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A Genetic Manipulation System for Oceanic Cyanobacteria of the Genus *Synechococcus*

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Unicellular cyanobacteria of the genus *Synechococcus* are among the most abundant members of the picoplankton in the open ocean, and their contribution to primary production is considerable. While several isolates have been used for physiological, biochemical, and molecular studies of their unique adaptations to the marine environment, it has become necessary to develop molecular genetic methods for one or more model open-ocean cyanobacteria in order for studies of these organisms and their unique properties to progress. A number of molecular tools for the genetic manipulation of *Synechococcus* sp. strains WH7803, WH8102, and WH8103 have been developed. These include a plating technique for obtaining isolated colonies at high efficiencies and a conjugation method for introducing both a replicative vector and a suicide vector. In addition, a method for the generation of random, tagged chromosomal insertions (N. Dolganov and A. R. Grossman, J. Bacteriol. 175:7644–7651, 1993; N. F. Tsinoremas, A. K. Kutach, C. A. Strayer, and S. S. Golden, J. Bacteriol. 176:6764–6768, 1994) has been applied to these organisms.

Unicellular cyanobacteria of the genus *Synechococcus* occupy an important position at the base of the marine food web: they are among the most abundant members of the picoplankton in the open ocean (32), and their contribution to primary production has been estimated to be 5 to 30% (32, 33). Marine *Synechococcus* species possess a number of unique biological properties not found in any other cyanobacterial group. These include the ability of some strains to swim by a novel mechanism (35), the ability to acquire major nutrients and trace metals at the submicromolar concentrations found in the oligotrophic open ocean, and the ability to synthesize unique photosynthetic pigments (1, 22, 23). Furthermore, unlike *Synechococcus* species isolated from brackish waters or coastal areas, these oceanic *Synechococcus* species are obligately marine, having elevated growth requirements not only for Na^+ but also for Cl^-, Mg^{2+}, and Ca^{2+} (33). Throughout this article, these organisms will be referred to as *Synechococcus* sp. strains of marine cluster A (MC-A) as proposed by Waterbury and Rippka (30) and Carr and Mann (6).

The unique properties of this group of marine cyanobacteria have been the subject of a number of physiological and biochemical studies, including studies of motility and chemotaxis (35, 38, 39), growth and nutrient dynamics (14, 16), mechanisms of viral resistance (27, 31, 40), and photosynthesis (15, 41). More recently, these organisms have become the subjects of molecular biological analyses of their unique adaptations to the marine environment (reviewed in reference 6), and a number of genes have been cloned and sequenced, including those coding for components of the photosynthetic apparatus (36, 37), a periplasmic phosphate-binding protein (25), and proteins involved in phosphate regulation (6) and urea utilization (8). Although genetic transfer systems are available for quite a number of terrestrial, freshwater, and coastal cyanobacteria (10, 24, 26, 28), such systems have not been developed for *Synechococcus* spp. of MC-A, in large part because these organisms have been difficult to grow on solid media as isolated colonies.

As part of an effort to address the novel mechanism of swimming exhibited by some *Synechococcus* spp. of MC-A, a number of molecular tools for the genetic manipulation of these organisms have been developed. I describe here a plating method for obtaining isolated colonies of *Synechococcus* sp. strains WH7803, WH8102, and WH8103 at high efficiencies; a method for introducing, via conjugation, both a replicative vector and a suicide vector into these organisms; and the application of a method for the inactivation of genes and for the generation of random, tagged chromosomal insertions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (18). Ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (10 μg/ml) were used, when appropriate, for the selection and maintenance of plasmids in *E. coli*. Cyanobacterial strains were grown in medium SN (34) prepared with seawater obtained from Scripps Pier (Scripps Institution of Oceanography, La Jolla, Calif.). Fifty-milliliter cultures in 125-ml flasks were incubated without shaking at 25°C and at a constant light intensity (10 microeinsteins $\cdot m^{-2} \cdot s^{-1}$). SN agar medium (34) was prepared by using Difco Bacto Agar further purified as described in reference 34. Prior to being added to the sterile seawater and agar, the mineral salts and trace metals were filter sterilized.

