Nitrogen-Regulated Hypermutator Strain of *Synechococcus* sp. for Use in In Vivo Artificial Evolution

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Artificially evolved variants of proteins with roles in photosynthesis may be selected most conveniently by using a photosynthetic organism, such as a cyanobacterium, whose growth depends on the function of the target protein. However, the limited transformation efficiency of even the most transformable cyanobacteria wastes much of the diversity of mutant libraries of genes produced in vitro, impairing the coverage of sequence space. This highlights the advantages of an in vivo approach for generating diversity in the selection organism itself. We constructed two different hypermutator strains of *Synechococcus* sp. strain PCC 7942 by insertionally inactivating or nutritionally repressing the DNA mismatch repair gene, *mutS*. Inactivation of *mutS* greatly increases the mutation rate of the cyanobacterium’s genes, leading to an up-to-300-fold increase in the frequency of resistance to the antibiotics rifampin and spectinomycin. In order to control the rate of mutation and to limit cellular damage resulting from prolonged hypermutation, we placed the uninterrupted *mutS* gene in the cyanobacterial chromosome under the transcriptional control of the cyanobacterial *nirA* promoter, which is repressed in the presence of NH₄⁺ as an N source and derepressed in its absence. By removing or adding this substrate, hypermutation was activated or repressed as required. As expected, hypermutation caused by repression in *PnirA-mutS* transformants led to an accumulation of spectinomycin resistance mutations during growth.

Artificial evolution (sometimes called directed or laboratory evolution) has, in recent years, been applied to a wide range of individual genes, successfully changing properties of encoded proteins ranging from thermostolerance, to catalytic specificity, to total activity (reviewed in references 3, 21, 29, 35, and 39). Using artificial evolution, whole organisms also can be evolved rapidly to exhibit new and complex traits (25, 40).

Techniques for artificial evolution typically entail a mutagenesis step, in which sequence diversity is generated in particular genes, and a step in which phenotypic expression of variant genes confers the desired characteristic that is selected. These techniques range from entirely in vitro procedures (for example, in vitro co-compartmentalization of an evolved gene and its protein product in aqueous droplets emulsified in oil [10]) to fully in vivo procedures. One of the major factors that limit the efficacy of artificial evolution is the number of permutations of a particular sequence that can be screened for the desired functional characteristic (sequence-space coverage). In many artificial evolution protocols, mutagenesis is performed in vitro and selection is performed in vivo. When the selection must be done in a photosynthetic organism, such as a cyanobacterium, to select variants of proteins with roles in photosynthesis, for instance, transformation efficiencies are typically low (8), and this wastes much of the generated sequence diversity. This loss can be avoided by generating the sequence diversity in vivo in the same organism that is to be used for selection. In such systems, the mutagenesis need not remain restricted to a single gene or group of genes, but can encompass the whole genome. Furthermore, selection in vivo ensures that the genes remain evolved to function in an in vivo environment.

Genetic methods for in vivo mutagenesis have largely replaced classical methods, such as treatment with chemicals or UV, since the latter are discontinuous, nonrandom, and can lead to significant cell damage. Such in vivo mutagenesis systems (hypermutator strains) have been developed in *Escherichia coli* and *Escherichia blattae* (9, 25). No such strains, however, have been constructed in a photosynthetic bacterium or can be applied specifically to photosynthetic genes. Here we describe construction of a novel hypermutator strain in the cyanobacterium *Synechococcus* sp. strain PCC 7942. Cyanobacteria such as *Synechococcus* sp. strain PCC 7942 are frequently chosen as model organisms to manipulate and study photosynthesis due to their amenability to molecular manipulation and the similarity of their photosynthetic apparatus to that of higher plants.

We targeted the *mutS* gene, which encodes a key protein in the DNA mismatch repair system (MMR), to construct our hypermutator strain because of previous suggestions that it suppressed hypermutation (23). One of the central roles of the MMR system is in the correction of postreplication DNA errors (11, 26). We show that disruption of this gene in *Synechococcus* sp. strain PCC 7942 leads to a hypermutator phenotype. In order to control the rate and duration of artificial evolution, we constructed a second hypermutator strain by placing the undisrupted *mutS* gene under the transcriptional control of the promoter of the *nirA* gene of *Synechococcus* sp. strain PCC 7942 (17, 33). This promoter controls transcription from the *nirA* operon. It is regulated by the NtcA protein (16), and

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therefore transcription from it is strongly repressed in the presence of NH$_4^+$ as an N source and derepressed when NO$_3^-$ is the sole N source. Thus, by varying N nutrition, hypermutation can be modulated and suppressed before and after selection to minimize unwanted mutations unrelated to those conferring the selected characteristic.

