Gene replacement in cyanobacteria mediated by a dominant streptomycin-sensitive *rps12* gene that allows selection of mutants free from drug resistance markers

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Chromosomal gene replacement in cyanobacteria often relies upon the availability of drug resistance markers, and thus multiple replacements have been restricted. Here, a versatile gene replacement system without this restriction is reported in a unicellular cyanobacterium, *Synechococcus* sp. PCC 7942. The system is based upon the dominance of a streptomycin-sensitive *rps12* gene encoding a ribosomal S12 protein over a streptomycin-resistant *rps12-R43* allele with a Lys-43 → Arg substitution. To demonstrate the utility of this method, a cassette consisting of the wild-type *rps12* gene and a *kan* gene conferring kanamycin resistance was integrated into the *rps12-R43* mutant at the *psbAI* locus encoding photosystem II D1 protein, resulting in streptomycin-sensitive merodiploids. Despite spontaneous gene conversion in these merodiploids to produce streptomycin-resistant progeny at frequencies ranging from 1×10^{-5} to 5×10^{-5} , homologous recombination could be induced by transforming the merodiploids with template plasmids carrying *psbAI* 5' and 3' non-coding sequences flanking the D1 coding sequence, which was then replaced by either the *gfp* ORF for a green fluorescent protein or a precise deletion. Depending on the replication ability of the template plasmids, at most 3–16% of streptomycin-resistant progeny of the merodiploids after transformation were homogenote recombinants with concomitant loss of the *kan* gene, even in these polyploid cyanobacteria. The *rps12*-mediated gene replacement thus makes it possible to construct mutants free from drug resistance markers and opens a way to create cyanobacterial strains bearing an unlimited number of gene replacements.

Keywords: homologous recombination, *Synechococcus*, gene conversion, merodiploid, *psbAI*

INTRODUCTION

Cyanobacteria occupy particular niches in the hydrosphere and adapt to varying environments. They carry out oxygenic photosynthesis similar to that operating in green plant chloroplasts, while the simple genomic organization and ease of genetic manipulation of these micro-organisms attract in-depth biochemical and genetic studies of as yet unsolved biological processes.

Recently, cyanobacteria have been revealed as one of the best experimental models to investigate the structure and function relationships of membranous pigment–protein complexes called photosystem I and photosystem II (for reviews, see Nixon & Diner, 1994; and Pakrasi, 1995). To genetically analyse these multisubunit complexes, a versatile method is needed to replace any chromosomal genes with *in-vitro*-constructed mutant genes without limitation of the number of manipulatable targets.

Some cyanobacterial strains, such as *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, are naturally transformable with externally added DNAs, and both integrative and replicative transformation systems are

Abbreviations: Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant; Cm^s, chloramphenicol-sensitive; Km^r, kanamycin-resistant; Km^s, kanamycin-sensitive; Sm^r, streptomycin-resistant; Str^r, streptomycin-resistant (chromosomal); Str^s, streptomycin-sensitive (chromosomal).

available in these strains (Chauvat *et al.*, 1986; Kuhlemeier *et al.*, 1983; Williams, 1988). However, due to the fact that homologous recombination mediated by *recA* function is essential for cell viability in cyanobacteria (Murphy *et al.*, 1990), recombinant plasmids containing any sequences homologous with their host chromosome are not stably maintained in cyanobacterial cells. Consequently, these homologous sequences are integrated with the aid of dominant drug resistance markers into non-essential sites of the chromosome conventionally termed platforms (Geerts *et al.*, 1995) or neutral sites (Bustos & Golden, 1992). Although this method works well, one of the drawbacks is that the dominant drug resistance marker, once integrated, cannot be eliminated from the genome, and thus a subsequent genetic manipulation in the same strain is limited by the availability of other drug resistance markers.

Another difficulty in manipulating cyanobacterial genes arises from their polyploidy; i.e. the multiple copies of genomic DNA in some strains (Binder & Chisholm, 1990). It is therefore of great importance to have a selection scheme that allows one to obtain mutants carrying homogeneous mutant genes on every copy of genomic DNA. Such homogenote (or homoallelic) mutants are only available for genes that are not essential for growth; otherwise heterogenotes containing the mutant as well as wild-type genes are formed (e.g. Ghassemian & Straus, 1996; Murphy *et al.*, 1990). In this regard, a dominant drug-sensitive marker should be easier to eliminate from the chromosome after its integration since even a single copy of the marker would reduce the ability of the polyploid host to survive in the presence of the drug.

It is well known that the *rpsL* gene encoding an S12 subunit protein of the 30S ribosome in *Escherichia coli* gives rise to *strA* mutations conferring the streptomycin-resistant (Str^r) phenotype (Funatsu & Wittmann, 1972; Timms *et al.*, 1992). When the mutant and wild-type alleles coexist in a cell, the resulting merodiploid shows a streptomycin-sensitive (Str^s) phenotype; i.e. the *strA* mutation is recessive (Lederberg, 1951). The cloned *rpsL* gene thus behaves as a dominant Str^s marker and has been utilized in a variety of genetic methods, including enforced cloning of foreign DNAs (Dean, 1981; Hashimoto-Gotoh *et al.*, 1993), a detection system for specific mutations (Gondo *et al.*, 1996) and a gene replacement system in a *recD* mutant (Russell & Dahlquist, 1989). These methods depend upon the logic that only when the *rpsL* gene is inactivated in an *rpsL/rpsL* (*strA*) merodiploid strain can the cells grow in the presence of streptomycin.

In this work, we have developed a methodology capable of introducing multiple gene replacements into the obligate photoautotrophic cyanobacterium *Synechococcus* sp. PCC 7942 based on the same strategy as the *rpsL* system in *E. coli*. For this purpose, the photosystem II *psbAI* gene encoding the D1 protein was chosen as a target, since the presence of three *psbA* genes in this strain permitted us to inactivate only the *psbAI* gene

without affecting photoautotrophic growth (Golden *et al.*, 1986). We first demonstrated that an *rps12* gene encoding an S12 ribosomal protein acts as a dominant Str^s marker in this cyanobacterium. We then applied the cloned *rps12* gene to systematically modify the *psbAI* ORF without leaving any externally incorporated drug resistance markers in the final mutants. This method is thus well suited for the construction of multiple gene replacements in cyanobacteria in order to analyse complex biological processes such as photosystems I and II.

