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RESEARCH ARTICLE

Engineering Nitrogen Fixation Activity in an Oxygenic Phototroph

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ABSTRACT Biological nitrogen fixation is catalyzed by nitrogenase, a complex metalloenzyme found only in prokaryotes. N_2 fixation is energetically highly expensive, and an energy-generating process such as photosynthesis can meet the energy demand of N_2 fixation. However, synthesis and expression of nitrogenase are exquisitely sensitive to the presence of oxygen. Thus, engineering nitrogen fixation activity in photosynthetic organisms that produce oxygen is challenging. Cyanobacteria are oxygenic photosynthetic prokaryotes, and some of them also fix N_2. Here, we demonstrate a feasible way to engineer nitrogenase activity in the nondiazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 through the transfer of 35 nitrogen fixation (*nif*) genes from the diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142. In addition, we have identified the minimal *nif* cluster required for such activity in *Synechocystis* 6803. Moreover, nitrogenase activity was significantly improved by increasing the expression levels of *nif* genes. Importantly, the O_2 tolerance of nitrogenase was enhanced by introduction of uptake hydrogenase genes, showing this to be a functional way to improve nitrogenase enzyme activity under micro-oxic conditions. To date, our efforts have resulted in engineered *Synechocystis* 6803 strains that, remarkably, have more than 30% of the N_2 fixation activity of *Cyanothece* 51142, the highest such activity established in any nondiazotrophic oxygenic photosynthetic organism. This report establishes a baseline for the ultimate goal of engineering nitrogen fixation ability in crop plants.

IMPORTANCE Application of chemically synthesized nitrogen fertilizers has revolutionized agriculture. However, the energetic costs of such production processes and the widespread application of fertilizers have raised serious environmental issues. A sustainable alternative is to endow to crop plants the ability to fix atmospheric N_2 *in situ*. One long-term approach is to transfer all *nif* genes from a prokaryote to plant cells and to express nitrogenase in an energy-producing organelle, chloroplast, or mitochondrion. In this context, *Synechocystis* 6803, the nondiazotrophic cyanobacterium utilized in this study, provides a model chassis for rapid investigation of the necessary requirements to establish diazotrophy in an oxygenic phototroph.

KEYWORDS cyanobacteria, N2 fixation, O2 tolerance, photosynthesis, synechocystis

The ability to introduce into crop plants the machinery to fix their own nitrogen via direct transfer of nitrogen fixation (*nif*) genes is envisioned to be key for the next agricultural revolution (1–3). However, engineering diazotrophic plants, however attractive a proposition, is an extreme challenge, due to the complexities in the biosynthesis of active nitrogenase, the enzyme that catalyzes nitrogen fixation, and the difficulty of coupling plant metabolism to supply energy and reducing power for the nitrogen fixation process (4). An additional impediment in the scenario is that photosynthesis produces O_2, which is highly toxic with respect to the synthesis and activity of nitrogenase (5).

Diazotrophy occurs only in limited species of bacteria and archaea (6). Nitrogen fixation is mainly catalyzed by an iron- and molybdenum-dependent nitrogenase

Received 8 May 2018 **Accepted** 14 May 2018 **Published** 5 June 2018

Citation Liu D, Liberton M, Yu J, Pakrasi HB, Bhattacharyya-Pakrasi M. 2018. Engineering nitrogen fixation activity in an oxygenic phototroph. mBio 9:e01029-18. https://doi.org/10.1128/mBio.01029-18.

Editor Eleftherios T. Papoutsakis, University of Delaware

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This article is a direct contribution from a Fellow of the American Academy of Microbiology. Solicited external reviewers: Terry Bricker, Louisiana State University; Louis Sherman, Purdue University.

enzyme complex, with two enzymatic components, an iron protein dinitrogenase reductase (NifH) and an iron-molybdenum protein dinitrogenase (NifDK) (7, 8). Three metal-dependent cofactors, the F cluster, P cluster, and M cluster, are necessary to form the holoenzyme for electron transfer to reduce atmospheric N_2 to form ammonia, the biologically available form of N_2 (9, 10). A significant number of additional *nif* genes are required for the biosynthesis of these metallocluster cofactors and for the maturation of nitrogenase to form a fully functional enzyme (11, 12).

Transferring nitrogen fixation to nondiazotrophs has been attempted for decades. To date, the heterotrophic bacterium *Escherichia coli* has been successfully engineered for nitrogen fixation activity through transfer of *nif* genes from various diazotrophic species (13–17). Attempts to engineer eukaryotic species for heterologous nitrogen fixation activity, including the yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii*, have been unsuccessful. Limited success was reached only in expressing the NifH component as an active moiety in *Chlamydomonas reinhardtii* (18). While all the Nif components have been successfully expressed in yeast cells, the formation of a fully functional nitrogenase complex has not yet been achieved (19–22).

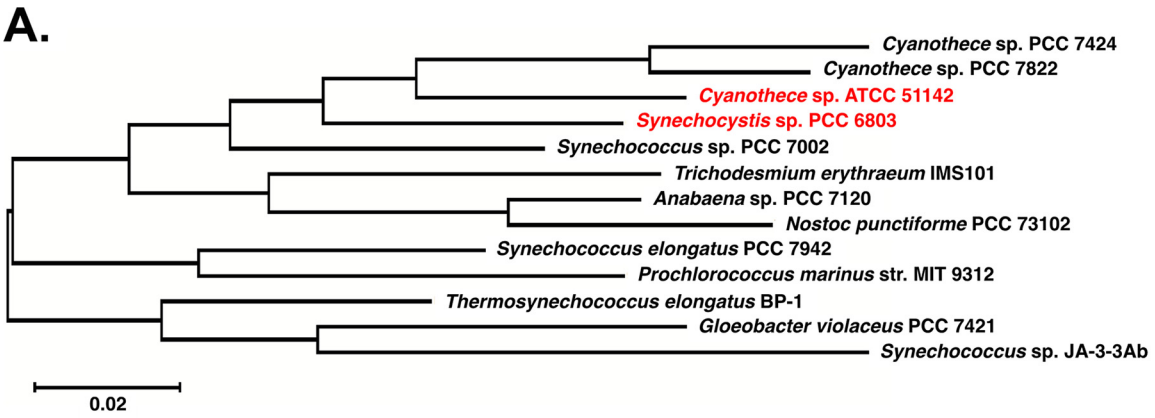
Expression of nitrogenase components in plants has also been attempted in a few studies. Individual expression of 16 Nif proteins targeted to the plant mitochondria has been reported recently, but none of the structural components showed enzymatic activity (23). Another recent study showed that an active NifH component can be formed in tobacco chloroplasts (24), indicating that expression of active nitrogenase in chloroplasts might be a viable way to engineer crop plants to fix nitrogen in the future (25). Since it is widely accepted that a cyanobacterial ancestor was the progenitor of chloroplasts (26), engineering a cyanobacterium to fix nitrogen may pave the way to achieving the final goal of engineering nitrogen-fixing ability into crop plants. We have utilized the nondiazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (here *Synechocystis* 6803) as a chassis to engineer nitrogen fixation activity into an oxygenic photosynthetic organism.