**MATERIALS AND METHODS**

**Growth of Synechococcus sp. strain PCC 7942.** The unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (also known as *Synechococcus elongatus* PCC 7942, *Synechococcus* iio, and *Anacystis nidulans* R2) was grown photoautotrophically at 30°C under continuous illumination provided by fluorescent lamps (30 µmol quanta m$^{-2}$ s$^{-1}$). Cultures of *Synechococcus* sp. strain PCC 7942 were maintained in BG11 medium, which contains 18 mM NaNO$_3$ and no NH$_4^+$ (2). Where exclusively NH$_4^+$–containing or NO$_3^+$–containing medium was required, BG11 was modified to exclude nitrogen (32), and 3.75 mM (NH$_4$)$_2$SO$_4$ or 15 mM KNO$_3$, respectively, was added to this basal medium. To transfer cultures between these modified media, cells were pelleted by centrifugation at 5,000 × g for 5 min at 25°C and washed three times by resuspension in new medium followed by recentrifugation. The same procedure was used to transfer cultures grown on solid medium to modified liquid medium, except that the cells were suspended in the liquid medium first. Cultures on plates were maintained in 2% (vol/vol) CO$_2$ in air. Liquid cultures were sparged with air. Where appropriate, kanamycin was added to media at a final concentration of 30 µg ml$^{-1}$, spectinomycin was added at 20 µg ml$^{-1}$, and rifampin was added at 50 µg ml$^{-1}$.

**Isolation and analysis of DNA.** DNA manipulations and DNA blotting were performed according to standard protocols (24). Genomic DNA was prepared from *Synechococcus* sp. strain PCC 7942 by using a standard miniprep procedure (22). Plasmid DNA was prepared from *E. coli* by using a standard miniprep procedure based on Qiagen protocols (Qiagen booklet). Preparative PCR was performed with recombinant Taq DNA polymerase (MBI Fermentas).

**Isolation of sequence 5′ to the known mutS fragment from *Synechococcus* sp. strain PCC 7942.** A 402-bp sequence with high homology to part of the protein-encoding region of *mutS* was obtained previously from a random insertional mutant of *Synechococcus* sp. strain PCC 7942 (23) (GenBank accession no. U95756). To obtain sequence flanking this fragment, inverse PCR (36) was employed on ligated, BamHI-digested genomic DNA from *Synechococcus* sp. strain PCC 7942 with the primers IPCRF (5′-GGCGAATTATTGGGCACGATTGAGTAACT-3′) and IPCRR (5′-TTTTTCTCTTCTGCCCTGTG-3′). Three products were obtained, only one of which contained *mutS* sequence detectable on DNA blots probed with a fragment of *Synechococcus* sp. strain PCC 7942 *mutS* amplified with the primers *mutS*PrF (5′-GGGTTAATCCGCGATCGCAGTCTGGAGT-3′) and *mutS*PrR (5′-GGCAAGCTCTCTCCGCAAGT-3′) (data not shown). This 3.5-kb fragment was extracted from a gel, A-tailed, and cloned into pGEM-T Easy (Promega technical manual). Sequencing revealed that the inverse PCR product had been amplified from two BamHI fragments that had ligated together, rather than from a single circularized fragment. Primer IPCRR apparently had bound to an alternative, perhaps homologous, site outside *mutS*, and, therefore, only *mutS* sequence downstream of the previously known *mutS* fragment was obtained. This additional sequence (0.7 kb), together with the 0.4-kb sequence previously known, was sufficient for insertional inactivation of *mutS*.

