Identification of two new genes involved in twitching motility in Pseudomonas aeruginosa

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Mu transposition complexes were used for transposon mutagenesis of Pseudomonas aeruginosa strain PA68. Mu DNA transposition complexes were assembled with MuA transposase and an artificial mini-Mu transposon in vitro, and introduced into Pseudomonas aeruginosa by electroporation. Eight mutants deficient in twitching motility were isolated. Southern blotting confirmed that the insertions had occurred as single events. DNA sequencing of the region flanking the insertion in the twitching-motility mutants revealed that the mini-Mu transposon had inserted into six different genes, PAO171, PA1822, PAO413, PA4959, PA4551 and PA5040. Four of these have previously been proven to be needed for twitching motility, whereas the PA1822 and PAO171 genes have not previously been shown to be required for twitching motility. The twitching-motility defect in the PA1822 mutant was partially complemented by providing the PA1822 gene in trans, and the defect in the PAO171 mutant was fully complemented when PAO171 was provided. A PAO171 mutant and a PA1822 mutant were constructed by gene replacement in the P. aeruginosa PA01 strain. These mutants were deficient in twitching motility, showing that both the PA1822 and the PAO171 gene are involved in twitching motility.

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen of humans. It usually causes persistent infection in immune-compromised patients (Simpson & Speert, 2000). Lower respiratory tract infections of bronchiectasis patients by P. aeruginosa are particularly problematic. Once acquired, it is extremely difficult to eradicate the organism from the bronchus or the lungs.

The colonization and establishment of infection by P. aeruginosa are dependent on the production of a number of virulence factors, including lipases, proteases, exopolysaccharides, alkaline phosphatases and type IV pili (Beatson et al., 2002). Type IV pili are flexible surface filaments about 6 nm in diameter produced at the poles of the bacterial cell (Beatson et al., 2002; Mattick, 2002); they are essential for the attachment of the pathogen to host epithelial tissues and also mediate a form of surface translocation known as twitching motility (Bradley, 1980; Mattick, 2002). Twitching motility has been shown to be required for the initial attachment and development of a biofilm by P. aeruginosa (O’Toole & Kolter, 1998; Costerton et al., 1999; Mcbride, 2001; Whiteley et al., 2001; Whitchurch et al., 2002). Once a biofilm is developed, cells growing in the biofilm can become 10–1000 times more resistant to the effects of antibiotics than their planktonic counterparts (Mah & O’Toole, 2001). Biofilm bacteria embedded in an extracellular polymeric matrix cannot be eradicated even with the most aggressive antibiotics. Mutants that either lack type IV pili or are twitching-motility deficient show loss of ability for biofilm initiation (O’Toole & Kolter, 1998) and reduced infectivity (Kang et al., 1997; Whitchurch et al., 2002).

P. aeruginosa pili are polymers of a single gene product, called PilA or pilin (Mattick, 2002), but their assembly and function require the products of almost 40 additional genes (Alm & Mattick, 1997; Mattick, 2002). The regulation of twitching motility, and the role and the signals of the signal transduction systems involved are still obscure.

Because twitching motility is crucial for systemic infection and biofilm formation inside a host, identification of genes involved in twitching motility may be helpful to develop new drugs in the future, which will help the host to eradicate P. aeruginosa more readily. In this study, we created a transposon mutant pool using Mu transposition complexes in P. aeruginosa PA68, and isolated mutants.
deficient in twitching motility. Analysis of these transposon insertion mutants identified two new genes required for twitching motility.