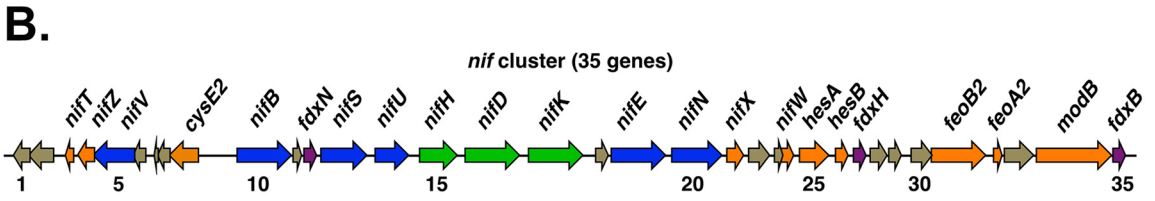
The unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 (here *Cyanothece* 51142) uses temporal separation as its strategy to protect nitrogenase from O_2 produced by photosynthesis (27, 28). Within *Cyanothece*, the two conflicting processes, photosynthesis and N_2 fixation, occur sequentially during the diurnal periods, such that photosynthesis and O_2 evolution are performed during the day whereas N_2 is fixed at night (29). The energy requirements for nitrogenase are met in *Cyanothece* by the catabolism of glycogen. Glycogen is accumulated in the light as the storage form of fixed CO_2 and is later degraded in the dark to provide energy for nitrogenase. The provision of energy coupled with high rates of respiration ensures a low-oxygen intracellular environment and sufficient supplies of energy for N_2 fixation (30). The *Cyanothece* 51142 genome contains the most complete contiguous set of nitrogen fixation and related genes forming the *nif* cluster, which contains 35 genes (cce_0545 to cce_0579), encoding structural proteins, metal cofactor synthesis proteins, ferredoxins, and proteins with unknown but necessary functions (Fig. 1). All 35 genes exhibit similar oscillating diurnal patterns of transcription during light/dark cycles, showing a high level of transcription in the dark and notably reduced levels in light (27). Such synchronized transcriptional patterns also confirm that all of these genes are related to nitrogen fixation.

In the current study, we successfully transferred and expressed this large *nif* gene cluster in nondiazotrophic *Synechocystis* 6803 with resultant N_2 fixation activity. Subsequent engineering of the cluster and its expression levels have led to nitrogenase activities as high as 30% of that in *Cyanothece* 51142.

RESULTS AND DISCUSSION

Introduction of *nif* genes into *Synechocystis* 6803. *Synechocystis* 6803 has a close phylogenetic relationship with *Cyanothece* 51142 (Fig. 1A) (31). The large *nif* cluster from *Cyanothece* 51142 (28.34-kb region of DNA) was transferred into wild-type *Synechocystis* 6803 on a single extrachromosomal plasmid. This large plasmid, pSyNif-1,





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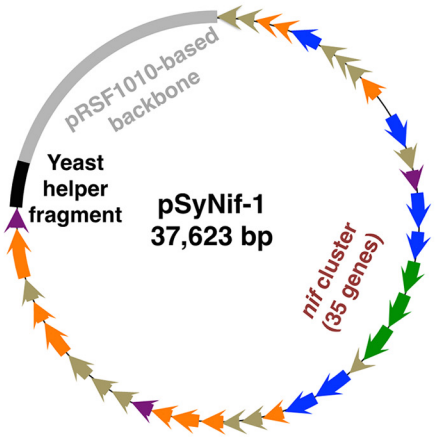
Gene	Role of gene product
nifH	Nitrogenase Fe protein (γ subunit), provides electrons and ATP for catalysis
nifDK	Nitrogenase MoFe protein (α and β subunits), catalyzes reduction of nitrogen to ammonia
nifV	Homocitrate synthase, involved in FeMo-cofactor synthesis
nifB	Nitrogenase cofactor biosynthesis protein, required for FeMo-cofactor synthesis
nifS	Cysteine desulfurase, transfers sulfur from cysteine to NifU
nifU	Fe–S cluster assembly scaffold protein
nifEN	FeMo-cofactor assembly proteins, putative function as a scaffold for synthesis of FeMo-cofactor
nifT	Unknown function, nitrogen fixation related protein
nifZ	Putative Fe-S cofactor synthesis protein
cysE2	Serine *O*-acetyl transferase, involved in biosynthesis of cysteine, a sulfur source of Fe–S cluster biosynthesis
nifX	Putative involved in FeMo-cofactor synthesis
nifW	Putative involved in stability of MoFe protein
hesA	Putative molybdenum cofactor biosynthesis
hesB	Putative involved in Fe-S cluster biosynthesis
feoB2	Ferrous iron transport protein B
feoA2	Ferrous iron transport protein A
modB	Putative molybdate ABC transporter
fdxN	4Fe-4S ferredoxin
fdxH	2Fe-2S ferredoxin
fdxB	4Fe-4S ferredoxin



