A cyanobacterial strain with all chromosomal rRNA operons inactivated: a single nucleotide mutation of 23S rRNA confers temperature-sensitive phenotypes

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The presence of a multicopy chromosome, with each copy containing two rRNA operons (*rrnA* and *rrnB*), has been an obstacle to analysing mutated rRNA in *Synechococcus* PCC 7942. To create a system for expressing homogeneous mutated rRNA, the chromosomal *rrn* operons were sequentially inactivated and a final strain was successfully obtained with all the chromosomal *rrn* operons inactivated but carrying a replaceable multicopy plasmid containing a single *rrn* operon. The lag time required for growth response on dark/light shift of mutant strains with chromosomal *rrnA* or *rrnB* inactivated was increased 50 % over that of the wild-type strain; however, the presence of the plasmid-borne *rrn* operon restored the lag time. The doubling time of mutant strains carrying only a functional *rrnB* operon, but not strains carrying only a functional *rrnA* operon, was significantly longer than that of the wild-type strain. A strain in which essentially all the cellular 23S rRNA contained the mutation C2588A was temperature sensitive at 16 °C and 45 °C. Position C2588 is equivalent to C2611 of the peptidyltransferase centre in domain V of *Escherichia coli* 23S rRNA.

INTRODUCTION

Cyanobacteria are prokaryotic micro-organisms that carry a complete set of genes for higher-plant-like oxygenic photosynthesis (Nakamura *et al.*, 1998). *Synechococcus* PCC 7942 is a prime example of photoautotrophic cyanobacteria. *Synechococcus* PCC 7942 (NZ_AADZ000000000) and *Synechococcus* PCC 6301 (NC_006576) possess nearly identical genomic sequences and therefore are considered as the same species. Results obtained with *Synechococcus* PCC 7942 or PCC 6301 would most likely be applicable to both strains. *Synechococcus* PCC 7942 and PCC 6301 (hereafter referred to as *Synechococcus*) have approximately 10 copies of the chromosome (Binder & Chisholm, 1990; Mori *et al.*, 1996). Each chromosome contains two rRNA operons, *rrnA* and *rrnB* (Tomioka & Sugiura, 1984), which are separated by 600 kb and transcribed oppositely (Kaneko *et al.*, 1996). The *rrnA* and *rrnB* operons have 100 % identical coding sequences organized in the following order: tandem promoters (P1, P2 and P3) (Asato, 2003), 16S gene, tRNA^{Ile} gene, tRNA^{Ala} gene, 23S gene, 5S gene and terminator region. Many bacteria also possess multiple *rrn* operons; for example there are seven *rrn* operons in *Escherichia coli* (Kiss *et al.*, 1977), ten in *Bacillus subtilis* (LaFauci *et al.*, 1986) and six in *Lactococcus lactis* (Tulloch *et al.*, 1991).

rRNAs play an important role in protein synthesis. A growing number of regions in both 16S and 23S rRNA have

been identified as having specific functions (for a review, see Green & Noller, 1997). It has been shown that 23S rRNA is a ribozyme that catalyses the peptidyltransferase step of protein synthesis (Nissen *et al.*, 2000). In *Synechococcus*, rRNA synthesis is stimulated by a light-activated DNA-binding factor in the light but not in the dark (Asato, 1998). However, current knowledge concerning the structure–function relationship of rRNA in cyanobacteria is still limited, due to the lack of a genetic system for mutational analysis. The presence of a multicopy chromosome in cyanobacteria has restricted the mutational analysis of rRNA and the *in vivo* production of pure mutant ribosome populations. Genetic systems for expressing homogeneous mutated rRNA have been developed in the yeast *Saccharomyces cerevisiae* (Chernoff *et al.*, 1994) and in *E. coli* (Asai *et al.*, 1999).