METHODS

Bacterial strains, plasmids and media. PA68 was the initial strain isolated from the sputum of a patient with bronchiectasis by bacterial culture using LB agar in the Second Teaching Hospital, Tianjin Medical University in 1995 (Shan et al., 2004). Randomly amplified polymorphic DNA (RAPD) analysis of subsequent isolates from this patient indicated that this strain had chronically colonized this patient for at least eight years (data not shown). All bacterial strains and plasmids are listed in Table 1. The bacteria were cultured in Luria broth medium (10 g tryptone l$^{-1}$, 5 g yeast extract l$^{-1}$, 5 g NaCl l$^{-1}$), adjusted to pH 7.0 with NaOH) or on LB agar (1.5%) plates, with or without antibiotics. The antibiotics used were as follows: for *Escherichia coli*, 100 μg ampicillin ml$^{-1}$, 30 μg kanamycin ml$^{-1}$, 50 μg tetracycline ml$^{-1}$ and 10 μg gentamicin ml$^{-1}$ for *P. aeruginosa*, 50 μg kanamycin ml$^{-1}$, 200 μg tetracycline ml$^{-1}$ and 30 μg gentamicin ml$^{-1}$.

Enzymes and chemicals. T4 DNA ligase, *Taq* DNA polymerase, all restriction enzymes and DNA molecular mass markers were purchased from TaKaRa; tryptone, yeast extract and granulated agar were purchased from Difco. An artificial mini-Mu transposon (Km–Mu) used in this study is defined as a segment of DNA that contains 50 bp of Mu R-end DNA as inverted repeats at each end, and a kanamycin resistance gene between the two ends; it was used for selection of transformants. A random insertion library was constructed by using Mu DNA transposition complexes, or MuA transpososomes, which were assembled with artificial mini-Mu transposons and MuA transposase in vitro and analysed by agarose gel electrophoresis, as previously reported (Lamberg et al., 2002). The MuA transpososomes were introduced into *P. aeruginosa* PA68 by electroporation. Mutants were selected on LB agar plates containing 50 μg kanamycin ml$^{-1}$.

Electroporation. Electroporant cells were prepared as described by Smith & Iglewski (1989). Electroporation was carried out at the following settings: capacitance, 25 μF; electrical field strength, 13 kV cm$^{-1}$; resistance, 200 Ω. DNA used for electroporation was prepared by the alkaline lysis procedure (Sambrook et al., 1989). For the construction of a mini-Mu transposon insertion library, 50–100 ng MuA transpososomes were electroporated into electrocompetent *P. aeruginosa* PA68 cells. For gene replacement experiments, the

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
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<tr>
<td><em>Escherichia coli</em></td>
<td>hsdR recA lacZYA Φ80 lacZ ΔM15</td>
<td>Sino-American</td>
</tr>
<tr>
<td>PA01</td>
<td>Laboratory strain, motile</td>
<td>Jin, S</td>
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<tr>
<td>PA68</td>
<td>Clinical strain, motile</td>
<td>This study</td>
</tr>
<tr>
<td>K2</td>
<td>PA68 PA1822::Mu (Km$^+$), non-motile</td>
<td>This study</td>
</tr>
<tr>
<td>K2-1822</td>
<td>K2 with plasmid pDN18F (Km$^+$, Tc$^-$), motile</td>
<td>This study</td>
</tr>
<tr>
<td>PA01-200</td>
<td>PA01 PA1822::Gm$^+$, non-motile</td>
<td>This study</td>
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<td>PA68 PA0171::Mu (Km$^+$), non-motile</td>
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<td>C54 with plasmid pDN18S (Km$^+$, Tc$^-$), motile</td>
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<tr>
<td>PA01-NS</td>
<td>PA01 PA0171::Gm$^+$, non-motile</td>
<td>This study</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
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<tr>
<td>Plasmid</td>
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<td>PCR cloning vector, Ap$'$, LacZ$'$</td>
<td>TakaRa</td>
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<td>pMD18 containing a 0–6 kb PCR product with the complete PA0171 gene</td>
<td>This study</td>
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<td>pUC7G</td>
<td>Gm$'$ cassette excisable with restriction enzymes PstI, Sall, BamHI and EcoRI</td>
<td>Jin, S</td>
</tr>
<tr>
<td>pDN18</td>
<td>Broad-host-range plasmid, IncP, Tc$'$</td>
<td>Jin, S</td>
</tr>
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<td>pDN18 containing a 0–6 kb PCR product with the complete PA0171 gene in a <em>BamHI</em>/HindIII site</td>
<td>This study</td>
</tr>
<tr>
<td>pDN18F</td>
<td>pDN18 containing a 1–7 kb PCR product with the complete PA1822 gene in a <em>BamHI</em> site in positive orientation relative to T7 promoter</td>
<td>This study</td>
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<td>pMD18Sgp</td>
<td>pMD18S with a gentamicin-resistance gene inserted in the Ndel site of the PA0171 gene</td>
<td>This study</td>
</tr>
<tr>
<td>PUC18SFGp</td>
<td>pUC18S with a gentamicin-resistance gene inserted in the PstI site of the 0–6 kb PA1822 fragment</td>
<td>This study</td>
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</table>

