

Role of Flagella in Host Cell Invasion by *Burkholderia cepacia*

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***Burkholderia cepacia* is an important opportunistic human pathogen that affects immunocompromised individuals, particularly cystic fibrosis (CF) patients. Colonization of the lungs of a CF patient by *B. cepacia* can lead not only to a decline in respiratory function but also to an acute systemic infection, such as bacteremia. We have previously demonstrated that a CF clinical isolate of *B. cepacia*, strain J2315, can invade and survive within cultured respiratory epithelial cells. In order to further characterize the mechanisms of invasion of *B. cepacia*, we screened a transposon-generated mutant library of strain J2315 for mutants defective in invasion of A549 respiratory epithelial cells. Here we describe isolation and characterization of a nonmotile mutant of *B. cepacia* with reduced invasiveness due to disruption of *fliG*, which encodes a component of the motor-switch complex of the flagellar basal body. We also found that a defined null mutation in *fliI*, a gene encoding a highly conserved ATPase required for protein translocation via the flagellar type III secretion system, also resulted in loss of motility and a significant reduction in invasion. Both mutants lacked detectable intracellular flagellin and failed to export detectable amounts of flagellin into culture supernatants, suggesting that disruption of *fliG* and *fliI* impaired flagellar biogenesis. The reduction in invasion did not appear to be due to defective adherence of the flagellar mutants to A549 cells, suggesting that functional flagella and motility are required for full invasiveness of *B. cepacia*. Our findings indicate that flagellum-mediated motility may facilitate penetration of host epithelial barriers by *B. cepacia*, contributing to establishment of infection and systemic spread of the organism.**

Over the last several decades *Burkholderia cepacia* has emerged as an important opportunistic human pathogen of the lower respiratory tract that affects immunocompromised individuals, particularly cystic fibrosis (CF) patients (18). Chronic colonization of the lungs of a CF patient by *B. cepacia* can lead not only to a decline in respiratory function, due to the onset of a necrotizing pneumonia, but also to an acute systemic infection, such as bacteremia or septicemia (11, 46). In addition to being an invasive pathogen that is capable of entering deeper tissues and becoming blood borne, *B. cepacia* can survive in epithelial cells and macrophages (4, 35, 42, 48), which may contribute to the persistence of the organism in the host. The rapid clinical decline due to *B. cepacia* colonization is known as *cepacia* syndrome, and this decline leads to mortality in approximately 20 to 35% of chronically colonized individuals (25, 47). Furthermore, colonization by *B. cepacia* reduces the life expectancy of a CF patient by 50%, from 30 to 15 years (24). The inherent resistance of *B. cepacia* to multiple antibiotics makes eradication of this pathogen from the lungs of infected individuals especially difficult. Despite the known association of *B. cepacia* with fatal pulmonary infections in CF patients, the pathogenic mechanisms and virulence determinants responsible for *B. cepacia* infection are only beginning to be elucidated (38).

B. cepacia currently encompasses at least six genotypically distinguishable genomovars (genomovars I to VI), which are collectively known as the *B. cepacia* complex (7, 50). Recently, genomovars II, IV, and V have been reclassified into distinct

species (*Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia vietnamiensis*, respectively) (50, 51). While strains representing all six genomovars of *B. cepacia* have been associated with opportunistic infections in humans, strains belonging to genomovar III have been most commonly linked to epidemic outbreaks and fatal infections in CF patients (50, 51).

B. cepacia is a motile organism, and motility is mediated by polar flagella (3, 21). Little is known about the structure and function of *B. cepacia* flagella, although attempts to type strains based on the elaborated flagellin protein have been made. Two major flagellin types were detected in a survey of *B. cepacia* strains; these types were distinguished on the basis of molecular mass (45 and 55 kDa) (20). The corresponding flagellar genes, designated *fliC*, encode proteins that exhibit high levels of homology to the flagellin of *Burkholderia pseudomallei*, another closely related human pathogen (9).

In *Escherichia coli*, as well as in other bacteria, expression of functional flagella requires more than 40 structural and regulatory genes, and biogenesis proceeds via a complex, hierarchical pathway (33). The extracellular components of the flagellum (namely, the hook and filament) are extended by addition of new structural subunits at the distal end of the growing organelle. The flagellar structural proteins are translocated from the cytoplasm into the growing structure via a highly conserved secretion system, designated the type III system (32). Components of the flagellar type III system exhibit high levels of homology to the components of type III secretion systems utilized by a number of bacterial pathogens to secrete virulence determinants into host cells (23).

