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Hongtao Min

Louis A. Sherman
Purdue University, lsherman@purdue.edu

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Genetic Transformation and Mutagenesis via Single-Stranded DNA in the Unicellular, Diazotrophic Cyanobacteria of the Genus *Cyanothece*▽

Hongtao Min and Louis A. Sherman*

Purdue University, Department of Biological Sciences, 201 S. University St., West Lafayette, Indiana 47907

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We describe a genetic system for producing specific gene knockouts in *Cyanothece* sp. strain PCC 7822 using a single-stranded DNA technique (B. Zorin, P. Hegemann, and I. Sizova, Eukaryot. Cell 4:1264–1272, 2005). The first fully segregated mutant was a Δ*nifK* mutant, and it was unable to grow on medium lacking combined nitrogen and produced virtually no hydrogen.

Cyanobacteria are important model organisms for the study of many biological processes, including photosynthesis, N_2 fixation, and gene regulation of metabolism. This has led to the development of genetic systems in key organisms such as *Synechococcus elongatus* (10, 11, 16), *Synechocystis* sp. strain PCC 6803 (30, 31, 33), and *Anabaena* sp. strain PCC 7120 (6, 7, 8, 9, 12, 13, 25, 34, 35, 36) and in some other bacteria (14).

We wish to develop a genetic system for a strain within the genus *Cyanothece* (19), large (3- to 8-μm) unicellular, diazotrophic cyanobacteria. When cells are grown in the absence of combined nitrogen under 12-h light-dark conditions, *Cyanothece* sp. strain ATCC 51142 has an extreme temporal regulation that coordinates photosynthesis during the daytime and N_2 fixation at night (3, 4, 19–22). This is a very valuable system for the study of photosynthesis, N_2 fixation, cellular morphology, and gene regulation (24, 27, 28, 32). Because of these properties, six *Cyanothece* genomes have been sequenced, starting with *Cyanothece* sp. strain ATCC 51142 (32). Subsequently, five other *Cyanothece* strains have been sequenced through the Department of Energy (DOE) Joint Genome Initiative (http://www.jgi.doe.gov/), including *Cyanothece* sp. strain PCC 7822 (29).

Cyanothece sp. strain ATCC 51142 was grown on ASP2, and PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802 were grown on BG11 as previously described (19). *Cyanothece* strains were grown until late log phase (~7 days), and cells were electroporated essentially as described previously (26), with the pulse controller set to 200 Ω. The cells were transformed with 1 μl DNA (5 pg to 0.5 μg) for 0.4 s and plated on either ASP2 plates (0.5% Phytagel) for *Cyanothece* sp. strain ATCC 51142 or BG11 plates (1.5% agar) for all of the other strains. The next day, after about 20 h of growth in continuous dim light (10 microeinsteins m^{-2} s^{-1}), spectinomycin (Sp; 10 μg/ml), streptomycin (1 μg/ml), or kanamycin (Km; 25 μg/ml) was underlaid under the agar.

Cyanothece genes were PCR amplified using hybrid primers with an upstream 21-nucleotide sequence matching either the upstream region of the EcoRI site or the downstream region of the HindIII site on pUC19 (Table 1). pUC19 was double digested by EcoRI and HindIII. Linearized pUC19 and PCR products of *Cyanothece* genes were transformed into XL-1 Blue (18).

The *nifK* gene was amplified by PCR using primers 7822 NifK1 and 7822 NifK2 (Table 1). Asymmetric PCR was carried out on pHM54 and pHM55 using the regular amount of 7822 NifK1 and 1/50 of the regular amount of 7822 NifK2 to produce single-stranded DNA (ss DNA) (37). The PCR product of ssDNA was boiled for 5 min and put into ice immediately for electroporation.

The putative Δ*nifK* mutant was analyzed for growth, acetylene reduction activity, and H_2 production as previously described (17).

