

Transposon Mutagenesis in a Marine *Synechococcus* Strain: Isolation of Swimming Motility Mutants

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Certain marine unicellular cyanobacteria of the genus *Synechococcus* exhibit a unique type of swimming motility characterized by the absence of flagella or any other obvious organelles of motility. While the abundant cell surface-associated 130-kDa glycoprotein SwmA is known to be required for the generation of thrust, identification of other components of the motility apparatus has, until recently, been unsuccessful. Here we report on the development of a transposon mutagenesis system for use with marine *Synechococcus* sp. strain WH8102, a model organism for which the genome has been sequenced. Utilizing this mutagenesis technique, we have isolated 17 independent mutants impaired in swimming motility. These 17 transposon insertions are located in nine open reading frames, which cluster in three separate regions of the genome. Included within these clusters are several multicomponent transport systems as well as a number of glycosyltransferases.

The unicellular marine cyanobacteria of the genus *Synechococcus* are ubiquitous in the world's oceans and comprise a major fraction of the photosynthetic marine picoplankton (29–31). As such, they are important contributors to global primary production and may generate as much as 30% of total oceanic primary production (29). A genetic manipulation system has been described previously (5, 6) which has allowed molecular investigation into the adaptations of this globally important organism. Here we describe an addition to the molecular tools available for use with marine *Synechococcus* spp. with the development of a transposon mutagenesis technique. We have applied this method of mutagenesis to investigate the unique swimming motility exhibited by marine *Synechococcus* spp.

Among the adaptations exhibited by marine *Synechococcus* spp. is the ability of certain strains to swim through their liquid environment without the use of flagella (33). Transmission electron microscopy techniques, such as negative staining and quick-freeze fixation, fracture, and etching as well as high-intensity dark-field microscopy, have been employed in attempts to visualize structures, without success (35). Additionally, shearing experiments and motility-dependent amplitude spectra confirm the lack of flagella or other extracellular appendages used for motility (35). Both jet propulsion and self-electrophoresis have been ruled out as possible mechanisms for motility (24). This leaves the cell surface itself as the only remaining structure with potential for generating a propulsive force. Ehlers et al. have proposed a model by which cells could move at the observed speeds by propagating longitudinal waves across their surfaces (9).

Nonflagellar motility is not limited to marine *Synechococcus* spp. A diverse array of bacteria (16), including other cyanobacteria (12), exhibit nonflagellar motility, but in virtually every

case this movement is not swimming but rather a movement associated with surfaces. Marine *Synechococcus* spp. are different in that in spite of their cells' clear lack of flagella, they swim through their liquid environment and do not move along surfaces. With the exception of the helical, wall-less bacteria of the genus *Spiroplasma*, which swim by means of conformational deformations of each cell's helical cytoskeletal filament (11, 36), *Synechococcus* spp. are the only bacteria known to swim without flagella. For a review of swimming motility in marine *Synechococcus* spp., see the work of Brahamsha (7).

