Roles of Specific Amino Acids in the N Terminus of *Pseudomonas aeruginosa* Flagellin and of Flagellin Glycosylation in the Innate Immune Response

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The Toll-like receptor 5 (TLR5) binding site has been predicted to be in the N terminus of the flagellin molecule. In order to better define the interaction between the N-terminal amino acids of *Pseudomonas aeruginosa* flagellin and TLR5, site-specific mutations were generated between residues 88 and 97 of *P. aeruginosa* PAK flagellin as well as outside of this region. The mutant flagellins were expressed in *Escherichia coli* BL21(plysS), purified by affinity chromatography, and passed through a polymyxin B column to remove contaminating lipopolysaccharide (LPS). Their ability to stimulate interleukin-8 (IL-8) release from A549 cells was examined. The cloned mutated genes were used to complement a PAK *flhC* mutant in order to test for effects on motility and on IL-8 release by purified flagellar preparations. All the mutations, single or double, in the predicted TLR5 binding region reduced IL-8 signaling to less than 95% of the wild-type flagellin levels, but the single mutation outside the binding region had no effect. Changes made at two amino acid sites resulted in loss/reduction of motility; however, changes made at single sites, i.e., Q83A, L88A, R90A, M91A, L94A, and Q97A, had no effect on motility. The mutated genes encoding two of the motile but poorly signaling flagellins had no compensatory mutations to allow motility. Thus, while it is speculated that pathogen-associated molecular patterns (PAMPs) have evolved in locations that are essential to maintain function, it appears that there is tolerance for at least single amino acid changes in the PAMP of *P. aeruginosa* flagellin. The purpose of flagellin glycosylation in *P. aeruginosa* is unknown. In order to examine its role, if any, in signaling an inflammatory response, we used whole flagella from the motile chromosomal mutant strains PAK *rfbC* and PAO1 *rfbC*, which are defective in flagellin glycosylation. IL-8 release from A549 cells stimulated with nonglycosylated flagellar preparations (having less than 1 picogram of LPS/ μ g) was significantly reduced compared to their respective wild-type flagellar preparations, indicating a role of flagellar glycosylation in the proinflammatory action of *Pseudomonas* flagellin. The basis of the latter activity is unknown, since the glycosylation sites are found in the D3 domain of flagellins and the TLR5 binding site is located in the D1 domain. Thus, *P. aeruginosa* flagellin has evolved additional flagellar signaling mechanisms over that described for *Salmonella* flagellin.

Pseudomonas aeruginosa is a common opportunistic human pathogen that is responsible for fatal infections in compromised individuals and chronic colonization of the lungs of patients with cystic fibrosis, leading to their death. This organism is motile via a single polar flagellum that has structural properties very similar to those of enteric gram-negative bacteria, with the added structural feature of being glycosylated (4, 39). The flagellum contains a relatively complex basal body and hook structure attached to a filament primarily enriched in assembled flagellin subunit protein. The role of flagellar motility in the virulence of many pathogenic microorganisms, including *P. aeruginosa*, has been well established (14, 33, 34). A *P. aeruginosa* strain carrying normal flagellin, upon infecting Calu-3 human airway epithelial cells, results in the upregulation of genes involved in innate immune response, as ascertained by microarray analysis. There is, however, no such increase in gene expression with mucoid *P. aeruginosa* strains, suggesting that flagellin is a critical proinflammatory determi-

nant (8). Recent in vitro investigations have indicated that flagellin induces the expression of proinflammatory mediators by monocytes (28, 35), intestinal epithelial cells (11, 43, 46), and airway epithelial cells (1), resulting from the activation of the transcription factor NF- κ B (12, 22, 26). The signal transduction pathway which mediates these effects has been recently identified by Hayashi and colleagues (22), who underscore the importance of flagellin as a unique ligand for Toll-like receptor 5 (TLR5). Through TLR5, flagellin activates macrophages, dendritic cells, and corneal, airway, and intestinal epithelial cells to produce inflammatory mediators (11, 14, 15, 18, 27, 28).

TLR5 is a member of a family of innate immune receptors that recognize specific pathogen-associated molecular patterns (PAMPs), which are conserved motifs unique to microorganisms. TLRs consist of an extracellular domain with a leucine-rich repeat, one or two cysteine-rich regions, and a cytoplasmic region called the Toll/interleukin-1 (IL-1) receptor domain. Flagellin has also been reported to interact with a putative leucine-rich repeat in human TLR5 (32). Ten different TLRs have been identified in humans (24). One group of pathogens is not exclusively recognized by one TLR (e.g., both TLR2 and TLR4 recognize gram-positive organism-derived PAMPs). In addition, the same TLR may respond to many structurally

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unrelated ligands, which are often derived from different groups of pathogens; for example, TLR2 may recognize both peptidoglycans from gram-positive organisms and lipopolysaccharide (LPS) from a variety of gram-negative bacteria (13). In addition, TLR2 has been reported to recognize lipoproteins, mycobacterial cell wall lipoarabinomannan, glycosylphosphatidylinositol lipid from *Trypanosoma cruzi*, a phenol-soluble modulins produced by *Staphylococcus epidermidis*, yeast cell walls, and some endogenous molecules, such as heat shock proteins Hsp60, Hsp70, Gp96, and also a high-mobility group box 1 protein (HMGB1) (29, 48). Other TLRs, such as TLR3, -5, and -9, have been reported to be more ligand specific and to recognize only one type of ligand (29). In addition to animals, plants and insects have also evolved innate immune recognition systems for bacterial flagellin. Plants recognize bacterial flagellin through FLS2, the flagellin sensitivity locus two-gene product, which is apparently a functional equivalent of TLR5. FLS2 binds to a conserved domain of flagellin represented by the peptide flg22 (21). This recognition event triggers a systemic disease resistance response (21, 45).