We made numerous unsuccessful attempts to construct specific knockout mutants of *Cyanothece* sp. strain ATCC 51142 (*nifH*, *nifD*, *ntcA*, and *kaiA* mutants) using single and double recombination procedures. The failure of single and double recombinations suggested to us that the Sp^r cassette was inserted randomly into *Cyanothece* sp. strain ATCC 51142 at a very high frequency. To confirm this, we used the Sp^r cassette alone, along with the *kaiA* knockout construct, to transform *Cyanothece* sp. strain ATCC 51142 in order to compare the levels of transformation. The Sp^r cassette alone gave rise to as many transformants as the *kaiA* knockout construct. The Sp^r cassette was recovered from the transformants that were produced by the Sp^r cassette (along with its flanking sequences) and sequenced. The sequencing results (see below) from three transformants indicated that the Sp^r cassette was inserted into the *Cyanothece* sp. strain ATCC 51142 genome randomly.

Sequencing results. Three results, as follows, were found. (i) The Sp^r cassette was modified by direct repeats (TGCTTGCTGCTTGCTGCTTGCTGCTTGCTGCTTGCTGCTTGCTGCTTGC) and then inserted between amino acids (aa) 66 and 67 on cce_3669, a hypothetical protein. (ii) The Sp^r cassette was inserted between aa 192 and 193 on cce_4555, a putative polysaccharide pyruvyl transferase. (iii) The Sp^r cassette was inserted between aa 126 and 127 on cce_1116, a putative biopolymer transport protein.

From such experiments, we concluded that nonhomologous recombination was significantly more likely than homologous recombination and that *Cyanothece* sp. strain ATCC 51142

* Corresponding author. Mailing address: Purdue University, Department of Biological Sciences, 201 S. University St., West Lafayette, IN 47907. Phone: (765) 494-8106. Fax: (765) 496-1496. E-mail: lsherman@purdue.edu.

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant genotype, description, or sequence	Source or reference
E. coli strains		
KC8	Km^r $RecA^+$ *lac*Δ	Clontech
XL-1 Blue	Tc^r nalidixic acid resistant	Agilent Technologies
Cyanothece sp. strains		
ATCC 51142	Isolated from intertidal area near Port Aransas, TX	Laboratory collection
PCC 7424	Isolated from rice field soil, Senegal, 1972	Laboratory collection
PCC 7425	Isolated from rice field soil, Senegal, 1972	Laboratory collection
PCC 7822	Isolated from rice field soil at Central Rice Research Institute, Cuttack, Orissa, India	Laboratory collection
PCC 8801	Isolated from rice field soil (during spring), Ping-Tong District, southern Taiwan, as *Synechococcus* sp. strain RF-1	Laboratory collection
PCC 8802	Isolated from rice field soil (during spring), Ping-Tong District, southern Taiwan, as *Synechococcus* sp. strain RF-2	Laboratory collection
Plasmids		
pUC19	Cloning vector	Laboratory collection
pRL1383a	RSF1010-derived broad-host-range vector, Sp^r and Sm^r, accession no. AF403426	10
pAM1037	Transposon Tn*5* derivative (Km^r)	19
pRL448	Plasmid carrying Km^r cassette	8
pRL453	Plasmid carrying Sp/Sm Ω cassette	8
pHM54	NifK knockout construct for PCC 7822 with Sp^r cassette going against *nifK*	This study
pHM55	NifK knockout construct for PCC 7822 with Sp^r cassette going with *nifK*	This study
Primers		
7822 NifK1	GCTATGACCATGATTACGCCAAGACCACGTTGAATTATTCC	This study
7822 NifK2	GTTGTAAAACGACGGCCAGTGTACGATCGATATCTTCAAACAGAG	This study
7822 NifK3	CGGCTGTCTTACCATGTAACCAAGC	This study
Sp/Up	CCAAGGATCGGGCCTTGATG	This study
Sp/11Up	CGTAACGCGCTTGCTGCTTG	This study

likely contained a recombination system that could insert such a cassette randomly throughout the genome.

Cyanothece sp. strains PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802 were successfully transformed by electroporation with a Tn*5* derivative (pAM1037) and pRL448 to kanamycin resistance and by pRL1383a and pRL453 to Sp^r. All of the strains, except PCC 7822, yielded many Sp^r colonies by nonhomologous recombination (Table 2). Since *Cyanothece* sp. strain PCC 7822 had the best ratio of legitimate transformation versus nonhomologous illegitimate recombination, it was chosen for mutagenesis by insertional inactivation.