METHODS

Strains and plasmids. *Synechococcus* sp. PCC 7942 R2-SPc, which had been cured of an indigenous plasmid, pUH24, and a shuttle vector plasmid, pUC303, carrying a streptomycin-resistant (Sm^r) gene and a chloramphenicol-resistant (Cm^r) gene (Kuhlemeier *et al.*, 1983) were obtained from Dr Y. Inoue, RIKEN, Japan. A plasmid, pEXE1, carrying the 5' and 3' non-coding sequences of the *psbAI* gene from *Synechococcus* sp. PCC 7942, was constructed previously (Sakai *et al.*, 1997). Plasmids pUC18, carrying an ampicillin-resistant (Ap^r) gene, and pUC4K, carrying a kanamycin-resistant (Km^r) gene, were purchased from Pharmacia. A plasmid, pGFPuv, carrying the *gfp* gene (UV version), encoding a green fluorescent protein was obtained from Clontech Laboratories. *E. coli* strain JM109 was used for routine cloning. *E. coli* GM33 (*dam-3*) was used for the preparation of plasmids without *dam* methylation. Plasmids and cyanobacterial strains constructed in this work are described in Table 1.

Culture conditions. Cells of *Synechococcus* sp. strain PCC 7942 R2-SPc and its derivatives (Table 1) were grown photoautotrophically in liquid BG-11 medium in an incubator equipped with fluorescent lamps under continuous illumination of 4.4×10^{-4} J cm⁻² s⁻¹ at 28 °C. *E. coli* cells were grown in LB medium at 37 °C. Appropriate antibiotics were added at concentrations of 50 µg kanamycin ml⁻¹, 10 µg streptomycin ml⁻¹, 50 µg ampicillin ml⁻¹ or 10 µg chloramphenicol ml⁻¹. For plating the cells, media were solidified with 1.5 % agar.

PCR and DNA manipulations. Standard methods (Sambrook *et al.*, 1989) were applied for digestion of DNA by restriction enzymes and cloning of DNA fragments into plasmids. PCR and inverse PCR were performed as described by Saiki *et al.* (1987) and Triglia *et al.* (1988), respectively, with the GeneAmp PCR system 2400 (Perkin Elmer-Applied Biosystems) and the oligonucleotide primers listed in Table 2. AmpliTaq GOLD (Perkin Elmer-Applied Biosystems) was used for most of the amplifications of DNA fragments. PCR fragments to be cloned into plasmids were amplified with Vent DNA polymerase (New England BioLabs), purified with Wizard PCR Preps (Promega) and cloned into pUC18 (SureClone Ligation Kit; Pharmacia). Nucleotide sequences were determined with a 310 Genetic Analyzer and reagents for BigDye Terminator Cycle Sequencing (Perkin Elmer-Applied Biosystems), as recommended by the supplier.

Sequencing of the *rps12* gene region. Two degenerate PCR primers (nos 1 and 2) were used to amplify a part (361 bp) of the *rps12* ORF. Both the fragments amplified from PCC 7942 R2-SPc (wild-type) and TEV1002-1 (a spontaneous Str^r mutant) strains were cloned into pUC18, and the nucleotide sequences were determined. Fragments containing sequences outside the *rps12* ORF were obtained by inverse PCR using primers 3, 4, 5 and 6 after cutting the chromosomal DNA with