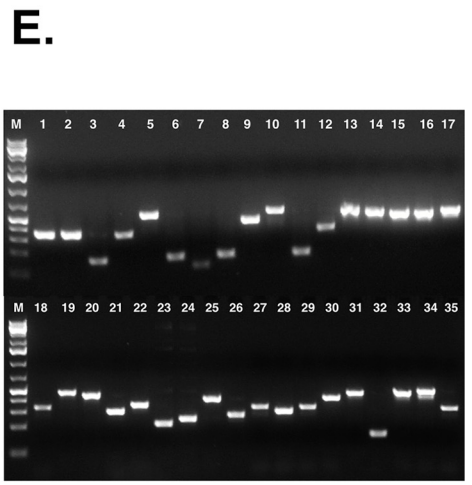


FIG 1 Introduction of *nif* genes into *Synechocystis* 6803. (A) Maximum likelihood 16S rRNA gene phylogeny of cyanobacteria. (B and C) Schemes showing the genetic organization of the *nif* cluster (B) and the role of each gene product (C) in *Cyanothece* 51142. Shown are the genes for the three structural proteins (*nifHDK*; green), necessary cofactors (blue), accessory proteins (orange), ferredoxins (purple), and hypothetical proteins

(Continued on next page)

containing the entire *nif* cluster with 35 genes (Fig. 1D), was constructed using the DNA assembler method (32). The chassis of this vector was based on pRSF1010 (33), and this self-replicating pSyNif-1 plasmid was transferred into *Synechocystis* 6803 by conjugation, generating the engineered strain TSyNif-1. Remarkably, over the past 4 years since its introduction into the heterologous host, pSyNif-1 has been stably maintained in its entirety in *Synechocystis* (see Fig. S1 in the supplemental material). Furthermore, all introduced *Cyanothece* genes were transcribed (Fig. 1E) as detected by reverse transcription-PCR (RT-PCR), indicating that native promoters in the *nif* cluster from *Cyanothece* 51142 can drive transcription of genes in *Synechocystis* 6803. An acetylene reduction assay method for nitrogen fixation detected nitrogenase activity under 12-h light/12-h dark conditions for strain TSyNif-1 (Fig. 2). Nitrogen fixation reached an activity level of 2% relative to that in *Cyanothece* 51142 grown under similar conditions. This is the first time that a nondiazotrophic phototroph has been engineered for biosynthesis of a fully functional nitrogenase enzyme and has been found to exhibit detectible and stable nitrogen fixation activity.

The minimal required gene cluster for nitrogen fixation activity. Gene expression parameters for *Synechocystis* 6803 are not as well understood as those for *E. coli*. Thus, the refactoring of *nif* genes as performed in *E. coli* and *Klebsiella* (16, 34) to determine the minimal requirement of genes for nitrogen fixation in *Synechocystis* 6803 is impractical at this stage. Therefore, we approached the identification of a minimal *nif* cluster for nitrogen fixation using a "top-down" method, which determines the influence of a gene on nitrogenase activity by selectively removing individual genes from the *nif* cluster (Fig. 2). Extrapolating the genetic requirements for nitrogen fixation activity observed in studies in which *nif* genes were introduced in *E. coli* (14, 15), we determined that genes for all homologous proteins introduced into *E. coli* are present in the *Cyanothece* 51142 *nif* cluster between gene *nifT* and *hesB* (Fig. 1). Hence, our second plasmid, pSyNif-2, contains 24 genes in the *nif* cluster between *nifT* and *hesB* (Fig. 2). Eleven genes were removed that presumably encode three metal transporter proteins, two ferredoxins, and six proteins of unknown function, none of which have been analyzed previously, although they are associated with nitrogen fixation. Intriguingly, this second engineered TSyNif-2 strain with a reduced cluster of 24 genes has a 3-fold increase in nitrogen fixation activity compared to strain TSyNif-1 (Fig. 2). Although plasmids pSyNif-1 and pSyNif-2 have the same plasmid backbone (Fig. S2), the transcriptional levels of the structural genes *nifH*, *nifD*, and *nifK* are higher in the TSyNif-2 strain (Fig. 3). This improvement in nitrogenase activity could be the result of removal of one or more regulatory genes, which may encode a protein(s) that represses expression of genes in the *nif* cluster.

However, further removal of genes from both directions resulted in a decrease of nitrogen fixation activity by more than 10-fold for strains TSyNif-3 to TSyNif-6, from which genes *hesB*, *hesAB*, *nifT*, and *nifTZ* were removed, respectively (Fig. 2). Thus, this "top-down" approach determined that an essential minimal cluster from *nifT* to *hesB* is required for nitrogen fixation activity in *Synechocystis* 6803. Additionally, we investigated the removal of two more genes in the cluster, *nifX* and *nifW*, generating the two strains TSyNif-7 and TSyNif-8. Deletion of these two genes did not affect expression of surrounding genes (Fig. S3). However, nitrogen fixation activity levels dropped 100-fold and 10-fold, respectively, in these engineered strains (Fig. 2). We conclude that both *nifX* and *nifW* are important genes for nitrogen fixation. Notably, *nifX* exhibited a positive influence on N_2 fixation in cyanobacteria, while it functions as a negative regulator for N_2 fixation in the heterotrophic diazotroph *Klebsiella oxytoca* (35).

FIG 1 Legend (Continued)
(brown). Gene names and annotations are from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Cyanobase (http://genome.microbedb.jp/cyanobase). (D) A schematic map of plasmid pSyNif-1 containing the entire *nif* cluster. The backbone (gray) is from broad host plasmid pRSF1010, which can replicate in *Synechocystis* 6803. The yeast helper fragment (black) contains *CEN6* and *ARS* as an *ori* and *ura3* as a selection marker. (E) Transcription of all 35 genes in engineered *Synechocystis* 6803. Each lane represents a gene in the *nif* cluster, as numbered in panel B. Total RNA was extracted from cells cultured in $BG11_0$ medium under 12-h light/12-h dark conditions, and cDNA was used as the template for PCR.

