Inducible expression of heterologous genes targeted to a chromosomal platform in the cyanobacterium *Synechococcus* sp. PCC 7942

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High-level, inducible expression of heterologous genes in the cyanobacterium *Synechococcus* sp. strain PCC 7942 was obtained using the *Escherichia coli* *trc* promoter and *lacI* repressor. The *petE* gene of *Anabaena* sp. strain PCC 7937 encoding plastocyanin precursor protein and the *E. coli* *uidA* gene encoding β -glucuronidase were initially placed under the control of the *trc* promoter and *lacI* repressor by cloning into the *E. coli* pTrc99C expression vector and were introduced into the chromosomal platform for integration in *metF* (PIM) of the *Synechococcus* R2-PIM9 recipient strain. These pTrc99C-derived constructs often gave rise to transformants that did not contain a complete insert gene, probably because of gene conversion events. Selection of the desired *Synechococcus* R2-PIM9 transformants was vastly improved using the new pTrcIS vector that contains the *aadA* gene encoding streptomycin resistance as an extra antibiotic resistance marker. The influence of IPTG concentration and induction time on gene expression with the *E. coli* *trc/lacI* system in *Synechococcus* was determined using β -glucuronidase as a reporter. The *Anabaena* PCC 7937 *petE* gene in *Synechococcus* was expressed to a high level upon induction with IPTG as shown by RNA and immunoblot analysis. The general usability of pTrcIS as a cloning vector for inducible heterologous gene expression in *Synechococcus* was confirmed by the introduction of several more genes.

Keywords: *Synechococcus* sp. PCC 7942, cyanobacterium, heterologous gene expression, PIM, *trc* promoter/*lacI* repressor gene

INTRODUCTION

The cyanobacteria represent an ancient and diverse group of prokaryotes possessing a number of unique characteristics (Stanier & Cohen-Bazire, 1977). Evolutionarily linked to chloroplasts (Gray, 1989), they are the simplest organisms capable of performing oxygenic photosynthesis, with a thylakoid apparatus remarkably similar to that of higher plant chloroplasts.

Cyanobacteria provide a simple but powerful experimental system for the analysis of the complex photo-

synthesis process because of the possibility of combining biophysical and biochemical procedures with genetic manipulation techniques (Bryant, 1992; Vermaas, 1993). Several unicellular cyanobacterial species have a natural capability to take up heterologous DNA (Porter, 1986). Other cyanobacterial strains can be transformed using conjugation with *Escherichia coli* (Wolk *et al.*, 1984) or electroporation (Thiel & Poo, 1989), but until recently (Cai & Wolk, 1990; Kuritz *et al.*, 1993) techniques for gene transfer could only be exploited fully in the naturally transformable strains. These strains all belong to the genera *Synechococcus* or *Synechocystis* (Rippka *et al.*, 1979).

To study protein transport in cyanobacteria, in the work described here we introduced the genes encoding several precursor proteins from different cellular compartments into a cyanobacterial host. The unicellular strain *Synechococcus* sp. strain PCC 7942 was chosen as the host, because it is both physiologically and genetically well-

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Abbreviations: GUS, β -glucuronidase (EC 3.2.1.31); MCS, multiple cloning site; MU, 4-methylumbelliferone; MUG, 4-methylumbelliferone- β -D-glucuronide; PC, plastocyanin; PCC, Pasteur Culture Collection; PIM, platform for integration in the *metF* locus; RBS, ribosome-binding site.

characterized and highly transformable (Porter, 1986), either with vectors capable of autonomous replication (reviewed by Houmard & Tandeau de Marsac, 1988) or by integration of heterologous DNA into the chromosome through homologous recombination. Using the latter approach, the major type of transformant normally results from 'gene replacement', an event in which a sequence in the recipient chromosome is replaced by a sequence from the donor DNA molecule (van der Plas *et al.*, 1990; Williams & Szalay, 1983).

The PIM (platform for integration in the *metF* locus) system of *Synechococcus* PCC 7942 has been described previously (van der Plas *et al.*, 1990). In the *Synechococcus* R2-PIM9 recipient strain used in this study, the chromosomal *metF* locus is interrupted by an integration platform, consisting of a promoterless *E. coli bla* gene (encoding Ap^R) and the pMB1 *oriV*, both from pBR322, separated by the *neo* gene (encoding Km^R). The platform enables integration of many pBR322-derived donor plasmids by homologous recombination at the *bla* and pMB1 *oriV* sequences that are present in both the donor plasmid and the integration platform, independent of the insert DNA on the plasmid. The *metF* locus, normally involved in methionine biosynthesis, is a neutral site: it can be inactivated (e.g. by integration of heterologous DNA) without affecting the viability of the cells when the strain is grown on medium supplemented with methionine (van der Plas *et al.*, 1990). By using a chromosomal expression system rather than a plasmid-bound one, copy-number effects (Scanlan *et al.*, 1990; Elhai, 1993) were prevented, allowing careful comparisons of the different transgenic *Synechococcus* strains.

A system for inducible gene expression avoids possible harmful effects on the cyanobacterial host cell by constitutive expression of a heterologous gene, and also prevents selection for gene inactivation by host insertion sequences (Cai & Wolk, 1990). We describe here the use of the *E. coli trc* promoter to obtain inducible, high-level expression of heterologous genes in *Synechococcus* PCC 7942 using control by the *E. coli* Lac repressor. This system was chosen because: (i) in *E. coli* the *trc* promoter is one of the strongest and best studied promoters (Brosius, 1987) and is tightly regulated by the Lac repressor (Amann *et al.*, 1988); (ii) the *trc* promoter-*lacI* repressor combination has been successfully used in a wide range of both Gram-negative (Labes *et al.*, 1990) and Gram-positive bacteria (Yansura & Henner, 1984); (iii) *E. coli* consensus promoters like *trc* are efficient in cyanobacteria (Ferino & Chauvat, 1989; Elhai, 1993); and (iv) the *trc* promoter can be induced by the addition of IPTG, which ought not to influence the metabolism of the cells.