Table 1. Plasmids and cyanobacterial strains used in this study

Plasmid or strain	Description or genotype*	Reference or source
Plasmids		
pEXE1	A 1.8 kb fragment upstream of the <i>psbAI</i> ORF and a 0.5 kb fragment downstream of the <i>psbAI</i> ORF were PCR-amplified and cloned into the <i>Bam</i> HI site of pUC19. The two fragments were joined with a <i>Not</i> I site which was adjacent to a <i>Ban</i> III site at the 5' terminus of the <i>psbAI</i> ORF; Ap ^r , <i>psbAI</i> ΔORF	Sakai <i>et al.</i> (1997)
pEXE2	A 1.0 kb <i>efe</i> gene ORF encoding <i>Pseudomonas syringae</i> ethylene-forming enzyme was PCR-amplified and cloned into the <i>Ban</i> III/ <i>Not</i> I sites of pEXE1 to produce a precise replacement of <i>psbAI</i> ORF with the <i>efe</i> ORF. Ap ^r , <i>psbAI</i> :: <i>efe</i> ORF	Sakai <i>et al.</i> (1997)
pIEK2	A 1.25 kb fragment from pUC4K containing the <i>kan</i> gene was cut out with <i>Hinc</i> II, ligated with <i>Not</i> I linkers and cloned into the <i>Not</i> I site of pEXE2. The transcriptional direction of the <i>kan</i> gene was the same as that of the <i>efe</i> gene. Ap ^r Km ^r , <i>psbAI</i> :: <i>efe</i> ORF- <i>kan</i>	Our laboratory stock (unpublished)
pEXR10	A 579 bp fragment containing the <i>rps12</i> gene with its promoter sequence was amplified by PCR using primers 9 and 10 with a 5' <i>Ban</i> III extension and cloned into the <i>Ban</i> III site of pEXE1. The transcriptional direction of the <i>rps12</i> gene was the same as that of the <i>psbAI</i> promoter on the pEXE1 vector. Ap ^r , <i>psbAI</i> :: <i>rps12</i>	This study
pEXR11	A 1.25 kb <i>kan</i> gene fragment from pIEK2 was cloned into the <i>Not</i> I site of pEXR10. The transcriptional direction of the <i>kan</i> gene was opposite to that of the <i>rps12</i> gene. Ap ^r Km ^r , <i>psbAI</i> :: <i>rps12-nak</i>	This study
pEXR12	A 1.25 kb <i>kan</i> gene fragment from pIEK2 was cloned into the <i>Not</i> I site of pEXR10. The transcriptional direction of the <i>kan</i> gene was the same as that of the <i>rps12</i> gene. Ap ^r Km ^r , <i>psbAI</i> :: <i>rps12-kan</i>	This study
pEXR20	A 375 bp ORF for the <i>rps12</i> gene was PCR-amplified with <i>Ban</i> III and <i>Not</i> I linkers at the 5' and 3' termini, respectively, using primers 11 and 12. The fragment was cloned into the <i>Ban</i> III/ <i>Not</i> I sites of pEXE1 to produce a precise replacement of the <i>psbAI</i> ORF with the <i>rps12</i> ORF. Ap ^r , <i>psbAI</i> :: <i>rps12</i> ORF	This study
pEXR21	A 1.25 kb <i>kan</i> gene fragment from pIEK2 was inserted into the <i>Not</i> I site of pEXR20. The transcriptional direction of the <i>kan</i> gene was the same as that of the <i>rps12</i> gene. Ap ^r Km ^r , <i>psbAI</i> :: <i>rps12</i> ORF- <i>kan</i>	This study
pEXGFP	A 717 bp <i>gfp</i> gene ORF fragment was PCR-amplified from pGFPuv using primers 15 and 16 and was cloned into the <i>Ban</i> III/ <i>Not</i> I sites of pEXE1 to produce a precise replacement of the <i>psbAI</i> ORF by the <i>gfp</i> ORF. Ap ^r , <i>psbAI</i> :: <i>gfp</i> ORF	This study
pEXGFPK	A 1.25 kb <i>kan</i> gene fragment from pIEK2 was inserted into the <i>Not</i> I site of pEXGFP. The transcriptional direction of the <i>kan</i> gene was opposite to that of the <i>gfp</i> gene. Ap ^r Km ^r , <i>psbAI</i> :: <i>gfp</i> ORF- <i>kan</i>	This study
pUC3032	A 1.45 kb <i>Sac</i> I- <i>Sal</i> I fragment containing 5' and 3' non-coding regions of the <i>psbAI</i> gene from pEXE1 was isolated and ligated with a 8.1 kb <i>Sac</i> I- <i>Xho</i> I fragment from pUC303. The resulting 9.55 kb plasmid, pUC3032, contains the <i>psbAI</i> ΔORF gene in place of the plasmid-encoded Sm ^r gene on pUC303. Cm ^r , <i>repAB psbAI</i> ΔORF	This study
Strains		
TEV1002	<i>Synechococcus</i> sp. PCC 7942 R2-SPc whose <i>psbAI</i> gene ORF was replaced with the <i>kan</i> gene in the opposite orientation. The <i>kan</i> gene was flanked on both sides by χ and RS, which represent the <i>E. coli</i> <i>chi</i> site and the recognition site for site-specific endonuclease I- <i>Tev</i> I, respectively. <i>psbAI</i> :: χ RS- <i>nak</i> -RS χ	Our laboratory stock (unpublished)
TEV1002-1	A spontaneous Str ^r mutant of TEV1002. <i>psbAI</i> :: χ RS- <i>nak</i> -RS χ <i>rps12</i> -R43	This study
GRPS1	A derivative of <i>Synechococcus</i> sp. PCC 7942 R2-SPc which received a PCR-generated <i>rps12</i> -R43 gene fragment from TEV1002-1 and exhibited streptomycin resistance. <i>rps12</i> -R43	This study
GRPS11	A derivative of GRPS1 in which the <i>psbAI</i> gene was replaced by the sequence from pEXR11. <i>rps12</i> -R43 <i>psbAI</i> :: <i>rps12-nak</i>	This study
GRPS12	A derivative of GRPS1 in which the <i>psbAI</i> gene was replaced by the sequence from pEXR12. <i>rps12</i> -R43 <i>psbAI</i> :: <i>rps12-kan</i>	This study
GRPS21	A derivative of GRPS1 in which the <i>psbAI</i> gene was replaced by the sequence from pEXR21. <i>rps12</i> -R43 <i>psbAI</i> :: <i>rps12</i> ORF- <i>kan</i>	This study
GLC1	A Km ^r transformant of PCC 7942 R2-SPc which received a copy of <i>psbAI</i> :: <i>gfp</i> ORF- <i>nak</i> from pEXGFPK. <i>psbAI</i> :: <i>gfp</i> ORF- <i>nak</i>	This study

* *nak* represents a *kan* gene inserted in the opposite transcriptional direction to that of the upstream gene.

Table 2. Sequences of oligodeoxynucleotides used for PCR primers

No.	Mer	Base sequence*	Target sequence†
1	26	ATGCCAACTATCCAGCARYTNATHCG	<i>rps12</i> N terminus (sense)
2	24	GTTTGGTGCCGTATTTNGANCGNC	<i>rps12</i> C terminus (antisense)
3	23	GGTCAAAGACCTACCCGGTGTGC	<i>rps12</i> C terminus (sense)
4	22	TCGTGGTGTAGACGCGAGTGCA	<i>rps12</i> N terminus (antisense)
5	23	GCAGTTGACCTTGCTCGTTCCAG	<i>rps12</i> 3' non-coding (sense)
6	22	TTCGCGTTCGTGCGAATCAGC	<i>rps12</i> N terminus (antisense)
7	24	GTCTTTTCCAAGAGCAGCCGAACC	<i>rps12</i> 5' non-coding (sense)
8	23	TGGAACGAGCAAGGTCAACTGCC	<i>rps12</i> 3' non-coding (antisense)
9	32	CCC <u>ATCGATG</u> TTCCAGAGAGGCTACCTGGGGC	<i>rps12</i> 5' non-coding (sense)
10	32	CCC <u>ATCGATG</u> TGGAACGAGCAAGGTCAACTGCC	<i>rps12</i> 3' non-coding (antisense)
11	35	CCC <u>ATCGAT</u> ATGCCTACAATCCAGCAGCTGATTGC	<i>rps12</i> N terminus (sense)
12	32	TTT <u>GCGGCCG</u> CCTAGGCCTTCGGACGCTTCGC	<i>rps12</i> C terminus (antisense)
13	24	GAGCACTGTAGCCTTGGGCGATCG	<i>psbA1</i> 5' non-coding (sense)
14	25	GGGCAATGCGTTGTTGCAGTACCTC	<i>psbA1</i> 3' non-coding (antisense)
15	40	CCCATCGATATGAGTAAAGGAGAAGAACTTTTCACTGGAG	<i>gfp</i> N terminus (sense)
16	38	TTT <u>GCGGCCG</u> CTTATTTGTAGAGCTCATCCATGCCATG	<i>gfp</i> C terminus (antisense)

* N = A, C, G or T; R = A or G; Y = C or T; H = A, C or T; restriction sites used for cloning are denoted by solid underlining (*Ban*III) or dotted underlining (*Not*I).