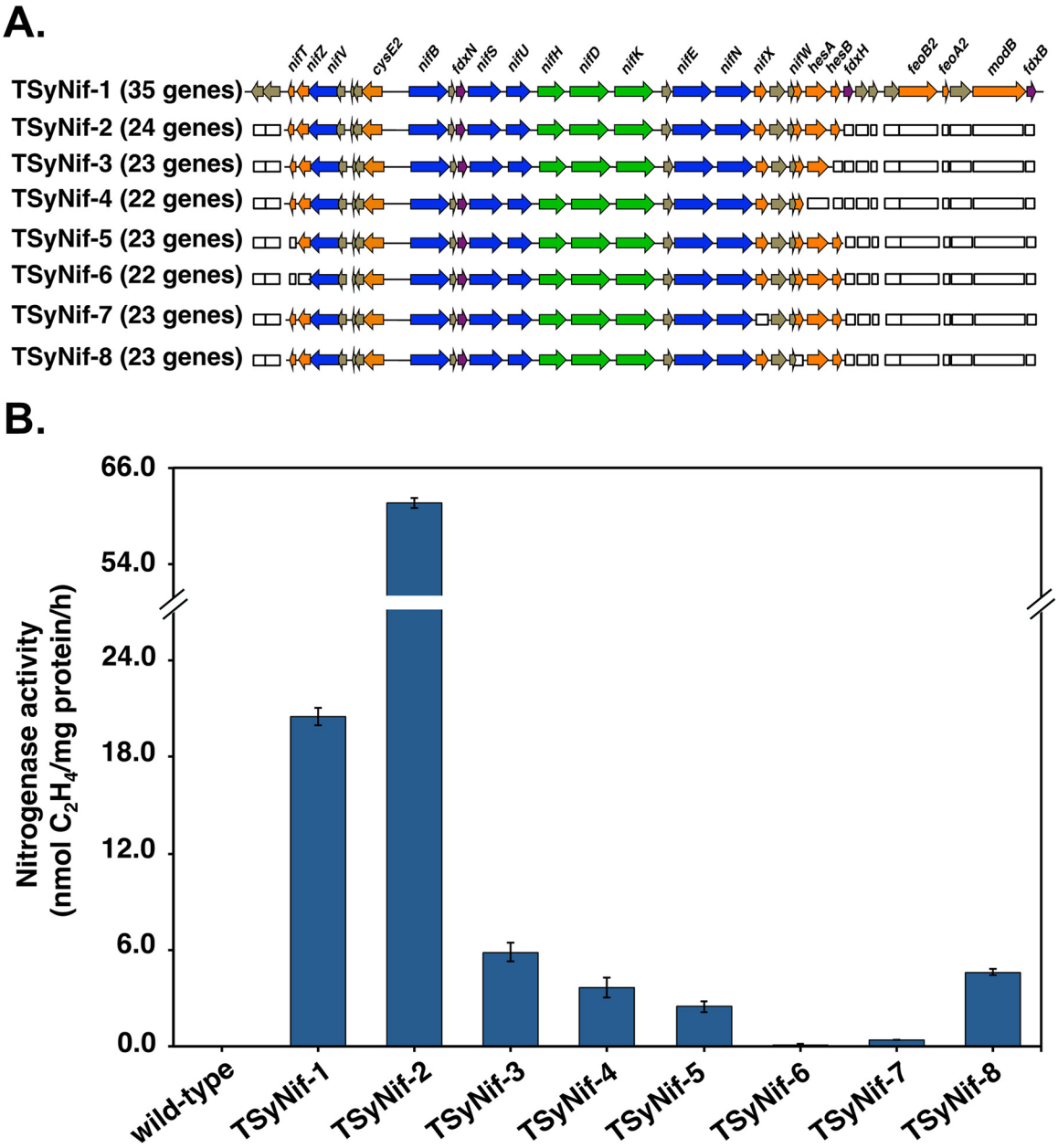


FIG 2 The minimal *nif* cluster required for nitrogen fixation activity in *Synechocystis* 6803. (A) Scheme showing the top-down method to determine the minimal *nif* cluster. The hollow rectangles represent the genes deleted from the cluster, and the colored rectangles represent the remaining genes. (B) Nitrogen fixation activity in engineered strains. Samples were collected from cultures under 12-h light/12-h dark conditions in $BG11_0$ medium. Nitrogen fixation activity was assayed by acetylene reduction, and error bars represent the standard deviations observed from at least three independent experiments.

Improvement of nitrogen fixation activity. To increase the RNA expression levels of the nitrogenase-related genes, we took advantage of three small endogenous plasmids in *Synechocystis* 6803: pCA2.4, pCB2.4, and pCC5.2. The heterologous genes harbored in these endogenous plasmids maintained higher transcriptional levels than those in a pRSF1010-based plasmid, because of the higher copy numbers of these three plasmids within *Synechocystis* (36, 37). First, we replaced the RSF1010 backbone of plasmid pSyNif-2 by the entire DNA segment of each of these endogenous episomes and then transferred the plasmids to *Synechocystis* 6803, generating three strains, TSyNif-9, TSyNif-10, and TSyNif-11, with the chassis of pCA2.4, pCB2.4, and pCC5.2, respectively (Fig. 3A; see also Fig. S4). As expected, genes *nifH*, *nifD*, and *nifK* in strain