In this work, we describe a strategy for developing a genetic system for expressing homogeneous mutated rRNA in cyanobacteria. We sequentially constructed and characterized *Synechococcus* mutant strains with chromosomal *rrnA* or *rrnB* inactivated and a final strain with all chromosomal *rrn* operons inactivated but carrying a replaceable multicopy plasmid containing a single *rrn* operon. Here we report an application of our system. We constructed and characterized a *Synechococcus* strain in which essentially all cellular 23S rRNA contained the mutation C2588A encoded by a

plasmid. Position C2588 is equivalent to C2611 of the peptidyltransferase centre in domain V of *E. coli* 23S rRNA.

METHODS

Strains, growth conditions and gene transfer procedures.

E. coli strains DH5 α (Hanahan, 1983) and RL443 (Elhai & Wolk, 1988b) were grown in LB broth or on agar (Sambrook & Russell, 2001). *Synechococcus* PCC 7942 strain R2-SPc (Kuhlemeier *et al.*, 1983) was grown in liquid or on solid (1.5% Difco Bacto Agar) BG-11 medium (Williams, 1988) under constant illumination of 3000 lx (i.e. 38 $\mu\text{E m}^{-2} \text{s}^{-1}$). *Synechococcus* cell growth was monitored as OD₇₃₀. The OD₇₃₀ of cell suspensions was linearly related to cell density (determined by flow cytometry) over the range of values used in the experiments (OD₇₃₀ < 0.55). The doubling time of exponentially growing cultures at 30 °C was calculated from OD₇₃₀ data. *Synechococcus* was transformed as described by Kuhlemeier *et al.* (1983). Triparental conjugation, used to transfer plasmid into *Synechococcus*, was performed according to Elhai & Wolk (1988b). Antibiotics were added at the following concentrations when

required: 10 μg chloramphenicol (Cm) ml^{-1} , 20 μg erythromycin (Em) ml^{-1} , 10 μg kanamycin (Km) ml^{-1} and 50 μg spectinomycin (Spc) ml^{-1} .

Construction of chromosomal *rrn*-inactivated strains. In order to construct strains harbouring $\Delta rrnA::\text{Spc}^R$ (designated A1) and $\Delta rrnB::\text{Cm}^R$ (designated B1), pDA-Spc and pDB-Cm were linearized with *Nde*I and separately transformed into *Synechococcus* wild-type strain (WT). Since *Synechococcus* contains multiple copies of the chromosome, the transformants containing heterogeneous chromosomal *rrn* operons (i.e. wild-type and inactivated *rrn* operons) were segregated on BG-11 agar containing Spc at 50–150 $\mu\text{g ml}^{-1}$ or Cm at 10–30 $\mu\text{g ml}^{-1}$. The resulting A1 and B1 strains harbouring homogeneous chromosomal *rrn* operons were transformed with pRN-A or pRN-B to obtain strains A2 ($\Delta rrnA::\text{Spc}^R/\text{pRN-A}$), A3 ($\Delta rrnA::\text{Spc}^R/\text{pRN-B}$) and B2 ($\Delta rrnB::\text{Cm}^R/\text{pRN-B}$). Strain A3 was transformed with *Nde*I-linearized pDB-Cm to obtain strain AB1 ($\Delta rrnA::\text{Spc}^R \Delta rrnB::\text{Cm}^R/\text{pRN-B}$). The resulting strains harbouring homogeneous inactivated *rrn* operon(s) with or without plasmid-borne *rrn* operon are shown in Table 1.

Southern blot analysis of chromosomal *rrn*-inactivated strains. Total DNA of *Synechococcus* was isolated as described by