Jacchieri et al. (23) used an *in silico* search to predict the complementary region common to the flagellins of *Salmonella enterica* serovar Typhimurium (20), *Listeria monocytogenes* (38), and *P. aeruginosa* (47) that interacts with TLR5. This region has been confirmed to be involved in TLR5 recognition in *Salmonella* (23, 36, 41), and recently it has been demonstrated that flagellin of ϵ - and α -*Proteobacteria* is not recognized by TLR5 (2). These organisms are reported to possess specific changes in the TLR5 recognition site on flagellin that prevent TLR5 recognition and demonstrate compensatory amino acid changes in their flagellin molecules that preserve motility.

A short stretch of 10 amino acids (amino acids 88 to 97; LQIRDLALQ) in the N-terminal region of the flagellin of *P. aeruginosa* strain PAO1 was predicted to be important for binding to TLR5. More specifically, it was predicted that the TLR5-flagellin interactions involved Glu552 (TLR5)-Gln89 (flagellin), Asp555 (TLR5)-Arg92 (flagellin), and Arg558 (TLR5)-Asp93. However, alanine-scanning mutagenesis of *Salmonella* flagellin suggested that a number of other amino acids in this region might also be involved in TLR5 recognition (41). To test these predictions concerning *P. aeruginosa* and to identify the amino acids that are involved in the TLR5 interaction with *P. aeruginosa* flagellin, we have performed site-directed mutagenesis in this region and outside the predicted TLR5 binding region of the flagellin molecule of strain PAK. Part of our aim was to confirm the identity of the amino acids that have been implicated in interaction with TLR5 and that do not affect motility, since it is not possible to distinguish whether a loss of signaling function is due to loss of this interaction caused by the mutations introduced or whether it was due to improper filament folding. Indeed, it has been hypothesized that such PAMPs are unalterable for bacterial fitness (41). We therefore created different site-specific mutants in this region and examined them for their effects on bacterial motility and their abilities to induce an inflammatory response in A549 cells. Additionally, the flagellins of *P. aeruginosa* have been demonstrated to be glycosylated (4, 5), an attribute of unknown significance. However, flagellin glycosylation in *Pseudomonas syringae* pv. *glycinea* (45) is involved in the inflammatory

response of plants. By contrast, glycosylation of *Salmonella* flagellin produced in CHO cells was not necessary for TLR5 recognition (41). Thus, in order to examine the role of *Pseudomonas* flagellin glycosylation in inflammation, if any, flagella from glycosylation-defective mutants of strains PAK and PAO1 were also examined for their abilities to induce inflammatory responses. In this report, we show that the amino acids predicted to be involved in the *P. aeruginosa* flagellin-TLR5 recognition are essential for both motility and the inflammatory response. Besides these residues, there are other amino acids in the 10-amino-acid region that appear to be more specific for the inflammatory response, whereas full motility is retained and the IL-8 response is reduced by >95% when they are mutated. The mutation outside the putative TLR5 binding region had no effect on motility or IL-8 response. We also find that flagellin glycosylation plays a role in the inflammatory response caused by *P. aeruginosa* flagellin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains, plasmid vectors, and derivatives thereof used in this study are shown in Table 1. The bacteria were propagated in liquid Luria-Bertani (LB) broth or on LB agar plates (1.7% agar) with or without antibiotics. The antibiotics (and concentrations) used were as follows: for *Escherichia coli*, ampicillin (200 μ g/ml), and for *P. aeruginosa*, carbenicillin (150 μ g/ml or 300 μ g/ml).

Site-directed mutagenesis. Strain PAK was used to PCR amplify the 1.2-kb *fljC* insert as an NdeI-BamHI fragment, which in turn was cloned into pET15bVP vector. The resulting pET/*fljC* construct was used to PCR amplify the 1.2-kb *fljC* insert as an EcoRI-BamHI fragment with the appropriate primer set. This fragment was further cloned into the EcoRI-BamHI sites of pBluescript KS(+) (Stratagene), resulting in the construction of pBS/*fljC*. A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) was used to generate different site-specific mutants of the *fljC* gene according to the protocol provided in the kit. Briefly, 50 ng of column-purified (plasmid Mini kit; QIAGEN, Valencia, Calif.) plasmid template (pBS/*fljC*) was used in a 50- μ l amplification reaction mixture consisting of 1 μ l of a solution containing each deoxynucleoside triphosphate at a concentration of 10 mM, 1 μ l of *Pfu* polymerase, 1.25 μ l of a 20 μ M solution of each primer, and 5 μ l of 10 $\times$ reaction buffer. The mixture was subjected to a cycling profile consisting of initial denaturation for 30 s at 95°C, followed by 13 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 68°C for 10 min. The reaction mixture contents were then treated with DpnI to digest the original plasmid template. One microliter of the postdigestion amplification reaction mixture was used to transform *E. coli* XL1-Blue cells, and transformants were selected on LB agar plates containing ampicillin. Several clones were sequenced, and in each case, a clone with the desired site-specific mutation was subsequently used for further subcloning. For all the single site- or double site-specific mutants created, single sets of primer pairs were used. Using this method, the following amino acid changes were engineered in the *FljC* protein, singly or in combination with others: Q83A, L88A, Q89N, Q89A, R90A, M91A, R92K, R92E, L94A, and Q97A (Table 1). To ensure that no secondary compensatory mutation had occurred in certain specific mutants, the entire flagellin genes, e.g., PAKCM3 and PAKCM7, were sequenced.