Other inducible expression systems that have been described for use in cyanobacteria include the heat-shock-induced bacteriophage λ P_LP_R promoter-cl857^{ts} repressor system in *Synechococcus* PCC 7942 and PCC 6301 (Friedberg & Seijffers, 1986; Mermet-Bouvier & Chauvat, 1994), and the light-responsive *Synechocystis* PCC 6803 LR1 promoter in *Synechococcus* PCC 7942 and PCC 6301 (Marraccini *et al.*, 1993). However, heat shock leads to

drastic changes in protein synthesis and content (Borbély *et al.*, 1985), and in the composition of intracellular membranes (Wada & Murata, 1990), and readily affects photosynthetic activity by inactivation of photosystem II (Berry & Bjorkman, 1980). Light also has many effects on gene expression and physiology in cyanobacteria (Tandeau de Marsac & Houmard, 1993). Since we study cellular processes such as protein transport and photosynthesis, we wanted to avoid alteration of endogenous gene expression caused by the use of normal cellular activators such as light and temperature. The previously described systems therefore did not meet the criteria stated above.

METHODS

Organisms and culture conditions. The organisms used in this study are described in Table 1. *E. coli* strains were grown as described by Sambrook *et al.* (1989), and cyanobacterial strains were grown as described by Geerts *et al.* (1994). *Synechococcus* R2-PIM9 transformation and transformant selection were performed according to the method of van der Plas *et al.* (1990). Recombinant *Synechococcus* R2-PIM9 strains containing construct pX are called *Synechococcus* R2-X.

Materials. Enzymes and oligonucleotides used for DNA cloning and sequencing procedures were purchased from Pharmacia. Radiolabelled nucleotides and Hybond-N membranes were from Amersham. Nitrocellulose filters and TLC plates were from Schleicher & Schuell. Chemicals needed to prepare BG11 medium were from Merck. Plant agar was from Duchefa. BSA, Tween 20 and Triton X-100 were from Sigma. Equipment and chemicals for SDS-PAGE experiments were from Bio-Rad. Immobilon PVDF membrane was from Millipore. The Western Blot AP system was from Promega. 4-Methylumbelliferone (MU) fluorescence was measured with the Hoefer TKO 100. All other chemicals were from Boehringer.

DNA cloning, sequencing procedures and plasmid construction. The plasmids used in this study are listed in Table 1. Oligo site-directed mutagenesis was performed using *E. coli* HB2154 according to the method of Carter *et al.* (1985). DNA sequence analysis was performed using the T7 DNA sequencing kit (Pharmacia) according to the manufacturer's recommendations. Other DNA manipulations were as described previously (van der Plas *et al.*, 1989; Geerts *et al.*, 1994). All plasmid constructs were checked by DNA restriction and sequence analysis.

Construction of plasmids

pTrcIS. pTrcIS (6250 bp, see Fig. 4a) is similar to pTrc99C but contains Sm^R as an additional marker. It was constructed from pTrc97C, which contains a *Bgl*II site in place of the *lacI*^a gene in pTrc99C. pTrcIS was assembled by ligation of a 2059 bp *Eco*RI fragment accommodating the *aadA* gene (encoding Sm^R) from pUC7S together with a 1247 bp *Eco*RI fragment containing the *lacI*^a gene from pPAFI²⁰ into the *Bgl*II site of pTrc97C (2944 bp) after blunting of all three fragments with Klenow DNA polymerase. Transformants were selected for an Ap^R Sm^R phenotype by plating, and subsequently for the presence of the *lacI* gene by colony blot hybridization (Sambrook *et al.*, 1989) using a specific DNA probe (see below). The orientation of the *aadA* and *lacI*^a genes is antiparallel to that of the *bla* gene. The sequence of pTrcIS is: bp 1–2938, pTrc97C (bp 1–2938); bp 2939–2950, AATTCAGCTTGC (filled-in *Eco*RI site; *Eco*RI–*Hind*III linker); bp 2951–4172, *lacI*^a gene (pTrc99C, bp 4170–2939); bp 4173–4189, GCAAGCTTGAATTAATT (*Eco*RI–*Hind*III linker; filled-in *Eco*RI site; filled-in *Eco*RI site);

