The chaperone/usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation

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***Pseudomonas aeruginosa*, an important opportunistic human pathogen, persists in certain tissues in the form of specialized bacterial communities, referred to as biofilm. The biofilm is formed through series of interactions between cells and adherence to surfaces, resulting in an organized structure. By screening a library of Tn*5* insertions in a nonpiliated *P. aeruginosa* strain, we identified genes involved in early stages of biofilm formation. One class of mutations identified in this study mapped in a cluster of genes specifying the components of a chaperone/usher pathway that is involved in assembly of fimbrial subunits in other microorganisms. These genes, not previously described in *P. aeruginosa*, were named *cupA1–A5*. Additional chaperone/usher systems (CupB and CupC) have been also identified in the genome of *P. aeruginosa* PAO1; however, they do not appear to play a role in adhesion under the conditions where the CupA system is expressed and functions in surface adherence. The identification of these putative adhesins on the cell surface of *P. aeruginosa* suggests that this organism possess a wide range of factors that function in biofilm formation. These structures appear to be differentially regulated and may function at distinct stages of biofilm formation, or in specific environments colonized by this organism.**

Pseudomonas aeruginosa, a common environmental Gram-negative bacterium, is also an opportunistic human pathogen and is responsible for serious damage to the respiratory tract of cystic fibrosis patients (1). Nosocomial pneumonia in intubated and mechanically ventilated patients is the second most common infection of hospitalized patients, and *P. aeruginosa* is the key etiological agent of hospital-acquired infections (2). The ability of *P. aeruginosa* to attach to abiotic surfaces, to host tissues, or to each other, and the subsequent differentiation of the microorganisms into biofilm, can be considered a major virulence trait in a variety of infections (3). Biofilm formation can take place on a variety of surfaces, such as medical instruments, leading to many types of nosocomial infections, and *P. aeruginosa* has been shown to persist in biofilm in the lungs of cystic fibrosis patients (4). Biofilms are characterized by a complex, highly structured, bacterial organization (5). They are initiated by the attachment of a single planktonic cell on a surface. Multiplication and the development of microcolonies separated by water-filled channels follow this event. The ability to form biofilm endows the bacteria with several important characteristics, including a marked increase in resistance to antibiotics (6).

A genetic screen, developed by Kolter and coworkers (7, 8) provided some of the tools to study the genetic determinants of biofilm formation. By characterizing several *P. aeruginosa* mutants defective in distinct stages of biofilm formation, a number of components that participate in the initiation of biofilm formation and progression to microcolonies and mature biofilm were identified (9). Through the analysis of the phenotypes of different *P. aeruginosa* mutants, it was proposed that type IV pili and flagella, two organelles of motility, may play an important role during the initial interaction of the bacterial cell with the surface by counteracting repulsive forces. Furthermore, type IV pili appeared to be required for the initial differentiation by promoting cell aggregation and the formation of microcolonies. Once the bacterial population reached a given threshold, cell–cell communication by means of the quorum-sensing regulatory systems, more particularly by means of the *lasI*-dependent *N*-(3-oxododecanoyl)homoserine lactone, programmed the differentiation process and the maturation into biofilm (10). The stabilization of the biofilm community is usually accomplished by production of an exopolysaccharide (11), such as alginate in the case of *P. aeruginosa*. Several physiological factors, such as the global carbon metabolism regulator (Crc) of *P. aeruginosa* (12), also play a role in biofilm formation. The availability of carbon/energy sources was also reported as an important signaling information determining the initiation of biofilm formation in *Pseudomonas fluorescens* (7).

In this study we adapted the method described by O'Toole and Kolter (9) for identifying biofilm-defective mutants. We screened a collection of Tn*5* mutants in a strain of *P. aeruginosa* that was unable to produce type IV pili. Expression of pili is important during various stages of biofilm formation, but bacteria lacking these organelles can still interact with abiotic surfaces (9). This screen, therefore, identifies genes specifying new adhesion factors. Among the series of biofilm-defective mutants obtained, one was affected in a gene encoding a protein similar to periplasmic chaperones of the chaperone/usher pathway in a variety of bacteria (13). This gene belongs to a cluster that we called *cupA*. We proposed that the *cupA* genes encode the components of a new class of *P. aeruginosa* adhesins, related to the adhesins in other microorganisms (14). We showed that mutants devoid of a functional CupA are defective in the formation of biofilm, in a manner that is independent of the presence of type IV pili. Using the available sequence of the PAO1 genome, we identified additional *cup* gene clusters and tested their involvement in biofilm formation. The CupB and CupC systems identified do not appear to play a role in biofilm formation under the conditions tested. These observations suggest that multiple factors are available to *P. aeruginosa* to facilitate its binding to various surfaces and for interbacterial adhesion as well. The wide variety of such attachment mechanisms, which are apparently differentially regulated, may reflect the complex needs of this organism during the colonization of widely diverse environmental niches.

