JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2116–2125 Vol. 179, No. 7
0021-9193/97/$04.00+0

Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: Isolation of Motility Mutants and Molecular Characterization of the Flagellin Structural Gene

DAVID DESHAZER, PAUL J. BRETT, ROBERT CARLYON, AND DONALD E. WOODS*

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1

Received 14 November 1996/Accepted 17 January 1997

***Burkholderia pseudomallei* is a human and animal pathogen in tropical regions, especially Southeast Asia and northern Australia. Currently little is known about the genetics and molecular biology of this organism. In this report, we describe the mutagenesis of *B. pseudomallei* with the transposon Tn*5*-OT182. *B. pseudomallei* 1026b transposon mutants were obtained at a frequency of 4.6×10^{-4} per initial donor cell, and the transposon inserted randomly into the chromosome. We used Tn*5*-OT182 to identify the flagellin structural gene, *fliC*. We screened 3,500 transposon mutants and identified 28 motility mutants. Tn*5*-OT182 integrated into 19 unique genetic loci encoding proteins with homology to *Escherichia coli* and *Salmonella typhimurium* flagellar and chemotaxis proteins. Two mutants, MM35 and MM36, contained Tn*5*-OT182 integrations in *fliC*. We cloned and sequenced *fliC* and used it to complement MM35 and MM36 in trans. The *fliC* transcriptional start site and a σ^F-like promoter were identified by primer extension analysis. We observed a significant difference in the expression of two distinct *fliC-lacZ* transcriptional fusions during bacterial growth, suggesting the presence of a latent intragenic transcriptional terminator in *fliC*. There was no significant difference in the virulence of 1026b compared to that of MM36 in diabetic rats or Syrian hamsters, suggesting that flagella and/or motility are probably not virulence determinants in these animal models of *B. pseudomallei* infection. A phylogenetic analysis based on the flagellins from a variety of bacterial species supported the recent transfer of *B. pseudomallei* from the genus *Pseudomonas* to *Burkholderia*.**

Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*) was first described in 1912 by Whitmore and Krishnaswami in Rangoon, Burma (70). The organism is a gram-negative bacillus that is motile by means of a polar tuft of two to four flagella. It is a soil saprophyte and is the causative agent of the disease melioidosis in tropical regions, especially Southeast Asia and northern Australia (16). Infection is probably due to soil or water contamination of skin abrasions or inhalation of *B. pseudomallei* from an environmental source. As a result, a relatively high percentage of the cases in Thailand are in farmers that work in rice fields without protective clothing (14). *B. pseudomallei* can disseminate from sites of localized infection, such as the lungs or the skin, to virtually any other organ of the body (16). Acute septicemic melioidosis is the most severe form of disease and is responsible for much morbidity and mortality, especially in northeastern Thailand (14).

B. pseudomallei produces a number of factors that may be involved in pathogenesis. These include secreted products such as protease, lipase, lecithinase, and a heat-labile toxin as well as cell-associated products such as flagella, capsule, and pili (4, 11, 63). The outcome of the host-pathogen interaction during infection may also be influenced by the ability of *B. pseudomallei* to resist killing by normal human serum (63), survive within phagocytic cells, and invade nonphagocytic cells (31).

Little is known about the molecular biology of the putative virulence determinants of *B. pseudomallei*, as it is poorly characterized genetically. We are interested in identifying and characterizing those factors that are responsible for *B. pseudomallei* pathogenesis at the molecular level. Our goal is to use a combination of an animal model of infection and transposon mutagenesis to study potential virulence factors of *B. pseudomallei*. We have previously described the use of two animal models of acute *B. pseudomallei* infection, diabetic rats (73) and Syrian hamsters (10). The transposon that we selected for use in *B. pseudomallei* is a Tn*5*-derivative termed Tn*5*-OT182 (42). Tn*5* is well characterized genetically, and it transposes with relatively high frequencies in many gram-negative bacteria and generally integrates with little sequence specificity (8). Tn*5*-OT182 contains a tetracycline resistance determinant which is a useful selective marker in *B. pseudomallei*. In addition, this transposon contains the pBR325 origin of replication which allows the "self-cloning" of DNA immediately flanking the site of Tn*5*-OT182 integration (42). Finally, the presence of a promoterless *lacZ* reporter gene on this transposon allows the formation of *lacZ* transcriptional fusions when Tn*5*-OT182 integrates downstream of a functional promoter.

In this report, we describe the mutagenesis of *B. pseudomallei* with Tn*5*-OT182. We have used this transposon to identify multiple genes involved in motility, including the flagellin structural gene, *fliC*. We describe the cloning, sequencing, and transcriptional analysis of *fliC*. In addition, MM36 (*fliC*) and 1026b (wild type) were compared in the diabetic rat and Syrian hamster models of acute *B. pseudomallei* infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *B. pseudomallei* and *Escherichia coli* were grown at 37°C on Luria-Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. For animal studies, *B. pseudomallei* cultures were grown at 37°C in TSBDC medium (10). When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin, 25 μg of kanamycin, 25 μg of chloramphenicol, 12.5 μg of tetracycline, 100 μg of streptomycin, and 1.5 mg of trimethoprim per ml for *E. coli* and 50 μg of tetracycline, 100 μg of streptomycin, and 100 μg of trimethoprim per ml for *B. pseudomallei*. A 100-mg/ml stock solution of trimethoprim was prepared in *N*,*N*-dimethyl-

* Corresponding author. Phone: (403) 220-2564. Fax: (403) 283-5241.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km^r Sm^s	61
SURE	e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5 uvrC* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*] Km^r Tc^r	Stratagene
DH5α	F$^-$ ϕ80d*lacZ* Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
B. pseudomallei		
E203	Environmental isolate; Sm^r Tc^s	D. A. B. Dance[a]
316c	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
319a	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
304b	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
1026b	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
U7/mel	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
666a	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
406e	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
199a	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
307e	Clinical isolate; Sm^r Tc^s	D. A. B. Dance
PBS1	1026b::pPBS1; Tc^r	This study
Plasmids		
pSKM11	Positive selection cloning vector; IncP mob; ColE1 ori; Ap^r Tc^s	49
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp^r	59
pOT182	pSUP102(Gm)::Tn*5*-OT182; Cm^r Gm^r Ap^r Tc^r	42
pMM35E	8.5-kb *Eco*RI fragment from MM35 obtained by self-cloning; Ap^r Tc^r	This study
pMM36E	8.0-kb *Eco*RI fragment from MM36 obtained by self-cloning; Ap^r Tc^r	This study
pPBS1	pSKM11 containing 1.6-kb *Hin*dIII-*Eco*RI fragment from pMM36E; Ap^r Tc^r	This study
pPBS2H	8.0-kb *Hin*dIII fragment from PBS1 obtained by self-cloning; Ap^r Tc^r	This study
pDD400	pUCP28T containing 2.0-kb *Hin*dIII-*Eco*RI from pPBS2H; Tp^r; *rpsU*$^+$ *fliC*$^+$	This study
pDD401	pDD400 derivative lacking the 0.7-kb *Pst*I fragment; *rpsU*$^+$ *fliC*	This study

[a] London School of Hygiene and Tropical Medicine, London, England.

acetimide. Plasmids were purified from overnight cultures by using Wizard *Plus* Minipreps (Promega).

Tn*5*-OT182 mutagenesis and plasmid conjugations. Tn*5*-OT182 was delivered to *B. pseudomallei* via conjugation with SM10(pOT182) by using a membrane filter mating technique. Briefly, SM10(pOT182) was inoculated into 2 ml of LB broth containing kanamycin, chloramphenicol, and tetracycline and grown at 37°C for 18 to 20 h with shaking (250 rpm). *B. pseudomallei* was also grown under these conditions but without antibiotic selection. One hundred microliters of each saturated culture was added to 3 ml of sterile 10 mM $MgSO_4$, mixed, and filtered through a 0.45-μm-pore-size nitrocellulose filter, using a 25-mm Swinnex filter apparatus (Millipore). Control assays, using donor and recipient alone, were also performed. Filters were placed on LB plates supplemented with 10 mM $MgSO_4$ and incubated for 8 h in a 37°C incubator. The filters were washed with 4 ml of sterile 0.85% NaCl, and 100 μl aliquots were spread onto LB plates containing 100 μg of streptomycin and 50 μg of tetracycline per ml. Tetracycline-resistant (Tc^r) streptomycin-resistant (Sm^r) transconjugants were identified after 24 to 36 h incubation at 37°C. No colonies grew on the control plates because streptomycin selected against the donor strain and tetracycline selected against the recipient strain.

Plasmids pSKM11 and pUCP28T were likewise conjugated to *B. pseudomallei* by using *E. coli* SM10 as the donor strain except that conjugations were allowed to proceed for 5 and 3 h, respectively.

Motility plate assay. Tn*5*-OT182 mutants were screened for their motility phenotypes by transferring individual colonies with sterile toothpicks to a petri plate containing LB with 0.3% agar, tetracycline, and streptomycin. Motility plates were incubated for 1 to 3 days at 37°C, and the motility phenotype of each mutant was assessed. Motile strains swam through the semisolid agar away from the site of inoculation, resulting in a larger area of growth than for nonmotile strains.

