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Gene Transfer in *Leptolyngbya* sp. Strain BL0902, a Cyanobacterium Suitable for Production of Biomass and Bioproducts

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**Abstract**

Current cyanobacterial model organisms were not selected for their growth traits or potential for the production of renewable biomass, biofuels, or other products. The cyanobacterium strain BL0902 emerged from a search for strains with superior growth traits. Morphology and 16S rRNA sequence placed strain BL0902 in the genus *Leptolyngbya*. *Leptolyngbya* sp. strain BL0902 (hereafter *Leptolyngbya* BL0902) showed robust growth at temperatures from 22°C to 40°C and tolerated up to 0.5 M NaCl, 32 mM urea, high pH, and high solar irradiance. Its growth rate under outdoor conditions rivaled *Arthrospira* ("pirulina") strains. *Leptolyngbya* BL0902 accumulated higher lipid content and a higher proportion of monounsaturated fatty acids than *Arthrospira* strains. In addition to these desirable qualities, *Leptolyngbya* BL0902 is amenable to genetic engineering that is reliable, efficient, and stable. We demonstrated conjugal transfer from *Escherichia coli* of a plasmid based on RSF1010 and expression of spectinomycin/streptomycin resistance and yemGFP reporter transgenes. Conjugal efficiency was investigated in biparental and triparental matings with and without a "elper"plasmid that carries DNA methyltransferase genes, and with two different conjugal plasmids. We also showed that *Leptolyngbya* BL0902 is amenable to transposon mutagenesis with a Tn5 derivative. To facilitate genetic manipulation of *Leptolyngbya* BL0902, a conjugal plasmid vector was engineered to carry a trc promoter upstream of a Gateway recombination cassette. These growth properties and genetic tools position *Leptolyngbya* BL0902 as a model cyanobacterial production strain.

**Introduction**

Great interest is being focused on photosynthetic microorganisms for their ability to convert solar energy and CO\textsubscript{2} into fuels and other bioproducts. Cyanobacteria provide an excellent platform for the production of renewable biofuels and other products [1,2]. Cyanobacterial carbohydrate and lipid metabolism has been studied by several laboratories but much remains to be understood [3–5]. Cyanobacteria typically accumulate glycogen and polyhydroxyalkanoates rather than lipids as stored energy, but their photosynthetic membranes are rich with glycolipids and they naturally produce hydrocarbons [4,6], the major constituents of gasoline, diesel, and jet fuel [7].

Cyanobacteria have been a major component of our biosphere for over 2.5 billion years [8]. Architects of our atmosphere, these photosynthetic organisms still play an essential role in biogeochemical transformations, particularly in the oceans where they may account for more than 50% of phytoplankton biomass and primary production [9]. With a wide range of metabolic capabilities and few nutritional demands, cyanobacteria live in diverse environmental conditions [8]. Some fix nitrogen, reducing the need for nitrogen fertilizer and the associated production of nitrous oxide, a major greenhouse gas [10,11]. Most species tolerate high pH and some tolerate high salt concentration, conditions that help to control contaminants and predators in outdoor ponds. Certain cyanobacteria produce a mucilaginous envelope for protection against predators and desiccation. Regulation of photosynthetic antenna complexes called phycobilisomes allow cyanobacteria to adapt to changes in light quality and to extremely low light levels [12], and extracellular and intracellular screening pigments protect them against high light or UV radiation [13]. Cyanobacteria usually have higher growth rates than other phytoplankton under low light [14]. In addition to their physiological and ecological variety, cyanobacteria are also...
diverse in terms of morphology, including multicellular filamentous species that may biofloculate or float to the surface of a pond for easier harvesting. These characteristics reflect their genetic diversity and make them good sources for gene mining.

As prokaryotic, gram-negative bacteria, cyanobacteria are easy to manipulate genetically. Extensive genetic tools have been developed for a variety of different species. DNA can be introduced into cyanobacteria by transformation, conjugation, and electroporation and then propagated in the strain if carried on a replicating plasmid or if integrated into the host chromosome [15,16]. However, genetic approaches have been developed for only a limited number of model strains used to investigate fundamental processes such as photosynthesis, nitrogen fixation, and circadian rhythm [16–18]. Productivity, particularly outside of the highly regulated environment of the laboratory, and the ability to grow in a wide range of ecological conditions were not determining factors in the selection of these strains for laboratory studies.

Research featuring genetically engineered cyanobacteria for the production of liquid biofuels including ethanol [19], isobutylaldehyde and isobutanol [20], and free fatty acids [21] has recently flourished. Although using cyanobacteria as cell factories has become more common, studies are still carried out with standard laboratory model organisms rather than with potential production strains. For their desirable growth qualities, much consideration has been given to strains of the genus Arthrospira (“paralina”), which are grown at industrial scale mostly as a nutritional supplement. However, several attempts to transform Arthrospira strains have had only limited success [22,23], and to our knowledge there is no reliable genetic system for the stable transformation of Arthrospira spp.

We have identified and characterized the cyanobacterial strain Leptolyngbya sp. strain BL0902 (hereafter Leptolyngbya BL0902), which emerged from a screen of cyanobacterial strains for superior growth traits, and show that it is amenable to genetic manipulation. Leptolyngbya BL0902 has good growth characteristics when compared to two common outdoor production strains of the genus Arthrospira. We show that Leptolyngbya BL0902 can receive and maintain conjugal shuttle vectors, express an antibiotic resistance gene, and be subjected to transposon-tagging mutagenesis.

### Results

#### Morphological description and identification

*Leptolyngbya* BL0902 is a filamentous cyanobacterium without heterocysts, akinetes, or true or false branching; filaments are composed of single trichomes (chains of cells) that are straight to wavy and lack conspicuous motility. Trichomes are cylindrical and usually unsheathed, but a very thin hyaline sheath might be observed at trichome breakage; necridic cells are absent. Trichomes are slightly constricted at the cross-walls; cells are 1.3 to 3.3 times longer than wide with an average size of 1.42±0.15 (1.12 – 1.66) μm wide, 3.11±0.57 (2.09 – 4.18) μm long; and end cells are rounded. The cytoplasm is homogeneous with a few granules but no gas vesicles (Fig. 1).

#### Molecular identification based on 16S rRNA gene and ITS

Based on 16S rRNA data, *Leptolyngbya* BL0902 may be considered novel. The top hit identified by BLAST was *Spirulina laxisima* SAG 256.80 with 97.8% identity (Table 1) and to which no detailed morphological description is associated, leaving the possibility of misidentification. The uniqueness of *Leptolyngbya* BL0902 was verified by the Internal Transcribed Spacer (ITS) between the 16S and 23S rRNA genes, which shared only 89% identity with the first hit identified by BLAST (Table 2).

