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Identification of Pilus-Like Structures and Genes in *Microcystis aeruginosa* PCC7806

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Four putative type IV pilus genes from the toxic, naturally transformable *Microcystis aeruginosa* PCC7806 were identified. Three of these genes were clustered in an arrangement which is identical to that from other cyanobacterial genomes. Type IV pilus-like appendages were also observed by electron microscopy.

Microcystis aeruginosa PCC7806 is a toxic, unicellular cyanobacterium that is naturally transformable (4, 15). Microcystin (a hepatotoxin) production by this cyanobacterium involves a large gene cluster that encodes its synthesis via a polyketide synthetase and nonribosomal peptide pathway (4, 15). The arrangement of genes in this cluster and its primary structure show high similarity to those of two other toxin clusters in cyanobacteria of different orders: the filamentous *Planktothrix* and *Nodularia* (3, 11). The microcystin gene cluster from *Anabaena* sp. strain 90 is also known, and the individual genes show high sequence similarities to the corresponding ones from the other cyanobacteria (14). The association of transposases with this conserved gene cluster in all four organisms suggests that lateral gene transfer may have facilitated the distribution of an ancient genomic locus which, through time, has evolved to produce different toxin isoforms in these organisms.

Type IV pili (Tfp) are present in many gram-negative bacterial systems and function in a number of physiological processes such as cell adhesion, motility, and natural transformation (10). The apparent distribution of Tfp across vastly different bacterial systems suggests that it may be an ancient apparatus. Since Tfp and natural transformation are linked in a number of bacterial systems, including cyanobacteria (18), we have initiated studies of Tfp systems in the bloom-forming genus *Microcystis*. This has led to the identification of four Tfp-like genes and pilus-like structures in *M. aeruginosa* PCC7806. Because cyanobacteria are believed to be an ancient group of organisms, the presence of a Tfp system in *Microcystis* (the third reported cyanobacterial case after *Synechocystis* sp. strain PCC6803 and, putatively, *Thermosynechococcus elongatus* BP-1 [2, 9]) would further our knowledge regarding Tfp, lateral transfer of toxic gene clusters, and the evolution of prokaryotic genomes.

Identification of pilus-like structures by electron microscopy. Transmission electron microscope (TEM) observations of *M. aeruginosa* PCC7806 cells led to the identification of numerous pilus-like structures on the cell surface (Fig. 1). Exponential-phase cells grown in liquid BG-11 medium (17) as batch cultures under continuous light (25 μmol photons m^{-2} s^{-1}), supplied by cool white fluorescent lamps at 28°C ± 1°C, were harvested at room temperature at 4,000 × *g* for 15 min, washed once in fresh BG-11, and resuspended in 100 μl of BG-11. Cells from BG-11 agar plate cultures were scraped off and resuspended in 100 μl of BG-11. Cell suspensions dropped onto Formvar-coated copper grids were either air dried or wicked off with filter paper after standing for 10 min. Grids were stained with 1% phosphotungstic acid (pH 7.0) for 30 seconds, excess stain was removed with filter paper, and grids were allowed to air dry before being observed under a Hitachi model H-7000 TEM at 75 kV.

There was a notable difference between the piliation characteristics of cells from liquid preparations (Fig. 1A) and agar plates (Fig. 1B and 1C). Cells from liquid preparations exhibited thin and flexible pilus structures. The diameters of these structures were 6 to 10 nm; however, it was difficult to discern their lengths as they were highly intertwined and numerous. These pilus structures are comparable to the thick-pilus measurements reported by Bhaya et al. (2). Cells from agar plate preparations also exhibited numerous pilus-like structures. However these appeared more rigid and thicker, with diameters of 20 to 35 nm. A dense network of these rigid filaments was observed between clumps of cells from agar plates, which appeared to span several tens of micrometers (Fig. 1C). Interspaced between these rigid structures, however, were filaments with diameters similar to those observed with cells from liquid preparations (Fig. 1B). The rigid pilus-like structures appeared to consist of bundles of thinner filaments (Fig. 1B, arrows), which could be an artifact of growth on solid media. In an aqueous environment, the thinner, more-flexible pilus structures may lack a solid support on which stable interactions may facilitate bundling. Yoshihara et al. observed bundles of pili (diameters less than 45 nm) which appeared to consist of the more typical pili (diameter of 5 nm) in *Synechocystis* sp. strain PCC6803, whereas Bhaya et al. observed only the latter pilus morphology (2, 18). The method of preparation of cells for electron microscopy in the former study was from agar plates in contrast to preparation from liquid culture in the latter study. From these observations, the method of preparation could influence the morphology of the pili, and the results in this study are comparable to those in previous reports.