For pour plating of single colonies, *Synechococcus* sp. strains WH7803, WH8102, and WH8103 were serially diluted in SN medium and 0.1 ml of the dilution was added to 35 ml of SN medium containing 0.3% (wt/vol) agar at 37°C and poured immediately into a Falcon (Becton Dickinson, Lincoln Park, N.J.) plastic petri dish. For plates that were to be spread, the agar concentration was 0.6% (wt/vol). All plates were incubated at 25°C at a light intensity of 10 microeinsteins $\cdot m^{-2} \cdot s^{-1}$ for 24 h and then moved to a higher light intensity of 20 to 30 microeinsteins $\cdot m^{-2} \cdot s^{-1}$. The initial incubation in lower-intensity light is critical for high plating efficiencies.

Conjugation. For biparental matings, *E. coli* MC1061(pRK24, pRL153) was grown in LB broth supplemented with the appropriate antibiotics overnight at 37°C with vigorous aeration. One milliliter of culture was centrifuged, washed twice in 1 ml of LB medium, and resuspended in 500 μl of SN containing 10% (vol/vol) LB broth. Twenty-five milliliters of exponentially growing cyanobacterial culture (typically containing 7×10^7 to 4×10^8 cells per ml as determined by direct cell counts) was centrifuged at 20°C and resuspended in 150 μl of SN. Ten to fifty microliters of cyanobacterial suspension was mixed with 50 μl of *E. coli* strains and spotted immediately on a week-old SN plate containing 0.6% (wt/vol) agar. Once the liquid had been absorbed (approximately 2 h at room temperature), the plate was incubated at a light intensity of 10 microeinsteins $\cdot m^{-2} \cdot s^{-1}$ for 48 h at 25°C. The inoculated areas, which generally were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference[a]
Strains		
E. coli		
MC1061	Host for pRK24, pRL153, and interposon bank; used as donor in conjugations	7
DH5α	Recipient in transformations	BRL
Synechococcus sp.		
WH7803	Recipient in conjugations; nonmotile	J. Waterbury
WH8102	Recipient in conjugations; motile	J. Waterbury
WH8103	Recipient in conjugations; motile	J. Waterbury
Plasmids		
pRK24	Tc^r Ap^r; conjugal plasmid; derivative of RK2	20
pRL153	Kan^r; RSF1010 derivative consisting of bp 680–2516 of Tn*5* ligated to bp 2118–7770 of RSF1010	P. Wolk
pRL528	Cm^r; helper plasmid; carries *mob*	10
pBR322	Ap^r Tet^r; carries *bom*	NEB
pUC4K	Kan^r Ap^r; source of Kan^r cassette	Pharmacia
pMUT100	Kan^r Tet^r; suicide vector consisting of pBR322 containing Kan^r cassette from pUC4K in *Pst*I site	This work

[a] BRL, Bethesda Research Laboratories, Gaithersburg, Md.; NEB, New England Biolabs, Beverly, Mass.

approximately 5 mm in diameter, were cut out of the agar with a sterile spatula and placed in 2 to 10 ml of SN. Serial dilutions were then plated in SN pour plates containing 0.3% (wt/vol) agar and 25 μg of kanamycin per ml. Isolated colonies were picked from the agar by being aspirated into a pipette tip with a Pipetman (Rainin Instruments, Woburn, Mass.) and were inoculated into 2 ml of SN medium containing kanamycin (15 μg/ml). To check for the presence of *E. coli*, portions of the SN liquid culture were spotted on LB plates or inoculated into LB broth and incubated at 37°C in the dark. Portions of the SN cultures were also spotted on SN plates supplemented with glucose (0.2%) and tryptone (0.05%) and incubated at 25 and 37°C in the dark to further ensure that there was no contamination.