Nevertheless, *Leptolyngbya* BL0902 belongs to a relatively tight cluster of thin oscillators (Fig. 2), mostly assigned to the genus *Leptolyngbya*, including the strains OBB30802, UTEX 2910, ANT.ACE.V6.1, OBB10812, MXI_0BB24S04, PCC 7104 (formerly identified as belonging to the LPP group B [24]), UTEX 2910, OBB32602, and Kovacik 1999/1. Based on morphological features, “*scillatoria neglecta*” IAM M-82 corresponds more likely to an unknown species of the genus *Leptolyngbya* Anagnostidou & Komárek 1988 (R. Rippka, personal communication). No morphological descriptions of *Spirulina laxisima* SAG 256.80, *Phormidium* sp. 195-A12, *Phormidium* sp. MBC10025, *Phormidium* sp. SAG 61.90, or *Oscillatoria* sp. [AJ133106] are available. The different taxonomic assignments might be related to either the use of other taxonomic systems or misidentifications.

#### Characterization of growth traits

Growth traits including ranges of tolerance for temperature, salinity, pH, light, and urea were determined for *Leptolyngbya* BL0902 and two strains of *Arthrospira*, *A*. platensis BL0909, and *A*. maxima CS-328 (Table 3) as well as 40 other strains (data not shown). *A*. platensis BL0909 had a strict requirement for bicarbonate addition and was unable to grow in BG-11 medium that did not contain bicarbonate. *Leptolyngbya* BL0902 was more versatile with respect to growth media and grew well in both BG-11 and Zarrouk media. All three strains grew well in the 22–40 °C temperature range and tolerated up to 0.5 M NaCl, high pH up to 11, and high solar irradiance. Unlike *A*. maxima, *Leptolyngbya* BL0902 was able to tolerate urea at 32 mM, which is commonly used in algal outdoor growth ponds for control of rotifer and amoebae predators.

#### Growth rate and productivity measurements

The doubling time of *Leptolyngbya* BL0902 was measured and compared to *Arthrospira* strains under laboratory and outdoor growth conditions (Table 4). *Leptolyngbya* BL0902 grew faster than both *Arthrospira* species under laboratory conditions. In outdoor open-pond conditions, *Leptolyngbya* sp. BL0902 outperformed *A*. maxima CS-328 and had productivity on par with *A*. platensis BL0909. Importantly, *Leptolyngbya* BL0902 showed good culture stability during 3 months of continuous growth in 1-acre cultivation ponds during the summer 2009 season (Fig. 1c). Additionally, *Leptolyngbya* BL0902 formed long filaments that could be harvested with a vibrating screen similarly to *Arthrospira* spp. (Fig. 1f).

#### Heterotrophic growth

We tested *Leptolyngbya* BL0902 for heterotrophic growth with glycerol and 8 different sugars: glucose, fructose, sucrose, lactate, galactose, arabinose, maltose, and mannose. To prevent growth of potential contaminants, we used a genetically engineered *Leptolyngbya* BL0902 strain expressing the *aadA* gene and supplemented the media with Sp and Sm to a final concentration of 2 μg/ml for each of those antibiotics. The engineered *Leptolyngbya* BL0902 was incubated in the presence of glycerol and each of the 8 sugars at 10 mM final concentration, kept in complete darkness for over 3 weeks or incubated in the light in the presence of the photosynthesis inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-di-methylurea) at 10 μM final concentration. In both conditions, none of the 8 sugars or glycerol supported growth of *Leptolyngbya* BL0902, demonstrating that *Leptolyngbya* BL0902 cannot grow heterotrophically or photoheterotrophically under these conditions. However, in the presence of glucose, fructose, or sucrose,
survival of Leptolyngbya BL0902 was improved in the tested conditions.

Cellular composition and fatty acid profile

The composition of major cellular components (protein, carbohydrate, fat, ash, fiber, moisture, and fatty acid methyl ester [FAME]) was determined for A. maxima CS-328 and Leptolyngbya BL0902 (Table 5). Calculated as ash-free dry weight, Leptolyngbya BL0902 produced 28.8% FAME compared to 15.6% for A. maxima CS-328. Fatty acid profiles are shown in Figure 3. A. platensis BL0909 and A. maxima CS-328 both contained high levels of tri-unsaturated fatty acids, whereas Leptolyngbya BL0902 contained a higher proportion of monounsaturated fatty acids.

Antibiotic sensitivity

The antibiotic sensitivity of Leptolyngbya BL0902 was evaluated for nine antibiotics in BG-11 liquid culture media and on nitrocellulose filters on BG-11 agar plates, which mimics conditions used for genetic conjugations (Table 6). Leptolyngbya BL0902 was sensitive to low concentrations of Sp, Sm, Em, and Cm and moderate concentrations of Nm. Leptolyngbya BL0902 was somewhat resistant to Km, Gm, and G418 at commonly used concentrations; therefore, these antibiotics could be used to prevent growth of other organisms in laboratory settings. These data provide a panel of antibiotics that could be used as selective markers in genetic manipulations with Leptolyngbya BL0902.

Conjugal transfer and maintenance of RSF1010-based plasmids

Conjugation from E. coli donor cells has been used to introduce DNA into a wide variety of cyanobacteria, and broad-host-range plasmids derived from RSF1010 have been shown to replicate in many strains [18]. To determine whether these methods could be used with Leptolyngbya BL0902, we performed biparental matings with Leptolyngbya BL0902 and a conjugal E. coli donor strain (AM4338) that contained the cargo plasmid pRL1393a, the conjugal plasmid pRL443, and the helper plasmid pRL623. Transconjugant colonies became apparent on selective mating plates after about one week and showed robust growth after transfer to fresh selective plates. Control conjugations without the cargo plasmid never showed any antibiotic resistant colonies.

The ability to genetically modify Leptolyngbya BL0902 was further demonstrated by the heterologous expression of the yemGFP gene. The recombinant plasmid pAM4413 carrying the yemGFP gene was electroporated into AM1359, and the resulting strain was conjugated with Leptolyngbya BL0902. After one week, isolated transconjugant colonies were restreaked on fresh selective plates, and isolated colonies were then patched to fresh selective plates. Liquid cultures were grown in selective BG-11 medium. Expression of yemGFP was observed by fluorescence microscopy (Fig. 4).

Our initial conjugation experiments were performed with donor strains carrying the helper plasmid pRL623, which carries 3 methylase genes. The methylase genes are required for efficient conjugation into Anabaena recipient strains [25]. To assess the necessity of these genes for Leptolyngbya BL0902 conjugation, we determined the efficiency of conjugal transfers in biparental and triparental matings with and without the helper plasmid pRL623 and with two different conjugal plasmids: pRL443 and pRK2013 (Table 7). The conjugation protocol was modified as described in the methods to yield more reproducible data for transconjugant colony forming units (CFUs).

Approximately 3% of potential colony-forming units were transformed by conjugal transfer in bi- or tri-parental matings, which was increased about two-fold when a methylase-expressing helper plasmid was included (Table 7). We did not observe significant differences between bi-parental and tri-parental matings or between the conjugal plasmids pRL443 and pRK2013 (Table 7).