Construction of plasmids to assess flagellar functions and morphology. In order to express the wild-type *fljC* gene and the *fljC* site-specific mutant genes in *P. aeruginosa* from the inducible *tac* promoter of the vector pMMB67EH, the 1.2-kb *fljC* inserts lacking a promoter but with different amino acid mutations were excised from their respective pBluescript II/KS(+) (pBS) constructs and were then cloned into EcoRI-BamHI sites of the vector pMMB67EH (Table 1). The resulting plasmids containing the PAK/*fljC* genes (wild type and mutants) were used to complement a PAK/*fljC* mutant, PAKAC (9). The complemented PAKAC strains thus created were named PAKC (PAK-pMMB-*fljC*), PAKCM1 (PAK-pMMB-*fljC*^{Q89N, R92K}), PAKCM2 (PAK-pMMB-*fljC*^{Q89A, R92E}), PAKCM3 (PAK-pMMB-*fljC*^{L88A}), PAKCM4 (PAK-pMMB-*fljC*^{M91A}), PAKCM5 (PAK-pMMB-*fljC*^{L88A, M91A}), PAKCM6 (PAK-pMMB-*fljC*^{R90A}), PAKCM7 (PAK-pMMB-*fljC*^{L94A}), PAKCM8 (PAK-pMMB-*fljC*^{Q97A}), and PAKCM9 (PAK-pMMB-*fljC*^{Q83A}).

Motility assay and electron microscopy. Chemotactic motilities of the strains carrying pMMB constructs with site-specific mutations in the *fljC* gene were assessed by qualitative analysis of the zone formed by the bacteria on a 0.3% LB

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	<i>hsdR recA lacZYA</i> ϕ 80 Δ lacZ Δ M15	GIBCO BRL
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Z Δ M15 Tn10 (Tet ^r)]	Stratagene
BL21(pLysS)	F ⁻ <i>ompT hsdS</i> _B (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3), T7 lysozyme gene on pLysS	Novagen, Inc.
<i>P. aeruginosa</i>		
PAK	Wild-type laboratory strain, motile	D. Bradley
PAK Δ C	PAK flagellin mutant, <i>fliC</i> gene deleted	9
PAO1	Wild-type laboratory strain, motile	M. Vasil
PAK Δ <i>rfbC</i>	PAK with <i>rfbC::gent</i>	3
PAO1 Δ <i>rfbC</i>	PAO1 with <i>rfbC::gent</i>	5
Plasmids		
pBluescript II/KS(+)	General-purpose cloning vector, Ap ^r	Stratagene
pMMB67EH	Broad-host-range low-copy-number cloning vector, Cb ^r , makes single-stranded DNA	9
pET15bVP	Expression vector, T7 promoter, His tag coding sequence, Amp ^r , pBR322 origin, contains a broad-host-range origin of replication <i>oriV</i>	9
pBS- <i>fliC</i>	pBluescript II/KS(+) containing PAK <i>fliC</i> gene as a 1.2-kb EcoRI-BamHI insert	This study
pBS- <i>fliC</i> ^{Q89N, R92K}	pBS- <i>fliC</i> with the mutations Q89 $\rightarrow$ N and R92 $\rightarrow$ K	This study
pBS- <i>fliC</i> ^{Q89A, R92E}	pBS- <i>fliC</i> with the mutations Q89 $\rightarrow$ A and R92 $\rightarrow$ E	This study
pBS- <i>fliC</i> ^{L88A}	pBS- <i>fliC</i> with the mutation L88 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{M91A}	pBS- <i>fliC</i> with the mutation M91 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{L88A, M91A}	pBS- <i>fliC</i> with the mutations L88 $\rightarrow$ A and M91 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{R90A}	pBS- <i>fliC</i> with the mutation R90 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{L94A}	pBS- <i>fliC</i> with the mutation L94 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{Q97A}	pBS- <i>fliC</i> with the mutation Q97 $\rightarrow$ A	This study
pBS- <i>fliC</i> ^{Q83A}	pBS- <i>fliC</i> with the mutation Q83 $\rightarrow$ A	This study
pMMB- <i>fliC</i>	pMMB67EH containing PAK <i>fliC</i> gene as a 1.2-kb EcoRI-BamHI insert	This study
pMMB- <i>fliC</i> ^{Q89N, R92K}	pMMB- <i>fliC</i> with the mutations Q89 $\rightarrow$ N and R92 $\rightarrow$ K	This study
pMMB- <i>fliC</i> ^{Q89A, R92E}	pMMB- <i>fliC</i> with the mutations Q89 $\rightarrow$ A and R92 $\rightarrow$ E	This study
pMMB- <i>fliC</i> ^{L88A}	pMMB- <i>fliC</i> with the mutation L88 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{M91A}	pMMB- <i>fliC</i> with the mutation M91 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{L88A, M91A}	pMMB- <i>fliC</i> with the mutations L88 $\rightarrow$ A and M91 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{R90A}	pMMB- <i>fliC</i> with the mutation R90 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{L94A}	pMMB- <i>fliC</i> with the mutation L94 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{Q97A}	pMMB- <i>fliC</i> with the mutation Q97 $\rightarrow$ A	This study
pMMB- <i>fliC</i> ^{Q83A}	pMMB- <i>fliC</i> with the mutation Q83 $\rightarrow$ A	This study
pET- <i>fliC</i>	1.2-kb PAK <i>fliC</i> gene inserted as an NdeI-BamHI fragment into pET15bVP	This study
pET- <i>fliC</i> ^{Q89N, R92K}	pET- <i>fliC</i> with the mutations Q89 $\rightarrow$ N and R92 $\rightarrow$ K	This study
pET- <i>fliC</i> ^{Q89A, R92E}	pET- <i>fliC</i> with the mutations Q89 $\rightarrow$ A and R92 $\rightarrow$ E	This study
pET- <i>fliC</i> ^{L88A}	pET- <i>fliC</i> with the mutation L88 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{M91A}	pET- <i>fliC</i> with the mutation M91 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{L88A, M91A}	pET- <i>fliC</i> with the mutations L88 $\rightarrow$ A and M91 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{R90A}	pET- <i>fliC</i> with the mutation R90 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{L94A}	pET- <i>fliC</i> with the mutation L94 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{Q97A}	pET- <i>fliC</i> with the mutation Q97 $\rightarrow$ A	This study
pET- <i>fliC</i> ^{Q83A}	pET- <i>fliC</i> with the mutation Q83 $\rightarrow$ A	This study