DNA manipulation and electroporation. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean II kit (Bio 101). Chromosomal DNA was isolated from *B. pseudomallei* by using a previously described protocol (71). For the self-cloning of *B. pseudomallei* DNA from Tn*5*-OT182 mutants and the cointegrate strain PBS1, approximately 5 μg of chromosomal DNA was digested with restriction enzyme, boiled for 5 min, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol. The chromosomal fragments were then washed with 70% ethanol, air dried, and resuspended in 60 μl of distilled H_2O. Twenty microliters of this suspension was ligated in a 25-μl reaction volume for 1 h at 22°C or overnight at 16°C. The ligation mix (2 μl) was electroporated into *E. coli*, using a GenePulser II/Pulse Controller Plus apparatus (Bio-Rad) as instructed by the manufacturer.

DNA sequencing. Automated DNA sequencing was performed by the University Core DNA Services (University of Calgary) with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System and AmpliTaq DNA polymerase (Perkin-Elmer). DNA sequencing reactions were analyzed with an ABI373A DNA Sequencer. The oligodeoxyribonucleotide OT182-RT (5′-ACATGGAAGTCAGA TCCTGG-3′) was used to initiate DNA sequence reactions with plasmids obtained from Tn*5*-OT182 mutants by self-cloning with *Bam*HI, *Cla*I, *Sst*I, or *Eco*RI. The oligodeoxyribonucleotide OT182-LT (5′-GATCCTGGAAAACGG GAAAG-3′) was used to initiate DNA sequence reactions with plasmids obtained from Tn*5*-OT182 mutants by self-cloning with *Sal*I, *Sma*I, *Hin*dIII, *Not*I, or *Xho*I. Insert DNA from plasmid pDD400 was sequenced on both strands by using a primer walking strategy. Custom oligodeoxyribonucleotides were synthesized at the University Core DNA Services.

Sequence and phylogenetic analysis. DNA and protein sequences were analyzed with GeneJockey version 1.20 software for the Macintosh and the University of Wisconsin Genetics Computer Group package (20). The blastx and blastp programs were used to search the nonredundant sequence database for homologous proteins (40). The homologous proteins in Table 2 were identified by using blastx and in most cases had the lowest *P* values and high scores of greater than 100. For phylogenetic analysis, the amino acid sequences of 44 flagellins were aligned with the program PILEUP, using the default parameters. The aligned sequences were edited so that there were no gaps, and the highly variable internal domain was removed. The conserved N- and C-terminal sequences retained for subsequent analysis corresponded to amino acids 3 to 101 and 319 to 384 in the *B. pseudomallei* flagellin, respectively. The program DISTANCES was used to calculate a matrix of the pairwise evolutionary distances between the aligned sequences. The Kimura method was used to correct the distances for multiple substitutions at a site. The distances were expressed as substitutions per 100 amino acids. GROWTREE was used to reconstruct a phylogenetic tree from

the distance matrix by using the neighbor-joining method. The RpsU amino acid sequences were aligned with the program PILEUP, using the default parameters.

β-Gal assays. Overnight *B. pseudomallei* cultures were diluted 1:100 in LB broth, and 1-ml aliquots were removed at timed intervals and assayed for β-galactosidase (β-Gal) as described previously (43). The optical density at 600 nm was also determined at each time point.

Primer extension analysis. RNA was isolated from a logarithmic-phase culture of *B. pseudomallei* 1026b by using a S.N.A.P. Total RNA Isolation kit (Invitrogen). The oligodeoxyribonucleotide *fliC*-PE (5′-GTTGCTGTTGATTCCGAGC AT-3′) was used for the primer extension. This primer is complementary to the 5′ end of *fliC*. The primer was labeled at its 5′ end with T4 polynucleotide kinase (GibcoBRL) and [γ-^{32}P]ATP (Amersham), and approximately 10^6 cpm of primer was added to 10 μg of RNA. The synthesis of cDNA was performed with a cDNA Cycle kit (Invitrogen). The primer extension product was loaded onto a gel containing 6% polyacrylamide, 7 M urea, and 30% formamide. A DNA sequence reaction with the same primer and pDD400 was loaded next to the primer extension product for comparison.

Animal studies. The animal models of acute *B. pseudomallei* infection were described in detail elsewhere (10, 73). Briefly, Syrian hamsters (6 to 8 weeks) and streptozotocin-treated infant Sprague-Dawley rats (average weight of 30 g) were injected intraperitoneally with 100 μl of one of a number of serial dilutions of logarithmic-phase cultures adjusted appropriately with sterile phosphate-buffered saline. Five animals were used per dilution in the Syrian hamster experiment, and six animals were used per dilution in the diabetic rat experiment. The 50% lethal doses (LD_{50}s) at 2 days (Syrian hamsters) and 7 days (diabetic rats) were calculated as previously described (10, 73).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers U73848 (*rpsU-fliC*) and U73849 (Tn*5*-OT182).

RESULTS

Tn*5*-OT182 mutagenesis of *B. pseudomallei*. The transposon used in this study, Tn*5*-OT182, is depicted schematically in Fig. 1A. Based on published reports (42, 62) and our own sequencing data, we have determined the entire nucleotide sequence of Tn*5*-OT182. The transposon consists of 10,705 bp. It is worth noting that the gene order of the tetracycline resistance determinants is reversed with respect to the initial description of this transposon (42). In addition we identified from the sequence data 10 unique restriction sites, *Bsi*WI, *Csp*45I, *Xmn*I, *Sca*I, *Vsp*I *Apa*I, *Stu*I, *Avr*II, *Nhe*I, and *Not*I, that should enhance the self-cloning capability of this transposon.

We randomly chose 10 *B. pseudomallei* strains (Table 1) and tested them in the Tn*5*-OT182 mutagenesis procedure. Six of the ten strains formed Tc^r Sm^r transconjugants, suggesting that Tn*5*-OT182 mutagenesis of *B. pseudomallei* is strain dependent under these conditions. *B. pseudomallei* E203, 316c, 319a, and 304b did not form Tc^r Sm^r transconjugants. For strains that were successfully mutagenized with Tn*5*-OT182, the number of Tc^r Sm^r transconjugants per plate varied from 14 (666a) to 150 (1026b). This result indicates that the efficiency of Tn*5*-OT182 mutagenesis can vary as much as 10-fold between different *B. pseudomallei* strains.

B. pseudomallei 1026b, a strain isolated from the blood of a patient with melioidosis, was used in further studies. Tc^r Sm^r transconjugants were obtained at a frequency of 4.6×10^{-4} per initial donor cell. Ten Tc^r Sm^r transconjugants were chosen at random, and the chromosomal DNA from these mutants was isolated and digested with *Eco*RI. These chromosomal fragments were separated by electrophoresis, blotted to a nylon membrane, and probed with radioactively labeled pOT182. All 10 mutants contained a single copy of Tn*5*-OT182 in a unique location (data not shown), suggesting that Tn*5*-OT182 inserts randomly into the 1026b chromosome.

Isolation and characterization of *B. pseudomallei* 1026b motility mutants. One of the goals of this study was to isolate and characterize the flagellin structural gene of *B. pseudomallei*. Approximately 50 genes are required for flagellar synthesis and chemotaxis in *E. coli* and *Salmonella typhimurium* (9). *B. pseudomallei* probably contains a similar number of genes for flagellar synthesis and chemotaxis. Thus, the strategy that we chose for specifically identifying the flagellin structural gene involved (i) mutagenesis with Tn*5*-OT182, (ii) screening for nonmotile mutants on motility agar plates, (iii) eliminating siblings by Southern blot analysis, (iv) isolating the DNA flanking Tn*5*-OT182 in each mutant by self-cloning (42), (v) performing a single-strand sequence reaction (SSSR) on each clone by using a Tn*5*-OT182-specific primer, and (vi) identifying homologous sequences in the sequence database by using the local alignment search tool blastx (40).

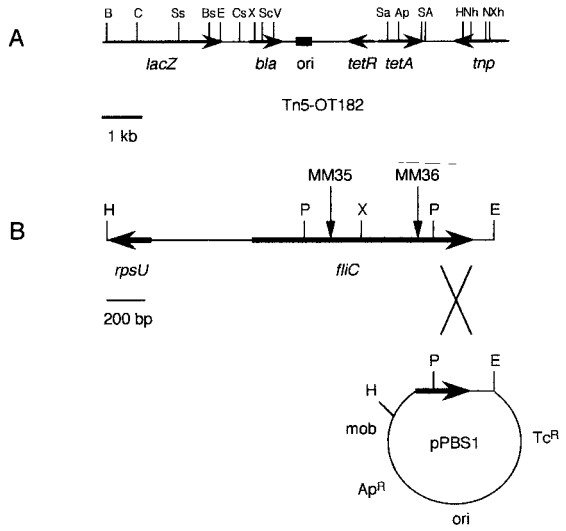

FIG. 1. Schematic representation of Tn*5*-OT182 and the *rpsU-fliC* locus. (A) Physical and genetic map of Tn*5*-OT182. The arrows represent the locations and directions of transcription of genes. The black box represents the pBR325 origin of replication. *lacZ*, promoterless gene encoding β-Gal; *bla*, β-lactamase; ori, pBR325 origin of replication; *tetRA*, tetracycline resistance genes from RP4; *tnp*, transposase; B, *Bam*HI; C, *Cla*I; Ss, *Sst*I; Bs, *Bsi*WI; E, *Eco*RI; Cs, *Csp*45I; X, *Xmn*I; Sc, *Sca*I; V, *Vsp*I; Sa, *Sal*I; Ap, *Apa*I; S, *Stu*I; A, *Avr*II; H, *Hin*dIII; Nh, *Nhe*I; N, *Not*I; Xh, *Xho*I. (B) Physical and genetic map of the *rpsU-fliC* locus and strategy used to clone the 2.0-kb *Hin*dIII (H)-*Eco*RI (E) fragment. The locations and directions of transcription of *rpsU* and *fliC* are represented by black arrows. The locations of Tn*5*-OT182 in MM35 and MM36 are shown. The cointegrate strain PBS1 was created by integration of the suicide vector pPBS1 into the chromosome of *B. pseudomallei* 1026b. The large × represents the location of homologous recombination between pPBS1 and the 1026b chromosome. The 2.0-kb H-E fragment was isolated from PBS1 by self-cloning with H. *rpsU*, gene encoding ribosomal protein S21; *fliC*, gene encoding flagellin protein; mob, RP4 origin of transfer; Ap^R, ampicillin resistance; Tc^R, tetracycline resistance; ori, ColE1 origin of replication; P, *Pst*I; X, *Xho*I.

