

Novel Motility Mutants of *Synechocystis* Strain PCC 6803 Generated by In Vitro Transposon Mutagenesis†

DEVAKI BHAYA,^{1*} AKIKO TAKAHASHI,² PAYAM SHAHI,³ AND ARTHUR R. GROSSMAN¹

Department of Plant Biology, Carnegie Institution, Stanford, California 94305¹; Riken Plant Science Center, Laboratory for Metabolic Compartmentation, 2–1 Hirosawa, Wako, Saitama 351-0198, Japan²; and Department of Biological Sciences, San Jose State University, San Jose, California 95192³

Received 18 May 2001/Accepted 13 July 2001

We screened for transposon-generated mutants of *Synechocystis* sp. strain PCC 6803 that exhibited aberrant phototactic movement. Of the 300 mutants generated, about 50 have been partially characterized; several contained transposons in genes encoding chemotaxis-related proteins, while others mapped to novel genes. These novel genes and their possible roles in motility are discussed.

Cyanobacteria such as *Synechocystis* sp. strain PCC 6803 exhibit surface-dependent phototactic motility which requires type IV pili (2, 4). To identify structural and regulatory components important for motility, we screened for mutants with aberrant phototactic movement (i.e., nonmotile or negatively phototactic under conditions in which wild-type cells show positive phototaxis).

A *Synechocystis* genomic library was constructed that contained approximately 10⁵ clones with an average insert size of 6 to 8 kbp (3). The library of clones was used for in vitro transposon mutagenesis, and mutagenized clones were used for transformation of wild-type *Synechocystis* cells. Following transformation, cells were spread on 0.6% agar plates (10 µg of kanamycin/ml) and placed under a directional light source. Colonies that were nonmotile or exhibited an inverted response were picked, rescreened, and maintained for further analysis. Approximately 300 independent mutants (90% were nonmotile and 10% exhibited negative phototaxis) from a population of 8,000 transformants were identified. This screen required robust cell growth, thus eliminating mutants that might have appeared nonmotile because of growth defects. To identify the site of transposon insertion, we used plasmid rescue or direct sequencing of genomic DNA using primers that anneal to the ends of the transposon-kanamycin resistance gene cassette. Of the 50 or so mutants characterized so far, several contained transposons in genes encoding chemotaxis-related proteins (3). We also identified transposons in several novel genes, described in this report, that provide us with clues concerning functional and possibly architectural features that are important for motility.

Although we have not yet confirmed the phenotypic results by insertionally inactivating all of the genes found to be disrupted by transposons in the mutant strains, a number of findings strongly argue that the insertion of the transposon cassette is responsible for the mutant phenotype. First, based on Southern blot hybridizations, all of the mutants examined

appeared to have a single transposon insertion (data not shown). Second, in several cases, independent transposition events led to the insertion of the transposon into different positions within the same gene or genetic locus; in all cases, the insertions always yielded the same phenotype. Since the entire *Synechocystis* genome has been sequenced, the positions of the inserted transposon can be easily mapped, and the phenotypes of all of the mutants generated can be readily confirmed by targeted inactivations. The third and most convincing argument is that in all cases in which we have specifically inactivated a target gene identified by the transposon mutagenesis (10 cases to date), the targeted and transposon-generated mutants yielded identical phenotypes (2, 3, 4).

Table 1 shows the phenotypes of various mutants isolated from our screen (it excludes the chemotaxis-related genes which are described in Bhaya et al. [3]) and gives the positions of the independent transposon insertions (relative to the open reading frame), the size of the disrupted open reading frames (in amino acids and nucleotides), the gene designation (as given in Cyanobase), and the putative function of the gene product. Three nonmotile mutants were shown to harbor transposons in the *pilT1* and *pilC* genes, which are located adjacent to each other on the genome. We have recently demonstrated that disruption of either of these genes results in a nonmotile phenotype (2). This provides evidence that the method of mutant generation is robust and can be used to isolate novel motility mutants. Four independent transposon insertions led to the disruption of a gene encoding a putative DnaK-like chaperone (sll0058). There are three DnaK-like genes on the *Synechocystis* genome, of which sll0170 and sll1932 are likely to be the major chaperones (based on theoretical predictions), while sll0058 may be a minor chaperone dedicated to particular aspects of pilus biogenesis (10). In *Myxococcus xanthus* it has been shown that a nonessential DnaK-like chaperone is required for social motility and the production of fibrils (15), although its exact role in these processes has yet to be established.

We identified a nonmotile mutant disrupted for an ABC transporter gene (sll0415), and the phenotype of the mutant strain was confirmed by specific targeted inactivation. Interestingly, the mutant strain appears to have a normal complement of thin and thick pili (P. Shahi, D. Bhaya, and A. R. Grossman,

* Corresponding author. Mailing address: Department of Plant Biology, Carnegie Institution, 260 Panama Street, Stanford, CA 94305. Phone: (650) 325-1521. Fax: (650) 325-6857. E-mail: devaki@andrew2.stanford.edu.