Transposon mutagenesis

To determine if transposon mutagenesis could be used as a genetic tool with Leptolyngbya BL0902, biparental matings were carried out with the E. coli strain AM4353, which harbors the SpR

Figure 1. Photomicrographs of wild-type Leptolyngbya BL0902 (a, b, c, and d), and its growth in a microalgae farm in Imperial Valley, California (e and f). Bright field (a and b), differential interference contrast (c), and phase contrast microscopy (d). 1-acre paddle-wheel raceway microalgae pond (e) and filamentous cyanobacteria collected with a vibrating screen (f). Scale bars, 5 μm.
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Sm’ Em’ Tn5-692 transposon on the suicide plasmid pRL692, as well as helper and conjugal plasmids (Table 8). Hundreds of Sp’ Sm’ transconjugant colonies were obtained on mating plates after incubation for a week, and selected Sp’ Sm’ colonies grew normally on fresh Sp’ + Sm’ BG-11 plates and in liquid medium. However, no colonies grew on medium containing Em at concentrations as low as 1.25 µg/ml. BL0902 is very sensitive to Em, and the Tn5-692 Em’ gene may not be expressed in Leptolyngbya BL0902. A repetition of the transposon-tagging experiment produced similar results.

The integration of the Tn5-692 transposon into the Leptolyngbya BL0902 chromosome was confirmed by a set of PCR assays.

Table 1. BLAST results obtained by querying the 16S rRNA gene of Leptolyngbya BL0902 with GenBank, and geographical and ecological origins of the hits.

| Description* | Accession | Query coverage | Score | E-value | % Identity | Origin of the strain or clone | Reference |
|---------------|-----------|----------------|-------|---------|------------|-----------------------------|-----------|
| Spirulina laxissima strain SAG 256.80 | DQ393278 | 100% | 2257 | 0 | 97.8 | Lake Nakuru, natron lake, Kenya | - |
| Uncultured bacterium clone DP10.3.11 | FJ612370 | 100% | 2239 | 0 | 97.3 | Dongping Lake, China | - |
| Uncultured bacterium clone GBI-83 | GQ441263 | 100% | 2167 | 0 | 96.4 | Marine microbial mats from a sandy intertidal beach, Schiermonnikoog, The Netherlands (53.48 N 6.13 E°) | - |
| Leptolyngbya sp. strain 08B30502 | A639892 | 100% | 2161 | 0 | 96.5 | Bubano Basin, Imola, Italy | [60] |
| Pseudoanabaenaceae cyanobacterium DPG1-KKS | EF654067 | 100% | 2161 | 0 | 96.4 | | [61] |
| Phormidium sp. strain 195-A12 | EU282429 | 100% | 2159 | 0 | 96.7 | Siberian permafrost, borehole 1/95, 2.4–2.45 m, Kolyma Lowland, Siberia, Russia | - |
| Uncultured bacterium clone GBI-87 | GQ441350 | 100% | 2158 | 0 | 96.6 | Marine microbial mats from a sandy intertidal beach, Schiermonnikoog, The Netherlands (53.48 N 6.13 E°) | - |
| Leptolyngbya antarctica ANT.ACE.1 | AY493588 | 100% | 2152 | 0 | 96.4 | Ace lake, Vestfolds hills, Antarctica | [62] |
| Uncultured cyanobacterium clone R8-R56 | DQ181691 | 100% | 2146 | 0 | 96.3 | Lake Rauer 8, Rauer Island, Antarctica | [63] |
| Phormidium sp. strain MBIC10025 | AB183566 | 95% | 2145 | 0 | 97.3 | Pacific Ocean, 27.03N-141.54E | - |
| Leptolyngbya sp. strain 08B19512 | A639895 | 100% | 2145 | 0 | 96.3 | Bubano Basin, Imola, Italy | [60] |
| Leptolyngbya sp. strain MX1 | GQ848193 | 100% | 2134 | 0 | 96.1 | Lake Taihu, Shangai, China | - |
| Leptolyngbya sp. strain PCC 7104 | A0839012 | 100% | 2128 | 0 | 96.1 | Rock at shoreline, Montauk Point, Long Island, New York, U.S.A | [24] |
| Leptolyngbya sp. strain 08B24504 | A639893 | 100% | 2128 | 0 | 96 | Bubano Basin, Imola, Italy | [60] |
| Phormidium sp. strain SAG 61.90 | EU624415 | 100% | 2128 | 0 | 95.9 | River Meuse near Tihange, Belgium | [61] |
| Uncultured cyanobacterium clone A132 | DQ181668 | 100% | 2124 | 0 | 96 | Ace lake, Vestfolds hills, Antarctica | [63] |
| Leptolyngbya nodulosa UTEX 2910 | EF122600 | 100% | 2117 | 0 | 95.7 | South China Sea | [64] |
| Uncultured Leptolyngbya sp. clone EHF51_S05b | EU071483 | 100% | 2117 | 0 | 95.7 | ESTEC HYDRA facility | - |
| Uncultured cyanobacterium clone A5-45-2 | FJ666611 | 100% | 2117 | 0 | 95.7 | Submerged sinkhole | - |
| Leptolyngbya sp. strain 08B32502 | A639894 | 100% | 2111 | 0 | 95.7 | Bubano Basin, Imola, Italy | [60] |
| Oscillatoria neglecta IAM M-82 | AB003168 | 100% | 2100 | 0 | 95.9 | | [65] |
| Oscillatoria sp. | AJ133106 | 100% | 2091 | 0 | 95.4 | Lake Loosdrecht, The Netherlands | - |
| Leptolyngbya sp. Kovacik 1999/1 | GQ495618 | 100% | 2074 | 0 | 95.9 | Biofilm from interior wall, Cartusian Monastery Ruins, National Park Slovak Paradise, Klastorisko, Slovakia | - |

*Redundant closely related clones originating from the same place were removed.

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Table 2. BLAST results obtained by querying the ITS of *Leptolyngbya* BL0902 with GenBank, and geographical and ecological origins of the hits.

| Description* | Accession | Query coverage | Score | E value | Identity | Origin of the strain or clone | Reference b |
|--------------|-----------|----------------|-------|---------|----------|------------------------------|-------------|
| Uncultured cyanobacterium isolate DGGE band #38 | AY827768 | 96% | 650 | 0 | 89% | Lake Klinckenberg, The Netherlands | [66] |
| *Oscillatoria* sp. strain CCME 416 | AM398957 | 87% | 477 | 3.00E-131 | 82% | Marble point, Antarctica | [67] |
| *Leptolyngbya antarctica* ANT.ACEV6.1 | AY493632 | 87% | 464 | 2.00E-127 | 82% | Lake Ace, Vestfold Hills, Antarctica | [62] |
| Uncultured Antarctic cyanobacterium clone TM2FOCH9 | EU852532 | 87% | 462 | 6.00E-127 | 81% | Forilidas Pond, Forilidas Valley, Transantarctic Mountains, Antarctica | - |
| *Leptolyngbya antarctica* TM1FOS73 | EU852528 | 87% | 461 | 2.00E-126 | 81% | Forilidas Pond, Forilidas Valley, Transantarctic Mountains, Antarctica | - |
| *Leptolyngbya antarctica* ANT.ACE 1 | AY493633 | 87% | 461 | 2.00E-126 | 81% | Lake Ace, Vestfold Hills, Antarctica | [62] |
| Uncultured cyanobacterium clone R8-R60 | DQ181762 | 87% | 461 | 2.00E-126 | 81% | Lake Rauer 8, Rauer Island, Antarctica | [63] |
| Uncultured cyanobacterium clone A5-45-2 | FJ866611 | 94% | 448 | 1.00E-122 | 81% | Submerged sinkhole ecosystem | - |
| *Oscillatoriales* cyanobacterium 2Dp86E | GU265558 | 87% | 444 | 2.00E-121 | 80% | Hosted by Dynamena pumila L. | [68] |
| Uncultured Antarctic cyanobacterium clone S334-8 | EU032374 | 87% | 443 | 6.00E-121 | 82% | Dry Valleys, Antarctica | [69] |
| *Pseudanabaenaceae* cyanobacterium DP1-KKS | EF654067 | 87% | 437 | 2.00E-119 | 81% | Desert crust, on sand dunes, Nizzara, Israel | [61] |
| *Phormidium* sp. strain AA | AM398947 | 87% | 437 | 2.00E-119 | 81% | Marble point, Antarctica | [67] |

*Redundant closely related clones originating from the same place were removed.