Approximately 3,500 transposon mutants were screened for their motility phenotypes on motility agar plates, and 39 nonmotile mutants were identified and named MM1 to MM39. All 39 mutants were rescreened; 8 were found to have attenuated motility, while the other 31 were still completely nonmotile. For this study, we were interested only in nonmotile mutants; therefore, the eight mutants displaying attenuated motility were not analyzed further. Chromosomal DNA was isolated from the 31 nonmotile mutants, and Southern blot analysis was performed with radioactively labeled pOT182. Three siblings were identified and were not analyzed further (data not shown). Thus, a total of 28 unique nonmotile mutants were isolated from the 3,500 transposon mutants screened (Table 2).

The DNA flanking Tn*5*-OT182 in each motility mutant was isolated by self-cloning (42). Chromosomal DNA was digested with *Eco*RI, ligated, and used to transform *E. coli* SURE. A primer specific to the right end of Tn*5*-OT182 (as depicted in Fig. 1A) was used to perform an SSSR on each of the 28 plasmids isolated by self-cloning. The resulting sequences were

TABLE 2. Genetic loci associated with Tn*5*-OT182 integrations in *B. pseudomallei* motility mutants and the functions of the homologous proteins that they encode

Motility mutant	Homolog[a]	Function
MM1		
MM2	Tsr	Sensory transducer protein
MM3	FlgH[b]	L ring
MM4	CheA	Chemotaxis protein
MM6	FliM	Switch
MM7	FlhB[b]	Flagellar assembly
MM9		
MM14	MotA	Flagellar motor protein
MM15	FlgB	Rod protein
MM16	FliM	Switch
MM18		
MM19	FliK	Regulation of hook length
MM20	FliR	Flagellar subunit export
MM21	FliP	Flagellar subunit export
MM22	CheW	Chemotaxis protein
MM24		
MM25	FliQ	Flagellar subunit export
MM26	GalU	UDP-glucose pyrophosphorylase
MM27	FlgE[b]	Hook
MM28	FliN	Switch
MM31	FlgD	Hook scaffolding protein
MM32	FlhA	Flagellar subunit export
MM33	FlgL	Hook-associated protein
MM34	FliE	Flagellar assembly
MM35	FliC	Flagellin
MM36	FliC	Flagellin
MM37		
MM39		

[a] Homologous protein present in *E. coli* and/or *S. typhimurium* (9, 69).
[b] Tn*5*-OT182 is located immediately downstream of the gene encoding the homologous protein in this strain.

approximately 300 bp in length and were analyzed by using the local alignment search tool blastx (2, 40). This program compares a nucleotide query sequence translated in all six reading frames against a nonredundant protein sequence database. The sequences obtained from 22 of the 28 motility mutants contained homologous sequences in the database (Table 2). On the other hand, the sequences obtained from MM1, MM9, MM18, MM24, MM37, and MM39 did not contain any homologous sequences in this database. Most of the homologous sequences identified with blastx were proteins involved in flagellin synthesis and operation in *E. coli* and *S. typhimurium* (Table 2). These include switch proteins (FliM and FliN), flagellar export proteins (FliR, FliP, FliQ, and FlhA), flagellar assembly proteins (FlhB and FliE), a rod protein (FlgB), the L-ring protein (FlgH), the hook protein (FlgE), a hook-associated protein (FlgL), a hook scaffolding protein (FlgD), a protein involved in regulation of hook length (FliK), a flagellin motor protein (MotA), and the flagellar subunit protein (FliC) (9). There were also chemotaxis protein homologs identified in MM2 (Tsr), MM4 (CheA), and MM22 (CheW) (Table 2). We conclude that synthesis and operation of the flagellar apparatus in *B. pseudomallei* requires multiple genes that presumably encode proteins with functions similar to those found in *E. coli* and *S. typhimurium*. The fact that such a variety of motility genes were identified by this mutagenesis procedure demonstrates the random nature of integration of Tn*5*-OT182 in the *B. pseudomallei* 1026b chromosome.

MM6 and MM16 both contained a copy of Tn*5*-OT182 integrated in *fliM* but in separate locations. This was also the case for *fliC* in MM35 and MM36 (Table 2; Fig. 1B). The site of Tn*5*-OT182 integration in MM3, MM7, and MM27 was immediately downstream of the genes encoding their respective motility proteins. The nonmotile phenotype displayed by these mutants was probably due to disruption of an adjacent motility gene or to a polar effect on the expression of a downstream motility gene(s). In all other motility mutants, the Tn*5*-OT182 integration site was within the gene encoding the corresponding motility protein. In MM26, the transposon integrated within a gene encoding a protein that was homologous to GalU (69). GalU, UDP-glucose pyrophosphorylase, is the enzyme that catalyzes the conversion of UTP and glucose-1-phosphate into UDP-glucose and pyrophosphate.

Tn*5*-OT182 contains a promoterless *lacZ* reporter gene that allows for the formation of promoter fusions when the transposon integrates downstream of a functional promoter (Fig. 1A) (42). *B. pseudomallei* strains do not produce β-Gal, the protein product encoded by *lacZ* (15). We have confirmed this result with strain 1026b. We performed β-Gal assays on all of the nonmotile mutants in Table 2 and found that 57% produce β-Gal and therefore contain *lacZ* transcriptional fusions (data not shown). In all of the nonmotile mutants, the orientation of the predicted gene as determined by using blastx was confirmed with the β-Gal assays. These results indicate that the promoterless *lacZ* of Tn*5*-OT182 functions as a promoter probe in *B. pseudomallei* and should allow the analysis of transcription of genes in this organism. In addition, β-Gal assays should provide a convenient way of predicting the orientation of a mutated gene with respect to Tn*5*-OT182, assuming that the growth conditions are conducive to expression of the gene being analyzed.

Cloning of the flagellin structural gene and complementation of MM35 and MM36. MM35 and MM36 contain Tn*5*-OT182 integrations within *fliC*, the flagellin structural gene (Table 2). No flagellar filaments were identified when MM35 and MM36 were negatively stained and examined with a transmission electron microscope (data not shown). The Tn*5*-OT182 integrations in MM35 and MM36 were mapped to the 5′ and 3′ ends, respectively, of *fliC* (Fig. 1B). In addition, β-Gal assays and SSSR results indicated that the orientation of Tn*5*-OT182 integrations in both strains resulted in *fliC-lacZ* transcriptional fusions. The strategy that we used to clone a wild-type copy of *fliC* is shown schematically in Fig. 1B. Plasmid pSKM11 contains a ColE1 origin of replication and is unable to replicate in *B. pseudomallei* (49). Therefore, the only way derivatives of this plasmid can be maintained is by homologous recombination between cloned *B. pseudomallei* DNA and the corresponding region on the bacterial chromosome. The 1.6-kb *Hin*dIII-*Eco*RI fragment from pMM36E was cloned into the corresponding sites of pSKM11, creating plasmid pPBS1 (Fig. 1B). The cloned fragment contained the 3′ end of *fliC* and approximately 100 bp downstream of this gene. We conjugated pPBS1 into *B. pseudomallei* 1026b and selected for Tc^r cointegrates (1026b::pPBS1). Several Tc^r colonies were identified, and one, PBS1, was chosen and used in further studies. The wild-type *fliC* was cloned from PBS1 by using a self-cloning procedure similar to that described for Tn*5*-OT182 mutants (42). Chromosomal DNA from PBS1 was isolated, digested with *Hin*dIII, ligated, and used to transform *E. coli* SURE. The resulting plasmid, pPBS2H, contained a 2.0-kb *Hin*dIII-*Eco*RI fragment that was subsequently cloned into the broad-host-range plasmid pUCP28T (Fig. 1B) (59). The pUCP28T derivative, pDD400, was able to complement the mutations in MM35 and MM36 in trans (Fig. 2A). As expected, the negative controls MM35(pUCP28T) and MM36(pUCP28T) were nonmotile. These results indicate that pDD400 contains a wild-

