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Transformation and Conjugal Transfer of Foreign Genes into the Filamentous Multicellular Cyanobacteria (Subsection V) *Fischerella* and *Chlorogloeopsis*

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Abstract Cyanobacteria of subsection V grow as filaments with asymmetrical cell divisions that can generate a true-branching phenotype. Members of the genera *Fischerella* and *Chlorogloeopsis* furthermore differentiate akinetes (spore-like resting stages), heterocysts (specialized in nitrogen fixation) and hormogonia (cell aggregates with gliding motility for colonization and dispersal). Genetic approaches to studying the complex morphology and differentiations of these prokaryotes require transformation techniques. For *Fischerella* and *Chlorogloeopsis* reliable protocols for introducing foreign genes are lacking. Here, we explored conjugation, electroporation, and biolistic DNA transfer methods in *Fischerella* and *Chlorogloeopsis*, using the cyanobacterial replicon pRL25C as a marker. We successfully transformed *Fischerella muscicola* PCC 7414 and *Chlorogloeopsis fritschii* PCC 6912 and were able to express the GFP reporter protein under two different promoters: the nitrogen regulated $_{p}$*glnA* and the strong *E. coli* hybrid $_{p}$*trc*. For *Fischerella* all methods worked, for *Chlorogloeopsis* electroporation was unsuccessful. For both strains conjugation delivered the most reproducible results, whereby partial removal of the exopolysaccharide sheath by salt washing was a critical step.

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Introduction

Cyanobacteria are important as primary producers and nitrogen fixers. They can be engineered for production of diverse biofuels [31] and they are a rich source of bioactive metabolites, including antitumoral and antimicrobial agents [5]. Based on morphology, they were traditionally classified into five sections [33], later placed as "subsections I–V" into the phylum cyanobacteria [8]. Subsection V contains the most complex members, all of which are filamentous and differentiate heterocysts, oxygen impermeable cells where nitrogen fixation takes place. Some species develop hormogonia, motile filaments that serve as spreading agents, and some differentiate akinetes, spore-like cells. Based on those criteria, subsection V cyanobacteria resemble members of subsection IV (such as *Nostoc* or *Anabaena*). The key difference of subsection V is their ability to undergo multiplanar cell division, thereby generating multiseriate filaments or filaments perpendicular to the primary trichomes that are termed "true-branches". The ancestor of subsections IV–V is estimated to be 2,400–2,100 Mya in age [44]; hence, this group evolved multicellularity early in evolution. Many subsection V members isolated so far are thermophiles; inhabiting acidic–iron-rich thermal springs [7, 12].

The morphology of cyanobacteria belonging to subsection V was studied in the early 1980s by electron and laser scanning microscopy [15, 26, 27, 38], as was sheath envelope composition [30, 35]. However, genetic studies of subsection V are almost nonexistent. To our knowledge, only one isolated report on gene transfer in *Fischerella muscicola* UTEX 1829 exists [13].

The incorporation of foreign DNA through lateral gene transfer (LGT) has contributed substantially to bacterial diversification. Three means of LGT exist in nature and can be exploited in the laboratory: transformation, conjugation, and

transduction. Transformation or the uptake of naked DNA into a recipient genome can occur by natural competence or be induced in the laboratory for example, by electroporation. Some cyanobacteria, all of them so far found to be unicellular are naturally competent, among them, *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6803, and *Thermosynechococcus elongatus* BP-1 [14, 29, 36, 37]. Conjugation is the transfer of plasmid DNA from a donor to a recipient bacterium through cell-to-cell contact, and transduction is the transfer of genetic material by phage infection. For cyanobacteria outside subsection V, many approaches to genetic manipulation exist, including mainly transformation, electroporation, and conjugation [18, 42]. One of the main barriers to gene transfer are endogenous restriction endonucleases, which are particularly abundant in filamentous cyanobacteria [20, 48]. Pre-methylation of plasmid DNA or deletion of the predicted restriction sites targets of the cyanobacteria in question have helped to increase efficiency of transformation [10, 41, 49]. A number of plasmids are available for gene expression and homologous recombination in cyanobacteria [4, 49]. Natural transformation and conjugal transfer from *Escherichia coli* are generally the standard method for unicellular and filamentous cyanobacteria, respectively, although electroporation has been employed in filamentous cyanobacteria with success [32, 41]. DNA bombardment (biolistics), a system that accelerates DNA-coated particles into different tissues/organisms, was originally developed for plants, but currently widely used for gene transfer in other eukaryotic organisms and in bacteria [17] has been used to transform a marine of *Synechococcus* strain [23].

