The role of an alternative sigma factor in motility and pilus formation in the cyanobacterium Synechocystis sp. strain PCC6803

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ABSTRACT Disruption of a gene for an alternative sigma factor, designated sigF, in the freshwater, unicellular cyanobacterium Synechocystis sp. strain PCC6803 resulted in a pleiotropic phenotype. Most notably, this mutant lost phototactic movement with a concomitant loss of pili, which are abundant on the surface of wild-type cells. The sigF mutant also secreted both high levels of yellow–brown and UV-absorbing pigments and a polypeptide that is similar to a large family of extracellular proteins that includes the hemolysins. Furthermore, the sigF mutant had a dramatically reduced level of the transcript from two tandemly arranged pilA genes (sll1694 and sll1695), which encode major structural components of type IV pilus. Inactivation of these pilA genes eliminated phototactic movement, though some pili were still present in this strain. Together, these results demonstrate that SigF plays a critical role in motility via the control of pilus formation and is also likely to regulate other features of the cell surface. Furthermore, the data provide evidence that type IV pili are required for phototactic movement in certain cyanobacteria and suggest that different populations of pili present on the Synechocystis cell surface may perform different functions.

The evolutionarily ancient cyanobacteria may have been one of the first groups of organisms to evolve directional motility. While motility has been extensively studied in some microorganisms, the nature of motility in cyanobacteria has been considered enigmatic because neither the mechanism nor the environmental factors that control movement have been elucidated.

Several species of both filamentous and unicellular cyanobacteria are motile (1, 2). The filamentous cyanobacteria show gliding motility, which requires contact with a solid surface and occurs in a direction parallel to the long axis of the filament. Interestingly, nonmotile Phormidium uncinatum cells are deficient in components of the cell surface, including extracellular slime and helically arrayed fibrils that are comprised of a rod-shaped glycoprotein called oscillin (3, 4). Oscillin has been proposed to play a passive role in motility with propulsion resulting from shear forces generated between the oscillin fibrils on the cell surface and slime as it is exuded from cells (“surface mucilage” hypothesis). In certain marine unicellular cyanobacteria, motility in an aqueous environment has been termed “swimming” (5). In Synechococcus WH8102 the generation of thrust for swimming requires SwmA, which is an extracellular protein that exhibits similarity to oscillin and is associated with the outer membrane (6). Directional motility in both the unicellular and filamentous cyanobacteria appears to depend on Ca2+, although the exact role of this cation in motility is not clear (1, 2, 7).

Neither P. uncinatum nor the marine Synechococcus species that have been examined have obvious cell appendages, such as flagella or pili (3–5), that have been shown to facilitate movement in other microbes. However, pil-like structures have been observed on the surface of certain unicellular and filamentous cyanobacteria (8–11). No function has been demonstrated for these appendages, and there is no understanding of the cellular mechanisms or environmental factors that control their biosynthesis. In this study we have inactivated a gene encoding an alternative sigma factor (gene number slr1564 in the Cyanobase), which we have designated sigF because of its similarity to the group III sigma factors SigF of Bacillus coagulans and SigB of Bacillus subtilis. We demonstrate that it is required (either directly or indirectly) for the biosynthesis of pil and that specific pilA genes (sll1694 and sll1695) are needed for directional motility of the cyanobacterium Synechocystis sp. strain PCC6803 (henceforth referred to as Synechocystis).

METHODS

Culture and Growth Conditions. The motile strain of Synechocystis sp. strain PCC 6803 was originally from the Pasteur Culture Collection but was obtained from Young Mok Park (Korean Basic Science Institute, Teajon, Korea). Cells were grown in BG-11 medium in moderate light (70 μmol photons m−2 s−1). When appropriate, antibiotics were included in the medium at a final concentration of 2 μg/ml of ampicillin, 25 μg/ml of spectinomycin, or 10 μg/ml of kanamycin. Whole-cell spectra from 350 to 800 nm were measured with a Beckman DU70 spectrophotometer (Fullerton, CA).

Motility Assays. Five to 10 microliters of log-phase cells were streaked as an approximately 1-mm thick line onto BG-11 solid medium containing 0.4% agar and supplemented with 15 mM glucose. The cells, maintained at 30°C, were exposed to unidirectional incandescent light of 40 μmol photons m−2 s−1.