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source/reference
Strain		
<i>E. coli</i> PC2495	<i>hsdS recA</i> (JM101 derivative)	van der Plas <i>et al.</i> (1988)
<i>E. coli</i> HB2154	<i>mutL</i>	Carter <i>et al.</i> (1985)
<i>Anabaena</i> sp. PCC 7937		PCC
<i>Synechococcus</i> PCC 7942-R2-PIM9	Recipient strain containing PIM platform Ap ^R Km ^R Sm ^S Met ⁻	van der Plas <i>et al.</i> (1990)
Plasmid		
pACYC184	<i>cat</i> gene, Cm ^R , 4.2 kb	Rose (1988)
pEMBL18	Ap ^R , 4.0 kb	Dente & Cortese (1987)
pEMBL19	Ap ^R , 4.0 kb	Dente & Cortese (1987)
pPAFI ⁹ 20	<i>lacI^q</i> gene, Ap ^R , 5.2 kb	Pilon <i>et al.</i> (1990)
pPCV6	<i>Anabaena</i> PCC 7937 <i>petE</i> gene, Ap ^R , 6.4 kb	van der Plas <i>et al.</i> (1989)
pRA81	<i>Synechococcus</i> PCC 7942 <i>petF</i> gene, Ap ^R , 5.1 kb	van der Plas <i>et al.</i> (1990)
pRAJ275	<i>E. coli uidA</i> gene, Ap ^R , 4.6 kb	Sleat <i>et al.</i> (1987)
pTrc97C	pMB1 <i>oriV</i> , <i>P_{trc}</i> , Ap ^R , 2.9 kb	Amann <i>et al.</i> (1988)
pTrc99C	pMB1 <i>oriV</i> , <i>P_{trc}</i> , <i>lacI^q</i> gene, Ap ^R , 4.2 kb	Amann <i>et al.</i> (1988)
pUC7S	<i>aadA</i> gene, Ap ^R , Sm ^R , 3.9 kb	van der Plas <i>et al.</i> (1990)
pTrcIS	pMB1 <i>oriV</i> , <i>P_{trc}</i> , <i>lacI^q</i> , Ap ^R Sm ^R , 6.3 kb	This study
pMG1	Intermediate in <i>petE</i> gene construction, Ap ^R , 4.0 kb	This study
pMG2	Intermediate in <i>petE</i> gene construction, Ap ^R , 4.8 kb	This study
pMG59	Intermediate in <i>petE</i> gene reconstruction, Ap ^R , 4.6 kb	This study
pMG77A	<i>Anabaena</i> PCC 7937 <i>petE</i> gene in pTrc99C, Ap ^R , 4.8 kb	This study
pMG95A	pMG77A with interrupted <i>lacI^q</i> gene, Ap ^R Cm ^R , 6.1 kb	This study
pMG139A	<i>E. coli uidA</i> gene in pTrc99C, Ap ^R , 6.0 kb	This study
pMG140A	pMG139A with interrupted <i>lacI^q</i> gene, Ap ^R Cm ^R , 7.4 kb	This study
PMG145A	<i>petE-amyL</i> gene fusion in pTrc99C, Ap ^R , 6.0 kb	This study
pMG77B	<i>Anabaena</i> PCC 7937 <i>petE</i> gene in pTrcIS, Ap ^R Sm ^R , 6.9 kb	This study
pMG95B	pMG77B with interrupted <i>lacI^q</i> gene, Ap ^R Cm ^R Sm ^R , 8.2 kb	This study
pMG139B	<i>E. coli uidA</i> gene in pTrcIS, Ap ^R Sm ^R , 8.1 kb	This study
pMG140B	pMG139B with interrupted <i>lacI^q</i> gene, Ap ^R Cm ^R Sm ^R , 9.4 kb	This study
pMG145B	<i>petE-amyL</i> gene fusion in pTrcIS, Ap ^R Sm ^R , 8.0 kb	This study

Fig. 1. Mutagenesis and reconstruction of the *Anabaena* PCC 7937 *petE* gene. Creation of a *Bsp*HI site spanning the ATG start codon of the *Anabaena petE* gene by use of adaptor 87–95/96. For details see Methods. Capital letters indicate the coding sequence of the *petE* gene. Overlined sequences indicate restriction sites used. Position and direction of the *trc* promoter in pTrc99C are indicated by an arrow. The *lacZ* RBS is underlined.

bp 4190–6240, *aadA* gene [Prentki *et al.* (1991), bp 2067–16]; bp 6241–6250, AATTGATCTG (filled-in *Eco*RI site; filled-in *Bgl*II site). Because of sites present in the *lacI^q* and *aadA* genes, the *Pst*I, *Sph*I and *Hind*III sites in the multiple cloning site (MCS) are not unique. Unique sites in the MCS are *Nco*I, *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I and *Sal*I.

pMG77/pMG95. A 760 bp *Sau*3AI–*Pst*I fragment containing the entire *Anabaena* PCC 7937 *petE* gene from pPCV6 was ligated into pEMBL19 cut with *Bam*HI and *Pst*I, resulting in pMG59. The *Sau*3AI site is 104 bp upstream of the *petE* gene, and the *Pst*I site downstream from the *petE* gene is from the MCS of

pPCV6. To allow insertion of the *petE* gene into the *Nco*I site of pTrc99C or pTrcIS so that a translational fusion was established, a *Bsp*HI site spanning the ATG start codon of *petE* gene was created. *Bsp*HI is compatible with *Nco*I and the creation of this site does not result in changes in the nucleotide sequence of the *petE* gene coding region. The *Bsp*HI site was made using adaptor 87–95/96 consisting of 22 bp of the *petE* gene (from –4 to the *Hind*III site at +18), flanked by *Eco*RI and *Hind*III sites and with a *Bsp*HI site spanning the ATG codon (Fig. 1). The adaptor was ligated into pEMBL19 cut with *Eco*RI and *Hind*III, resulting in pMG1. The *petE* gene was reconstructed in pMG1

by ligation of a 635 bp *Hind*III fragment from pMG59 into pMG1 cut with *Hind*III, resulting in pMG2. The *petE* gene was cloned into pTrc99C or pTrcIS cut with *Nco*I and *Pst*I by ligation of the 650 bp *Bsp*HI-*Pst*I fragment from pMG2, resulting in pMG77A or pMG77B, respectively. For interruption of the *lacI* gene, a 1330 bp *Xmn*I-*Bcl*I fragment containing the *cat* gene (encoding Cm^R) from pACYC184 was blunted with Klenow DNA polymerase and ligated into pMG77A or pMG77B cut with *Pvu*II, resulting in pMG95A or pMG95B, respectively. The orientation of the *cat* gene in pMG95A and pMB95B has not been determined.