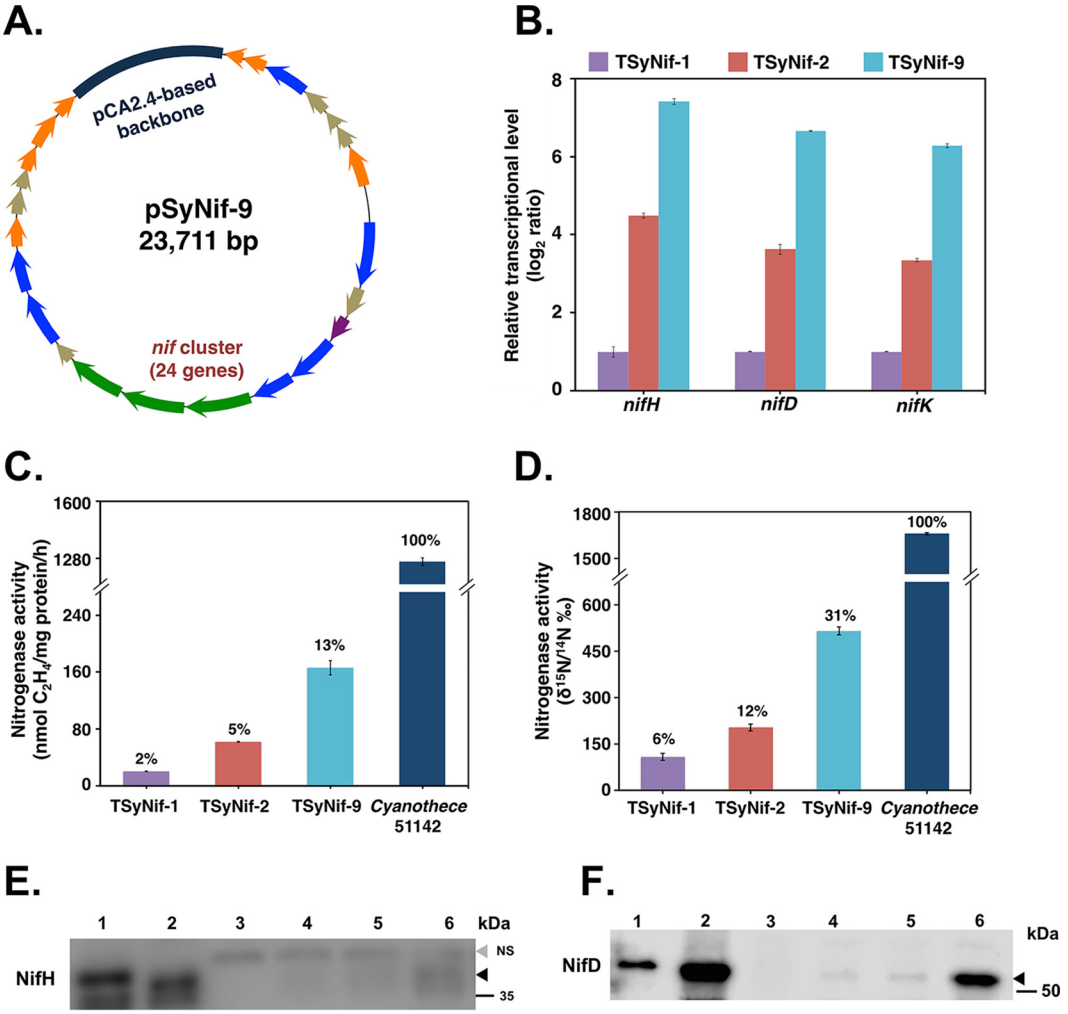


FIG 3 Enhancement of transcription levels of *nif* genes leads to higher nitrogen fixation activity. (A) A schematic map of plasmid pSyNif-9 containing the *nif* cluster with 24 genes from *nifT* to *hesB*. The backbone (dark blue) is from endogenous plasmid pCA2.4 of *Synechocystis* 6803. (B) Comparison of transcription levels of the *nif* structural genes in engineered strains through quantitative PCR (q-PCR). (C and D) Comparison of nitrogen fixation activities in engineered strains, as measured by C_2H_2 reduction assay (C) as well as ^{15}N assimilation assay (D). (E) Western blot showing the presence of NifH protein in engineered *Synechocystis* 6803 strains. Lanes 1 to 6 represent 2.0 μg purified NifH-His protein from *E. coli* and 15 μg whole-cell extracts of *Cyanothece* 51142, the *Synechocystis* 6803 wild-type strain, TSyNif-1, TSyNif-2, and TSyNif-9, respectively. The black triangle indicates the band for NifH protein, while the gray one indicates the nonspecific band. (F) Western blot showing the presence of NifD protein in engineered *Synechocystis* 6803 strains. Lanes 1 to 6 represent 0.5 μg purified NifD-His protein from *E. coli* and 15 μg whole-cell extracts of *Cyanothece* 51142, the *Synechocystis* 6803 wild-type strain, TSyNif-1, TSyNif-2, and TSyNif-9, respectively. Cyanobacterial samples were collected from cultures under 12-h light/12-h dark conditions in $BG11_0$ medium. Error bars represent the standard deviations of results from at least three independent experiments.

TSyNif-9 showed transcription levels that were severalfold higher than in TSyNif-2 (Fig. 3B). In addition, nitrogen fixation activity was increased by another 2- to 3-fold, reaching 13% for the acetylene reduction activity in TSyNif-9 relative to that observed in *Cyanothece* 51142 (Fig. 3C). Next, nitrogenase activity was directly assayed in the engineered strains using a ^{15}N assimilation assay method. Remarkably, the highest activity obtained was from strain TSyNif-9, reaching 31% of ^{15}N assimilation relative to *Cyanothece* 51142 (Fig. 3D). The activity data presented here is comparable to published data from studies on nitrogen fixation activity in engineered *E. coli* strains (Table 1). Additionally, both the NifH and NifD nitrogenase structural proteins were detected in whole-cell extracts via Western blotting by using antisera against the NifH and NifD proteins of *Rhodospirillum rubrum*, respectively (Fig. 3E and F). Although the protein level in *Cyanothece* 51142 is significantly higher than in the engineered *Synechocystis* 6803 strains, the NifD protein level in strain TSyNif-9 reached 10% of total

TABLE 1 Nitrogen fixation activity in diazotrophs and engineered strains

Strain	Nitrogenase activity (nmol C_2H_4/mg protein/h)	% activity (based on C_2H_2 reduction assay)	% activity (based on ^{15}N assimilation assay)	Source or reference
Diazotrophs				
Cyanothece 51142	1,262	100	100	This study
Azotobacter vinelandii	3,300	100	100	52
Paenibacillus sp. strain WLY78	3,050	100	100	14
Pseudomonas stutzeri A1501	1,050	100	ND[e]	53
Klebsiella oxytoca M5a1	3,708	100	ND	54
Engineered strains				
TSyNif-1	20	2	6	This study
TSyNif-9	166	13	31	This study
Engineered *E. coli*[a]	180	5	35	15
Engineered *E. coli*[b]	300	10	30	14
Engineered *E. coli*[c]	105	10	ND	53
Engineered *E. coli*[d]	740	20	ND	16

[a]Nitrogen fixation genes from *A. vinelandii* and *K. oxytoca*.
[b]Nitrogen fixation genes from *Paenibacillus*.
[c]Nitrogen fixation genes from *Pseudomonas*.
[d]Nitrogen fixation genes from *K. oxytoca*.
[e]ND, not determined.

cellular proteins (Fig. S4). It was also evident that the nitrogenase activities in the engineered strains were proportional to the level of nitrogenase structural proteins, which implied that optimizing the expression of nitrogenase proteins is critical for the activity. Most importantly, from an evolutionary standpoint, these results highlight the potential for engineering plant chloroplasts to fix nitrogen at a high level of activity, since oxygenic cyanobacteria are the progenitors of chloroplasts.