While the basic tools required for genetic manipulations in *Synechococcus* spp. have been described previously (5, 6), a functional transposon mutagenesis system would be a powerful additional tool for molecular and genetic studies. The integration of a transposon into the host chromosome creates an insertional mutation, which can be easily cloned and sequenced to determine the site of insertion. We used the delivery vector pRL27 (15), which has a mini-Tn5 derivative engineered to enhance transposition frequency, for transposon mutagenesis in *Synechococcus* sp. strain WH8102, and our results have shown that this tool is useful in identifying new genetic loci involved in swimming motility. As this was the first use of transposon mutagenesis in a marine *Synechococcus* sp., preliminary experiments were conducted to characterize the use of this delivery vector and transposon. When combined with an appropriate screen or selection, the method described here will be applicable to the study of various aspects of *Synechococcus* physiology. We have developed a screen to identify mutants impaired in the ability to swim, and this screen in combination with transposon mutagenesis has identified several regions of the chromosome that contain genes necessary for nonflagellar swimming motility.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains MC1061(pRK24, pRL528), DH5 α , BW20767 (15), and Transformax EC100D *pir*⁺ (Epicentre, Madison, WI) were grown in Luria-Bertani medium (28). When appropriate,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source ^b or reference
Strains		
<i>Synechococcus</i> sp. strain WH8102	Motile strain, recipient in conjugations with pRL27 and pMUT100 constructions	J. Waterbury
<i>E. coli</i>		
MC1061	Host for pRK24, pRL528; used as a donor in pJM construction conjugations	8
DH5 α	Recipient in transformations	BRL
BW20767	Used as donor for pRL27 conjugations	21
Transformax EC100D <i>pir</i> ⁺	Expresses <i>pir</i> gene product for propagation of vectors containing the R6K γ origin of replication	Epicentre
Plasmids		
pMUT100	Kan ^r , Tet ^r ; suicide vector	5
pRL27	Kan ^r ; mini-Tn5 plasmid (<i>oriR6K</i>) delivery vector	15
pRK24	Tc ^r , Amp ^r ; conjugal plasmid, RK2 derivative	5, 22
pRL528	Cm ^r ; helper plasmid, carries <i>mob</i>	5, 10
pCR2.1-TOPO	Kan ^r , Amp ^r ; PCR product cloning vector	Invitrogen
pJM37	pMUT100 containing SYNW0079 fragment of nt 78600 to 78939	This work
pBB1000	pMUT100 containing <i>swmA</i> fragment of nt 84507 to 84926	This work
pJM62	pMUT100 containing SYNW0087 fragment of nt 88793 to 89004	This work
pJM18	pMUT100 containing SYNW0088 fragment of nt 89414 to 89635	This work
pJM59	pMUT100 containing SYNW0192 fragment of nt 193443 to 193661	This work
pJM40	pMUT100 containing SYNW0193 fragment of nt 194020 to 194287	This work
pJM20	pMUT100 containing <i>swmB</i> fragment of nt 913153 to 913396	This work
pJM35	pMUT100 containing SYNW0957 fragment of nt 947811 to 948110	This work
pJM34	pMUT100 containing SYNW0960 fragment of nt 953811 to 954171	This work

^a nt, nucleotide. Numbering according to reference 23.

^b BRL, Bethesda Research Laboratories, Gaithersburg, MD; Invitrogen, Carlsbad, CA; Epicentre, Madison, WI.

ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), and chloramphenicol (10 μ g/ml) were used for the selection and maintenance of plasmids in *E. coli*. Cyanobacterial strains were grown either in SN medium (32) made with seawater obtained from the Scripps Pier (Scripps Institution of Oceanography, La Jolla, CA) or in SN medium prepared with synthetic ocean water (25). Cyanobacterial cultures were incubated at 25°C with a constant illumination of 25 microeinsteins m⁻² s⁻¹ and were maintained as either 4-ml cultures in 17- by 100-mm polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ) or as 50-ml cultures in 125-ml glass flasks without shaking. SN pour plates for obtaining isolated colonies were prepared as previously described (5), with the single modification of reducing the agar concentration to 0.2% (wt/vol), which aids in screening for nonmotile colonies. Kanamycin was added to a final concentration of 25 μ g/ml for pour plates and 20 μ g/ml for liquid cultures, where appropriate, to maintain selection for insertions.

Transposon mutagenesis. Biparental matings of *E. coli* and the *Synechococcus* sp. were carried out as previously described (5). *E. coli* strain BW20767, which harbors the plasmid delivery vector pRL27 (15), was used as the donor in conjugations with *Synechococcus* sp. strain WH8102. Following a 48-h incubation, cells were resuspended and plated in SN pour plates containing 0.2% agar and kanamycin (25 μ g/ml) to obtain isolated colonies. Following the appearance of colonies, which usually took from 2 to 3 weeks, the plates were screened visually for putative nonmotile mutants displaying a compact, dense colony morphology. These colonies were transferred into and grown in liquid SN medium and examined by phase-contrast microscopy to confirm the loss of swimming motility in liquid. The absence of the *E. coli* donor strain was verified as described previously (5).