Table 1. Plasmids and *Synechococcus* strains

Plasmid or strain	Description or genotype	Reference
Plasmids		
pHP45 Ω	Source of Spc^R cassette	Prentki & Krisch (1984)
pUC303	Source of Cm^R cassette	Kuhlemeier <i>et al.</i> (1983)
pDA-Spc	pUC18 containing a 2.0 kb Spc^R cassette flanked by 1.4 kb PCR product (primers AF1 and 16R1) of upstream <i>rrnA</i> and 1.8 kb PCR product (primers 23F2 and 5R1) of 5' end of 5S gene, $\text{Ap}^R \text{Spc}^R$, <i>rrnA::\text{Spc}^R</i>	This work
pDB-Cm	pUC18 containing a 1.0 kb Cm^R cassette flanked by 2.0 kb PCR product (primers BF1 and 16R1) of upstream <i>rrnB</i> and 1.8 kb PCR product (primers 23F2 and 5R1) of 5' end of 5S gene, $\text{Ap}^R \text{Cm}^R$, <i>rrnB::\text{Cm}^R</i>	This work
pKG	<i>E. coli</i> - <i>Synechococcus</i> shuttle vector, Km^R	Chungjatupornchai <i>et al.</i> (1999)
pRN-A	pKG containing a 6.5 kb PCR product (primers AF1 and AR1) of full-length <i>rrnA</i> replacing the β -glucuronidase gene, Km^R , <i>rrnA</i>	This work
pRN-B	pKG containing a 6.3 kb PCR product (primers BF2 and BR1) of full-length <i>rrnB</i> replacing the β -glucuronidase gene, Km^R , <i>rrnB</i>	This work
pKT210	RSF1010-derived broad-host-range plasmid, $\text{Cm}^R \text{Str}^R$ (kindly provided by C. P. Wolk, Michigan State University, USA)	Elhai & Wolk (1988a)
psbV/EmR	Source of Em^R gene (kindly provided by J. J. Eaton-Rye, University of Otago, New Zealand)	Morgan <i>et al.</i> (1998)
pKT-A	pKT210 containing a 6.5 kb PCR product (primers AF1-P and Nos-R) of full-length <i>rrnA</i> including Nos-ter, Cm^R gene replaced by an Em^R gene, $\text{Em}^R \text{Str}^R$, <i>rrnA</i>	This work
pKT-A ^{C2611A}	pKT-A containing C2611A mutation in 23S rRNA gene, $\text{Em}^R \text{Str}^R$, 23S ^{C2611A}	This work
Strains		
WT	Wild-type, <i>Synechococcus</i> PCC 7942 strain R2-SPc, <i>rrnA rrnB</i>	Kuhlemeier <i>et al.</i> (1983)
A1	WT $\Delta rrnA::\text{Spc}^R$	This work
A2	A1/pRN-A	This work
A3	A1/pRN-B	This work
B1	WT $\Delta rrnB::\text{Cm}^R$	This work
B2	B1/pRN-B	This work
AB1	WT $\Delta rrnA::\text{Spc}^R \Delta rrnB::\text{Cm}^R/\text{pRN-B}$	This work
AB2	WT $\Delta rrnA::\text{Spc}^R \Delta rrnB::\text{Cm}^R/\text{pKT-A}$	This work
AB3	WT $\Delta rrnA::\text{Spc}^R \Delta rrnB::\text{Cm}^R/\text{pKT-A}^{\text{C2611A}}$	This work

Draper & Scott (1988). Three micrograms of total DNA was digested with *Pst*I, electrophoresed on agarose gel and transferred to a nylon membrane (Hybond-N⁺, Amersham Biosciences). Probe I (485 bp) and probe II (490 bp) were amplified using primer sets 16F1 and 16R2; 23F1 and 23R1, respectively (Table 2, Fig. 1), and labelled with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche Applied Science). The hybridized membrane was treated with antidigoxigenin-alkaline phosphatase Fab fragments and luminescent substrate CSPD as recommended by the manufacturer (Roche Applied Science). The treated membrane was exposed to X-ray film.