Table 1. Bacterial strains and plasmids used in this study

PA68 was twitching motility. Analysis of these transposon insertion mutants identified two new genes required for twitching motility.

Construction of a mini-Mu insertion library in *P. aeruginosa* strain PA68. The artificial mini-Mu transposon (Km–Mu) used in this study is defined as a segment of DNA that contains 50 bp of Mu R-end DNA as inverted repeats at each end, and a kanamycin resistance gene between the two ends; it was used for selection of transformants. A random insertion library was constructed by using Mu DNA transposition complexes, or MuA transpososomes, which were assembled with artificial mini-Mu transposons and MuA transposase in vitro and analysed by agarose gel electrophoresis, as previously reported (Lamberg et al., 2002). The MuA transpososomes were introduced into *P. aeruginosa* PA68 by electroporation. Mutants were selected on LB agar plates containing 50 μg kanamycin ml$^{-1}$.

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plasmid DNA was linearized by a restriction enzyme (Arora et al., 2000), and about 2 μg linear plasmid DNA was electropropored into electrocompetent P. aeruginosa PA01 cells. For complementation experiments, 50–100 ng supercoiled or covalently closed, circular plasmid DNA was electropropored into the target strains.

**Twitching motility assay.** Twitching motility was assayed as described by Semmler et al. (1999). Briefly, cells were stab inoculated with a toothpick through a thin (approximately 3 mm) Difco LB agar (1% Difco granulated agar) layer to the bottom of the Petri dish. After overnight growth at 37°C, the zone of twitching motility between the agar and Petri dish interface was visualized by staining with Coomassie Brilliant Blue R250 (Sino-American Biotechnology).

**Recombinant DNA techniques and sequence analysis.** The preparation of plasmid and genomic DNA, restriction endonuclease digestion and ligation reactions were carried out using standard protocols (Sambrook et al., 1989). Briefly, genomic DNA of each twitching-motility-deficient mutant was digested with BamHI (there is no BamHI site in the artificial mini-Mu transposon), generating a fragment with a transposon attached to its genomic DNA flanks. These fragments were then cloned into the BamHI site of pUC18. DNA sequences of transposon borders were determined from these recombinant plasmids by using transposon-specific primers reading sequences outwards from within the transposon. The primers used for DNA sequencing were primer1 (5'-GCAACTGTCCATACTCTGA-3') and primer2 (5'-CCGTGGGTTTATGTCGCA-3'). DNA sequencing was performed by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd (Sangon). More than 500 nucleotides were sequenced on each flank of the insertion. The genomic locations of mini-Mu insertions were identified by using the flank sequences to do a BLAST search in the P. aeruginosa genome database (www.pseudomonas.com), or at the National Center for Biotechnology Information servers (www.ncbi.nlm.nih.gov). Domain analysis of genes was performed using SMART (http://smart.embl-heidelberg.de/) (Schultz et al., 2000) and the Pfam database (http://pfam.wustl.edu/) (Bateman et al., 2000). Function analysis of genes was performed using the Pseudomonas aeruginosa Community Annotation Project (PseudoCAP) (http://www.pseudomonas.com/GenomeSearchU.asp).

**Southern blotting.** Chromosomal DNA isolated from twitching-defective mutants was digested with restriction enzymes, and electrophoretically separated in 0.8% agarose gels; DNA was transferred onto a positively charged nylon membrane (Sino-American) and fixed by UV cross-linking. Southern analysis was performed using a PCR-generated 700 bp Km–Mu fragment as a probe. All probes were labelled with [32P]dCTP (Beijing Yahui Biotechnology Company) using a random primer labelling kit (TaKaRa) according to the manufacturer's instructions.