agar plate containing carbenicillin (150 μ g/ml) and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The plates were inoculated by stabbing the surfaces containing the wild-type PAKC strain and different mutant strains with a sterile toothpick. For electron microscopy (EM), bacterial strains were grown overnight statically in LB at 37°C with carbenicillin (150 μ g/ml). A drop of the culture was allowed to adhere to a carbon-coated grid for 10 s, and excess culture was drained off; the grid was then rinsed in a drop of saline, and adherent cells were negatively stained with a 2% aqueous solution of phosphotungstic acid for 10 s. Samples were examined with a Hitachi H-7000 transmission electron microscope.

Expression and purification of FliC. The mutated *fliC* genes were PCR amplified as NdeI-BamHI fragments using appropriate pBS/*fliC* templates and primers. These NdeI- and BamHI-digested PCR products of *fliC* were cloned into pET15bVP, yielding different expression clones, which were named pETC (pET-*fliC*), pETCM1 (pET-*fliC*^{Q89N, R92K}), pETCM2 (pET-*fliC*^{Q89A, R92E}), pETCM3 (pET-*fliC*^{L88A}), pETCM4 (pET-*fliC*^{M91A}), pETCM5 (pET-*fliC*^{L88A, M91A}), pETCM6 (pET-*fliC*^{R90A}), pETCM7 (pET-*fliC*^{L94A}), pETCM8 (pET-*fliC*^{Q97A}), and

pETCM9 (pET-*fliC*^{Q83A}). This process allowed the expression of *fliC* as a fusion protein with the histidine tag in *E. coli* BL21(pLysS) cells (Novagen) for further purification of flagellins from these strains. *E. coli* BL21(pLysS) contains the T7 polymerase gene on the chromosome under the control of the *lacUV5* promoter. Bacterial cultures were grown to A₅₅₀s of 0.4 to 0.5, and the T7 polymerase was induced by the addition of a 1.0 mM final concentration of IPTG. The cultures were grown for an additional 3 h and then pelleted. The cell pellets were resuspended in 1 $\times$ binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). Cell lysates were prepared by disrupting the cells in a French pressure cell at 16,000 lb/in² and then spun at 20,000 $\times$ g for 20 min to collect the inclusion bodies having insoluble His-FliC.

The inclusion bodies were resuspended in 1 $\times$ binding buffer with 6 M urea and incubated on ice for 1 h to dissolve the protein. Insoluble material was removed by centrifugation at 39,000 $\times$ g for 20 min. A small disposable column containing 2.5 ml of chelating Sepharose Fast Flow resin (Pharmacia Biotech, Inc., Piscataway, N.J.) was packed. The column was charged with 50 mM NiSO₄ according to the pET instruction manual (Novagen, Inc.). The supernatants were filtered and

FIG. 1. (A) Deduced amino acid sequence of PAK flagellin showing the location of the predicted TLR5 binding site (boxed). The numbering of the amino acids in the flagellin molecule has been done after exclusion of the first methionine. (B) The locations of mutated amino acids in *fliC*-expressing strains. The putative TLR5 binding regions (residues 88 to 97) are in italics.

loaded onto the nickel columns. Further steps in the purification of His-*fliC* were performed according to the pET instruction manual. All buffers contained 6 M urea in order to keep the His-*fliC* protein solubilized. His-*fliC* protein was finally eluted with 1× elution buffer (1 M imidazole, 0.4 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The protein was dialyzed against 1-liter volumes of phosphate-buffered saline with stepwise decreases in urea concentration (5 M→4 M→3 M→2 M→1 M→0.5 M→no urea). To remove any possible contaminant LPS, the purified flagellin preparations were passed through a polymyxin B column, resulting in an LPS-free flagellin preparation, which was analyzed by a *Limulus* amoebocyte lysate kit (BioWhittaker, Walkersville, MD). Purity of proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (37). Purified proteins were quantitated by a Bio-Rad protein assay (37).