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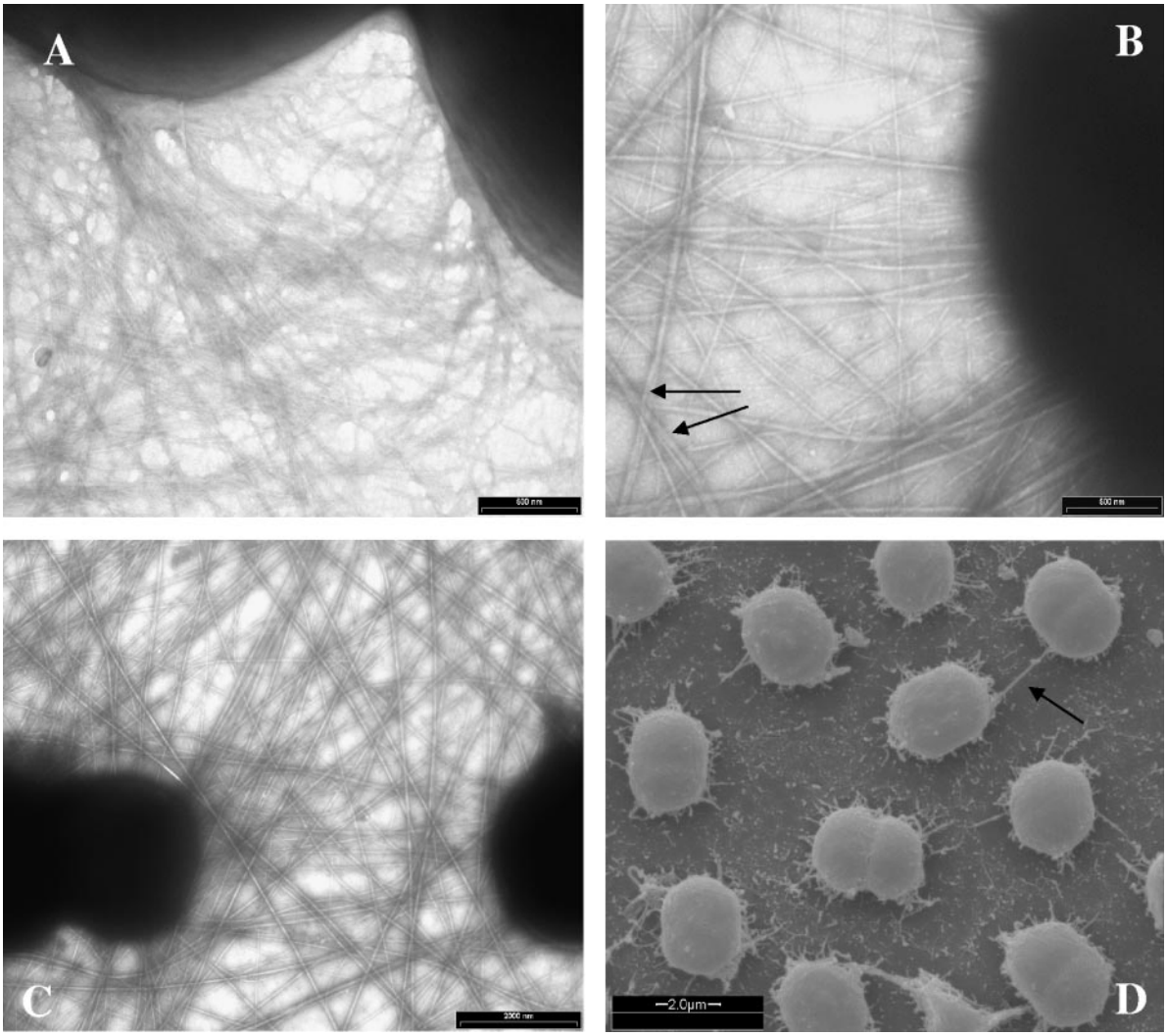