† Sense and antisense denote the coding and non-coding strands, respectively.

*Pvu*II and *Pst*I to generate 1.4 and 3.5 kb DNA fragments, respectively, and religation. Inverse PCR fragments were directly sequenced using the same primers. A 491 bp PCR fragment containing the *rps12* gene with 35 bp 5' and 81 bp 3' non-coding regions was prepared by using primers 7 and 8, whereas a 375 bp PCR fragment containing only the *rps12* ORF was amplified with primers 11 and 12.

Electroporation. Cyanobacterial cells were grown in BG-11 medium supplemented with appropriate antibiotics to a density of 10^8 cells ml⁻¹. The cells in 1.0 ml culture were collected by centrifugation in 1.5 ml sterile centrifuge tubes at 13000 g for 5 min at 4 °C, and the supernatant was discarded. The cells were suspended in 1.0 ml SH buffer (272 mM sucrose, 8 mM HEPES, pH 7.4), centrifuged, and the supernatant was removed. The cells were washed as above with 0.2 ml SH buffer, suspended in 20 µl 2 × SH buffer and cooled on ice. A 20 µl aliquot of cell suspension was transferred to a cuvette of 0.1 cm path length (Bio-Rad), and necessary DNA solutions and sterile water was added to a final liquid volume of 40 µl. The cuvette was capped and cooled on ice before electroporation at 900 V, 129 Ω, 50 µF, using an Electro Cell Manipulator (model 600M; BTX). Immediately after electroporation, 1.0 ml BG-11 medium was added into the cuvette, and the cells were suspended with a pipette, transferred into a sterile centrifuge tube and kept for 10 min on ice. The cells were then collected by centrifugation at 13000 g for 1 min at 4 °C, and the supernatant was discarded. The cells were again washed with 1.0 ml of BG-11 medium, resuspended in 1.0 ml BG-11 medium and transferred into an empty sterile glass test tube with a cotton plug. The tube was shaken on a reciprocal shaker at 100 r.p.m. in an illuminated incubator at 28 °C overnight. Finally, the cells were spread onto BG-11 agar plates and incubated at 28 °C under light for up to 2 weeks.

Curing of plasmids. For curing of plasmids from cyanobacterial transformants, cells were cultivated photoautotrophically in BG-11 medium in the absence of the antibiotic to which the resistance had been conferred by the plasmid. When the OD₇₃₀ of the culture exceeded 1.0, a portion of the

culture was transferred into a tube containing fresh BG-11 medium, and the cultivation was repeated until cells had been propagated for 32 generations. The culture was then diluted and spread on BG-11 medium. The colonies formed were then replicated with nylon membrane (Hybond N+; Amersham Pharmacia) onto BG-11 medium containing appropriate antibiotics. Cured strains that no longer grew in the presence of antibiotics made up 15–20% of the population after 32 generations.

Fluorescence microscopic observation. Cellular fluorescence was observed with a fluorescence microscope (Olympus OPTICAL model BX50 microscope; BX-FLA reflected light illuminator equipped with a mercury burner). Excitation was done at 360–370 nm, while 510–550 nm emission was observed through an absorption filter.

Southern blot analysis. Five to ten micrograms of chromosomal DNA were digested with restriction enzymes, electrophoresed on agarose gel and transferred onto a nylon membrane (Hybond N+) by vacuum suction (VacuGene XL; Amersham Pharmacia). DNA probes were prepared by labelling with digoxigenin using a PCR Dig Labeling Mix (Roche Diagnostics). Hybridization of the membrane with a probe was carried out in a buffer containing 5 × SSC (75 mM sodium citrate, pH 7.0, and 0.75 M NaCl) at 62–65 °C overnight. The hybridized membrane was treated with anti-digoxigenin-alkaline phosphatase Fab fragments (Roche Diagnostics) and luminescent substrate (Lumi-Phos 530; Wako Pure Chemical Industries) as recommended by the manufacturer, and exposed to X-ray film (XAR; Kodak).

RESULTS

Isolation and characterization of a streptomycin-resistant mutant

As a prerequisite for streptomycin selection of gene replacements, a well-defined Str^r mutant is essential. Although the spontaneous frequency for occurrence of

Table 3. Transformation of *Synechococcus* sp. GRPS1 with *psbAI* gene disruption plasmids

Plasmids (gene on fragment)*	Amount of DNA used (µg)	Number of Km ^r transformants	Ratio of Str ^s /Str ^r phenotypes†	Spontaneous gene conversion frequency‡
pEXR11/ <i>Bam</i> HI (<i>psbAI::rps12-nak</i>)	6	475	3/1	ND
	3	1110	1/3	4.4 × 10 ⁻⁵ (GRPS11)
pEXR12/ <i>Bam</i> HI (<i>psbAI::rps12-kan</i>)	6	321	3/1	ND (GRPS12)
	3	26	4/0	2.5 × 10 ⁻⁵
pEXR21/ <i>Bam</i> HI (<i>psbAI::rps12ORF-kan</i>)	8	60	4/0	1.7 × 10 ⁻⁵ (GRPS21)
	6	1	1/0	ND
	4	72	4/0	1.1 × 10 ⁻⁵
	2	80	4/0	5.4 × 10 ⁻⁵

* Plasmids were cut by *Bam*HI and used for electroporation without further purifying the fragments.

† Streptomycin sensitivity/resistance is represented as the ratio of colony numbers tested; for example, a ratio of 3/1 indicates that three transformants were Str^s while one was Str^r.

‡ Selected Km^r Str^s transformants (including GRPS11, 12 and 21 as indicated in parentheses) were grown in BG-11 medium, and cells were plated onto BG-11 media containing either kanamycin or streptomycin to calculate the fraction of spontaneous gene conversion to produce Str^r progeny. Values represent single measurements. ND, Not determined.