**Insertional inactivation of *mutS*.** The procedure used relied on double homologous recombination following transformation with a plasmid containing a partial *mutS* sequence interrupted and partly replaced by a Km$^R$ gene encoding neomycin phosphotransferase, which confers resistance to kanamycin (Fig. 1A, 1B). Based on the sequence of the inverse-PCR product, a 1.08-kb region of *mutS*—subsequently found to encompass nucleotides +196 to +1277 of the protein-encoding region (described below)—was PCR amplified from genomic DNA from *Synechococcus* sp. strain PCC 7942 with the primers *HindIII* SF and *HindIII* SR (Fig. 1A and 1B). This fragment was cloned into the EcoRI-*HindIII*-digested pUC19, producing pUC19*mutS*. A 1.2-kb Km$^R$ gene was excised from pUC4K by using HindIII and cloned into SacI-PstI-digested, Klenow-blunted pUC19*mutS*, producing pUC19*mutS*Km$^R$. pUC19*mutS*Km$^R$ was transformed into *Synechococcus* sp. strain PCC 7942, and *mutS*::Km$^R$ transformants (with a 130-bp fragment of *mutS* replaced by a Km$^R$ gene) were selected on kanamycin-containing plates. After several rounds of restreaking on solid medium in the presence of antibiotic, complete segregation of the insertion (produced by double-homologous recombination) throughout all genome copies was established by PCR with primers *ΔmutS*SF and *ΔmutS*SR: the wild type gave a single 1.1-kb band, while the *ΔmutS*::Km$^R$ transformants gave a single 2.3-kb band, as expected (Fig. 1B).
FIG. 2. Protocol used for plasmid rescue of the sequence upstream of the known mutS sequence (stipped boxes) (23). Double-homologous recombination of the entire pUC18Kan sequence into mutS in the cyanobacterial genome (depicted by the crossed lines) ensured no duplication of the mutS sequence and facilitated plasmid rescue following digestion of the genomic DNA of the transformant with Sacl. The boxes with jagged ends represent the unknown 5’ and 3’ regions of mutS.

CATGACAACCTCTGAGCTTCCGGGACG3’, BspHI site underlined) and nirA2 (5’GGGAAATTCTCTAGTCAGGATGGGACG3’, EcoRI site underlined), producing the product mutSPCR. The nirA1 primer was designed to incorporate the codon for the N-terminal Met of mutS into the BspHI site. Nucleotides +4 to −533 of the promoter region of the nirA gene of Synechococcus sp. strain PCC 7942 were amplified with primers nirA3 (5’GGGAAATTCTCTAGTCAGGATGGGACG3’, EcoRI site underlined) and nirA4 (5’GGGAAATTCTCTAGTCAGGATGGGACG3’, EcoRI site underlined), producing the product PnirAPCR. The natural Ncol site present in nirA3 contained the initiator Met codon of nitrite reductase. Nucleotides −8 to −160 of the mutS promoter region were amplified with primers mutS1 (5’GGGCCATGGTATTTTCAAAATTTATTCTAGTCAGGATGGGACG3’, SphI site underlined), producing the product PmutSPCR. The natural Ncol site present in mutS1 contained the initiator Met codon of nitrite reductase. Nucleotides +1 to −4 of the promoter region were amplified with primers mutS2 (5’GGGCCATGGTATTTTCAAAATTTATTCTAGTCAGGATGGGACG3’, SphI site underlined) and mutS3 (5’GGGCCATGGTATTTTCAAAATTTATTCTAGTCAGGATGGGACG3’, SphI site underlined), producing the product PmutSPCR. The natural Ncol site present in mutS2 contained the initiator Met codon of nitrite reductase. Nucleotides +8 to −161 of the mutS promoter region were amplified with primers mutS4 (5’GGGCCATGGTATTTTCAAAATTTATTCTAGTCAGGATGGGACG3’, SphI site underlined) and mutS5 (5’GGGCCATGGTATTTTCAAAATTTATTCTAGTCAGGATGGGACG3’, SphI site underlined), producing the product PmutSPCR. The natural Ncol site present in mutS4 contained the initiator Met codon of nitrite reductase.

pGEM-3Zf (+)PnirA-nirSmutS5’-PCR. Finally, a Km’ gene excised from pUC4K by using HindIII was cloned into the BamHI sites of pGEM-3Zf (+)PnirA-nirSmutS5’-PCR present at positions −323 and −368 of the nirA promoter region, such that transcription of the Km’ gene occurred in the opposite orientation to mutS transcription. This minimized the possibility of transcriptional readthrough from the Km’ gene into mutS. The completed construct (Fig. 3A) was sequenced with primers nirA1 to nirA6 to verify that no errors had been introduced by PCR and that the nirA promoter was appropriately fused to the mutS gene.

The construct was linearized by digestion with Sall and transformed into Synechococcus sp. strain PCC 7942, and transformants were selected with kanamycin [Φ(PnirA-mutS), a gene fusion construct containing mutS under the transcriptional control of the nirA promoter]. Ten transformants were selected for PCR present at positions −323 and −368 of the nirA promoter region, such that transcription of the Km’ gene occurred in the opposite orientation to mutS transcription. This minimized the possibility of transcriptional readthrough from the Km’ gene into mutS. The completed construct (Fig. 3A) was sequenced with primers nirA1 to nirA6 to verify that no errors had been introduced by PCR and that the nirA promoter was appropriately fused to the mutS gene.
correct insertion and complete segregation of the PairA::Km\(^{r}\)-mutS sequence (Fig. 3A).