† Carnegie Institution of Washington publication no. 1488.

TABLE 1. Mutants generated by transposon mutagenesis

Phenotype	Position(s) of transposon insertion(s)	Size of gene, amino acids (nucleotides)	Gene designation	Putative gene function and/or protein domain
Nonmover	-13, 776	369 (1,107)	slr0161	PilT1
Nonmover	521	405 (1,215)	slr0162	PilC
Nonmover	99, 448, 534, 1107	692 (2,076)	sll0058	Chaperone
Nonmover	617	354 (1,062)	sll0415	ABC transporter
Nonmover	276, 606, 664	324 (972)	sll0564	Ubiquinone methyltransferase-like
Nonmover	270	133 (399)	sll0565	E/Q-rich protein downstream of ubiquinone methyltransferase
Nonmover	911	416 (1,248)	sll1575	Eukaryotic-type serine/threonine protein kinase
Nonmover	953	337 (1,011)	slr1991	Adenylyl cyclase (<i>cya1</i>)
Nonmover	89	158 (474)	slr2016	Part of a gene locus (slr2015-2019) which includes an ABC transporter (slr2019) and <i>pilA</i> -like gene (slr2016)
Nonmover	83, 244, 583, 859	360 (1,080)	slr2017	Part of a gene locus (slr2015-2019)
Nonmover	78, 2280	799 (2,397)	slr2018	Part of a gene locus (slr2015-2019)
Slowmover	437	374 (1,122)	slr0358	E/P-rich
Nonmover	-3, 443, 1057	535 (1,605)	slr1301	E/Q-rich coiled coil
Nonmover	147	134 (402)	slr1964	Q-rich
Nonmover	64, 348, 502	259 (777)	sll0183	Pentapeptide repeat domain and tetratricopeptide domain
Slowmover	342	286 (858)	sll0414	Pentapeptide repeat domain
Nonmover	97	169 (507)	sll0301	Pentapeptide repeat domain

unpublished results). Of the approximately 42 putative ABC transporters of *Synechocystis* (<http://www.biology.ucsd.edu/~ipaulsen/transport/>), the one encoded by sll0415 has the highest similarity to an ABC transporter (PilH) required for motility in *M. xanthus* (38% identity and 60% similarity), although the role of the *M. xanthus* protein in motility is not known (16).

Three transposition events have been mapped to sll0564, whose gene product shows low but significant homology (25% identity and 36% similarity) to a methyltransferase of the ubiquinone methyltransferase type which uses *S*-adenosylmethionine as a methyl donor. The putative polypeptide encoded by sll0564 does not resemble the CheR polypeptide, which is a methyltransferase involved in methylation of the methyl-accepting chemoreceptor proteins (11). Therefore, we cannot assign a function to this putative polypeptide without detailed biochemical analyses. It is also notable that a transposon which mapped to the gene (sll0565) downstream of the putative methyltransferase also produced a nonmotile phenotype, raising the possibility that both genes are important for motility. The putative sll0565 protein does not show similarity to any protein in the database with an assigned function but has a relatively high proportion of glutamine (Q) and glutamic acid (E) residues (see below).

Transposons have also been mapped to genes encoding polypeptides with homology to a eukaryotic-type serine-threonine kinase (sll1575) and an adenylyl cyclase (slr1991); both of these polypeptides may participate in signal transduction events required for phototactic movement. It has been shown previously that a lesion in the adenylyl cyclase gene resulted in a nonmotile strain (13). There are three genes on the *Synechocystis* genome that encode polypeptides with homology to adenylyl cyclase; it is not known if the other two genes (sll1161 and sll0646) function in the regulation of motility. Kamei et al. have also demonstrated that sll1575 is required for motility; however, the precise function of this kinase in motility is unclear (8). It is interesting that a eukaryotic-type kinase is involved in this process; this suggests that signal transduction events that govern phototaxis may involve complex interactions.

A novel locus containing five genes (slr2015 to slr2019) is interesting because transposons mapped to three of the genes (slr2016, slr2017, and slr2018). The polypeptide encoded by slr2016 shows weak but significant homology to the family of PilA-like polypeptides (4), while the slr2017 and slr2018 gene products do not show obvious homologies to other polypeptides in the databases, nor do they contain obvious functional motifs. We are currently examining the function of this locus