DNA extractions and transformations. Chromosomal DNA was prepared from 50- to 500-ml cultures of *Synechococcus* sp. strains WH7803, WH8102, and WH8103 as follows. Cells were pelleted and washed once in TES (10 mM Tris-HCl, 1 mM Na_2EDTA, 200 mM NaCl [pH 8.0]) and resuspended in TE (10 mM Tris-HCl, 1 mM Na_2 EDTA [pH 8.0]). Lysozyme was added to a final concentration of 5 mg/ml, and the mixture was incubated at 37°C for 45 min. Proteinase K and sodium dodecyl sulfate were added to final concentrations of 100 μg/ml and 0.5%, respectively, and the solution was incubated at 55°C for 1 h. The solution was extracted once with equilibrated phenol (18), once with equilibrated phenol-chloroform, and once with chloroform. The nucleic acids were precipitated with ethanol and resuspended in TE. DNase-free RNase (Boehringer-Mannheim, Indianapolis, Ind.) was added to a final concentration of 10 μg/ml, and the mixture was incubated at 37°C for 30 min. As a final purification step, the DNA was extracted with hexadecyltrimethyl ammonium bromide as described in reference 2.

Chromosomal DNA minipreparations (from 5 ml or less) were carried out as described in reference 12 by using a GeneClean kit (Bio 101, San Diego, Calif.).

Plasmids were extracted from cyanobacterial strains by a modification of the alkaline lysis minipreparation method (18). Following resuspension in GTE (50 mM glucose, 10 mM Na_2EDTA, 25 mM Tris-HCl [pH 8.0]), lysozyme was added to a final concentration of 10 mg/ml and the mixture was incubated at 37°C for 30 min prior to alkaline lysis.

E. coli strains were transformed by the $CaCl_2$ procedure (18).

Interposon library. A library consisting of *Sau*3A fragments of genomic DNA from *Synechococcus* sp. strain WH7803, WH8102, or WH8103 ligated into the *Bam*HI site of pMUT100 (Table 1) was constructed as described in references 9 and 29. The genomic DNA was completely digested with *Sau*3A, yielding fragments in the size range of 0.15 to 1.3 kb. For all three of the cyanobacterial strains, the size of the majority of the *Sau*3A fragments was approximately 0.2 kb. *E. coli* MC1061(pRK24, pRL528) was transformed with the ligation mixture and plated on LB plates containing ampicillin (10 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (10 μg/ml). The resulting colonies (approximately 2,000 to 20,000, depending on the experiment) were pooled, washed twice in LB broth, and resuspended in SN medium containing 10% (vol/vol) LB broth, and aliquots were used in conjugations with the appropriate cyanobacterial recipient. Integrated plasmids were rescued from the cyanobacterial transconjugants as described in reference 9 by using chromosomal DNA prepared by the miniprep procedure described above that was cleaved with either *Sac*I or *Bst*EII, neither of which cleaves pMUT100. The digested DNA was circularized by ligation and used to transform *E. coli* DH5α, with selection for kanamycin resistance. Plasmids isolated from *E. coli* were digested with either *Sac*I or *Bst*EII, depending on which restriction enzyme had been used to rescue them. The size of the linearized plasmid was determined by agarose gel electrophoresis and compared with the size of the fragment hybridizing with pMUT100 in the genomic digest from which the plasmid was rescued.

RESULTS

Plating. A pour plating method was used to obtain isolated colonies of *Synechococcus* sp. strains WH7803, WH8102, and WH8103 at high efficiencies. Mid- to late-log-phase cultures containing 7×10^7 to 4×10^8 cells per ml were serially diluted and plated in SN medium containing 0.3% agar. Colonies became apparent in 12 to 14 days, at which point they could easily be picked. Figure 1 shows the appearance of 21-day-old plates of *Synechococcus* sp. strains WH7803, a nonmotile strain, and WH8103, a motile strain. The motile strain forms more diffuse colonies (Fig. 1). With this plating method, the plating efficiency, as determined by comparing direct cell counts with numbers of colonies formed, is high, ranging from 70 to 100% (data not shown). Totals of 1×10^8 to 4×10^8 CFU/ml are routinely obtained from late-log-phase cultures of WH8102, WH8103, and WH7803. On the other hand, the plating efficiency for spread plates is much lower, ranging from less than 0.2% for WH8102 and WH8103 to 8% for WH7803 (data not shown). Also, colony size on spread plates is about half that of colonies formed in soft agar.