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Table 1. Strains and plasmids used

Strains/plasmids	Relevant characteristics*	Source†
Strains		
Escherichia coli		
TG1	*supE* Δ(*lac–proAB*) *thi hsdR*Δ*5* (F′ *traD36 rpoA*$^+$ *B*$^+$ *lacI*^{q} *Z*Δ*M15*)	Lab collection
TOP10F′	F′(*lacI*^{q} Tn*10*(Tet^R)) *mrcA* Δ(*mrr–hsdRMS-mcrBC*) Φ80 *lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara–leu*)*7697 galU galK rpsL*(Str^R) *endA1 nupG*	Invitrogen
P. aeruginosa		
PAK	Wild type	D. Bradley
PAKΔpilA	Deletion of *pilA* in PAK	D. Nunn
PAK-NP	PAK *pilA*::Tet^R	S.L.
PAK-NF	PAK *fliC*::Gm^R	S.L.
PAN66	Tn*5*Gm derivative of PAKΔpilA, *cupA2*	This study
PAN67/PAN67B	PAK-NP/PAK, *cupA3*::Cb^R	This study
PAB3/PAB3B	PAK-NP/PAK, *cupB3*::Cb^R	This study
PAC3/PAC3B	PAK-NP/PAK, *cupC3*::Cb^R	This study
Plasmids		
pBBR1MCS4	Broad host range, Ap^R	M. E. Kovach
pBBRN66	pBBR1MCS4 containing *cupA2*	This study
pCR2.1	ColE1, f1 *ori*, Ap^R, Km^R	Invitrogen
pCRN67	DNA fragment from *cupA3* in pCR2.1	This study
pCRB3	DNA fragment from *cupB3* in pCR2.1	This study
pCRC3	DNA fragment from *cupC3* in pCR2.1	This study

*Tet^R, tetracycline resistance; Str^R, streptomycin resistance; Gm^R, gentamicin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Cb^R, carbenicillin resistance.
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Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains and plasmids used are listed in Table 1. Strains were grown at 37°C in L broth (LB) or on LB agar plates. Plasmids were introduced into *P. aeruginosa* by electroporation (15), and transformants were selected on *Pseudomonas* isolation agar (PIA) containing antibiotics. Antibiotics were used at the following concentrations (μg/ml): ampicillin, 50 (*Escherichia coli*); carbenicillin, 500; tetracycline, 200; and gentamicin, 50 (*P. aeruginosa*).

Construction of a Tn*5* Insertions Library in *P. aeruginosa*. A random insertion library was created by using Tn*5*G, a modified transposon Tn*5* (16). The transposon was introduced into *P. aeruginosa* PAKΔpilA, a strain containing a chromosomal deletion of the pilin gene, by pRK2013-mediated conjugation (17).

Biofilm Formation Assay and Quantification. The biofilm formation assay was performed according to O'Toole and Kolter (9) with slight modifications. Screening was performed in 96-well polystyrene microtiter dishes containing 100 μl of M63 minimal medium supplemented with 0.2% glucose, 1 mM $MgSO_4$, and 0.5% Casamino acids. The wells were inoculated with individual clones, taken with a toothpick, from the Tn*5* mutant collection isolated on agar plates, and the dishes were incubated at 30°C for 10–12 h. The phenotype of putative nonadherent clones was further checked in 24-well microtiter dishes. In those wells, 1 ml of M63-derived minimal medium was inoculated with 10^8 bacterial cells from an overnight inoculum. Bacterial cells bound to the wall of the wells were stained with crystal violet 1% (Sigma), and for quantification they were suspended in 400 μl of 95% ethanol followed by addition of 600 μl of water, and the OD_{600} was measured.

Microscopic Analysis of Biofilm Formation. Cells were grown in 4 ml of M63 minimal medium supplemented with glucose, $MgSO_4$, and Casamino acids, in a 50-ml Corning tube containing a glass cover slide. After 10–12 h of growth at 30°C without shaking, the glass slide was removed and rinsed. The remaining cells were visualized by phase-contrast microscopy using a Zeiss PhoMi III microscope. Images were captured with a camera and integrated with the IMAGE PRO PLUS software (Media Cybernetics, Silver Spring, MD).