Plasmid replacement in the strain with all chromosomal *rrn* operons inactivated. To generate plasmids containing site-directed mutation at nucleotide position C2588, equivalent to C2611 in *E. coli* numbering (hereafter referred to as nt 2611), the procedure was carried out as described in the QuickChange Mutagenesis Kit (Stratagene), using pKT-A as template and appropriate primer sets including CAF and CAR. The mutated C2611A/G/T in the resulting plasmids was confirmed by DNA sequencing. The cargo plasmids pKT-A, pKT-A^{C2611A}, pKT-A^{C2611G} and pKT-A^{C2611T} were separately transferred into strain AB1 by triparental conjugation. Em^R-Km^R conjugants harbouring both cargo plasmid and pRN-B were obtained. Replacement was enforced by segregating the conjugants on BG-11 containing Em to obtain Em^R-Km^S clones harbouring only cargo plasmid but not pRN-B. The resulting clones harbouring pKT-A and pKT-A^{C2611A} were designated AB2 and AB3, respectively. The presence of pKT-A and pKT-A^{C2611A} was confirmed by PCR and RT-PCR. The PCR was carried out using total DNA, with primers 23F3 and 23R3. The RT-PCR was performed essentially as described previously (Chungiatupornchai *et al.*, 2002; Plansangkate *et al.*, 2004). The cDNA of the 23S rRNA gene, synthesized from total RNA with primer 23R3, was used as template for amplification of PCR product using primers 23F3 and 23R3. The resulting PCR and RT-PCR products of 1.6 kb were digested with *Acl*I to generate fragments of 630 and 1002 bp. The 1002 bp fragment was further analysed by *Sau*96I digestion (see Results). The digested DNA was resolved in a 4.5 % agarose gel containing ethidium bromide. An

image of the gel was captured with UVP (Life Science). The mutated C2611A of PCR and RT-PCR products was confirmed by automated sequence analysis (Perkin-Elmer, ABI Prism 3100).

RESULTS

Inactivation of *Synechococcus* chromosomal *rrn* operons

We previously identified the upstream and downstream chromosomal flanking sequences of the *rrnA* and *rrnB* operons using the genome walking method. The resulting sequences, identical to parts of currently reported *Synechococcus* genome sequences (AADZ00000000 and NC_006576) (data not shown), were used to design primers for PCR to clone the full-length *rrnA* and *rrnB* operons. The two operons have identical coding sequences containing three rRNA genes (16S, 23S and 5S) and two tRNA genes (for Ile and Ala) (see Fig. 1). *Synechococcus* strains with the chromosomal *rrn* operons inactivated were sequentially constructed (see Methods). The chromosomal *rrnA* and *rrnB* operons were inactivated by deletion-insertion mutagenesis using cassettes encoding Spc and Cm resistance, respectively (Fig. 1). The resulting *Synechococcus* strains harbouring homogeneous inactivated *rrn* operon(s) with or without plasmid-borne *rrn* operon are shown in Table 1. Southern blot analysis using probes specific to regions of the 16S and 23S genes confirmed that strains with expected genotypes were obtained (Fig. 2). The intensity of hybridized bands was determined by densitometry. The results indicated that the ratio of a chromosomal *rrn* operon (either *rrnA* or *rrnB*) to a plasmid-borne *rrn* operon (either pRN-A or pRN-B)

Table 2. Primers

Locations of *rrn* primers are indicated in Fig. 1. The restriction sites *Eco*RI, *Bam*HI, *Pst*I and *Sal*I are underlined.

Primer	Sequence (5'–3')	Target sequence
16F1	GGTAGGGGTAGCGGGAATTC	16S
16R1	CGGGATCCTGTAGGCAGATTCTACGC	16S
16R2	GTGGGTTCGCTCGTTGCG	16S
23F1	GAGGATTTCGAATGGGGC	23S
23F2	CGGGATCCTGCGCCGAAAATGAACG	23S
23F3	AGGAGGTGGGAGTGCATAGACAACCAGG	23S
23R1	CGGCTCATTCTTCAACAGGC	23S
23R3	TTCTCCCCGGTCCTCTCGTACTAGGGAG	23S
5R1	CGGAATTCAGAGTGGTTCCATACCGCC	5S
AF1	ACGCGTCGACTCAAGATTGCGCGGAC	Upstream of <i>rrnA</i>
AF1-P	AAAACTGCAGACTCAAGATTGCGCGGAC	Upstream of <i>rrnA</i>
AR1	CGGGATCCGCAGAGGATTGGCTATTGG	Downstream of <i>rrnA</i>
BF1	AAAACTGCAGCTGCATTGCTCGGTGCG	Upstream of <i>rrnB</i>
BF2	ACGCGTCGACCGCACAGAGCTGTTTGAC	Upstream of <i>rrnB</i>
BR1	CGGGATCCGGGATAGTAACGCCTTTCCC	Downstream of <i>rrnB</i>
Nos-R	AAAACTGCAGATCTAGTAACATAGATGAC	Nos-ter in pKG
CAF	CGTGAGACAGTTCGGTCAATATCCGGTGCAGG	C2611A mutation
CAR	CCTGCACCGGATATTGACCGAACTGTCTCAGC	C2611A mutation