It has been demonstrated previously that flagella and flagellum-mediated motility contribute to the virulence of a number of pathogenic bacterial species. Mutations in flagellar biogenesis genes have been shown to attenuate the virulence of sev-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>supE44 lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories
S17-1	Integrated RP4-2, Tc::Mu Km::Tn7	44
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAB lacI</i> ^q Δ M15 Tn10)	Stratagene
<i>B. cepacia</i> strains		
J2315	CF clinical isolate, genomovar III	17
D9	<i>fliG</i> ::mini-Tn5Tc derivative of J2315	This study
CM58	<i>fliI</i> :: <i>cat</i> derivative of J2315	This study
CM100	CM58 with plasmid pJG7	This study
CM340	D9 with plasmid pMT46	This study
Plasmids		
pBluescript SK(-)	Cloning and single-stranded phagemid	Stratagene
pBluescript SK(+)	Cloning and single-stranded phagemid	Stratagene
pGEM-T Easy	TA cloning vector	Promega
pLAFR-5	Cosmid vector, Tc ^r	27
pCAT1	Source of <i>cat</i> cassette, Cm ^r	This study
pMR4	Broad-host-range vector, Tc ^r	C. Mohr and R. Roberts
pUCP18	Broad-host-range vector, Ap ^r	52
pCMT1	Cm ^r derivative of pUCP18	This study
pCM117	pLAFR-5 cosmid containing 20-kb <i>B. cepacia</i> genomic DNA insert encoding the <i>fliG</i> locus	This study
pAR1	3.5-kb <i>XhoI</i> fragment from pCM117 subcloned into pBluescript SK(+)	This study
pAR2	1.3-kb <i>PstI</i> fragment containing <i>fliI</i> , subcloned from pAR1 into pBluescript SK(+)	This study
pAR3	1.3-kb <i>XhoI-PstI</i> fragment from pAR2 subcloned into pBluescript SK(+)	This study
pAR4	pAR3 containing <i>fliI</i> gene inactivated with <i>cat</i> cassette, Cm ^r /Ap ^r	This study
pJG7	1.6-kb <i>PstI</i> fragment encoding <i>fliI</i> truncated at codon 476, cloned in pMR4	This study
pMT38	1.5-kb PCR product encoding <i>fliG</i> cloned into pGEM-T Easy	This study
pMT46	1.5-kb <i>fliG</i> PCR product cloned into pCMT1	This study

eral human pathogens, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Campylobacter jejuni*, and *Helicobacter pylori* (12, 14, 37, 39, 40). In some bacteria, components of the flagellum, including the mucin-binding flagellar cap protein of *P. aeruginosa* (1) and flagellin proteins of *C. jejuni* (55), may act as adhesins and implicate flagella in facilitating colonization through direct interactions with host ligands. The adhesive properties of some flagella, however, do not fully account for the observed reduction in virulence of flagellar mutants, and active motility is often required for full pathogenesis. This hypothesis is supported by studies which showed that flagellar motility can enhance invasion of host cells by *C. jejuni*, *P. mirabilis*, *Vibrio anguillarum*, and other pathogenic species (37, 41, 55).

Several laboratories have demonstrated that *B. cepacia* is capable of invading and surviving within cultured respiratory epithelial cells (4, 28, 35, 48). *B. cepacia* invasion has also been observed in vivo in a murine model of infection (6). Together, these findings suggest that invasion may be an important virulence factor in the pathogenesis of *B. cepacia*. Invasion may be a mechanism by which *B. cepacia* breaches the epithelial barrier to enter deeper tissues and ultimately becomes blood borne, which results in systemic infection. We have previously shown that *B. cepacia* strain J2315, a clinical isolate associated with epidemic outbreaks and mortality in CF patients, can invade cultured human respiratory epithelial cells (35). In order to further characterize the mechanisms employed by *B. cepacia* to invade respiratory epithelial cells, we screened a transposon-generated mutant library of *B. cepacia* strain J2315 for mutants with reduced ability to enter A549 respiratory epithelial cells. Here we describe the isolation and character-

ization of a nonmotile mutant of *B. cepacia* defective in invasion due to disruption of *fliG*, which encodes a component of the motor-switch complex of the flagellar basal body. Additionally, we found that a defined null mutation in *fliI*, a gene encoding a highly conserved ATPase required for protein translocation via the flagellar type III secretion system, results in a loss of motility, which is coupled with a significant reduction in invasion of A549 respiratory epithelial cells. Our findings indicate that flagellum-mediated motility may play a role in the pathogenesis of *B. cepacia* by facilitating penetration of the host epithelial cell barriers and contributing to the onset of systemic spread of the organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown with aeration at 37°C in Luria-Bertani (LB) broth or on LB agar plates supplemented with ampicillin (100 μ g/ml), tetracycline (12 μ g/ml), or chloramphenicol (30 μ g/ml) as necessary. *B. cepacia* strain J2315 is a CF clinical isolate belonging to *B. cepacia* genomovar III (17). Strain J2315 was grown with aeration at 37°C in LB broth or peptone-yeast extract (PYE) (13) supplemented with tetracycline (50 μ g/ml) or chloramphenicol (30 μ g/ml) or on LB agar plates supplemented with tetracycline (500 μ g/ml), kanamycin (50 μ g/ml), or chloramphenicol (300 μ g/ml) as necessary. For quantitative invasion and adherence assays *B. cepacia* was grown in PYE.

Transposon mutagenesis. The mini-Tn5Tc transposon (8, 22) was introduced into *B. cepacia* strain J2315 by conjugation using *E. coli* donor strain S17-1 (44). For mating, 250- μ l portions of an *E. coli* overnight culture and 500- μ l portions of a *B. cepacia* overnight culture were mixed on sterile nitrocellulose filters. Following incubation of the filters on LB agar for 4 to 5 h at 37°C, the bacteria were resuspended in LB broth and plated on LB agar containing 300 μ g of tetracycline per ml and 50 μ g of kanamycin per ml to select against wild-type *B. cepacia* and the *E. coli* donor strain, respectively. Southern blot hybridization

analysis of a subset of individual transposon-generated mutants indicated that the mini-Tn5Tc transposon inserted randomly into the *B. cepacia* chromosome.