Transformations and electroporations. Transformations of *E. coli* DH5α and *E. coli* BL21(plysS) were performed using a standard protocol (9). Electroporations were performed by using a modification of the protocol of Smith and Iglewski (40). The DNA used for the electroporations was prepared by the alkaline lysis procedure (7). For complementation experiments, 50 to 100 ng of supercoiled, covalently closed circular plasmid DNA was electroporated into the target strains.

Purification of flagella. Flagella were purified from *Pseudomonas* strains grown overnight in LB broth. Flagella were mechanically sheared from the surface of the bacteria and collected by ultracentrifugation as previously described (39). Whole flagellar preparations were also passed through a polymyxin B column to remove LPS contaminants.

Cell culture. A human lung carcinoma cell line (A549) was obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (HyClone), 200 U/ml penicillin, and 200 μg/ml streptomycin (17) in 100-mm plates and also in six-well plates for the measurement of interleukin-8 (Pierce and Endogen, Woburn, MA).

IL-8 assay. A549 cells were seeded in six-well plates and grown to confluence before stimulation with wild-type and mutant flagellins or flagella at a concentration of 4.0×10^{-10} M for 24 h. At the 4.0×10^{-10} M concentration, maximum IL-8 response was produced in A549 cells, as determined by a dose-response

assay (see Fig. 4). IL-8 concentration in the culture medium was determined using an enzyme-linked immunosorbent assay kit (Pierce and Endogen, Woburn, MA) according to the instructions provided by the manufacturer.

Statistical analysis. Where applicable, results are expressed as the means ± standard deviations (SD) of three to four experiments. Student's *t* test was used to compare mean values. Differences were considered significant when *P* values were <0.05.

RESULTS

Functional characterization of site-directed flagellin mutants. The 10-amino-acid region of *P. aeruginosa* (PAK) flagellin that has been predicted to interact with TLR5 is shown in Fig. 1A. In order to define the contribution of this predicted site in binding to TLR5, we generated different site-specific mutants experimentally (as listed in Table 1). To show the specificity of the putative TLR5 binding site on flagellin, we have created a site-specific mutation outside the predicted binding site. The locations of and the changes made in the amino acids of the flagellin molecule are shown in Fig. 1B. In order to test whether these mutations affect flagellar function, the *fliC* mutant complemented with the various plasmid-borne mutated *fliC* genes was examined for motility and flagellum production. The plasmids carrying the wild-type PAK *fliC* gene, mutated *fliC*, and the vector control (pMMB67EH) without the *fliC* insert were electroporated into the *fliC* mutant strain, PAKΔC. The resulting strains were tested for their motility phenotypes on motility plates containing carbenicillin (150 μg/ml) and 1 mM IPTG, as the flagellin in these strains was expressed under the control of the *tac* promoter in

TABLE 2. Effects of different site-specific mutations in flagellin on bacterial motility and IL-8 response^a

Mutant	Motility ^b	IL-8 response
PAKC	++	33,000 ± 116
PAKΔC	–	150 ± 28
PAKCM1	–	971 ± 19.0
PAKCM2	–	1,194 ± 23.8
PAKCM3	+++	7,535 ± 151
PAKCM4	++	535 ± 10.0
PAKCM5	+	516 ± 25
PAKCM6	+++	7,569 ± 151.3
PAKCM7	++	6,519 ± 130
PAKCM8	++	9,049 ± 181
PAKCM9	++	27,834 ± 347

^a A549 cells stimulated with purified flagellar preparations at a concentration of 4.0×10^{-10} M purified from the motile and nonmotile/partially motile TLR5 binding site mutants show a significant ($P < 0.001$) reduction in IL-8 levels compared to that of the wild type. However, the IL-8 response produced by flagella purified from the motile non-TLR5 binding site mutant at a 4.0×10^{-10} M concentration was not significantly different from that of the wild-type flagella. Data shown are means ± SD of four different experiments.

^b Bacterial motility scores: ++, wild-type motility; +++, >25% wild-type motility; +, 25 to 50% wild-type motility; –, <5% wild-type motility.

pMMB67EH. The double site-specific mutants PAKCM1 and PAKCM2 were nonmotile (Table 2), indicating that they are unable to make functional flagella; however, PAKCM5 (Table 2) showed reduced motility when compared to PAKC, suggesting that the double mutations do not affect the motility phenotype uniformly. The motility zones of single-site mutants PAKCM3,

PAKCM4, PAKCM6, PAKCM7, PAKCM8, and PAKCM9 were comparable to that of PAKC (Table 2), indicating that the mutations in these strains did not affect flagellar assembly or motility. Thus, an initial examination suggests that the double mutants were either nonmotile or partially motile, whereas single mutants were motile, indicating that the amino acid changed in single mutants was not essential for motility. Western blot analysis showed the presence of flagellin in all of the mutants (Fig. 2A). One of the nonmotile mutants, PAKCM2, was subjected to EM studies in order to examine the appearance of its flagellum. The flagellum of this mutant strain (Fig. 3C and D) did not have the usual wave pattern seen in the strain complemented with the wild-type gene (Fig. 3A), which suggests that the motility defect may have been due to an improperly assembled flagellum that possibly led to a more rigid structure. These flagella seem to be fragile, as we saw many broken pieces floating in the medium (Fig. 3C and E). By contrast, PAKCM3, a fully motile strain containing the mutation L88A, whose amino acid change will be discussed later, demonstrated a fully formed, normal flagellum (Fig. 3B) that was similar to that of the wild type (Fig. 3A). The sequences of the entire flagellin genes from two fully motile strains, PAKCM3 and PAKCM7, showed that there were no compensatory mutations, which may have allowed the retention of full motility.