**PCR amplification and primers.** PCR was performed in a DNA Petitre Thermal Cycler PTC-200 (MJ-research) to obtain specific amplification products. The reactions were performed in a final volume of 25 μl. Each reaction mixture contained 20 ng DNA template, 1-25 U LA Taq polymerase (TaKaRa), 1-5 mM MgCl2, 0-1 mM deoxynucleoside triphosphates mix and 0-2 μM primers. Thirty cycles were run, each consisting of incubation for 1 min at 94°C, 1 min at 55°C, and 1–4 min at 72°C (depending on the length of the amplified segments). The primers used for PCR amplification were purchased from Sangon. Restriction enzyme recognition sites were added to the ends of primers (shown in bold type, below) to facilitate subsequent cloning of the PCR products if desired. Additional nucleotides were added to the 5' ends (shown in italic) to ensure efficient cleavage. The following primers were used for PCR. Primer S1 (5'-AAGCTTCTTCTTAAACACGGGCG-3'), with a HindIII site, was used as the 5' end primer, and primer S2 (5'-GGATCCCATCCTGATCTCGACTC-3'), with a BamHI site, was used as the 3' end primer to amplify the complete PA0171 gene from P. aeruginosa PA68 or PA01. Primer F1 (5'-TCCAAAGGATCCGGAGGGCGCTATAACC-3'), with a BamHI site, and primer F2 (5'-CCCCAGGATCCCATCAGGGGCCGACCG-3'), with a BamHI site, were used for the amplification of the complete PA1822 gene from P. aeruginosa PA68. Forward primer FPI (5'-GGATCCCTCTTCGCTCAAGAAGCTTGCT-3') (BamHI site in bold) and the reverse primer FP2 (5'-AACGTTGCTTGGCTGCCATTGGATT-3') (HindIII site in bold) were used to amplify a 620 bp PA1822 segment from P. aeruginosa PA01. This fragment contains only one Psl site, while the complete PA1822 gene contains four Psl sites, which facilitated subsequent plasmid construction for insertional inactivation of the PA1822 gene. Primer Mu1 (5'-GGCGGTGATCTCAATAGA-3') and primer Mu2 (5'-TCCCAACAGCTTATATACCT-3') were used for amplification of a 700 bp mini-Mu transposon fragment from the plasmid carrying the artificial mini-Mu transposon. This fragment was used as probe in Southern hybridization.

**Electron microscopy.** Carbon-coated copper grids were gently placed on the surface of the colony grown after 12 to 15 h at 37°C on LB agar plates; after 1 min, the grids were carefully removed, rinsed twice with distilled water, and stained with 1% phosphotungstic acid. The negatively stained cells were visualized with a Philips EM400-ST transmission electron microscope.

**Construction of plasmids and mutant strains.** A 593 bp amplification product was obtained by PCR using primers S1 and S2 with PA01 genomic DNA as template. This fragment, containing the complete PA0171 gene, was cloned to the PCR cloning vector pMD18, yielding pMD18S. pMD18S was linearized at the unique Ncol site present in the PA0171 gene, and a gentamicin-resistance gene cassette excised from pUC7G and blunt-ended by a fill-in reaction was inserted at that site, leading to the construction of pMD18SGm. This plasmid was utilized to generate a chromosomal mutation in the P. aeruginosa PA0171 gene by marker exchange in strain PA01. Using the same strategy, a 620 bp PA1822 fragment was obtained by PCR with the primers FP1 and FP2, with PA01 genomic DNA as template, and cloned into vector pMD18. This PA1822 fragment was subcloned into BamHI/HindIII site in pUC18, which removed a Psl site in pUC18, yielding pUC18FP. A gentamicin-resistance gene cassette excised from pUC7G was inserted into the unique Psl site in the PA1822 fragment, generating pUC18FPGm. This construct, which cannot replicate in P. aeruginosa, was used to generate a chromosomal mutant of PA01 PA1822 by gene replacement. Plasmid pDN18S, which was obtained by cloning a 593 bp PCR fragment carrying the complete PA68 PA0171 gene using primers S1 and S2 into the BamHI and HindIII sites of the broad-host-range vector pDN18, was used for complementation of PA68 PA0171 mutant C54. Plasmid pDN18F, used for complementation of the PA1822 mutation in K2, was constructed by cloning a 1733 bp PCR fragment containing the PA68 PA1822 gene into the BamHI site of pDN18 in positive orientation relative to the T7 promoter. This fragment was amplified by PCR using primers F1 and F2 from the chromosomal DNA of PA68. Plasmids pDN18S and pDN18F were also used as templates for DNA sequencing of the PA68 PA0171 and PA1822 genes, respectively.