Fig. 1. Chromosomal status of *rps12* merodiploids. Chromosomal DNAs from selected Km^r transformants exhibiting Str^s and Str^r phenotypes were analysed by Southern hybridization using the *rps12* ORF probe. Hybridization was performed in 5×SSC at 62 °C. Lanes 2–11 contained 5 µg chromosomal DNAs digested with *Hind*III. Samples in lanes 3–8 were from Str^s transformants, whereas those in lanes 9–11 were from Str^r transformants. Lanes: 1 and 12, digoxigenin-labelled, *Hind*III-cut λ DNA marker; 2, GRPS1; 3, 4, 5, 9 and 10, transformants of GRPS1 obtained with pEXR11 carrying *psbAI::rps12-nak* insertion; 6, 7, 8 and 11, transformants obtained with pEXR12 carrying a *psbAI::rps12-kan* insertion. Since *Hind*III cuts the *kan* gene nearly symmetrically, a slight mobility difference between *rps12-nak* and *rps12-kan* fragments was not evident here. Sizes of hybridizing bands are indicated on the right.

conferring kanamycin resistance at the *psbAI* locus (Table 1). The Km^r Str^r mutant strain was designated TEV1002-1, and its *rps12* gene encoding the S12 ribosomal protein was compared with the wild-type *rps12* gene as described in Methods. A contiguous 1428 bp nucleotide sequence including the *rps12* gene and a part of the *rps7* gene downstream of the *rps12* gene was determined, and found to be identical to the sequence (GenBank accession X17442) reported by Meng *et al.* (1989) in *Synechococcus* sp. PCC 6301, formerly called *Anacystis nidulans*. This result was not unexpected since PCC 7942 and PCC 6301 are nearly identical strains (Golden *et al.*, 1989).

Within the regions sequenced, the only difference between the wild-type and the mutant *rps12* genes is a single base transition from A to G at position 128 of the ORF. The point mutation occurred at the second position of the 43rd codon, resulting in a replacement of a lysine (AAA) with an arginine (AGA) codon. Accordingly, the mutant allele was designated *rps12-R43*. We verified that the mutation in the *rps12-R43* gene is the sole change determining the Str^r phenotype of the mutant by transforming *Synechococcus* sp. PCC 7942 R2-SPc to streptomycin resistance with a PCR-generated 375 bp DNA fragment of the *rps12-R43* ORF. An Str^r transformant was obtained and was found by sequencing of its *rps12* gene to have the same point mutation as the *rps12-R43* allele. This strain was named GRPS1, and it was used as the standard *rps12-R43* mutant. Using an *rps12* probe, a single band was detected in genomic Southern hybridization with several restriction digests, indicating a single-copy *rps12* gene (data not shown).

Str^r mutants in *Synechococcus* sp. PCC 7942 R2-SPc was less than 10⁻⁸, we found by chance a spontaneous Str^r mutant from strain TEV1002 carrying the *kan* gene

Fig. 2. Schematic representation of gene replacement utilizing the dominant *rps12* gene. Chromosomal and plasmid DNAs are shown by wavy and ellipsoidal lines, respectively. Individual genes or ORFs are represented by rectangular boxes, whereas the *psbAI* 5' and 3' flanking non-coding sequences are represented by hatched boxes. Possible routes for recombination between homologous sequences are shown by dotted lines. Only relevant restriction sites are shown. (a) Top, Chromosomal state in a *Str^r* strain carrying the *rps12-R43* allele; bottom, plasmid used to disrupt the *psbAI* gene by *rps12* and *kan* genes. (b) Top, Chromosomal state in a *Str^s Km^r* merodiploid strain carrying the *rps12-R43* and *psbAI::rps12-kan* alleles; bottom, template plasmid carrying a *gfp* gene within the *psbAI* 5' and 3' flanking sequences. (c) Chromosomal state in a *Str^r Km^s* recombinant after gene replacement at the *psbAI* locus. (d) Chromosomal state in a *Str^r Km^r* merodiploid strain after gene conversion of the *rps12* gene inserted at the *psbAI* locus into the *rps12-R43* allele.

Merodiploid analysis for the recessiveness of *rps12-R43* allele

We tested the dominance/recessiveness of the *rps12-R43* allele by constructing merodiploid strains in which a wild-type *rps12* gene was inserted into a non-essential *psbAI* locus in the *rps12-R43* background. For this purpose, pEXE1, carrying the *psbAI* 5' and 3' flanking sequences with a precise deletion of the D1 protein coding sequence, was used to clone the wild-type *rps12* gene with its own promoter or the *rps12* ORF fragment followed by the *kan* gene, generating the gene disruption plasmids pEXR11, pEXR12 and pEXR21 (Table 1). These plasmids were linearized by *Bam*HI digestion prior to transformation of strain GRPS1 to facilitate two-point homologous recombination (see Fig. 2a) rather than a single-point insertion of the whole plasmid into the *psbAI* locus.

The results presented in Table 3 show that *Km^r* transformants of strain GRPS1 were *Str^s* using pEXR21, while *Str^s* and *Str^r* phenotypes segregated among transformants using pEXR11 and pEXR12. The latter phenomenon could be explained by intrachromosomal recombination leading to gene conversion of the wild-type *rps12* to the *rps12-R43* allele because even *Km^r Str^s* transformants spontaneously segregated *Str^r* progeny at frequencies of 1.1×10^{-5} – 5.4×10^{-5} . In addition, Southern hybridization of both *Str^s* and *Str^r* transformants showed that they were in fact merodiploids,

exhibiting two *rps12* bands, the 3.6 kb *Hind*III fragment of which corresponds to the *rps12* gene inserted at the *psbAI* locus (Fig. 1). We concluded that the *Str^r rps12-R43* allele is recessive to the *Str^s* wild-type *rps12* gene, and that the dominant *Str^s rps12* gene inserted at the *psbAI* locus is liable to be converted into the resistant allele spontaneously. This premise was confirmed by the direct sequencing of PCR fragments of the *psbAI* region, which showed that an *Str^r* merodiploid carried an *rps12-R43* allele at the *psbAI* locus (data not shown).