DNA Manipulation, RNA Isolation, and Northern Blot Hybridization Analysis. Molecular techniques were performed according to standard procedures (12). DNA was isolated from Synechocystis according to the method of Tandeau de Marsac et al. (13). RNA was isolated from pelleted cells frozen at −80°C by using a slight modification of the method of de Saizieu et al. (14) in which 100 mg of glass beads (0.1 μm average diameter) were added to the cells after the addition of the acid phenol and the suspension was vortexed for 1 min. This procedure yielded between 150 and 250 μg of RNA from a typical 50-ml culture (cell density approximately 5 × 108 cells).

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Gene Inactivation. The sigF gene (srl1564 and originally designated rpoF in the Cyanobase) was identified by examination of the Cyanobase (http://zearth.kazusa.or.jp/cyanobase/). It was isolated by PCR amplification using the forward primer 5'-GGCTCAGATGACCAATGCCC-3' (GG clamp shown in italics, XbaI site in bold followed by the first 12 bases of the sigF gene encoding amino acids 1–4) and the reverse primer 5'-GGATGATATTAAAAATTATTAACCC-3' (GG clamp shown in italics, ClaI site in bold, followed by the last 12 bases of the sigF gene encoding the stop codon and amino acids 258–256). The PCR product was cloned into the XbaI/ClaI site of Bluescript KS−. The sigF gene was interrupted at a unique Psrl site (base 463) with the aphHII gene cassette that confers kanamycin resistance to Synechocystis cells. The Bluescript KS− vector containing the interrupted sigF gene was used to transform Synechocystis, and kanamycin-resistant transformants that were ampicillin sensitive were selected to ensure replacement of the wild-type gene by a double homologous recombination event. DNA was isolated from individual transformants, and PCR analysis was used to check that all copies of the chromosome in the cell carried the interrupted sigF gene. To ensure that the phenotype observed in the sigF mutant was not the result of a polar effect, we inactivated the gene downstream of sigF, designated srl1565 in the Cyanobase. The strain carrying this inactivated gene exhibited a wild-type phenotype (data not shown). Both the mutant and wild-type cells were grown in BG-11 medium in moderate light (70 µmol photonm−2s−1).

To inactivate the tandemly arranged genes encoding PilA1 and PilA2 (sll1694 and sll1695), a single 1.1-kbp PCR product containing these genes was generated by using the forward primer 5'-ATGCGATTAATTTTAAA-3' (encoding the first six amino acid residues of sll1694) and the reverse primer 5'-TCACAAAAAGCTTAAT-3' (encoding the last five amino acid residues and stop codon of sll1695). The PCR product was cloned into pGEM T vector (Promega). A 0.38-kbp fragment (excised by HpaI and KpnI) was replaced with a 2-kbp fragment that contains the gene conferring spectinomycin resistance (which is flanked by short inverted repeats carrying transcription and translation termination signals) (15), resulting in a vector construct that had both genes disrupted. This vector was used to transform Synechocystis, and the same strategy as described above was used to ensure that all copies of the chromosome in the cell carried the interrupted pilA genes.

Protein Isolation and SDS/PAGE. Cells were grown for 6 days in 50 ml of BG-11 medium in moderate light and pelleted by centrifugation at 3,600 × g for 5 min at 4°C. The centrifugation step was repeated on the growth medium to ensure complete removal of whole cells and cellular debris. The absorbance of the growth medium was measured, and proteins in the medium were precipitated by adding trichloroacetic acid to a final concentration of 10%, incubating on ice for 2 h, and then centrifuging for 15 min at 3,600 × g, 4°C. The protein pellet was washed twice with ice-cold 90% acetone, air-dried, and resuspended in 1 × loading buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 10 mM DTT, 0.005% bromophenol blue). Ten or 20 µl of solubilized sample was analyzed by 8% SDS/PAGE in Laemmli buffer (16) and stained either with Coomassie brilliant blue (R-250) or by periodic acid Schiff staining (17) for the detection of glycoproteins.