**RESULTS AND DISCUSSION**

**Strain identification, construction of a mini-Mu insertion library of the PA68 strain, and screening for twitching-motility-deficient mutants**

Strain PA68, which was originally isolated from a bronchiectasis patient, was identified to be a P. aeruginosa strain based on physiological characteristics (results not
shown); this was confirmed by its 16S rDNA sequence (GenBank accession number AY521230) in that it was more than 99% identical with sequences from members of the \textit{P. aeruginosa} group.

Mu transpososome mutagenesis is an efficient mutagenesis strategy, and has been used for the functional analysis of some bacterial genomes, such as \textit{E. coli} and \textit{Yersinia enterocolitica} (Lamberg et al., 2002). In this study, MuA transpososomes were electroporated into \textit{P. aeruginosa} PA68 using the protocol described in Methods. High transformation efficiency was achieved (up to $3.66 \times 10^4$ transformants per $\mu$g transposon DNA), and a pool of approximately 6000 Mini-Mu insertion mutants of PA68 was made. These Mu transposon mutants were screened for defects in twitching motility, using the subsurface stab assay. When cells are stabbed through an
(-Mu) in sequence column means mini-Mu transposon with kanamycin-resistance gene. Target site duplications are in bold capital letters. Genetic locations were determined from the comparison sequences results for clinical strain PA68 and the complete PAO1 genome. Gene names, gene numbers and sections are all from PAO1 genome database; the PAO1 genome is composed of 529 sections. The functions of genes PA5040 (pilQ), PA4551 (pilV) and PA4959 (fimX) have been demonstrated in P. aeruginosa (Martin et al., 1993; Alm & Mattick, 1995; Huang et al., 2003). The function of PA0413 (PilL) was proposed based on the presence of conserved amino-acid motifs and structural features (Mattick, 2002). PA0171 and PA1822 (fimL) ORFs encode hypothetical proteins. No function has yet been assigned to them.

<table>
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<th>Mutant</th>
<th>Sequence</th>
<th>Genetic location</th>
<th>Product</th>
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<tr>
<td>A13</td>
<td>GGCCCCGGCCGAGCC(Km-Mu)GAGCCTGGCCCTGTCC</td>
<td>PA0413 (pilL) 7470–7474 39</td>
<td>Probable component of chemotactic signal transduction system</td>
</tr>
<tr>
<td>C68</td>
<td>CCCGCTGGAATAAGG(Km-Mu)ATGGACCGCTATTCG</td>
<td>PA0413 (pilL) 11572–11577 39</td>
<td>Probable component of chemotactic signal transduction system</td>
</tr>
<tr>
<td>F57</td>
<td>GGAAGGCGGCTTGGG(Km-Mu)CTGGCCGTCAGCCGG</td>
<td>PA0413 (pilL) 9540–9544 39</td>
<td>Probable component of chemotactic signal transduction system</td>
</tr>
<tr>
<td>C54</td>
<td>CAGCCATGGCGAGCA(Km-Mu)CAGCAAGTCTCAGGT</td>
<td>PA0171 1012–1016 16</td>
<td>Component of chemotaxis system?</td>
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<tr>
<td>F9</td>
<td>GATACGCCAGCCGC(Km-Mu)GCCGAGGCCGCTCG</td>
<td>PA5040 (pilQ) 5479–5483 478</td>
<td>Type 4 fimbrial biogenesis outer-membrane protein PilQ precursor</td>
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<tr>
<td>G93</td>
<td>CGCCCCAGCCACCTT(Km-Mu)ACCCTCAGCATCGGC</td>
<td>PA4959 (fimX) 11568–11572 469</td>
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</tr>
<tr>
<td>K2</td>
<td>GCTGCGCTGGCCGG(Km-Mu)GGCGATCGGCTGCT</td>
<td>PA1822 (fimL) 10946–10950 169</td>
<td>Signal transduction protein?</td>
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<tr>
<td>L16</td>
<td>CCGCGGACAGACGAGC(Km-Mu)CAGCGGCGAGACCTC</td>
<td>PA4551 (pilV) 1719–1723 430</td>
<td>Type 4 fimbrial biogenesis protein PilV</td>
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</table>