Understanding the basis of differentiation and morphology in cyanobacteria from subsection V requires techniques for their genetic manipulation. Here, we have investigated gene transfer methods in the two model strains *Fischerella muscicola* PCC 7414 and *Chlorogloeopsis fritschii* PCC 6912, the heterocysts, hormogonia and akinetes of which are shown in Fig. 1. Their morphology, however, differs in that *Fischerella*, like all other subsection V genera, forms true branches, whereas in *Chlorogloeopsis* asymmetric cell division only produces multiseriate (more than one filament in a row) filaments and aseriate aggregations. Filaments of both strains are enclosed by a thick exopolysaccharide layer visible by light microscopy (Fig. 1). Protocols for conjugation, electroporation and DNA bombardment for these strains are reported.

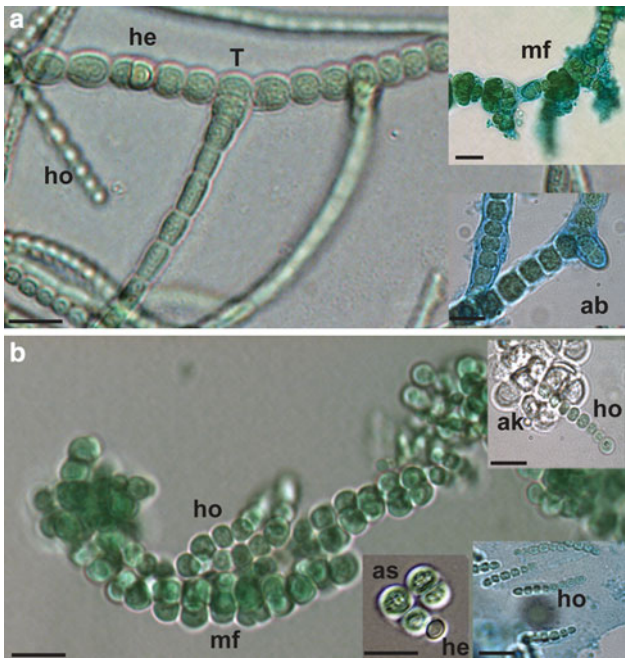

Fig. 1 Brightfield microscopy of *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912. **a** A characteristic filament from *F. muscicola* PCC 7414 may present all cell stages, true T-branches (*T*), hormogonia (*ho*), and mature filaments. Intercalated heterocysts (*he*) are easily spotted. Stationary phase trichomes can also be seen as multiseriate filaments (*mf*). **b** *C. fritschii* PCC 6912 trichomes are seen as hormogonia and multiseriate filaments; true branches are not observed. Hormogonia rise from akinetes (*ak*). Aseriate aggregates (*as*), resembling the division stages of subsection II cyanobacteria, are as abundant as trichomes. *Scale bars* represent 10 µm. Note the thick polysaccharide layer surrounding and within cells in a filament as visualized with 0.01 % alcian blue staining (*ab*)

Materials and Methods

Bacterial Strains and Culture Conditions

Chlorogloeopsis fritschii PCC 6912 and *Fischerella muscicola* PCC 7414 were obtained from the Pasteur Culture Collection (PCC) of cyanobacteria, France. Stock cultures were grown photoautotrophically in liquid BG11 medium [33], supplied with or without ($BG11_0$) combined nitrogen at 37 °C and a light intensity of 30 µmol m^{-2} s^{-1}. After conjugation, electroporation or DNA bombardment, cells were incubated on 1 % agarose A&A plates [1] supplemented with NH_4Cl or $NaNO_3$ as described below. Estimation of biomass was based on total chlorophyll from 1 ml methanolic extracts [21]. *E. coli* strains XL1-blue, HB101, and ED8654 were used for cloning and conjugation. Antibiotic selection for cyanobacteria was neomycin (Nm) 30 µg ml^{-1}.

Plasmids and GFP Constructs

Cargo plasmid pRL25C carrying the cyanobacterial replicon pDU1 from *Nostoc* sp. PCC 7524, an *E. coli* origin of replication and transference and an antibiotic resistance cassette for selection in *E. coli* and cyanobacteria (Nm^R, Km^R) [50], and conjugal plasmid pRL443 (Ap^R, Tc^R) [9] were kindly provided by Enrique Flores. In addition, the

Table 1 Primers used in this study

Primer	Sequence (5′–3′)
glnAPF-*Bam*HI	CATGGATCCTCCTTTTTCTCCCAATCGTTCCT
glnAPR_gfp	TCCTTTACTCATGGTTACTCCTTC tct acc gtt tta g
GFPF_glnAP	gaa gga gta acc ATGAGTAAAGGAGAAGAACTTTTCAC
GFPR_glnAT	CTAGCCCCTTTCACTTGTATAGTTC atc cat gcc atg
glnATF_gfp	gaa cta tac aag TGAAAGGGGCTAGGGAC
glnATR_*Eco*RI	CATGAATTCATAATTTAGAGGTTGATGGTTGG
nptF	CAGGGGCGCCCGGTTCTTTT
nptR	TCGCCGCCAAGCTCTTCAGC
qglnAF	CTGCAAGATATGCGGACAGA
qglnAR	AAGCCCAATTCACACTGACC

Restriction sites are underlined and sequences for overhang PCR are shown in lower case

methylation plasmid pRL623 (Cm^R), provided by Peter Wolk [10], was employed as helper and to protect foreign DNA from the cyanobacterial restriction modification (RM) system. Plasmid pRL153-GFP (Km^R), a RSF1010 derivative was provided by Wolfgang Hess [43].