pMG139/pMG140. The *E. coli uidA* gene in pRAJ275 has an *Nco*I site spanning the ATG start codon. To make a translational fusion of the *uidA* gene, a 1855 bp *Nco*I-*Eco*RI fragment from pRAJ275, containing the *E. coli uidA* gene, was ligated into pTrc99C or pTrcIS cut with *Nco*I and *Eco*RI, resulting in pMG139A or pMG139B, respectively. The *lacI* gene in pMG139A and pMG139B was interrupted as described above, resulting in pMG140A and pMG140B, respectively. The orientation of the *cat* gene in pMG139B and pMG140B has not been determined.

pMG145. pMG86 is a construct containing the *Anabaena* PCC 7937 *petE* gene sequence encoding the PC signal peptide fused to the *Bacillus licheniformis amylL* gene sequence encoding α -amylase mature protein (D. Geerts and others, unpublished results). The *petE*-*amylL* gene fusion was cloned into pTrc99C or pTrcIS cut with *Nco*I and *Sst*I by ligation of a 1785 bp *Nco*I-*Sst*I fragment from pMG86, resulting in pMG145A or pMG145B, respectively.

***Synechococcus* colony blot hybridization.** *Synechococcus* transformants were screened for the presence of a heterologous gene by colony blot hybridization using a specific DNA probe (see below). Cells were streaked on nitrocellulose filters on top of BG11 plates as well as directly on BG11 master plates and grown for 4–7 d. Lysis of the cell streaks using SDS and NaOH was as described by Sambrook *et al.* (1989), except that the incubation with 2 $\times$ SSC was omitted. After baking, the filters were shaken at 37 °C in 2 $\times$ SSPE, 0.1% (w/v) SDS and 50 μ g ml⁻¹ proteinase K for 1 h, and in 2 $\times$ SSPE, 0.1% (w/v) SDS for 30 min to remove cell debris. The filters were pre-hybridized for 1 h at 42 °C in 50% (v/v) formamide, 5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.1% (w/v) SDS and 0.1 mg denatured salmon-sperm DNA ml⁻¹ (Sambrook *et al.*, 1989). Hybridization was initiated by the addition of denatured probe DNA to the pre-hybridization medium, followed by incubation at 42 °C for 16 h. Blots were washed twice for 5 min at room temperature and twice for 15 min at 42 °C in 1 $\times$ SSPE, 0.1% (w/v) SDS. Autoradiography was for 4–16 h at -70 °C. The *Anabaena* PCC 7937 *petE* probe used was a 400 bp *Hind*III-*Nhe*I fragment containing most of the *petE* gene coding region from pPCV6, and the *E. coli uidA* probe was a 1180 bp *Bcl*I-*Ssp*I fragment internal to the *uidA* gene from pRAJ275. Radiolabelling was performed as described by Feinberg & Vogelstein (1983).

RNA isolation and RNA:DNA hybridization. RNA isolation and RNA:DNA blot hybridization were performed as described by Bovy *et al.* (1992). RNA dot blots were prepared on Hybond-N membranes (Amersham) according to the manufacturer's instructions. The probe used for *petE* mRNA is described above, and a 360 bp *Sst*I-*Eco*RI fragment containing most of the *Synechococcus* PCC 7942 *petF* gene coding region from pRA81 was used as a probe for *petF* mRNA. A 620 bp *Bst*EII-*Nsi*I fragment internal to the *lacI* gene from pTrc99C was the probe for *lacI* mRNA. Radiolabelling was performed as described above.

Protein isolation and immunoblot analysis. Cultures of *E. coli* cells harbouring pMG constructs were grown to mid-exponential phase by 1:100 dilution of an overnight culture into 100 ml Luria-Bertani medium followed by growth at 37 °C for 2–3 h. Cultures were divided into 50 ml batches and grown for an additional 3 h in the presence or absence of 1 mM IPTG. Cells were harvested at 4 °C by centrifugation (10 min at 4000 g), washed in 10 mM Tris/HCl (pH 8.0), and resuspended in 1 ml of the same buffer. Cultures of *Anabaena* and *Synechococcus* R2-PIM9 derivatives were grown to 15 μ g chlorophyll *a* ml⁻¹ as described by Geerts *et al.* (1994). Induction and harvest were as described above, but induction was for the last 16 h of growth and harvesting was at room temperature. Protein concentration was determined as described by Geerts *et al.* (1994). Protein (50 μ g) was separated on 15% (w/v) SDS-PAGE gels as described by Laemmli (1970). Preparation and screening of immunoblots and polyclonal antiserum 8806 against *Anabaena* PCC 7937 PC were as described by Geerts *et al.* (1994). Polyclonal antiserum against *E. coli* LacI protein was a gift from Dr Ponzy Lu (Department of Chemistry, University of Pennsylvania, PA, USA).

β -Glucuronidase (GUS) assay. Cultures (10 ml) of *E. coli* cells harbouring pMG139A or pMG140A, and *Synechococcus* R2-MG139A or R2-MG140A were grown and harvested as described above, washed in 50 mM sodium phosphate buffer (pH 7.0), and resuspended in 0.25 ml phosphate buffer containing 10 mM EDTA and 10 mM β -mercaptoethanol. Cells were lysed by sonication. Sarkosyl and Triton X-100 were added to 0.1% (v/v), the suspension was mixed, incubated for 10 min on ice, and cell debris was removed by centrifugation for 10 min at 6000 g. GUS activity in the extracts was assayed using 4-methylumbelliferone- β -D-glucuronide (MUG) as a substrate according to the method of Vorst *et al.* (1990). The values obtained were converted into specific activity using a calibration curve with known amounts of MU product (relative fluorescence 10000 μ mol⁻¹).