Flagellar Motility Assay. Bacteria were inoculated with a toothpick on minimal agar plate (M63 with glucose, $MgSO_4$, and Casamino acids) containing 0.3% agar. The plates were then incubated at 30°C, and a halo corresponding to the spreading of bacteria from the point of inoculation was observed.

Inverse PCR. PCR amplification of DNA regions bordering the Tn*5* in selected PAKΔpilA mutants was performed by using two divergent oligonucleotides: OTn1, 5′-GCGCGGATCCTG-GAAAACGGGAAAG-3′; and OTn2, 5′-CCATCTCATCA-GAGGGTAGT-3′. Chromosomal DNA from each mutant was prepared by using the Nucleospin C + T kit (Macherey-Nagel), digested with *Alu*I, *Taq*I, or *Xho*I, and religated, and the circular DNA was used as matrix for PCR amplification. The DNA fragments thus amplified were directly cloned into the PCR2.1 vector with the TA cloning kit (Invitrogen) and transformed into *E. coli* TOP10F′ (Invitrogen). DNA sequencing was performed by MWG Biotech (Ebersberg, Germany).

Genome Sequence Analysis. The DNA sequences obtained were analyzed with the BLASTN program and compared with the PAO1 genome at http://www.pseudomonas.com. The putative ORFs were then analyzed with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The annotated genome was used on release to update our analysis.

Plasmids Constructions and Molecular Techniques. The *ladN66/cupA2* gene was PCR amplified with the High Fidelity polymerase (Boehringer), from the chromosomal DNA of *P. aeruginosa* PAK, using oligonucleotides ON1 (5′-CCAACGGAGCCAC-CAGCACCA-3′) and ON2 (5′-CAGGAAGAGCCGAGCAA-CAG-3′). The DNA fragment was cloned into PCR2.1 and subcloned into the broad host range vector pBBR1MCS4 as a *Hin*dIII/*Xba*I fragment, yielding pBBRN66. Internal DNA fragments from the *cupA3*, *cupB3,* and *cupC3* genes were PCR amplified by using the oligonucleotide pairs OMP1/OMP2 (5′-TCCAACCTACACCTATTCCCGCTAC-3′/5′-CCGTCG-TAGAAATCGCTGGAGGAG-3′), MUB1/MUB2 (5′-CCT-GTCTGCTGGCACTGTTTC-3′/5′-AATAGCTGGGCAC-CGAGACATA-3′), and MUT1/MUT2 (5′-AGGTGTCCG-TCTATTCCAGGT-3′/5′-GGTACGGTTGCTACTGAACT-

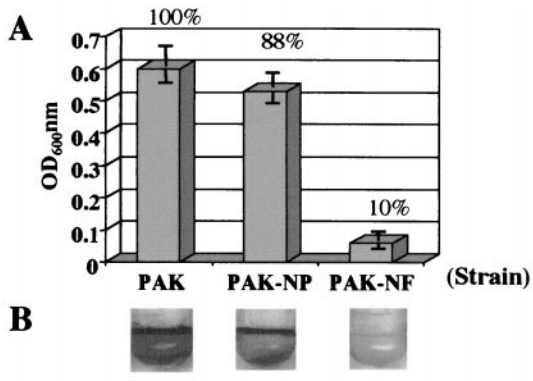

Fig. 1. Biofilm formation of wild-type PAK strain, PAK-NP (*pilA*), and PAK-NF (*fliC*). (*A*) Bars represent the measure of OD_{600} as a result of four distinct experiments. The level of attachment of the mutant strains is indicated as a percentage of attachment of the wild-type PAK strain. (*B*) Visualization of biofilm formation in wells of a microtiter dish after crystal violet staining.

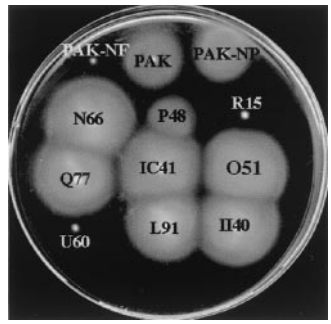

Fig. 2. Flagellar motility. Motility of strains PAK, PAK-NP, and PAK-NF are compared with motility of PAKΔpilA Tn*5* mutant derivatives. Strains R15, U60, and P48 appeared to be mutants affected in flagellum biogenesis (see Table 2), whereas IC41, O51, Q77, L91, II40, and N66 are affected in genes unrelated to flagellar biogenesis that were called *lad*. N66 (also named PAN66) is affected in a gene that we renamed *cupA2*. In the case of strain P48, for which slight motility is observed, the mutated gene corresponds to *motY,* which has a role in the function of the flagellum but not its assembly.