The $_{p}$*glnA-gfp* fusion was performed by overhang PCR using as template DNA plasmids pGREG576 (*gfp*) [16], and *C. fritschii* PCC 6912 genomic DNA with the primers depicted in Table 1. The 280 bp region downstream of *C. fritschii* PCC 6912 *glnA*, containing a predicted terminator sequence, was included at the end of the *gfp* sequence. The fusion $_{p}$*glnA-gfp*-terminator fragment was cloned into pJET (Fermentas, Germany), exscinded with *Bam*HI and *Eco*RI and cloned into pRL25C. A fragment containing the *trc* promoter fused to the *gfp-mut3.1* gene was exscinded from pRL153-GFP [43] with *Nhe*I and cloned into the unique *Nhe*I site of pRL25C.

Triparental Matings

Conjugal transfer of plasmid DNA was performed by triparental matings between *E. coli* HB101 carrying the conjugative plasmid pRL443, *E. coli* HB101 carrying the cargo plasmid pRL25C or carrying pRL25C and the helper plasmid pRL623 with the recipient *Chlorogloeopsis* or *Fischerella*. Pre-methylation was conferred by helper plasmid pRL623, who besides mobilizing plasmids carrying the oriT of pMB1, such as pRL25C, encodes for the methylases M.*Ava*I, M.*Eco*47II, and M.*Eco*T22I [10]. To disrupt the polysaccharide layers in the cyanobacteria, as well as to increase the contact surface of donor–recipient, 50 ml of cyanobacterial cultures were subject to 3× 2–5 min rounds of sonication in a water bath. After filament disruption, cells were returned to the growth chamber and left for 24 h to recover. The day of conjugation cells were incubated for 1 h with NaCl 0.5–2 M with agitation (150 rpm), followed by four washes with BG11. After the last wash, cells were concentrated by centrifugation to a chlorophyll concentration of 150–200 μg ml^{-1} and left for 4–6 h to recover under light. Triparental matings were performed as described in Elhai and Wolk [9] with some modifications. Cargo and helper *E. coli* HB101 strains were mixed in equal proportions at the moment of mating and concentrated to OD_{600} of 9–10. 1 ml of the *E. coli* suspension was mixed with 2 ml of cyanobacteria and incubated 1.5 h under light. After incubation, 500 μl of cells were plated on nitrocellulose filters (Hybond-C extra, Amersham Biosciences, Germany) on 1 % agarose A&A plates supplemented with 2.5 mM NH_4Cl and 0.5 % LB for 24 h under dim light at 37 °C, transferred to A&A supplemented with 2.5 mM NH_4Cl plates for another 24 h. After 72 h of recovery, filters were transferred to A&A antibiotic plates.

Electroporation

Electroporation was carried out with cultures previously sonicated and concentrated to total chlorophyll concentration of 150–200 μg ml^{-1}. 10 μg of plasmid DNA was placed in a 0.1-mm gap electroporation cuvette and pulsed once in a Gene Pulser Controller (BioRad, Germany). For all electro-transferences, the capacitor was kept at 25 μF and the time constant varied by changing the resistors. Field strengths and resistor values are shown in Table 2. After electroporation cells were recovered in 3 ml A&A/4 supplemented with 2.5 mM NH_4Cl and 5 mM MOPS for 24 h. Antibiotics were added after 24 h recovery to the liquid medium; transformants, observed as green filaments in a dead cell background, were seen after 10–15 days. Cells were then collected by centrifugation and transferred to A&A/NO_3 selective plates. Single colonies were picked.

DNA Bombardment

For DNA bombardment, 1 ml of concentrated cells prepared as described above was laid on a sterile nitrocellulose filter (SCWP, Millipore, Germany). Biolistic transfer of plasmid DNA was performed with the PDS-1000 He

Table 2 Electro-transformation parameters

Field strength (kV cm^{-1})	Resistance (Ω)	Time constant (ms)
0.6	200	5
0.9	200	5
1.3	200	5
1.3	600	15
1.6	600	15
1.9	600	15

Capacitance was set at 25 μF

System (BioRad, Germany). Bombardment parameters were as recommended by the manufacturer with the exception that 5–10 μg of vector DNA was coated in 0.6 μm gold particles instead of tungsten. DNA coating was performed following the protocol described in Burns and Cassidy-Hanley [6]. After bombardment, cells were resuspended in 5 ml BG11 without antibiotics and left to recover for 24 h. Antibiotic selection was on liquid media until transformants appeared (10–15 days). Transformants were then transferred to A&A/NO_3 selective plates and single colonies picked.