Evaluation of the purity and minimum dose of purified flagellar preparations/recombinant purified flagellin proteins

FIG. 2. (A) Immunoblot of bacterial samples from complemented PAKΔC strains showing the expression of FliC with anti-FliC antiserum. Left margin shows molecular size markers (in kDa). (B) 10% Coomassie-stained SDS-PAGE gels demonstrating the purities of flagellin preparations from the wild type and different mutant strains. Left margin shows molecular size markers (kDa). The minor contaminating bands seen come from the BL21 background and do not contribute towards IL-8 signaling, as confirmed by doing the assay with an equivalent amount of total protein [4.0×10^{-10} M flagellin] purified from BL21 cells through a nickel column (data not shown).

FIG. 3. Electron micrographs showing the expression of flagella in wild-type PAKC, motile mutant PAKCM3 (L88A), and nonmotile mutant PAKCM2 (Q89A, R92E). (A) Wild-type (WT) strain PAKC demonstrates a fully formed, normal, wavy flagellum. (B) Mutant PAKCM3 demonstrates a fully formed, normal flagellum similar to that of the wild type. (C and D) EM shows the presence of a deformed and fragile flagellum on PAKCM2. Many broken pieces of fragile flagella were seen floating in the medium as depicted in panel C and at higher magnification in panel E.

FIG. 4. Dose-response curve. A549 cells stimulated with different concentrations of flagellin purified from pETC demonstrated a significantly higher IL-8 production at 4.0×10^{-10} M flagellin concentration compared to those at the other concentrations used ($P < 0.001$). Similarly, the purified flagella from the PAK wild-type strain and the PAO1 wild-type strain produced maximum IL-8 responses at a concentration of 4.0×10^{-10} M in comparison to those at the other concentrations used ($P < 0.001$). The means $\pm$ SD were determined from at least three independent experiments.

required to produce maximum stimulation of A549 cells. As a first step in the analysis of the FliC signaling mechanism, we evaluated the effect of purified flagella from complemented strains and also of recombinant FliC produced in *E. coli* BL21(plysS) on IL-8 production by A549 cells. We purified and quantified the mutated flagella/flagellin proteins and analyzed them by SDS-PAGE and Coomassie blue staining to assess their purity (Fig. 2B and data not shown). The minor contaminants coming from the BL21 background do not contribute towards IL-8 signaling, as the IL-8 levels were similar to that seen in the cell control. No contaminating bands were seen in the PAK Δ C background. To ensure that we would be measuring responses induced by FliC and not those induced by

contaminating LPS, polymyxin B resin was used to remove LPS. This treatment reduced the LPS content of the FliC preparations used for the assay to less than 1 pg/ μ g, as measured by a *Limulus* assay (16).

Wild-type recombinant monomeric flagellin is nonglycosylated, as it runs at a lower molecular weight than purified flagella (data not shown). The difference between the migration rate of some of the purified flagellin bands in Fig. 2B and that seen in Fig. 2A could also be attributed to the fact that flagella expressed by different complemented strains can have variable degrees of glycosylation; moreover, monomeric flagellin coming from the *E. coli* background is not glycosylated at all. Both wild-type flagellin/flagella purified from PAK/PAO1 were very potent stimuli of IL-8 synthesis, as indicated by the IL-8 responses obtained from the A549 cells incubated with flagellin/flagella (Fig. 4). This experiment was done to ascertain the minimum dose of flagellin [4.0×10^{-10} M] that would give maximum IL-8 response.

IL-8 responses of A549 cells stimulated with purified flagella/flagellin from the wild type and different site-specific mutants. The putative TLR5 binding site mutants created for the present study could be divided into two broad categories: those that had TLR5-binding site mutations and were motile and those that had TLR5-binding site mutations and were nonmotile or partially motile. Flagellin purified from the putative TLR5 binding site mutants which are motile (pETCM3, pETCM4, pETCM6, pETCM7, and pETCM8) demonstrated an almost 80-fold reduction in IL-8 response compared to the wild-type flagellin from pETC (Fig. 5). These mutations were sufficient to abrogate flagellin-TLR5 interaction without having an effect on flagellum-mediated motility. Similarly, there was a significant reduction in the IL-8 response produced by the nonmotile mutants pETCM1 and pETCM2 and the partially motile pETCM5 compared to that of the wild-type flagellin (Fig. 5). Among the flagellins that retained motility, pETCM4, pETCM6, pETCM7, and pETCM8 were more attenuated in IL-8 production than pETCM3. Among the non-

FIG. 5. IL-8 responses of A549 cells incubated with flagellins purified from motile and nonmotile/partially motile site-specific mutants. A549 cells stimulated with flagellin at a concentration of 4.0×10^{-10} M purified from the motile and nonmotile/partially motile TLR5 binding site mutants show significant ($P < 0.001$) reductions in IL-8 levels compared to that of the wild type (WT). IL-8 response produced by flagellin purified from a motile non-TLR5 binding site mutant at 4.0×10^{-10} M concentration was not significantly different from that produced by wild-type flagellin. Data shown are means $\pm$ SD of four different experiments.

