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Animal Protection and Structural Studies of a Consensus Sequence Vaccine Targeting the Receptor Binding Domain of the Type IV Pilus of *Pseudomonas aeruginosa*

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Abstract

One of the main obstacles in the development of a vaccine against *Pseudomonas aeruginosa* is the requirement that it is protective against a wide range of virulent strains. We have developed a synthetic-peptide consensus-sequence vaccine (Cs1) that targets the host receptor-binding domain (RBD) of the type IV pilus of *P. aeruginosa*. Here, we show that this vaccine provides increased protection against challenge by the four pilated strains that we have examined (PAK, PAO, KB7 and P1) in the A.BY/SnJ mouse model of acute *P. aeruginosa* infection. To further characterize the consensus sequence, we engineered Cs1 into the PAK monomeric pilin protein and determined the crystal structure of the chimeric Cs1 pilin to 1.35 Å resolution. The substitutions (T130K and E135P) used to create Cs1 do not disrupt the conserved backbone conformation of the pilin RBD. In fact, based on the Cs1 pilin structure, we hypothesize that the E135P substitution bolsters the conserved backbone conformation and may partially explain the immunological activity of Cs1. Structural analysis of Cs1, PAK and K122-4 pilins reveal substitutions of non-conserved residues in the RBD are compensated for by complementary changes in the rest of the pilin monomer. Thus, the interactions between the RBD and the rest of the pilin can either be mediated by polar interactions of a hydrogen bond network in some strains or by hydrophobic interactions in others. Both configurations maintain a conserved backbone conformation of the RBD. Thus, the backbone conformation is critical in our consensus-sequence vaccine design and that cross-reactivity of the antibody response may be modulated by the composition of exposed side-chains on the surface of the RBD. This structure will guide our future vaccine design by focusing our investigation on the four variable residue positions that are exposed on the RBD surface.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that can cause serious infections in individuals with compromised host defenses.¹ It has been suggested that adhesins play key roles in the initial stages of infection, where they mediate attachment of the bacteria to host epithelial-cell surfaces, allowing for subsequent colonization and potential invasion.

Because of its length, the type IV pilus has been suggested to mediate initial attachment of the bacteria to host surfaces before other adhesins secure the attachment. Once attached, the coordinated expression of numerous other virulence factors facilitates invasion of the surface by the bacteria.² Because of its role early in the pathogenesis of infection, the type IV pilus has been proposed as an attractive vaccine target.

Type IV pili are multimeric, non-branching, filamentous structures of only 6 nm in diameter that can reach lengths of several micrometers.³ They are the longest extension from the surface of *P. aeruginosa* and can be many times longer than the bacterium itself. The pilus fiber is composed of thousands of identical pilin protein monomers, encoded by the PilA gene.⁴ Atomic resolution structures have been determined for four type IVa pilins (*P. aeruginosa*: PAK (29–144),⁵ PAK (1–144),⁶ K122-4 (29–150);⁷ *Neisseria gonorrhoeae* MS11 (1–150)).⁸ Truncation of residues 1–28 of the N-terminal α -helix allows for soluble expression of the pilin monomer, but does not disrupt the structure of the globular head domain of the pilin protein. The structure of the full-length pilin monomer and cryo-electron microscopy studies have given rise to a detailed structural model of the pilus fiber.⁹

The N-terminal α -helix of the pilin monomers is highly conserved among all type IV pilins and functions in pilus assembly. In contrast, the globular domain, comprised of β -sheet and loop regions, shows much greater sequence diversity, with approximately 30% sequence homology among strains.⁵ Though the assembly is common between type IVa and IVb pilins, the receptor binding domain (disulfide loop region) in *P. aeruginosa* strains is dramatically different from other type IVa and IVb pilins. For example, the disulfide loop region is 65 residues between cysteine residues in the type IVb pilin of *Vibrio cholerae*, 29 residues in *N. gonorrhoeae* MS11, and 12 residues in the majority of *P. aeruginosa* strains (PAK, PAO, KB7, K122-4 and CD4; Figure 1).⁹ In addition, there is little sequence homology between the disulfide loop regions of *P. aeruginosa* and other pilins. Thus, it is highly unlikely to have antibody cross-reactivity between pilins from *P. aeruginosa* and pilins from other species.

Pili bind tracheal, buccal, and corneal epithelial cells specifically through interaction with a disulfide-loop region found at the C terminus of the pilin monomer called the receptor-binding domain (RBD).¹⁰ Antibodies specific for the RBD can block pilus mediated adhesion and are the basis for this vaccine development project.¹¹ The importance of pili in the binding of *P. aeruginosa* to epithelial cell surfaces has been shown by a 90% decrease in the ability of non-piliated strains of *P. aeruginosa* to bind human A549 type II pneumocytes.^{12,13} In another study using a neonatal mouse model of *P. aeruginosa* pneumonia, non-piliated strains caused 28% to 96% fewer cases of pneumonia compared to piliated control strains.¹⁴ These results show that pili are important for establishing infection by *P. aeruginosa*.

The C-terminal receptor binding domain of *P. aeruginosa* pilin is a strong candidate for a peptide vaccine. The 14 amino acid residue loop that is bridged by the disulfide bond represents a continuous protective epitope that can be conveniently represented by a single peptide. Peptide analogs of the RBD are also well-structured in solution, exemplified by the presence of two β -turns within the loop.^{15,16} The conformational constraints imposed by the disulfide bridge contribute to the peptide structure. There is structural similarity between the free peptide and the RBD in the full length native pilin protein⁶ as well as truncated monomeric pilin proteins.^{5,15,17} Finally, peptide analogs retain receptor-binding activity to host epithelial cells. This functional analogy was shown by the ability of disulfide-bridged synthetic peptide derivatives of the pilin RBD to competitively inhibit pili binding to cultured epithelial cells.¹⁸ Antibodies raised against peptide conjugates can inhibit adhesion of *P. aeruginosa* to cultured human epithelial cells and have been shown to be protective

against bacterial challenge. RBD-specific antibodies have been shown to only bind the tip of the pilus.¹⁹ This is consistent with the current model of pilus assembly, where the RBD is partially obscured along the sides of the pilus by the $\alpha\beta$ -loop.⁹

Type IV pili from all strains of *P. aeruginosa* share a common receptor, but there is considerable sequence heterogeneity among the strains in the RBD regions. This sequence diversity in an important epitope presents a significant obstacle to the development of a broadly protective vaccine targeting the type IV pilus. As shown in Figure 1, six of 12 positions within the disulfide loop are highly variable (positions 130, 132, 133, 135, 136 and 138). The other six residues within the loop have conserved identities or show conservative substitutions. The two cysteine residues (Cys129 and Cys142) and proline 139 are strictly conserved among all strains and are required to form the disulfide loop. Residues 131, 134, 137, 140, and 141 are semi-conserved. These positions show only conservative substitutions among the strains (e.g. Ser for Thr and Phe for Tyr at positions 131 and 137, respectively). We have hypothesized that the conserved and semi-conserved residues are framework residues that define the RBD. It is unclear what effect the remaining variable positions have on the receptor binding domain functionally or structurally. In regard to vaccine design, it is possible that the variation of these residues decreases the chances that more than one strain would be recognized by the immune system.