Amino Terminal Sequencing. The 200-kDa polypeptide, present in the medium in which the sigF mutant was grown, was resolved by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes and stained with amido black. The N-terminal amino acid sequence was determined by the Protein and Nucleic Acid facility, Stanford University Medical School. The first 15 aa of the polypeptide [LSPNV(1)/A]AALQIMYT] exactly matched the deduced N-terminal encoded by hlyA (sll1951).

Electron Microscopy. Log-phase cells were negatively stained with 1% phosphotungstic acid and examined by using a Phillips CM12 microscope.

RESULTS
As shown in Fig. 1A, the Synechocystis SigF polypeptide is most similar to SigB (also known as RpoF or Sigma 37) of B. subtilis and Bacillus licheniformis (31% identical and 54% similar to both). It is also similar to the sporulation-specific sigma factor F of Bacillus coagulans and to a sigma factor from Streptomyces setonii that is involved in controlling carotenoid biosynthesis, as well as more distantly to RpoS from various organisms (data not shown). SigB of B. subtilis has been designated a regulator of "general stress" responses and is involved in activation of more than 40 genes. The activity of this sigma factor is modulated by a signal transduction network composed of sigF gene products. This network responds to stress signals generated by (a) reduced energy supplies resulting from nutrient limitation or entry into stationary phase and (b) environmental stresses such as heat shock (18–20). Genes with homology to the rsb genes of B. subtilis are present on the Synechocystis genome, which has been completely sequenced (21).

We analyzed the phenotype of a mutant of Synechocystis in which the sigF gene was disrupted. This mutant produced yellowish-brown and UV-absorbing (absorbance maxima at 397, 363, and 335 nm) pigments that were released from the cells and accumulated in the growth medium (Fig. 2A, traces a, c, and e). This pigmentation was barely detectable in cultures of wild-type cells (Fig. 2A, traces b, d, and f). UV-absorbing pigments have been associated with the cyanobacterial cell sheath and often increase when cells are exposed to elevated levels of UV light (22, 23). In addition, the medium in which the sigF mutant was grown accumulated a glycoprotein (based on Schiff staining, data not shown) of molecular mass of approximately 200 kDa (and less abundant lower molecular mass polypeptides) (Fig. 2B, lane 3). This protein is not readily detectable in the culture medium from wild-type cells (Fig. 2B, lane 2). The N-terminal sequence of the 200-kDa polypeptide established its identity as the 1,741-aa polypeptide designated HlyA (sll1951). The C-terminal half of HlyA (the last 1,000 aa) is similar to a diverse family of cell surface/extracellular Ca2+-binding proteins that includes the pore-forming cytolsins typified by the hemolysins of Escherichia coli (24). This protein class is characterized by tandem arrays of a 9-aa motif rich in glycine, aspartic acid, and asparagine [LXGXXG(N/D)DX], which is involved in Ca2+-binding. The Synechocystis HlyA protein also shows similarity to the motility-related glycoproteins oscillin (30% identity and 60% similarity), and SwmA (25% identity and 50% similarity), both of which contain several repeats of the 9-aa motif and are cell-surface associated (3, 4, 6). The elevated levels of extracellular pigments and HlyA polypeptide in the growth medium of cultures of the sigF mutant suggested that this strain had a defect in structuring or synthesizing components of its extracellular surface layers.

Because the hemolysin-like SwmA and oscillin polypeptides have been linked to motility in cyanobacteria, we examined the response of wild-type cells and the sigF mutant to a unidirectional light source. Exposure of wild-type cells to unidirectional incandescent light of 40 µmol photons−2s−1 resulted in a phototactic response. Finger-like projections of cells migrating toward the direction of the light source were visible within 1–2 days of streaking the cells onto agar plates (Fig. 3A, WT). Under these conditions the rate of movement was calculated to be approximately 70 µm/h. Photophobic movement was observed when the cells were exposed to light intensities of above 50 µmol photons·m−2·s−1 (D.B. and A.R.G., unpublished data). In striking contrast, the sigF mutant showed no directional motility whatsoever (Fig. 3A, sigF). Furthermore, wild-type cells when grown under a uniform...
light source formed spreading colonies (indicative of motility) whereas the sigF mutant formed tight colonies that did not spread (data not shown). Thus the sigF mutant appears unable to move under a uniform light source, which suggests that the motility process itself is impaired; as a consequence there is no phototactic movement.