FIG. 1. Transmission and scanning electron micrographs of *M. aeruginosa* PCC7806 cells displaying pilus-like structures. (A) TEM of representative sample of cells stained from liquid culture. (B and C) TEM of representative samples of cells stained from solid agar culture. (D) SEM of representative samples of cells from solid agar culture. Scale bars in panels A, B, C, and D represent 500 nm, 500 nm, 2,000 nm, and 2,000 nm, respectively. Cells for TEM were stained with 1% phosphotungstic acid (pH 7.0) for 30 seconds. Arrows in panel B indicate thick rigid pilus-like structures consisting of thinner filaments. The arrow in panel D shows an extracellular appendage connecting two cells.

Scanning electron microscopy (SEM) also revealed the presence of extracellular structures that appeared to interconnect cells (Fig. 1D, arrow). For SEM, 1.3-mm-diameter coverslips coated with poly-L-lysine were touched against cells from agar plates and fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were washed three times for 5 min with 0.1 M sodium cacodylate (pH 7.2) buffer and postfixed in 1% osmium tetroxide for 1 h, followed by another buffer wash for 10 min. Samples were dehydrated in ethanol and critical point dried with carbon dioxide. Dried samples were mounted onto stubs, sputter coated with gold, and examined with a FEI Company Quanta 200 scanning electron microscope at 15 kV. Due to the resolution of SEM, the structures seen in Fig. 1D probably represented the thick rigid pilus-like structures observed from TEM, rather than the thinner flexible structures.

From TEM and SEM observations, a dense array of appendages appeared to exist between *M. aeruginosa* PCC7806 cells (Fig. 1C and 1D). Although these micrographs represent cells from solid agar instead of a liquid suspension, they suggest that the pilus-like structures may be involved in the cohesion or aggregation of *M. aeruginosa* PCC7806 cells, especially if they are surrounded by a network of these filaments which may restrain dispersion of clumped cells. In *Synechocystis* sp. strain PCC6803, Tfp are often seen to form links between cells, although the significance of these connections is not known (2).

Identification of putative pilus genes in *M. aeruginosa* PCC7806. Degenerate PCRs, followed by partial-inverse and adaptor-mediated PCR methods (11), led to the identification of four open reading frames (ORFs) from *M. aeruginosa* PCC7806 which showed high similarity to those encoding known PilA, PilB, PilC, and PilT proteins. Twenty-five primers were used, and these sequences are available upon request.