TG-3′), respectively. The fragments were cloned into PCR2.1, yielding pCRN67, pCRB3, and pCRC3, respectively. Those plasmids, which do not replicate in *P. aeruginosa*, were introduced by electroporation into strain PAK or the nonpiliated PAK-NP strain, and mutants were selected on PIA plates containing carbenicillin. The positions of the plasmid insertion, within the chromosome of these mutant strains, were checked by PCR with appropriate oligonucleotide pairs, including ON1, UBV (5′-CCCTCCGTTTCCCCGCTTTTTA-3′) or UTV (5′-TCAGAAGAGCAGAGCAGAGCAG-3′), for *cupA3*, *cupB3,* or *cupC3*, respectively, and the universal or reverse M13 primers.

Results and Discussion

Screening Procedure and Strategy for Identification of New Adhesins. Recent advances in genetic studies on biofilm formation by microorganisms resulted in substantial progress in the understanding of the molecular mechanisms involved in this process (8). In *P. aeruginosa,* the involvement of two distinct surface appendages has clearly been established (9). Those structures include the primary organelles of motility: the flagellum and the type IV pili. In *P. aeruginosa,* type IV pili appear to be involved in several functions, including adhesion to epithelial cells (18, 19) and a particular form of motility, called twitching motility (20). Their biogenesis and function in twitching motility involve the assembly of PilA monomers into a filament, a process that requires the products of a number of accessory genes (21, 22).

Type IV pili also appear to be important for adherence to abiotic surfaces as well, because mutations in three genes associated with pilus formation (*pilB, pilC,* and *pilY1*) yielded *P. aeruginosa* strains defective in attachment to polyvinyl chloride (PVC) (9). These mutants formed monolayers on plastic, but they lacked the ability to form microcolonies and to differentiate into biofilm. To uncover adhesive phenotypes that may function under various conditions, we modified the original assay of O'Toole and Kolter (9), by growing the bacteria at lower temperature (30°C) in an enriched minimal medium. A modest difference was observed between binding to abiotic surfaces by wild-type *P. aeruginosa* PAK and the isogenic nonpiliated mutant (PAK-NP). Indeed, the nonpiliated mutant could attach to plastic with an efficiency of 88%, relative to the wild type (Fig. 1), suggesting that under these conditions, the contribution of type IV pili to overall adhesion was reduced, allowing the identification of a novel adhesive system.

We constructed and screened a collection of Tn*5* mutants derived from the nonpiliated *P. aeruginosa* strain PAKΔpilA. Each individual clone was grown in Casamino acids/glucose medium, and bacteria were allowed to bind to the wells of a polystyrene microtiter dish for a period of 10–12 h at 30°C. The mutants that were not able to form a ring on the walls at the air–liquid interface, as revealed by crystal violet staining, were considered nonadherent. In the conditions used for this screen, a mutant that lacks flagella because of a mutation in the *fliC* gene, which encodes the structural subunit of the flagellum, PAK-NF, was unable to attach efficiently (Fig. 1). This observation prompted us to analyze all of the mutants selected by our screen onto plates containing 0.3% agar, to determine whether the mutation affected flagellar motility (Fig. 2). Out of 4,140 clones tested, we isolated 46 nonadherent mutants. From those, 29 were also deficient in motility, as compared with the wild-type strain. The remaining 17 mutants had thus lost adherence independent of the function of type IV pili and flagella. These mutants were named *lad* for *l*ost *ad*herence.

Characterization of the Nonmotile Mutants. The mutants were analyzed by inverse PCR, using two oligonucleotides, OTn1 and OTn2, to amplify adjacent DNA fragment to the transposon insertion site, followed by sequencing of the cloned product (see *Materials and Methods*). To verify this approach, five of the nonmotile mutants were analyzed first. Each insertion was located in genes involved in flagellar biogenesis (Table 2) (23). Among those strains, mutation in genes essential for flagellum structure (*flgF*) (24), flagellum assembly (*flhB*) (25), or regulation of flagellar gene expression (*fleS*) (26) were found. O'Toole and Kolter (9) also previously showed that a nonmotile mutant possessing an incomplete flagellum (*flgK*) was unable to adhere to the wall of a microtiter dish. The adhesion defect was severe