Due to complications arising from antigenic competition, a multivalent pilus-based vaccine representing multiple strains of *P. aeruginosa* is not effective at generating immunity against multiple strains, so we have pursued a consensus sequence approach to vaccine design.^{20,21} The feasibility of this approach has been demonstrated, where single amino acid substitutions in peptide immunogens was shown to modulate the cross-reactivity of the antibody response in rabbits.²⁰ A consensus-sequence immunogen has been designed in this laboratory based on the hypothesis that making substitutions of the PAO sequence into the PAK sequence would increase cross-reactivity of antibodies raised against it.²¹ A series of peptide immunogens with one, two, or three substitutions of the PAO sequence into the PAK sequence was designed. It was found that certain di-substituted immunogens generated antisera with increased cross-reactivity for other *P. aeruginosa* strains.²¹ One promising PAK analog with two substitutions, T130K-E135P, was chosen as a putative consensus sequence and designated Cs1 (Figure 1).

Here, we show that the Cs1 peptide immunogen has an improved immunological activity in comparison to native-strain immunogens. Animal protection studies show that Cs1 can induce an immune response that can be protective against challenge by four pilated strains of *P. aeruginosa*. To understand how the substitutions in the consensus sequence affect the RBD epitope as it is presented to the immune system, we have determined the high resolution X-ray crystal structure of the Cs1 sequence in the context of a monomeric pilin protein. Our structural analyses provide insight into the mechanism by which the Cs1 sequence may generate antibodies of increased cross-reactivity.

Results

Active immunization with Cs1

Active immunization studies were performed to characterize the protection provided by the Cs1 peptide against challenge by four pilated strains of *P. aeruginosa*. The A.BY/SnJ mouse strain has a competent humoral immune response, but genetic defects make it naturally susceptible to infection by *P. aeruginosa* and provide a convenient mouse model of infection that does not require additional immunosuppressive procedures.²² For these experiments, peptides derived from the receptor-binding domains of the PAK and PAO pilins as well as the Cs1 sequence were conjugated to tetanus toxoid (TT) to increase the immunogenicity of

the peptides. Groups of ten A.BY/SnJ mice were immunized with the peptide conjugates and subsequently challenged with lethal doses of four different strains of *P. aeruginosa*.

Figure 2 shows the results of the protection studies. When challenged with PAK strain bacteria, mice immunized with the PAO peptide TT conjugate are not as well protected as mice immunized with the Cs1 peptide TT conjugate (Figure 2(a)). Only two out of ten mice immunized with the PAO peptide conjugate survived in comparison to five out of ten mice that survived that were immunized with the Cs1 peptide conjugate. Likewise, when challenged by PAO strain bacteria, animals immunized with the PAK peptide TT conjugate are not as well protected as mice immunized with the Cs1 peptide TT conjugate (Figure 2(b)). In this case, four of ten mice immunized with the PAK peptide conjugate survived challenge by the PAO bacteria in comparison to seven out of ten mice immunized with the Cs1 peptide conjugate that survived. These results show that when immunized with native strain sequences, mice challenged with heterologous strains are not as well protected as when mice are immunized with the Cs1 immunogen. The Cs1 peptide TT conjugate was also shown to provide increased protection against challenge by KB7 and P1 strain bacteria when compared to immunization with adjuvant alone (Figure 2(c) and (d)). This is significant because the amino acid sequences of the RBD regions of the KB7 and P1 strains are quite different from the Cs1 sequence, which is based only on the PAK and PAO sequences (Figure 1). We have also immunized mice with Cs1 peptide TT conjugate and challenged with bacteria (strains PAK, PAO and P1) and compared the results to a control immunization with scrambled Cs1 peptide TT conjugate (Figure 2(e)–(g)). The scrambled Cs1 peptide TT conjugate represents the most rigorous approach to demonstrating the effectiveness of the consensus sequence in generating cross-protective protection. We are not attempting to claim that the consensus sequence will necessarily be more protective than the native pilin receptor binding domain sequence for protection against the homologous strain challenge, but rather that the consensus peptide vaccine is “better” in the sense that a single peptide sequence elicits a broadly cross-reactive immune response that confers protection against heterologous strain challenges. This eliminates the need for a large number of antigens (which would elicit a limited response against only a few of the immunogens) and yet still confers protection. Thus, the term “better” does not imply that the consensus sequence will elicit a more protective response against a homologous strain, but to indicate a broadly cross-reactive and protective response is obtained. For example, immunization with PAO sequence does not confer protection against KB7 challenge whereas Cs1 does. To summarize, these protection data have shown that the Cs1 immunogen has increased protection in two respects. First, the heterologous protection conferred by Cs1 is greater than protection conferred by the native strain sequences (Figure 2(a) and (b)). Second, Cs1 induces an immune response that is truly cross-protective against infection by a range of strains of *P. aeruginosa* (Figure 2(c)–(g)). In fact, it is interesting that Cs1 antibodies are protective against challenge by strain P1 where the RBD has 17 residues in the disulfide loop compared to only 12 residues in Cs1 (Figure 1).

Structure of Cs1 pilin

The animal protection data show that the substitutions used to create the Cs1 sequence have produced a novel immunological activity that is distinctly different from the activity of the native PAK strain sequence upon which it was based. This makes Cs1 a strong vaccine candidate. On the basis of these data, we undertook structural studies to determine how the Cs1 sequence differed from the native strain sequences. Since the PAK monomeric pilin protein had been crystallized,⁵ the Cs1 sequence was engineered into the PAK pilin protein, to create the double mutant pilin protein called Cs1 pilin. Cs1 pilin was crystallized. The structure was solved by molecular replacement, and refined to a resolution of 1.35 Å, with

good stereochemistry and crystallographic statistics (Table 1). High quality electron density was observed for most of the model (Figure 3), except for pilin residue 144.

As expected, the three-dimensional structure of Cs1 monomeric pilin was similar to the reported structure of PAK monomeric pilin,⁵ indicating that alteration of the RBD region did not alter the overall structure of the protein (Figure 4). The monomeric pilin structure is composed of a major β -sheet, flanked on one side by a long α -helix and a minor two-stranded β -sheet, with the C terminus forming the RBD. Least-squares fitting of the main-chain atoms of the Cs1 pilin and PAK pilin structures revealed two regions that deviate by more than 2 Å between the two structures. Gly60, which is in the loop region preceding the minor β -sheet also showed a deviation between the PAK and Cs1 structures. These differences may be attributed to differences in crystal contacts, and exclusion of these residues from the alignment resulted in an overall r.m.s.d. value of 0.57 Å, which is higher than would be expected for two identical structures (Figure 4(b)). The Cs1 pilin was similar to the globular head domain of the full length PAK pilin,⁶ where the r.m.s.d. for main-chain atoms was 0.65 Å for residues 29–143.