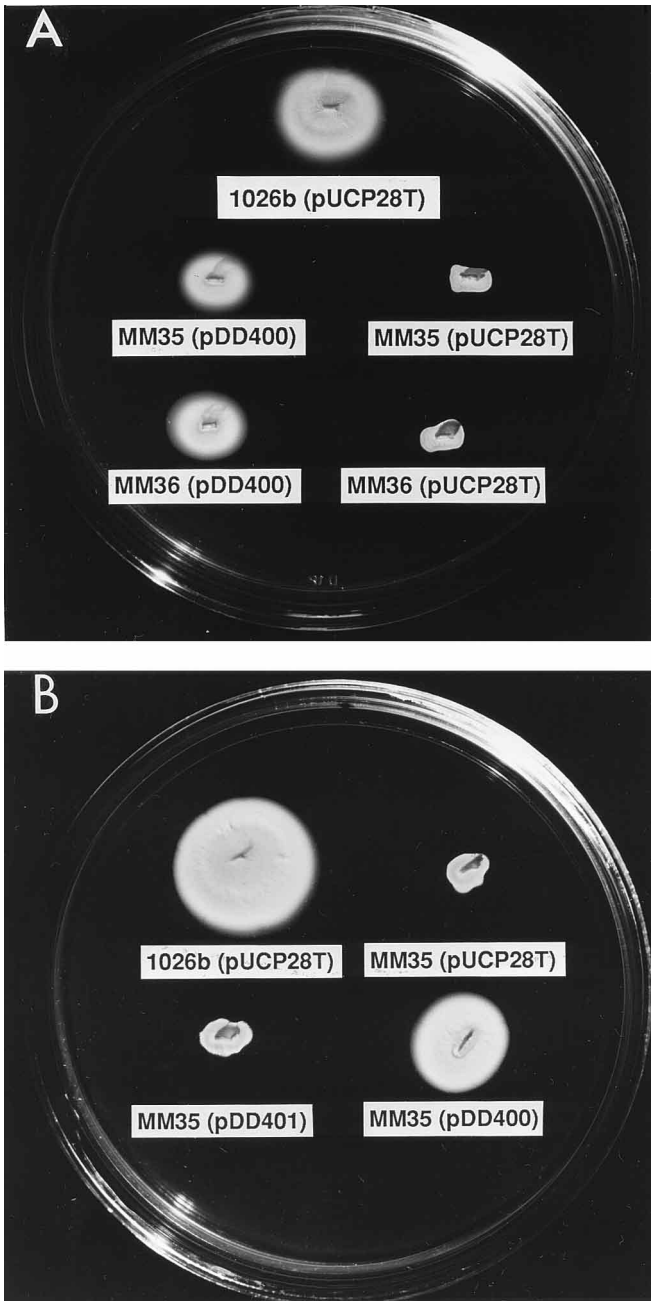

FIG. 2. Motility plate assays. (A) Complementation of MM35 and MM36 with pDD400 (*rpsU*$^+$ *fliC*$^+$). The plate was incubated at 37°C for 2 days, and the relative motilities of 1026b (wild type), MM35 (*fliC*), and MM36 (*fliC*) were assessed by the visualization of a halo originating from the site of inoculation. The vector pUCP28T served as a negative control in this experiment. (B) Plasmid pDD401 (*rpsU*$^+$ *fliC*) does not complement MM35. The plate was incubated at 37°C for 3 days.

```
                                                                HindIII
                                                                AAGC TTA GTG GAG   13
                                                                     *** His Leu

CTT CTT CGG CAG CAT CTG GCT GCG CAG GCC CTT GTG CAG GCG CTT CAC CGC AGC CGC   70
Lys Lys Pro Leu Met Gln Ser Arg Leu Arg Lys His Leu Arg Lys Val Ala Ala Ala

TTT CTT GCG CTT GCG CAC GGC GGT CGG CTT CTC GTA TGC CTG GCG CTC GCG CAG CTC  127
Lys Lys Arg Lys Arg Val Ala Thr Pro Lys Glu Tyr Ala Gln Arg Glu Arg Leu Glu

GGC GAT CAG GCC GTT CTT CTC GAT CGC GCG GCG GAA GCG GCG AAT CGC CAC TTC GAA  184
Ala Ile Leu Gly Asn Lys Glu Ile Ala Arg Arg Phe Arg Arg Ile Ala Val Glu Phe
                                                  SD
CGG CTC GTT TTC TTT CAA AAG AAT CGT CGT CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAA  249
Pro Glu Asn Glu Lys Leu Leu Ile Thr Thr Met     ← rpsU
CGTAGCGGCCAAGCGCCACGCACCGGCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCG  324

GAGGCCGGAAAAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACTGTCAG  399

CAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATTCAGCCCGCCATCATAACAGG  474

TAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGCGGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTT  549

GCGGGCCGCCGGACGGCGCGCCGTTCGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACA  624
                                  -35          -10          +1
CAAAAAATTCGGGCCGAATCTCGCCCTGCCCCCTCAAGTTTTTTGGCGTACGCCGAAATCCTGCACAAGGCGGC  699
                                                                SD fliC →
CGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATACAGCTCAATTTGGAGATTTTC  774

ATG CTC GGA ATC AAC AGC AAC ATT AAC TCG TTG GTC GCT CAA CAG AAC CTC AAC GGC  831
Met Leu Gly Ile Asn Ser Asn Ile Asn Ser Leu Val Ala Gln Gln Asn Leu Asn Gly

TCG CAA GGC GCC CTG TCC CAA GCG ATC ACC CGC CTG TCG TCG GGC AAG CGC ATC AAC  888
Ser Gln Gly Ala Leu Ser Gln Ala Ile Thr Arg Leu Ser Ser Gly Lys Arg Ile Asn

AGC GCG GCG GAC GAT GCG GCC GGC CTC GCG ATC GCC ACC CGG ATG CAA ACG CAG ATC  945
Ser Ala Ala Asp Asp Ala Ala Gly Leu Ala Ile Ala Thr Arg Met Gln Thr Gln Ile

AAC GGC CTG AAC CAG GGC GTG TCG AAC GCG AAC GAC GGC GTG TCG ATC CTG CAA ACG 1002
Asn Gly Leu Asn Gln Gly Val Ser Asn Ala Asn Asp Gly Val Ser Ile Leu Gln Thr
                                                PstI
GCA TCG AGC GGC CTG ACC TCG CTC ACC AAC AGC CTG CAG CGT ATC CGC CAG CTC GCC 1059
Ala Ser Ser Gly Leu Thr Ser Leu Thr Asn Ser Leu Gln Arg Ile Arg Gln Leu Ala

GTG CAG GCC TCG AAC GGC CCG CTG AGC GCG AGC GAC GCG TCG GCG CTG CAA CAG GAA 1116
Val Gln Ala Ser Asn Gly Pro Leu Ser Ala Ser Asp Ala Ser Ala Leu Gln Gln Glu
                                                                MM35 V
GTC GCG CAG CAG ATC TCG GAA GTG AAC CGT ATC GCT TCG CAG ACG AAC TAC AAC GGC 1173
Val Ala Gln Gln Ile Ser Glu Val Asn Arg Ile Ala Ser Gln Thr Asn Tyr Asn Gly

AAG AAC ATC CTC GAC GGC TCG GCA GGC ACG CTG AGC TTC CAG GTC GGC GCG AAC GTC 1230
Lys Asn Ile Leu Asp Gly Ser Ala Gly Thr Leu Ser Phe Gln Val Gly Ala Asn Val

GGC CAG ACG GTC TCC GTC GAC CTC ACG CAA AGC ATG TCG GCG GCG AAG ATC GGC GGC 1287
Gly Gln Thr Val Ser Val Asp Leu Thr Gln Ser Met Ser Ala Ala Lys Ile Gly Gly
                                                XhoI
GGC ATG GTT CAG ACG GGC CAG ACG CTC GGC ACG ATC AAG GTG GCG ATC GAC TCG AGC 1344
Gly Met Val Gln Thr Gly Gln Thr Leu Gly Thr Ile Lys Val Ala Ile Asp Ser Ser
—
GGC GCG GCC TGG TCG TCG GGC AGC ACC GGC CAG GAG ACG ACG CAG ATC AAC GTC GTG 1401
Gly Ala Ala Trp Ser Ser Gly Ser Thr Gly Gln Glu Thr Thr Gln Ile Asn Val Val

TCG GAC GGC AAG GGC GGC TTC ACG TTC ACC GAT CAG AAC AAC CAG GCG CTG TCG TCG 1458
Ser Asp Gly Lys Gly Gly Phe Thr Phe Thr Asp Gln Asn Asn Gln Ala Leu Ser Ser

ACG GCC GTG ACC GCC GTG TTC GGC TCG TCG ACC GCC GGC ACG GGC ACG GCG GCC TCG 1515
Thr Ala Val Thr Ala Val Phe Gly Ser Ser Thr Ala Gly Thr Gly Thr Ala Ala Ser

CCG TCG TTC CAG ACG CTG GCG CTG TCG ACT TCG GCA ACC AGC GCG CTG TCC GCG ACC 1572
Pro Ser Phe Gln Thr Leu Ala Leu Ser Thr Ser Ala Thr Ser Ala Leu Ser Ala Thr

GAC CAG GCG AAC GCC ACG GCG ATG GTT GCG CAG ATC AAC GCG GTC AAC AAG CCG CAA 1629
Asp Gln Ala Asn Ala Thr Ala Met Val Ala Gln Ile Asn Ala Val Asn Lys Pro Gln
  V MM36
ACG GTC TCG AAC CTC GAC ATC AGC ACG CAG ACG GGC GCG TAC CAG GCG ATG GTA TCG 1686
Thr Val Ser Asn Leu Asp Ile Ser Thr Gln Thr Gly Ala Tyr Gln Ala Met Val Ser
                                        PstI
ATC GAC AAC GCA CTC GCG ACG GTC AAC AAT CTG CAG GCA ACG CTC GGC GCG GCG CAA 1743
Ile Asp Asn Ala Leu Ala Thr Val Asn Asn Leu Gln Ala Thr Leu Gly Ala Ala Gln

AAC CGG TTC ACC GCG ATC GCG ACG ACG CAG CAA GCC GGC TCG AAC AAC CTC GCG CAG 1800
Asn Arg Phe Thr Ala Ile Ala Thr Thr Gln Gln Ala Gly Ser Asn Asn Leu Ala Gln

GCG CAA TCG CAA ATC CAG AGC GCG GAC TTT GCT CAG GAA ACC GCG AAC CTG TCG CGC 1857
Ala Gln Ser Gln Ile Gln Ser Ala Asp Phe Ala Gln Glu Thr Ala Asn Leu Ser Arg

GCG CAA GTG CTC CAG CAA GCC GGC ATC TCG GTG CTC GCG CAA GCG AAC TCG CTG CCG 1914
Ala Gln Val Leu Gln Gln Ala Gly Ile Ser Val Leu Ala Gln Ala Asn Ser Leu Pro

CAG CAA GTG CTG AAG CTC CTG CAA TAA CGTGTACCGAACGACGGGCCGCGCTGCCAGGCGGCCCGC 1980
Gln Gln Val Leu Lys Leu Leu Gln ***
                                                              EcoRI
CTTTGTGCGGCGGAGCAACACCCTCGTGGCCGCCGCACGTTTTTGCTTTCTGATTCTTCCGAATTC          2046
```