**Measurement of relative mutation rate.** Wild-type, ΔmutS::Km\(^{r}\), and Φ(PairA-mutS) cells were grown in liquid medium (containing kanamycin for the insertion mutants, but not for the wild type) with NO\(_{3}^{−}\) as the nitrogen source [and also with NH\(_{4}^{+}\) as a nitrogen source in the case of Φ(PairA-mutS)]. The numbers of cells in mid-exponential-phase cultures were estimated by measuring the apparent \(A_{750}\) (Apparent absorbance was related to the number of cells per unit volume by calibration with a hemocytometer.) Concentrated cultures \((<10^{10}\) cells\) were plated onto solid media containing NO\(_{3}^{−}\) and rifampin or spectinomycin. After several weeks, antibiotic-resistant colonies were counted, and the frequency of antibiotic-resistant cells in the original culture was calculated. This frequency was taken as a measure of the relative mutation rate. Similar platings onto antibiotic-free media revealed that the plating efficiencies were similar for the wild type, ΔmutS::Km\(^{r}\), and Φ(PairA-mutS) strains grown on either NO\(_{3}^{−}\) or NH\(_{4}^{+}\). Ninety to 120\% of the cells plated (estimated as above) yielded colonies regardless of genotype or prior growth condition. A plating efficiency of 100\% was assumed when calculating the mutation rate.

**Nucleotide sequence accession number.** The sequence of the mutS fragment obtained by Ronen-Tarazi et al. (23) can be obtained from GenBank under accession no. U95756. The extended sequence determined in this publication has also been deposited in GenBank (accession no. AY191320).

### RESULTS

**Phenotype of ΔmutS::Km\(^{r}\) transformants.** Using a combination of a novel plasmid-rescue procedure and inverse PCR, a 1.8-kb segment of the coding and 5′ noncoding regions of mutS was isolated (see Materials and Methods). The mutS sequence isolated from *Synechococcus* sp. strain PCC 7942 was highly homologous to the mutS sequences of the other cyanobacteria, *Synechocystis* sp. strain PCC 6803 and *Thermosynechococcus elongatus* BP-1 (Cyanobase: http://www.kazusa.or.jp/cyano/cyano.html). To assess whether inactivation of mutS in *Synechococcus* sp. strain PCC 7942 resulted in a hypermutator phenotype, the relative rates of appearance of resistance to rifampin and spectinomycin were compared in three separate ΔmutS::Km\(^{r}\) transformants and in the wild type.

Resistance to rifampin and spectinomycin results from spontaneous point mutations in genes encoding RNA polymerase and ribosomal components, respectively. With both antibiotics, a greater mutation rate was observed in the mutants (Table 1). In different experiments, the increases ranged from 30- to 300-fold (mean, 132 ± 124) for rifampin and 6- to 30-fold (mean, 15 ± 11) for spectinomycin. The difference between the two antibiotics may reflect differences in the frequency of resistance-conferring mutable sites in the target genes. For the ΔmutS::Km\(^{r}\) strain, the variability between experiments is likely to be caused by variation in the number of generations that the cells had passed through between the transformation event that caused insertional inactivation of mutS and plating on rifampin- or spectinomycin-containing medium. Since many of these generations will have occurred during growth of the first colony of the single initial transformant cell, this variation is not controllable. This lack of control is an inherent problem when hypermutation derives from insertional inactivation. Nevertheless, we concluded that disruption of mutS does cause a hypermutator phenotype and that this gene can be used as a basis of a hypermutator strain.

We do not know what genes flank mutS in the *Synechococcus* sp. strain PCC 7942 genome, or whether mutS is monocistronic. However, in fully sequenced cyanobacterial genomes, mutS is flanked by seemingly unrelated genes (analysis not shown). Therefore, it seems unlikely that disruption of another transcriptionally linked gene contributes to the phenotype of ΔmutS::Km\(^{r}\). Despite the hypermutator phenotype, ΔmutS::Km\(^{r}\) grew photoautotrophically at a rate similar to that of the wild type (described below).

