

Pseudomonas aeruginosa Pili as Ligands for Nonopsonic Phagocytosis by Fibronectin-Stimulated Macrophages

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Fibronectin is capable of activating macrophages for enhanced nonopsonic phagocytosis of *Pseudomonas aeruginosa* grown in vivo in rats or mice or in vitro on nutrient agar plates. In this study it was determined that while fibronectin was able to significantly increase phagocytosis of organisms grown in static broth, uptake of agitated bacteria could not be promoted. Agitated *P. aeruginosa* cultures were proven to lack surface pili expression, as assessed by electron microscopic studies. A pilus-deficient *pilA::Tn501* mutant of *P. aeruginosa* PAO was constructed by gene replacement techniques. Phagocytosis of this mutant could not be enhanced by fibronectin regardless of growth conditions. Furthermore, 60 µg of exogenously added *Pseudomonas* pili per ml was capable of abrogating the enhanced phagocytosis of the wild-type strain observed with fibronectin-stimulated macrophages. It is concluded that *Pseudomonas* pili were the bacterial ligands required for attachment to fibronectin-stimulated macrophages in the initial stages of nonopsonic phagocytosis.

Macrophages are involved in defense against bacterial colonization and infection (7, 20). In addition to their role in bacterial phagocytosis, they play an important part in amplification of the host immune response. Through release of potent immunomodulators, inflammation and wound healing occur at an accelerated rate (25). Furthermore, antigen processing and presentation by macrophages is involved in T-cell recognition of antigens in the cellular and humoral immune responses.

In previous studies (11, 14) it was determined that *Pseudomonas aeruginosa* cells taken directly from an in vivo peritoneal chamber growth system were significantly more susceptible to phagocytosis by macrophages than were the same cells after being washed in buffer. The phagocytosis-promoting factor was isolated from the supernatant of centrifuged in vivo-grown bacteria and was determined to be fibronectin (14). This molecule was capable of increasing nonopsonic uptake of *P. aeruginosa* grown in vivo in rats or mice or in vitro on agar plates. It was subsequently demonstrated that promotion by fibronectin of nonopsonic phagocytosis was mediated by the direct action of fibronectin on the macrophages (13). The tetrapeptide arginine-glycine-aspartate-serine (RGDS), from the eucaryotic cell-binding domain of fibronectin, was also capable of stimulating macrophages for enhanced phagocytosis of *P. aeruginosa* (13).

As fibronectin-stimulated phagocytosis of *P. aeruginosa* occurred in the absence of opsonins, the resulting uptake must be considered nonopsonic. Speert and colleagues have presented evidence that nonopsonic phagocytosis of *P. aeruginosa* by human neutrophils and monocyte-derived macrophages was higher in three piliated, hydrophobic *P. aeruginosa* isolates than in two nonpiliated, hydrophilic isolates (26). However, these strains were not isogenic, and further investigation revealed a weakly piliated isolate that was not susceptible to nonopsonic phagocytosis (16). Nevertheless, addition of exogenous pili inhibited nonopsonic phagocytosis of *P. aeruginosa* by human neutrophils (16). It was tentatively concluded that pili may play a role in

nonopsonic phagocytosis of *P. aeruginosa*. To facilitate examination of this proposal with respect to fibronectin-stimulated nonopsonic phagocytosis, we have constructed, by gene replacement techniques, a Tn501 mutant of *P. aeruginosa* PAO that lacked surface pili. Using this strain, we have demonstrated that pili are essential for fibronectin-stimulated nonopsonic phagocytosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All strains and plasmids used are described in Table 1. For phagocytosis assays, strains were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) without (strains H103, BLP3, and PAO1-leu) or with [strain BLP3(pBP161)] carbenicillin (300 µg/ml). Prior to assay, cells were inoculated from these plates and grown at 37°C for 20 h either with vigorous shaking or without agitation (i.e., statically) in Trypticase soy broth or on fresh Trypticase soy agar plates. Cells were washed in phosphate-buffered saline (PBS), pH 7.2, and resuspended to a concentration of 10⁹ cells per ml prior to phagocytosis experiments. For genetic experiments, Luria-Bertani (LB) medium (14) was used. *Pseudomonas* isolation agar (PIA) (Difco Laboratories, Detroit, Mich.) was prepared as recommended by the manufacturer. Antibiotic concentrations were 50 µg/ml for ampicillin and chloramphenicol, 150 µg/ml for tetracycline, and 300 µg/ml for carbenicillin. The mercuric chloride concentration was 15 µg/ml.