Conjugation. Conjugation has been used successfully to introduce DNA from *E. coli* into a wide variety of cyanobacteria (10, 17), including certain coastal marine species (26). Furthermore, a number of vectors based on the broad-host-range plasmid RSF1010 have been shown to replicate in a variety of coccoid unicellular cyanobacteria (19, 26). To determine whether these methods could be used with *Synechococcus* species of MC-A, biparental matings consisting of a cyanobacterial recipient and an *E. coli* donor carrying the conjugative plasmid pRK24 and the replicative plasmid pRL153 were carried out. For *Synechococcus* sp. strains WH7803, WH8102, and WH8103, the conjugation efficiencies with pRL153 as the replicative plasmid were comparable, ranging from 3.7×10^{-3} to 6.1×10^{-3} (Table 2).

The putative transconjugant colonies were picked and grown in SN medium containing kanamycin (15 μg/ml). The liquid cultures were tested for the presence of *E. coli* as described above. No viable *E. coli* cells were ever detected. After several

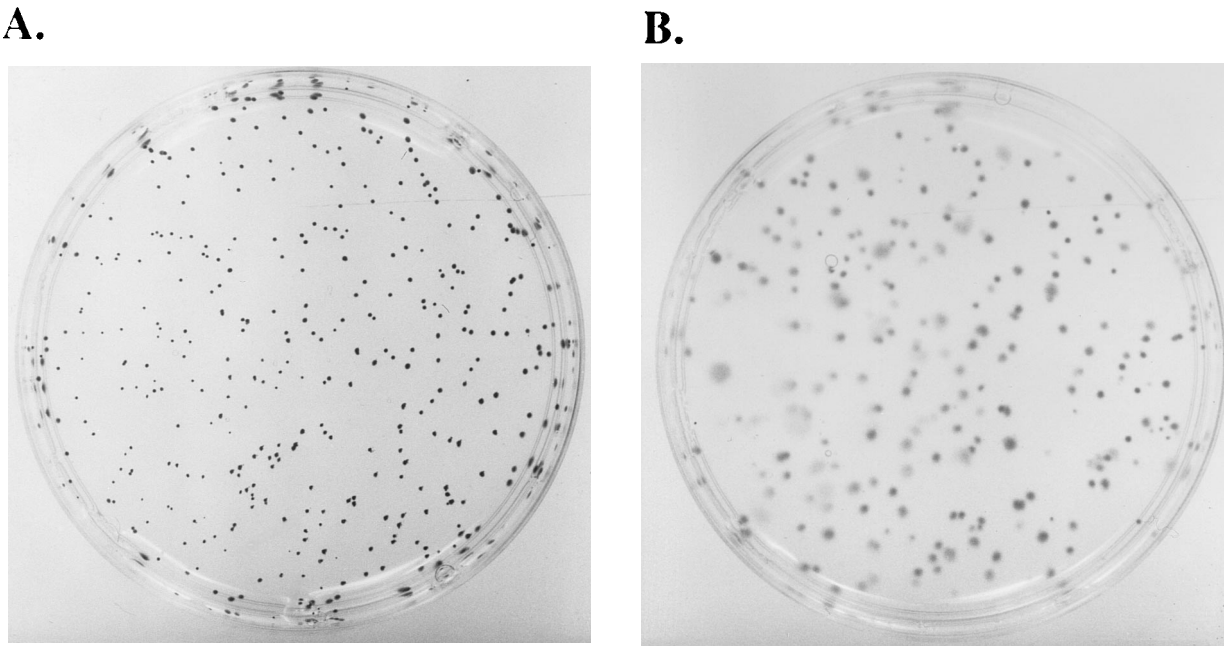

FIG. 1. Pour plates of two *Synechococcus* sp. strains. (A) Isolated colonies of *Synechococcus* sp. strain WH7803, a nonmotile strain; (B) isolated colonies of *Synechococcus* sp. strain WH8103, a motile strain.