In contrast, in comparison to Cs1 pilin, the α -helix and β -sheet of K122-4 pilin are in different orientations with respect to each other. However, the helical region and the β -sheet region of the two proteins can be overlaid individually to show the structural similarities. The α -helix of Cs1 pilin and K122-4 pilin (residues 29–46) can be superimposed, giving an r.m.s.d. value of 0.55 Å for the helical region. Likewise, the major β -sheet and RBD regions (including residues 98–105, 107–121, and 127–142 of Cs1 and residues 93–100, 104–118 and 127–142 of K122-4) can be superimposed to give an r.m.s.d. value of 1.41 Å for the β -sheet region. These r.m.s.d. comparisons demonstrate the similarity of the overall structures among the native and Cs1 monomeric pilins. In addition, the most significant structural deviations of Cs1 pilin are not in the RBD, so they should not interfere with our analysis of the Cs1 sequence.

Cs1 substitutions do not alter the secondary structure elements of the RBD

The purpose of this structural study was to analyze the RBD of Cs1 monomeric pilin to gain insight into the novel immunological activity of the Cs1 sequence. First, we compared the backbone conformations of the RBD region of Cs1 pilin to the available structures of native monomeric pilins (Figure 5(b) and (c)). The r.m.s.d. value of the Cs1 and PAK pilin protein regions outside of the RBD (residues 29–127) which have identical amino acid sequence is 0.51 Å. When the RBD regions (128–143) of Cs1 pilin and truncated PAK pilin5 are superimposed, the main-chain r.m.s.d. value is 0.70 Å, indicating minor differences in the RBD regions. Likewise, the main-chain r.m.s.d. value between the RBD regions (128–143) of Cs1 pilin and the full-length PAK pilin6 is 0.65 Å. The r.m.s.d. value between the RBD regions (128–144) of Cs1 and K122-4 is 1.41 Å, which indicates greater differences between the RBD of these two structures. The RBD is composed of three secondary structure elements: one β -strand (residues 128–132), one β -turn (residues 134–137) and a second β -turn (residues 139–142). The calculated r.m.s.d. values for the superposition of the three elements range from 0.09 Å whereas the r.m.s.d. values range from 0.20 Å to 0.49 Å when comparing the Cs1 and K122-4 pilins (Table 2). These r.m.s.d. values are well within the range expected for identical structures, and thus the Cs1 substitutions preserve the general RBD features.

The way in which the RBD is positioned by the pilin protein core is conserved among the pilin protein structures. The first five residues of the RBD (positions 128 to 132) that form the β -strand region of the RBD anchor it to the major β -sheet of the pilin protein through a conserved network of main-chain hydrogen bonds with 114 to 119 of the neighboring β -strand (Figure 6(a)). The r.m.s.d. values for residues 128–132 is 0.15 Å and 0.26 Å in the

comparison of Cs1 pilin to PAK and K122-4 pilins, respectively. We suggest that the interactions defining the β -strand secondary structure stabilize the conformation of this N-terminal region of the receptor binding domain and make this conformation tolerant to sequence diversity. Lys130 of Cs1 makes van der Waals contacts with the side-chain of Thr117, but the Lys130 side-chain remains in an extended conformation, and this substitution does not alter the β -strand backbone conformation (Figure 5(c)). The T130K consensus-sequence substitution adopts phi and psi dihedral angles of -139.2° and 151.2° , respectively (-12.1° and 1.7° for $\Delta\phi$ and $\Delta\psi$ from PAK pilin, respectively). These torsion angles are well within the allowed ranges of dihedral angles for β -sheet secondary structure, indicating that the β -strand has not been disrupted by the Cs1 substitutions.

Following the β -strand, the two conserved β -turns define the backbone conformation of the RBD. The first β -turn is a type I turn that spans residues 134 to 137 and is observed in Cs1 pilin as it is in PAK and K122-4 pilins. The second consensus-sequence substitution, E135P, occupies the $i + 1$ position of the type I β -turn. Pro135 adopts phi and psi dihedral angles of -57.8° and -26.3° , respectively (-15.6° and 16.7° for $\Delta\phi$ and $\Delta\psi$ from PAK pilin, respectively). These phi and psi angles are very near the ideal dihedral angles for the $i + 1$ position of a type I β -turn (-60° and -30° , respectively).²³ Therefore, the E135P substitution in the Cs1 sequence may actually stabilize the type I β -turn and its side-chain does not make any contact with the rest of the pilin protein (Figure 5(c)). The second β -turn spans residues 139 to 142 and does not include either of the consensus-sequence substitutions. In the structures of Cs1 and PAK pilin this is a type II β -turn, but was found to be a type III β -turn in the K122-4 pilin. The transition from a type II to a type III β -turn may have come about because of the presence of threonine at position $i + 2$ of the K122-4 sequence.⁷ The β -branched threonine is more restricted in its possible main-chain dihedral angles. The larger r.m.s.d. value of K122-4 is consistent with the transition from the type II to the type III turn (Table 2). In the Cs1 pilin and PAK pilin structures, the phi and psi angles of the type II β -turn are close to ideal, showing that no major changes have occurred as a result of the consensus sequence substitutions.

Identification of hinge residues influence the conformation of the RBD

Whereas all three of the secondary structure elements of the RBD in isolation have highly similar and conserved structures, there are larger differences in the conformations of the RBD regions overall. Figure 6(a) shows that when the three pilin structures are superimposed along the β -strand element (128–132), differences in the positions of first β -turn become apparent. The $i + 1$ and $i + 2$ residues of this β -turn deviate from Cs1 pilin by 0.94–1.17 Å and 2.71–3.10 Å for the PAK and K122-4 pilins, respectively (Table 3). Furthermore, when the structures are superimposed along residues of the first β -turn, the deviations of the second β -turn are obvious (Figure 6(b)). In this case, the $i + 1$ and $i + 2$ residues of the first β -turn deviate from the Cs1 pilin by 1.14–1.89 Å and 5.91–5.96 Å for the PAK and K122-4 pilins, respectively (Table 3). The residues located at positions between the three secondary structure elements, positions 133 and 138 have differences in backbone conformation, which lead to the deviations in each of the pilin RBD regions. Therefore, positions 133 and 138 are hinge residues that account for the slightly different orientations of the β -strand and β -turns within the RBD.