FIG. 3. Nucleotide sequence of the 2,046-bp *Hin*dIII-*Eco*RI fragment containing *rpsU* and *fliC*. The deduced amino acid sequences are shown beneath the corresponding nucleotide sequences in the three-letter code. The amino acid sequence of S21 was derived from the reverse complement. Putative SD sequences and the −35 and −10 sequences of the σ^F-like promoter are underlined. The transcriptional start site of *fliC* is also underlined and indicated as +1. The locations of Tn*5*-OT182 integrations in MM35 and MM36 are indicated above the nucleotide sequence (V). Relevant restriction endonuclease recognition sites are indicated above the sequence. ***, stop codon.

type copy of *fliC* and that the mutations in MM35 and MM36 do not have a polar effect on downstream genes.

Nucleotide sequence analysis of pDD400. The nucleotide sequence of the pDD400 insert was determined on both strands and was found to be 2,046 bp in length (Fig. 3). The G+C content of this region was 63%, which was lower than the 68% G+C content of the *B. pseudomallei* ATCC 23343 genome (74). We identified an open reading frame (ORF) of 1,167 bp beginning with the ATG at position 775 and ending with the TAA at position 1939 (Fig. 3). The exact locations of Tn*5*-OT182 integrations in MM35 and MM36 were identified within this ORF, strongly suggesting that this ORF was the flagellin structural gene, *fliC*. There was a putative Shine-Dalgarno (SD) sequence, GGAG, immediately upstream of *fliC*. We did not identify any obvious rho-independent transcriptional terminators in the 105 bp immediately downstream of *fliC*. The protein encoded by *fliC* was predicted to be 388

amino acids in length, and amino acids 2 to 17 were identical to the N-terminal sequence obtained from the flagellin purified from *B. pseudomallei* 319a (11). Even though the *fliC* sequence predicted a methionine at position 1 in the flagellin protein, it was not identified in the purified protein (11) (Fig. 3). The N-terminal methionine is commonly removed posttranslationally from bacterial flagellins, and this appears to be the case with the *B. pseudomallei* flagellin (25, 26, 29, 32, 36, 58, 66). The calculated molecular mass of FliC (after removal of the N-terminal methionine) was 39.1 kDa, similar to the apparent molecular mass (43.4 kDa) estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Furthermore, the predicted amino acid composition of FliC was similar to that determined biochemically (11). There were no predicted codons for histidine or cysteine in FliC (Fig. 3).

We also identified an ORF 558 bp upstream of *fliC* on the opposite strand (Fig. 1B and 3). This ORF was preceded by a putative SD sequence and predicted a protein product of 70 amino acids (8.4 kDa). We used the predicted protein product to search the protein database for homologous proteins. The results of this search indicated a high degree of similarity to the ribosomal protein S21 from *E. coli* (70%), *Myxococcus xanthus* (66%), *Haemophilus influenzae* (64%), and *Anabaena variabilis* (63%) (12, 17, 22, 57). The S21 proteins from these organisms were aligned with the predicted *B. pseudomallei* protein; all were similar in length (62 to 71 amino acids) and contained 14 conserved amino acids. Based on this evidence, we named the ORF and its predicted protein product *rpsU* and S21, respectively.

We investigated the possibility that complementation of MM35 and MM36 by pDD400 was due to *rpsU*. We deleted the 700-bp *Pst*I fragment from pDD400, creating pDD401 (Fig. 1B and 3). This deletion was completely within *fliC* and did not affect *rpsU* or the 558-bp *rpsU-fliC* intergenic region. As shown in Fig. 2B, MM35(pDD400) was motile but MM35(pUCP28T) and MM35(pDD401) were not. Thus, the plasmid containing *rpsU* alone (pDD401) was unable to complement the mutation in MM35 whereas the plasmid containing a wild-type copy of *fliC* (pDD400) was able to complement MM35 (Fig. 2B).

Analysis of *fliC* expression and identification of the transcriptional start site. Several bacterial species differentially regulate flagellin gene transcription in response to growth temperature (1, 21, 26, 29, 33). In general, these bacteria are motile at room temperature but are nonmotile at 37°C due to a decrease in the transcription of the flagellin gene and/or genes required for its expression. We found that there was no significant difference in *B. pseudomallei* motility at room temperature compared to 37°C. Therefore, temperature probably plays little or no role in *fliC* transcription in this organism.

As mentioned above, both MM35 and MM36 contain *fliC-lacZ* transcriptional fusions. We investigated the expression of the *fliC-lacZ* fusions in MM35 and MM36 during bacterial growth in LB broth at 37°C (Fig. 4). While there was no difference in the growth rates of 1026b, MM35 and MM36 under these conditions, 1026b did not produce β-Gal at any of the time points examined (data not shown). The levels of production of β-Gal by MM35 and MM36 were similar until the cultures reached middle to late logarithmic phase (approximately 8 h). β-Gal production by MM36 peaked at about 970 Miller units and slightly decreased thereafter. MM35, on the other hand, produced a significantly higher amount of β-Gal (1,500 Miller units) as the bacterial culture entered the late logarithmic to early stationary phase of growth (Fig. 4). This result was surprising since the *lacZ* fusions in MM35 and MM36 are in the same gene and are separated by only 462 bp (Fig. 1 and 3). These results were reproducible and suggest

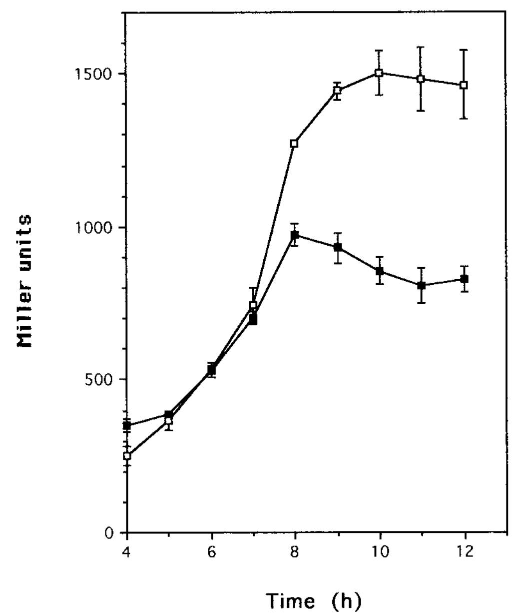

FIG. 4. β-Gal production by MM35 (□) and MM36 (■) during bacterial growth. Each numerical value for β-Gal production is the mean of one experiment performed in triplicate ± standard deviation (bars).

that some *fliC* transcripts may terminate in this 462-bp region as the bacterial culture reaches the late logarithmic to early stationary phase of growth.

We isolated RNA from 1026b and performed a primer extension in order to determine the start site of *fliC* transcription (Fig. 5). The start site of transcription was mapped to an A residue 83 bp upstream of the *fliC* ATG start codon (Fig. 3 and

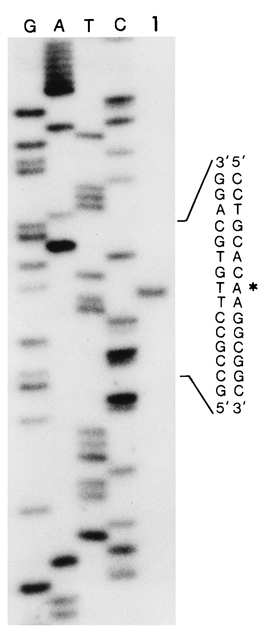

FIG. 5. Primer extension analysis of *B. pseudomallei fliC* mRNA. The oligodeoxyribonucleotide used for reverse transcription was complementary to the 5′ end of *fliC*. The primer extension product (lane 1) was loaded onto a gel with a sequence ladder (lanes G, A, T, and C) made with the same primer as that used for the primer extension. The complementary nucleotide sequence flanking the start site of transcription (*) is shown.

