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tonB3 Is Required for Normal Twitching Motility and Extracellular Assembly of Type IV Pili

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Three mutants with Tn*5*-B21 insertion in *tonB3* (PA0406) of *Pseudomonas aeruginosa* exhibited defective twitching motility and reduced assembly of extracellular pili. These defects could be complemented with wild-type *tonB3*.

Twitching motility in *Pseudomonas aeruginosa* is a form of surface motility mediated by type IV pili, which are located at the pole of the cell and are responsible for attachment to and translocation across host epithelial cells (10). Around 40 genes at various genomic loci have been identified as being involved in the biogenesis and function of type IV pili in *P. aeruginosa*, which are central to host colonization and the formation and maturation of biofilms (10).

P. aeruginosa inhabits a wide variety of environments including soil, water, and plant and animal tissues (18). To obtain iron from these environments, *P. aeruginosa* has multiple iron acquisition systems (9). It produces pyoverdin and pyochelin as key siderophores (6) and synthesizes specific high-affinity transporters, FpvA and FptA, which concentrate ferripyoverdin and ferric-pyochelin ligands, respectively, at the cell surface (3, 11). The subsequent translocation of the ferriligands into the periplasm is mediated by TonB (16).

In the *P. aeruginosa* genome, two *tonB* genes have been identified, *tonB1* and *tonB2*. Disruption of *tonB1* inhibits siderophore-mediated iron uptake and heme uptake (12, 20). Inactivation of *tonB2* has no adverse effect on iron or heme acquisition, but *tonB1-tonB2* double mutants are more compromised with regard to growth in iron-restricted medium than a single *tonB1* knockout mutant (21). Here we report the identification and characterization of the third *tonB*-like gene of *P. aeruginosa*, *tonB3*, whose product is required for twitching motility.

Three independent Tn*5*-B21 mutants of *P. aeruginosa* with defective twitching motility (S38, S281, and S311) were identified, with the insertions at a locus designated PA0406 in the *P. aeruginosa* genome (15). The Tn*5*-B21 insertions in these mutants were at nucleotide positions 431, 470, and 578 bp from the start codon in the open reading frame. *P. aeruginosa* with a mutation of this gene, which we have designated *tonB3* (see below), has been previously reported to have defective twitching motility and exhibit reduced virulence in the fruit fly *Drosophila melanogaster* (7), although the defect in twitching motility was not further characterized. Interestingly, mutants that are impaired in core aspects of the biogenesis of type IV pili (*pilD* and *pilQ*) and are completely defective in twitching motility retain wild-type virulence in the fruit fly model (7).

In the standard subsurface assay (1), mutants S38 (Fig. 1C), S281, and S311 (data not shown) exhibited twitching motility that was reduced but not completely impaired compared to that of the pilin-negative PAKΔ*pilA* mutant (Fig. 1B). The growth rates of the mutants were the same as that of the PAK parental strain in Luria-Bertani broth (data not shown), suggesting that the reduced twitching motility was not simply due to a growth defect. The micromorphology of the twitching zone edge on S38 was also examined by light microscopy and showed that compared to the wild type, S38 exhibited significantly reduced outward movement of the rafts and a lack of lattice-like networks, whereas these features were completely lacking from nonmotile *pilA* mutants (Fig. 1I to K). Both the macroscopic and microscopic twitching motility phenotypes of these mutants were restored by complementation with pBH36, which contains a 1.6-kb NotI-SalI fragment spanning the *tonB3* coding sequence (from 311 bp upstream of the start codon to 343 bp downstream of the stop codon) cloned into the vector pUCPSK (17) (Fig. 1D and L), indicating that the twitching motility defect in S38 is due to a mutation of *tonB3* and not due to polar effects on neighboring genes or to a secondary mutation elsewhere on the genome.