The side-chains of variable positions 133 and 138 appear to be important in determining the RBD conformation. In the Cs1 and PAK structures, the side-chain of Gln133 is directed toward the core of the pilin protein on the buried face of the RBD region. The side-chain of Gln133 is in the center of an inferred hydrogen bond network with the sidechains of Asn111 and Ser131 (Figure 7(a)). The hydrogen bond between the side-chain of Gln133 and the side-chain of Asn111 anchors the RBD on the core of the pilin protein. The hydrogen bond between the side-chain of Gln133 and Ser131 contributes to the conformation of the receptor

binding domain in the PAK and Cs1 structures. The side-chain of Ile138 forms a small hydrophobic core around which the rest of the RBD is organized. The Cs1 pilin structure shows that there is no reorganization of the side-chains of these residues in comparison to PAK pilin as a result of the consensus-sequence substitutions. Furthermore, the Cs1 substitutions (T130K, E135P) do not affect interactions of the RBD with the core of the pilin protein, as the backbone dihedral angles are preserved and the side-chains are solvent exposed at both of the substitution sites. Therefore, the subtle differences in backbone conformation that we have observed in the Cs1 structure compared to the PAK pilin structure can be most likely be attributed to differences in crystal packing interactions. Notably, residues 134–136 of Cs1 pilin make crystal contacts that are not present in the PAK pilin structure.

In contrast, differences in K122-4 pilin at positions 133 and 138 give rise to the more pronounced deviations in the backbone conformation of the RBD. The presence of alanine at position 133 precludes the formation of hydrogen bonds with residues in the core of the pilin protein (Figure 7(b)). Instead, the side-chain of Ala133 makes a van der Waals contact with Leu33 and Leu111 (equivalent to Leu108 in the numbering system of K122-4, but equivalent structurally to position 111 in the Cs1 and PAK structures) (Figure 7(b)). Thus, the hydrogen bonding residues observed in the Cs1 and PAK structures (Gln133, Asn111 and Ser131) are replaced by hydrophobic side-chains (Ala133, L111 and Leu33), which contact each other to form a hydrophobic cluster. This suggests that the amino acid sequence of the RBD and in distant regions of the protein (positions 33 and 111) contribute in different ways to define the conformation of the RBD in the Cs1 and PAK pilins in comparison to K122-4 pilin. This is significant because although the sequences of the β -sheet region are generally variable among strains, the positions of the hydrophobic regions are highly conserved. Both configurations (hydrogen-bond network versus hydrophobic cluster) serve the same purpose of anchoring the RBD to the core of the pilin protein and defining the backbone conformation of the RBD, but accomplish this through different types of interactions.

The different interactions that side chain 133 makes in Cs1 pilin versus K122-4 pilin may explain the different RBD conformations observed in Figure 6 and Table 3. In K122-4, the space occupied by the side-chain of Gln133 in the Cs1 and PAK structures is partially replaced by the side-chains of Leu138 and Leu33. Interestingly, in the Cs1 and PAK sequences, Leu is replaced by Gly at position 33, which precludes the hydrophobic interaction between the side-chains of positions 33 and 138. Figure 6(b) shows that differences in dihedral angles around position 138 result in changes in the orientations of the two β -turns in the K122-4 structure compared to Cs1 and PAK. In part, this may be explained by the presence of the β -branched Ile138 in PAK and Cs1 versus Leu138 in K122-4. The different identities of residues at positions 133 and 138 may lead to the deviations observed in the K122-4 RBD. In addition, the different side-chain configurations at these positions in the K122-4 pilin may account for the larger r.m.s.d. value when compared to Cs1 pilin.

Surface residues in Cs1 and PAK RBD recognition

Given that the Cs1 substitutions do not appear to alter the conformation of the RBD, and the hinge residues of PAK and Cs1 are the same, we sought an explanation for the observed differences in immunogenicity. As discussed earlier, the identities of residues 130, 132, 133, 135, 136 and 138 vary among strains and antibodies specific for these variable residues may result in strain-specific protection. Therefore, we examined the solvent accessible surface of the exposed face of the RBD region that is altered due to the consensus sequence substitutions. The solvent accessible surface of the RBD of Cs1 pilin has features that are attributed to conserved residues. As shown in Figure 8(a), only the side-chains of the

conserved residues Asp134 and Lys140 are completely exposed on the surface of the RBD. The side-chain of Phe137 is partially solvent accessible and the side-chains of Cys129 and Cys142 that form the conserved disulfide bond are mostly buried. The side-chains of residues of Ser131 and Pro139 are completely buried. While the side-chains of the most of the conserved residues are at least partially buried, the side-chains of solvent accessible conserved residues are located toward the periphery of the RBD. The remaining surface of the receptor binding domain, 230.4 Å², is accounted for by backbone atoms, shown in Figure 8(a), and a role for these in receptor binding has been proposed.^{5,7} We also have hypothesized that antibodies that are cross-reactive among pilin proteins from multiple strains of *P. aeruginosa* may recognize an epitope consisting of conserved residues and backbone atoms.

There is a striking contrast in the positions of the conserved side-chains compared to the variable side-chains. The positions of the variable position side-chains on the surface of the Cs1 pilin protein shown in Figure 8(b). The side-chains of variable residues Lys130, Asp132, Pro135 and Gln136 are exposed on the RBD surface. Only 17% of the surface area of Ile138 is solvent exposed, because it is in the center of the RBD and packs against other residues. The side-chain of Gln133 is completely buried. The variable residues Lys128 and Ser143, though not within the disulfide loop, are also solvent exposed (Figure 8(b)). Therefore, four out of six variable residues of the RBD of Cs1 pilin are solvent accessible and are arranged prominently in a line across the exposed face of the RBD. In comparison to the surface of the RBD of PAK pilin, the side-chains of the same residues are solvent exposed and only differ in the identities of residues 130 and 135 in Cs1 pilin. This seems to indicate that the presence of Lys at position 130 and Pro at 135 can influence the cross-reactivity of antibodies raised against the RBD. The substitutions in the Cs1 pilin create changes in the electrostatic surface and potential electrostatic interactions in comparison to PAK pilin. The combination of the threonine to lysine and glutamic acid to proline substitutions result in a change in net charge of + 2 in the receptor binding domain and creates a new basic region on the protein surface. This difference in electrostatic potential could have significant implications on antibody recognition, as electrostatic interactions are often attributed to determination of antibody specificity.

Discussion

Over the past four decades, many approaches have been explored in the design of a vaccine against *P. aeruginosa*.^{24–26} One of the main obstacles has been to develop a protective vaccine that is active against a wide range of virulent strains. We have pursued the development of a synthetic-peptide vaccine that specifically targets the RBD of the type IV pilus of *P. aeruginosa*. Previous studies have shown that antibodies raised against a synthetic peptide derived from the RBD of the type IV pilus can provide protection against challenge by the strain from which the sequence was derived, but weaker protection against heterologous strain challenge.²¹ To produce a vaccine that is protective against a variety of strains, we have taken a novel approach in the use of a consensus-sequence immunogen. Here, we have characterized a consensus sequence (Cs1) that has been designed to induce an antibody response that is protective against multiple strains of *P. aeruginosa*.