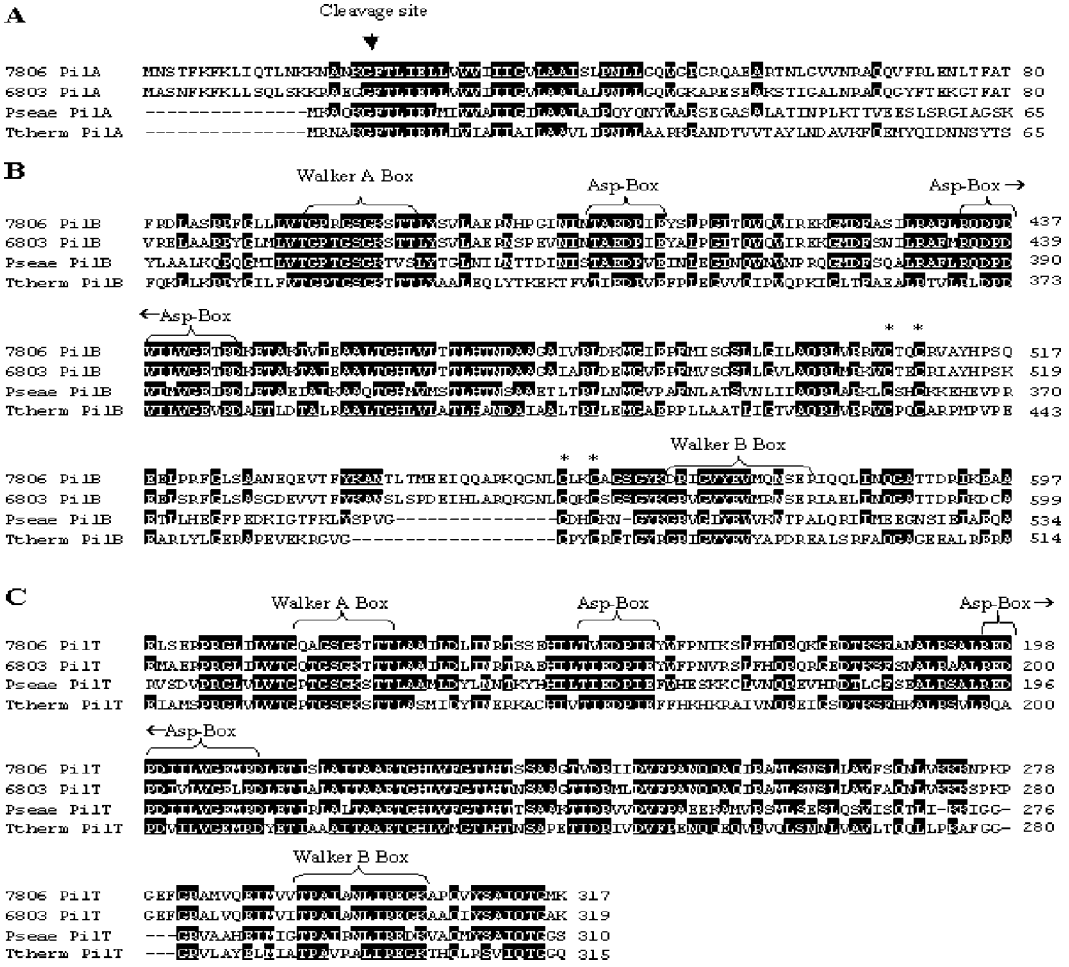

FIG. 2. Multiple alignments of the conserved regions of the deduced pilus proteins from *M. aeruginosa* PCC7806 to known pilus proteins. Identical residues are shown in the highlighted boxes. (A) Alignment of the PCC7806 PilA to known PilA proteins. The arrowhead indicates the putative site of cleavage by a leader peptidase. (B) Alignment of the PCC7806 PilB to characterized PilB proteins. Asterisks indicate conserved cysteine residues. (C) Alignment of the PCC7806 PilT to characterized PilT proteins. Abbreviations: 7806, *M. aeruginosa* PCC7806; 6803, *Synechocystis* sp. strain PCC6803; Pseae, *P. aeruginosa* PAO1; Ttherm, *Thermus thermophilus* HB27.