(generally three to six) transfers in SN medium containing kanamycin (15 μg/ml), plasmids were extracted. Because the yield was generally low, as has been noted for other unicellular cyanobacterial strains carrying RSF1010 derivatives (17), the presence of the plasmid in cyanobacterial extracts was ascertained either by Southern blotting using pRL153 as a probe or by transformation of *E. coli*, as was done previously (17). Plasmids were recovered from all three strains, even after 6 months of transfer in liquid medium containing kanamycin (15 μg/ml), indicating that pRL153 can replicate in these hosts. Plasmids isolated from cyanobacteria and passed through *E. coli* were digested with *Bst*EII, *Bst*XI, *Eco*RV, *Pst*I, and *Bst*EII and *Eco*RV. The restriction pattern was compared with that of pRL153. No differences in the restriction patterns between plasmids recovered from cyanobacteria and pRL153 were detected, indicating that pRL153 does not appear to undergo deletion or rearrangement in these three cyanobacteria.

Interposon insertions. Chromosomal recombination has been used widely for the complementation of mutations, for the insertional inactivation of specific genes, and for the generation of random, tagged mutations. To determine whether this technique would be applicable to *Synechococcus* MC-A strains, a kanamycin-resistant derivative of pBR322, pMUT100, was constructed. This plasmid retains the *bom* site and can be efficiently mobilized into *Synechococcus* sp. strains WH7803, WH8102, and WH8103 by the RP4 derivative pRK24 and the helper plasmid pRL528. pMUT100 cannot replicate in these cyanobacterial hosts and therefore can serve as a suicide vector. Genomic libraries consisting of *Sau*3A fragments ligated into the *Bam*HI site of the suicide vector pMUT100 were conjugated into *Synechococcus* sp. strains WH7803, WH8102, and WH8103, and selection for kanamycin resistance was applied. Kanamycin-resistant colonies arose at frequencies of 10^{-4} for WH7803 and 10^{-5} for WH8102 and WH8103.

To determine whether the kanamycin resistance of these colonies was the result of integration of the plasmid into the chromosome, *Bst*EII or *Sac*I genomic digests of some of the kanamycin-resistant colonies of *Synechococcus* sp. strains WH7803, WH8102, and WH8103 were probed with pMUT100 (Fig. 2). In each digest, a single hybridizing band was present, indicating that integration of the vector had occurred and that it had occurred at a single site (Fig. 2). Furthermore, for each *Synechococcus* strain, the hybridizing bands were of different

TABLE 2. Conjugation frequencies

Recipient	Replicative plasmid	Frequency (10^{-3})[a]
Synechococcus sp. strain WH7803	pRL153	3.7
Synechococcus sp. strain WH8102	pRL153	4.3
Synechococcus sp. strain WH8103	pRL153	6.1

[a] The conjugation frequency is expressed as the number of transconjugants per recipient. This ratio was determined by counting the colonies formed in the presence and in the absence of kanamycin. The values represent the means for three to six independent experiments.

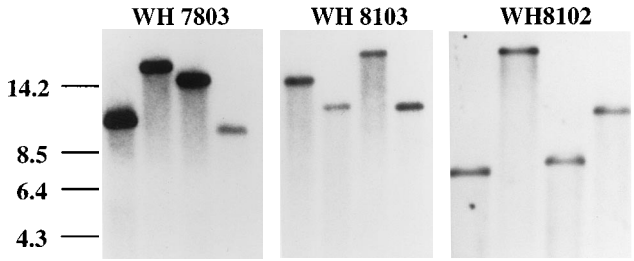

FIG. 2. Southern blots of chromosomal digests of kanamycin-resistant isolates of *Synechococcus* sp. strains WH7803, WH8103, and WH8102 obtained by interposon insertions. The probe was pMUT100. Each lane in each panel represents an independent isolate. Chromosomal DNA was digested with *Sac*I (WH7803) or *Bst*EII (WH8102 and WH8103). The migration of molecular weight standards is indicated on the left in kilobase pairs.