Gene replacement in *rps12* merodiploids triggered by non-replicating template plasmids

Since *rps12-R43/rps12* merodiploids exhibit a *Str^s* phenotype due to the presence of the dominant wild-type *rps12* gene inserted at the *psbAI* locus (Fig. 2b, top), removal of the wild-type *rps12* gene from the merodiploids should restore the *Str^r* phenotype inherent from the *rps12-R43* allele. Gene replacement at the *psbAI* locus was initiated by introducing appropriate template DNAs bearing terminal regions of homology with *psbAI* 5' and 3' flanking sequences, thus inducing homologous recombination via double crossing-over (Fig. 2b, bottom). In principle, such a recombinant with a successful gene replacement can be detected by their *Str^r* as well as kanamycin-sensitive (*Km^s*) phenotype because of the simultaneous elimination of the *kan* gene (Fig. 2c). On the other hand, spontaneous gene conversion of the

Table 4. Gene replacement in *rps12* merodiploids by transformation with template plasmids

Plasmid (amount used)*	Recipient strain	Number of Str ^r colonies	Number of Km ^r colonies	Frequency of Str ^r clones per Km ^r population†	Number of Km ^s transformants per Str ^r clone tested‡
pEXGFP (1 µg)	GRPS11	1.54×10^4	1.36×10^8	1.1×10^{-4}	3/96
pEXGFP (2 µg)/ <i>EcoRI</i> + <i>SphI</i>		1.38×10^4	1.20×10^8	1.2×10^{-4}	0/96
pEXE1 (1 µg)		2.40×10^4	1.85×10^8	1.3×10^{-4}	0/96
pEXE1 (2 µg)/ <i>Bam</i> HI		2.40×10^4	2.59×10^8	9.3×10^{-5}	0/96
pEXGFP (1 µg)	GRPS21	1.56×10^3	7.32×10^8	2.1×10^{-6}	3 (1)/96
pEXGFP (2 µg)/ <i>EcoRI</i> + <i>SphI</i>		1.30×10^3	5.26×10^7	2.5×10^{-5}	0/96
pEXE1 (1 µg)		4.97×10^3	2.23×10^8	2.2×10^{-5}	1 (0)/96
pEXE1 (2g)/ <i>Bam</i> HI		3.42×10^3	1.97×10^8	1.7×10^{-5}	0/96
pUC3032 (0.5 µg)	GRPS11	NA	NA	NA	3/100
	GRPS21	NA	NA	NA	16/100

* Plasmids were used as closed circular molecules or digested with restriction enzyme(s) prior to electroporation.

† Numbers represent single measurements.

‡ Km^s phenotypes of 96 and 100 randomly picked Str^r colonies were tested in liquid and solid BG-11 medium containing kanamycin, respectively. In the case of transformation of GRPS21, two and one Str^r Km^s recombinants obtained by using pEXGFP and pEXE1, respectively, were heterogenotes segregating Km^r cells in subcultures and were therefore subtracted, as shown by the numbers in parentheses.

NA, Not applicable because transformants were selected by Cm^r phenotype, and then Str^r clones from a plasmid-bearing transformant were recovered.

rps12 gene at the *psbAI* locus into the *rps12-R43* allele will lead to Str^r merodiploid cells that are still Km^r (Fig. 2d). Thus, products of true gene replacement can be distinguished from those of gene conversion by their kanamycin phenotype among the Str^r population.

At first, we employed plasmids unable to replicate in cyanobacteria as template DNAs to replace the *psbAI* gene with foreign sequences. One of the template plasmids, pEXGFP, was used to precisely replace the *psbAI* ORF with the *gfp* ORF encoding a green fluorescent protein (GFPuv), whereas pEXE1 bearing the *psbAI*ΔORF gene was employed to introduce a precise deletion of the *psbAI* ORF. Two Str^r merodiploid strains, GRPS11 and GRPS21 (Tables 1 and 3), were selected and electroporated with pEXGFP or pEXE1. The results of four independent experiments showed that the frequencies of Str^r Km^s recombinants were low, reaching a maximum of only 3/96 using 1 µg intact plasmid (Table 4, last column). No Str^r Km^s recombinant was detected among 96 Str^r colonies using 2 µg linearized plasmid.

The seven Str^r Km^s recombinant clones obtained above were analysed for their chromosomal structure at the *psbAI* locus by PCR using primers that amplify DNA segments from outside the *psbAI* ORF (Fig. 3). Six Str^r Km^s recombinants obtained by using pEXGFP (Fig. 3, lanes 4–9) gave rise to 1.3 kb DNA bands corresponding to the *psbAI*::*gfp*ORF, while a 0.6 kb band corresponding to the deletion of *psbAI* ORF was amplified from one Str^r Km^s recombinant obtained by using

pEXE1 (Fig. 3, lane 10). These PCR fragment sizes are in good agreement with the expected gene replacement products at their *psbAI* loci. When these Str^r Km^s recombinants with potential gene replacement were analysed further by Southern hybridization, the *rps12* or *rps12* ORF insertions at the *psbAI* locus were eliminated in seven recombinants and replaced by *gfp* ORF fragments in six recombinants (Fig. 4).

Heterogenote formation in the *psbAI* locus

Although the above results of PCR and Southern hybridization showed that the gene replacement was feasible utilizing the *rps12* merodiploid strains and the appropriate template plasmids, a possibility still remained that the *psbAI* locus might be heterogeneous in some of the Str^r Km^s transformants. Careful inspection of Southern hybridization results disclosed that traces of hybridizing bands were present at 4.4 or 4.2 kb positions in a few transformants (e.g. Fig. 4a, lanes 10 and 11). Should the *kan* gene remain inserted at a fraction of *psbAI* loci, they would revive some Km^r cells. Indeed, three out of seven recombinants described above (Table 4) segregated a small number of growing colonies on BG-11 medium containing kanamycin after prolonged incubation. Consequently, these three recombinants were regarded as heterogenotes at the *psbAI* locus containing both the replaced (*gfp*ORF or *psbAI*ΔORF) and unreplaced (*rps12*ORF-*kan*) insertions. PCR tests also disclosed the presence of the *kan* gene in their chromosomal DNAs (data not shown). In con-

Fig. 3. PCR analysis of cyanobacterial recombinants with potential gene replacement at their *psbAI* loci. Chromosomal DNAs from *Str^r* *Km^s* recombinants obtained from *rps12* merodiploid strains were analysed by PCR using primers 13 and 14, which anneal to the 5' and 3' non-coding regions of the *psbAI* gene, respectively. Amplified DNAs were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. Lanes: 1 and 11, *HindIII*-cut λ DNA marker; 2, GRPS1; 3, GRPS21; 4–6, *Str^r* *Km^s* transformants from GRPS11 obtained with pEXGFP; 7–9, *Str^r* *Km^s* transformants from GRPS21 obtained with pEXGFP; 10, *Str^r* *Km^s* transformant from GRPS21 obtained with pEXE1. In lanes 4–9, 1.5 kb bands of unknown origin were seen in addition to the expected 1.3 kb bands. Sizes of the amplified DNA bands are indicated on the right.

clusion, transformation of the *rps12* merodiploid strains with non-replicating template plasmids finally resulted in four *Str^r* *Km^s* homogenote recombinants, in which all copies of the *psbAI* ORF were replaced by the *gfp* ORF.