Fig. 1. Deletion mutations introduced into the *Synechococcus* chromosomes. The physical maps of chromosomal *rrn* operons including the promoters (P) and terminator (ter) are shown. The numbers of nucleotides are with respect to the +1 transcription start site of the *rrn* operon. Locations of primers and probes used in this study are indicated. Deletion regions of *rrnA* and *rrnB* operons were replaced with spectinomycin (*Spc*^R) and chloramphenicol (*Cm*^R) resistance gene cassettes, respectively.

Fig. 2. Southern blot analysis of chromosomal *rrn*-inactivated strains. The total DNA of *Synechococcus* (3 µg) digested to completion with *Pst*I was hybridized with DIG-labelled probes (see Fig. 1). The unlabelled probes at 1 and 3 ng (lanes 1 and 3, respectively) were used as control. (a) Inactivation of the 16S rRNA gene. The DNA was hybridized with probe I. (b) Inactivation of the 23S rRNA gene. Probe I was removed from the membrane shown in (a), and the DNA was rehybridized with probe II.

was 1 : 3 in strains A2, A3 and B2 (Fig. 2). The band intensity ratio of 1 and 3 ng of unlabelled probes used as control was also 1 : 3. We obtained the final strain AB1 with all chromosomal *rrn* operons inactivated but carrying the replaceable multicopy plasmid pRN-B containing a single *rrnB* operon (Fig. 2).

Growth response of *rrn*-inactivated strains on dark/light shift

Strains A1 and B1, harbouring an inactivated *rrnA* or *rrnB* operon respectively, were viable, indicating that the remaining one chromosomal *rrn* operon was enough for cell survival. To investigate the growth response on dark/light shift, exponential cultures were incubated in the dark and then re-exposed to the light. It has been shown that a shift from light to darkness will cause all *Synechococcus* cells to arrive at a common rest point and that upon returning to light, the cells start to grow from the common rest point (Asato, 2003). The lag time that was required to initiate cell growth upon returning to light was determined. The lag time of strains A1 and B1 was 3 h as compared to 2 h for the WT, representing a 50 % increase; however, the presence of plasmid-borne *rrn* operon in strains A2 and B2 restored the lag time to 2 h (Fig. 3). The results indicated that the two chromosomal *rrn* operons were necessary for *Synechococcus* to adapt rapidly for initiating cell growth on dark/light shift.

Effect of *rrn* inactivation on doubling time of cell growth

The *Synechococcus* mutant strains used in this study (Table 1) survived with no apparent deleterious effects. In order to investigate the effect of *rrn* inactivation on cell

Fig. 3. Growth of chromosomal *rrn*-inactivated strains on dark/light shift. Exponential cultures of strains WT (wild-type), A1 ($\Delta rrnA::Spc^R$), A2 ($\Delta rrnA::Spc^R/pRN-A$), B1 ($\Delta rrnB::Cm^R$) and B2 ($\Delta rrnB::Cm^R/pRN-B$) were incubated in the dark for 14 h and then re-exposed to the light. Cell growth was measured as OD₇₃₀. The data are the means of three independent experiments. The maximum standard deviation was 0.02. At least two strains of each type were tested in each growth condition and found to behave similarly.

growth, the doubling time of the mutant strains was measured. This revealed that inactivation of chromosomal *rrnA* (strain A1) prolonged the doubling time (Table 3). The presence of plasmid-borne *rrnA* (strain A2), but not plasmid-borne *rrnB* (strain A3), restored the doubling time. In contrast, inactivation of chromosomal *rrnB* (strain B1) in the presence of plasmid-borne *rrnB* (strain B2) did not affect the doubling time. Similarly, the presence of only plasmid-borne *rrnB* in strain AB1 prolonged the doubling time, whereas only plasmid-borne *rrnA* in strain AB2 did not. The results suggested that the *rrnA* operon might produce a higher amount of rRNA and tRNA molecules

than the *rrnB* operon. In addition, the doubling time of strain AB3 producing C2611A-mutated 23S rRNA was significantly longer than those of the AB2 and WT strains (Table 3).