Cs1 peptide vaccine has novel immunological activity

In animal protection studies, we have shown that immunization with Cs1 induced a response that increases survival of mice challenged by four pilated strains of *P. aeruginosa*. Immunogens derived from native strain sequences provided less protection against heterologous challenge than immunization with Cs1. The scale of the animal protection studies presented here were limited by cost and logistics (ten animals per group), which has limited the statistical significance of the conclusions that could be made. We believe that the

trends in the results presented here clearly show that Cs1 is a strong vaccine candidate. Additional studies with larger sample sizes would be necessary to determine the exact significance of the protection. The efficacy of this consensus sequence construct was impressive enough to warrant progression of Cs1 to phase I clinical safety trials in humans that demonstrated not only safety but that normal human volunteers could generate an immune response to the RBD region when immunized with the synthetic peptide tetanus toxoid conjugate (R.S.H., unpublished results).

Effect of consensus-sequence substitutions on backbone conformation

Structural studies have provided insight into the basis for this novel immunological activity. Here, we have characterized the structure of the Cs1 consensus sequence in the context of the truncated PAK pilin protein. Previously, NMR spectroscopy has been used to study synthetic peptides corresponding to the PAK, PAO, KB7 and P1 strain receptor-binding domains.^{15,27–31} These solution structures retained certain structural features seen in the crystal structures of the PAK and K122-4 pilin proteins, notably the two β -turns. In comparison to the crystal structures, however, differences in conformation and flexibility of the peptides made it unclear how the solution structures of the peptides related to the structures of identical sequences in the intact pilin proteins. Since our goal was to understand the relationship of the consensus sequence to the analogous regions in pilin proteins, we engineered the Cs1 sequence into the RBD of PAK pilin. The crystal structure of Cs1 pilin is very similar to the PAK pilin structure, despite the fact that Cs1 pilin crystallized in a different space group than the PAK pilin.⁵ Focusing on the RBD region, we found that the backbone of the consensus sequence adopts a conformation that is similar to the PAK and K122-4 strain pilin proteins. The similarities among these three RBD structures are most apparent at the level of secondary structure, where the β -strand and two β -turns overlay very closely. The differences in conformations of the RBD appear to be due to the types of interactions made by two hinge residues, 133 and 138. While even subtle changes in backbone conformation can result in changes in interactions such as antibody recognition, at this time we have no data to suggest that the observed changes in backbone conformation would be significant for antibody recognition.

The conserved positions of the receptor binding domain most likely provide a framework that can tolerate substitution at the variable positions. The most obvious examples of this are the two conserved cysteine residues, Cys129 and Cys142, which form a disulfide bond and limit dramatically the possible conformations of the receptor binding domain. The identities of other conserved positions of the RBD region may be important for formation of the two β -turns. In fact, based on structural and sequence data from the RBD region, the amino acid sequence of positions 134 to 142 of the RBD region appear to be optimized for β -turn formation. In studies of β -turns, Asp and Asn are the most frequently observed residues at position $i+2$, and we find that Asp134 is conserved at the i position of the type I β -turn of the RBD. At position $i+1$, proline has the highest positional potential in type I β -turns, followed by glutamic acid.³³ Interestingly, Pro and Glu occupy position 135 in the PAO and PAK sequences, respectively. The amino acid compositions of positions 136 and 137 do not match identities of the most preferred residues of positions $i+2$ and $i+3$ of type I β -turns, which may indicate that these residues play roles other than stabilization of the β -turn and may be important for establishing tertiary structure or in receptor binding. Phe137 is implicated in establishing tertiary structure because it makes a conserved van der Waals contact with Ala29 and Arg30 in the N-terminal α -helix in all three structures and it is unlikely to play a role in receptor recognition because it was not shown to be critical for binding of RBD peptide analogs to the eukaryotic receptor.³⁴

The β -turn that spans residues 139 to 142 in the RBD region is also composed of residues with high positional potentials for type II β -turns. Positions i , $i+2$, and $i+3$ are occupied

by residues with the highest propensities at those positions (Pro, Gly and Cys, respectively).³³ This suggests that the requirement of a second β -turn in the RBD is dictating the amino acid composition of these positions. Residues occupying position $i + 1$ of the type II β -turn in pilin RBD regions, Lys and Asn, do not have significantly high positional potentials for this position,³³ suggesting that this position has an important functional role not related to stabilization of the β -turn motif.

The strong propensity of the pilin RBD region to form two β -turns has led us to the hypothesis that the consensus sequence substitution of Glu to Pro at position 135 in Cs1 stabilizes the type I β -turn that spans residues 134 to 137. Stabilization of the β -turn may enhance its cross-reactivity as an immunogen because it makes the peptide more likely to adopt a native-like conformation. As one of the limitations of peptide immunogens is their inherent flexibility, stabilization of native-like secondary structure is likely to be beneficial. Much like the disulfide bridge aids the immunogenicity of the peptide immunogen by imposing conformational constraints, optimization of the primary structure of the peptide immunogen may increase its ability to generate RBD-specific antibodies by bolstering the formation of the conserved β -turn structure. Structural data have allowed us to propose roles for each of the conserved positions in the RBD region, as shown in Table 4. Only one conserved position, Lys140, does not have a clear role in defining the backbone structure of the RBD region. Therefore, we can hypothesize that it plays a role in receptor recognition. Although we did not express a full-length Cs1 pilin in *P. aeruginosa* to determine whether it could form intact pili, previous experiments have shown that pilin with a scrambled RBD will form pili, but are defective in adhesion.¹³

Impact of hinge residues on the RBD epitope

Interestingly, certain variable positions also appear to stabilize the conserved backbone conformation. We observe that a hydrogen bond network involving variable residue Gln133 in Cs1 and PAK pilins appears to define the conformation of the RBD and to anchor it to the core of the pilin protein. In contrast, this hydrogen bond network is absent in the K122-4 pilin, and the RBD is anchored on the pilin by formation of a hydrophobic cluster involving Ala133 (Figure 7). These two configurations serve the same purpose, which is to define RBD conformation through interactions with the core of the pilin protein, producing very similar backbone conformations.