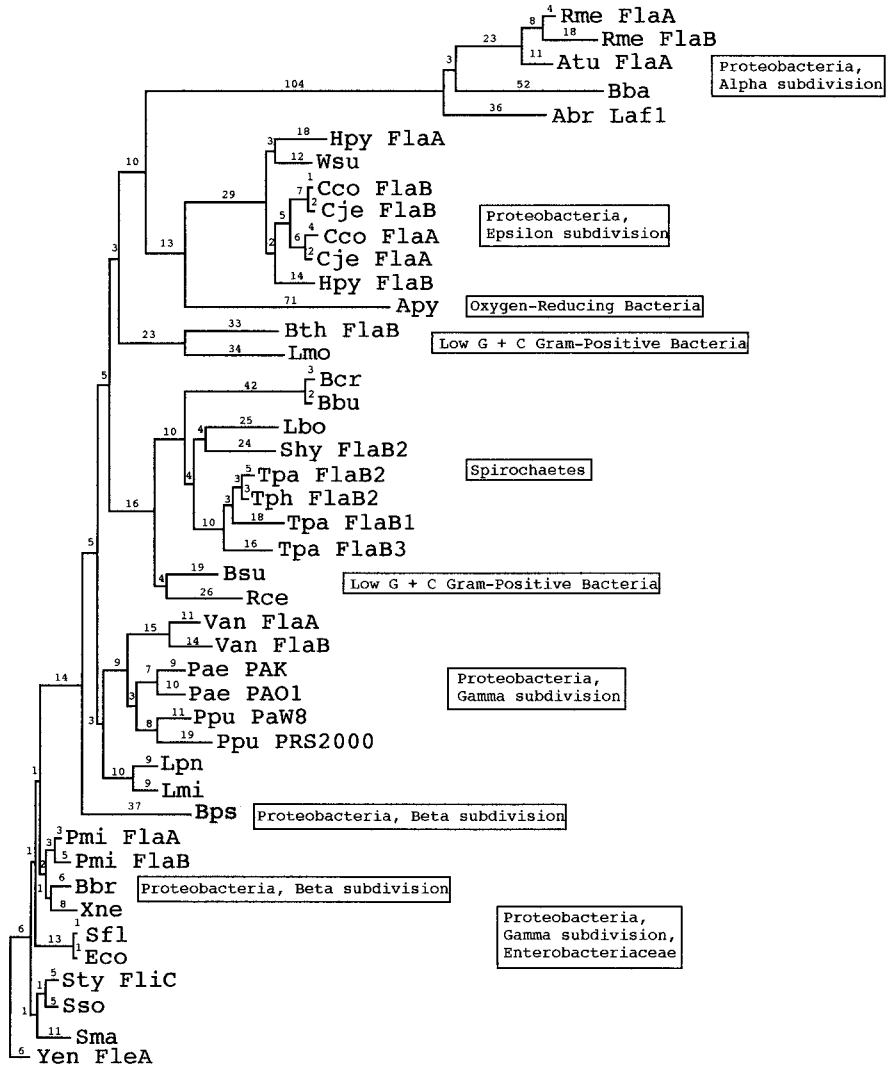

FIG. 6. Phylogenetic analysis based on the conserved N- and C-terminal domains of flagellin proteins. The conserved domains used in the analysis corresponded to amino acids 3 to 101 and 319 to 384 in the *B. pseudomallei* flagellin. The units on the horizontal branches are substitutions per 100 amino acids. The boxes indicate the phylogenetic group to which each organism belongs based on 16S rRNA sequences. The flagellins used in the analysis were *Rhizobium meliloti* 10406 FlaA (Rme FlaA) (55), *R. meliloti* 10406 FlaB (Rme FlaB) (55), *Agrobacterium tumefaciens* FlaA (Atu FlaA) (60), *Bartonella bacilliformis* (Bba) (3), *Azospirillum brasilense* Sp7 Laf1 (Abr Laf1) (47), *Helicobacter pylori* 898-1 FlaA (Hpy FlaA) (36), *Wolinella succinogenes* 1740 (Wsu) (58), *Campylobacter coli* VC167 FlaB (Cco FlaB) (25), *C. jejuni* 81116 FlaB (Cje FlaB) (52), *C. coli* VC167 FlaA (Cco FlaA) (38), *C. jejuni* 81116 FlaA (Cje FlaA) (52), *Helicobacter pylori* 85P FlaB (Hpy FlaB) (64), *Aquifex pyrophilus* DSM6858 (Apy) (6), *Bacillus thuringiensis* Bt75 FlaB (Bth FlaB) (39), *Listeria monocytogenes* 12067 (Lmo) (21), *Borrelia crocidurae* (Bcr) (51), *B. burgdorferi* GeHo (Bbu) (23), *Leptospira borgpetersenii* L171 (Lbo) (46), *Serpulina hyodysenteriae* C5 FlaB2 (Shy FlaB2) (34), *Treponema pallidum* Nichols FlaB2 (Tpa FlaB2) (54), *T. phagedenis* Kazan 5 FlaB2 (Tph FlaB2) (37), *T. pallidum* Nichols FlaB1 (Tpa FlaB1) (13), *T. pallidum* Nichols FlaB3 (Tpa FlaB3) (13), *Bacillus subtilis* I168 (Bsu) (45), *Roseburia cecicola* GM (Rce) (41), *Vibrio anguillarum* NB10 FlaA (Van FlaA) (44), *V. anguillarum* NB10 FlaB (Van FlaB) (44), *Pseudomonas aeruginosa* PAK (Pae PAK) (66), *P. aeruginosa* PAO1 (Pae PAO1) (68), *P. putida* PaW8 (Ppu PaW8) (72), *P. putida* PRS2000 (Ppu PRS2000) (72), *Legionella pneumophila* Corby (Lpn) (29), *L. micdadei* (Lmi) (5), *Burkholderia pseudomallei* 1026b (Bps), *Proteus mirabilis* BB2000 FlaA (Pmi FlaA) (7), *P. mirabilis* BB2000 FlaB (Pmi FlaB) (7), *Bordetella bronchiseptica* GP1SN (Bbr) (1), *Xenorhabdus nematophilus* F1 (Xne) (24), *Shigella flexneri* IID642 (Sfl) (65), *Escherichia coli* K-12 (Eco) (35), *Salmonella typhimurium* SL877 FliC (Sty FliC) (32), *Shigella sonnei* IID969 (Sso) (65), *Serratia marcescens* 274 (Sma) (26), and *Yersinia enterocolitica* 8081 FleA (Yen FleA) (33).

6). Immediately upstream of the transcriptional start site we identified an *E. coli* σ^F-like promoter (28, 50). σ^F, encoded by *fliA*, is a member of the σ^{28} family of alternative sigma factors and is responsible for the specificity of the RNA polymerase that transcribes level 3 flagellar and chemotaxis genes (9, 27). The consensus promoter sequence recognized by σ^F is TAAA $GTTN_{12}GCCGATAA$ (28, 50). The *B. pseudomallei fliC* promoter, $TCAAGTTN_{12}GCCGAAAT$, closely resembled the σ^F consensus promoter (Fig. 3). These results suggest that *fliC* is probably transcribed by RNA polymerase containing the alternative sigma factor σ^F.

Relative virulence of 1026b and MM36 in two animal models of acute *B. pseudomallei* infection. We have previously shown that polyclonal antiflagellin antisera obtained from rabbits immunized with purified flagellin passively protected diabetic rats from an intraperitoneal challenge with *B. pseudomallei* (11). Our interpretation of this result is that motility and/or the presence of a flagellum may be important virulence determinants of this organism and that the flagellum may serve as a protective antigen. We investigated the role of flagella and motility in the pathogenesis of *B. pseudomallei* in two animal models of acute *B. pseudomallei* infection, Syrian hamsters (10)

and diabetic rats (73). The LD_{50}s of 1026b were <2 in Syrian hamsters (2 days) and 2×10^4 in diabetic rats (7 days). These LD_{50}s were similar to those reported previously (10, 73). The LD_{50}s of MM36 were 8 in Syrian hamsters and 8×10^3 in diabetic rats. Thus, the 1026b and MM36 LD_{50} results were not significantly different in either animal model of infection. The animals in both groups died of a distemper-like illness and were found to be bacteremic at the time of death. MM36 was reisolated from the blood of several infected animals and was found to be nonmotile and Tc^r, suggesting that the Tn*5*-OT182 integration in MM36 was stable in the absence of selection. We conclude that flagella and/or motility are probably not *B. pseudomallei* virulence determinants in these experimental animal models of infection.

Phylogenetic analysis based on flagellin sequences. The flagellin encoded by *B. pseudomallei fliC* has considerable homology with other bacterial flagellins, especially in the N- and C-terminal regions. These conserved regions are important for the export and polymerization of flagellin (30). Flagellins also contain an internal domain that is of variable length and amino acid composition. This domain is surface exposed and contains the flagellar antigenic determinants (23, 32). There are approximately 100 flagellin structural genes currently in the sequence databases. We aligned the amino acid sequences of 44 flagellins from a variety bacterial species by using the program PILEUP (20). The highly variable internal domain was edited out of the alignment before DISTANCES and GROWTREE were used to calculate a distance matrix and create a phylogenetic tree (20). In general, the phylogenetic tree based on the N- and C-terminal flagellin sequences was consistent with the prokaryotic phylogeny based on 16S rRNA sequences (Fig. 6) (53). The location of *B. pseudomallei* on the phylogenetic tree was distinct from those of *Pseudomonas aeruginosa* and *P. putida*, which supports the transfer of this organism from the genus *Pseudomonas* to the new genus *Burkholderia* (74). Based on 16S rRNA sequence data, *B. pseudomallei* is a member of the beta subdivision of the proteobacteria group. The only other member of the proteobacteria, beta subdivision, in Fig. 6 is *Bordetella bronchiseptica*. It is interesting that *B. bronchiseptica* was localized to the grouping with the *Enterobacteriaceae* (proteobacteria, gamma subdivision) (Fig. 6). In a previous report, Akerley and Miller pointed out the similarity of the *B. bronchiseptica* flagellin to those of *E. coli* and *S. typhimurium* (1). These authors also mentioned that the *S. typhimurium* flagellin was more similar in amino acid sequence to the *B. bronchiseptica* flagellin than to the *E. coli* flagellin. The phylogenetic tree depicted in Fig. 6 supports these findings.