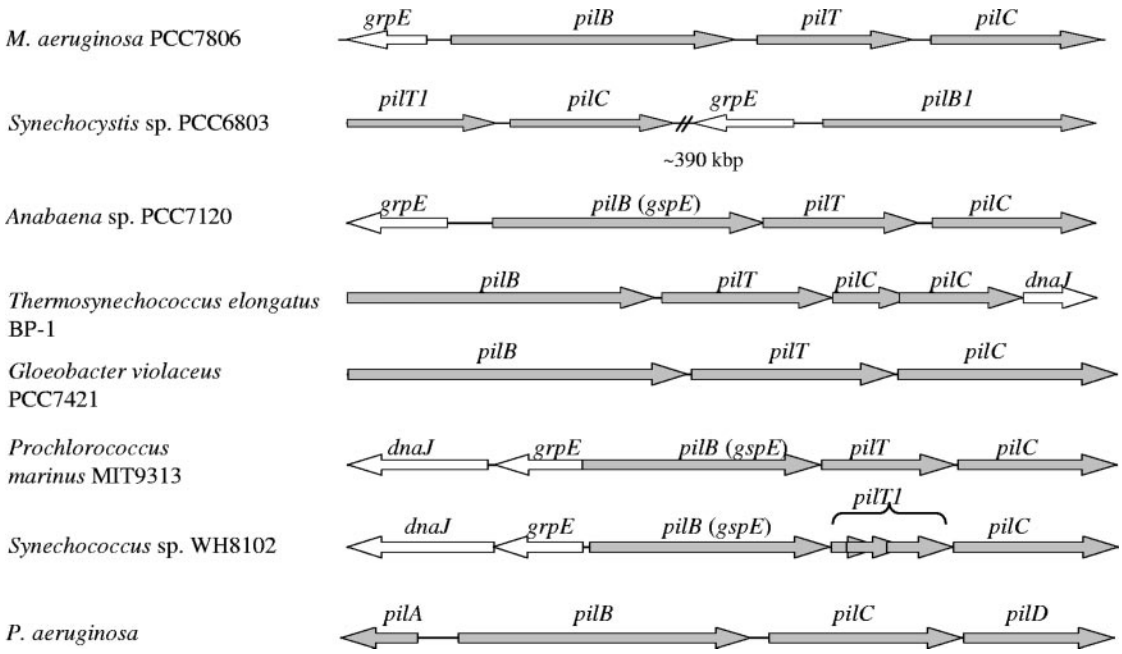

FIG. 3. Comparison of the *pilBCT* gene cluster organization from cyanobacterial genomes and the organization of *pilA*, *pilB*, *pilC*, and *pilD* from *P. aeruginosa*.