Based on the amino acid sequences of pilins from other strains (Figure 1), we expect that the PAO pilin will form a similar hydrogen bonding network to the Cs1 and PAK (Gln133, Asn111 and Gly33) pilins, whereas the KB7 pilin will form a hydrophobic cluster similar to the K122-4 (Val133, Ile111 and Val33) pilin (Figure 7). These findings have tremendous significance for vaccine design. Although we have identified these two different modes of interaction involving positions 33, 111, 133 and 138, the interactions among these residues take place on the buried face of the RBD and both result in similar backbone conformations. Therefore, we do not expect the differences between the two types of interaction to impact the way in which the RBDs bind the host receptor or how they are presented as immunogens. On this basis, we have concluded that the variability at positions 133 and 138 can be disregarded in vaccine design.

Because we have observed a conserved conformation of the RBD in the Cs1, PAK and K122-4 pilins, we have hypothesized that an effective consensus sequence must maintain the native backbone conformation. In addition, the tolerance of the conformation of the receptor binding domain to diversity at variable positions in native strains has led us to hypothesize that non-native substitutions will perturb the conformation of the RBD whereas substitutions derived from native strains at variable positions will maintain the conformation. As such, future consensus sequences should be derived from the residues that occur in native strains

at each position. Preliminarily, this hypothesis has been confirmed by molecular dynamics simulations (D. Osguthorpe, personal communication). Prior to solving the structure of Cs1 pilin, He and coworkers³⁵ had shown that mutations in secondary structural elements rarely change the overall conformation of proteins. However, they did describe large deviations in the solvent exposed loops (3–4.3 Å) because of changes in hinge-bending angles. Since the receptor binding domain of *P. aeruginosa* involves a solvent exposed loop with two β -turns within the 14-residue loop, careful analysis was required (the two turns involve eight residues of the 14 residues in the loop). In fact, it was through this detailed analysis that we showed the dramatic effect of variable positions 133 and 138 where 133 is before the first turn (134–137) and residue 138 is the only residue between the two turns (second turn 139–142). Thus, this situation is dramatically different from the examples in T4 lysozyme. It was very important to determine whether the turns were identical or not.

The similarity of the backbone conformations that we observed implies that the cross-reactive immune response is influenced by the correct combination of solvent accessible side-chains. These side-chains may affect the degree to which the antibodies recognize the exposed backbone structure. For example, the greater the importance of backbone recognition, the more cross-reactive the antibody would be. We hypothesize that backbone recognition is more important in antibodies raised against our consensus sequence than native sequences.

Key surface residues important for vaccine design

The structures of Cs1, PAK and K122–4 pilin show us that the side-chains of the variable positions define the solvent exposed face of the receptor binding domain. Mapping of the variable and conserved positions of the receptor binding domain onto the molecular surface of Cs1 pilin reveals the solvent exposure of the side-chains of most of the variable positions (Figure 8). In contrast, the side chains of the conserved positions are either on the buried face of the RBD or are located toward the periphery of the exposed face of the RBD. In terms of vaccine design, this arrangement of variable and conserved positions has significant implications. The epitopes of most neutralizing antibodies are on the surfaces of proteins. Because so many of the variable position side-chains are on the surface of the receptor binding domain, we believe that the composition of the variable positions will play a major role in determining the specificity of antibodies that recognize the receptor binding domain. While it has been suggested that main-chain atoms may define a surface for binding the eukaryotic receptor,⁵ we believe it may be difficult to design a vaccine construct that will generate a response primarily to backbone atoms because of the variable position side-chains that are prominently located at the center of the receptor binding domain surface. Interestingly, a contiguous patch of surface exposed atoms can be constructed from a combination of the two consensus sequence substitutions and backbone atoms (Figure 8).

According to our current hypothesis, a consensus sequence immunogen must maintain the conserved conformation of the receptor binding domain while displaying a particular combination of variable residues on its surface that will induce antibodies that are cross-reactive. Our analysis has shown that the side-chains of four residues are prominent on the exposed face of the receptor binding domain: Lys130, Asp132, Pro135, and Gln136. The presence of proline at position 135 in Cs1 may bolster the β -turn propensity of the RBD region so we propose to maintain at position 135 in the structure-based design of a consensus sequence immunogen. Therefore, we will shift our focus from six variable residues to three residues (Lys130, Asp132 and Gln136), as these are the variable positions that are clearly presented on the pilin surface and do not appear to play other structural roles in the pilin RBD. We believe that the other consensus-sequence substitution, T130K, has demonstrated that the crossreactivity of the antibody response can be modulated by proper selection of residues at the positions where their side-chains are solvent accessible. While

there are still gaps present in our understanding of the immunological activity of the consensus sequence, our structural analyses will help focus future research.

The facile crystallization and structure determination of Cs1 pilin has demonstrated that the PAK pilin protein may be useful as a scaffold for structural analysis of pilin receptor binding domain vaccine constructs as well as native pilin receptor binding domain sequences. The substitutions made into the PAK pilin protein structure to create the consensus sequence have not disrupted the stability of the overall protein fold. The robustness of the PAK pilin protein fold makes it an attractive scaffold for analysis of future consensus sequences. As shown by the crystallization and structure determination of the Cs1 pilin, it is a relatively simple process to use mutagenesis to engineer the receptor binding domain sequence of PAK pilin for structural determination of other analogs. Insertion of receptor binding domain sequences from heterologous strains may also make it possible to crystallize hard-to-purify pilin proteins, such as the PAO strain pilin. This technology will be useful for straightforward and rapid analysis of structure–function relationships in vaccine design as well as for investigations of receptor binding.

Methods

Animal protection studies

The synthetic-pilin peptides were prepared by standard methods of solid phase peptide synthesis (SPPS).³⁶ Specific methods for SPPS, purification and characterization of the peptides have been described.³⁷ Peptides were synthesized with a photo-reactive group, benzoyl benzoic acid, attached to the N terminus.^{38–40} These peptides were conjugated to the carrier protein tetanus toxoid (TT). Briefly, solutions of peptide and TT were prepared in phosphate-buffered saline (PBS) at pH 7.2 (5–10 mg/ml of peptide and 20 mg/ml of TT). This solution was irradiated at 350 nm for 1 h at 4 °C in a RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middleton, CT). Unconjugated peptide was removed by extensive dialysis against PBS (pH 7.2). Peptide incorporation was determined by amino acid analysis.

A. By/SnJ mice at 10–12 weeks of age were used for these studies. Groups of ten mice were actively immunized i.p. at weeks zero, 2 and 4 with peptide conjugate (25 µg peptide content per injection) and 100 µg Adjuvax adjuvant in 50 µl of PBS. Mice were challenged i.p. with $5 \times \text{LD}_{50}/\text{mouse}$ (1×10^6 – 5×10^6 colony-forming units (CFU)). The effectiveness of the vaccine was determined by observing survival over 48–72 h following challenge. The time of 48 h was initially selected for the protocol before the experiments were carried out without knowledge of the survival results of individual mice and all mice were sacrificed at the end of this time period. In later experiments, the time period was increased to 72 h to address the question of survival over a longer time period. All experiments were performed in compliance with Canadian Council on Animal Care animal welfare guidelines as approved by the University of Alberta Animal Health and Welfare Committee. In accordance with animal care guidelines, animals were sacrificed when their condition deteriorated to a point where they objectively met euthanasia criteria set to minimize suffering.