Bacterial growth in vivo. Chambers for implantation into rats were constructed from 3-ml polypropylene syringe barrels as previously described (12). Briefly, washed bacteria were inoculated into plastic chambers which were sealed on either end with 0.22-µm-pore-size filters. When these chambers were inserted into the peritoneal cavity of laboratory animals, the filters allowed free exchange of peritoneal fluids and small bacterial products while prohibiting bacterial escape or direct immune cell access to the organisms. Bacterial cells were grown for 72 h in vivo prior to harvesting. Unwashed in vivo-grown *Pseudomonas* cells were counted in a Petroff-Hausser bacterial counting chamber

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or property	Source or reference
<i>E. coli</i>		
JM 83	<i>lacZ</i> ΔM15	31
MM294(pRK2013)	Contains mobilizing plasmid	22
<i>P. aeruginosa</i>		
H103	PAO1 wild type	10, 12
PAO1-leu	Leu auxotroph of PAO1; spontaneous mutant	This study
BLP3	<i>pilA</i> ::Tn501 mutant of PAO1-leu	This study
Plasmids		
pLAFR1	Tc ^r , IncP cosmid cloning vector	8
pBP310	PAK <i>pilA</i> clone in pLAFR1	Pasloske et al. ^a
pBP311	<i>pilA</i> ::Tn501 in pB310	Pasloske et al. ^a
pMR5	Temperature-sensitive RP1 plasmid, Cb ^r Tc ^r , IncP	21
pUC19	Ap ^r , ColE1 cloning vector	21
pKT210	Cm ^r , broad-host-range vector	1
pBP101	Ap ^r , PAO <i>pilA</i> clone	23
pB161	Ap ^r Cm ^r , PAO <i>pilA</i> clone in pK210	This study

^a B. L. Pasloske, D. G. Scraba, and W. Paranchych, manuscript submitted.

(Hausser Scientific, Blue Bell, Pa.) and maintained on ice until use. Washed organisms were centrifuged at 1,200 × *g* and gently resuspended in PBS twice prior to assay. The first decanted supernatant from these cells was saved for assessment of phagocytosis enhancement (in vivo supernatant).

Maintenance of macrophage cell line. Mouse macrophage cell line P388_{D1} was maintained at 37°C in 10% CO₂ in Nunc flat-bottomed flasks (Gibco, Burlington, Ontario) with supplemented RPMI-1640 medium (Gibco) as described previously (14). Prior to assay, macrophages were grown for 16 h in Nunclon petri dishes (Gibco; 35 by 10 mm) at a concentration of 10⁶ cells per dish. Nonadherent cells were removed by gently washing the monolayer with unsupplemented RPMI-1640 (phagocytosis buffer).

Phagocytosis assay. The visual assay for phagocytosis was performed as described previously (2). Briefly, 1 ml of phagocytosis buffer was added to a washed macrophage monolayer to give a final concentration of 10⁶ cells per ml. To assess enhancement of phagocytosis, PBS, in vivo supernatant (100 μl), 50 μg of bovine plasma fibronectin (Sigma Chemical Co., St. Louis, Mo.) or 50 μg of the tetrapeptide RGDS (Sigma) was added to the assay volume. No additional opsonins or macrophage activators were included in the system. The bovine fibronectin was demonstrated by the *Limulus* amoebocyte lysate assay to contain less than 0.1 ng of endotoxin per 50 μg of fibronectin. Bacterial cells were used at a *P. aeruginosa*-macrophage ratio of 20:1, and phagocytosis was allowed to occur for 90 min at 37°C in a 10% CO₂ atmosphere. Uptake was assessed visually following Diff-quick staining (Canlab, Vancouver, Canada). When used, 60 μg of purified PAO1 pili per ml, prepared as described previously (17), was incubated with in vivo supernatant-, fibronectin-, or RGDS-activated macrophages for 15 min at 37°C in 10% CO₂ prior to the addition of bacteria. This concentration of pili was chosen on the basis of analogous experiments with human neutrophils (18).