Plasmid rescue. The plasmid pRL27 allows for one-step cloning of a transposon insertion and its flanking DNA (15). Chromosomal DNA was prepared from a 50-ml culture of a *Synechococcus* transconjugant as described previously (5). This purified DNA was digested with BamHI, which does not cut within the transposon sequence. The resulting fragments were ligated using T4 DNA ligase (Roche Applied Science, Indianapolis, IN) to generate a transposon junction plasmid consisting of the transposon and the flanking chromosomal DNA. The material from the ligation reaction was then electroporated into *E. coli* strain Transformax EC100D *pir*⁺ (Epicentre, Madison, WI) following the manufacturer's recommendations. Following electroporation, the *E. coli* isolate was plated on Luria-Bertani plates containing kanamycin. Plasmid DNA was isolated from the transformants using a QIAprep spin miniprep kit (QIAGEN, Valencia, CA).

The site of insertion of the transposon was determined by sequencing, utilizing the outward-directed transposon-specific primer tprNL 17-1 (5'-AACAGCCAGGGATGTAACG-3') (15). DNA sequencing was performed with Megabace reagents (Amersham, Piscataway, NJ) on a Megabace 500 sequencer.

Directed mutagenesis. Directed inactivations were accomplished by cloning a completely internal fragment of a gene into the suicide vector pMUT100 as previously described (5). Twenty-mer oligonucleotide primers (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify DNA fragments (details given in Table 1) for cloning into pMUT100. These constructions were introduced into *Synechococcus* sp. strain WH8102 by conjugation with *E. coli* followed by subsequent selection of exconjugants on solidified media containing kanamycin. Clonal isolates were grown in liquid medium to confirm the mutant phenotype of the original transposon mutant. Complete segregation of mutant chromosomes was confirmed by Southern blotting and by PCR. For the PCR test, chromosomal DNA from the cyanobacterial mutant strain was isolated using a DNeasy tissue kit (QIAGEN, Valencia, CA) with the following modification: prior to proteinase K treatment, cells were incubated in a solution containing 20 mM Tris-Cl (pH 8.0), 2 mM Na₂EDTA, 1.2% Triton X-100, and 50 mg/ml lysozyme for 30 min at 37°C. This DNA was then used as a template for PCR analysis utilizing primers flanking the fragment used for inactivation. Primer pairs used in these tests were confirmed to amplify a fragment of the expected size from wild-type DNA. Failure to amplify a fragment of the wild-type size from a mutant strain's DNA confirms the absence of the intact gene among the clonal population of mutant cells. This same DNA sample was used as a template in another PCR, utilizing primers directed to another open reading frame (ORF) as a positive control to confirm that the DNA was of sufficient quality for PCR amplification.

Genome information. Complete genomic sequence information for *Synechococcus* sp. strain WH8102 is available at http://genome.ornl.gov/microbial/syn_wh/ (23).

RESULTS

Initial characterization. After an initial conjugation of *Synechococcus* sp. strain WH8102 with *E. coli* strain BW20767 (containing the plasmid delivery vector pRL27), 11 kanamycin-resistant transconjugants were selected randomly without

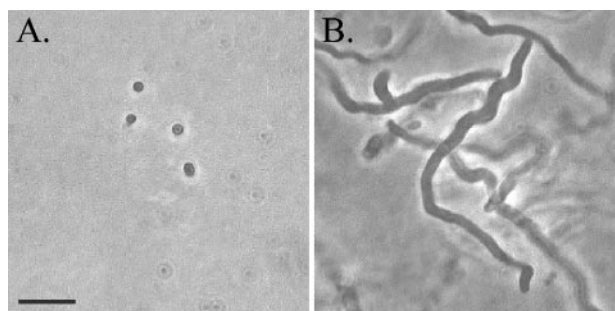


FIG. 1. Phase-contrast micrographs of wild-type WH8102 (A) and *minD* mutant (B) cells. Scale bar, 10 μ m (both panels).