Plasmid Isolation from *Chlorogloeopsis* and *Fischerella*

Plasmid DNA was isolated from 50 ml of culture collected by centrifugation, frozen in liquid nitrogen and grinded with mortar and pestle. Ground cells were resuspended in resuspension buffer (Fermentas, Germany) with 2 mg ml^{-1} lysozyme, cell lysates were incubated at 37 °C for 30 min. Plasmids were isolated with the GeneJET Plasmid Miniprep Kit (Fermentas, Germany). Plasmid DNA was visualized in agarose gels and retransformed into *E. coli*. The presence of plasmid DNA was confirmed by PCR of the *npt* (neomycin/kanamycin resistance) gene (Table 1).

RNA Isolation and qPCR

Samples for qPCR experiments were harvested 3, 8, and 24 h after nitrogen depletion by filtering (8 μm pore diameter, SCWP, Millipore, Germany), rapidly frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using Concert Plant RNA Reagent (Invitrogen, Germany) according to the manufacturer's instructions and treated with DNase I 2 U μg^{-1} RNA (DNA-*free*, Ambion, Germany). One microgram of total RNA was used for single-strand cDNA synthesis with iScript (BioRad, Germany).

Quantitative RT-PCRs were performed with primers on Table 1 in the StepOne Real-Time PCR System with Power SYBR Green PCR master mix (Applied Biosystems, Germany) and 50 ng template cDNA. All samples were run in technical triplicates. Transcript levels of the target gene were normalized to the respective transcript level of *rnpB* and the final relative levels of *glnA* mRNA were calculated by the $\Delta\Delta Ct$ method.

Protein Extraction and Immunoblots

Protein extracts were obtained from 20 to 50 ml of culture centrifuged 10 min 3,250×*g* at 4 °C. Cell pellets were resuspended in 0.1 v/v culture of lysis buffer (50 mM HEPES, 0.2 M NaCl, 10 mM $MgCl_2$, 5 % glycerin, 1 % Triton X 100 and 1 mM DTT) and lysed with ultrasound 3 × 30 s with 30 s intervals on ice. Cell debris was removed by centrifugation 10 min 16,000×*g* at 4 °C. Protein concentration was measured with Bradford reagent (BioRad, Germany). 15–30 μg protein was loaded on a 12 % SDS gel, transferred to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, Germany) and western blots were performed with the primary rabbit anti-GFP antibody (ab290, Abcam, UK) at a 1:5,000 dilution. The secondary antibody (Anti rabbit IgG peroxidase conjugated, Thermo Scientific, Germany) was used at a 1:10,000 dilution. Detection was performed using the Supersignal West pico chemiluminescent kit (Thermo Scientific, Germany), and recorded with a radiographic film.

Confocal Microscopy

Live cultures of *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 were visualized with a Plan-Apochromat 63x/1.40 Oil DIC M27 objective in a LSM-710 confocal laser scanning microscope (Carl Zeiss, Germany). GFP was exited with an argon ion laser at 488 nm and fluorescent emission narrowed to a 500–530 nm window. Cyanobacterial autofluorescence was recorded at 630–700 nm.

Results

Transfer of pRL25C to Subsection V Strains

The exopolysaccharide layer of *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 can be disrupted by sonication. Two rounds of 2–5 min sonication in a water bath disrupted *Chlorogloeopsis* cell clumps into four-celled units and *Fischerella* layers to small sheath sections 40,000–160,000 μm^2 in size. Sonication was also applied before electroporation and conjugation to increase the accessible cell surface for DNA uptake or contact with the *E. coli* donor strain.