A hyphen “” indicates sequences not published in a research article.

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Carried out on three putative transconjugant clones. The clones were grown in BG-11 liquid culture, which resulted in the loss of all viable donor *E. coli* cells. The absence of *E. coli* was confirmed by a lack of colony formation when transconjugant cyanobacterial samples were inoculated on BG-11 plates supplemented with 0.04% (wt/vol) glucose and 5% (vol/vol) LB broth and incubated in the dark at 30°C, or on LB plates incubated at 37°C. Two pairs of primers were used for the PCR assays. The primer pair pRL692-2118F/2418R amplifies a 347-bp fragment within the origin of transfer (OriT) of the plasmid backbone from pRL692 (Table 9) amplifies a 421-bp fragment of primers were used for the PCR assays. The primer pair pRL692 transposon can be used for transposon tagging in *Leptolyngbya* BL0902.

**Construction and testing of the pAM4418 expression vector**

To facilitate the ability to introduce and express genes or noncoding and antisense RNAs in *Leptolyngbya* BL0902 and other cyanobacterial strains, we constructed plasmid pAM4418 based on the conjugal vector pRL1383a (Fig. 6). pAM4418 contains an *E. coli lacI* gene and the inducible *trc* promoter upstream of a Gateway recombination cassette. Genes of interest that are cloned in a pENTR vector can be introduced into pAM4418 by an LR recombination reaction. We monitored the expression of yemGFP as fluorescence emission intensity in *Leptolyngbya* BL0902 harboring pAM4418-yemGFP for two days following induction with IPTG. The reporter was constitutively expressed at moderately high levels, but there was no significant increase in yemGFP fluorescence intensity with IPTG addition at final concentrations ranging from 0.1 to 10 mM. We conclude that the *trc* promoter functions well in *Leptolyngbya* BL0902, but that either the lacI gene is not expressed or the LacI protein fails to repress expression from the *trc* promoter on pAM4418.

**Discussion**

*Leptolyngbya* BL0902 provides a new experimental model for cyanobacterial research that is focused on the goal of outdoor commercial production. Its growth traits related to harvestability, temperature range, and tolerance of high salt, pH, and light, paired with facile genetic manipulation, make *Leptolyngbya* BL0902 a potential commercial production platform strain. *Leptolyngbya* BL0902 growth rates in the laboratory and in outdoor ponds were similar to those of *Arthrospira* spp. that are currently grown at commercial scales, and large-scale outdoor pond cultures showed...
Figure 2. Phylogenetic tree inferred from 16S rRNA gene sequences (E. coli positions 110–1440) by maximum likelihood (Likelihood = $-58560.00282$); branch support values are indicated at the node. Clusters observed using at least 3 construction methods were collapsed or indicated with a black spot at the node. The E. coli sequence was used as out-group. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bars (0.1 mutation per position). The box in the upper right corner displays a subtree comprising sequences not included in the main figure and that share more than 95% similarity with Leptolyngbya BL0902.

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excellent stability during 3 months of growth in the summer of 2009. This is noteworthy because 13 out of 15 tested strains failed attempts to scale up to 1-acre growth ponds (unpublished observations).

Morphology and molecular data (16S rRNA and ITS gene sequences) place Leptolyngbya BL0902 as a novel isolate of this genus, within a cluster of thin oscillators isolated from a variety of biotopes and locations, which suggests a high resilience and competitiveness in a range of environmental conditions. The Leptolyngbya genus is heterogeneous and polyphyletic with a high genotypic diversity hidden behind a simple morphology. Specimens have been reported from hypersaline, marine, and freshwater habitats ranging from Antarctic lakes to hot springs. Most would have originally been identified as species of Lyngbya Agardh 1824, Phormidium Kützing 1843, Plecotomia Thuret 1875, or Oscillatoria Vaucher 1803, and were grouped under the name LPP [24–26]. This group was later revised to form a new genus, Leptolyngbya [27].

Leptolyngbya BL0902 accumulated higher FAME content and a higher proportion of mono-unsaturated fatty acids, preferable for a biodiesel feedstock, than two strains of Arthrospira spp.; the latter have high levels of tri-unsaturated fatty acids, preferable for nutritional applications but not desirable for fuel applications due to low oxidative stability. FAME recovery by a proprietary direct conversion process (Inventure Chemical, Inc.) for Leptolyngbya BL0902 and other cyanobacterial strains was significantly higher than has been reported by standard Bligh-Dyer extraction for cyanobacterial strains [28]. Further improvement of the Leptolyngbya BL0902 fatty acid profile may be achieved by overexpressing the native or a heterologous Δ9-acyl-lipid desaturase to increase the proportion of monounsaturated fatty acids.

Microalgal industrial production strains will need to be genetically manipulable. At least thirty-three different strains of cyanobacteria have been transformed, and a variety of genetic tools have become available since the unicellular cyanobacterium S. elongatus PCC 7942 (formerly Anacystis nidulans R2) was transformed four decades ago [18,29]. While transformation and electroporation are used for some strains, including a few naturally transformable cyanobacteria [16], conjugation, first shown in Anabaena sp. strain PCC 7120 [30], is generally the most successful and efficient method for gene transfer into cyanobacteria [31]. Conugal plasmids derived from the related IncP plasmids RP4 and RK2 [32], including pRL443 and pRK2013, have been used to mediate transfer of engineered cargo plasmids into several strains [16]. We demonstrated that broad host range plasmid vectors based on RSF1010 can be efficiently transferred to and stably maintained in Leptolyngbya BL0902. Previous studies have found that pRK2013 and its Km2 derivative pRK2073 promote increased conjugal transfer efficiencies in 3 strains of Chroococcidiopsis species and Nostoc punctiforme ATCC 29135 [33,34], but pRL443 and pRK2013 performed similarly in our study.