Table 2. Gene characterization from *P. aeruginosa* mutants affected in flagellar motility

Mutant	Homologous gene product	Identity/similarity, %	Function of the homologous protein
R15	FlgF/*E. coli*	38/56	Flagellum proximal rod protein
U60	FlhB/*E. coli*	36/55	Flagellar export apparatus protein
A86	FleS/*P. aeruginosa*		Regulation of flagellar synthesis
P48	MotY/*Vibrio cholerae*	26/48	Component of the flagellar motor
X24	MotA/*Pseudomonas putida*	82/90	Stator of the flagellar motor

in *P. aeruginosa,* since microscopic analysis revealed a complete absence of bacteria adhering to plastic (9). In contrast, the lack of functional flagella in *E. coli* does not totally prevent surface attachment (27). Interestingly, our screen identified genes that are necessary either for the assembly of flagella or for flagellar rotation (*motA* and *motY*) (28, 29). The requirement for flagellar rotation is an indication that during formation of biofilm by *P. aeruginosa*, the most important function of the flagellum is not in adhesion, but in facilitating bacterial motility.

Characterization of the *lad* Mutants. Among the 17 motile mutants obtained from the Tn*5* library, most of the transposons were found in genes that were annotated as hypothetical unknown genes. One gene encoded a putative component of regulatory systems, the product of another was similar to a permease from an ABC transporter, whereas another putative *lad* gene product shared 39% identity with the putative protein Y4iJ from *Rhizobium* sp. NGR234 (30). Finally, one of the *lad* genes (*ladN66)* was identified through two different Tn*5* insertions in the same coding sequence. This gene encodes a protein belonging to a family of periplasmic chaperones involved in pilus assembly via the so-called chaperone/usher pathway (31). Those chaperones are periplasmic proteins that adopt an Ig-like fold for recognition and further assembly of pilin subunits (32). Surface exposure required the function of a second accessory component, called usher, that forms a channel into the outer membrane through which the pilin subunits are sequentially assembled (33). This highly conserved pathway in Gram-negative bacteria has been involved in the assembly of more than 30 adhesive organelles (14). These organelles are mostly fimbrial structures that may adopt different morphologies. Two main classes could be discerned. The thick and rigid pili (7 nm in diameter), such as the P or type 1 pili from *E. coli*, and the thin and flexible pili (2–5 nm in diameter), such as the F17 pili from *E. coli*. In addition, very thin or afimbrial adhesins, also called atypical structures, are assembled via the chaperone/usher pathway. The exact composition and the architecture of those structures has not been well characterized, but they are most probably composed of monomers or simple oligomers of the afimbrial subunit assembled at the cell surface. Similar structures have been proposed for *Yersinia* species, namely the Caf1 antigen of *Yersinia pestis* (34).

The periplasmic chaperones involved in the chaperone/usher pathway could be distinguished into two structurally and functionally distinct subfamilies. These two groups differ by the length of the loop that connects the F1 and G1 β-strands of domain 1 (14). Chaperones of the FGL family have a loop with a length of over 20 aa, whereas chaperones from the FGS family have a shorter loop. The FGL chaperones assemble atypical or nonfimbrial adhesins, whereas the FGS chaperones assemble fimbriae. The amino acid sequence analysis revealed that the *ladN66* gene product belongs to the FGS family, which suggests that it may be involved in pili formation rather than in assembly of nonfimbrial adhesins. So far, we have not clearly identified LadN66-dependent fimbrial structures by electron microscopic analysis (data not shown).

The *ladN66* gene was amplified by PCR using the primers ON1 and ON2 and was cloned under control of the *lac* promoter into pBBR1MCS4, yielding pBBRN66. This plasmid was then introduced into the corresponding mutant PAN66 by electroporation. This strain was analyzed for its ability to attach to inert surfaces as described previously. We observed that it attaches even more efficiently as compared with the PAK-NP strain (data not shown), indicating that the *lad* phenotype of the PAN66 mutant strain was complemented by the introduction of this unique gene. The lack of attachment of the mutant PAN66 was therefore due solely to the disruption of the *ladN66* gene by the transposon.

Biofilm Formation Phenotype of PAN66 Mutant. More detailed analysis of the defects conferred by the mutations in the *ladN66* gene was obtained through microscopic analysis of bacteria attached to glass cover slides (see *Materials and Methods*). As illustrated in Fig. 3, only a few cells of PAN66, which could be found in clusters, are attached to the glass cover slide, whereas PAK-NP is capable of forming a significant biofilm on this support. The direct visual inspection of the biofilm formation phenotype of the PAN66 mutant revealed that, compared with the parental strain, only very few bacteria could make a stable attachment with the abiotic surface, and they are not able to form a structured biofilm. This observation thus confirmed the results of our initial screening procedure in microtiter dishes.