DISCUSSION

The transposon mutagenesis procedure described in this report should facilitate the genetic analysis of *B. pseudomallei* and allow the identification and characterization of potential virulence determinants. In addition to the motility genes described here, we have used this transposon to identify *B. pseudomallei* genes responsible for secretion of exoproducts, serum resistance, invasion of A549 cells, and aminoglycoside resistance (19). The inability of some *B. pseudomallei* strains to be mutagenized by Tn*5*-OT182 may be due to inefficient transfer of pOT182 to recipient cells as a result of restriction systems, surface exclusion phenomena, or lack of essential envelope functions (18). We found that mutagenesis was most efficient with *B. pseudomallei* 1026b. This strain was isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue, and spleen involvement. It has all of the typical phenotypic characteristics of *B. pseudomallei* (10) and thus should be useful for future studies.

We identified 28 unique motility mutants containing Tn*5*-OT182 integrations in a variety of genes encoding proteins with homology to flagellar and chemotaxis proteins in *E. coli* and *S. typhimurium* (Table 2) (9). This strongly suggests that flagellar synthesis and operation in *B. pseudomallei* is similar to that found in these organisms. In addition, the *B. pseudomallei* genes *cheAW* and *fliPQR* are linked and have the same transcriptional orientation as the corresponding genes in *E. coli* and *S. typhimurium*. These genes may also be regulated similarly since *B. pseudomallei fliC* contains a σ^F-like promoter that resembles the promoter upstream of the *E. coli* and *S. typhimurium* flagellin structural genes (Fig. 3) (27). When SSSRs were performed on cloned DNA from MM1, MM9, MM18, MM24, MM37, and MM39, no homologous proteins were identified with blastx. The Tn*5*-OT182 integrations in these mutants may be in regulatory or intergenic regions and may have polar effects on the expression of adjacent motility genes. Alternatively, these mutants may have Tn*5*-OT182 integrations in novel motility genes. In MM26, the transposon integrated within a gene encoding a protein that was homologous to GalU, an enzyme required for UDP-glucose synthesis (69). In *E. coli*, UDP-glucose is a glycosyl donor for cell envelope polysaccharide synthesis, including lipopolysaccharide and capsular polysaccharide (69). In *E. coli, galU* mutants exhibit decreased levels of flagellin mRNA and protein compared to parental strains (33a). It is currently unknown if the *B. pseudomallei* GalU homolog is important for motility or if the lack of motility in MM26 is due to a polar effect of Tn*5*-OT182 on an adjacent motility gene.

Upstream of *fliC* we identified a divergently transcribed gene that encodes a protein with homology to the ribosomal protein S21 from several bacterial species. This gene (*rpsU*) is usually found as the first gene in an operon that also contains *dnaG* and *rpoD*, the genes that encode primase and σ^{70}, respectively (67). The *rpsU-dnaG-rpoD* operon is termed the macromolecular synthesis (MMS) operon because the gene products are responsible for the initiation of protein, DNA, and RNA synthesis. The gene order of the MMS operon is conserved in many gram-negative bacteria (67). Since the 2.0-kb *Hin*dIII-*Eco*RI fragment that we sequenced in this study only contains 4 bp downstream of *rpsU*, we do not know if the MMS operon is conserved in *B. pseudomallei*. The genetic organization described here is unique; however, as in *E. coli*, *S. typhimurium*, and *Proteus mirabilis*, *fliD* is found upstream of *fliC* (7, 9).

We investigated the expression of *fliC* during bacterial growth in two strains that contain *fliC-lacZ* transcriptional fusions separated by 462 bp. We found that as the bacterial cultures approached the late logarithmic phase of growth, the expression of the upstream *fliC-lacZ* fusion in MM35 was 1.5-fold greater than the expression of the downstream *fliC-lacZ* fusion in MM36 (Fig. 4). This observation suggests the presence of a latent intragenic transcriptional terminator in the 462-bp region separating the *fliC-lacZ* fusions. Previous studies have shown that intragenic transcriptional terminators are present in some *E. coli* genes and that they may play important roles in preventing the synthesis of unused transcripts during metabolic stress or starvation (56). Intragenic terminators are generally dependent on rho factor and do not contain a recognizable consensus sequence. A current model suggests that rho may access rho binding sites in newly transcribed RNA only when transcription and translation become uncoupled (56). Under such conditions, rho may prevent the transcription of RNA by RNA polymerase by displacing it from the DNA

template. Our current hypothesis is that there is a rho factor binding site in the aforementioned 462-bp region of *fliC* that is recognized by rho factor as the bacterial culture enters the late logarithmic phase of growth. The production of flagella is relatively costly metabolically, requiring approximately 2% of biosynthetic energy expenditure (48). It may be more feasible metabolically to prevent the synthesis of unused transcripts under such circumstances and use energy stores more prudently. Further studies are required to fully understand this interesting observation.

In this study, we found that there was no significant difference in the virulence of MM36 (*fliC*) and 1026b (wild-type) in diabetic rats or Syrian hamsters. On the other hand, we previously demonstrated that polyclonal rabbit antisera raised against purified flagellin passively protected diabetic rats from challenge with *B. pseudomallei* (11). Taken together, these results indicate that while flagella and/or motility are probably not virulence determinants in these animal models of infection, purified flagellin may still serve as a protective immunogen against *B. pseudomallei* infection.

The N- and C-terminal domains of *B. pseudomallei* FliC are homologous to the corresponding regions of other flagellar subunit proteins. We used these conserved flagellin domains to perform a phylogenetic analysis on a variety of motile bacteria. In general, the phylogenetic tree is similar to that generated by using 16S rRNA sequences (53). The phylogenetic tree presented in Fig. 6 supports the transfer of *B. pseudomallei* from the genus *Pseudomonas* to *Burkholderia* (74). Once more *Burkholderia* species flagellin gene sequences become available, it will be interesting to see where they fit on this tree.

ACKNOWLEDGMENTS

This work was supported by the Canadian Bacterial Diseases Network of Centers of Excellence and by Canadian Department of National Defense contract W7702-5-R512/01-XSG. D.D. is the recipient of an Alberta Heritage Foundation for Medical Research Fellowship award.

We thank Ron Read for critically reading the manuscript.

REFERENCES

1. **Akerley, B. J., and J. F. Miller.** 1993. Flagellin gene transcription in *Bordetella bronchiseptica* is regulated by the BvgAS virulence control system. J. Bacteriol. **175:**3468–3479.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Arevalo-Jimenez, J. I., M. Williams, K. Marks, and G. M. Ihler.** 1993. Unpublished data. GenBank accession number L20677.
4. **Ashdown, L. R., and J. M. Koehler.** 1990. Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei*. J. Clin. Microbiol. **28:**2331–2334.
5. **Bangsborg, J., and P. Hindersson.** 1995. Unpublished data. GenBank accession number X84699.
6. **Behammer, W., Z. Shao, W. Mages, R. Rachel, K. O. Stetter, and R. Schmitt.** 1995. Flagellar structure and hyperthermophily: analysis of a single flagellin gene and its product in *Aquifex pyrophilus*. J. Bacteriol. **177:**6630–6637.
7. **Belas, R., and D. Flaherty.** 1994. Sequence and genetic analysis of multiple flagellin-encoding genes from *Proteus mirabilis*. Gene **148:**33–41.
8. **Berg, D. E.** 1989. Transposon Tn*5*, p. 185–210. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
9. **Blair, D. F.** 1995. How bacteria sense and swim. Annu. Rev. Microbiol. **49:**489–522.
10. **Brett, P. J., D. DeShazer, and D. E. Woods.** Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. Epidemiol. Infect., in press.
11. **Brett, P. J., D. C. Mah, and D. E. Woods.** 1994. Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. Infect. Immun. **62:** 1914–1919.
12. **Burton, Z. F., C. A. Gross, K. K. Watanabe, and R. R. Burgess.** 1983. The operon that encodes the sigma subunit of RNA polymerase also encodes ribosomal protein S21 and DNA primase in *E. coli* K12. Cell **32:**335–349.
13. **Champion, C. I., J. N. Miller, M. A. Lovett, and D. R. Blanco.** 1990. Cloning, sequencing, and expression of two class B endoflagellar genes of *Treponema pallidum* subsp. *pallidum* encoding the 34.5- and 31.0-kilodalton proteins. Infect. Immun. **58:**1697–1704.
14. **Chaowagul, W., N. J. White, D. A. Dance, Y. Wattanagoon, P. Naigowit, T. M. Davis, S. Looareesuwan, and N. Pitakwatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. **159:**890–899.
15. **Dance, D. A., V. Wuthiekanun, P. Naigowit, and N. J. White.** 1989. Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API 20NE. J. Clin. Pathol. **42:**645–648.
16. **Dance, D. A. B.** 1996. Melioidosis, p. 925–930. *In* G. C. Cook (ed.), Manson's tropical diseases, 20th ed. W. B. Saunders Company Ltd., London, England.
17. **Davis, J. M., J. Mayor, and L. Plamann.** 1995. Unpublished data. GenBank accession number U20669.
18. **De Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stabile phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
19. **DeShazer, D., P. J. Brett, A. L. Jones, R. A. Moore, and D. E. Woods.** Unpublished data.
20. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
21. **Dons, L., O. F. Rasmussen, and J. E. Olsen.** 1992. Cloning and characterization of a gene encoding flagellin of *Listeria monocytogenes*. Mol. Microbiol. **6:**2919–2929.
22. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
23. **Gassmann, G. S., E. Jacobs, R. Deutzmann, and U. B. Gobel.** 1991. Analysis of the *Borrelia burgdorferi* GeHo *fla* gene and antigenic characterization of its gene product. J. Bacteriol. **173:**1452–1459.
24. **Givaudan, A., A. Lanois, and N. Boemare.** 1995. Unpublished data. GenBank accession number X91047.
25. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172:**1853–1860.
26. **Harshey, R. M., G. Estepa, and H. Yanagi.** 1989. Cloning and nucleotide sequence of a flagellin-coding gene (*hag*) from *Serratia marcescens* 274. Gene **79:**1–8.
27. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. **5:**2875–2882.
28. **Helmann, J. D., and M. J. Chamberlain.** 1987. DNA sequence analysis suggests that expression of flagellar and chemotaxis genes in *Escherichia coli* and *Salmonella typhimurium* is controlled by an alternative sigma factor. Proc. Natl. Acad. Sci. USA **84:**6422–6424.
29. **Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Luck, K. H. Mann, R. Marre, M. Ott, and J. Hacker.** 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infect. Immun. **63:**2499–2507.
30. **Homma, M., H. Fujita, S. Yamaguchi, and T. Iino.** 1987. Regions of *Salmonella typhimurium* flagellin essential for its polymerization and excretion. J. Bacteriol. **169:**291–296.
31. **Jones, A. L., T. J. Beveridge, and D. E. Woods.** 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect. Immun. **64:**782–790.
32. **Joys, T. M.** 1985. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparison with other flagellins. J. Biol. Chem. **260:**15758–15761.
33. **Kapatral, V., and S. A. Minnich.** 1995. Co-ordinate, temperature-sensitive regulation of the three *Yersinia enterocolitica* flagellin genes. Mol. Microbiol. **17:**49–56.