The presence of the helper plasmid pRL623, which carries three restriction methylase genes and is necessary to overcome restriction barriers in Anabaena sp. strain PCC 7120 [25], increased conjugation efficiency in Leptolyngbya BL0902 by only two-fold. Restriction systems usually result in order-of-magnitude differences in conjugation efficiencies, but our results indicated little protective role for the methyltransferases carried by pRL623. Therefore, restriction systems do not appear to pose a significant barrier to genetic manipulation of Leptolyngbya BL0902, and the conjugation efficiency with or without a helper plasmid is on par with the efficiency reported for Anabaena sp. strain PCC 7120 [25]. Triparental and biparental matings involving the same set of plasmids performed similarly. Triparental matings, in which the conjugal and mobilizable cargo plasmids are not in the same cell at

**Table 3. Growth traits of A. maxima, A. platensis, and Leptolyngbya BL0902.**

| Strain                        | Temperature (°C) | NaCl (M) | pH | Light intensity (μmol photons m⁻² s⁻¹) | Urea (mM) |
|------------------------------|------------------|----------|----|-------------------------------------|-----------|
|                              | 10 | 22 | 30 | 40 | 0° | 0.1 | 0.25 | 0.5 | 1 | Std² | 8 | 9 | 10 | 11 | 15 | 125 | 250 | 500 | 0° | 8 | 16.7 | 32 |
| *Arthrospira maxima* CS-328  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| *Arthrospira platensis* BL0909 | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| *Leptolyngbya* L0902         | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |

²Std, corresponds to BG-11 medium.

+, robust growth; +/-, some growth; –, no significant growth; nd, not determined due to inability of strain BL0909 to grow in BG-11 medium.

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**Table 4. Doubling time under laboratory conditions and productivity measurements in outdoor ponds.**

| Strain                        | Doubling time (h) | Productivity (g m⁻² day⁻¹) |
|------------------------------|------------------|---------------------------|
| *A. maxima* CS-328            | 32               | 20                        |
| *A. platensis* L0909          | 24               | 20–30                     |
| *Leptolyngbya* L0902         | 23               | 20–25                     |

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**Table 5. Cellular composition and FAME content as measured by Inventure Chemical.**

|                  | *A. maxima* CS-328 | *Leptolyngbya* BL0902 |
|------------------|--------------------|-----------------------|
| moisture         | 11.8               | 8.1                   |
| protein          | 55.5               | 35.4                  |
| fat              | 6.8                | 5.3                   |
| fiber            | 2.2                | 3.7                   |
| carbohydrates    | 9.2                | 13.3                  |
| ash              | 17.3               | 34.3                  |
| FAME %* (Inventure) | 12.9             | 18.9                  |

*FAME/total dry weight.

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the start of mating [35] allow the use of plasmids from the same incompatibility group or that carry the same selectable markers.

Transposon mutagenesis is a powerful tool for gene discovery. As in the heterocystous and unicellular cyanobacterial strains Anabaena variabilis ATCC 29413 and S. elongatus [36], Tn5-692 is capable of transposition in Leptolyngbya BL0902. The high frequency of stable antibiotic-resistant colonies indicate that transposon mutagenesis will be a useful method for identifying new genes in Leptolyngbya BL0902 that are involved in traits related to large-scale growth, such as growth rate in open ponds and resistance to predators and pathogens. Gene discovery in Leptolyngbya BL0902 will be enhanced by the availability of a complete genome sequence, which is underway. Application of these genetic tools can lead to rapid strain modifications for improved growth properties, and the production of biomass and desired molecules such as renewable biofuels. Leptolyngbya species are not generally known to produce toxins, however there is a report of a toxin-related gene in a marine Leptolyngbya strain [37]. Identification and targeted inactivation of toxin genes would be another obvious goal for engineered strain improvement. Our work also provides a basis for developing gene transfer methods and genetic engineering tools for new strains of cyanobacteria that possess desirable characteristics for growth in a variety of different conditions and geographic locations.

Materials and Methods

Ethics Statement

This research involved field studies of algal strains grown in outdoor ponds at Carbon Capture Corporation’ Algae Research Center in Imperial Valley, California, which was leased by Biolglight Harvesting, Inc. during the field studies described in this work. No specific permits were required for the described field studies, which were performed at a leased commercial facility, and which did not involve endangered or protected species.

Strain isolation

Plasmids and strains related to this work are listed in Table 8. Leptolyngbya BL0902 was isolated from an open pond at the Carbon Capture Corporation Algae Research Center in Imperial Valley, CA. A sample of the pond water was serially diluted and incubated in 96 well plates at 30°C under 100-mmol photons m⁻² s⁻¹ constant light in BG-11 or Zarrouk medium. Unialgal wells were subcultured and, following visual examination, the best-growing non-redundant cultures were chosen as representatives of the strains present in the open ponds. Leptolyngbya BL0902 was one of the isolates. An axenic culture of Leptolyngbya BL0902 was obtained by picking isolated and “lean” filaments under a dissecting microscope and repeatedly streaking on agar-solidified BG-11 and BG-11 supplemented with 0.04% (wt/vol) glucose and 5% (vol/vol) LB (Lennox broth) (BG-11 Omni medium) followed by repeated serial dilution of the culture in liquid BG-11. To verify that the strain was axenic, cloned isolates were inoculated into 4 different solid and liquid media: (1) BG-11 Omni medium, (2) BG-

| Table 6. Antibiotic sensitivity of Leptolyngbya BL0902. |
|-----------------------------------------------|
| **Antibiotic** | x1 final concentration (µg/ml) | Serial dilutions of the antibiotic |  |  |  |  |  |  |  |  |  |  |
| | | x1/4 | x1/2 | x1 | x2 | x4 | | | | | |
| | | Liquid | Plate | Liquid | Plate | Liquid | Plate | Liquid | Plate | Liquid | Plate |
| Chloramphenicol (Cm) | 7.5 | – | – | – | – | – | – | – | – | – |
| Erythromycin (Em) | 20 | – | – | – | – | – | – | – | – | – |
| G418 | 10 | nd | + | + | + | nd | +/− | nd | +/− | nd |
| Gentamicin (Gm) | 2 | + | + | + | + | – | +/− | – | +/− | – |
| Kanamycin (Km) | 5 | + | + | + | + | + | – | +/− | – | – |
| Neomycin (Nm) | 25 | − | +/− | – | − | +/− | – | – | – | – |
| Spectinomycin (Sm) | 2 | – | nd | – | nd | – | nd | – | nd | – |
| Streptomycin (Sp) | 2 | – | nd | – | nd | – | nd | – | nd | – |
| Sp×Sm | 2 each | – | – | – | – | – | – | – | – | – |

+, robust growth; +/−, some growth; −, no significant growth; nd, not determined; x1 final concentration corresponds to an antibiotic concentration commonly used for model strains Anabaena sp. PCC 7120 and Synechococcus elongatus PCC 7942.

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Figure 3. Fatty acid profiles of A. maxima CS-328, Leptolyngbya BL0902, and A. platensis BL0909.

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11 supplemented with 0.01% (wt/vol) glucose, yeast extract (Difco), and Bacto-Peptone (Difco), (3) Gram-negative broth (GNB) medium (Difco), and (4) LB. Solid and liquid cultures were incubated at room temperature, 30°C, and 37°C in the dark. If no growth of heterotrophic bacteria was observed under any of the conditions after incubation for 1 month, the cultures were judged axenic. The isolates were also checked for contamination by differential interference contrast (DIC) and fluorescence microscopy after being stained with DAPI (4′,6-diamidino-2-phenylindole) at 10 μg ml^{-1}. Strains were stored at -20°C in medium supplemented with 8% DMSO.