Nitrogen fixation activity in *Synechocystis* 6803. Despite the additional metabolic load of expressing large cohorts of 35 or 24 genes related to nitrogen fixation being introduced in *Synechocystis* 6803, remarkably, the expression and activities of these heterologous proteins did not affect the growth of the engineered strains under diurnal light/dark conditions (Fig. S1). We used strain TSyNif-2 to assess the influence of oxygen and exogenous nitrate on nitrogenase activity under four conditions, BG11, $BG11_0$ (BG11 without nitrate), BG11 with 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1 dimethylurea] (no O_2 evolution), and $BG11_0$ with 10 μM DCMU (Fig. S5). Interestingly, the transcript levels of the *nifH*, *nifD*, and *nifK* genes in the TSyNif-2 strain were downregulated by nitrate, which is similar to the results seen with *Cyanothece* 51142. Specifically, the depletion of nitrate improved the nitrogenase activity over 30-fold in $BG11_0$ with DCMU (Fig. S5). Nitrogen fixation activity was obtained only in an anaerobic environment when DCMU was added to the testing culture, although the headspace of all cultures was flushed with pure argon. These data indicate that oxygen generated by photosynthesis directly blocks nitrogenase activity in TSyNif-2, highlighting that one of the biggest challenges for engineering nitrogen fixation in oxygenic phototrophs is the sensitivity of nitrogenase to oxygen.

Improvement of O_2 tolerance by introduction of hydrogenase uptake. In order to test the oxygen sensitivity of nitrogen fixation activity in TSyNif-2, a measured amount of oxygen was added to the headspace of cultures grown in $BG11_0$ media to generate micro-oxic conditions of 0.5% and 1.0% of O_2 in the sealed testing bottles. The activity dropped more than 10-fold and 60-fold (Fig. 4A), respectively, demonstrating that, as expected, nitrogen fixation activity in engineered *Synechocystis* 6803 is highly sensitive to O_2. To enhance O_2 tolerance under the same conditions, genes coding for the uptake hydrogenase enzyme from *Cyanothece* 51142 were introduced into the chromosome of the TSyNif-2 strain. The uptake hydrogenase is conserved in diazotrophic cyanobacteria (38) and has been shown to be necessary for nitrogen fixation under aerobic conditions in *Cyanothece* (39). The uptake hydrogenase may utilize the H_2 produced by the nitrogenase, but it may have three other beneficial functions for

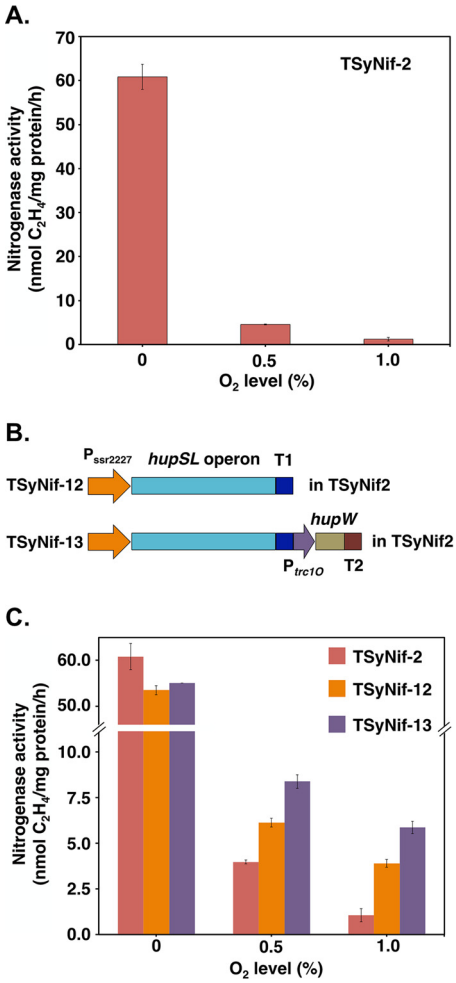


FIG 4 Expression of uptake hydrogenase improves O_2 tolerance of nitrogenase. (A) Effect of O_2 on nitrogen fixation activity of the TSyNif-2 strain. (B) Schematic showing the insertion of uptake hydrogenase genes *hupSL* and *hupW* from *Cyanothece* 51142 into the chromosome of the TSyNif-2 strain. (C) Comparison of nitrogen fixation activities under different micro-oxic conditions. Samples were collected from cultures under 12-h light/12-h dark conditions in $BG11_0$ medium. Nitrogen fixation activity was assayed by acetylene reduction, and error bars represent the standard deviations of results from at least three independent experiments.

the organism: supplying the organism with ATP via the oxyhydrogen (Knallgas) reaction; removing oxygen from nitrogenase, thereby protecting it from inactivation; and providing electrons (reducing power) to nitrogenase and other enzymes (39). The structural genes for this hydrogenase, *hupS* and *hupL*, present together in a single operon in *Cyanothece* 51142, were transformed into TSyNif-2, generating strain TSyNif-12 (Fig. 4B). In addition to the structural genes *hupSL*, a protease encoded by *hupW* is also present in *Cyanothece* 51142. HupW is required for the maturation of HupL protein through the processing of its C terminus (40). Thus, the *hupSLW* genes organized in two operons were transformed into TSynif-2 to generate the TSyNif-13 strain (Fig. 4B). The expression of *hup* genes in TSyNif-12 and TSyNif-13 was assessed by RT-PCR (Fig. S6). The introduction of the uptake hydrogenase did not affect nitrogen fixation activity under anaerobic conditions (Fig. 4C). Interestingly, under micro-oxic conditions, nitrogen fixation activity markedly improved with the expression of uptake hydrogenase, especially for strain TSyNif-13, with 2-fold and 6-fold increases in TSyNif-2 for O_2 levels of 0.5% and 1.0%, respectively. The results described above suggest that expression of uptake hydrogenase proves to be highly effective in enhancing O_2 tolerance of nitrogen fixation activity in the engineered *Synechocystis* strain.