Database searches showed that TonB3 has strong homology over its entire sequence to the TonB protein of *Pseudomonas syringae* (69% identity and 83% similarity; GenBank accession no. NP_794769). TonB3 also has significant homology to TonB1 (23% identity and 43% similarity) and TonB2 (25% identity and 46% similarity) of *P. aeruginosa* (12, 21). Interestingly, TonB3 has a similar level of homology to TolA of *P. aeruginosa* (28% identity and 47% similarity), which is part of another energy-coupled active iron import system (5). TonB3 also has the same functional domains as those of TonB1, TonB2, and TolA, notably an N-terminal transmembrane domain (http://www.cbs.dtu.dk/services/TMHMM/) and C-terminal conserved TolA and TonB domains (http://smart.embl-heidelberg.de).

The effects of mutations in *tonB1* and *tonB2* on type IV pilus biogenesis and twitching motility in *P. aeruginosa* were not assessed in previous studies (12, 21). We therefore constructed

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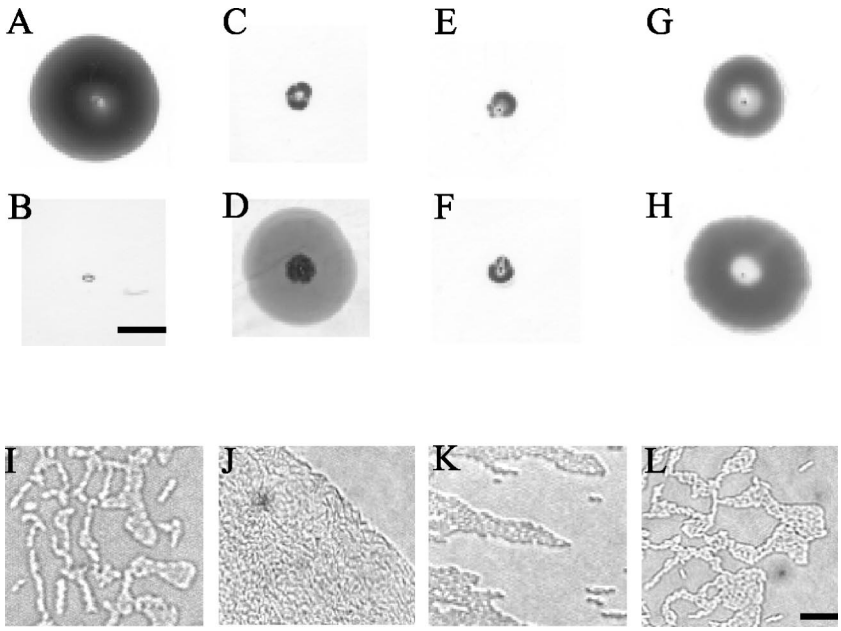

FIG. 1. Macroscopic and microscopic examination of twitching motility in *tonB3* mutants. (A to H) Twitching zones observed in the subsurface stab assay on agar plates after 24 h of growth (bar, 1 cm). (I to L) Light microscopy of the edge of the twitching zone obtained at the interstitial surface between the glass coverslip and GelGro medium (magnification, ×200; bar, 10 μm). (A and I) PAK wild-type; (B and J) PAKΔ*pilA* mutant; (C and K) *tonB3* mutant S38; (D and L) S38 + pBH36; (E) S38 + pBH195 (*tonB1*); (F) S38 + pBH182 (*tonB1*); (G) MKL (PAK*tonB1*::Gm); (H) MKE (PAK*tonB2*::Tc). Similar results were obtained for complementation of mutants S281 and S311 (data not shown).

tonB1 and *tonB2* allelic exchange insertion mutants (2). These mutants exhibited the same growth phenotypes in both rich and iron-depleted media as previously reported (12, 21). Twitching motility in the *tonB1* mutant was reduced, presumably only as a result of its slow growth (Fig. 1G). The twitching zone of the *tonB2* mutant was indistinguishable from that of the wild type (Fig. 1H), and both *tonB1* and *tonB2* mutants showed wild-type levels of pilin production and surface assembly (Fig. 2A to C), indicating that neither TonB1 nor TonB2 is required for pilus biogenesis. Moreover, cloned *tonB1* and *tonB2* constructs could not restore twitching motility to *tonB3* mutants (Fig. 1E and F).