Sequence analysis of the flanking DNA of the inserted mini-Mu transposons

DNA sequencing of the regions flanking the insertions of the twitching mutants isolated revealed that the sequences of the clinical strain PA68 obtained were 97–99% identical compared to the corresponding sequences of P. aeruginosa PAO1. It has been reported that Mu transposition produces a 5 bp target-site duplication in the host DNA flanking the insertion (Allet, 1979; Lamberg et al., 2002). Such duplications were also identified in the twitching mutants isolated (Table 2). In the eight twitching mutants, the mini-Mu transposon had inserted into six different genes. Three insertions were in gene PA0413, and only one insertion had occurred in PA0171, PA1822, PA4050, PA4551 and PA4959 (Table 2; gene numbers are from the Pseudomonas Genome Project). DNA sequence analysis suggested that PA5040 (gene name, pilQ) encodes a precursor of PilQ, which is an outer-membrane protein for type 4 fimbrial biogenesis (Martin et al., 1993); PA4551 (gene name, pilV) encodes a type 4 fimbrial biogenesis protein, PilV (Alm & Mattick, 1995); PA0413 (alternative name, pilL or chpA) lies within a

Fig. 1. Stab assay for twitching motility of P. aeruginosa. Cells from an overnight LB agar (1·5 % w/v) plate were inoculated with a toothpick to the bottom of the LB agar (1 % granulated agar, w/v) plate and incubated for 24 h at 37 °C. The twitching zone of different strains obtained at the interstitial surface of the agar and the plate was visualized after removing the agar layer and staining with Coomassie Brilliant Blue R 250. (A) Screening twitching-motility-deficient mutants. WT, Wild-type strain PA68; 1–8, twitching motility deficient mutants A13, C54, C68, F9, F57, G93, K2 and L16. (B) Trans-complementation of PA0171 mutant C54 (i) and PA1822 mutant K2 (ii). (i) 1, wild-type strain PA68; 2, PA0171 mutant C54; 3, C54 complemented with pDN18S (carrying the PA0171 gene); 4, C54 containing vector control pDN18B. (ii) 1, Wild-type PA68; 2, PA1822 mutant K2; 3, K2 complemented with pDN18F (carrying the PA1822 gene); 4, K2 containing vector control pDN18B. (C) Twitching-motility assay of P. aeruginosa PAO1 and its derivatives. 1, PAO1; 2, PAO1 PA1822 mutation strain PAO1-NF; 3, PAO1 PA0171 mutation strain PAO1-NS.
gene cluster predicted to encode a chemosensory phospho-transfer signal transduction (chp) system; this cluster is homologous to the frz cluster from Myxococcus xanthus that is involved in controlling twitching motility (Mattick, 2002). The
chp system is similar to, but substantially more complex than, the chemotactic (che) system, which controls flagella rotation in swimming motility in a variety of bacteria (Ward & Zusman, 1997; Wall & Kaiser, 1999; Mattick, 2002). The
PA0413 gene encodes a very large protein (2742 amino acids). Domain analysis indicated that this gene probably encodes a component of the chp system (Mattick, 2002), but there was no strong experimental evidence; PA4959 (alternative name, fimX) was recently proven to be involved in motility and attachment (Huang et al., 2003). The functions of PA0171 and PA1822 (alternative name, fimL) (PseudoCAP) were not reported previously.