sizes, indicating that integration had occurred at different sites. No free plasmid DNA was detected in any of the kanamycin-resistant transconjugants as judged by transformation of *E. coli* with DNA extracts prepared from the transconjugants, indicating that the pMUT100 derivatives were not likely to be replicating in these strains but rather had integrated into the chromosome. However, when genomic DNA from the transconjugants was digested with *Bst*EII or *Sac*I, circularized by ligation, and used to transform *E. coli*, plasmids were recovered. The size of the plasmids linearized with either *Sac*I or *Bst*EII was consistent with the size of the *Sac*I or *Bst*EII fragment hybridizing with pMUT100 in the genomic digest from which the plasmids were rescued (data not shown).

DISCUSSION

The pour plating method, employing a very low concentration of washed agar, was used to obtain high plating efficiencies for three strains of *Synechococcus* MC-A, including WH7803, a strain of low phycourobilin content characteristic of shelf waters, and two phycourobilin-rich strains (WH8102 and WH8103) representative of open-ocean environments (6, 21). None of these strains grew well on agar surfaces, possibly because these organisms normally grow suspended in seawater and thus may be sensitive to desiccation. This plating technique also allows discrimination between motile and nonmotile colonies and will thus be of utility in studies of the novel mechanism of swimming motility exhibited by some strains.

A conjugation system based on the mobilization properties of plasmid RP4 (10) was used to transfer plasmid pRL153 from *E. coli* to *Synechococcus* sp. strains WH7803, WH8102, and WH8103 at high frequencies. pRL153 is a derivative of the broad-host-range plasmid RSF1010 (3) and confers kanamycin resistance. It replicates and is stably maintained in *Synechococcus* sp. strains WH7803, WH8102, and WH8103. This RSF1010 derivative was used as the replicating plasmid because a screen of a number of open-ocean *Synechococcus* strains did not detect any endogenous plasmids that could have served as the basis for the construction of vectors (5). RSF1010 derivatives have been shown to replicate stably in other unicellular cyanobacteria (17), including certain coastal marine species (26), as well as in a wide range of gram-negative bacteria (11) and some gram-positive bacteria (13). pRL153 should be of utility in the cloning of genes into these strains (for the complementation of mutations, for example).

The ability to generate random, tagged mutations as well as to inactivate specific genes is a crucial component of a system for the genetic manipulation of an organism. Because efforts at obtaining Tn*5* transposition in *Synechococcus* sp. strains WH7803 and WH8102 were for the most part unsuccessful, a recombinational method of mutagenesis described in references 9 and 29 was used to obtain insertions into the chromosomes of *Synechococcus* sp. strains WH7803, WH8102, and WH8103. This method is based on homologous recombination between a genomic bank of restriction fragments cloned in a suicide vector and their counterparts on the chromosome. If a restriction fragment is internal to a particular gene, then a single homologous recombination event results in duplication and truncation of both copies and hence inactivation of that gene. As long as selection for the plasmid-encoded antibiotic resistance is maintained, the insertion remains in the chromosome. This method has many of the advantages of transposon mutagenesis: it results in tagged mutations that are easily rescued, it results in a single insertion, and it is essentially random. This technique has yielded motility as well as pigment mutants of *Synechococcus* sp. strain WH8102 (5), suggesting that it will be of utility as a mutagenesis tool. Furthermore, by use of pMUT100 as a suicide vector, the gene encoding a motility-associated major outer membrane protein in *Synechococcus* sp. strain WH8102 has been insertionally inactivated (4), indicating that homologous recombination can be used to target specific genes in these marine cyanobacteria.

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