**Microscopy, morphological description, and identification**

Bright field, DIC, and phase contrast photomicroscopy were carried out with a Zeiss Axioskop microscope equipped with Plan-Neofluar 40x/0.75 and 100x/1.30 objectives and a SPOT RT3 25.4 2 Mp Slider camera. DIC and fluorescence microscopy were carried out and images were captured on a Delta Vision (Applied Precision, Inc.) microscope system composed of an Olympus IX71 inverted microscope equipped with an Olympus UPlanSApo 100x/1.40 objective and a CoolSNAP HQ2/ICX285 camera. Tetramethylrhodamine isothiocyanate (TRITC) filters (S555/25

![Figure 4. Photomicrographs of wild-type *Leptolyngbya* BL0902 (a, b, and c) and a strain of *Leptolyngbya* BL0902 harboring pAM4418-yemGFP expressing the yemGFP gene (d, e, and f). (a, d) Differential interference contrast (DIC); (b, e) DIC and green fluorescence; (c, f) Chlorophyll (red) and green fluorescence. Scale bar, 10 μm. doi:10.1371/journal.pone.0030901.g004](image)

**Table 7.** Comparison of conjugal transfer efficiencies in *Leptolyngbya* BL0902 mating experiments.

| Exp. | *E. coli* cargo strain | *E. coli* conjugal strain | Mating type | Efficiency (Colonies/CFU)* |
|------|------------------------|---------------------------|-------------|---------------------------|
| 1    | AM4413 (pAM4413), Sp^r^ Sm^r^ | AM4416 (pRK2013), Km^r^ | Triparental | 0.031±0.011               |
| 2    | AM4413 (pAM4413), Sp^r^ Sm^r^ | AM4415 (pRL443), Ap^r^ Tc^r^ | Triparental | 0.029±0.011               |
| 3    | AM4417 (pAM4413, pRL623), Cm^r^ Sp^r^ Sm^r^ | AM4416 (pRK2013), Km^r^ | Triparental | 0.063±0.020               |
| 4    | AM4417 (pAM4413, pRL623), Cm^r^ Sp^r^ Sm^r^ | AM4415 (pRL443), Ap^r^ Tc^r^ | Triparental | 0.063±0.005               |
| 5    | AM4414 (pAM4413, pRL623, pRL443), Cm^r^ Sp^r^ Sm^r^ Ap^r^ Tc^r^ | | Biparental, positive control | 0.060±0.026 |
| 6    | pAM4413 (pAM4413), Sp^r^ Sm^r^ | | Biparental, negative control | 0.000±0.000 |

*Mean±S.D. (N=3). doi:10.1371/journal.pone.0030901.t007
| Strain or plasmid | Derivation and/or relevant characteristic | Source or reference |
|-------------------|------------------------------------------|---------------------|
| **Plasmids**      |                                          |                     |
| pAM2255           | Expression vector carrying a trc (IPTG inducible) promoter, Ap<sup>+</sup> | H. Iwasaki, [70]    |
| pAM4413           | pRL1383a vector carrying the yemGFP open reading frame downstream of trc constitutive promoter, Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| pAM4418           | Conjugal destination vector pRL1383a carrying an IPTG inducible trc promoter upstream of a Gateway recombination cassette, Sp<sup>r</sup> Sm<sup>r</sup> Km<sup>r</sup> | This study          |
| pAM4418-yemGFP     | Expression plasmid resulting from a Gateway LR recombination reaction of pENTR-SD-yemGFP and pAM4418, Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| pENTR-M3          | Gateway destination vector optimized for Synechococcus elongatus PCC 7942 and carries lac<sup>+</sup> and trc promoter upstream of a recombination cassette. Ap<sup>r</sup> Km<sup>r</sup> | This study          |
| pENTR-SD/D-TOPO    | Gateway entry vector with Shine-Dalgarno sequence; Km<sup>r</sup> | Invitrogen          |
| pENTR-SD-yemGFP    | pENTR-SD/D-TOPO carrying the yemGFP open reading frame, Km<sup>r</sup> | This study          |
| pEXP_1ax-yemGFP    | Expression vector carrying yemGFP downstream of an IPTG inducible promoter, Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| pJS151            | Expression plasmid containing yemGFP, a GFP allele with F64L, S65T, and A206K mutations and that has been codon-optimized for expression in yeast. The first two mutations correspond to mut1GFP [71], and the third mutation interferes with GFP dimerization [72] | J. Hasty            |
| pRK2013           | Conjugal plasmid, derivative of RK2, Km<sup>r</sup> | J. Meeks, [34,53]   |
| pRL443            | Conjugal plasmid, Km<sup>r</sup> derivative of RP4, Ap<sup>r</sup> Tc<sup>r</sup> | [25]                |
| pRL623            | Helper plasmid carrying Mob<sub>ColE1</sub> and methylase genes M:Aval, M:Eco47II, M:EcoT22I, Cm<sup>r</sup> | [25]                |
| pRL692            | Transposon mutagenesis plasmid carrying the mobile element Tn5-692 that contains a pMB1 oriV, Sp<sup>r</sup> Sm<sup>r</sup> Em<sup>r</sup> | (GenBank Accession No. AF424805) [36] |
| pRL1383a          | Mobilizable, broad host range plasmid derived from RSF1010, Sp<sup>r</sup> Sm<sup>r</sup> | (GenBank Accession No. AF403426) [54] |
| **E. coli strains**|                                          |                     |
| DH5<sup>z</sup>   | Cloning host                            | Gibco BRL          |
| DH10B             | Cloning host                            | Gibco BRL          |
| One Shot ccdB Survival<sup>TM</sup> (T1R) | Cloning host                           | Invitrogen         |
| One Shot TOP10    | Cloning host                            | Invitrogen         |
| AM1358            | DH10B harboring pRL623, Cm<sup>r</sup> | [52]                |
| AM1359            | DH10B harboring pRL623 and pRL443, Cm<sup>r</sup> Ap<sup>r</sup> Tc<sup>r</sup> | [73]                |
| AM4438            | AM1359 harboring pRL1383a, Cm<sup>r</sup> Ap<sup>r</sup> Tc<sup>r</sup> Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| AM4353            | AM1359 harboring pRL692, Cm<sup>r</sup> Ap<sup>r</sup> Tc<sup>r</sup> Sp<sup>r</sup> Sm<sup>r</sup> Em<sup>r</sup> | This study          |
| AM4389            | DH10B harboring pENTR-SD-yemGFP, Km<sup>r</sup> | This study          |
| AM4413            | DH10B harboring pAM4413, Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| AM4414            | AM1359 harboring pAM4413, Cm<sup>r</sup> Ap<sup>r</sup> Tc<sup>r</sup> Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| AM4415            | DH10B harboring pRL443, Ap<sup>r</sup> Tc<sup>r</sup> | This study          |
| AM4416            | DH10B harboring pRK2013, Km<sup>r</sup> | This study          |
| AM4417            | AM1358 harboring pAM4413, Cm<sup>r</sup> Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| AM4503            | One Shot ccdB Survival<sup>TM</sup> harboring pDEST_M3, Km<sup>r</sup> Ap<sup>r</sup> Cm<sup>r</sup> | This study          |
| AM4517            | DH5<sup>z</sup> harboring pAM4418-yemGFP, Sp<sup>r</sup> Sm<sup>r</sup> | This study          |
| **Cyanobacterial strains** |                                      |                     |
| BL0902            | Wild type                              | This study          |
| BL0909            | Wild type                              | This study          |
| CS-328            | Wild type                              | D. Bryant           |
| PCC 7120          | Wild type                              | Laboratory collection |
| PCC 7942 (AMC006) | Wild type                              | Laboratory collection |