In this study, introduction of the *nif* gene cluster from *Cyanothece* 51142 enabled nitrogen fixation activity in *Synechocystis* 6803. The minimal cluster for 24 genes (Fig. 2) for nitrogenase activity will provide a useful framework for future studies to further enhance such activity by refactoring genes as previously done in *Klebsiella oxytoca* (34). Although uptake hydrogenase is a complex enzyme, introduction of its structural genes and a protease works as a mechanism providing protection from the toxicity of O_2. The O_2 toxicity to nitrogenase is likely the most difficult aspect to overcome to achieve nitrogen fixation activity under aerobic conditions.

A fully functional nitrogenase holoenzyme requires 8 electrons and 16 ATPs to reduce one molecule of N_2 to ammonia. Thus, metabolism within cells needs to be adjusted to supply enough reducing power and energy for nitrogen fixation. Biosynthesis of fully functional nitrogenase is a complex process. This complexity increases the difficulty of finding the minimal genes and the best ratios of proteins expressed from the *nif* cluster in *Synechocystis* 6803. Genes with the same designations in different species occasionally have alternative functions (see Table S1 in the supplemental material). An example is the *nifX* gene, which functions as a negative regulator in *Klebsiella oxytoca* (35). Gene *nifX* is of importance in *Cyanothece*, since deletion of *nifX* affects nitrogenase activity.

Although multiple challenges and many barriers exist with respect to enabling plants to efficiently fix atmospheric nitrogen, we have engineered an oxygenic photosynthetic cell to fix N_2 by reconfiguring the genetic processes for nitrogen fixation from *Cyanothece* 51142 to function in *Synechocystis* 6803. Our studies to date have established the highest rate of engineered nitrogen fixation activity in any nondiazotrophic oxygenic organism.

MATERIALS AND METHODS

Microorganisms, culture conditions, and media. All cyanobacterial strains, including *Cyanothece* 51142, *Synechocystis* 6803, and engineered strains (see Table S2 in the supplemental material), were cultured in 100-ml flasks of fresh BG11 medium (41) with appropriate antibiotics (20 μg/ml kanamycin, 15 μg/ml chloramphenicol, or 20 μg/ml spectinomycin). As a preculture, cells were grown at 30°C, with 150 rpm shaking, and under conditions of 50 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ constant light. For the nitrogen fixation activity assay, unless otherwise stated, precultured cells were collected and washed with fresh $BG11_0$ medium (BG11 medium without nitrate) and resuspended in 500 ml fresh $BG11_0$ medium. Cells were grown at 30°C with air bubbling under 12-h light/12-h dark conditions with 50 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ of light. Yeast and *E. coli* strains (listed in Table S2) used for construction of recombinant plasmids were grown with 200 rpm shaking in yeast extract-peptone-dextrose plus adenine (YPAD) medium (42) and LB medium at 30°C and 37°C, respectively.

Construction of recombinant plasmids and engineered strains. Plasmids and strains used in this study are listed in Table S2, and all primers used in this study are listed in Table S3. Two methods were used to construct the plasmids: DNA assembler (32) and Gibson assembly (43). For building the large pSyNif-1 and pSyNif-2 plasmids containing the *nif* genes, genomic DNA from *Cyanothece* 51142 was used as the template for PCR, and all DNA fragments were combined using the DNA assembler method to form the plasmids in yeast. For the other recombinant plasmids listed in Table S2, Gibson assembly was the method used to construct them with DNA fragments amplified by PCR. Genomic DNAs from *Cyanothece* 51142 and the large pSyNif-2 plasmid were used as the templates for PCR to construct the plasmids for backbone replacement, the plasmids containing the uptake hydrogenase genes, or the plasmids used to remove specific *nif* genes. The sequences of all of the plasmids constructed in this study were verified (Genewiz, NJ).

Plasmids pSyNif-1 and pSyNif-2 were introduced into the *Synechocystis* 6803 wild-type strain through the method of triparental conjugation (44) to form strains TSyNif-1 and TSyNif-2, respectively. The other recombinant plasmids were transformed into *Synechocystis* 6803 by natural transformation (45), and double homologous recombination integrated the fragments into the chromosome (for the uptake hydrogenase genes) or the plasmid (for the plasmid backbone replacement and for the specific removal of *nif* genes).

Reverse transcription-PCR (RT-PCR) and quantitative PCR (q-PCR). RT-PCR analysis was performed using RNA samples isolated from culture grown in $BG11_0$ medium at time point D1 (1 h into the dark period) under light/dark conditions. After extraction and quantification of RNA (46), 100 ng of DNase-treated RNA samples and Superscript II reverse transcriptase and random primers (Invitrogen) were used for reverse transcription according to the manufacturer's instructions. cDNA generated after reverse transcription was used as the template for PCR to validate the transcription of genes.

q-PCR was performed on RNA samples extracted from culture grown in $BG11_0$ medium under light/dark conditions as previously described (27). Briefly, QRT-PCR Sybr green dUTP mix (ABgene) was used for the assay on an ABI 7500 system (Applied Biosystems). Each reaction was performed in three

replicates, and the average threshold cycle (C_T) value was used to calculate the relative transcriptional levels for the amounts of RNA. All primers used for RT-PCR and q-PCR are listed in Table S3.