Complementation of PA0171 and PA1822

The plasmids carrying the genes PA0171 (pDN18S) or PA1822 (pDN18F), and their vector controls without the insert (pDN18), were electroporated into the PA0171 and PA1822 mutant strains C54 and K2, respectively. The resulting strains were tested for twitching motility. The assays showed that the non-motile C54 mutant had twitching motility restored by pDN18S essentially to wild-type levels (Fig. 1B). Full complementation was not achieved with the PA1822 gene in PA1822 mutant strain K2, as twitching motility of the K2 mutant was only partially restored by plasmid pDN18F (Fig. 1B). When the longer trans-complementation time was used, a larger twitching zone could be observed, while the phenotype of plasmid control did not change (data not shown). These data suggest that both PA0171 and PA1822 are required for twitching motility. One explanation for the partial complementation of the PA1822 mutant with the pDN18F plasmid could be that the expression was weak and slow.

Construction of PA0171 and PA1822 mutants in the PAO1 strain

PA68 is a clinical P. aeruginosa strain. To confirm that PA0171 and PA1822 are also required for twitching motility in strain PAO1, a PA0171 mutant of strain PAO1 was constructed by gene replacement. The PA01 PA0171 gene located on a 593 bp BamHI/HindIII PCR-generated fragment was inactivated by inserting a Gmr cassette into the unique Nael site in this gene. The insertionally inactivated PA0171 gene on a non-replicating plasmid (pMD18SGm) was introduced into PAO1 by electroporation, where it replaced the corresponding chromosomal copy of the PA0171 gene by double reciprocal recombination, giving rise to a PA0171 mutant strain, PAO1-NS. The replacement of the wild-type PA0171 in PAO1-NS was confirmed by PCR. A 2.4 kb fragment was amplified using primers S1 and S2 combined with PAO1-NS genomic DNA as template (0.6 kb PA0171 gene with a 1.8 kb gentamicin-resistance gene insertion), while a 0.6 kb fragment was amplified using primers S1 and S2, and PAO1 genomic DNA as template. PAO1-NS was non-motile on 1% LB agar (Fig. 1C). The PA1822 mutant strain PAO1-NF was constructed by using the same strategy and it was also deficient in twitching motility (Fig. 1C). These results indicated that PA0171 and PA1822 are also needed for twitching motility in P. aeruginosa PAO1.

Alignment analysis and putative function of PA0171 and PA1822 genes

The complete nucleotide sequences of PA0171 were determined (GenBank accession number AY502957). Alignment of the sequences of this gene in PA68 and PAO1 using BLAST analysis showed that the two sequences are more than 99% identical at the nucleotide level, and 92% identical at the deduced amino acid level. The sequences of the deduced protein of PA0171 show 38% identity with a conserved hypothetical protein of Geobacter sulfurreducens PCA.

PA0171 encodes a relatively short ORF (543 nucleotides). The gene and the putative protein have no significant homology with any gene or protein with known function in P. aeruginosa or other bacteria. However, a chemotaxis-like system, designated chemotaxis gene cluster 4 (Croft et al., 2000), is located upstream of this gene. This chemotaxis system is entirely novel and has no known function (Croft et al., 2000); it is composed of PA0179, PA0178, PA0177,

Fig. 2. Organization of P. aeruginosa PA0171 and its flank genes. Individual ORFs are shown in boxes and their orientation is denoted by arrows. The predicted class of protein encoded by each ORF is indicated within the boxes (Y, CheY; W, CheW). The gene numbers from the P. aeruginosa PA01 genome database (http://www.pseudomonas.com) are indicated below each ORF box. PA0180 to PA0173 compose a putative chemotaxis system of unknown function (Croft et al., 2000). Domain analysis indicates that the predicted product of PA0172 contains a HAMP domain. PA0169 contains a DUF1 (or DDGFF) domain.