The level of intracellular pilin expression and the amount of surface pili of *tonB* mutants were analyzed with semiquantitative Western blotting and quantitative enzyme-linked immunosorbent assays (ELISAs) (1, 13), respectively, using rabbit anti-PAK pilus antibodies. S38 exhibited relatively normal levels of intracellular pilin (Fig. 2A), but Western blotting and quantitative ELISAs showed that the amount of surface pili was significantly reduced (Fig. 2B and C), giving results similar to those obtained with the *pilV* mutant, which is known to have normal intracellular pilin expression but largely lacks surface pili (1). Complementation of S38 with cloned *tonB3* restored surface pili to levels that appeared quantitatively higher than that of wild-type PAK by ELISA (Fig. 2C), suggesting overcompensation by higher gene dosage. Although the Ton and Tol systems are regarded as major energy-coupled active import systems for gram-negative bacteria, the data here indicate that TonB3 may be involved in some aspect of the transport and secretion (Fig. 2A and C) of the pili or of a component required for their formation in the same manner as TonB provides energy to efflux systems to export antibiotics and toxic

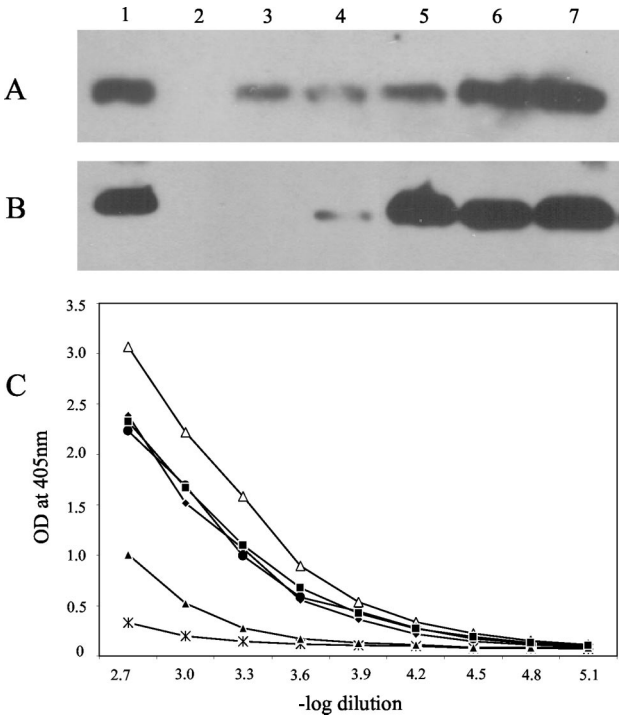

FIG. 2. Western blot analyses and ELISAs for pilus production in *tonB* mutants. (A) Western blotting on the whole-cell proteins to detect the expressed pili from PAK (lane 1), PAKΔ*pilA* (lane 2), PAKΔ*pilV* (lane 3), S38 (lane 4), S38 + pBH36 (lane 5), MKL (PAK*tonB1*::Gm) (lane 6), and MKE (PAK*tonB2*::Tc) (lane 7). (B) Western blotting on the surface pili from the same strains as in panel A. (C) Quantitative analysis of the level of surface pili by ELISA from PAK (◆); PAKΔ*pilA* (∗); S38 (▲); S38 + pBH36 (△); MKL (PAK*tonB1*::Gm) (●); and MKE (PAK*tonB2*::Tc) (■).

solvents out of the cell (8, 19). *Vibrio cholerae* does not require either of its two TonB systems for type IV pilus biogenesis (4). Free iron sequestration by lactoferrin and other iron binding compounds has been shown to increase twitching motility (14), suggesting that iron uptake per se is not required for twitching motility.

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