Functional expression of the *gfp* ORF integrated at the *psbAI* locus

To investigate whether or not the gene replacement at the *psbAI* locus led to expression of the *gfp* ORF under the control of the constitutive *psbAI* promoter, we analysed GFPuv protein in cyanobacterial cells under a fluorescence microscope. GFPuv absorbs ultraviolet light at 360–400 nm, and emits green fluorescence with a maximum at 509 nm (Cramer *et al.*, 1996). Since cyanobacterial chlorophylls also absorb ultraviolet light, and emit red fluorescence with a maximum at 668 nm, only emission in the range 510–550 nm was visualized through an absorption filter. Moreover, the intracellularly emitted light might have been quenched by various absorbing pigment molecules. Nonetheless, the above cyanobacterial recombinants with a *psbAI::gfpORF* genotype, as well as a control strain GLC1 (Table 1) with a *psbAI::gfpORF-nak* genotype did emit the dim fluorescence which was absent in the parental GRPS1 strain (data not shown). We also detected the expression of green fluorescent protein by Western blotting of cell-free extracts from the recombinant cyanobacterial strains (data not shown). These data clearly demonstrated that the *gfp* ORF inserted at

Fig. 4. Genomic Southern hybridization of gene replacement recombinants obtained with non-replicating template plasmids. Lanes 2–11 contain 5 µg *Scal*-digested chromosomal DNA. Hybridization was carried out with $5 \times$ SSC at 65 °C using either (a) an *rps12* ORF probe, or (b) a *gfp* ORF probe. Lanes: 1 and 12, digoxigenin-labelled, *HindIII*-cut λ DNA marker; 2, GRPS1; 3, GRPS11; 4, GRPS21; 5–7, potential *psbAI::gfpORF* gene replacement recombinants from GRPS11; 8–10, potential *psbAI::gfpORF* gene replacement recombinants from GRPS21; 11, potential *psbAI*ΔORF recombinant from GRPS21. Sizes of hybridizing bands are indicated on the right. The 4.4 and 4.2 kb bands in lanes 3 and 4 correspond to *psbAI::rps12* and *psbAI::rps12ORF* fragments, respectively.

the *psbAI* locus is intact and functional after gene replacement.

Transformation of *rps12* merodiploids with replicating template plasmids

The frequency at which gene replacement products are established from *rps12* merodiploids depends upon the availability of template DNA within the cyanobacterial cells. When a template plasmid replicates after incorporation into a cell to supply multiple copies of templates, then the probability of homologous recombination between the plasmid DNA and the chromosomal DNA should be elevated as compared with a non-replicating template plasmid which is diluted out during cell propagation. To test the above hypothesis, a pUC303-derived plasmid, pUC3032 (Table 1), carrying the *psbAI*ΔORF gene and cyanobacterial replication machinery was constructed. pUC3032 is devoid of the plasmid-borne *Sm^r* gene but carries the *Cm^r* gene and *repAB* genes necessary for autonomous replication in PCC 7942 or its cognate strains lacking endogenous pUH24 (van der Plas *et al.*, 1992). This plasmid was used to introduce a deletion of the *psbAI* gene ORF by gene replacement in the *rps12* merodiploid strains.

As shown in the last row of Table 4, transformation of strains GRPS11 and GRPS21 using pUC3032 resulted in

Fig. 5. Southern hybridization of recombinant cyanobacteria from *rps12* merodiploids obtained after transformation with replicating template plasmid. All the chromosomal DNAs (5 µg) were cut by *ScaI*. A digoxigenin-labelled 625 bp fragment consisting of joined *psbAI* 5' and *psbAI* 3' non-coding sequences was prepared by PCR with pEXE1 as a template using primers 13 and 14, and was used as a probe. Hybridization was carried out in 5 × SSC at 65 °C. Lanes: 1, digoxigenin-labelled, *HindIII*-cut λ DNA marker; 2, GRPS1; 3, GRPS11; 4, GRPS21; 5–7, potential *psbAI*ΔORF recombinants from GRPS11; 8–10, potential *psbAI*ΔORF recombinants from GRPS21. Sizes of hybridizing DNA bands are shown on the right.

three and sixteen recombinants exhibiting the Cm^r Str^r Km^s phenotype from GRPS11 and GRPS21, respectively, among 100 Str^r clones tested. These recombinants were homogenotes as judged from their inability to grow on BG-11 medium containing kanamycin. The apparent frequencies of Km^s recombinants among Str^r clones (3 and 16 %) using a replicating plasmid were improved in comparison with those (less than 3 %) using non-replicating plasmids.

Three clones of each Cm^r Str^r Km^s recombinant from GRPS11 and GRPS21 were cured for their pUC3032 (see Methods), and one chloramphenicol-sensitive (Cm^s) strain was recovered from each clone. When these Cm^s Str^r Km^s recombinant strains were analysed by PCR using the same primers as in Fig. 3, DNA fragments of an expected size (0.6 kb) were amplified (data not shown). Southern hybridization of *ScaI*-digested chromosomal DNA from the above six cured strains using a probe specific for the *psbAI* 5' and 3' non-coding sequences detected the same 2.6 kb bands corresponding to a 1.08 kb deletion of the *psbAI* gene ORF, as compared with a 3.7 kb band in GRPS1 and 4.4/4.2 kb bands in strains GRPS11 and GRPS21, depending on the insertions (*rps12-nak* or *rps12ORF-kan*) which they carry at the *psbAI* locus (Fig. 5). This result provides direct evidence that the *psbAI*ΔORF gene is transferred from a replicating plasmid to the chromosomal *psbAI* locus with a concomitant loss of *rps12* and *kan* genes at the same locus.