To determine whether inactivation of the sigF gene resulted in gross cell surface changes, we viewed the cyanobacterial cells by transmission electron microscopy. Wild-type *Synechocystis* had numerous, peritrichously arranged pili or fimbriae on cell surfaces by transmission electron microscopy. Wild-type cells accumulated high levels of a 1.1-kbp transcript during growth in moderate light (Fig. 4B, WT, lane 1), suggesting that the two *pilA* genes, each with a coding region of approximately 500 nt, are transcribed as a single operon. In contrast, no pili were observed on the surface of the *sigF* mutant (Fig. 3B, WT, lane 1). Northern blot hybridization, which showed that the sigF* mutant had no detectable *pilA* transcript accumulation and suggest that the release of cell surface pigments and proteins in cultures of the strain to form pili and/ or other cell surface structures.

In a number of Gram-negative bacteria, twitching and gliding motility are associated with the presence of type IV pili. Several genes required for pilus formation, assembly, and for coupling energy to pilus movement have been identified in *Myxococcus xanthus* and pathogenic bacteria (25, 26). The *pilA* gene is transcribed as a single operon, which is essential for motility, and gene products are required for pilus formation, assembly, and cell surface changes (25, 26). Analysis of the *pilA* gene as a probe. We isolated RNA from both wild-type cells and the sigF* mutant and examined the level of transcripts from the *pilA* gene as a probe. Wild-type cells accumulated high levels of a 1.1-kbp transcript during growth in moderate light (Fig. 4B, WT, lane 1), suggesting that the two *pilA* genes, each with a coding region of approximately 500 nt, are transcribed as a single operon. In contrast, no pili were observed on the surface of the *sigF* mutant (Fig. 3B, WT, lane 1). Northern blot hybridization, which showed that the sigF* mutant had no detectable *pilA* transcript accumulation and suggest that the release of cell surface pigments and proteins in cultures of the *sigF* mutant may be the consequence of the inability of this strain to form pili and/ or other cell surface structures.

To determine whether the *pilA* gene products were required for motility, we constructed a strain in which both the *pilA1A2* mutant and examined the level of transcripts from the *pilA* gene as a probe. Wild-type cells accumulated high levels of a 1.1-kbp transcript during growth in moderate light (Fig. 4B, WT, lane 1), suggesting that the two *pilA* genes, each with a coding region of approximately 500 nt, are transcribed as a single operon. In contrast, no pili were observed on the surface of the *sigF* mutant (Fig. 3B, WT, lane 1). Northern blot hybridization, which showed that the sigF* mutant had no detectable *pilA* transcript accumulation and suggest that the release of cell surface pigments and proteins in cultures of the *sigF* mutant may be the consequence of the inability of this strain to form pili and/ or other cell surface structures.

To determine whether the *pilA* gene products were required for motility, we constructed a strain in which both genes were disrupted by insertion of a spectinomycin cassette at a position 300 bp downstream of the *pilA* gene as a probe. Wild-type cells accumulated high levels of a 1.1-kbp transcript}
was truncated in the mutant (Fig. 4B, lane 2). The size of the truncated transcript, estimated to be about 0.7 kbp, suggests that transcription might initiate about 150 bp upstream of pilA1, continue through the truncated pilA1 gene, and terminate at the terminator positioned upstream of the spectinomycin cassette. The pilA1A2 mutant, like the sigF mutant, exhibited no phototactic movement (Fig. 3A, pilA1A2). However, as revealed by transmission electron microscopy, peritrichously arranged pili were still present on the surface of the cell (Fig. 3B, pilA1A2), although they were generally not as abundant as in wild-type cells (based on observations of several different fields). These results suggest that PilA1 and PilA2 are involved in motility, probably forming motility-specialized pili on the cell surface. The pili that remain in the pilA1A2 mutant may be encoded by the other pilA-like genes (Fig. 1B), and we speculate that they are involved in processes such as conjugation and adhesion, both of which have been observed in cyanobacteria (27, 28).