FIG. 6. IL-8 responses of A549 cells stimulated with flagellin purified from pETC, pETCM3, and flagellin mixed in equimolar quantities of pETC and pETCM3. Cells incubated with the mutant flagellin [4.0×10^{-10} M] from pETCM3 demonstrated a significant ($P < 0.001$) decrease in IL-8 levels compared to cells incubated with flagellin from pETC at the same concentration. On the other hand, the cells stimulated with a mixed flagellin sample (pETC+pETCM3) showed a significant ($P < 0.001$) increase in IL-8 response in comparison to cells incubated with flagellin at the 4.0×10^{-10} M concentration from pETCM3 alone. Data shown are means $\pm$ SD of four different experiments.

motile/partially motile flagellins, pETCM1 was more attenuated than pETCM2 and pETCM5. Thus, certain amino acids in this region appeared to be more important in stimulating IL-8 release than others, but all that were mutated led to reduced signaling. Hence, some sites appear to be more specific for interacting with TLR5 rather than to be essential structural amino acids. The IL-8 response produced by fully motile mutant pETCM9 was not significantly different from that of the wild type (Fig. 5), indicating that the 10-amino-acid region essentially harbors the TLR5 binding site.

To examine the specificity of the signaling, one representative mutant flagellin, pETCM3, was tested again by mixing it with equimolar quantities of the wild-type pETC flagellin to ascertain whether the mutation resulted in loss of binding to TLR5 and hence in IL-8 signaling. As expected, we observed a significant increase in IL-8 response in the mixed sample in comparison to that of pETCM3 (Fig. 6), indicating that loss of signaling with this mutant flagellin was consistent with the hypothesis that the mutation resulted in loss of interaction with the receptor rather than by some other mechanism.

Effect of flagellar glycosylation mutants on IL-8 release. The flagellin of *P. aeruginosa* is known to be glycosylated at two specific sites (3, 4, 5, 39) that are different in the type a and type b flagellins. The function(s) of these modifications is unknown, but such modifications have been shown to play a role in the hypersensitivity response of plant cells to *P. syringae* pv. *glycinea* (44, 45). In order to examine whether these modifications in *P. aeruginosa* play a role in inflammation, flagella with a defect in glycosylation were isolated from *P. aeruginosa*, purified, and tested for their ability to stimulate IL-8 production from A549 cells. Flagella from glycosylation mutants PAK r fb c (3) and PAO1 r fb c (5) showed IL-8 responses significantly reduced compared to those of wild-type flagella (Fig. 7), suggesting a role for flagellin glycosylation in the inflammatory process. Also of interest was the observation that PAK flagellin, whose two glycan chains make up about 10% of the observed mass (approximately 4.0 kDa) (39), is significantly more stimulatory than PAO1 flagellin ($P < 0.001$), where the two glycan chains are together about 1.4 kDa (5). Moreover, comparison

FIG. 7. IL-8 responses of A549 cells incubated with a 4.0×10^{-10} M flagellar preparation purified from PAK wild type (PAKWT), PAO1 wild type (PAO1WT), PAK r fb c , and PAO1 r fb c . A549 cells incubated with the flagellar preparation from glycosylation-defective mutant strains PAK r fb c and PAO1 r fb c showed a significant ($P < 0.001$) reduction in IL-8 response compared to those of their respective wild-type strains. Data shown are means $\pm$ SD of four different experiments.

of the nonglycosylated mutants with each other showed that there are no differences in the levels of stimulatory activity, which again indicates the role of glycosylation in the proinflammatory activity of *P. aeruginosa* flagellin.

DISCUSSION

Several studies have demonstrated that flagellin signaling for the release of inflammatory mediators occurs through the interaction of flagellin and TLR5 (19, 22, 26, 31, 32). However, it has been suggested that *Pseudomonas* flagellin also binds to TLR2 and to asialoGM1 to incite inflammation (1, 42). Information enabling examination of the TLR5 flagellin interaction in more detail is available, since the site on flagellin that interacts with TLR5 is known to be located in the D1 domain of flagellins and the location has been predicted by in silico studies (23) and verified experimentally (10, 11, 31). We have taken advantage of these predictions to more precisely define amino acid moieties that are involved in signaling, presumably through TLR5, and that are not critical for flagellar structure.

The basic structure of flagellin is reportedly constituted of two conserved domains (D1 and D2) at the N and C termini and of a central hypervariable domain (D3) (25), which is of variable length among bacterial species. The region in flagellin which stimulates the inflammatory response has been localized to the constant N- and C-terminal domains by Eaves-Pyles et al. (11) and Donnelly and Steiner (10). Donnelly and Steiner (10) experimentally identified two regions required for TLR5 activation in the D1 domain of *E. coli* flagellin. One region, on the N-terminal side, includes amino acids 57 to 190, and the other region is on the C-terminal side (amino acids 461 to 509) (10). Neither this region nor the 411-to-419 region suggested by Murthy et al. (36) exist in *P. aeruginosa* type a flagellin used in this study (47). In an in silico study, Jacchieri et al. (23) located the TLR5 binding site of flagellin within the constant

domain D1 at residues 88 to 97; this location, therefore, coincides with that experimentally found by Donnelly and Steiner (10). Jacchieri et al. also suggested that the side chains of Gln 89, Arg 92, and Glu 93 on the surface of the flagellin are optimally positioned to interact with the side chains of Glu 552, Asp 555, and Arg 558, respectively, on the surface of TLR5. Based on the above studies, we made site-specific mutations between residues 88 and 97 of the D1 domain of the *P. aeruginosa* type a flagellin molecule and also one site-specific mutation at residue 83 outside the putative binding domain.