Reasoning that thick exopolysaccharide layers would impede gene transfer to *Fischerella* and *Chlorogloeopsis*

Table 3 Frequencies of conjugation and transformation success in *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912

	Frequencies %	
	F. muscicola PCC 7414	*C. fritschii* PCC 6912
Conjugation		
pRL25C (pRL443)	0 (0/8)	0 (0/7)
pRL25C (pRL443, pRL623)	0 (0/2)	0 (0/2)
pRL25C (pRL443, pRL623), NaCl	83 (5/6)	67 (2/3)
Electroporation		
pRL25C	0 (0/2)	0 (0/5)
pRL25C (pRL623)	18 (2/11)	0 (0/5)
DNA bombardment		
pRL25C	0 (0/4)	7 (1/14)
pRL25C (pRL623)	67 (2/3)	0 (0/2)

Frequencies represent the ratio of successful experiments (seen as appearance of resistant cyanobacterial colonies/patches in the selection plates) from the total of performed experiments

strains, we incorporated a simple wash and centrifugation step (called salt treatment hereafter) using various NaCl concentrations to reduce the exopolysaccharides (see "Materials and Methods"), 1 M NaCl gave the highest frequency of success, seen as proportion of successful conjugation events (growth on the selection plates). Conjugal transfer of plasmid DNA was performed by triparental matings with conjugal plasmid pRL443 and with or without helper plasmid pRL623. Ex-conjugants (green colonies/patches on the selection plates) were observed for both *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 only when conjugal transfer was performed with helper plasmid pRL623 and after salt treatment (Table 3). Since *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 did not grow in the presence of Nm above 10 μg ml^{-1} (not shown), selection was with Nm 30. Ex-conjugants appeared on the antibiotic plates after 10–14 days of selection. Due to the growth style of *Fischerella* (filament networks) and *Chlorogloeopsis* (cell aggregates), quantification of ex-conjugants is not always possible. Furthermore *Fischerella* and *Chlorogloeopsis* produce hormogonia, which can spread on plates. In *F. muscicola* PCC 7414, these glide rapidly and might affect transformation efficiency estimates. In *Chlorogloeopsis* hormogonia do not disperse on the plates, therefore aggregations might be taken as a single transformant. We expressed results based on frequency of obtaining resistant colonies (Table 3). Experiments were repeated numerous times.

Electro-transformation of *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 with pRL25C was tested under several varied parameters, including field strength and resistance (Table 2). These parameters were selected based on their success in cyanobacteria harboring thick exopolysaccharide layers, as *Chroococcidiopsis* spp. and *Nostoc punctiforme* ATCC 29133 [3, 39]. *F. muscicola* PCC 7414 transformants were observed in Nm selection medium only when a field strength of 1.6 kV cm^{-1}, resistance of 600 Ω and time constant of 15 ms was applied (Table 3). Success of electroporation depended on pre-methylation of plasmid DNA by pRL623. Independent of the parameters set, or pre-methylation of plasmid DNA, no transformants were observed in *C. fritschii* PCC 6912.

For DNA bombardment, we used small gold particles (0.6 μm) for DNA coating, considering that mature vegetative cell diameters of *Fischerella* and *Chlorogloeopsis* may extend to 8 μm (Fig. 1). DNA bombardment success, like electroporation, was dependent on pre-methylation of pRL25C for *F. muscicola* PCC 7414 but not *C. fritschii* PCC 6912. However, the reproducibility of this method was low, and modifying DNA concentration and vacuum pressure, as well as distance to the launch assembly did not yield transformants (not shown). The presence of pRL25C in transformants *C. fritschii* PCC 6912 was confirmed by isolation of plasmid DNA, transformation of *E. coli* and amplification of the neomycin transferase gene (Fig. 2). Confirmation of *F. muscicola* PCC 7414 transformants and ex-conjugants was confirmed by western blots as described in the section below.

Expression of the Reporter *gfp* Directed by a Cyanobacterial and *E. coli* Promoter

In order to establish a reporter system for cyanobacteria from subsection V, we explored the expression of GFP driven by the cyanobacterial *glnA* and *E. coli trc* promoters. The glutamine synthetase (GS) is a key enzyme in nitrogen metabolism, which catalyzes the synthesis of glutamine from ammonia and glutamate. In order to examine the regulatory pattern of *glnA* in *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912, *glnA* expression was quantified by RT-qPCR at 3, 8, and 24 h after N-stepdown, from cultures adapted to grow on nitrate. During this time frame heterocysts are developed and diazotrophic growth is established.

The activity of the *glnA* promoter was assessed in untransformed cells. The increase of *glnA* transcript levels was evident in both strains after 3 h of N-stepdown but