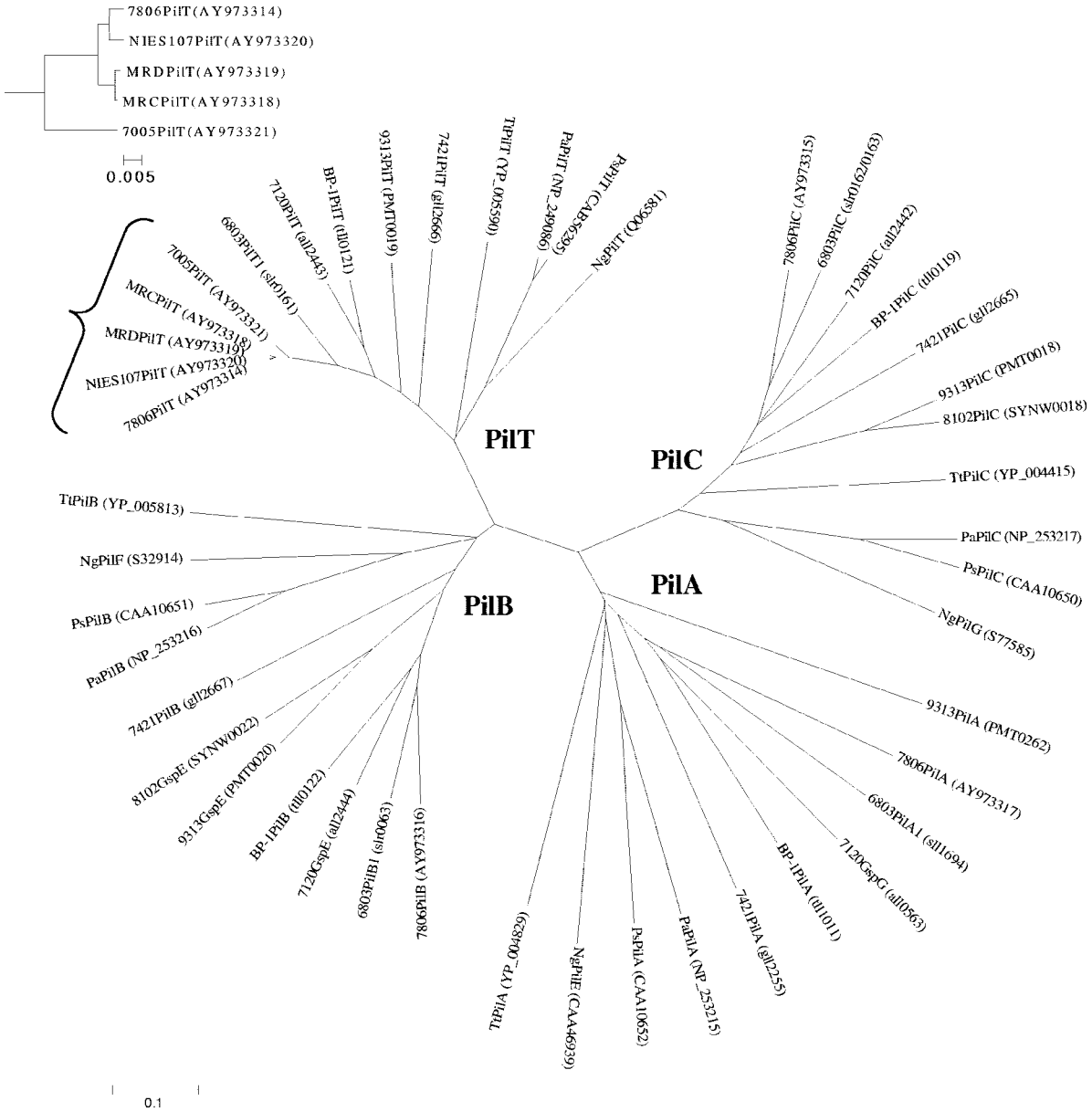

FIG. 4. Unrooted phylogenetic tree of cyanobacterial and noncyanobacterial PilA, PilB, PilC, and PilT proteins. The tree was generated using ClustalX 1.83 and reconstructed using TreeExplorer. The scale bar represents 1 substitution per 10 amino acids. The subtree of *Microcystis* PilTs is shown in the upper left corner (scale bar represents 5 substitutions per 1,000 amino acids). GenBank accession and CyanoBase accession numbers are indicated in parentheses. Abbreviations: 7806, *M. aeruginosa* PCC7806; MRC and MRD, *M. aeruginosa* UWOCC MRC and MRD, respectively; 7005, *M. aeruginosa* PCC7005; NIES107, *M. wesenbergii* NIES107; 6803, *Synechocystis* sp. strain PCC6803; 7120, *Anabaena* sp. strain PCC7120; BP-1, *Thermosynechococcus elongatus* BP-1; 9313, *Prochlorococcus marinus* MIT9313; 8102, *Synechococcus* sp. strain WH8102; 7421, *Gloeobacter violaceus* PCC7421; Pa, *P. aeruginosa* PAO1; Ps, *Pseudomonas stutzeri* JM300; Ng, *Neisseria gonorrhoeae*; Tt, *Thermus thermophilus* HB27.

The four ORFs were putatively named *pilA*, *pilB*, *pilC*, and *pilT*, according to their similarity to the corresponding bacterial counterparts. Multiple alignments revealed conserved motifs characteristic for each group of pilus proteins. The PilA sequence possessed a putative cleavage site recognized by PilD peptidases, followed by a phenylalanine, which is the first amino acid of the mature pilin (8) (Fig. 2A). The PilB and PilT (Fig. 2B and C) sequences both possessed nucleotide-binding motifs, known as the Walker A and B boxes, found in many ATPases (16). There were also two aspartate box domains between the Walker boxes, which are common for PilB and PilT homologs but not found in other proteins with ATP-binding sites (5, 12). In addition, a distinguishing feature of the PilB class of proteins is the presence of a tetracysteine motif, which resembles the zinc-binding motifs found in many zinc-dependent enzymes (13). Multiple alignments and motif searches in PilC revealed two general secretion pathway F signature domains at positions 69 to 192 and 272 to 395, which is typical of this group of pilus proteins.