PCR-amplified *nifK* was cloned into pUC19, and *Cyanothece* sp. strain PCC 7822 was transformed by electroporation using ssDNA made from pHM54 and pHM55 (Table 1 and Fig. 1). Twenty Sp^r colonies were picked from each group for segregation by stepwise transfer onto fresh Sp plates three times, and 3 out of 20 colonies in each group demonstrated the mutant phenotype; i.e., they could not grow in the absence of combined nitrogen. We focused on three mutants from the pHM54 group. Colony PCR confirmed that two out of these three colonies had the Sp^r cassette inserted in *nifK* as shown in Fig. 1. DNA sequencing was performed on the PCR product from one colony, and the results (Fig. 2) indicated that the Sp^r cassette was inserted into the EcoRI site of *nifK* as designed and sketched in Fig. 1. Colony PCR on the Δ*nifK* mutant after 1 month of continuous growth in the presence of antibiotics indicated that chromosomal segregation was complete.

The phenotype of the Δ*nifK* mutant was demonstrated both on plates and in liquid culture (Fig. 3A and B). When the Δ*nifK* mutant and the wild type were spotted onto plates containing Sp, the wild type died, as seen after 5 weeks (Fig. 3B, left plate), whereas the Δ*nifK* mutant always grew well. When

TABLE 2. Transformation efficiency[a]

Cyanothece sp. strain	No. of transformants/CFU, 10^4		Km^r ratio (pAM1037/pRL448)	No. of transformants/CFU, 10^4		Sp^r ratio (pRL1383a/pRL453)
	pAM1037 (Km^r)	pRL448 (Km^r)		pRL1383a (Sp^r)	pRL453 (Sp^r)	
ATCC 51142	2.0	2.0	1.0	2.0	2.0	1.0
PCC 7424	1.0	1.0	1.0	1.0	1.0	1.0
PCC 7425	2.0	2.0	1.0	2.0	2.0	1.0
PCC 7822	1.0	0.2	5.0	1.0	0.01	100.0
PCC 8801	1.0	1.0	1.0	1.0	1.0	1.0
PCC 8802	1.0	1.0	1.0	1.0	1.0	1.0

[a] Six *Cyanothece* strains were transformed by transposon Tn*5* derivative pAM1037 or broad-host-range plasmid pRL1383a in comparison with suicide vector pRL448 or pRL453, respectively. Only *Cyanothece* sp. strain PCC 7822 demonstrated a significantly lower background (transformation by suicide vectors) to evoke future mutagenesis by homologous recombination.

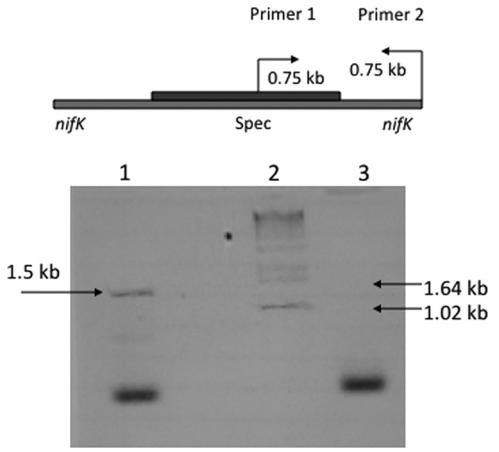

FIG. 1. PCR confirmation of the *Cyanothece* sp. strain PCC 7822 Δ*nifK* mutant. PCR with primer 1 in the Sp^r cassette (Sp/11Up) and primer 2 in the *nifK* gene (7822 NifK2) produced a band of about 1.5 kb from Δ*nifK* mutant DNA (lane 1) but not from wild-type DNA (lane 3). Lane 2 is the 1-kb DNA ladder, and the 1.64- and 1.02-kb bands are highlighted. The 1.5-kb band in the Δ*nifK* mutant then was sequenced to demonstrate that the Sp^r cassette (Spec) was located within the *nifK* gene as shown in the scheme at the top.

the two strains were spotted onto plates lacking combined nitrogen, the wild type always grew whereas the Δ*nifK* mutant slowly died (Fig. 3B, right plate). The phenotype was noticeable by 1 week, and by 5 weeks, the culture was completely bleached (Fig. 3A). This phenotype was demonstrated numerous times and has remained stable for >1 year. These results strongly suggested that the Δ*nifK* mutant was incapable of growth on media lacking combined nitrogen and presumably was defective in N_2 fixation. We then checked the mutant for both hydrogen production and nitrogenase activity. As shown in Table 3, the Δ*nifK* mutant produced little hydrogen when incubated either in air or under argon.