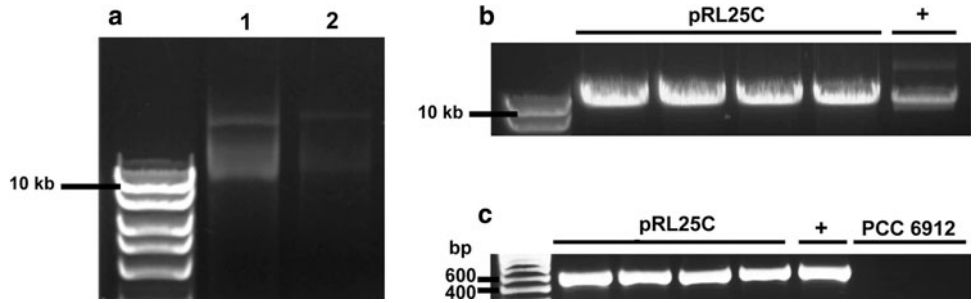

Fig. 2 Confirmation of plasmid DNA in transformants obtained by bombardment. **a** Plasmid DNA was isolated from two *C. fritschii* PCC 6912 transformants carrying pRL25C. **b** Screening of four *E. coli* clones retransformed with *C. fritschii* PCC 6912 pRL25C, showed the expected size of *Eco*RI linearized pRL25C (10.14 kb). **c** Amplification of a gene fragment from the neomycin transferase (581 bp) of pRL25C isolated from *E. coli* confirmed the presence of the plasmids in *E. coli* retransformed clones. Positive controls (+) represent pRL25C from *E. coli* ED8654 (source strain); *C. fritschii* PCC 6912 genomic DNA was used as negative control

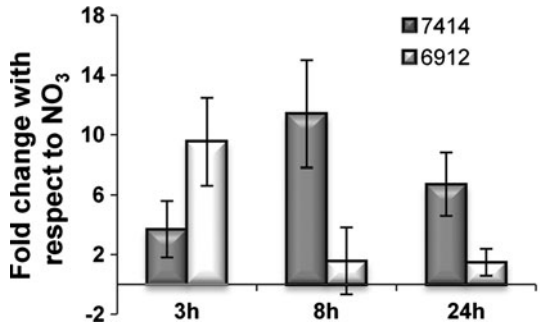

Fig. 3 Relative quantification of *glnA* transcripts in cultures subject to nitrogen step-down. *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 cultures grown under combined nitrogen (NO_3) were washed and resuspended in N-free medium. *glnA* expression was detected at 0, 3, 8, and 24 h after N-depletion by qRT-PCR. *Ct* values were normalized against *rnpB*. *Bars* represent $\Delta\Delta Ct$ of tx versus 0 h (NO_3). Results are expressed as fold changes with respect to the internal control. Values for all measurements are represented as the mean ± standard deviation (SD)

continued to increase in *F. muscicola* PCC 7414 until 8 h to a maximum of 12-fold and remained at least fivefold induced after 24 h. In *C. fritschii* PCC 6912, transcript levels returned to basal levels after 8 h of N-deprivation and remained low (Fig. 3).

Constructs of $_p$*glnA-gfp* (promoter derived from *C. fritschii* PCC 6912) were transferred to *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 by triparental matings with helper plasmid pRL623 and after salt treatment as described above. The *C. fritschii* PCC 6912 *glnA* promoter modulated the expression of GFP in *F. muscicola* and *C. fritschii* under nitrate and diazotrophic growth. Under combined nitrogen, fluorescence was observed in vegetative cells and mature heterocysts were not visible (Fig. 4). After 24 h and in cultures adapted to diazotrophic growth GFP fluorescence was still located in vegetative cells but also detected in heterocysts (Fig. 4). Immunodetection of GFP confirmed the expression of GFP in nitrate- and N_2-fixing cultures.

The synthetic constitutive *E. coli* promoter $_p$*trc* fused to the *gfpmut3.1* gene ($_p$*trc-gfp*) construct was replicated in HB101 carrying pRL623. Premethylated plasmid DNA was transferred to *F. muscicola* PCC 7414 by electroporation. GFP fluorescence was observed in *F. muscicola* PCC 7414 along the complete filament (Fig. 5) and GFP expression were confirmed by western blots. The signal intensity surpassed that driven by the *glnA* promoter.

Discussion

We show here that *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 can incorporate exogenous DNA by transformation and conjugation as shown by Nm resistance, plasmid re-isolation and western blots for expressed foreign gene products. We anticipated problems stemming from the exopolysaccharide layers of both strains (Fig. 1). In *F. muscicola* PCC 7414, the sheath is composed of neutral sugars and lipopolysaccharides and minor amount of amino acids, sulfate, phosphate, and fatty acids [30]; in *C. fritschii* PCC 6912 is composed of two types of polysaccharides, one apparently covalently linked to proteins, but no lipopolysaccharides [35]. In *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 success of conjugal transfer of pRL25C, and constructs based on it, was dependent upon salt treatment (wash with 1 M NaCl), we assume that the effect of the NaCl wash is exopolysaccharide removal. Concentrated NaCl is commonly used in DNA isolation protocols in combination with cationic surfactants such as CTAB, where it aids in the removal of polysaccharides, although NaCl alone can remove polysaccharides from DNA contaminated samples [11]. This pre-treatment with NaCl might increase the efficiency of transformation of other species with thick exopolysaccharide layers for example, isolated from cyanobacterial mats.