Screening for invasion-defective mutants. Individual transposon-generated mutants of *B. cepacia* were grown overnight in 200- μ l portions of LB broth in 96-well microtiter plates, subcultured into new microtiter plates, and grown to the mid-exponential phase (A_{630} , ~0.4). Aliquots (20 μ l) were used to infect confluent monolayers of A549 respiratory epithelial cells. The A549 cell line (American Type Culture Collection, Manassas, Va.) is a human alveolar epithelial carcinoma cell line. The A549 cells were seeded 24 h prior to infection in 96-well microtiter plates at a level of 8.8×10^4 cells per well. The bacteria were centrifuged onto the monolayers (165 \times g for 5 min) and incubated at 37°C in an atmosphere containing 5% CO₂ for 30 min to allow bacterial entry. Killing extracellular bacteria and quantification of intracellular bacteria were performed as previously described (35). Approximately 4,000 transposon-generated mutants were screened. The phenotypes of invasion-defective mutants identified in the primary invasion screening analysis were confirmed by quantitative invasion assays.

Quantitative invasion assays. Invasion assays were performed essentially as described previously (35), with the following modifications. Bacterial strains were grown to the exponential phase in PYE, and where indicated below, the centrifugation step was omitted. The bacterial strains were allowed to invade for 2 h. All quantitative invasion assays were performed in triplicate wells, with two samplings per well.

Cosmid library construction. Cosmid vector pLAFR-5 (27) was used to construct a cosmid library of *B. cepacia* strain J2315. Genomic DNA was extracted from strain J2315 with a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.) used according to the manufacturer's instructions. Approximately 3 μ g of J2315 DNA was partially digested with *Sau*3A, and 20- to 40-kb fragments were eluted and combined with 1.5 μ g of pLAFR-5 linearized with *Bam*HI and *Sca*I in a 22- μ l ligation mixture. Following overnight incubation at 14°C, 4 μ l of the ligation mixture was packaged into bacteriophage lambda by using Gigapack III Plus packaging extract (Stratagene, La Jolla, Calif.). The resulting phage extract was transfected into *E. coli* XL-1 Blue. Approximately 2,100 cosmids (average insert size, ~28 kb) were picked into 96-well plates, grown overnight, and replica plated onto LB agar. After overnight growth, the bacterial grids were replica plated onto nylon membranes (Hybond N; Amersham Pharmacia Biotech, Piscataway, N.J.). Dimethyl sulfoxide (10%) was added to each well of the 96-well plates, and the plates were stored at -70°C.

Nucleotide sequencing. Nucleotide sequencing was performed at the Advanced Genetic Analysis Center at the University of Minnesota, using the dideoxy chain termination method and an ABI 1371A DNA sequencer (Applied Biosystems, Foster City, Calif.). The oligonucleotide primers used for sequencing were standard forward and reverse (T3 and T7) pBluescript primers or custom oligonucleotides synthesized by Integrated DNA Technologies (Coralville, Iowa). The nucleotide sequences of both strands were determined. Double-stranded sequences were aligned and assembled by using the EditSeq and SeqMan components of a demonstration version of the Lasergene sequence analysis software package (DNASTAR, Inc., Madison, Wis.). For nucleotide and amino acid sequence searches and analysis we utilized the BLASTX and BLASTP programs of the National Center for Biotechnology Information.

DNA manipulations. DNA-modifying enzymes, including restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase, were obtained from Roche (Indianapolis, Ind.), New England Biolabs (Beverly, Mass.), and Gibco BRL (Rockville, Md.). Plasmid DNA was isolated by the boiling lysis method (43) or with a QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, Calif.). Recombinant plasmids were introduced into *E. coli* and *B. cepacia* strain J2315 by electroporation with a Gene Pulser II (Bio-Rad, Richmond, Calif.), as previously described by Burns and Hedin (5). Southern blot and colony hybridization analyses were generally performed as described by Sambrook et al. (43) using Hybond N nitrocellulose membranes, probes labeled with [α -³²P]dCTP (Amersham Pharmacia Biotech), and the random primer method.

To clone the DNA flanking the site of the mini-Tn5Tc insertion in mutant D9, genomic DNA was digested with a series of restriction enzymes and examined by Southern blot hybridization analysis in which probes internal to the mini-Tn5Tc transposon were used. A 4-kb *Pst*I cross-hybridizing fragment encoding mini-Tn5Tc and flanking DNA was identified and cloned by generating a subgenomic library of *Pst*I fragments ranging from 3 to 6 kb long in pBluescript SK(+) and plating the library on LB agar containing 12 μ g of tetracycline per ml to select for mini-Tn5Tc-encoded tetracycline resistance.

For complementation of *B. cepacia* mutant D9, the *fliG* gene was amplified by performing PCR with oligonucleotide primers *fli*18 (5'-CAAGGCGGCGGAG GAAC-3') and *fli*19 (5'-GATGCGAGATCGTGTTCG-3'), a PCR Sprint thermocycler (Hybaid, Franklin, Mass.), and *Taq* DNA polymerase (Promega, Mad-

ison, Wis.). The PCR was carried out for 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, concluding with an additional 5 min of extension at 72°C after the 35 cycles. The 1.5-kb *fliG* PCR product, containing the 3' end of *fliF*, *fliG*, and the 5' end of *fliH*, was cloned into the pGEM-T Easy (Promega) TA cloning vector (pMT38) and subsequently excised as an *Eco*RI fragment and cloned into the pCMT1 broad-host-range vector to generate pMT46. Plasmid pMT46 was introduced into D9 by electroporation, which resulted in strain CM340.