Similarly, the nitrogenase activity of the Δ*nifK* mutant differed from that of the wild type, but in an interesting fashion. As shown in Table 3, acetylene reduction by the Δ*nifK* mutant

GATTGATCCGGTGGATGACCTTTTGAATGACCTTTAATAGATTATATTAC
TAATTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAAATTTTTT
CACAAAACGGTTTACAAGCATAAAGCTTGCTCAATCAATCACCGGATCCC
CGGGTACCGAGCTC**GAATTC**AAGATGTACCAAGGCGGTACAACTTTAGAG
GAAGGGGCTGATTCCATCAACGCAGAAGCTACCATCACCCTACAAACCTA
CCCCACCGTTAAAACTCGGGAATATATCGAGGAAAAATGGGGACAAAAAA
CCTTTACCTATCGTCCTTGGGGTGTCAAAGGAACAGATGAGTTCTTAATG
GGACTTTCTGAACTGACCGGTAATCCTATCCCTGCTGAATTAGAACTCGA
AAGAGGACGCGCAGTTGACGCGATGACCGATAGCCATGCTTGGTTACATG
GTAAGACAGCC

FIG. 2. The Sp^r cassette was inserted into *nifK* at the EcoRI site as designed. The PCR product from the Δ*nifK* mutant was sequenced from a primer within the Sp^r cassette, Sp/Up (Table 1). The sequence underlined is the Sp^r cassette, and the rest is *nifK*. The GAATTC sequence shown in bold is the EcoRI site. Another sequencing result using a primer with *nifK* (7822 NifK3, Table 1) confirmed the insertion of the Sp^r cassette into *nifK* as well (data not shown).