The success of electroporation and DNA bombarding on *F. muscicola* PCC 7414 depended pre-methylation of

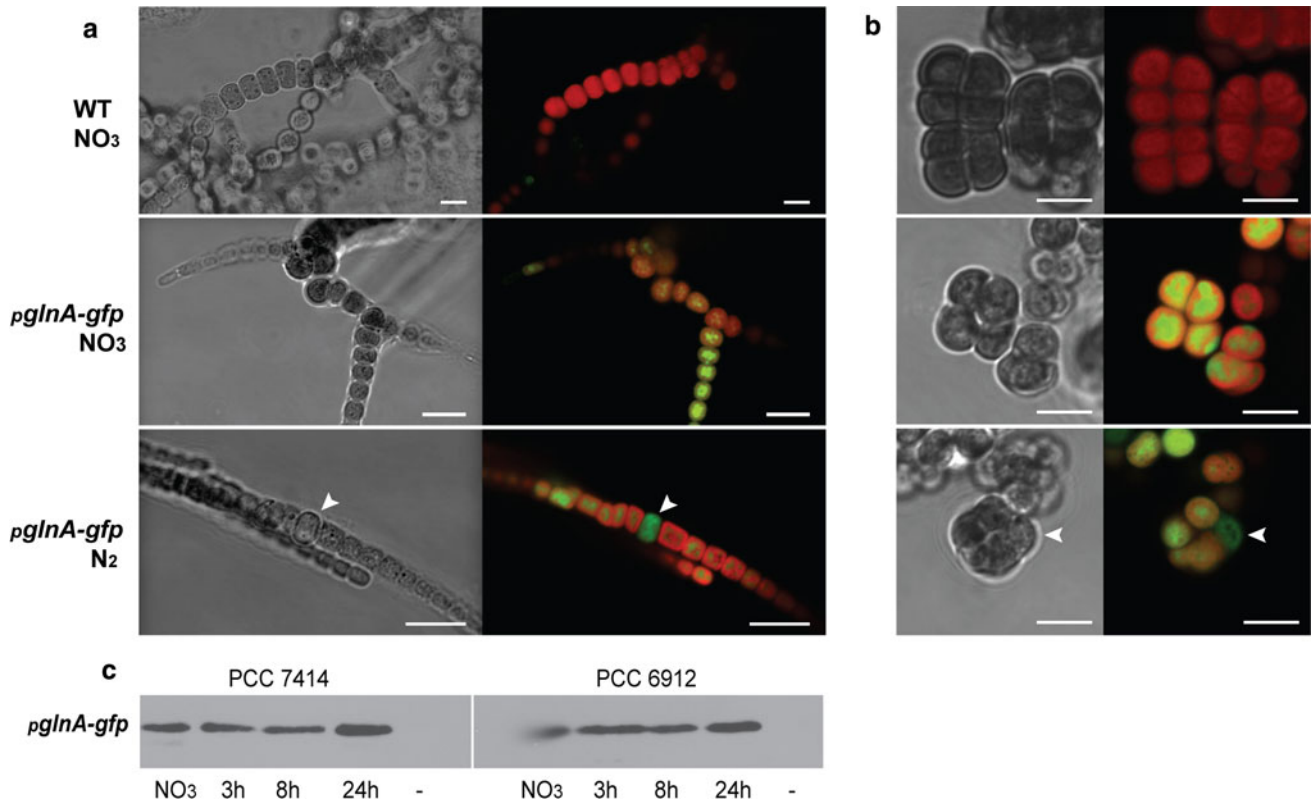

Fig. 4 Ectopic expression of GFP from the *glnA* promoter. Brigthfield images (*left*) and merged GFP and chlorophyll fluorescence (*right*) of **a** *F. muscicola* PCC 7414 and **b** *C. fristchii* PCC 6912 wild type (WT-NO_3), expressing $_p$*glnA-gfp* under nitrate (GFP-NO_3) and diazotrophic growth (GFP-N_2). *White arrows* indicate heterocysts. *Scale bars* represent 10 µm. **c** Cultures grown under combined nitrogen (NO_3) were washed and resuspended in N-free medium. GFP expression was detected after 3, 8 and 24 h of N-depletion by immunoblotting with an anti-GFP antiserum