Protein expression and purification

The Cs1 monomeric pilin gene was created through site-directed mutagenesis targeting the coding sequence of the PAK receptor-binding domain. Mutagenesis of the truncated (monomeric) PAK pilin $\Delta(1-28)$ in the plasmid pRLD was performed (Quick-Change; Stratagene, La Jolla, CA). Two oligonucleotides were used (Oligo1: 5′-CTC TGG AAG TGC AAA AGT GAT CAG GAT GAG; Oligo2: 5′-CAA AAG TGA TCA GGA TCC

GCA GTT TAT TCC G) to introduce the T130K and E135P substitutions. Cs1 pilin was expressed in *Escherichia coli* as described.⁵ The periplasmic fraction was extracted by osmotic shock. The crude protein sample was then lyophilized for purification. The lyophilized periplasmic fraction was resuspended in 0.05 to 0.1 of the original culture volume in 0.2% (v/v) aqueous trifluoroacetic acid (TFA). Insoluble material was removed by centrifugation at 20,000g and passed through a 0.2 μ m filter. Cs1 pilin was purified from the crude periplasmic fraction by reversed-phase chromatography (RP-HPLC) using an Zorbax 300SBC8 250 mm $\times$ 9.4 mm ID column (5 μ m particle size, 300 Å pore size; Agilent Technologies, Palo Alto, CA). A preparative RP-HPLC method was devised using a linear AB gradient (2% B/min at a flow rate of 2.0 ml/min, where eluent A is 0.2% aq. TFA and eluent B is 0.18% TFA in acetonitrile) up to 30% acetonitrile, followed by a shallow gradient of 0.1% acetonitrile/min up to 50% acetonitrile. Fractions were collected over 1 min intervals. Fractions containing pure protein were identified by SDS–PAGE and electrospray-TOF mass spectrometry. Acetonitrile and TFA were removed by repeated lyophilization.

Crystallization and structure determination

Cs1 pilin was crystallized under the same conditions described by Hazes et al.⁵ Crystallization was performed using the vapor-diffusion hanging drop method. The mother liquor consisted of 60% saturated ammonium sulfate, 0.1 M Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 8.1). Large, single crystals were obtained after microseeding of hanging drops. Crystals formed in the $P2_12_12_1$ space group with unit cell dimensions of $a = 28.472$ Å, $b = 52.891$ Å, $c = 63.872$ Å, $\alpha = \beta = \gamma = 90^\circ$. There was one protein molecule per asymmetric unit. The X-ray diffraction data were collected at the APS 19ID beamline at Argonne National Laboratory. The structure was solved by molecular replacement using the program Phaser⁴¹ with the PAK monomeric pilin (PDB ID, 1DZO,⁵) as a search model. Refinement of the molecular replacement model was performed using Refmac (CCP4)⁴² and O⁴³ with all data to a resolution 1.35 Å. Diffraction data and refinement summary statistics are shown in Table 1. The R and R_{free} values of the final refined model were 17.1% and 19.7%, respectively. PROCHECK analysis showed that 93.8% of residues were in the most favored regions of the Ramachandran plot and the remaining 6.2% of residues fell into additional allowed regions. The r.m.s.d. values of bond lengths and bond angles were 0.015 Å and 1.6°, respectively.

r.m.s.d. calculations and structural alignments were performed using LSQMAN.⁴⁴ Molecular diagrams were made using PyMOL (DeLano Scientific, San Carlos, CA). Coordinate files used for structural comparisons were PDB ID, 1DZO⁵ (PAK pilin) and 1QVE 7 (K22-4 pilin). The full-length PAK pilin structure has also been solved and was very similar to the structures of the monomeric pilins.⁶ The main-chain r.m.s.d. value between the globular head domain of (residues 29–144) of truncated PAK pilin and full-length PAK pilin is 0.45 Å and the r.m.s.d. between the RBD (128–144) of truncated PAK pilin and full-length PAK pilin is 0.17 Å. In this study, we have drawn comparison to the structure of PAK monomeric pilin reported by Hazes et al.⁵, but because of the similarity between the full-length and truncated PAK pilin structures in the RBD region, our analyses are valid for the full-length pilins as well.

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Figure 1. Amino acid sequences of the Cs1 consensus sequence and the receptor-binding domains from six strains of *P. aeruginosa*
Alignment of amino acid sequences of the receptor-binding domain from *P. aeruginosa* strains PAK, PAO, KB7, K122-4, CD4 and P1. Boxed residues show identical positions. Shaded residues indicate positions with conservative substitutions.

Figure 2. Protection of A.BY/SnJ mice from challenge by different strains of *P. aeruginosa* after active immunization by pilin peptide-tetanus toxoid conjugates
 (a) Challenge by PAK strain bacteria. (b) Challenge by PAO strain bacteria. (c) Challenge by KB7 bacteria. (d) Challenge by P1 bacteria. (e) Challenge by PAK bacteria. (f) Challenge by PAO bacteria. (g) Challenge by P1 bacteria. For panels (e)–(g) the open squares denote Cs1-TT and filled triangles denote the scrambled Cs1 peptide-TT (Scr-TT). The strain used for challenge is indicated in the top left of each panel. TT indicates animals immunized with tetanus toxoid alone and Adjuvax indicates animals that received adjuvant alone. A scrambled Cs1 peptide conjugated to tetanus toxoid was used as a control to demonstrate dramatic protection of the peptide beyond the adjuvant effect of tetanus toxoid alone (unpublished results).

Figure 3.
Representative sigmaA-weighted 2 mFo-Fc electron density map of Cs1 pilin, contoured at 2 σ , showing the quality of X-ray diffraction data for Cs1 pilin.

Figure 4. Ribbon diagrams showing similarities among Cs1, PAK and K122-4 monomeric pilin structures

(a) Cs1 pilin. The α -helix spanning residues 25–51 and the four strands of the major β -sheet are labeled. The receptor binding domain is boxed. Residue 29 is the start of the monomeric pilin construct. Residues 1–28 were removed to prevent polymerization. C denotes the C-terminal of the pilin. (b) Overlay of Cs1 and PAK pilin. (c) Overlay of Cs1 and K122-4 pilin. The superimposed region of Cs1 and K122-4 includes the major β -sheet and the RBD is shown (as discussed in the text). In (b) and (c), the regions highlighted in red are the regions of the two proteins that deviate by 2 Å or less (and used for r.m.s.d. calculations).

