, G.F., Keizer, D.W., Bautista, D.L., Slupsky, C.M., *et al.* (2005) DNA binding: a novel function of *Pseudomonas aeruginosa* type IV pili. *J Bacteriol* **187:** 1455–1464.

Schweizer, F., Jiao, H., Hindsgual, O., Wong, W., and Irvin, R.T. (1998) Interaction between the pili of *Pseudomonas aeruginosa* PAK and its carbohydrate receptor β-D-GalNAc (1–4) β-D-Gal analogs. *Can J Microbiol* **44:** 307–311.

Sheth, H.B., Lee, K.K., Wong, W.Y., Srivastava, G., Hindsgaul, O., Hodges, R.S., *et al.* (1994) The pili of *Pseudomonas aeruginosa* strains PAK and PAO bind specifically to the carbohydrate sequence β GalNAc (1,4) βGal found in glycosphingolipids asialo-GM1 and asialo-GM2. *Mol Microbiol* **11:** 715–723.

Sheth, H.B., Glasier, L.M.G., Ellert, N.W., Cachia, P., Kohn, W., Lee, K.K., *et al.* (1995) Development of an anti-adhesive vaccine for *Pseudomonas aeruginosa* targeting the C-terminal region of the pilin structural protein. *Biomed Pept Proteins Nucleic Acids* **1:** 141–148.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407:** 762–764.

Sreekumari, K.R., Nandakumar, K., and Kikuchi, Y. (2001) Bacterial attachment to stainless steel welds: significance of substratum microstructure. *Biofouling* **17:** 303–316.

Stanley, P.M. (1983) Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel. *Can J Microbiol* **29:** 1493–1499.

Starnbach, M.N., and Lory, S. (1992) The fliA (rpoF) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis. *Mol Microbiol* **6:** 459–469.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton, J.W. (2002) Biofilms as complex differentiated communities. *Ann Rev Microbiol* **56:** 187–209.

Suci, P.A., and Geesey, G.G. (2001) Comparison of adsorption behavior of two *Mytilus edulis* foot proteins on three surfaces. *Colloids Surf B Biointerfaces* **22:** 159–168.

Traverso, C.E., De Feo, F., Messas-Kaplan, A., Denis, P., Levartovsky, S., Sellem, E., *et al.* (2005) Long term effect on IOP of a stainless steel glaucoma drainage implant (EXPRESS) in combined surgery with phacoemulsification. *Br J Ophthalmol* **89:** 425–429.

Tredget, E.E., Shankowsky, H.A., Joffe, A.M., Inkson, T.I., Volpel, K., Paranchych, W., *et al.* (1992) Epidemiology of infections with *Pseudomonas aeruginosa* in burn patients: the role of hydrotherapy. *Clin Infect Dis* **15:** 941–949.

VanHaecke, E., Remon, J.P., Moors, M., Raes, F., De Rudder, D., and Van Peteghem, A. (1990) Kinetics of *Pseudomonas aeruginosa* adhesion to 304 and 316-L stainless steel: role of cell surface hydrophobicity. *Appl Environ Microb* **56:** 788–795.

Watnick, P., and Kolter, R. (2000) Biofilm, city of microbes. *J Bacteriol* **182:** 2675–2679.

Wong, W.Y., Irvin, R.T., Paranchych, W., and Hodges, R.S. (1992) Antigen–antibody interactions: elucidation of the epitope and strain-specificity of a monoclonal antibody directed against the pilin protein adherence binding domain of *Pseudomonas aeruginosa* strain K. *Protein Sci* **1:** 1308–1318.

Wong, W.Y., Campbell, A.P., McInnes, C., Sykes, B.D., Paranchych, W., Irvin, R.T., and Hodges, R.S. (1995) Structure-function analysis of the adherence-binding domain on the pilin of *Pseudomonas aeruginosa* strains PAK and KB7. *Biochemistry* **34:** 12963–12972.

Wozniak, D.J., Wyckoff, T.J., Starkey, M., Keyser, R., Azadi, P., O'Toole, G.A., and Parsek, M.R. (2003) Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci USA* **100:** 7907–7912.

Yu, L., Lee, K.K., Paranchych, W., Hodges, R.S., and Irvin, R.T. (1996) Use of synthetic peptides to confirm that the *Pseudomonas aeruginosa* PAK pilus adhesin and the *Candida albicans* fimbrial adhesin possess a homologous receptor-binding domain. *Mol Microbiol* **19:** 1107–1116.

Zuo, R., Örnek, D., and Wood, T.K. (2005) Aluminum- and mild steel-binding peptides from phage display. *Appl Mirobiol Biotechnol*. **68:** 505–509.