**Phenotype of the Φ(PairA-mutS) transformants.** To control the expression of mutS, and thus the degree of hypermutation, the mutS gene was placed under the transcriptional control of the promoter of the nirA operon of *Synechococcus* sp. strain PCC 7942. This promoter has been well characterized. Repression in the presence of 7.5 mM NH\(_{4}^{+}\) results in a dramatic reduction in transcript abundance (17, 20, 33). Three Φ(PairA-mutS) transformants, which had been maintained on NO\(_{3}^{−}\)-replete medium were in the range observed for wild-type cultures (Table 1). The numbers of doublings since the transfer, estimated from the measured growth rates (see Results), are plotted on the right ordinate (dashed lines): □, NH\(_{4}^{+}\); ■, NO\(_{3}^{−}\). The arrows indicate the times at which the cultures were diluted.

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**TABLE 1.** Frequencies of antibiotic-resistant cells in wild-type and ΔmutS::Km\(^{r}\) cultures

| Expt | Frequency of resistant cells \((10^{−16} \text{no})\) | Rifampin (50 μg ml\(^{−}\)) | Spectinomycin (20 μg ml\(^{−}\)) |
|------|---------------------------------|----------------|----------------|
| Wild type | | | |
| 1 | 1.6 ± 3.1 | 6.3 ± 3.2 |
| 2 | 23.4 ± 12.8 | 44.6 ± 14 |
| 3 | 15 ± 1.73 | 41.6 ± 5.7 |
| ΔmutS::Km\(^{r}\) | | | |
| 1 | 489 ± 74.9 | 187 ± 29 |
| 2 | 1,380 ± 135 | 468 ± 113 |
| 3 | 445 ± 38.3 | 229 ± 30 |

\(a\) Values are means ± standard deviation for four replicate plates. The unpaired \(t\) test \(P\) values for wild type versus ΔmutS::Km\(^{r}\) are 0.068 for rifampin and 0.041 for spectinomycin (calculated by using frequencies derived from all individual plates).

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**FIG. 4.** Frequencies of spectinomycin-resistant cells (left ordinate, solid lines) in Φ(PairA-mutS) cultures after transfer in triplicate from NO\(_{3}^{−}\)-replete solid medium to either NH\(_{4}^{+}\)-replete (C) or NO\(_{3}^{−}\)-replete (●) liquid medium. Each point is an average of the three replicates (± standard deviation). The frequency of resistant cells \((\text{no per ml})\) is plotted on the right ordinate (dashed lines): □, NH\(_{4}^{+}\); ■, NO\(_{3}^{−}\). The arrows indicate the times at which the cultures were diluted.
containing solid medium, were inoculated into liquid cultures of NH$_4^+$-replete and NO$_3^-$-replete medium (containing kanamycin). These cultures grew at similar rates (described below) and were diluted each week with the same medium to a final $A_{750}$ of 0.05 to 0.1. The frequency of spectinomycin-resistant cells was measured every 2 weeks.

After transfer of NO$_3^-$-grown inocula to NH$_4^+$-replete media, the cells grew more slowly for approximately 1 week before normal growth commenced. This lag period, which was observed in the wild type as well as in the $\Phi$(PnirA-mutS) strain (data not shown), may be an adaptation phase, and no increase in the frequency of spectinomycin resistance occurred during it (Fig. 4). Subsequently, the frequency of resistant mutants in the $\Phi$(PnirA-mutS)(NH$_4^+$) culture increased by approximately 4-fold at week 3 and 20-fold at week 5. Meanwhile, no increase was observed in the $\Phi$(PnirA-mutS)(NO$_3^-$) culture, which maintained a mutation rate within the range observed for the wild type (Fig. 4 and Table 1). This demonstrates that control of hypermutation when using the $\Phi$(PnirA-mutS) system is effective. It also establishes that the nitrA promoter drives expression of mutS at an adequate level in the presence of NO$_3^-$.

Relationship between growth and hypermutation. The three genotypes grew at similar rates in liquid culture with kanamycin, regardless of nitrogen source. The doubling times were as follows: wild type (no kanamycin), 33.4 ± 1.7 h; ΔmutS:Km$, 30.4 ± 1.7 h; $\Phi$(PnirA-mutS)(NO$_3^-$), 29.7 ± 6.0 h; $\Phi$(PnirA-mutS)(NH$_4^+$), 27.9 ± 3.9 h. With $\Phi$(PnirA-mutS), the growth lag during the first week after switching the nitrogen source from NO$_3^-$ to NH$_4^+$ increased the average doubling time during this period to 66 ± 7 h. For this strain, the frequency of spectinomycin-resistant mutations correlated roughly with the number of cell doublings since repression of mutS (Fig. 4).