B. cepacia J2315 *fliI* mutant strain (CM58) was generated by allelic exchange mutagenesis. Briefly, the chloramphenicol resistance cassette (*cat*) encoded in plasmid pCAT1 was excised as an *Eco*RI fragment and inserted into the unique *Eco*RI site located in the *fliI* coding sequence on plasmid pAR3. The resulting construct, designated pAR4, was introduced into strain J2315 by electroporation, and the transformants were plated on LB agar containing 300 μ g of chloramphenicol per ml. Chloramphenicol-resistant colonies were stabbed into swarm agar, and mutants whose motility was impaired were examined by Southern blot hybridization analysis to confirm that the *cat* cassette had integrated into the *fliI* gene and that a double-crossover event had occurred. For complementation of CM58, a 1.6-kb *Pst*I fragment encoding amino acids 1 to 475 of the *fliI* gene product was cloned into the pMR4 broad-host-range vector, generating pJG7. The pJG7 plasmid was introduced into CM58 by filter mating, which resulted in strain CM100.

Motility assays. For motility assays LB agar swarm plates containing 0.25% (wt/vol) agar were stab inoculated with overnight cultures of *B. cepacia* and incubated for 24 h at 37°C.

Immunoblot analysis. Cytoplasmic and supernatant protein fractions were prepared from exponential-phase cultures as previously described (45). Briefly, *B. cepacia* strains were grown to the mid-exponential phase in 50 ml of PYE. For whole-cell protein analysis, 1 ml of each culture was harvested by centrifugation, and the bacterial pellets were resuspended in Laemmli buffer (30). Protein samples were boiled, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting. For supernatant analysis, 25-ml portions of mid-exponential-phase cultures were harvested, and the supernatants were filtered through a 0.45- μ m-pore-size filter. Each supernatant protein fraction was precipitated overnight at 4°C by adding ammonium sulfate to a concentration of 50% (wt/vol). The precipitated protein samples were centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 30 min, and the pellets were washed with 70% ethanol, resuspended in Laemmli buffer, boiled, and analyzed by immunoblotting.

Immunoblotting on SDS-polyacrylamide gels was performed as described by Jenal et al. (26). The flagellin-specific rabbit polyclonal antiserum used was a generous gift from D. Woods and was raised against purified *B. pseudomallei* flagellin (2). Blots were probed with the primary flagellin-specific antiserum at a dilution of 1:10,000 and then with secondary antibody (goat anti-rabbit immunoglobulin G) at a 1:2,500 dilution. Western blots were developed with a Renaissance chemiluminescence kit (DuPont NEN, Boston, Mass.) used according to the manufacturer's instructions. The protein standards used were prestained SDS-PAGE low-range standards (Bio-Rad).

Adherence assays. Mid-exponential-phase bacteria were added to confluent A549 monolayers at a multiplicity of infection of 10:1. The bacteria were centrifuged onto the monolayers (165 \times g for 5 min) and incubated at 37°C in the presence of 5% CO₂ for 1 h to allow bacterial adherence. The monolayers were washed five times with phosphate-buffered saline to remove nonadherent bacteria. The adherent bacteria were enumerated by lysing A549 cells with 0.25% Triton X-100 and determining the viable cell counts on LB agar. While this assay does not allow discrimination between adherent and intracellular bacteria, under these conditions the subpopulation of internalized bacteria accounts for approximately 5% of the total number of bacteria recovered from the assay (data not shown). Therefore, the vast majority of the CFU recovered in this assay represent adherent, noninternalized bacteria. All assays were performed in triplicate wells, with two samplings per well.

Statistical analysis. The statistical significance of the observed differences in mean invasion frequencies was determined by calculating the *P* values using the two-tailed Student *t* test for unpaired data sets.

Nucleotide sequence accession number. The nucleotide sequence of the *fliG* locus has been deposited in the GenBank database under accession no. AF453480.

RESULTS

Identification of a *B. cepacia* mutant defective in invasion.

We have previously shown that a CF clinical isolate of *B. cepacia*, strain J2315, is able to invade cultured A549 respira-

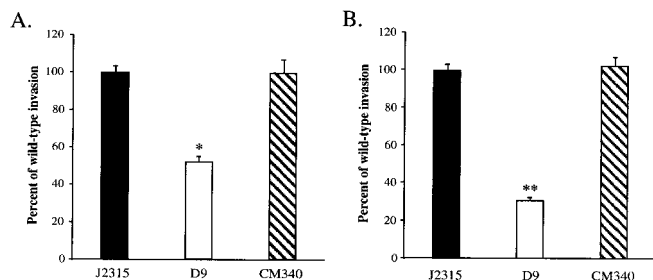


FIG. 1. Invasion of A549 cell monolayers by *B. cepacia* strain J2315 (wild type), mutant D9, and D9 complemented with *fliG* (strain CM340). (A) Invasion of A549 cell monolayers with centrifugation. Quantitative invasion assays were performed in triplicate as described in Materials and Methods. The invasion values were calculated by determining the percentages of the bacterial inocula that survived after 2 h of antibiotic treatment. The values were normalized to the value for wild-type strain J2315, which was arbitrarily set at 100%. The actual invasion frequency for strain J2315 was $0.67\% \pm 0.03\%$. The asterisk indicates that the level of invasion by mutant D9 was significantly less than the level of invasion by the parent strain ($P < 0.000002$). (B) Invasion of A549 cell monolayers without centrifugation. The actual invasion frequency for strain J2315 without centrifugation was $0.31\% \pm 0.01\%$. The double asterisks indicate that the P value was < 0.000002 .