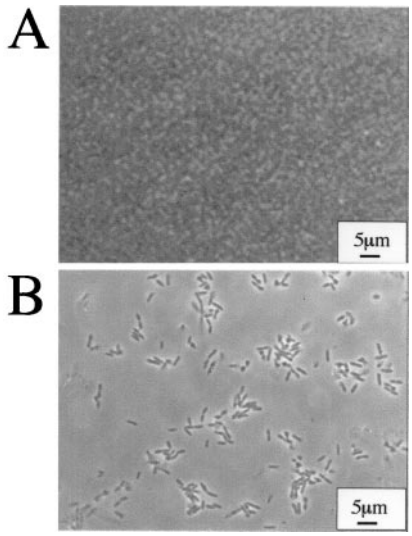

Fig. 3. Microscopic analysis of biofilm formation. Bacteria grown in M63 medium, supplemented with glucose, $MgSO_4$, and Casamino acids, were allowed to attach to glass cover slides. The development of biofilm on a solid surface was visualized by phase-contrast microscopy. (*A*) PAKΔpilA. (*B*) PAN66 (*ladN66*/*cupA2*).

Characterization of the *cupA* Gene Cluster. The availability of the *P. aeruginosa* PAO1 genome sequence (http://www.pseudomonas.com) allowed us to identify the gene cluster associated with *ladN66*. Flanking *ladN66* were genes that encode additional components of the chaperone/usher pathway in other bacteria, including genes encoding two pilin-like subunits, an usher, and a second chaperone (Fig. 4). Because of these similarities the genes belonging to this cluster have been renamed *cup* for "*c*haperone–*u*sher *p*athway." This gene cluster contains five genes (*cupA1–cupA5*), with *cupA2* being the previously identified *ladN66* gene.

Comparison of the deduced protein sequences of the *cupA* cluster showed sequence relatedness to other adhesive system; however, the most closely related proteins were not always from the same bacterium (Table 3). For example, CupA1 shares sequence similarity with the F17A fimbrial subunit of *E. coli*, whereas the sequence of CupA4 shares sequence similarity with a protein from *Yersinia pestis*, which is homologous to the HifA fimbrial subunit from *Haemophilus influenzae* (35). CupA2 and CupA5 are both related to the members of the periplasmic chaperone family, with the closest identity to F17a-D of *E. coli*, and FhaD of *Bordetella pertussis*, respectively. CupA3 appears to be the usher component of the system, based on its high similarity to FocD from *E. coli*. Since the analysis of the relatedness of the CupA proteins to other fimbrial systems with defined structures, such as the thick or thin filaments (14), revealed no consistent pattern, we should consider the possibility that the CupA proteins participate in the assembly of a type of adhesive organelle not previously recognized.

We also examined the organization of genes that specify adhesins of the chaperone/usher family in other bacteria. We observed that a gene cluster in *Y. pestis* (*yp36–40*) showed strictly

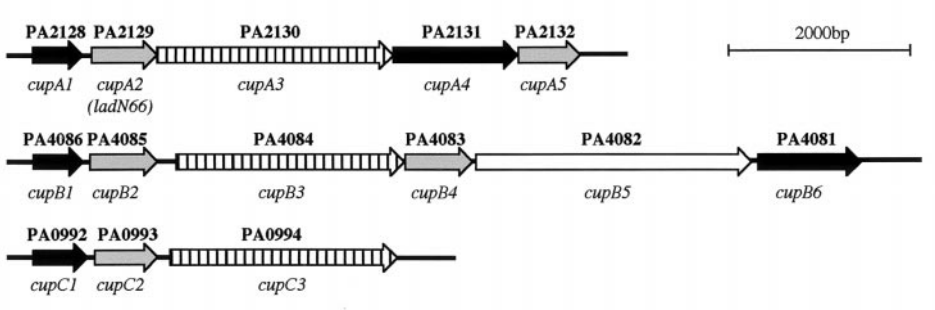

Fig. 4. Organization of the *cup* gene clusters. The three *cup* gene clusters have been represented to scale. Genes encoding fimbrial subunits are represented as black arrows, genes encoding periplasmic chaperones are represented as gray arrows, and genes encoding ushers are represented with striped arrows. The *cupB5* gene, encoding a protein similar to Fha, is represented in white. The numbers indicated above each gene correspond to the genome annotation (http://www.pseudomonas.com).

the same genetic organization as the *cupA* gene cluster (36). Moreover, each of the *yp* genes encoded components having precisely the same homologies as those found with the CupA proteins, including the unique sequence of one of the putative fimbrial subunit (Yp39). Interestingly, the *yp36–40* locus is located upstream of a high-pathogenicity island (HPI).