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PA0176, PA0175, PA0174, PA0180 and PA0173, which encode homologues of CheY, CheA, CheW, CheR, CheD, CheB, and two associated MCPs, respectively (Fig. 2). PA0172 is located directly upstream of the PA0171 gene, and is predicted to encode a protein containing a HAMP domain, which is found in bacterial sensor and chemotaxis proteins (Pfam); accordingly, we predict that PA0172 is involved in this chemotaxis system. PA0169 is located downstream of PA0171, and is predicted to encode a protein containing a DUF1 (or DDGFF) domain; other proteins with a similar domain structure include the Caulobacter crescentus protein PleD, which is part of a signal transduction pathway controlling cell differentiation (Hecht & Newton, 1995; Aldridge & Jenal, 1999). In P. aeruginosa, it probably participates in a phosphotransfer-dependent signal transduction pathway involving this chemotaxis-like system. In this study, the insertion in PA0171 not only caused twitching-motility deficiency, but also affected swimming and swarming motility, and all of the motility defects in the PA0171 mutant could be complemented by a plasmid harbouring the PA0171 gene (data not shown), suggesting that PA0171 could be part of this chemotaxis operon. Examination of morphology by transmission electron microscope showed that the type IV pili of the PA68 PA0171 mutant could not be detected, and that the type IV pili could be restored by trans-complementation (Fig. 3). These data indicated that PA0171 is involved in the regulation of both motility and type IV pilus biogenesis.

The complete nucleotide sequence of PA1822 was also determined (GenBank accession number AY502958). The sequences of the PA1822 genes in PA68 and PAO1 are 98 % identical at nucleotide level, and 99 % identical when the deduced proteins are compared. The deduced amino-acid

![Fig. 3. Transmission electron microscopic visualization of P. aeruginosa type IV pili. Cells were stained with phosphotungstate and viewed at different magnifications. Bars in A, B and D, 100 nm; bar in C, 500 nm. Black arrows indicate the flagellum; white arrows point to type IV pili. (A) Wild-type PA68; (B) PA68 PA1822 mutant; (C) PA68 PA0171 mutant; (D) PA68 PA0171 mutant complemented with pDN18S (carrying PA0171 gene).](http://mic.sgmjournals.org)
The suitability of the new genes, organisms

sequence showed significant similarity and identity to CheY-like receiver and PilL of other organisms (Table 3). CheY is a signal transduction protein of the bacterial chemotaxis system, which acts as a molecular switch to alter the swimming behaviour of the bacterium (Halkides et al., 1998). Some genes required for twitching motility in P. aeruginosa have been shown to have significant homology to CheY protein or to contain a CheY-like domain (Darzins, 1993; Huang et al., 2003). Upstream of PA1822 is an ORF (PA1821) which is predicted to encode an enoyl-CoA hydratase/isomerase; PA1823, with a mutT domain, is located directly downstream of this gene (Pfam). The MutT domain is a small protein responsible for removing an oxidatively damaged form of guanine from DNA and the nucleotide pool (Pfam). Neither PA1821 nor PA1823 has high homology to genes involved in type IV pilin biogenesis or function. Mini-Mu transposon insertion into PA1822 caused twitching motility deficiency, but type IV pili were detectable in the PA1822 mutant by electron microscopy (Fig. 3), suggesting that PA1822 (fimL) is probably involved in the regulation of twitching motility in response to environmental cues.

Identifying components required for twitching is important, as all such proteins are potential targets for designing drugs to control infections caused by P. aeruginosa by interfering with biofilm formation. The suitability of the new genes, identified by us to be required for twitching, as drug targets, needs to be elucidated in future studies.

ACKNOWLEDGEMENTS

We thank Dr. Harri Savilahti providing us with mini-Mu transposon DNA and MuA transposase; Professor Shouguang Jin (University of Florida) for technical assistance, and Professor Shiyi Guo (Nankai University) for electron microscopy. This work was supported by the National Natural Science Foundation of China (No. 30270075).

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