tory epithelial cells (35). In order to identify the genetic elements required for invasion, we developed a method to screen large numbers of transposon-generated mutants of *B. cepacia* strain J2315 for defects in the ability to invade A549 cell monolayers. Initially, we screened approximately 4,000 mutants, and the ability of one of these mutants, designated mutant D9, to enter A549 cells appeared to be reduced. In order to confirm the invasion defect, mutant D9 and wild-type *B. cepacia* strain J2315 were compared using quantitative invasion assays. The mutant D9 was consistently reduced in ability to invade A549 respiratory epithelial cells, with approximately 45% reduced cell invasion compared to the wild-type strain J2315 (Fig. 1A). These findings suggested that the transposon insertion in mutant D9 disrupted functions required for optimal invasion by *B. cepacia*.

Characterization of invasion-defective mutant D9. Southern blot hybridization analysis in which mini-Tn5Tc DNA was used as a probe indicated that there was a single transposon insertion in invasion-defective mutant D9 (data not shown). In order to identify the transposon insertion site, DNA flanking the mini-Tn5Tc transposon was cloned and sequenced. Sequence analysis revealed that the site of Tn5 insertion in D9 disrupted an open reading frame homologous to the *fliG* gene family encoding components of the flagellar basal body (Fig. 2). The transposon insertion site was determined to be between codons 203 and 204 of the *B. cepacia fliG* gene. The *B. cepacia fliG* homolog encodes a putative polypeptide consisting of 331 amino acids, which is 57% identical and 76% similar to FliG of *Salmonella enterica* serovar Typhimurium (Fig. 2).

In order to further delimit the extent of the *fliG* locus, a strain J2315 cosmid library was constructed and screened for clones encoding *fliG*. Subclones from a *fliG* cross-hybridizing cosmid were generated and sequenced. Additional genes with high levels of homology to known flagellar genes were identified. The 6.5-kb flagellar locus of *B. cepacia*, shown in Fig. 2,

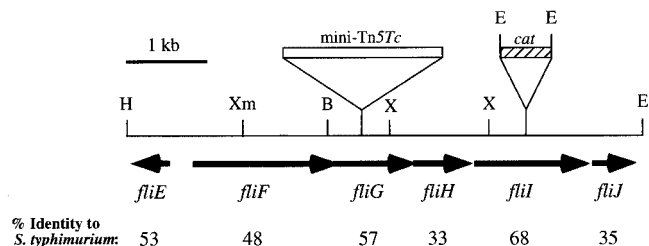


FIG. 2. Physical map of the *B. cepacia fli* locus. The open box indicates the site of mini-Tn5Tc insertion in *fliG* mutant D9, and the cross-hatched box indicates the site of insertion of the *cat* cassette in *fliI* null strain CM58. The arrows indicate the directions of transcription. The numbers below the open reading frames indicate the levels of amino acid identity to the corresponding flagellar homologs in *S. enterica* serovar Typhimurium. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xho*I; Xm, *Xma*I.

encodes at least six genes, including *fliFGHIJ* and *fliE* in divergent orientations. All of these genes encode homologs of early structural and regulatory components of the flagellar assembly pathway in *E. coli* and *Salmonella* spp. (Fig. 2). The FliF, FliG, FliH, and FliI proteins of *S. enterica* serovar Typhimurium are components of a flagellum-specific type III secretion system that is utilized for export and assembly of flagellar protein substrates (23). The *fliE* gene encodes a structural component of the flagellar basal body (36), while it has been proposed that the *fliJ* gene product functions as a molecular chaperone, facilitating flagellar protein export and assembly (45). The genetic organization of the *B. cepacia fliE-fliJ* locus identified thus far is identical to the gene arrangement in the corresponding loci of *E. coli* and *S. enterica* serovar Typhimurium. The *B. cepacia fli* gene products exhibit high levels of amino acid identity to the *S. enterica* serovar Typhimurium flagellar proteins (range, 33 to 68%) (Fig. 2), suggesting that the *B. cepacia fli* locus is also involved in flagellar biogenesis.

Analysis of the FliG protein in *S. enterica* serovar Typhimurium has shown that FliG is required for flagellar morphogenesis and plays a central role in mediating interactions within the motor-switch complex of the flagellar basal body (54). Insertional inactivation of *fliG* in *S. enterica* serovar Typhimurium renders cells nonmotile and results in a block in flagellar assembly (54). In order to examine the effect of disruption of the *B. cepacia fliG* gene on motility, we analyzed the D9 mutant by performing swarm agar motility assays. As shown in Fig. 3A, the D9 mutant was nonmotile in swarm agar. Additionally, the nonmotile phenotype of mutant D9 was confirmed by light microscopy. There was no significant difference in growth kinetics between wild-type strain J2315 and the D9 mutant (data not shown), indicating that the observed motility and invasion defects were not due to delayed growth of D9. Motility was restored to mutant D9 by providing a wild-type copy of the *B. cepacia fliG* gene in *trans* on plasmid pMT46 (Fig. 3A). This construct also complemented the invasion defect of the D9 mutant (Fig. 1). The plasmid vector alone did not restore motility or wild-type invasion levels to the *fliG* null strain. Successful complementation of both motility and invasion functions with *fliG* alone suggests that the observed invasion defect in D9 is due to its nonmotile phenotype and that the *B. cepacia fliG* gene is essential for flagellar biogenesis.