Figure 5. Stereo images of stick representations showing the similarities in backbone conformation of the entire receptor binding domain (128–143) from the crystallographically determined structures of the three pilin proteins

The view has been rotated by 180° about the horizontal and 90° about the vertical axes of the page relative to the orientation in Figure 4. (a) Cs1 pilin protein (PDB ID, 2PY0). (b) Overlay of the RBD (128–143) of Cs1, PAK (PDB ID, 1DZO) and K122-4 (PDB ID, 1QVE) pilins, showing only main-chain atoms. (c) Overlay of the RBD (128–143) of Cs1, PAK, and K122-4 pilins, showing main-chain and side-chain atoms. The r.m.s.d. between residues 128–143 truncated and full-length forms of PAK pilin is $0.17 \text{ \AA}$ for main-chain atoms, so only the truncated pilin structure has been shown. In each diagram, the backbone of Cs1 pilin is shown in light blue, PAK pilin in light green and K122-4 in orange.

Figure 6. Stick representations showing the differences in backbone conformation of the RBD region in each of the three pilin proteins

(a) Cs1, PAK and K122-4 pilin are superimposed by the backbone atoms of residues 128–132. The differences in position of the first β -turn (residues 134–137) are shown when the RBD regions are superimposed in this way. Hydrogen bonds are shown by red dashes. Stick and ball models are used to show the side-chains of residues 132 and 114, which form hydrogen bonds with main-chain atoms to terminate the β -strand of the RBD. (b) Cs1, PAK and K122-4 pilin are superimposed by the backbone atoms of residues 134–137. The differences in position of the second β -turn (residues 139–142) are shown when the RBD regions are superimposed in this way. In both panels, the backbone carbon atoms of Cs1 pilin are shown in light blue, PAK pilin in light green and K122-4 in orange.

Figure 7. Stick diagrams showing the interactions of buried side-chains of the RBD
 (a) Cs1 pilin, showing the hydrogen bonding of the side-chain of Gln133 with Ser131 and Asn111. The side-chain of Ile138 is shown as a space-filling model. The same interactions shown in (a) are observed in the PAK pilin structure. (b) K122-pilin, showing the side-chain of Ala133. The side-chains of Leu138, Leu33 and Leu111 (equivalent to Leu108 in the K122-4 sequence alignment) are shown as space-filling models to demonstrate the hydrophobic interactions on the buried face of the RBD of K122-4 pilin. Both panels show the conserved hydrogen bond of the side-chain of Ser131 with the backbone nitrogen of residue 33.

Figure 8. Molecular surfaces showing the positions of variable and conserved residues on the solvent accessible surface of the Cs1 pilin receptor binding domain

(a) The contribution of conserved position side-chains are shown in red, green and blue. Red indicates an acidic residue side-chain (Asp134). Green indicates a hydrophobic residue side-chain (Phe137). Blue indicates a basic residue side-chain (Lys140). Yellow indicates the disulfide bond. The surface contributions from backbone atoms are highlighted in orange. Light grey highlights variable position side-chains (Lys130, Asp132, Pro135, Gln136, Ile138 and Ser143). (b) The contributions of residues in the RBD region to the molecular surface are shown in blue. The side-chains of variable positions Asp132 and Gln136 are shown in red. Lys128 and Ser143, which are outside of the disulfide loop, are also shown in red, as their identities vary among strains. The contributions of the side-chains to the molecular surface of the consensus sequence substitution sites 130 and 135 are shown in orange. The contribution of Ile138 is shown in white because although exposed on the RBD surface, 83% of the surface area of Ile138 is buried and we do not expect this side-chain to be significant in antibody recognition. The side-chain of variable position Gln133 is buried, and therefore is not visible on the molecular surface. In both panels, dark gray denotes region of the molecular surface from residues not in the RBD region. The solvent accessible surface was calculated using a 1.4 Å probe.

Table 1

Crystallographic data collection and refinement statistics for Cs1 monomeric pilin.

a, b, c (Å)	28.47, 52.89, 63.87
α , β , γ (°)	90, 90, 90
Resolution (Å) (Highest resolution shell)	50 – 1.35 (1.385 – 1.35)
Space group	P2 ₁ 2 ₁ 2 ₁
Observed reflections	168,031 (16,624)
Unique reflections	21,824 (2,159)
Completeness (%)	99.7 (99.8)
Redundancy	7.7 (7.7)
R _{sym} (%)	5.1 (37.5)
$\langle I/\sigma \rangle$	42.8 (7.0)
R _{cryst} (%)	17.1 (20.7)
R _{free} (%)	19.7 (25.9)
No. of reflections used	20,721 (1,577)
Coordinate Error (R _{free}) (Å)	0.135
Protein (atoms)	948
Solvent (atoms)	229
HEPES (atoms)	15
Mean B-Value	14.9
Ramachandran plot	
Most favored regions	93.8 %
Additionally allowed regions	6.2 %
Generously allowed regions	0 %
rms deviations from ideality	
Bond lengths (Å)	0.015
Bond angles (°)	1.6

Table 2

An r.m.s.d. comparison of the three secondary structural elements of the RBD between Cs1, PAK, and K122-4 pilin proteins.

	β -strand ^a	β -turn 1 ^a	β -turn 2 ^a
	Res. 128–132 ^b	Res. 134–137 ^b	Res. 139–142 ^b
Cs1 to PAK	0.15 Å ^c	0.11 Å ^c	0.09 Å ^c
Cs1 to K122-4	0.26 Å ^c	0.20 Å ^c	0.49 Å ^c

^aSecondary structural element being compared.

^bPositions of residues used to superimpose the two proteins and for r.m.s.d. calculation.

^cCalculated r.m.s.d. for the main chain atoms of the residues listed.

Table 3

Distances between equivalent residues in the β -turns of the RBD after superposition along the β -strand or the first β -turn.

	Superposition: 128–132 ^a		Superposition: 134–137 ^a	
	135 ^b	136 ^b	140 ^b	141 ^b
Cs1 to PAK	0.94 Å ^c	1.17 Å ^c	1.14 Å ^c	1.89 Å ^c
Cs1 to K122-4	3.10 Å ^c	2.71 Å ^c	5.96 Å ^c	5.91 Å ^c

^aPilin protein structures were superimposed along these residue using a least squares fit.

^bPosition of residue being compared

^cDistance between the C α of equivalent positions in the two superimposed proteins being compared.

Table 4

Proposed functions of the conserved positions of the pilin receptor binding domain.

Residue	Identity	Function
129	Cys	Disulfide bond
131	Ser/Thr	Stabilization of backbone conformation.
134	Asp	β -turn formation
137	Phe/Tyr	Stabilization of tertiary structure
139	Pro	β -turn formation
140	Lys	Receptor recognition (Hypothetical)
141	Gly	β -turn formation
142	Cys	Disulfide bond, β -turn formation