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excitation and S630/60 emission) were used to image autofluorescence of photosynthetic pigments, and GFP filters (S484/16 excitation and S515/30 emission) were used to image GFP fluorescence. Image acquisition, deconvolution, and analysis (cell measurements) were performed using Resolve3D softWoRx-Acquire (Version 4.0.0) and Adobe Photoshop CS4.

Morphological description and identification were based on the taxonomic work of Komárek and Anagnostidis [38].

Characterization of growth traits

Ranges of tolerances for temperature, salinity, pH, light intensity, and urea concentration were determined for *Leptolyngbya* BL0902 and compared with 40 other cyanobacterial strains (data not shown) including two strains of *Arthrosira*, *A. platensis* BL0909 and *A. maxima* CS-328. Traits were assessed in 6- or 24-well plates containing liquid BG-11 or BG-11 supplemented with 20 mM NaHCO₃ for *Arthrosira spp.*. Unless temperature or light intensity was being investigated, cultures were maintained at 30°C under continuous light with an intensity of 125 μmol photons m⁻² s⁻¹ as measured with a QSL-100 Quantum light meter (Biospherical Instruments, Inc.). The temperature effect on growth was evaluated at 10°C, 22°C, 30°C, and 40°C, and the effect of light intensity was evaluated at 15, 125, 250, and 500 μmol photons m⁻² s⁻¹. To assess growth at various NaCl concentrations, BG-11 medium containing 20 mM HEPES (pH 8.0) was adjusted to final concentrations of 0.1, 0.25, 0.5, 1, and 2 M NaCl. The influence of pH on growth was investigated with culture media adjusted to pH 8.0 with 10 mM HEPES, pH 9.0 and 10.0 with 10 mM CHES, and pH 11.0 with 10 mM CAPS. Unbuffered BG-11 (pH~7.5) was used as a control. Tolerance to urea was determined by addition of urea to final concentrations of 8, 16.7, 32, 64, and 128 mM. All experiments included control BG-11 samples.

Cultures were incubated for 2 weeks except for growth at 10°C, for which plates were incubated for up to one month. Growth was determined by visual assessment.

The doubling time of *Leptolyngbya* BL0902 was measured and compared to *A. platensis* BL0909 and *A. maxima* CS-328 under both laboratory and outdoor growth conditions. Laboratory cultures were grown in 100 ml Zarrouk medium in 250 ml flasks on orbital shakers illuminated with 100 μmol photons m⁻² s⁻¹ in 12:12 h light:dark and 35±5°C temperature cycles. Optical densities at 750 nm were used to determine doubling times. *Leptolyngbya* BL0902 cultures were also grown in outdoor raceway ponds of an algae farm located in the Imperial Valley, CA, USA for more than 5 months continuously during the summer of 2009. The biggest ponds were about 1.2 acres and 15 cm deep, with a paddle wheel-driven flow speed of about 9 m/min (Fig. 1c). Daily average air
temperatures in the Imperial Valley during the summer of 2009 (June 1 - September 30) were between 25.6°C and 40.8°C, with the lowest and highest temperatures being 16.6°C and 46.6°C, respectively. During this period, there was between 12 and 14 h of daylight with no significant precipitation and little to no cloud cover. Cyanobacterial filaments were harvested using a sloped 50 μm vibrating screen (Fig. 1f). The slurry from the screen was rinsed with fresh water, dewatered using the vibrating screen, and then spread on a cement slab to dry for two days.

Heterotrophic growth

*Leptolyngbya* BL0902 was tested for heterotrophic growth with glycerol and 8 different sugars: glucose, fructose, sucrose, lactose, galactose, arabinose, maltose, and mannose. To suppress growth of bacterial contaminants, these experiments were performed with a *Leptolyngbya* BL0902 strain containing pRL1383a, and 2 μg/ml each of spectinomycin and streptomycin were added to the growth media. The strain was incubated in the presence of glycerol or each of the 8 sugars at 10 mM final concentration and either kept in complete darkness for over 3 weeks or incubated in the light in the presence of the photosynthesis inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) at 10 μM final concentration.

Cellular composition and fatty acid profile

Proportions of the major cellular components including protein, carbohydrate, fat, ash, fiber, and moisture were determined by New Jersey Feed Lab. Inventure Chemical determined the percentage of fatty acid methyl ester (FAME) using 100 g dried samples collected from an outdoor open pond.

To determine fatty acid profiles, lipids were isolated from cell pellets using a modified Bligh-Dyer extraction [50] followed by transesterification with sodium methoxide, and GC-MS analysis. Samples (5 ml) of an exponentially growing culture (OD750, 0.8) were collected by centrifugation and resuspended in 0.8 ml of H2O. 3 ml CHCl3:MeOH (1:2) was added and the vials were vortexed for 1 min. After 1 h incubation at 60°C, 1 ml of CHCl3 was added and the vials were vortexed for 1 min. Then, 1 ml of H2O was added, the vials were vortexed for 1 min and briefly centrifuged. The lower layer was recovered into a fresh vial and solvent was removed under a stream of nitrogen. 1 ml of 0.5 M sodium methoxide in MeOH was used to resuspend the dried crude lipid and the reaction was incubated for 30 min at room temperature. The reaction was quenched with 1 ml of H2O and the resulting methyl esters were recovered into 2 ml of hexane by vortexing for 1 min. The hexane layer was clarified by
centrifugation and sampled for GCMS analysis. The extracts were analyzed on an Agilent 6890N GC equipped with a DB-FAP column (30 m length, 0.25 mm ID, and 0.50 μm film thickness) coupled to a 5973 inert mass selective detector (Agilent Technologies, Inc.). Helium was used as the carrier gas with a flow rate of 1.2 ml/min, and 1 μl was injected into the column with a 50:1 split ratio. The column temperature was held at 100°C for 5 min and then ramped at 10°C/min to 250°C and held for 10 min. The total run time was 30 min. Identification of the fatty acids was based on retention times and fragmentation patterns of standards.

Antibiotic sensitivity evaluation

The antibiotic sensitivity of _Leptolyngbya_ BL0902 was evaluated against a panel of antibiotics in BG-11 liquid culture media and on 25 mm nitrocellulose filters laid on BG-11 agar. The tested antibiotic concentrations were ¼, ½, 1, 2, and 4 times the concentrations commonly used in our laboratory for the selection of recombinant cyanobacterial strains: 5 μg/ml kanamycin (Km), 2 μg/ml gentamycin (Gm), 20 μg/ml erythromycin (Em), 7.5 μg/ml chloramphenicol (Cm), 25 μg/ml neomycin (Nm), and 10 μg/ml G418. 2 μg/ml each of streptomycin (Sp) and spectinomycin (Sm) were used together for Sp'/Sm' strains to limit the appearance of spontaneous resistant mutants.