The *pilB*, *pilC*, and *pilT* genes are clustered, similar to other

cyanobacterial genomes possessing Tfp genes (Fig. 3). Sequencing upstream of *pilB* in *M. aeruginosa* PCC7806 revealed a putative heat shock gene, annotated as *grpE* here due to its high similarity to *grpE* in *Synechocystis* sp. strain PCC6803. There are also heat shock genes present at one end of the *pilBCT* cluster in other cyanobacterial genomes (Fig. 3). In *Pseudomonas aeruginosa*, the *pilB* and *pilC* genes are clustered with *pilA* and the leader peptidase gene *pilD*, instead of *pilT*. The organization of the *pilBCT* genes in other heterotrophic bacteria is similar to that in *P. aeruginosa* (5). From these data, it is apparent that the arrangement of the cyanobacterial *pilBCT* gene cluster may be different from that of other bacteria.

Phylogenetic analysis of cyanobacterial and noncyanobacterial PilA, PilB, PilC, and PilT proteins showed that, within all pilus clusters, each of the putative *M. aeruginosa* PCC7806 pilus proteins was predicted to share a common ancestor with *Synechocystis* sp. strain PCC6803 (Fig. 4). With the exception of PilA from *Prochlorococcus marinus* MIT9313, there was a clear distinction between the cyanobacterial and noncyanobacterial pilus protein sequences, suggesting that the organismal ancestor of cyanobacteria possessed a progenote Tfp system. The PilA protein itself appeared to have undergone the most significant substitutions among the pilus proteins studied here. Apart from the leader peptide regions harboring the cleavage sites, the remainders of the PilA sequences do not show much conservation (Fig. 2). Using primers designed for structural analysis of the *pilT* genomic loci, amplification of orthologs was also achieved from the toxigenic *M. aeruginosa* UWOCC MRC and UWOCC MRD, *Microcystis wesenbergii* NIES107, and the nontoxigenic *M. aeruginosa* PCC7005 (data not shown). The *Microcystis* PilT sequences formed a monophyletic cluster (Fig. 4).

This study describes the presence of putative Tfp genes in *M. aeruginosa* PCC7806, whose products show high sequence similarity to other cyanobacterial Tfp proteins. Numerous pilus-like appendages on the cell surface are also reported. The morphology resembles that seen in *Synechocystis* sp. strain PCC6803, the Tfp system of which has been studied quite extensively in terms of phototactic motility and natural transformation (1, 18). We have initiated studies of the *M. aeruginosa* PCC7806 *pilT*, homologs of which are thought to be important for the DNA uptake process due to its retraction functionality (6, 7). DNA bound to the surfaces of cells around the pilus apparatus could concurrently be taken up when retraction of the pilus structure occurs. For these reasons, this Tfp component may represent a critical step in the process of lateral gene transfer, which is the uptake of DNA from the environment. In addition to the presence of putative Tfp genes in *M. aeruginosa* PCC7806, putative *pilT* genes have also been identified in other toxic and nontoxic *M. aeruginosa* strains. This finding is important in that it lends support to the hypothesis that lateral gene transfer (natural transformation via Tfp) may have facilitated the dissemination of toxin gene clusters among strains of the same species and may preempt the acquisition of toxigenicity by nontoxic strains.

Nucleotide sequence accession numbers. Nucleotide sequences of eight putative pilus genes reported in this paper have been submitted to GenBank under the accession numbers AY973314 to AY973321. The putative heat shock gene is under accession number AY987043.

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