RESULTS

Plasmid construction and expression in *E. coli*

pTrc99C (Fig. 2a) is a 4.2 kb pBR322-derived plasmid that contains the *E. coli trc* promoter followed by the *lacZ* ribosome-binding site (RBS) upstream from a unique *Nco*I site comprising the ATG start codon of the *lacZ* gene. The *Nco*I site is followed by the pEMBL18 MCS and the *E. coli rrnB* transcription terminator sequence. Insertion of a gene into this *Nco*I site so that the *Nco*I site spans its ATG start codon allows expression of the corresponding protein translated under the control of the *lacZ* RBS. Additionally, the plasmid contains the *lacI* gene encoding the Lac repressor for controlled expression of the insert gene. The *lacI* gene itself is constitutively expressed at a high level by its *lacI*^q promoter 'up'-mutation. In the absence of IPTG, the Lac repressor blocks the *trc* promoter. Addition of IPTG results in inactivation of the Lac repressor, derepression of the *trc* promoter, and subsequent high-level expression of the insert gene. The insert gene and the *lacI* gene are flanked by the *E. coli bla* gene (encoding Ap^R) and the pMB1 *oriV* sequence, a prerequisite for legitimate recombination into the integration platform of the *Synechococcus* R2-PIM9 recipient strain (Fig. 2b). pTrc99C-derived expression plasmids in this study are called pMGx-A (Table 1).

Fig. 2. Integration of a pMGx-A construct in the PIM of *Synechococcus* R2-PIM9. (a) The pTrc99C vector. Specific genes studied were placed under the control of the trc promoter by insertion in the NcoI site in the MCS of the vector. pTrc99C-derived constructs were called pMGx-A. The lacI gene product renders the trc promoter repressed in the absence of IPTG. The bla gene encodes Ap^R. Arrows indicate the direction of transcription. (b) Integration of pMGx-A in the platform of *Synechococcus* R2-PIM9 (Ap^S Km^R) via homologous recombination at the bla and pMB1 oriV regions. The neo gene (encoding Km^R) in the platform is replaced by the expression cassette (trc promoter, insert gene, rrnB terminator, and lacI gene) present in pMGx-A. The crosses depict the cross-over events. The absence of the bla promoter is indicated by ΔP. pMGx-A is drawn as a linear structure for reasons of presentation. The dashed lines indicate that plasmid DNA used to transform *Synechococcus* was in fact circular. The desired recombinant has an Ap^R Km^S phenotype.

The *Anabaena* PCC 7937 *petE* gene (van der Plas *et al.*, 1989) encodes the precursor protein of plastocyanin (PC), a copper protein active in the thylakoid lumen as electron donor to photosystem I. The *petE* gene was cloned into the NcoI site of pTrc99C, creating a translational fusion, as depicted in Fig. 1. The resulting plasmid was called pMG77A. As a control, the *lacI* gene in pMG77A was interrupted by insertion of a *cat* gene (encoding Cm^R), resulting in pMG95A. Expression of the *Anabaena petE* gene in *E. coli* was determined by immunoblotting using a specific antiserum (Fig. 3). In cells containing pMG95A, expression of the *Anabaena petE* gene was efficient and independent of the presence of IPTG (lanes 1 and 2). Cells with pMG77A showed expression of the *Anabaena petE* gene only after IPTG induction (lanes 3 and 4). In *E. coli* cells containing the pTrc99C vector, no PC could be detected (lanes 5 and 6).

Likewise, the *E. coli uidA* gene encoding GUS (Jefferson *et al.*, 1986) was cloned into pTrc99C, creating a translational fusion. This resulted in plasmids pMG139A (with an intact *lacI* gene) and pMG140A (with an interrupted *lacI* gene). Expression of the *uidA* gene in *E. coli* was tested by determination of GUS activity. In cells containing pMG139A, the level of GUS activity was 10–20 times higher in induced cells than in non-induced cells. GUS activity in cells containing pMG140A was of the same order as that in induced pMG139A cells, and was independent of the presence of IPTG.

Fig. 3. Expression of the *Anabaena petE* gene in *E. coli*. Cultures of *E. coli* clones harbouring pMG77A, pMG95A or pTrc99C were grown, induced with IPTG and harvested as described in Methods. Immunoblots were screened with antiserum specific for *Anabaena* PC. The position of PC is marked with an asterisk. Lane M represents the positive control, mature PC isolated from *Anabaena*.

Transfer of expression constructs to *Synechococcus* R2-PIM9

The pMGx-A expression plasmids and pTrc99C (as a control) were transferred to *Synechococcus* R2-PIM9. The recombination event leading to chromosomal integration in R2-PIM9 using a pMGx-A construct is represented in Fig. 2(b). Recombination results in restoration of a

Table 2. Transfer of pMGx-A constructs to *Synechococcus* R2-PIM9

Transformation was performed as described in Methods. Phenotypes were determined by plate screening. Gene presence was determined by colony blot hybridization, and inducible gene expression was determined by protein blotting, as described in Methods. Transformation efficiency is expressed in transformants per ng DNA and *n* indicates the number of experiments.

Vector	Selection	Transformation efficiency	N*	Ap ^R Km ^S transformants		
				Number	Insert gene	
					Present	Expressed
pMG77A	Ap ^R	118 ± 62 (<i>n</i> = 3)	148	6 (4 %)	6 (100 %)	0 (0 %)
pMG95A	Ap ^R	176 ± 148 (<i>n</i> = 4)	150	17 (11 %)	16 (94 %)	0 (0 %)
pMG139A	Ap ^R	35 ± 12 (<i>n</i> = 3)	171	43 (25 %)	12 (28 %)	3 (25 %)
pMG140A	Ap ^R	91 ± 51 (<i>n</i> = 3)	176	42 (24 %)	9 (21 %)	2 (22 %)
pMG145A	Ap ^R	101 ± 75 (<i>n</i> = 7)	48	9 (19 %)	4 (44 %)	0 (0 %)
pTrc99C	Ap ^R	208 ± 106 (<i>n</i> = 5)	129	13 (10 %)	NA	NA

NA, Not applicable.