Expression of homogeneous C2611A-mutated 23S rRNA in the strain with all chromosomal *rrn* operons inactivated

We have successfully constructed the AB1 strain with all chromosomal *rrn* operons inactivated but carrying a replaceable plasmid pRN-B (Fig. 2). To apply our system for experimental analysis of mutated rRNA, we constructed plasmids containing the mutation at position 2588 with respect to the first nucleotide of the 23S rRNA transcript (Douglas & Doolittle, 1984). Position C2588, equivalent to C2611 of the peptidyltransferase centre in domain V of *E. coli* 23S rRNA, is shown in Fig. 4(a). Plasmids containing wild-type C2611 (pKT-A) or mutated C2611 of the 23S rRNA gene (pKT-A^{C2611A}, pKT-A^{C2611G} and pKT-A^{C2611T}) were transferred separately into strain AB1 by conjugation to replace pRN-B. The resulting Em^R-Km^S strains are AB2 and AB3, harbouring pKT-A and pKT-A^{C2611A}, respectively. Strains AB2 and AB3 accounted for 35 % of the conjugants. However, attempts to select for an Em^R-Km^S strain harbouring pKT-A^{C2611G} or pKT-A^{C2611T}, but not pRN-B, were unsuccessful. The presence of wild-type C2611 and C2611A-mutated 23S rRNA was confirmed by PCR, RT-PCR (Fig. 4b, c) and DNA sequencing (data not shown). The results indicated that strains AB2 and AB3 expressed homogeneous wild-type and C2611A-mutated 23S rRNA, respectively.

Growth response of C2611A-mutated 23S rRNA strain on temperature shifts

In order to study the effect of *Synechococcus* C2611A-mutated 23S rRNA on temperature shifts, cell growth of

Table 3. Doubling time of chromosomal *rrn*-inactivated strains

The doubling time of exponentially growing cultures at 30 °C was calculated from OD₇₃₀ data. The data are the means of three independent experiments with standard deviations in parentheses. At least two strains of each type were tested and found to behave similarly. Data marked with an asterisk represent doubling times significantly different from that of the WT strain ($P < 0.01$, two-tailed paired *t* test).

Strain	Chromosomal <i>rrn</i> operon		Plasmid-borne <i>rrn</i> operon	Doubling time (h)
	Inactivated	Functional		
WT	None	<i>rrnA</i> , <i>rrnB</i>	None	13.62 (0.58)
A1	<i>rrnA</i>	<i>rrnB</i>	None	15.61 (0.77)*
A2	<i>rrnA</i>	<i>rrnB</i>	<i>rrnA</i>	13.13 (0.56)
A3	<i>rrnA</i>	<i>rrnB</i>	<i>rrnB</i>	15.06 (0.79)*
B1	<i>rrnB</i>	<i>rrnA</i>	None	14.58 (0.82)
B2	<i>rrnB</i>	<i>rrnA</i>	<i>rrnB</i>	14.18 (0.56)
AB1	<i>rrnA</i> , <i>rrnB</i>	None	<i>rrnB</i>	16.00 (1.31)*
AB2	<i>rrnA</i> , <i>rrnB</i>	None	<i>rrnA</i>	14.62 (0.65)
AB3	<i>rrnA</i> , <i>rrnB</i>	None	<i>rrnA</i> ^{C2611A}	17.36 (1.47)*