application of a specific phenotypic screen. Following plasmid rescue, the site of transposon integration was determined for each strain by sequencing outwards from the transposon into flanking chromosomal DNA. The transposon insertion site was unique for each isolate. Genomic DNA isolated from these strains was also subjected to digestion with *Sma*I and to Southern analysis utilizing a probe designed against a portion of the kanamycin resistance gene present within the transposon. For each strain assayed, the probe hybridized with a single band consistent in size with that predicted by the genome sequence (data not shown). The majority of these strains did not have a phenotype that was obviously different from that of the wild-type strain, with a few exceptions. Two nonmotile strains were isolated and are described in greater detail below. The cells of another isolate, while still motile, were extremely elongated, ranging from 10 to 100 times the typical cell length (Fig. 1). In this strain, transposon insertion was into a putative septum site-determining gene, *minD*, which is present on the chromosome in the typical operon arrangement *minCDE*. The proteins of the *minCDE* system act in concert as a negative regulator of septation site formation in *E. coli* (26), and the phenotype of the *Synechococcus minD* mutant is consistent with a similar role for the Min proteins in *Synechococcus*. Directed mutagenesis at this locus confirmed the filamentous phenotype observed in the transposon mutant; nevertheless, these elongated cells retain the ability to swim.

Nonmotile mutants. To screen for mutants with impaired ability to swim, a screen was developed to distinguish between motile and nonmotile isolates based on colony morphology. Solid SN medium prepared as described previously (5), with the single modification of reducing the agar concentration to 0.2%, allowed for the identification of nonmotile isolates. Nonmotile cells produce small, dense colonies while motile cells produce larger, more diffuse colonies (Fig. 2). Nonmotile isolates were grown for multiple transfers in liquid medium to confirm total loss of swimming motility.

Utilizing this screen, 17 nonmotile transposon mutant strains were obtained (Table 2). These 17 independent transposon insertions are located in nine ORFs, which cluster into three separate regions of the genome (Fig. 3). Eight transposon insertions are located in a 12-kb region (cluster 1) that includes the previously identified motility gene *swmA* (5). Another five transposon insertions are tightly grouped into a 1.4-kb region that appears to be the beginning of an operon containing four genes (cluster 2). The last four insertions are spaced out over

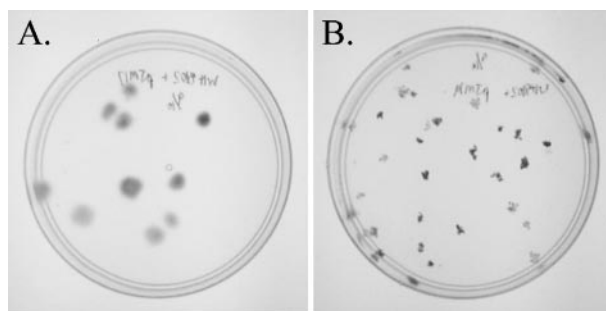


FIG. 2. Pour plates of *Synechococcus* sp. strain WH8102 transconjugants for isolation and screening of mutants. Shown are both a motile transconjugant (A) and a nonmotile transconjugant (B).

a 41-kb region that contains, among other ORFs, one large ORF of 32.4 kb (cluster 3).

To confirm that the phenotype observed for these mutants was the result of transposon insertion, directed mutations were constructed for each ORF identified by transposon mutagenesis as required for swimming motility. These directed mutations confirmed the motility phenotype of the original mutations. None of the mutants described here exhibits the attached rotating behavior of the *swmA* mutant. Two ORFs (SYNW0957 and SYNW0960) interrupted by transposon insertion were resistant to inactivation by directed mutagenesis. Multiple attempts were made to create a directed mutation near the site of transposon insertion of these problematic ORFs, and in no instance were we able to obtain transconjugants. DNA from the original transposon mutant isolates (strains Tr A4-5 and Tr D2-3) was analyzed by PCR, utilizing primers flanking the site of transposon insertion. Positive PCR amplification of the wild-type-sized product from these isolates confirmed that the original transposon mutants were incomplete segregants which still possessed a nondisrupted intact gene.

On the basis of their proximity to one another, some genes in which insertions occurred are likely to be arranged in operons, and hence polar effects on downstream genes are possible.