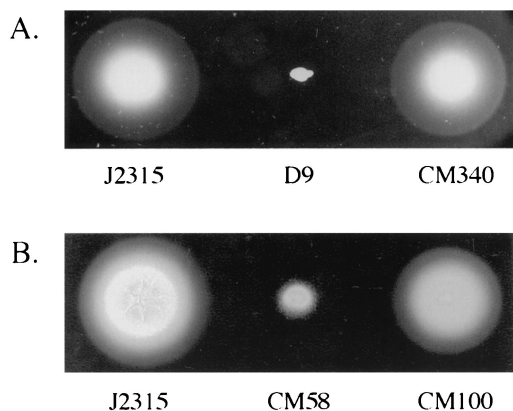


FIG. 3. Phenotypes of *B. cepacia* mutants with impaired motility. Strains were stabbed into semisolid LB medium plates (0.25% agar) and incubated at 37°C for 24 h. (A) Wild-type strain J2315, mutant D9 (*fliG::mini-Tn5Tc*), and D9 complemented with *fliG* (strain CM340). (B) Wild-type strain J2315, mutant CM58 (*fliI::cat*), and CM58 complemented with *fliI* (strain CM100).

To further define the role of the *B. cepacia fliG* gene in flagellar biogenesis, whole-cell and supernatant protein fractions of *fliG* mutant D9 were prepared and examined by immunoblot analysis for the presence of flagellin protein. Since antibodies to the *B. cepacia* flagellin protein were not available, we utilized a polyclonal antiserum to the flagellin protein of *B. pseudomallei*, whose amino acid sequence is 77% identical to the amino acid sequence of the 45-kDa flagellin protein of *B. cepacia* (20). Antibody to *B. pseudomallei* flagellin recognized a 45-kDa protein in both the whole-cell and supernatant fractions of wild-type *B. cepacia* strain J2315 (Fig. 4). In contrast, the levels of the 45-kDa protein were dramatically reduced in both the whole-cell and supernatant fractions of *fliG* mutant D9 (Fig. 4A). The presence of the 45-kDa protein was restored in both the whole-cell and supernatant fractions of the D9 mutant by complementation with the *fliG* gene on plasmid

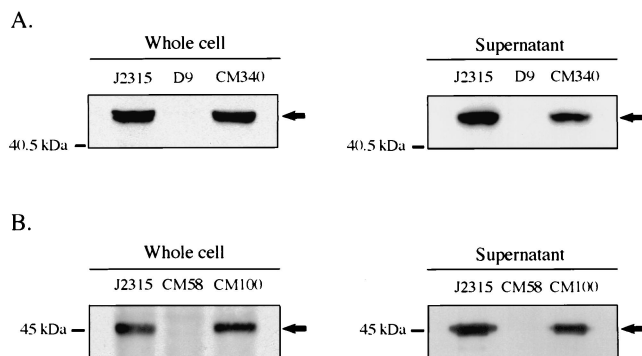


FIG. 4. Immunoblot analysis of flagellin protein in wild-type and flagellar mutant strains. Equal amounts of protein from whole-cell or supernatant fractions were separated on SDS-12.5% PAGE gels and immunoblotted with anti-flagellin antibody as described in Materials and Methods. (A) Wild-type strain J2315, D9 (*fliG::mini-Tn5Tc*), and CM340 (D9 complemented with *fliG*). (B) Wild-type strain J2315, CM58 (*fliI::cat*), and CM100 (CM58 complemented with *fliI*). The molecular masses of the protein standards are indicated on the left. The position of the 45-kDa flagellin protein band is indicated by an arrow.

pMT46 (strain CM340). These data suggest that the nonmotile phenotype of the D9 mutant is probably due to impaired formation of the flagellum structure.

In standard invasion assays, bacteria are typically brought into contact with host cell monolayers by centrifugation. We reasoned that centrifugation may bypass the role of motility in the initial stages of host cell invasion and that in the absence of centrifugation the *fliG* mutant strain should display a more pronounced invasion defect. When the D9 mutant and the wild-type strain were compared in quantitative invasion assays in the absence of centrifugation, the mutant was approximately 75% less invasive than the wild-type strain (Fig. 1B). Invasion was restored to wild-type levels by complementation of mutant D9 with plasmid pMT46. Therefore, flagellar biogenesis appeared to play an important role in the establishment of contact between *B. cepacia* and the A549 respiratory epithelial cell monolayers.