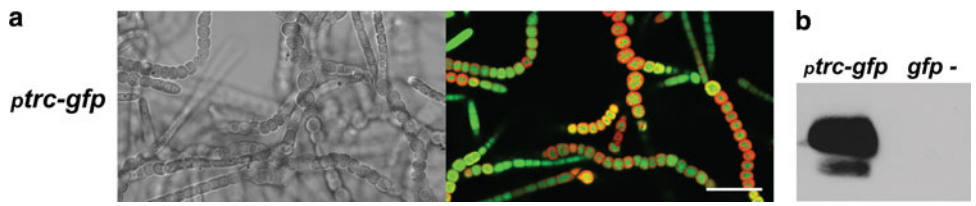

Fig. 5 GFP expression driven by the strong *E. coli* promoter $_p$*trc*. **a** Brigthfield image (*left*) and merged GFP and chlorophyll fluorescence (*right*) of *F. muscicola* PCC 7414 expressing GFP from the *trc* promoter. **b** Immunodetection of GFP in *F. muscicola* PCC 7414 expressing $_p$*trc-gfp* and wild type (*gfp*)

plasmid DNA. Helper plasmid pRL623 encodes for three methylases, M.*Ava*I, M.*Eco*47II, and M.*Eco*T22I, which protect against restriction of *Ava*I, *Ava*II, and *Ava*III, respectively [10]. Activity of these enzymes was not detected in *F. muscicola* PCC 7414 extracts [20]; endonuclease activity of the type *Asu*II (TT/CGAA) was reported instead. Information from the draft genome sequence of *F. muscicola* PCC 7414 (Dagan, unpublished data) revealed the presence of known endonucleases of the type *Fau*I ($CCCGCN_4$), *Pst*I (CTGCAG), and *Sph*I (GCATGC), all recognizing high GC content regions, whereas no enzyme similar to *Ava*I, *Ava*II, *Ava*III, *Asu*II or its isoschizomers was found (not shown). The activity of *F. muscicola* PCC 7414 might be encoded by a novel restriction endonuclease, which requires further purification and characterization assays.

Conjugation is the currently preferred method for gene transfer in filamentous cyanobacteria and is the only record of genetic transformation that exists for a strain of *Fischerella* [13]. Among the three methods tested, conjugation was the most reproducible in both *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912, whereas transformation by electroporation and biolistics was not highly reproducible. The reason why some cyanobacterial strains can be successfully transformed while other strains from the same species may not is normally attributed to the presence of restriction

endonucleases. However, this correlation is not always obvious when DNA can be transferred to a strain by conjugation but not electroporation as in *C. fritschii* PCC 6912. The reasons for this remain unclear, but it is a common observation among cyanobacteria [3, 22], prompting the investigation of gene transfer methods for a given strain.

Relative to conjugation, DNA bombardment has the advantages that it does not require disruption of cell aggregates and selection does not require the elimination of contaminating *E. coli*. However, reproducibility was low in both strains here, even after modifying several parameters. The pDU1 replicon from *Nostoc* contained in cosmid pRL25C has been shown to replicate in a broad range of cyanobacteria besides Nostocales [3, 32], our findings show that it was replicated in *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 as well, and that it could be isolated from *C. fritschii* PCC 6912 transformants (from bombardment) by simple miniprep preparation (Fig. 2), simplifying the screening process.

Expression of GFP tags has been shown in unicellular cyanobacteria [19, 43] and targeted to specialized cells of *Anabaena* and *Nostoc* [2, 46]. Here we show that they can be targeted to vegetative cells, hormogonia and heterocysts in *Fischerella* and *Chlorogloeopsis* as well. Promoter choice in cyanobacteria is not well established although several cyanobacterial and *E. coli* promoters have been utilized for heterologous expression in cyanobacteria [18, 34]. In the heterocystous cyanobacterium *Anabaena* sp. PCC 7120, the GS gene (*glnA*) is transcribed by at least three different promoters, two of which direct expression in response to nitrogen availability. mRNA levels from RNA_I (transcribed from the transcriptional start I) increase under nitrate and nitrogen deprivation [45, 47]. As in *Anabaena*, the *glnA* promoter modulated the expression of GFP in *C. fritschii* PCC 6912 and *F. muscicola* PCC 7414, at levels high enough to detect GFP fluorescence under nitrate and nitrogen deprivation, suggesting regulation of the *C. fritschii* PCC 6912 *glnA* promoter used. The *glnA* promoter was active in vegetative cells and heterocysts in both *F. muscicola* PCC 7414 and *C. fritschii* PCC 6912 (Fig. 4).

The hybrid *E. coli trc* promoter has been shown to promote constitutive GFP expression in the unicellular *Synechococcus* [24, 25], marine *Prochlorococcus* [43] and to be used in filamentous cyanobacteria [28, 40]. In *F. muscicola* PCC 7414, fluorescence was observed homogeneously along the filament. The *trc* promoter, if cloned together with the *Lac*I repressor can, in principle, lead to inducible gene expression, however, repression by *Lac*I was not detected in the cyanobacterium *Leptolyngbya* in a plasmid modified for the expression of *yemgfp* from the *trc* promoter [40]. The availability of gene transfer systems for subsection V cyanobacteria should aid the investigation of these morphologically complex prokaryotes.

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