TABLE 2. Transposon insertions yielding nonmotile mutants

Strain	ORF	Directed-mutant phenotype	Function
Tr C5-13	SYNW0079	Nonmotile	Conserved hypothetical
Tr B8-2	SYNW0085	Nonmotile	SwmA (cell surface S-layer protein [4])
Tr 3-4	SYNW0087	Nonmotile	Putative glycosyltransferase
Tr C10-3	SYNW0087		
Tr B7-3	SYNW0088		
Tr B5-2	SYNW0088	Nonmotile	Putative glycosyltransferase
Tr B1-4	SYNW0088		
Tr B3-10	SYNW0088		
Tr B8-5	SYNW0192	Nonmotile	Putative parvulin-like PPIase
Tr B6-4	SYNW0193	Nonmotile	Putative ABC transporter of the HlyB family
Tr A6-8	SYNW0193		
Tr A6-1	SYNW0193		
Tr B6-6	SYNW0193		
Tr A4-4	SYNW0953	Nonmotile	SwmB
Tr 15-2	SYNW0953		
Tr A4-5	SYNW0957	No transconjugants	Conserved hypothetical
Tr D2-3	SYNW0960	No transconjugants	Putative parvulin-like PPIase

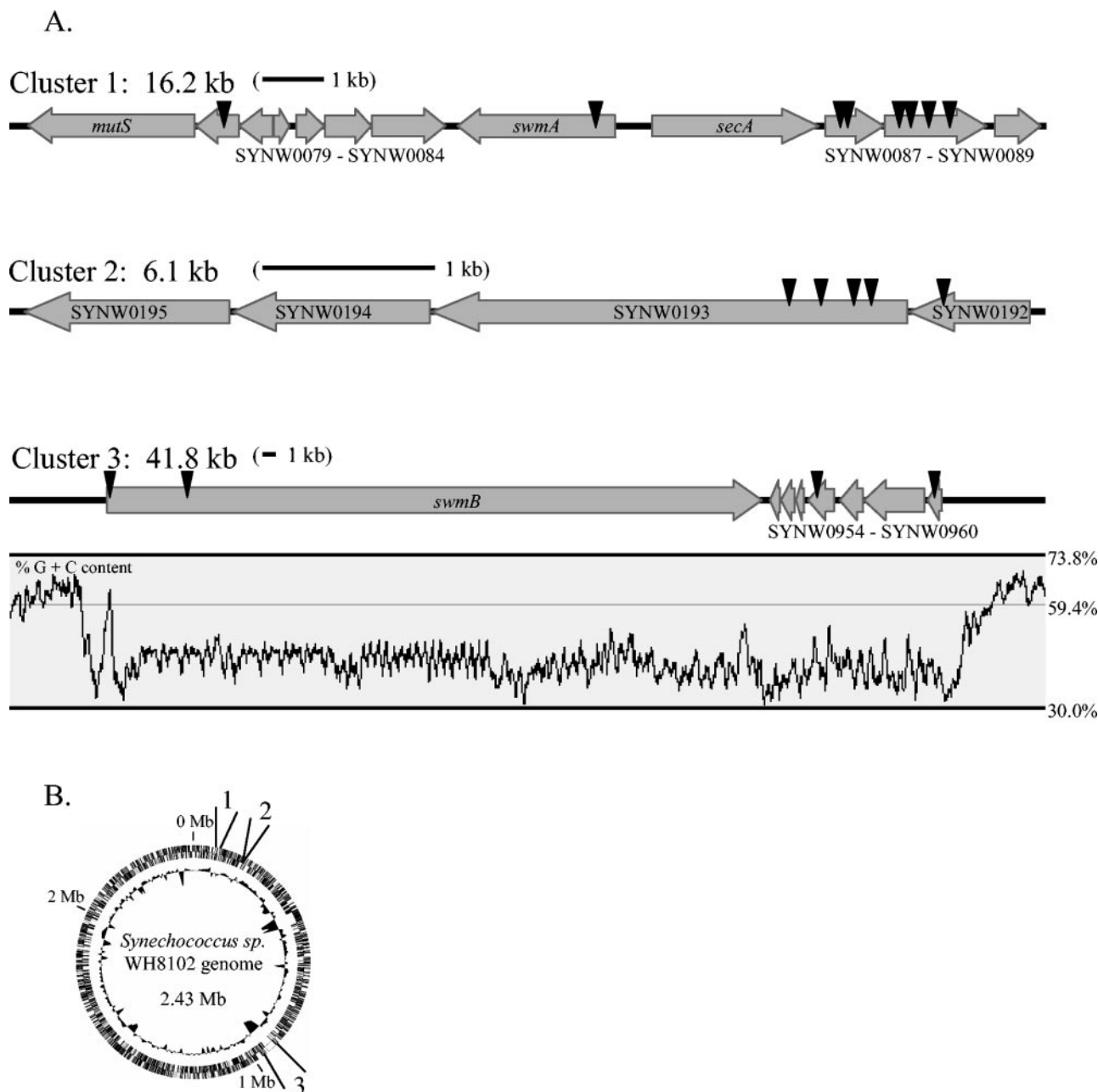


FIG. 3. Chromosomal regions containing genes involved in swimming motility as identified by transposon mutagenesis. (A) Gene arrangement and location of each transposon insertion (arrowheads) are shown. A 1-kb scale bar is included for each cluster. Percent G+C content (window = 200 bp) is included for the chromosomal region encompassing cluster 3. (B) Location of clusters 1 to 3 on a circular chromosome. The outer ring contains predicted ORFs for both strands, and the inner ring shows associated G+C content (deviation from average).

The start codon of SYNW0088 is 5 bp from the stop codon of SYNW0087. Likewise, in cluster 2, the stop codon of SYNW0192 overlaps the start codon of SYNW0193, and the stop and start codons of the next two downstream ORFs are 2 and 5 bp apart, respectively. On the other hand, neither *swmA* (SYNW0085) nor SYNW0953 is transcribed with other genes, while the intergenic distances between SYNW0079, SYNW0957, SYNW0960, and SYNW0088 and their downstream genes (159 bp, 59 bp, 118 bp, and 123 bp, respectively) indicate that they are unlikely to be cotranscribed. Determining which, if not all, of ORFs SYNW0087, SYNW0192,

SYNW0193, SYNW0194, and SYNW0195 are needed for motility will require complementation experiments. Unfortunately, these experiments are currently not possible with *Synechococcus* sp. strain WH8102, as kanamycin is the only selectable marker available. We are working to remedy this.

DISCUSSION

The transposon delivery vector pRL27 has been mobilized into a diverse array of bacterial species (15). As this was its first use in a marine *Synechococcus* sp., we report here on prelim-