Fig. 4. Analysis of C2611A-mutated 23S rRNA expressed in strain AB3. (a) Secondary structure of the peptidyltransferase centre in domain V of *Synechococcus* 23S rRNA (Douglas & Doolittle, 1984). Positions C2611 and G2057 of the *E. coli* numbering are indicated in parentheses. The circled and boxed nucleotides indicate the sequence polymorphisms of *E. coli* 23S rRNA and *Sacch. cerevisiae* mitochondrial 21S rRNA, respectively. The deletion polymorphism is indicated by a triangle. (b) The *Sau96I* restriction map of the region containing *Synechococcus* C2611. The presence of the C2611A mutation abolishes the *Sau96I* site as indicated by an arrow. (c) Image of a 4.5% agarose gel stained with ethidium bromide. Lanes 1 and 3 are *Sau96I*-digested PCR products of strains AB2 and AB3, respectively. The PCR products were amplified using total DNA including plasmids as template. Lanes 2 and 4 are *Sau96I*-digested RT-PCR products of strains AB2 and AB3, respectively. First-strand cDNA synthesis was performed using total RNA.

strains AB2 and AB3 was determined. The results revealed that the growth of strain AB3 producing C2611A-mutated 23S rRNA was significantly slower than that of the WT at 30 °C and almost ceased at 16 °C and 45 °C, whereas the growth of strain AB2 producing wild-type 23S rRNA was not significantly different from that of the WT (Fig. 5). The results indicated that C2611A-mutated 23S rRNA confers temperature-sensitive phenotypes in *Synechococcus*.

DISCUSSION

We constructed several strains with the chromosomal *rrn* operons inactivated and harbouring a multicopy plasmid containing a single *rrn* operon. Southern blot analysis revealed that the ratio of a chromosomal *rrn* operon (either *rrnA* or *rrnB*) to a plasmid-borne *rrn* operon (either pRN-A or pRN-B) was 1:3 in strains A2, A3 and B2 (Fig. 2). *Synechococcus* has approximately 10 copies of the chromosome (Binder & Chisholm, 1990; Mori *et al.*, 1996). Thus, the data suggested that there were approximately 30 copies of pRN-A or pRN-B in the cells. It has been shown that there were approximately 30 copies of RSF1010-derived plasmid in cyanobacterial cells (Ng *et al.*, 2000). Therefore, there were approximately 30 copies of the RSF1010-derived plasmids pKT and pKT-A^{C2611A} in strains AB2 and AB3, respectively. The data suggested that there were approximately 20 copies of the *rrn* operons in WT; 10 copies in strains A1 and B1; 40 copies in strains A2, A3 and B2; and 30 copies in strains AB1, AB2 and AB3.

Strains A1 and B1, harbouring an inactivated *rrnA* and *rrnB* operon respectively, survive with no apparent deleterious effects, similar to the findings of Golden *et al.* (1989). Analysis of the growth response on dark/light shift (Fig. 3) revealed that decreased *rrn* copy number (10 copies in strains A1 and B1) increased the lag time required for initiating cell growth upon returning to light, whereas increased *rrn* copy number (40 copies in A2 and B2) restored the lag time but did not achieve faster adaptation than the 20 copies in the WT. The results agree well with the feedback inhibition model in *E. coli*, in which increased *rrn* copy number does not lead to increased rRNA transcription; rRNA synthesis from individual operons is reduced to keep the total rRNA production unchanged (Jinks-Robertson *et al.*, 1983) and depletion of functional *rrn* operons causes increased expression of the remaining intact copies (Condon *et al.*, 1993).

The presence of 10 copies of the *rrnA* operon (strain B1) did not affect the doubling time, whereas the same copy number of the *rrnB* operon (strain A1) increased the doubling time. However, 40 copies of the *rrnB* operon (strain A3) did not restore the doubling time (Table 3). These results suggested that the *rrnA* operon might produce a higher amount of rRNA and tRNA molecules than the *rrnB* operon. The sequences downstream of nt -60, including the core promoter regions of the *rrnA* and *rrnB* operons, are 100% identical (NZ_AADZ00000000 and NC_006576). However, we observed that the upstream sequence of the *rrnA* operon,