* N, number of Ap^R transformants used in further testing.

functional *bla* gene, conferring an Ap^R phenotype upon the cell, and replacement of the *neo* gene of the platform by the insert gene present on the pMGx-A donor plasmid ('gene replacement'). *Synechococcus* transformants resulting from a gene replacement event are obtained by selection for an Ap^R phenotype, and subsequent screening for loss of the Km^R platform marker by plating and for the presence of the insert DNA by colony blot hybridization using a specific DNA probe.

Transformation of the R2-PIM9 strain with the pMGx-A constructs was very efficient (Table 2); 35–208 × 10³ Ap^R transformants were obtained per µg DNA, which was similar to data obtained by Williams & Szalay (1983). Only 4–25 % (depending on the construct transferred) of the Ap^R transformants had lost the Km^R marker gene function contained on the platform. The majority of the transformants (Ap^R Km^R) resulted from a single cross-over event at the *bla* gene sequence and consequently contained not only the insert gene in the chromosome, but also other parts of the donor plasmid ('plasmid addition'). The transformants were highly unstable and quickly lost parts of the insert from the chromosome, as described previously (Williams & Szalay, 1983).

Even transformants with the correct Ap^R Km^S phenotype did not necessarily have the proper genetic structure. Most Ap^R Km^S transformants did not contain the insert gene as judged by colony blot hybridization. Screening, by immunoblotting, of Ap^R Km^S transformants that did react with the probe failed to show synthesis of the encoded protein. Colony blot hybridization of the Ap^R Km^S transformants with DNA probes specific for small regions of the donor DNA confirmed that the DNA transferred to the recipient platform in the transformants varied in length, and most transformants did not contain a complete insert gene. This indicated that in these transformants a cross-over had occurred in the *bla* gene, followed by a second, illegitimate recombination event

proximal to the *oriV* sequence ('gene conversion' or non-reciprocal recombination). The yield of correct transformants was very different for the various constructs tested (Table 1 and results not shown), implying influence of the insert DNA on the recombination process. For some constructs we were not able to obtain the desired clones, although several hundred Ap^R transformants were screened.

To improve the selection of the desired *Synechococcus* transformants, the pTrc99C vector was modified. Recombination in the *bla* gene results in a changed phenotype (from Ap^S to Ap^R), whereas recombination in the *oriV* region does not. Rather than alter the PIM, we decided to insert a second antibiotic resistance marker between the *lacI* gene and the *oriV* sequence in the donor plasmid. Screening of *Synechococcus* transformants for resistance to this marker (in combination with selection for Ap^R) should lead to selection of transformants that had the complete expression cassette (*trc* promoter, insert gene, *rrnB* terminator sequence, and *lacI* gene) transferred to the chromosome. Insertion of the *aadA* gene encoding Sm^R into a precursor of the pTrc99C vector resulted in the construction of plasmid pTrcIS (Fig. 4a). Genes of interest were cloned into pTrcIS resulting in the pMGx-B expression constructs (Table 1). Fig. 4(b) is a schematic representation of the recombination events leading to chromosomal integration in R2-PIM9 using a pMGx-B construct as donor plasmid.

The efficiency of transformation of *Synechococcus* R2-PIM9 with pMG145B and pTrcIS (as a control) depended upon the selection applied (Table 3); 75–90 × 10³ Ap^R transformants were obtained per µg DNA. When the first selection of the transformants was for an Ap^R phenotype, only 2 or 9 % of the Ap^R transformants had acquired the Sm^R gene and lost the Km^R platform marker function (compare with Table 2). In contrast, selection of transformants directly for an Ap^R Sm^R phenotype gave a 74–

Fig. 4. Integration of a pMGx-B construct in the PIM of *Synechococcus* R2-PIM9. (a) In comparison to pTrc99C, pTrcIS contains the *E. coli* *aadA* gene encoding Sm^R as an additional marker gene. The *aadA* gene and *bla* gene flank the expression cassette. pTrcIS-derived constructs were called pMGx-B. (b) Integration of pMGx-B in the platform of *Synechococcus* R2-PIM9 via homologous recombination. The desired recombinant has an Ap^R Km^S Sm^R phenotype. Other details are as in Fig. 2.

Table 3. Transfer of pMGx-B constructs to *Synechococcus* R2-PIM9

See Table 2 legend for details.

Vector	Selection	Transformation efficiency	N*	Ap ^R Sm ^R Km ^S transformants		
				Number	Insert gene	
					Present	Expressed
pMG145B	Ap ^R	88 ± 72 (<i>n</i> = 9)	62	1 (2%)	0 (0%)	0 (0%)
	Ap ^R Sm ^R	0.2 ± 0.2 (<i>n</i> = 8)	66	52 (79%)	52 (100%)	52 (100%)
	Sm ^R	2.3 ± 0.7 (<i>n</i> = 8)	276	240 (87%)	† (100%)	† (100%)
pTrcIS	Ap ^R	74 ± 79 (<i>n</i> = 5)	22	2 (9%)	NA	NA
	Ap ^R Sm ^R	1.0 ± 1.1 (<i>n</i> = 4)	18	16 (89%)	NA	NA
	Sm ^R	7.9 ± 3.5 (<i>n</i> = 4)	99	97 (98%)	NA	NA

NA, Not applicable.

* N, number of Ap^R transformants used in further testing.