Characterization of bacterial cell envelopes and lipopolysaccharide samples. Bacterial cell envelopes were isolated from *P. aeruginosa* H103 as described previously (12). Sodium

dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (10, 12).

Determination of pili expression. *P. aeruginosa* H103 was grown for 20 h at 37°C in either rapidly agitated or static Trypticase soy broth. Plate-grown organisms were suspended in distilled water, while broth-grown cultures were centrifuged at 2,500 rpm for 10 min and then suspended in distilled water. Cells were allowed to adhere to carbon-coated copper grids for 30 s, washed in distilled water, stained for 10 s in 0.75% phosphotungstate, washed, and air dried. The presence of pili was assessed visually by electron microscopy as described previously (9).

Phage sensitivity assays. The *P. aeruginosa* isolate was lightly streaked on LB agar, and 2 μl (approximately 10⁷ PFU) of the pilus-specific bacteriophage PO4 (4) was pipetted onto the center of the streak. If a zone of clearing appeared after the cells were incubated overnight at 30°C, then lysis had occurred because the cells had assembled retractile pili.

Recombinant DNA and genetic techniques. Recombinant DNA techniques were performed as outlined previously (15, 18). Triparental matings were performed as described by Pasloske and Paranchych (19). The conjugation of pMR5 into *P. aeruginosa* was performed as described earlier (21).

Construction of pilin-minus, isogenic *P. aeruginosa* PAO1. A cosmid bank was obtained from *P. aeruginosa* PAK DNA by using the vector pLAFR1 (8). From this bank, a cosmid was isolated (pBP310) containing the PAK pilin gene. Through transposon mutagenesis, we disrupted the pilin gene within pBP310 by the integration of transposon Tn501 within the structural sequence as determined by DNA sequencing (B. L. Pasloske, D. G. Scraba, and W. Paranchych, manuscript submitted). The mutant cosmid (pBP311) was used in the gene replacement strategy described below to construct an isogenic *pilA*::Tn501 mutant of *P. aeruginosa* PAO1-leu.

A triparental mating was performed with MM294 (pRK2013) as the donor of the mobilizing plasmid to mobilize pBP311 into *P. aeruginosa* PAO1-leu, and the transconjugant was selected on PIA containing tetracycline (to select plasmid sequences) and Hg (to select the inserted Tn501). The cells were passaged every 12 h for 120 h with shaking at 37°C in LB broth and without antibiotic selection to allow gene replacement to occur through a double homologous recombination. A successful gene replacement would result in *P. aeruginosa* becoming resistant to the pilus-specific bacteriophage PO4. Therefore, to select for *pilA*::Tn501 mutants, the passaged cells were incubated with a 10,000-fold excess of PO4 for 30 min at 37°C and then grown at 30°C on LB agar containing Hg. Approximately 0.5% of these cells were phage-resistant, but they were also Tc^r, indicating that these mutants had retained pBP311.