***cupA3* Is Involved in Biofilm Formation.** To demonstrate that the whole chaperone/usher pathway is involved in biofilm formation, and that the nonadherent phenotype is not exclusively associated with a defect in the CupA2 chaperone, we introduced a mutation in the gene encoding the usher component (*cupA3*). An internal DNA fragment of the *cupA3* gene was amplified by PCR and cloned into a nonreplicative plasmid in *P. aeruginosa,* and, after electroporation and selection in *P. aeruginosa* PAK-NP, a mutant strain, PAN67, was obtained. This strain, carrying a disrupted *cupA3* gene, showed an adhesion-deficient phenotype, with only 10% of the bacteria adhering relative to the parental PAK-NP (Fig. 5), which is even lower than the adherence of PAN66 (14%). Interestingly, when the same mutation was introduced into the wild-type PAK strain (PAN67B), the level of attachment was also drastically reduced, comparable to the level observed with PAN67, despite the presence of type IV pili at the surface of those cells (data not shown). This result confirmed that the CupA-dependent attachment is independent of the presence of type IV pili. It also showed that CupA-dependent adhesins are more important, or required at an earlier stage than type IV pili, for attachment in these particular conditions. The involvement in biofilm formation of pili assembled by the chaperone/usher pathway has already been described for other organisms (37). Pratt and Kolter (27) reported that *E. coli* type I pili are essential for initial attachment, and that the lack of those structures abolished the attachment to the surface, in contrast to flagella-defective strains.

The Fimbrial Gene Clusters *cupB* and *cupC*, and Their Role in Biofilm Formation. By analyzing the PAO1 genome, we could identify two additional gene clusters encoding a complete set of components belonging to the chaperone/usher pathway. The *cupB* gene cluster contains six genes (Fig. 4). CupB1 and CupB6 are homologous to fimbrial subunits. CupB5 (1,018 aa) is homologous to particular type of adhesive molecules, such as the filamentous hemagglutinin (Fha) from *Bordetella pertussis* (38). CupB2 and CupB4 are chaperone-like proteins with their highest homology to FimB/FhaD precursor from *B. pertussis*. Finally, CupB3 belongs to the usher family (Table 3).

The *cupC* gene cluster contains three genes encoding CupC1, CupC2, and CupC3 (Fig. 4). CupC1 is homologous to fimbrial subunits, CupC2 to chaperone-like proteins, and CupC3 to usher proteins (Table 3).

Table 3. Homologies between Cup components and components of known chaperone/usher pathways

Gene name	PA number*	Protein size, aa†	Predicted function	Nearest homolog‡	Identity, %/residues§
cupA1	2128	183	Fimbrial subunit	F17A/*E. coli*	37/28–160
cupA2	2129	248	Chaperone	F17a–D/*E. coli*	35/28–239
cupA3	2130	872	Usher	FocD/*E. coli*	40/25–861
cupA4	2131	454	Fimbrial subunit		29/19–230
cupA5	2132	237	Chaperone	FhaD/*B. pertussis*	34/17–225
cupB1	4086	189	Fimbrial subunit	F17A/*E. coli*	33/16–180
cupB2	4085	248	Chaperone	FhaD/*B. pertussis*	37/13–246
cupB3	4084	844	Usher	F17a–C/*E. coli*	32/19–817
cupB4	4083	246	Chaperone	FhaD/*B. pertussis*	41/31–235
cupB5	4082	1018	Adhesive protein	HxuA/*H. influenzae*	34/55–398
cupB6	4081	381	Fimbrial subunit	LpfE/*S. typhimurium*	25/245–380
cupC1	0992	205	Fimbrial subunit	Type 1/*K. pneumoniae*	28/26–152
cupC2	0993	237	Chaperone	EcpD/*E. coli*	37/16–227
cupC3	0994	839	Usher	FimD/*E. coli*	34/3–775

*The PA number corresponds to the genome annotation (http://www.pseudomonas.com).

†The protein size is indicated in number of amino acids (aa).