† Only 50 transformants were tested.

or 440-fold lower yield in transformants per μg DNA, but 79 or 89% of these transformants had the desired Ap^R Sm^R Km^S phenotype, respectively. When transformants with this phenotype were analysed further, all contained

the complete expression cassette, as confirmed by the regulated expression of the insert gene. When the first selection of the transformants was for Sm^R, approximately 10-fold more transformants were obtained than when the

Fig. 5. GUS activity in *Synechococcus* as a function of IPTG concentration. *Synechococcus* R2-MG139A was grown as described and equal batches of culture were induced with varying concentrations of IPTG. Cells were harvested 4 h (open bars) or 24 h (filled bars) after the addition of IPTG, and GUS activity was determined. Vertical lines represent standard deviation, determined from five experiments.

first selection was for $\text{Ap}^{\text{R}} \text{Sm}^{\text{R}}$, whereas the percentage of transformants with an $\text{Ap}^{\text{R}} \text{Sm}^{\text{R}} \text{Km}^{\text{S}}$ phenotype was even higher. In some cases, e.g. with pMG145, the desired recombinant *Synechococcus* clone could only be isolated by transformation with a pMGx-B construct.

Analysis of expression conditions in *Synechococcus* using GUS as a reporter

The strength of the *E. coli trc* promoter and the optimum conditions for inducible gene expression in *Synechococcus* were determined using the *E. coli uidA* gene encoding GUS as a reporter. The strains studied were *Synechococcus* R2-MG139A and R2-MG140A. The conditions tested were IPTG concentration and induction time. Fig. 5 shows the results of induction experiments with R2-MG139A in which the IPTG concentration was varied, and cells harvested 4 or 24 h after the addition of IPTG. GUS activity reached a maximum level at concentrations of 0.1 mM IPTG and higher. After 4 h the increase in GUS activity was 4-fold compared to the level in non-induced cells, and after 24 h the increase was 16-fold. A time-course experiment (Fig. 6) showed that induced expression in R2-MG139A was maximum after 48 h, and 36-fold higher than in non-induced cells. Induced expression in R2-MG139A cells reached the level of constitutive expression in R2-MG140A cells.

Expression of the *Anabaena petE* gene in *Synechococcus*

Synechococcus R2-MG77B, R2-MG95B, R2-TrcIS (as a negative control) and *Anabaena* (as a positive control)

Fig. 6. GUS activity in *Synechococcus* as a function of induction time. *Synechococcus* R2-MG139A and R2-MG140A cultures were grown as described. Equal batches of R2-MG139A culture were induced with 1 mM IPTG (■) or not induced (●), and R2-MG140A was not induced (▲). Cells were harvested at various times of induction and GUS activity was determined. Standard deviation is presented as in Fig. 5.

Fig. 7. Expression of the *Anabaena petE* gene in *Synechococcus*: mRNA. Cultures of *Synechococcus* R2-MG77B, R2-MG95B, and R2-TrcIS and of *Anabaena* were grown as described in Methods and induced or not induced with IPTG. (a) RNA blot screened with a DNA probe specific for *Anabaena petE* mRNA. (b) Blot from (a) stripped and re-screened with a DNA probe specific for *Synechococcus* PCC 7942 *petF* mRNA.

were tested for expression of the *Anabaena petE* gene using RNA and immunoblot analysis. Fig. 7(a) shows an RNA blot probed with a DNA fragment carrying the *Anabaena petE* gene. In *Synechococcus* R2-MG95B cells,

Fig. 8. Expression of the *Anabaena petE* gene in *Synechococcus*: protein. (a) Immunoblot of the cells in Fig. 7 screened with antiserum specific for *Anabaena* PC. Lane M represents the positive control (see Fig. 3). The position of PC is marked with an asterisk. (b) SDS-PAGE gel as in (a) stained with Coomassie Brilliant Blue.

petE mRNA was present as expected in both the absence and presence of IPTG (lanes 1 and 2). In *Synechococcus* R2-MG77B cells, *petE* mRNA was detected only after induction with IPTG (lanes 3 and 4). *Synechococcus* R2-TrcIS cells did not contain *petE* mRNA (lanes 5 and 6). The *petE* mRNA detected in *Synechococcus* cells had two different sizes, one of approximately 740 bases, and a larger one of approximately 1030 bases. In *Anabaena* cells (lane M), a single *petE* transcript of about 740 bases long was present (Bovy *et al.*, 1992). To show that incubation of the cells with IPTG had no deleterious effect on the transcriptional capacity of the cells in general, the blot used in Fig. 7(a) was stripped and re-probed with a DNA fragment carrying the *Synechococcus* PCC 7942 *petF* gene encoding ferredoxin (van der Plas *et al.*, 1990). The results (Fig. 7b) showed that all lanes contained similar amounts of *petF* mRNA. Densitometric scanning confirmed that the concentration of *petF* mRNA differed only up to 15 %, with no apparent relation to the absence or presence of IPTG (results not shown).

Protein levels also varied as expected in response to IPTG. In cells of strain R2-MG95B, expression of *Anabaena* PC was independent of the addition of IPTG (Fig. 8a, lanes 1 and 2). The protein detected was the same size as mature PC protein in *Anabaena* cells (lane M),

Fig. 9. Expression of the *E. coli lacI* gene in *Synechococcus*. (a) RNA dot blot of the cells in Fig. 7 screened with a DNA probe specific for *E. coli lacI* mRNA. (b) Immunoblot of the cells in Fig. 7 screened with antiserum specific for the *E. coli* Lac repressor protein. The position of Lac repressor protein is marked with an asterisk. Lane M represents the positive control, *E. coli* PC2495 harbouring pTrc99C.

indicating that the precursor protein was recognized by the *Synechococcus* protein-sorting machinery, and processed. In strain R2-MG77B cells, protein detected by the PC antiserum was observed only after induction with IPTG (lanes 3 and 4). In *Synechococcus* cells containing pTrcIS, no PC could be detected (lanes 5 and 6). Fig. 8(b) shows the gel stained for total protein with Coomassie Brilliant Blue, displaying similar patterns in all lanes. In strain R2-MG77B cells, the *trc* promoter was efficiently shut off by the Lac repressor in the absence of IPTG. This was probably due to the efficient synthesis of the 38 kDa *lacI* gene product in *Synechococcus*, which was similar to that in *E. coli* (Fig. 9).