33a. **Komeda, Y., T. Icho, and T. Iino.** 1977. Effects of *galU* mutation on flagellin formation in *Escherichia coli*. J. Bacteriol. **129:**908–915.

34. **Koopman, M. B., E. Baats, O. S. de Leeuw, B. A. van der Zeijst, and J. G. Kusters.** 1993. Molecular analysis of a flagellar core protein gene of *Serpulina* (*Treponema*) *hyodysenteriae*. J. Gen. Microbiol. **139:**1701–1706.
35. **Kuwajima, G., J. Asaka, T. Fujiwara, T. Fujiwara, K. Node, and E. Kondo.** 1986. Nucleotide sequence of the *hag* gene encoding flagellin of *Escherichia coli*. J. Bacteriol. **168:**1479–1483.
36. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol. Microbiol. **6:**2863–2874.
37. **Limberger, R. J., L. L. Slivienski, D. B. Yelton, and N. W. Charon.** 1992. Molecular genetic analysis of a class B periplasmic-flagellum gene of *Treponema phagedenis*. J. Bacteriol. **174:**6404–6410.
38. **Logan, S. M., T. J. Trust, and P. Guerry.** 1989. Evidence for posttranslational

modification and gene duplication of *Campylobacter* flagellin. J. Bacteriol. **171:**3031–3038.
39. **Lovgren, A., M. Y. Zhang, A. Engstrom, and R. Landen.** 1993. Identification of two expressed flagellin genes in the insect pathogen *Bacillus thuringiensis* subsp. *alesti*. J. Gen. Microbiol. **139:**21–30.
40. **Madden, T. L., R. L. Tatusov, and J. Zhang.** 1996. Applications of network BLAST server. Methods Enzymol. **266:**131–141.
41. **Martin, J. H., and D. C. Savage.** 1988. Cloning, nucleotide sequence, and taxonomic implications of the flagellin gene of *Roseburia cecicola*. J. Bacteriol. **170:**2612–2617.
42. **Merriman, T. R., and I. L. Lamont.** 1993. Construction and use of a self-cloning promoter probe vector for gram-negative bacteria. Gene **126:**17–23.
43. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Milton, D. L., R. O'Toole, P. Horstedt, and H. Wolf-Waltz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178:**1310–1319.
45. **Mirel, D. B., and M. J. Chamberlin.** 1989. The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the sigma 28 form of RNA polymerase. J. Bacteriol. **171:**3095–3101.
46. **Mitchison, M., J. I. Rood, S. Faine, and B. Adler.** 1991. Molecular analysis of a *Leptospira borgpetersenii* gene encoding an endoflagellar subunit protein. J. Gen. Microbiol. **137:**1529–1536.
47. **Moens, S., K. Michiels, V. Keijers, F. Van Leuven, and J. Vanderleyden.** 1995. Cloning, sequencing, and phenotypic analysis of *laf1*, encoding the flagellin of the lateral flagella of *Azospirillum brasilense* Sp7. J. Bacteriol. **177:**5419–5426.
48. **Moens, S. M., and J. Vanderleyden.** 1996. Functions of bacterial flagella. Crit. Rev. Microbiol. **22:**67–100.
49. **Mongkolsuk, S., S. Rabibhadana, P. Vattanaviboon, and S. Loprasert.** 1994. Generalized and mobilizabile positive-selective cloning vectors. Gene **143:**145–146.
50. **Mytelka, D. S., and M. J. Chamberlin.** 1996. *Escherichia coli fliAZY* operon. J. Bacteriol. **178:**24–34.
51. **Noppa, L., N. Burman, A. Sadziene, A. G. Barbour, and S. Bergstrom.** 1995. Expression of the flagellin gene in *Borrelia* is controlled by an alternative sigma factor. Microbiology **141:**85–93.
52. **Nuijten, P. J., F. J. van Asten, W. Gaastra, and B. A. van der Zeijst.** 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265:**17798–17804.
53. **Olsen, G. J., C. R. Woese, and R. Overbeek.** 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176:**1–6.
54. **Pallesen, L., and P. Hindersson.** 1989. Cloning and sequencing of a *Treponema pallidum* gene encoding a 31.3-kilodalton endoflagellar subunit (FlaB2). Infect. Immun. **57:**2166–2172.
55. **Pleier, E., and R. Schmitt.** 1989. Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti*. J. Bacteriol. **171:**1467–1475.
56. **Richardson, J. P.** 1991. Preventing the synthesis of unused transcripts by Rho factor. Cell **64:**1047–1049.
57. **Sato, N.** 1994. A cold-regulated cyanobacterial gene cluster encodes RNA-binding protein and ribosomal protein S21. Plant Mol. Biol. **24:**819–823.
58. **Schuster, S. C., M. Bauer, J. Kellermann, F. Lottspeich, and E. Baeuerlein.** 1994. Nucleotide sequence of the *Wolinella succinogenes* flagellin, which contains in the antigenic domain two conserved regions also present in *Campylobacter* spp. and *Helicobacter pylori*. J. Bacteriol. **176:**5151–5155.
59. **Schweizer, H. P., T. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas* spp., p. 229–237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of pseudomonads. American Society for Microbiology, Washington, D.C.
60. **Shaw, C. H., G. J. Loake, A. P. Brown, C. S. Garrett, W. Deakin, G. Alton, M. Hall, S. A. Jones, M. O'Leary, and L. Primavesi.** 1991. Isolation and characterization of behavioral mutants and genes of *Agrobacterium tumefaciens*. J. Gen. Microbiol. **137:**1939–1953.
61. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: tranposon mutagenesis in gram negative bacteria. Biotechnology **1:**784–791.
62. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. Gene **80:**161–169.
63. **Smith, C. J., J. C. Allen, M. N. Embi, O. Othman, N. Razak, and G. Ismail.** 1987. Human melioidosis: and emerging medical problem. MIRCEN J. **3:**343–366.
64. **Suerbaum, S., C. Josenhans, and A. Labigne.** 1993. Cloning and genetic characterization of the *Helicobacter pylori* and *Helicobacter mustelae flaB* flagellin genes and construction of *H. pylori flaA*- and *flaB*-negative mutants by electroporation-mediated allelic exchange. J. Bacteriol. **175:**3278–3288.
65. **Tominaga, A., M. A. Mahmoud, T. Mukaihara, and M. Enomoto.** 1994. Molecular characterization of intact, but cryptic, flagellin genes in the genus *Shigella*. Mol. Microbiol. **12:**277–285.
66. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. J. Bacteriol. **172:**7188–7199.
67. **Versalovic, J., T. Koeuth, R. Britton, K. Geszvain, and J. R. Lupski.** 1993. Conservation and evolution of the *rpsU-dnaG-rpoD* macromolecular synthesis operon in bacteria. Mol. Microbiol. **8:**343–355.
68. **Wahl, S. A., and N. R. Baker.** 1996. Unpublished data. GenBank accession number U54775.
69. **Weissborn, A. C., Q. Liu, M. K. Rumley, and E. P. Kennedy.** 1994. UTP: α-D-glucose-1-phosphate uridylyltransferase of *Escherichia coli*: isolation and DNA sequence of the *galU* gene and purification of the enzyme. J. Bacteriol. **176:**2611–2618.
70. **Whitmore, A., and C. S. Krishnaswami.** 1912. An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon. Indian Med. Gaz. **47:**262–267.
71. **Wilson, K.** 1987. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
72. **Winstanley, C., J. A. Morgan, R. W. Pickup, and J. R. Saunders.** 1994. Molecular cloning of two *Pseudomonas* flagellin genes and basal body structural genes. Microbiology **140:**2019–2031.
73. **Woods, D. E., A. L. Jones, and P. J. Hill.** 1993. Interaction of insulin with *Pseudomonas pseudomallei*. Infect. Immun. **61:**4045–4050.
74. **Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36:**1251–1275.