‡For CupA4 see text. HxuA is the heme–hemopexin utilization protein from *Haemophilus influenzae*. CupB5 is also highly homologous to HMW1 (28% identity), the high molecular weight surface protein from *H. influenzae* and Fha, the filamentous hemagglutinin from *Bordetella pertussis* (23% identity). *S., Salmonella; K, Klebsiella.*

§The percentage identity is assigned to the region of the protein including the indicated amino acid residues.

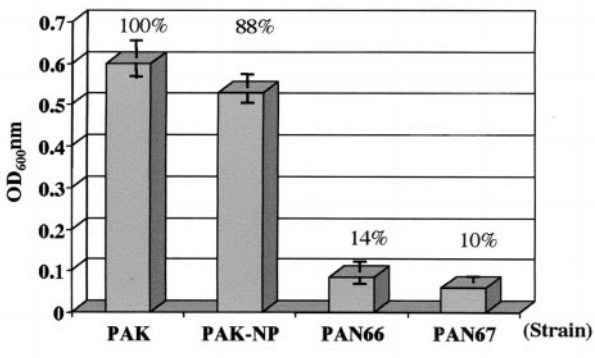

Fig. 5. Quantification of biofilm formation of the PAN66 and PAN67 strains. Bars represent the measure of OD_{600} as a result of four distinct experiments. The level of attachment of the mutant strains is indicated as a percentage of attachment of the wild-type PAK strain.

We subjected the genes of these two additional putative adhesion systems to mutagenesis by insertional interruption of the genes encoding the usher components, *cupB3* and *cupC3.* Internal DNA fragments were obtained by PCR, using MUB1/MUB2 primers for *cupB3* and MUT1/MUT2 primers for *cupC3*, and cloned into PCR2.1, yielding pCRB3 and pCRC3, respectively. Those plasmids were electroporated into the wild-type strain of *P. aeruginosa* PAK, and recombinant clones were selected on PIA plates containing carbenicillin. One mutant from each experiment was selected and named PAB3B and PAC3B, respectively. The position of the insertion was verified (see *Materials and Methods*) and the attachment phenotype was analyzed as previously described. In contrast to PAN67B, neither PAB3B nor PAC3B was altered in their ability to form a biofilm in microtiter dishes. The same observation was made when the mutations were introduced into the PAK-NP strain (PAB3 and PAC3). Thus, mutations within genes located in these two clusters did not affect the attachment capabilities of *P. aeruginosa.* Therefore, under the conditions of our assays, CupB and CupC systems do not participate in bacterial attachment to the polystyrene surface. Alternatively they may not be expressed, even in the wild-type *P. aeruginosa,* under the conditions that were used to grow the bacteria. It is worth noting that *E. coli* attachment and biofilm formation involve different surface appendages. Indeed, under growth conditions that were different from those promoting type I pili expression, curli filaments appear to predominate and play an essential role in early stages of adherence (39).

Conclusions

Bacterial cell-surface organelles, such as flagella and fimbrial structures, have a key role for attachment of microorganisms to surfaces. The identification of CupA2 as a chaperone of the chaperone/usher pathway brings a new insight into the *P. aeruginosa* strategies for attachment to surfaces. Indeed, assembly of fimbrial adhesins other than type IV pili has not previously been considered for this organism. Their function might not be redundant but synergic, specific for certain stages of attachment, or adapted to particular growth and environmental conditions. Interestingly, the *cupB* gene cluster encodes a protein with homology to filamentous hemagglutinin (Fha) from *B. pertussis*. This large protein, also known as fimbrial hemagglutinin, is clearly different from the pilin subunits but is also found attached to the surface of *B. pertussis* via the chaperone/usher pathway (38). Moreover, it was shown that Fha mediates attachment of *B. pertussis* to the upper respiratory tract (40). This observation also supports the idea that the three *cup* clusters identified here assemble different types of adhesins. These adhesins may provide *P. aeruginosa* with high adaptive capacities to colonize totally different surfaces. One may imagine that complex regulatory networks, that might involve two-component regulatory systems for sensing environmental stimuli, are participating in the differential expression of all these adhesive structures. In addition to elucidating the structure and the precise function of the CupA, CupB, and CupC-associated adhesins, full realization of the complexity and the specificity of the adherence mechanisms used by *P. aeruginosa* requires dissection of these networks controlling their expression. Infections with *P. aeruginosa* are a major burden for human health, and the understanding of the complete array of adhesive mechanisms used by the bacterium to colonize tissues as well as abiotic surface such as those of catheters or other medical devices is fundamental to understand the infection process.

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