**DISCUSSION**

The results presented here allow us to make four important conclusions. First, the sigF mutant of Synechocystis has a pleiotropic phenotype that includes the accumulation of ex-

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**Fig. 2.** (A) Spectra of wild-type (WT) and sigB cultures. Spectra (250–800 nm) were measured from 6-day-old cultures of the sigF mutant (a) and WT (b), the cell pellet of the sigF mutant (c) and WT (d), and the growth media of the sigF mutant (e) and WT (f). Spectra are offset along the ordinate and normalized to cell density as reflected by absorbance at 750 nm. (B) SDS/PAGE of polypeptides precipitated from the growth media from WT (lane 2) and sigF mutant (lane 3) cultures and stained with Coomassie blue. Molecular mass markers (in kDa) are shown in lane 1.

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**Fig. 3.** (A) Directional motility assay. Five to 10 microliters of logarithmically growing wild-type cells (WT) and the sigF and pilA1A2 mutants were streaked as an approximately 1-mm thick line onto solid (0.4% agar) BG-11 medium containing 15 mM glucose. The cells were subjected to unidirectional light (indicated by an arrow) of 40 μmol photons·m⁻²·s⁻¹ for 48 h. The temperature during the incubation was 30°C. Note the finger-like projections emerging from the WT streak. (B) Transmission electron microscopy of whole cells. Logarithmically growing WT (Left), sigF mutant (Upper Right) and pilA1A2 mutant (Lower Right) cells. The cells were negatively stained with 1% phosphotungstic acid and examined by using a Phillips CM12 microscope. The arrow in WT (Upper Left) points to a very long pilus while the arrow in WT (Lower Left) points to pili that appear to connect neighboring cells. The bars represent 0.28 μm (Upper Left), 0.24 μm (Upper Right), 0.74 μm (Lower Left), and 0.31 μm (Lower Right).
A reduction in the level of intracellular pigments and proteins, the loss of pili, and a marked repression of the pilA1A2 transcript in the mutant and the unassembled monomeric PilA in the cytoplasm controlling some aspect of the architecture of the cell surface, required for phototaxis in Synechocystis. For instance, the processing of the leader peptide of prepilin and the subsequent N-methylation is carried out by the bifunctional peptidase PilD, which also is used by the general secretory pathway. Furthermore, there are several PilA homologues that are required for the assembly of pili and the functioning of the general secretory pathway (26, 39). Thus, at this point we cannot exclude the possibility that some of the putative PilA-like polypeptides encoded on the genome of Synechocystis may play a role in type II secretion or in pilus assembly rather than being structural subunits of pili.

Fourth, the control of pilA genes by SigF is thus far unique to cyanobacteria. In other organisms that have been examined, which includes Vibrio species, P. aeruginosa, N. gonorrhoeae, and M. xanthus, the alternative sigma factor RpoN has been shown to be essential for motility (40–43). Mutants in P. aeruginosa that are null for rpoN expression cannot make pili, which reflects the inability of these strains to transcribe the gene encoding the pilin subunit. This sigma factor works in conjunction with PilR and PilS, which are members of a two-component regulatory system (44–47). However, it is not known which environmental cues modulate the phosphorylation state of these regulatory molecules and if these regulators control processes in addition to the production of pili. It is notable that our attempts to identify genes encoding possible homologues of RpoN, PilR, and PilS in Synechocystis, based on the whole genome sequence, were unsuccessful. On the other hand, the similarity of the Synechocystis SigF to the SigB of B. subtilis (which plays a crucial role in acclimation to a variety of stresses) and the presence of rsb-like genes on the Synechocystis genome raises the possibility that SigF participates in the acclimation of cyanobacteria to environmental stresses via its control of cell surface characteristics and/or pilin-dependent motility.

In conclusion, we have shown that a SigF mutant in Synechocystis lacks pili and appears to have other cell-surface related aberrations. As a result of the loss of pili these mutants lack motility and exhibit no phototaxis. Our findings provide an indication that type IV pili are involved in phototaxis. These mutants also may have other cell surface defects, including aberrations of the inner and outer cell membranes and alterations in the polysaccharide sheath. Furthermore, recent evidence suggests that type IV pili play a role in the formation of biofilms (48); these biofilms may provide an important growth habit for bacteria under specific environmental conditions. It is still unclear whether SigF directly regulates pilA1A2 gene expression or whether the marked reduction of pilA1A2 transcript is the outcome of a global effect of SigF on cell surface architecture. Analysis of this regulation may reveal how environmental factors impact on morphological and functional characteristics of the bacterial cell surface.

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