In the present study, it was found that changing amino acid residues Q89 and R92 to similar amino acids N and K or to different amino acids A and E, as in strains PAKCM1 and PAKCM2, interfered with motility. Additionally, changes to some other locations, e.g., L88A along with M91A, as in PAKCM5, also resulted in the reduction of motility. Thus, while residues Gln89 and Arg92 of flagellin may be optimally positioned for interaction with the side chains of Glu552 and Asp555, these sites are also important for the structure of the flagellum. It seems these sites are primarily involved in interacting with TLR5, and any mutation at these sites might result in altered folding of the protein (as seen in native gels [data not shown]) that could hinder flagellin TLR5 interaction.

In contrast, the single mutations at residues L88, R90, L94, and Q97, which resulted in a considerable reduction in IL-8 response of A549 cells, did not affect the motility phenotypes of the strains, indicating these amino acids do not have a role in flagellum assembly or function in motility. This result is further substantiated by EM analysis of the PAKCM3 strain that showed the appearance of a normal flagellum. Therefore these sites may be the critical ones for TLR binding, since they do not affect motility or, presumably, flagellin folding. Based on this observation, we agree that most individual point mutations as well as double mutations within the predicted site will profoundly reduce the innate immune recognition of flagellin; however, these data also point out some significant differences from the *Salmonella* flagellin. Whereas mutations L88A, R90A, and L94A do not affect motility in *P. aeruginosa*, they significantly reduce motility in *Salmonella* (41). While we have not mutated every amino acid in this area singly, these data establish the principle that single mutations may affect signaling without affecting motility and that PAMPs, at least those on flagellin, may undergo mutation. The mutation Q83A affected neither motility nor IL-8 signaling, indicating that the 10-amino-acid region is explicitly required for TLR5 binding.

A549 cells expressing TLR5 (46) were exposed to only one stimulant, flagellin, which is considered a very specific and potent ligand of TLR5 (26, 36, 41). Purified flagellin/flagella produced maximum IL-8 response at a concentration of 4.0×10^{-10} M, which is similar to the concentration reported by others for *Salmonella* flagellin (36).

Protein glycosylation in prokaryotes is now a well-established process, particularly in cell surface-associated or -secreted molecules (6, 30). Arora et al. (3) identified a cluster of 14 genes encoding the determinants of the flagellin glycosylation machinery in *P. aeruginosa* strain PAK. Subsequent to this report, Schirm et al. (39) have reported the PAK flagellin to be modified with a heterogeneous glycan comprising up to 11 monosaccharide units that were O linked through a rhamnose residue to the protein backbone. Their studies further

revealed that *orfA* and *orfN* genes were required for the attachment of a heterogeneous glycan and of the proximal rhamnose residue, respectively. The sites of modification in PAK flagellin T190 and S261 (39) and PAO1 flagellin S192 and S196 (5) were located in the central, surface-exposed, variable region rather than in the highly conserved N- and C-terminal regions of the protein, as is the case for *Campylobacter* and *Helicobacter* flagellins (39). The flagellin of the PAK $rfbC$ mutant was found to be predominantly nonglycosylated (39); similarly, the flagellin from the PAO1 $rfbC$ mutant strain was found to be unmodified (5). IL-8 signaling estimated from the flagella purified from the two $rfbC$ mutant strains is reduced up to 50% compared to the levels for their respective wild-type strains. Of note is the fact that the IL-8 stimulation seen with whole flagella was between 40 to 50% of that seen with similar amounts of recombinant flagellin, consistent with a role for monomeric flagellin being more active (41) or with the presence of more individual signaling molecules in the recombinant flagellin preparation. However, it should be noted that recombinant wild-type flagellin which is made in *E. coli* does not appear to be glycosylated, based on the apparent molecular weight. Therefore, the values obtained with recombinant and presumably monomeric flagellin actually may be less than the true values, since they lack glycosylation. Further support for a role of *Pseudomonas* flagellar glycosylation in inflammation comes from the observation that the nonglycosylated whole flagella from the two strains do not appear to differ in their stimulatory activities, whereas the glycosylated forms differ significantly. Whether flagellin glycan moieties would aid in the binding of flagellin to TLR5 is not known. This is possible but unlikely, since the glycan moieties are located at sites in the D3 domain that are surface exposed. Alternatively, the glycans may have some signaling activity of their own through another TLR or other cellular receptor.

Understanding the region of flagellin that interacts with TLR5 will provide insight into this very specific aspect of host-pathogen interaction and also aid in the future design of effective therapeutics and vaccines. Based on structural and functional studies, we conclude that TLR5 recognizes a combinatorial surface on flagellin that is determined by a large group of residues comprising the region from 88 to 97. Thus, the TLR5 binding site predicted in silico appears to be correct for *P. aeruginosa* flagellin. The findings suggest that the specific PAMP of *P. aeruginosa* flagellin has potential mutational tolerance, in contrast to what had been believed heretofore about PAMPs in general. Such poorly signaling motile mutants will be particularly useful in examining the contribution of flagellin to the pathogenesis of acute lung disease caused by *P. aeruginosa*. Furthermore, the significance of glycosylation of the flagellum of this organism appears to be clearer, as it is apparent that it is involved in some form of recognition that either aids in eliciting an inflammatory response or does so directly.

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