TABLE 2. Novel mutants with high E, Q, and P percentages or repeated motifs

Gene	Size		Insertion position(s)	Amino acid composition (% E/% Q/% P) ^a	Signal peptide	Coils	Transmembrane helices	PFAM designation(s)
	Residues	Nucleotides						
slr0358	374	1,122	437	13/4.3/10.4	No	No	No	No hits
sll0565	133	399	270	10.5/11.3/0.8	No	Yes	Yes	No hits
slr1964	134	402	147	5.2/ 10.4 /0.75	No	Yes	Yes	No hits
slr1301	535	1,605	3, 443, 1057	13.6/13.6/1.9	No	Yes	No	No hits
sll0183	259	777	64, 348, 502	5/4.6/3.1	Yes (18/19) ^b	No	Yes	00515, 00805
sll0414	286	858	342	4.5/9.1/3.5	No	No	No	00805
sll0301	169	507	97	3/2.4/1.2	Yes (32/33)	No	Yes	00805

^a Numbers in bold represent amino acid percentages higher than 10% (usual levels are 3 to 5%).

^b Numbers in parentheses represent putative signal peptide cleavage sites.

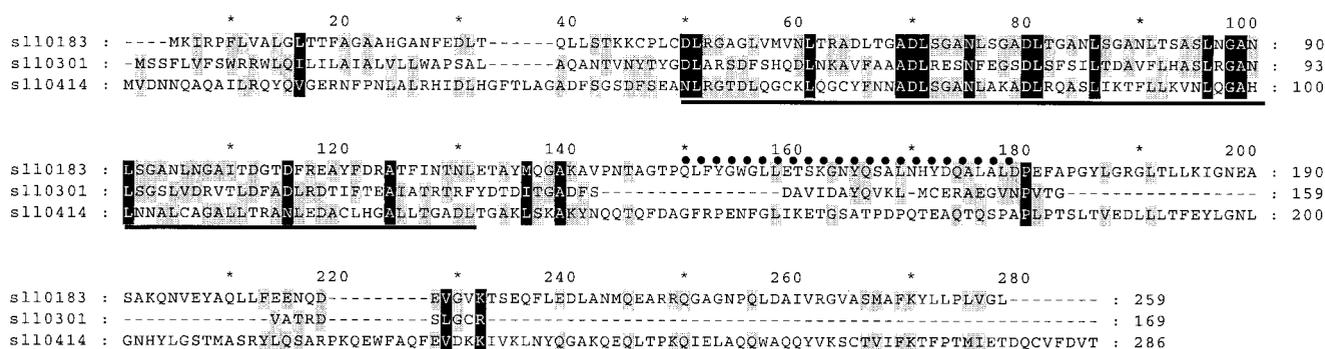


FIG. 1. Comparison of sll0183, sll0301, and sll0414, with black boxes indicating identical or conserved residues in all polypeptides and gray boxes indicating residues conserved in two polypeptides. The region with pentapeptide repeats is underlined; the tetratricopeptide motif of sll0183 is indicated by black circles.

by generating specific, nonpolar gene disruptions in each open reading frame and analyzing the effect of the disruptions on pilus morphology and biogenesis.

Table 2 lists seven genes encoding putative novel polypeptides that we have identified as being required for motility, based on phenotypes of strains mutated in these genes. These polypeptides fall into two distinct categories. The first category, represented by slr0358, sll0565, slr1964, and slr1301, are polypeptides that are relatively rich (defined here as being higher than 10%, usual levels being between 3 and 5%) in glutamic acid (E) or glutamine (Q) residues and that do not appear to have any recognizable motifs (based on analysis by Pfam [http://pfam.wustl.edu/index.html] or COG [http://www.ncbi.nlm.nih.gov/COG/] [1, 12]). While these four putative polypeptides appear to lack a signal sequence, sll0565 and slr1964 have potential transmembrane helices at the amino (N) or carboxy (C) terminus, respectively, based on DAS analysis (http://www.sbc.su.se/~miklos/DAS/) (7). Furthermore, all of these polypeptides except slr0358, which is proline (P) rich, have the possibility of forming coiled-coil structures, based on the predictions of Coils (http://www.ch.embnet.org/software/COILS_form.html) (9). The coiled-coil configuration of the putative slr1301 polypeptide may be especially extensive (data not shown). It has recently been demonstrated that FrzS of *M. xanthus* has an extensive coiled-coil structure and an FrzS mutant is incapable of social motility (14). Proteins rich in glutamate and glutamine residues and with the ability to form coiled coils may be involved in protein-protein interactions, often being part of a large macromolecular complex (6).