To cure the phage-resistant mutant of pBP311, the temperature-sensitive plasmid pMR5 (21) was conjugated into the resistant strain, and transconjugants were selected on PIA containing carbenicillin. Plasmids pMR5 and pBP311 belong to the same incompatibility group, IncP, and therefore cannot coexist in *P. aeruginosa*. Having expelled pBP311 with pMR5 from the phage-resistant strain, the bacteria were cured of pMR5 by growing the cells at 44°C for 18 h, since the plasmid is unstable at this temperature. Loss of pMR5 was determined by the mutant regaining its Cb^s and Tc^s phenotypes. One isolate (*P. aeruginosa* BLP3) which had been cured of pMR5 also retained its resistance to Hg and PO4 phage. Equivalent digests of genomic DNA from the mutant BLP3 and the wild-type strain PAO1 which were

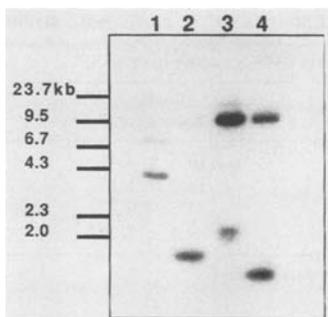


FIG. 1. Southern hybridization of *Pst*I- and *Hind*III-digested genomic DNA after probing with a 1.2-kilobase (kb) *Hind*III fragment containing the *P. aeruginosa* PAO pilin gene. Lane 1, strain PAO1-leu DNA digested with *Pst*I; lane 2, PAO1-leu DNA digested with *Hind*III; lane 3, strain BLP3 DNA digested with *Pst*I; lane 4, strain BLP3 DNA digested with *Hind*III. The altered mobility of the PAO1 pilin gene-reactive DNA fragment in strain BLP3 suggested that the pilin gene was interrupted, and the size of the *Pst*I fragment was consistent with insertion of Tn501 (which does not contain *Pst*I sites).

probed with a DNA pilin probe were found to have different restriction patterns (Fig. 1). The mutant had the restriction pattern expected from a Tn501 insertion into the pilin gene. Electron microscopy showed that these cells were bald of pili. Mobilizing pBP161 (carrying the PAO pilin gene) into the mutant also restored PO4 sensitivity, and pili were assembled by these transconjugants, as observed by electron microscopy.

Construction of broad-host-range plasmid expressing the PAO pilin gene. The PAO pilin gene clone pBP101 (19) was digested with *Hind*III and *Sal*I, and the 1,134-base-pair fragment carrying the PAO pilin gene was isolated by electrophoresis on 5% acrylamide (15). This fragment was cloned into the *Hind*III and *Sal*I restriction sites of pUC19 to yield plasmid pBP160. Both plasmids pBP160 and pKT210 were digested with *Eco*RI, ligated together, and transformed into *Escherichia coli* JM83, and transformants were selected on LB agar containing ampicillin and chloramphenicol. One of these clones (pBP161) was mobilized into *P. aeruginosa* BLP3 by a triparental mating, and transconjugants were selected on PIA containing carbenicillin.

RESULTS

In a previous investigation, it was determined that treating macrophages with fibronectin resulted in enhanced uptake of *P. aeruginosa* strains grown on agar plates but not of bacteria grown in agitated broth (14). These results were confirmed and extended by demonstrating that neither bovine nor rat fibronectin (the latter presented as an in vivo supernatant) could significantly stimulate macrophages for increased phagocytosis of bacteria grown in Trypticase soy broth with shaking ($P > 0.5$ by Student's *t* test). In contrast, both bovine and rat fibronectin significantly ($P < 0.005$) enhanced the uptake of bacteria grown on both Trypticase soy agar plates and Trypticase soy broth without shaking, compared with the uptake of bacteria by control macrophages (Table 2).

These data suggested that vigorous shaking during growth was capable of removing or suppressing expression of the bacterial ligand required for fibronectin-activated nonopsonic uptake by macrophages. Sodium dodecyl sulfate-polyacrylamide gels of outer membrane samples from cells

TABLE 2. Effect of growth conditions on stimulation of P388_{D1} macrophage phagocytosis by in vivo supernatant or bovine fibronectin

Growth condition (strain H103)	Phagocytosis stimulation index ^a	
	In vivo supernatant	Bovine fibronectin
Shaking broth	0	0.4
Static broth	7.3	3.8
Agar plate	10.1	6.4

^a The phagocytosis stimulation index was the number of bacteria per macrophage taken up by stimulated macrophages minus the number of bacteria per macrophage taken up by control macrophages (3.5 ± 0.6).

grown in the three growth conditions failed to indicate any detectable alterations in rapidly agitated cultures compared with statically or plate-grown cultures (data not shown).