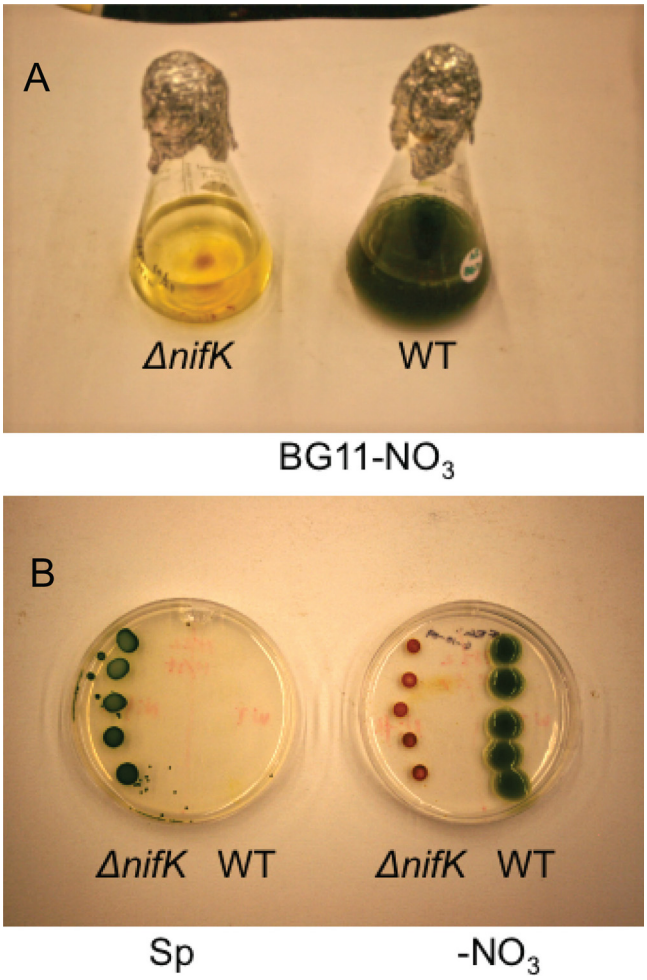

FIG. 3. Growth of the *Cyanothece* sp. strain PCC 7822 wild type (WT) and the Δ*nifK* mutant in liquid media (A) and on plates (B). (A) The wild type and the Δ*nifK* mutant were grown in BG11 medium without combined nitrogen for 5 weeks. The mutant was unable to fix nitrogen and began to appear bleached after 1 week. (B) The wild type and the Δ*nifK* mutant were spotted onto BG11 plates with Sp (left) to demonstrate that the mutant, but not the wild type, was antibiotic resistant. The plate on the right contained no combined nitrogen and demonstrated that the wild type, but not the Δ*nifK* mutant, could fix nitrogen.

was actually higher than that of the wild type when cells were incubated with either acetylene in air or acetylene in argon. The rate of acetylene reduction by the Δ*nifK* mutant under argon was ~1,000-fold greater than that by the wild type with acetylene in air.

This nitrogenase phenotype is not unique, and we must consider the relationship of H_2 evolution and acetylene reduction to the nitrogenase enzyme (1, 2). Hydrogen is always produced when nitrogenase reduces N_2 to NH_3, indicating that H_2 evolution is integral to the enzyme mechanism (23). Importantly, reduction of acetylene to ethylene is not accompanied by H_2 evolution and it is possible that nitrogenase reduces acetylene when only partially activated with no H_2 evolution. Acetylene reduction discharges nitrogenase before it ever reaches full activation. The current seven-stage mechanism for the fixation of N_2 to the production of $2NH_3$ also explains why

TABLE 3. Acetylene reduction activity and hydrogen production of *Cyanothece* sp. strain PCC 7822 and the Δ*nifK* mutant after incubation in air or argon

Cyanothece sp. strain PCC 7822	Incubation condition[a]	Avg relative acetylene reduction activity[b] ± SD	Avg hydrogen production rate[b] ± SD
Wild type	Air	1	5.1 ± 1.8
Δ*nifK* mutant	Air	2.7 ± 0.9	1.7 ± 2.5
Wild type	Argon	31.5 ± 10.6	58 ± 16
Δ*nifK* mutant	Argon	1,139 ± 363	2.4 ± 3.4

[a] Cultures were grown in medium containing N (2.5 mM NH_4NO_3) under low-light conditions for 10 days, washed with N-free medium twice, and grown in N-free medium for 3 days under low-light conditions. Then, 50 ml was added to 66-ml bottles. Some bottles were sparged with argon. Acetylene reduction assays were performed after the bottles were shaken under low-light conditions (30 μmol photons m^{-2} s^{-1}) for 22 h. After injection of 3 ml of acetylene, the bottles were also kept under light for 2 h.

[b] Activities were computed as milligrams of chlorophyll *a* per hour, and the acetylene reduction activities were normalized to the wild-type value obtained in air. Hydrogen production rates are in micromoles of H_2 per milligram of chlorophyll *a* per hour.

acetylene is a competitive inhibitor of N_2 fixation, whereas N_2 is a noncompetitive inhibitor of acetylene reduction (2, 5, 15). Consistent with this feature, air (79% N_2) inhibited acetylene reduction in the wild type and the Δ*nifK* mutant, but to a greater extent in the Δ*nifK* mutant. This result suggested that the MoFe center is present in the mutant (1, 2). Finally, N_2 cannot fully stop H_2 evolution, as we have also demonstrated in *Cyanothece* sp. strain PCC 7822 (17), whereas acetylene can (1). Thus, the phenotype of the Δ*nifK* mutant indicated that the metal cofactors (e.g., MoFe) were assembled and were likely poorly integrated into the abnormal nitrogenase complex in the mutant.

Cyanothece sp. strain PCC 7822 represents an excellent organism for further studies. It demonstrates cycling behavior of photosynthesis and nitrogen fixation, and like *Cyanothece* sp. strain ATCC 51142, it produces large quantities of organic acids, lipids, and polyhydroxyalkanoates (pHAs) and copious levels of hydrogen (17). The genomic sequence has been completed, and we have the opportunity to use this strain for metabolic enhancement of one or more of these important compounds.

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