Generation and characterization of a *B. cepacia fliI* null strain. In order to further characterize the role of flagellar biogenesis in *B. cepacia* host cell invasion, a defined null mutation in the *fliI* gene was generated (Fig. 2). The *fliI* gene product is a member of a family of ATP-binding proteins believed to provide energy for the export of flagellar protein substrates, as well as nonflagellar virulence factors, in a wide range of bacterial species. Members of this protein family are central components of bacterial type III secretion systems (23). The *B. cepacia* FliI amino acid sequence is 68% identical to the FliI amino acid sequence of *S. enterica* serovar Typhimurium and 43% identical to the amino acid sequence of YscN, the ATPase implicated in virulence protein secretion via the *Yersinia* spp. type III system (53). Insertional inactivation of the *B. cepacia fliI* gene impaired the motility of cells (Fig. 3B), and flagellin protein could not be detected in either the whole-cell or supernatant fractions of the *fliI* null strain, CM58 (Fig. 4B). Compared to the ability of wild-type *B. cepacia* strain J2315, the ability of CM58 to enter A549 respiratory epithelial cells was significantly reduced; invasion was reduced approximately 30 and 70% when the bacteria were centrifuged and not centrifuged onto A549 epithelial cell monolayers, respectively (Fig. 5). Motility, flagellin protein, and wild-type invasion levels were restored by *trans* complementation with plasmid pJG7 (strain CM100). None of these phenotypes was restored by the plasmid vector alone. The pJG7 plasmid encodes amino acids 1 to 475 of FliI, and complementation of all three phenotypes by this plasmid suggests that the C-terminal 26 amino acids of FliI are not essential for its function. The successful complementation of mutant CM58 with plasmid pJG7 indicates that impaired motility, loss of flagellin protein, and reduced invasion were due to disruption of *fliI* and not to polar effects (Fig. 3B, 4B, and 5).

Adherence to A549 respiratory epithelial cells. There is evidence that components of bacterial flagella can act as adhesins and mediate binding to host cells and mucosal surfaces (1, 55). In order to determine if the observed invasion defect of our flagellar mutants was due to reduced binding to A549 respiratory epithelial cells, we compared the adherence of these mutants to that of *B. cepacia* wild-type strain J2315. Bacteria were centrifuged onto A549 monolayers, and following incubation, the monolayers were washed repeatedly to remove nonadherent bacteria. Enumeration of the remaining adherent bacteria

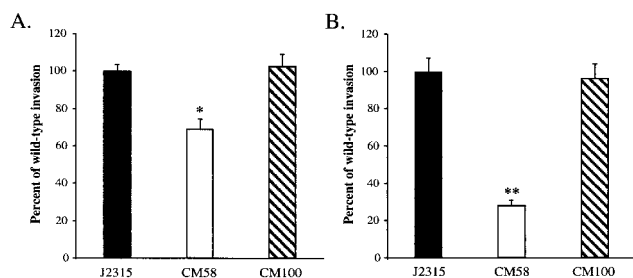


FIG. 5. Invasion of A549 cell monolayers by *B. cepacia* strain J2315 (wild type), mutant CM58 (*fliI::cat*), and CM100 (CM58 complemented with *fliI*) with (A) or without (B) centrifugation. The invasion values were calculated by determining the percentages of the bacterial inocula that survived after 2 h of antibiotic treatment and were normalized to the value for wild-type strain J2315, which was arbitrarily set at 100%. The actual invasion frequencies for strain J2315 with and without centrifugation were $0.67\% \pm 0.03\%$ and $0.26\% \pm 0.02\%$, respectively. The asterisk indicates that the *P* value was <0.0007 , and the double asterisks indicate that the *P* value was <0.00004 .

revealed a slight, but not statistically significant, increase in adherence of the flagellar mutants D9 and CM58 compared to the adherence of parent strain J2315 (Table 2). These results suggest that *B. cepacia* flagella do not function as adhesins in direct binding to A549 cells. Rather, the observed decreases in invasion of the *fliG* and *fliI* mutants are probably due to their impaired motility.

DISCUSSION

Motility has been shown to play an important role in the invasiveness of a number of bacterial pathogens. Active motility is required for optimal invasion of epithelial cells and translocation across polarized epithelial monolayers by *C. jejuni* (19, 55). The ability of a *P. mirabilis* nonmotile mutant to invade cultured human renal epithelial cells has been shown to be significantly reduced, even when the bacteria were centrifuged onto renal cell monolayers (37). Nonmotile mutants of *S. enterica* serovar Typhimurium have been shown to be less invasive in HeLa cells (29), while aflagellate mutants of *S. enterica* serovar Enteritidis are both less invasive in cultured intestinal epithelial cells (10, 49) and less adherent to these cells (10). In *S. enterica* serovar Typhimurium, mutations in flagellar regulatory components have also been shown to affect the expression of other genes required for invasion (31). *P. aeruginosa* nonmotile mutants have recently been shown to be defective for invasion of rabbit corneal epithelial cells, and similar to the findings reported here, centrifugation did not restore invasion to wild-type levels (15).

Our data are consistent with the results of previously de-

TABLE 2. Adherence of *B. cepacia* strain J2315 and isogenic nonmotile mutants to A549 respiratory epithelial cells

Strain	Genotype	% Adherence (mean \pm SE)
J2315	Wild type	3.7 \pm 0.4
D9	<i>fliG</i>	4.4 \pm 0.3
CM58	<i>fliI</i>	4.1 \pm 0.4

scribed studies in which it was found that flagellum-mediated motility contributes to the ability of bacteria to invade host cells. The abilities of both of the *B. cepacia* flagellar mutants generated in this study to invade were reduced even when the bacteria were brought in close contact with A549 cells by centrifugation, thus bypassing the role that motility plays in the initial establishment of contact between *B. cepacia* and host cells. The reductions in the ability to invade do not appear to be due to defective adherence of the motility mutants to A549 respiratory epithelial cells, although we cannot exclude the possibility that *B. cepacia* flagella may interact with other types of host cells and/or acellular ligands. Since more than 40 genes are required for flagellar biogenesis in other bacteria, it was somewhat surprising that additional motility mutants were not isolated from the invasion screening analysis. This may have been due to a lack of complete randomness in the targets for mini-Tn5 transposition within the *B. cepacia* chromosome. Alternatively, some motility mutants may not have been detected due to limited sensitivity of the invasion screening procedure. While the abilities of both the *fliG* and *fliI* mutants to invade were impaired, the invasion defect of the *fliI* mutant was less pronounced than that of the *fliG* mutant when the bacteria were centrifuged onto the A549 cell monolayers (Fig. 1A and 5A). After prolonged incubation of swarm agar plates inoculated with the *fliI* mutant, we observed a small but detectable swarm, which we did not observe on swarm agar plates inoculated with the *fliG* mutant. Motility in the absence of a functional *fliI* gene has been reported in other bacteria (16), although the genetic basis is not known. It is possible that the *B. cepacia fliI* mutant retains a low level of FliI activity. Alternatively, there is a second type III secretion homolog of *fliI*, which can at least partially compensate for the loss of the *fliI* gene.