Determination of the bacterial ligand for nonopsonic phagocytosis. In previous studies, it was suggested that *Pseudomonas* adherence to human epithelial cells (6, 29) and neutrophils and leukocytes (16, 26) was pilus mediated. Therefore, we considered the possibility that pili were the bacterial ligands involved in fibronectin-mediated macrophage stimulation. Electron microscopic observation of cells grown under the above conditions revealed that cells grown with rapid agitation were devoid of surface pili, whereas those grown in static broth contained 1 or more pili per bacterium, as previously observed for another *P. aeruginosa* strain (26).

This suggested surface pili as the potential bacterial ligand responsible for enhanced interaction of statically grown *P. aeruginosa* strains with fibronectin-stimulated macrophages. Therefore, a pilus-free mutant of *P. aeruginosa* PAO1-leu was created by cosmid cloning of the *P. aeruginosa* PAK pilin gene into *E. coli* (18), Tn501 mutagenesis of the cloned *pilA* gene, mobilization of the transposon-mutagenized gene (*pilA*::Tn501) into *P. aeruginosa* PAO1-leu by triparental mating, phage selection for homologous recombination into the chromosome, and plasmid curing (see Materials and Methods). The resultant mutant, BLP3, was mercury resistant and contained the *pilA*::Tn501 mutation in its chromosome, as revealed by Southern hybridization of a *pilA* gene probe with restriction digests of strain BLP3 genomic DNA. As a control, the pilin phenotype was reconstituted by mobilizing plasmid pBP161, containing the cloned *P. aeruginosa* PAO *pilA* gene, back into strain BLP3, to create strain BLP3(pBP161). Electron microscopic examination revealed that strain BLP3 was devoid of surface pili, and susceptibility testing revealed that it was resistant to the PAO1 pilus-specific phage PO4. Both the parent strain PAO1-leu and BLP3(pBP161) were piliated and phage PO4 sensitive. When grown on Trypticase soy agar plates, strains PAO1-leu and BLP3(pBP161) were significantly better taken up by macrophages stimulated with rat or bovine fibronectin or by the conserved fibronectin cell-binding tetrapeptide RGDS than they were by unstimulated macrophages (Table 3). In contrast, there was no significant difference in the extent of phagocytosis of the nonpiliated mutant BLP3 by stimulated or unstimulated macrophages. Similar data were obtained when these strains were grown in peritoneal chambers in mice.

To confirm pili as the bacterial ligand for nonopsonic phagocytosis, purified pili (60 μ g/ml) were added to in vivo supernatant or fibronectin- or RGDS-activated macrophages and incubated for 15 min prior to assay. Exogenously added

TABLE 3. Fibronectin-stimulated nonopsonic phagocytosis of piliated and nonpiliated *P. aeruginosa* strains

Strain	Phenotype	Avg no. of bacteria/P388 _{D1} macrophage ± SD			
		PBS control	In vivo supernatant	Bovine fibronectin	RGDS
H103	Piliated	6.0 ± 1.4	11.2 ± 2.2 ^a	13.5 ± 4.4 ^a	10.6 ± 3.2 ^a
PAO1-leu	Piliated	5.0 ± 1.4	10.1 ± 1.6 ^a	14.2 ± 1.1 ^a	10.7 ± 0.2 ^a
BLP3	Nonpiliated	5.2 ± 0.4	4.8 ± 0.1 ^b	4.7 ± 0.2 ^b	5.2 ± 0.5 ^b
BLP3(pBP161)	Piliated	4.8 ± 0.1	7.8 ± 2.2 ^a	9.5 ± 3.7 ^a	9.3 ± 1.0 ^a

^a $P < 0.005$ (Student's *t* test) compared with the PBS control (i.e., unstimulated macrophages) in repeated assays.