inary characterization of the behavior of this transposon delivery vector in this host. Sequence analysis of the first 11 isolated mutant strains indicates that each transposon insertion is unique, and Southern analysis of DNA isolated from these same mutant strains shows that for each strain, transposon insertion occurred only once, indicating that insertion of multiple transposons is likely to be rare.

Motile cells grown embedded in solidified media produce large, diffuse colonies among which nonmotile isolates can be identified by their small, dense colony morphology. Using this simple visual screen, we have isolated 17 nonmotile transposon mutants and characterized the site of transposon insertion for each. All motility genes identified to date cluster into three discrete regions on the chromosome. Various transporters and transport-related genes are present within these clusters. Both cluster 2 and cluster 3 contain a set of three genes that appear to encode a multicomponent transport apparatus. Moreover, these three genes are arranged in the same order in both clusters. Each set contains a gene for an ABC transporter of the protein-1 exporter (HlyB) family (SYNW0193 and SYNW0959) (27), a gene for a membrane fusion protein (SYNW0194 and SYNW0958) which is an auxiliary component possibly spanning the periplasm to connect the ABC transporter with an outer membrane component, and a gene whose product has limited similarity to peptidyl-prolyl isomerases (PPIases) (SYNW0192 and SYNW0960). Such PPIases have been shown to exhibit chaperone-like activity involved in the maturation of outer membrane porins in *E. coli* (2). Additionally, the SYNW0957 product shows very weak similarity to the MotA/TolQ/ExbB proton channel family (PFAM accession no. PF01618), which is also involved in protein translocation across both membranes of the gram-negative bacterial cell wall. In addition to multicomponent transporters, the products of several ORFs in these clusters (SYNW0087, SYNW0088, SYNW0084, and SYNW0195) show similarity to glycosyltransferases. Interestingly, the only previously known component of the motility apparatus, SwmA, is glycosylated (4) and has been shown to form an S-layer on the surface of these cells outside of the outer membrane (20). Both of these characteristics of SwmA correspond well with the types of genes found in the motility clusters. The presence of multiple transporters and multiple glycosyltransferases suggests that other components of the motility apparatus may be located outside the outer membrane and may be posttranslationally modified by glycosylation.