Our results indicate that flagellum-mediated motility may be required for optimal invasion of A549 cells during two distinct phases: establishment of contact with the host cell and bacterial entry once contact has been established. The motility of *B. cepacia* may be particularly important for initiation of contact between the bacteria and epithelial cells and may promote adherence to the airway epithelia of the lung and subsequently invasion of deeper tissues. Additionally, motility may allow *B. cepacia* to penetrate the viscous mucus that covers the airway epithelia, which is particularly abundant in the CF lung airways due to inefficient mucociliary clearance. The gastric pathogen *H. pylori* utilizes motility to breach the gastric mucosal barrier and persist within the mucosal lining (56). Similarly, *B. cepacia* may utilize motility to enter the viscous mucus layer in the CF lung airways, persist within it, or penetrate it to establish contact with the underlying epithelial tissues. Once the epithelial barrier is breached, motility may facilitate invasion of *B. cepacia* into underlying tissues, thus promoting entry of the bacteria into the bloodstream and systemic dissemination.

Our findings indicate that defective motility due to disruption of the *B. cepacia* flagellar export and assembly pathway is responsible for the reduced invasiveness of the *fliG* and *fliI* mutants. However, at this time we cannot rule out the possibility that other secretion defects contribute to the reduced invasion of the *fliG* and *fliI* mutants. It has recently been proposed that the flagellar type III secretion system has two functions, export of flagellar proteins and export of nonflagellar virulence determinants (57). For example, in *Yersinia en-*

terocolitica, components of the flagellar type III apparatus are required for export of a virulence-associated phospholipase, YplA (57). It is possible that nonflagellar virulence proteins exported via the *B. cepacia* flagellar secretion system also contribute to the ability of the organism to interact with and enter host cells.

We have demonstrated that the *B. cepacia* mutants with impaired motility lack detectable intracellular flagellin and do not export detectable amounts of flagellin into the supernatant. While it is possible that undetectable amounts of *B. cepacia* flagellin are expressed, it is clear that the levels of the *B. cepacia* flagellin in both D9 and CM58 are dramatically reduced compared to the level in parent strain J2315. Flagellar gene expression in a number of bacterial species is known to proceed through a hierarchical pathway. The genes encoding early structural components of the flagellum, as well as components of the flagellar type III secretion system, are transcribed first, while transcription of genes encoding later components, such as flagellin, are activated during the final stages of assembly (34). Mutations in early flagellar genes often block expression of genes encoding late flagellar components. Since the *B. cepacia* flagellar genes identified and characterized in this study are predicted to encode early structural components, as well as components of the export apparatus, it is likely that disruption of these genes in *B. cepacia* also leads to a block of flagellin gene expression. We cannot, however, exclude the possibility that the *B. cepacia* flagellin is expressed but subsequently degraded in the absence of either FliG or FliI.

In *E. coli*, the *fliFGHIJ(K)* genes are transcribed as an operon, which belongs to class II of the flagellar hierarchy. Transcription of the *fliFGHIJ(K)* operon in *E. coli* is induced by the class I FlhDC transcriptional activator complex (34). It is possible that the *B. cepacia fliFGHIJ* genes are also cotranscribed as an operon, as there are no identifiable internal promoter elements upstream of *fliG*, *fliH*, *fliI*, or *fliJ*. Additionally, the intergenic regions in the *fliFGHIJ* locus are 32 bp long (between *fliH* and *fliI*) and 5 bp long (between *fliI* and *fliJ*), while *fliF* and *fliG* overlap by 11 bp and *fliG* and *fliH* overlap by 8 bp. Therefore, if internal promoters do exist, they must overlap with the coding region of the gene immediately upstream. Interestingly, we identified a sequence (5'-CACGAT AA-3') that is 70 bp upstream of the *B. cepacia fliF* predicted start codon and matches at 6 of 8 bp the consensus sequence of the -10 region of *E. coli* flagellar class II core promoters (34). This observation suggests that the *B. cepacia fli* gene cluster may be regulated similarly. Studies aimed at elucidating the transcriptional organization and regulation of the *B. cepacia* flagellar gene locus are under way.

In conclusion, our data provide strong evidence that flagellum-mediated motility is a contributing factor in the ability of *B. cepacia* to invade respiratory epithelial cells. Thus, motility may be an important virulence determinant of *B. cepacia*, enhancing the pathogenicity of the organism in the human host. Future studies will focus on further defining the role of motility and flagellar biogenesis in *B. cepacia* pathogenesis using both chronic and acute animal models of *B. cepacia* infection and on further characterizing the mechanisms of invasion employed by *B. cepacia* to enter host cells.

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