The putative polypeptides encoded by sll0183, sll0414, and sll0301 are in a second category that is distinguished by the presence of a repeated pentapeptide (XGAD/NL) motif categorized in the Pfam database as pfam 00805 (ANLSGADLTGADLRGADLSGADLTGANLSGANLSGADLSG). The repeat of a leucine residue every fifth residue is reminiscent of a leucine zipper, but this region is not predicted to form an alpha helix (Fig. 1). There are 15 polypeptides encoded by the *Synechocystis* genome that contain this domain (slr1819, sll1446, sll0414, slr1851, slr1152, slr1519, slr0516, slr0967, sll0274, slr0719, sll0183, sll0577, sll0301, sll1011, and slr1697; genes in italic are transposon mutants). The proteins encoded by sll0183 and sll0301 may also contain signal sequences (the putative cleavage sites are shown in Table 2). The protein

encoded by sll0183 contains a second motif which is a tetratricopeptide repeat (amino acid 150 to 180) (Fig. 1). This motif (pfam 00515) represents an ancient module that functions in protein-protein interactions and participates in many cellular processes, including cell cycle progression, transcription, chaperone action, and protein transport (5). These findings raise the intriguing possibility that pilus biogenesis or function requires several polypeptides critical for protein-protein interactions, possibly having a scaffold or architectural function. We are currently addressing these questions.

The results of the mutant screen highlight two intriguing findings. First, there appear to be many polypeptides involved in cyanobacterial motility that have specific biochemical functions (based on homologies with other proteins), but their role in motility is relatively unexplored. We have evidence that two loci (*tax1* and *tax3*) which contain several *che*-like genes are involved in pilus-mediated phototaxis (3). These loci include histidine kinases as well as a chemoreceptor with a putative phytochrome-like chromophore-binding domain. We postulate that these proteins are involved in a complex signal transduction pathway that regulates phototaxis (3). In some cases analogous proteins have been found to be required for motility in other organisms, such as *M. xanthus*. Second, groups of polypeptides with distinct structural characteristics are required for motility, and although their role in motility is not known, they may serve to facilitate interactions among proteins in large complexes or be part of an intracellular matrix that is required to assemble and/or anchor the pilus-associated motor complex.

The work presented in this manuscript was supported in part by National Science Foundation grant MCB 9727836.

REFERENCES

- Bateman, A., E. Birney, R. Durbin, S. R. Eddy, K. L. Howe, and E. L. Sonnhammer. 2000. The Pfam protein families database. *Nucleic Acids Res.* **28**:263–266.
- Bhaya, D., N. R. Bianco, D. Bryant, and A. Grossman. 2000. Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp PCC 6803. *Mol. Microbiol.* **37**:941–951.
- Bhaya, D., A. Takahashi, and A. Grossman. 2001. Light regulation of Type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC 6803. *Proc. Natl. Acad. Sci. USA* **98**:7540–7545.
- Bhaya, D., N. Watanabe, T. Ogawa, and A. R. Grossman. 1999. The role of an alternate sigma factor in motility and pili formation in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Proc. Natl. Acad. Sci. USA* **96**:3188–3193.

5. **Blatch, G. L., and M. Lassel.** 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**:932-939.
6. **Burkhard, P., S. V. Strelkov, and J. Stetefeld.** 2001. Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.* **11**:82-88.
7. **Cserzo, M., E. Wallin, I. Simon, G. von Heijne, and A. Elofsson.** 1997. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng.* **10**:673-676.
8. **Kamei, A., T. Yuasa, K. Orikawa, X. Geng, and M. Ikeuchi.** 2001. A eukaryotic-type protein kinase, SpkA, is required for normal motility of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **183**:1505-1510.
9. **Lupas, A., M. Van Dyke, and J. Stock.** 1991. Predicting coiled coils from protein sequences. *Science* **252**:1162-1164.
10. **Mrazek, J., D. Bhaya, A. R. Grossman, and S. Karlin.** 2001. Highly expressed and alien genes of the *Synechocystis* genome. *Nucleic Acids Res.* **29**:1590-1601.
11. **Simms, S. A., A. M. Stock, and J. B. Stock.** 1987. Purification and characterization of the S-adenosylmethionine:glutamyl methyltransferase that modifies membrane chemoreceptor proteins in bacteria. *J. Biol. Chem.* **262**:8537-8543.
12. **Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin.** 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.* **29**:22-28.
13. **Terauchi, K., and K. Ohmori.** 1999. An adenylate cyclase, *cyaI*: regulates cell motility in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol.* **40**:248-251.
14. **Ward, M. J., H. Lew, and D. R. Zusman.** 2000. Social motility in *Myxococcus xanthus* requires FrzS, a protein with an extensive coiled-coil domain. *Mol. Microbiol.* **37**:1357-1371.
15. **Weimer, R. M., C. Creighton, A. Stassinopoulos, P. Youderian, and P. L. Hartzell.** 1998. A chaperone in the HSP70 family controls production of extracellular fibrils in *Myxococcus xanthus*. *J. Bacteriol.* **180**:5357-5368.
16. **Wu, S. S., J. Wu, Y. L. Cheng, and D. Kaiser.** 1998. The *pilH* gene encodes an ABC transporter homologue required for type IV pilus biogenesis and social gliding motility in *Myxococcus xanthus*. *Mol. Microbiol.* **29**:1249-1261.