Measurement of nitrogen fixation activity. Nitrogen fixation activity was measured by an acetylene reduction assay (47) modified from a previously published method (48). Unless otherwise stated, the activity assay was performed as follows. A 25-ml volume of cyanobacterial culture was grown in $BG11_0$ medium with air bubbling under light/dark conditions as mentioned above and was transferred to a 125-ml air-tight glass vial. DCMU (10 μM) was added to the culture, vials were flushed with pure argon, and cultures were incubated under 12-h light/12-h dark conditions. Cells in the sealed vials were cultured overnight, and, at the D1 time point, 5 ml acetylene was added into the sealed vials, followed by 3 h of incubation in light at 30°C. Two hundred microliters of gas was sampled from the headspace and injected into an Agilent 6890 N gas chromatograph equipped with a Porapak N column and a flame ionization detector, using argon as the carrier gas. The temperatures of the detector, injector, and oven were 200°C, 150°C, and 100°C, respectively.

Total protein levels were determined on a plate reader (Bio-Tek Instruments, Winooski, VT) using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Total chlorophyll *a* was subjected to methanol extraction and quantification on an Olis DW2000 spectrophotometer (OnLine Instrument Systems, Inc., GA).

***In vivo* $^{15}N_2$ incorporation assay.** All strains were grown under light/dark conditions as mentioned above, and 50-ml cultures were transferred into a 125-ml airtight glass vial. DCMU (10 μM) was added to the culture, vials were flushed with pure N_2, and cultures were incubated under light/dark conditions. Cells in the sealed vials were cultured overnight, and, at the D1 time point, 8 ml of headspace gas was removed followed by injection of 8 ml of $^{15}N_2$ gas (Cambridge Isotope Laboratories, Inc.) (98%$^+$). After 8 h of incubation at 30°C in light (50 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), the cultures were collected and dried in a laboratory oven at 50°C to 60°C for 24 h. The dried pellets were ground, weighed, and sealed into tin capsules. Isotope ratios were measured by elemental analyzer-isotope ratio mass spectrometry (EA-IRMS; Thermo Fisher Scientific), and values are indicated as $\delta^{15}N$ (‰), where the number represents a linear transform of the $^{15}N/^{14}N$ isotope ratios, representing the per-mille difference between the isotope ratios in a sample and in atmospheric N_2 (49). Data presented are mean values determined on the basis of results from at least two biological replicate cultures.

Western blot analysis. The *nifH* (cce_0559) gene and *nifD* (cce_0560) gene were subjected to individual PCR amplifications from the genomic DNA of *Cyanothece* 51142, using the primers shown in Table S3. The PCR fragment was ligated into expression vector pET28a cleaved by NdeI and BamHI. The resulting plasmids (pET28a-nifH and pET28a-nifD) were used to produce NifH and NifD proteins, each with an N-terminal His_6 tag. For overproduction of these proteins, *E. coli* BL21 (DE3) was transformed with plasmids pET28a-nifH and pET28a-nifD, respectively, and cultivated in LB medium at 37°C to an optical density at 600 nm (A_{600}) of 0.3. Protein expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside, and the culture was incubated for another 18 h at 20°C. After the cells were harvested, NifH and NifD were individually purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Briefly, harvested *E. coli* cells were resuspended in 20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl and in 2 mM β-mercaptoethanol supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Lysozyme was added to reach a concentration of 1 mg/ml, and the cells were lysed by freezing-thawing, followed by sonication. After cells were centrifuged at 13,000 rpm, the Tris-HCl buffer (pH 8.0) was added to the supernatant (final concentration, 50 mM), and it was loaded onto a Ni-NTA agarose column (0.2 ml). After bound proteins were washed with the starting buffer containing 1 M NaCl, they were eluted with 0.3 ml of the starting buffer containing 250 mM imidazole. The purified protein was stored at −20°C and used as the positive control for Western blot assay.

Cyanobacterial cells cultured in N-free medium under conditions of light/dark cycles were collected at time point D4 (4 h after the dark phase) and resuspended in 0.5 ml TG buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol) containing a protease inhibitor cocktail (Sigma-Aldrich). A 0.5-ml volume of sterilized, acid-washed glass beads was added to the cells, and the mixture was disrupted using a bead beater (BioSpec Products). The resultant mixture was centrifuged for 10 min at 7,500 $\times$ *g*, and the supernatant was transferred into a new tube. The amount of protein was determined using bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific).

A 15-μg (total) protein extract from each sample was solubilized with 8$\times$ sample buffer (10 ml of 0.5 M Tris [pH 6.8], 15 ml of 70% glycerol, 8 ml of 20% sodium dodecyl sulfate, 4 ml of β-mercaptoethanol, 4 ml of 0.1% bromophenol blue) at 70°C for 10 min and separated on a sodium dodecyl sulfate (0.1% [wt/vol])-polyacrylamide (12.5% [wt/vol]) gel by electrophoresis. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), blocked using 5% bovine serum albumin (BSA) for 2 h at room temperature, and then separately incubated with the primary rabbit antibodies raised against NifH and NifD protein of *Rhodospirillum rubrum* (50, 51) diluted in 1.5% BSA (1:2,000) overnight at 4°C. The horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) was diluted at 1:5,000 in 1.5% BSA. Immunodetection was performed using Western blotting Luminol reagent (Millipore).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01029-18.

FIG S1, PDF file, 0.05 MB.

FIG S2, PDF file, 0.3 MB.

FIG S3, PDF file, 4.3 MB.
FIG S4, PDF file, 0.5 MB.
FIG S5, PDF file, 0.1 MB.
FIG S6, PDF file, 1.3 MB.
TABLE S1, PDF file, 0.05 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Huimin Zhao and his research group (University of Illinois) for introducing us to the use of the DNA Assembler method; Lingxia Zhao, Xiujun Duan, and Alicia Lohman for expert technical assistance; and members of the research groups of H.B.P., Costas Maranas, Tae Seok Moon, and Fuzhong Zhang for critical scientific discussions. D.L., M.L., J.Y., H.B.P., and M.B.-P. designed the experiments; D.L., M.L., J.Y., and M.B.-P. performed the experiments; D.L., M.L., H.B.P., and M.B.-P. wrote the paper.

This study was supported by the National Science Foundation (MCB-1331194).

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