Mating and conjugal transfer of plasmid DNA

Transformations of _Leptolyngbya_ BL0902 through biparental and triparental conjugations followed published protocols [25,51,52] with minor modifications. Our standard biparental matings involved the cyanobacterial strain _Leptolyngbya_ BL0902 and an _E. coli_ strain (DH10B) that harbored the following three plasmids: (i) the conjugal plasmid pRL1443, an Ap'-Tc' Km' derivative of RP4 [51], or pRK2013, a Km' plasmid containing the transfer genes of RK2 cloned onto a CoeEl replicon [34,53], (ii) the “elper”plasmid pRL623, which carries the gene for Mobs3 and methylase genes encoding M.Aval, M.Eco47II, whose product methylates AvaII sites, and M.EcoT22I, an isoschizomer of M.AvaIII [25], and (iii) the cargo plasmid pRL1383a, pAM4413, or pRL692. Plasmid pRL1383a (GenBank Accession No. AF403426) is a Sp' Sm' derivative of RSFI1010 [54] and pRL692 (GenBank Accession No. AF424805) carries the Sp'/Sm' and Em' mobile element Tn–5692 [36]. Triparental matings involved the strain BL0902 and two _E. coli_ strains: a cargo strain carrying the cargo plasmid with or without a helper plasmid and a conjugal strain carrying a conjugal plasmid.

_E. coli_ strains were grown in 3 ml LB with the appropriate antibiotic(s) and incubated at 37°C overnight. Cells were harvested from 2 ml of each _E. coli_ culture by centrifugation and resuspended in 2 ml fresh LB. This step was repeated twice to wash the cells. After the third centrifugation, the cells were resuspended in 200 μl BG-11. Five milliliters of a growing _Leptolyngbya_ BL0902 culture by centrifugation and resuspended in 20 ml BG-11. Each mating contained 1 ml of _Leptolyngbya_ BL0902 concentrated cells and 0.2 ml of concentrated _E. coli_ cells. For each mating, 7.5 and 30 μl of the conjugation mixture, corresponding to about 3×10^6 and 1×10^7 short filaments (estimated microscopically with a hemocytometer), respectively, were adjusted to 150 mm nitrocellulose filters lying on mating plates using about 2 g of sterilized glass beads (2 to 4 mm diameter). To determine the total number of CFU in each conjugation mixture, 1 μl was serially diluted to 10^{-4} and 10^{-5}, and 150 μl of each dilution, corresponding to about 6×10^6 and 6×10^5 short filaments per ml, respectively, was plated and grown in parallel with the conjugation experiments.

Construction of recombinant plasmids based on the pRL1383a backbone

The pRL1383a backbone includes the following modules: multiple cloning site, SP6 promoter from pBAC108L (GenBank U51114), aadA promoter and gene conferring Sp' Sm', trpA terminator from Lorist6 (GenBank X98450), origin of replication, mob genes, rep genes, trpA terminator from Lorist6 (GenBank X98450), and T7 promoter from pBAC108L (GenBank U51114). To construct a pAM4413, a PCR fragment that included a lacP gene with an S289L mutation (pAM2255) and a trc promoter with an R801 mutation (pAM2255), a yemGFP (yeast-enhanced monomeric green fluorescent protein) gene with F64L, S65T, and A206K mutations, and a rep terminator from pAM2255 was amplified from the pEXP_lax-yemGFP plasmid with the primers lacz_F and rmb_B (Table 9) carrying the restriction sites EcoRI and AsaRI, respectively. The PCR fragment was gel purified and ligated into pRL1383a to replace the fragment between the EcoRI and AsaRI restriction sites.

The destination vector pAM4418 was constructed by ligation of a pDEST_M3 fragment and pRL1383a. The pDEST_M3 fragment included the following modules: trpA terminator [53],
lacP promoter with −35 to +1 region replaced by the conII synthetic promoter [56], lacP gene (synthetic ORF codon optimized for Synecococcus elongatus PCC 7942), hpp transcriptional terminator [57], r promoter (ends defined by the overlap between pTrcHis2-A for the 5’ end and pAM2255 for the 3’ end), Gateway cassette LR cloning frame A (chloramphenicol resistance) and cmdf DNA gyrase toxin [38] genes flanked by attR1 and attR2 recombination sites (Invitrogen), and the mB T2 terminator [59]. The pDEST_M3 fragment was isolated with NaeI and HindIII, treated with the T4 polymerase to generate blunt ends, and gel purified by electrophoresis. pRL1383a was linearized with HincII and dephosphorylated with CIP to prevent self-ligation. The ligation was transformed into One Shot cdbL Survival T1 Phage-Resistant (Tr) chemically competent E. coli (Invitrogen).

To construct pAM4418-based expression plasmids, the gene of interest needs to be amplified by PCR using a forward primer carrying a CACC motif at the 5’ end. The resulting PCR product can then be cloned into a pENTR-SD/D-TOPO vector (Invitrogen) and subsequently used in an LR recombination reaction (Gateway Technology, Invitrogen) with the pAM4418 vector. To test the pAM4418 vector, the yemGFP gene was amplified by PCR from pRL151 using the primer pair yemGFP F/yemGFP R and cloned as described above to make the plasmid pAM4418-yemGFP.

GenElute HP Plasmid Miniprep Kits (Sigma-Aldrich) were used for isolation of plasmid DNA from E. coli strains. Plasmids were digested with restriction endonucleases from New England BioLabs or other suppliers in buffers recommended by the suppliers. All plasmid constructs were first screened by restriction analysis, and one positive clone was confirmed by DNA sequencing. Sequences were deposited in GenBank under the following accession numbers: JN576076-JN376080.

IPTG induction of the trc promoter in pAM4418-yemGFP

Leptolyngbya BL0902 wild type and derivatives harboring the plasmid pAM4418-yemGFP or only pAM4418 were grown in BG-11 liquid medium, diluted to an OD750 of 0.15, and grown as 25 ml samples in 125 ml flasks on a shaker at standard conditions. After two days, the cultures were supplemented with IPTG to final concentrations of 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM for pAM4418-yemGFP and 1 mM for the control strains. The emission intensities of yemGFP from samples of the cultures were measured with a Tecan Infinite(R) M200 plate reader (TECAN) after induction for 0, 1.5, 3, 6, 12, 21, 24, 27, and 48 h. The excitation wavelength was set at 488 nm, and the emission was measured at 518 nm.

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Author Contributions

Conceived and designed the experiments: AT EL DMA GD SC SSG JWG. Performed the experiments: AT EL DMA GD SC. Analyzed the data: AT EL DMA GD SC SSG JWG. Contributed reagents/materials/analysis tools: AT EL DMA GD SC. Wrote the paper: AT EL DMA GD SC SSG JWG.

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