^b Not significantly different from the PBS control ($P > 0.5$).

pili prevented enhanced uptake of strain H103 by macrophages activated by any of the three preparations (Table 4).

DISCUSSION

The data presented in this paper demonstrate that the *P. aeruginosa* pilus was the bacterial ligand responsible for the observed enhanced nonopsonic phagocytosis by fibronectin-activated macrophages. Fibronectin promotion of macrophage phagocytosis was observed with *P. aeruginosa* grown on agar plates and in static broth, but not with rapidly agitated cultures (Table 1). These data correlated well with the absence of pili on bacteria grown with shaking. Furthermore, fibronectin was unable to enhance uptake of a constructed mutant strain of *P. aeruginosa* lacking surface pili (Table 2). In addition, exogenously added pili suppressed phagocytosis of *P. aeruginosa* PAO1 by fibronectin or RGDS-stimulated macrophages to the level observed with untreated macrophages.

The data presented in this paper are consistent with previous studies showing that heavily piliated strains of *P. aeruginosa* are generally more susceptible to nonopsonic phagocytosis by neutrophils than less-piliated strains (15, 25). It was also determined that exogenous pili were capable of inhibiting nonopsonic uptake of this organism by neutrophils (16). These studies utilized freshly isolated human peripheral blood neutrophils. Although these authors did not investigate the role of fibronectin, given the high concentrations of fibronectin in human serum (0.7 μM) and our own inability to reverse fibronectin effects by washing fibronectin-treated macrophages (13), we would predict that the phagocytes they used were already fibronectin stimulated.

P. aeruginosa pili have also been shown to mediate binding to epithelial (6, 29) and epidermal (24) cells. We propose that the role of pili in nonopsonic phagocytosis is to mediate initial bacterial attachment to macrophages and

neutrophils. While the mucoid alginate exopolysaccharide can also mediate binding to epithelial cells (5), it must be noted that the strains studied here were nonmucoid and that phage-selected mucoid derivatives of *P. aeruginosa* PAO1 were still susceptible to fibronectin-promoted nonopsonic phagocytosis (J. Kluffinger and R. E. W. Hancock, unpublished observations).

It has been suggested previously that fibronectin promotes phagocytosis of particles coated with C3b or C3bi via the macrophage receptors CR1 and CR3, respectively (30). This response occurs rapidly and is not accompanied by a change in the number of receptor molecules on the surface of the phagocyte (30). Recent data have suggested that the macrophage receptor for nonopsonic phagocytosis of *P. aeruginosa* is the mannose receptor (30). Interestingly, nonopsonic phagocytosis of *E. coli* also occurs via the mannose receptor and appears to be mediated by mannose-sensitive pili (3). Thus, it is of interest to determine exactly how fibronectin influences the mannose receptor to promote a functional interaction with *P. aeruginosa* pili.

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TABLE 4. Inhibition of fibronectin-mediated macrophage nonopsonic uptake of *P. aeruginosa* PAO1 strain H103 by exogenous PAO1 pili

Macrophage activator	Avg no. of bacteria/P388 _{D1} macrophage ± SD	
	Without pili	With added pili
None (PBS control)	7.1 ± 1.6	6.7 ± 1.1 ^a
In vivo supernatant	16.0 ± 3.2 ^b	6.7 ± 0.1 ^{a,c}
Fibronectin	19.1 ± 6.3 ^b	5.5 ± 1.2 ^{a,c}
RGDS	14.2 ± 5.1 ^b	5.0 ± 0.7 ^{a,c}

^a Not significantly different from the PBS control without added pili.

^b $P < 0.005$ (Student's *t* test), significantly higher than the PBS control without added pili.

^c $P < 0.005$ (Student's *t* test), significantly lower than the equivalent value in the absence of added pili.

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