DISCUSSION

Since the elucidation of the lethal effect of streptomycin on 30S ribosomes as causing erroneous protein synthesis (Gorini, 1974), mutational sites in Str^r mutants of

various organisms have been assigned to either the 30S ribosomal subunit protein S12 (Funatsu & Wittmann, 1972; Liu *et al.*, 1989; Timms *et al.*, 1992; Finken *et al.*, 1993) or 16S rRNA (Montandon *et al.*, 1985; Melancon *et al.*, 1988; Powers & Noller, 1991; Finken *et al.*, 1993). The same amino acid substitutions as found here have been reported in some Str^r mutant S12 proteins from *E. coli* (Funatsu & Wittmann, 1972; Timms *et al.*, 1992) and *Mycobacterium tuberculosis* (Finken *et al.*, 1993) at Lys-43 as well as Lys-88, both of which are phylogenetically conserved. The small size and well-defined Str^r mutational sites of the *rps12* gene imply a universal applicability of *rps12*-mediated gene replacement in other organisms amenable to transformation.

The dominant nature of the wild-type *rps12* gene over the *rps12-R43* allele was demonstrated unambiguously by the Str^s phenotype of merodiploid strains bearing the *rps12-R43* allele and the *rps12* gene inserted at the *psbAI* locus. The difference in the ability of promoters to express the ectopic *rps12* gene, i.e. the natural *rps12* promoter or the *psbAI* promoter, did not influence the apparent Str^s phenotype of the merodiploids, although the *psbAI* promoter seemed stronger than the *rps12* promoter. However, *rps12* gene and *rps12* ORF sequences have different tendencies to suffer gene conversion. Thus, the spontaneous segregation rate of Str^r progeny was about seven times higher in the GRPS11 merodiploid carrying the *rps12* gene than in the GRPS21 merodiploid carrying the *rps12* ORF (see Table 4, fifth column). Although its basis is not known exactly, this difference in gene conversion frequencies might arise from a site-specific recombination at a highly iterated palindromic sequence (HIP1), 5'-GCGATCGC-3', which has been found in many cyanobacteria (Robinson *et al.*, 1995, 1997; Akiyama *et al.*, 1998) and is present once in the promoter region of the 579 bp *rps12* gene but absent from the 375 bp *rps12* ORF. We therefore postulate that *rps12* merodiploids would be a good experimental system to study mechanistic details of intrachromosomal gene conversion in cyanobacteria.

The present study established a novel technique utilizing a dominant Str^s *rps12* gene to replace any non-essential chromosomal sequences in cyanobacteria with desired insertions, substitutions or deletions, yet generating the final mutants free from foreign drug resistance markers. Essentially, this method consists of an elimination of the chromosomally integrated *rps12* gene via homologous recombination with externally added template DNA. A similar strategy was employed previously to select plasmid-to-chromosome gene replacement in *E. coli* *recD rpsL* (*str*) mutants (Russell & Dahlquist, 1989). In *E. coli*, however, a strong exonucleolytic activity conferred by the *recBCD* gene product makes linear DNA fragments impractical for use as template DNA, and, therefore, it is still mandatory to use a *recBCD*-deficient mutant for efficient gene replacement (Murphy, 1998). Although cyanobacteria do not seem to degrade linear DNA so extensively, linearized template DNAs were much less effective than circular DNAs for gene replacement. Taking it for granted that *recA*-mediated

homologous recombination requires sufficient length of sequences flanking the sequence to be integrated, we employed template plasmids carrying the *psbAI* 5' and 3' non-coding sequences extending to more than 500 bp. Whether or not a specific sequence such as HIP1 is required for high frequency of recombination is not known, although both *psbAI* 5' and 3' flanking sequences used here contain at least one HIP1 site (unpublished results).

Compared with the haploid genome of *E. coli*, the polyploid nature of the cyanobacterial genome poses an additional difficulty in isolating homogenote recombinants. Available data indicate that the mean copy number of chromosomes per cell in *Synechococcus* spp. PCC 6301 and PCC 7942 is four to six, depending on growth rate and temperature (Binder & Chisholm, 1990). Accordingly, several rounds of successive replacements at *psbAI* loci in a polyploid genome should be necessary to establish homogenote recombinants. Considering the transient maintenance of non-replicating plasmids after incorporation into the cell, it is somewhat surprising that gene replacement was achieved at a frequency up to 3% of the Str^r progeny using a circular form of a non-replicating plasmid. This frequency corresponds to about 3×10^{-6} per cell, a value not impractical to screen for recombinants among 10^8 cells.

When the template plasmids replicate, gene replacement was induced via three steps: first, transformation with the Cm^r plasmid; second, induction of recombination to give Str^r Km^s progeny; and finally, curing of the plasmid. This procedure has an advantage in the availability of multiple copies of template plasmids in all transformant cells, resulting in an increased frequency of gene replacement of up to 16% among survivors. Although the curing of the plasmid is an added labour, most of the shuttle vector plasmids currently available in cyanobacteria are unstable and therefore could be removed relatively easily.

The ease with which gene replacements were screened is an urgent requirement for routine work with mutant constructions. So far, a conditional lethal *sacB* gene encoding levansucrase can cause death of host cells of Gram-negative bacteria on sucrose-containing media (Gay *et al.*, 1985), and has been utilized as a tool for construction of gene replacement mutants in a plant pathogen, *Erwinia chrysanthemi* (Ried & Collmer, 1987), and a filamentous cyanobacterium, *Anabaena* sp. PCC 7120 (Cai & Wolk, 1990). The *sacB* gene, however, suffers inactivation through spontaneous mutations, leading to false recombinants. In the *rps12*-mediated gene replacement, segregation of false Str^r recombinants from the *rps12/rps12-R43* merodiploids by gene conversion is unavoidable when using the 100% homologous *rps12* gene in the *recA*⁺ background. However, we expect to suppress the gene conversion frequency in merodiploids by using an *rps12* gene homologue such as the *E. coli rpsL* gene or by using a mutant strain deficient in an as yet unidentified gene conversion system. There is a tenable experimental basis for the heterologous expression of S12 proteins in different hosts (Liu *et al.*,

1989). We are at present exploring the feasibility of employing heterologous *rps12* genes to obtain cyanobacterial merodiploids with a stable Str^s phenotype.

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