DISCUSSION

Under unchanging conditions, bacterial populations typically maintain a very low mutation rate to suppress the mostly deleterious effects of random mutations (5). In times of change, however, natural hypermutator strains often can arise in such populations (15, 18, 27, 34). Such hypermutator strains are thought to play a role in evolutionary adaptation to a new environment, and many have disruptions in the MMR pathway. By placing the mutS gene of the MMR pathway of *Synechococcus* sp. strain PCC 7942 under the N-regulated control of the nitrA promoter, we have made this process experimentally controllable in a cyanobacterium suitable for selecting genes for photosynthesis-related proteins with specified altered properties.

Cyanobacteria, such as *Synechococcus* sp. strain PCC 7942, grow much more slowly than *E. coli*: >6-h doubling time even under optimal conditions (19). Therefore, generation of mutations takes weeks (Fig. 4) rather than the days required with similar *E. coli* hypermutators (9), but this does not cause serious difficulty. The cyanobacterial hypermutator that we have constructed is not as sophisticated as some hypermutator strains of *E. coli*, in which a variety of genes involved in the MMR pathway and other genes as well as mutS have been altered to enhance the rate of mutation (9, 25). Future cya-
nobacterial hypermutator strains may be engineered by using analogous target genes, a task now made easier by the availability of complete genome sequences for some cyanobacteria.

Our observations that disruption (Table 1) or controlled repression (Fig. 4) of mutS causes a hypermutator phenotype support the previous inference by Ronen-Tarazi et al. (23) that the high-CO$_2$-requiring phenotype of the IL-7 mutant of Synechococcus sp. strain PCC 7942 is caused by a secondary point mutation resulting from a primary insertional disruption of the mutS gene. They also indicate that there is a single functional copy of the mutS gene in this cyanobacterium, consistent with the single restriction fragment detected on DNA blots by using the mutS probe (Fig. 3B) and the available whole-genome sequences of other cyanobacteria (Cyanobase). Ronen-Tarazi et al. (23) observed that IL-7 cells sometimes became elongated—up to 5 to 15 times the length of wild-type cells. We found this elongated-cell phenotype to be quite common in strains of wild-type Synechococcus sp., including strain PCC 7942, when grown on solid media and are unsusp of its physiological significance.

Our data (Fig. 4) are consistent with the expectation that growth is required for hypermutation, presumably because DNA synthesis must occur for the defect caused by lack of the MutS protein to be expressed. This requirement must be incorporated into selection protocols. Selection conditions that do not permit growth of unmutated cells will need to follow a period of growth under permissive conditions during which mutations can accumulate. Such a strategy is illustrated schematically in Fig. 5.

The ϕ(PnirA-mutS) system will make a useful addition to the toolbox of molecular genetic techniques available for the study and manipulation of cyanobacteria (6, 13, 38). Completion of the genome-sequencing projects currently in progress on Synechococcus sp. strain PCC 7942 (Synechococcus elongatus PCC 7942 Functional Genomics Project: http://www.bio.tamu.edu/synecho/index.html) and its close relative, Synechococcus sp. strain PCC 6301 (Anacystis nidulans 6301 Genome Project: http://www.bio.nagoya-u.ac.jp:8001/~gene/CGR6301gp.html), will enhance this utility. Among other benefits, the genome sequence will facilitate location of mutation sites in the ϕ(PnirA-mutS) transformants evolved under specific conditions, using techniques such as genomic mapping by functional complementation (14). The biotechnological uses of cyanobacteria are numerous and wide ranging and include directed mutation of genes related to photosynthesis, bioremediation (28), and industrial hydrogen production (37). Systems such as ϕ(PnirA-mutS) could play a key role in adapting cyanobacteria to perform optimally in these tasks. Systems for the expression of foreign proteins also exist for Synechococcus sp. strain PCC 7942 (1, 7), allowing the artificial evolution of foreign genes in vivo.

Combination of the respective strengths of different techniques may also be a useful way of enhancing artificial evolution. For example, DNA shuffling in vitro (reassembly of genes from random fragments of a single gene or family of related genes) (4, 30) could be used to generate starting diversity in a library of target genes. Although much of this diversity will be lost when the library is transformed into a selection organism, such as ϕ(PnirA-mutS), this would nevertheless introduce a powerful combinatorial element to complement the wide sequence-space coverage and whole-genome approach of the subsequent in vivo mutagenesis.

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