One exceptional gene present in cluster 3 (SYNW0953), hereinafter called *swmB*, is more than 32 kb long and comprises more than 1% of the total genome of this organism. While the function of SwmB is still unclear, preliminary experiments have shown that this protein is associated with the outer membrane (J. McCarren and B. Brahamsha, unpublished). More than half of the sequence of *swmB* is composed of regions of large tandem repeats. Database searches against the SwmB sequence indicate limited similarity to bacterial RTX proteins (named for repeats in toxin, which are secreted toxins that possess tandem copies of a nine-amino-acid motif) (34) and that this similarity is restricted to the repetitive portions of SwmB. While SwmB shares similarity with RTX proteins, SwmB does not actually contain the RTX repeat. Cluster 3 is also exceptional in its percent G+C content compared to

that of the rest of the genome (Fig. 2). *Synechococcus* sp. strain WH8102 has a genome average G+C content of 59.4% (23) while the G+C content of cluster 3 is 42.2% and exhibits an abrupt departure from the genome average immediately before *swmB* and after SYNW0961. The dramatically different percent G+C contents suggest that these ORFs may have been acquired by a recent horizontal gene transfer event. While the ORFs in cluster 3 may be a recent acquisition, clusters 1 and 2 have percent G+C contents typical of *Synechococcus* sp. strain WH8102, suggesting that all of the components required for motility were not acquired in a single horizontal transfer event.

While swimming motility in marine *Synechococcus* spp. is distinct from the surface-associated gliding motility observed in other bacteria, it does share characteristics with the gliding motility observed in the *Cytophaga-Flavobacteria-Bacteroides* group, which occurs without slime extrusion or apparent appendages (17). Following chance attachment, both types of cells are observed to rotate about their point of attachment at a rate of 1 to 2 rotations per s (14, 35). Additionally, several parallels can be drawn between the motility genes of *Flavobacterium johnsoniae* identified in reports from the laboratory of McBride and coworkers (1, 13, 18, 19) and the types of genes identified in the data presented here. The first is the importance of transporters to both mechanisms of motility. Two sets of putative multicomponent ABC transporters have been identified in our current results. Similarly, several genes homologous to multicomponent ABC transporters (*gldA*, *gldF*, and *gldG*) and other transport-related genes (*secDF*) are all required for *F. johnsoniae* motility. A second similarity shared by both mechanisms of motility is that they employ PPIase-like proteins. While the PPIase-like ORF products required for *Synechococcus* and *F. johnsoniae* motility are from separate and evolutionarily unrelated families, the implication of functionally related proteins is of interest. Lastly, some genes with limited similarity to *swmB* have been implicated in *Cytophaga-Flavobacteria-Bacteroides*-type gliding motility. Recently, both a gene encoding an RTX autotransporter protein (T. Braun, S. Nelson, M. Uppal, and M. McBride, Abstr. Gen. Meet. Am. Soc. Microbiol. 2004, abstr. 104, 2004) and an exceptionally large and repetitive ORF (S. Nelson, personal communication) have been found to be involved in *F. johnsoniae* gliding motility. Also worthy of note in light of the low G+C content of cluster 3 genes, bacteria of the family *Flavobacteriaceae* have a characteristically low G+C content, ranging from 28 to 44% (3). How surface-associated gliding motility and nonflagellar swimming motility are related is difficult to envision, but the identification of similar types of genes involved in both phenomena suggests some possible relationship.

How the products of these recently identified genes function has yet to be determined. Exporting components of the motility apparatus to the cell surface appears to be important. In addition to allowing for the correct localization of the motility apparatus, perhaps these transporters play a more active role in generating swimming motility. Furthermore, a number of glycosyltransferases appear to be implicated. Whether these function to glycosylate protein components of the motility apparatus or whether they are involved in some other aspect of polysaccharide biosynthesis important for motility is unclear. Although how *Synechococcus* spp. are able to swim is still not

understood, these findings have begun to identify the genes required for swimming motility.

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