The general usability of pTrcIS as a cloning vector for inducible, heterologous gene expression in *Synechococcus* PCC 7942 was confirmed by the introduction of the genes encoding *Arabidopsis thaliana* and *Silene pratensis* PC, *Bacillus licheniformis* α -amylase, *Streptococcus mutans* fructosyltransferase and two *Anabaena* PC-*Bacillus* α -amylase fusion proteins. For all genes studied, the desired recombinant clones were procured efficiently, and high-level gene expression was found after induction with IPTG (results not shown). The transformants obtained were all stable for at least one year of continuous culturing without antibiotic selection.

DISCUSSION

Synechococcus R2-PIM9 was highly transformable by pTrc99C-derived pMGx-A plasmids, but many transformants arose through illegitimate means. van der Plas *et al.* (1990) found that the percentage of transformants with the desired phenotype depended on the gene transferred and could be well below 100 %. The inability to select for

the presence of the inserted gene diminished the general utility of the PIM. Consequently, the *aadA* gene encoding Sm^R as a second antibiotic-resistance marker was cloned into pTrc99C to force recombination in sequences flanking the inserted gene, resulting in vector pTrcIS. pTrcIK, containing the *neo* gene encoding Km^R , for use in *Synechococcus* R2-PIM8 ($\text{Ap}^S \text{Sm}^R$) (van der Plas *et al.*, 1990) was also constructed.

Using pTrcIS-derived pMGx-B plasmids we were able to correctly introduce *petE*, *uidA* and several other genes into the platform at high efficiency. The number of transformants with the desired phenotype using a pMGx-B plasmid is actually higher when the first screen is for an Sm^R phenotype instead of for an $\text{Ap}^R \text{Sm}^R$ phenotype (Table 3), implying that screening for an Ap^R phenotype results in the loss of transformants with the proper phenotype. *Synechococcus* R2-MGx-A or -B clones containing the same insert gene showed similar expression patterns.

Maximum induction of the *trc/lacI* system found with *E. coli uidA* as a reporter gene was 36-fold. With other genes tested, e.g. the *Anabaena petE* gene, the induction factor was even higher: densitometric scanning of protein blots of *Synechococcus* R2-MG77B showed about 100-fold induction (Geerts *et al.*, 1994). The induced GUS activity could reach the level of constitutive GUS activity (in cells without a functional *lacI* gene), showing that derepression by inactivation of the Lac repressor was virtually total. The *E. coli trc* promoter in the *uidA* gene fusion in *Synechococcus* was almost fourfold stronger than the *Anabaena* PCC 7937 *petF* promoter [400 vs 120 $\text{nmol MU min}^{-1} (\text{mg protein})^{-1}$], the strongest cyanobacterial promoter tested in *Synechococcus* PCC 7942 by Bovy *et al.* (1993).

The level of activity of the *E. coli uidA* gene in R2-MG139A and the *Anabaena petE* gene in R2-MG77B was very low in the absence of IPTG, meaning that there was saturating binding of the Lac repressor to the *lac* operator sequence. In a recently described plasmid-borne *tac* promoter/*lacI* repressor system used in *Anabaena* sp. PCC 7120, repression by LacI resulted in only about fivefold repression (Elhai, 1993). The successful repression in our system could be due to the efficient synthesis of the Lac repressor (Fig. 9). The high-level expression of the *lacI* gene in *Synechococcus* in our system was surprising, since it is transcribed from its own promoter. The gene also contains 19 codons that are rarely used in *Synechococcus* (J. van der Plas and others, TNO, Zeist, The Netherlands, unpublished results), and it has been suggested that the use of rare codons reduces *lacI* expression in heterologous hosts, as in *Zymomonas mobilis* (Arfman *et al.*, 1992).

Two different sizes of *petE* mRNA were found in *Synechococcus* R2-MG77B and R2-MG95B, one of the correct length of approximately 740 bases, and a larger one of about 1030 bases (Fig. 7a). These bands are most likely the result of transcription termination at either of two strong stem-loop structures located downstream of the *petE* gene: one (80 bp downstream of the *petE* gene, $\Delta G -18.2 \text{ kCal mol}^{-1}$; -76 kJ mol^{-1}) in the *petE* gene

itself, the second one (446 bp downstream of the *petE* gene, $\Delta G -36.0 \text{ kCal mol}^{-1}$; -151 kJ mol^{-1}) in the *E. coli rrnB* transcription terminator sequence in the vector. The *petE* mRNA is translated efficiently, resulting in accumulation of PC to a level similar to that found in *Anabaena* cells (Fig. 8a). *Synechococcus* cells expressing the *Anabaena petE* gene had a drastically enhanced photosynthetic activity, proving that the expressed PC was efficiently targeted to the thylakoid lumen, and properly folded with a functional copper centre (Geerts *et al.*, 1994).

In contrast to other inducible gene expression systems described for cyanobacteria (Friedberg & Seijffers, 1986; Marracini *et al.*, 1993; Mermet-Bouvier & Chauvat, 1994), the expression system described in this paper did not seem to have pleiotropic effects on the cellular metabolism upon induction. Addition of IPTG at concentrations of up to 4 mM did not result in any gross effect on the cell, as judged by Coomassie Brilliant Blue staining of total protein content (Fig. 8b), expression of the endogenous *petF* gene (Fig. 7b), growth rate (data not shown) or photosynthetic activity (Geerts *et al.*, 1994).

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