Fig. 5. Effect of C2611A-mutated 23S rRNA on growth following temperature shifts. Exponential cultures of strains WT (wild-type), AB2 ($\Delta rrnA::Spc^R \Delta rrnB::Cm^R/pKT-A$) and AB3 ($\Delta rrnA::Spc^R \Delta rrnB::Cm^R/pKT-A^{C2611A}$) grown at optimal temperature (30 °C) were diluted with BG-11 medium. The diluted cultures were then incubated at 30 °C as control (a), 16 °C (b) and 45 °C (c). Cell growth was measured as OD₇₃₀. The data are the means of three independent experiments. The maximum standard deviations in (a), (b) and (c) were 0.31, 0.06 and 0.14, respectively. At least two strains of each type were tested in each growth condition and found to behave similarly.

but not the *rrnB* operon, includes the sequence 5'-TGCA-TCTCC-AGCA-3' (nt -123 to -111), which is highly homologous to the binding site for the LysR-type transcriptional activator of *Synechococcus* TGCA-N₅-TGCA (Maeda *et al.*, 1998). Whether the presence of this putative binding site for a LysR-type transcriptional activator leads to higher expression of the *rrnA* operon remains to be investigated. *rrn* operons with different promoter strengths have been found in *E. coli* (Condon *et al.*, 1992).

We have successfully constructed the AB1 strain with all chromosomal *rrn* operons inactivated but carrying the replaceable plasmid pRN-B. Using this system, we obtained strains AB2 and AB3 carrying only pKT-A and pKT-A^{C2611A}, respectively. However, attempts to obtain a strain harbouring only pKT-A^{C2611G} or pKT-A^{C2611T}, but not pRN-B, were unsuccessful. Thus, the mutation C2611G/U in the absence of wild-type 23S rRNA may be lethal to cells. The C2611A-mutated 23S rRNA in strain AB3 prolongs doubling time (Table 3) and confers temperature-sensitive phenotypes at 16 °C and 45 °C (Fig. 5). It has been reported that in *Sacch. cerevisiae*, a single C3993A mutation in the peptidyltransferase region of mitochondrial 21S rRNA confers a cold-sensitive phenotype and blocks the assembly of the 54S ribosomal subunit at 20 °C but not at 32 °C, suggesting that the nucleotide at position 3993 or base pairing between positions 3993 and 1950 may influence the interaction between the rRNA and a ribosome protein (Cui & Mason 1989). Positions C3993 and G1950 are equivalent to C2611 and G2057 of *E. coli* 23S rRNA, respectively. Positions 2611 and 2057 of the peptidyltransferase centre in domain V of *E. coli* 23S rRNA, *Synechococcus* 23S rRNA and *Sacch. cerevisiae* 21S rRNA are shown in Fig. 4(a). Disruption of the 2611–2057 base pair, resulting in disruption of the rRNA structure at the end of the stem preceding the single-stranded portion of the peptidyltransferase region, was found to confer resistance to macrolide antibiotics including Em (Vester & Douthwaite, 2001). However, the mutation C2611A/G/U has little effect on ErmE methylation (Villsen *et al.*, 1999). Mutation at nt 2611 is associated with disparate phenotypes. Our results indicated that C2611G/U-mutated 23S rRNA seems to be lethal to *Synechococcus*, whereas the mutations C2611A/G/U in *Sacch. cerevisiae* mitochondria, C2611G/U in *Chlamydomonas reinhardtii* chloroplast, C2611A/G/U in *Streptococcus pneumoniae* and C2611U in *E. coli* cause a different pattern of resistance to macrolide antibiotics (Vester & Douthwaite, 2001; Franceschi *et al.*, 2004). We are not able to determine whether the C2611A-mutated 23S rRNA in AB3 strain confers resistance to Em, since pKT-A^{C2611A} contains an Em^R gene (*ermC*) as a selectable marker.

We have successfully constructed a *Synechococcus* strain for expression of homogeneous engineered rRNA in cyanobacteria; this strain has all chromosomal *rrn* operons inactivated but carries a replaceable multicopy plasmid containing a single *rrn* operon. This system provides potential for the study of the structure and function of rRNA in photoautotrophs.

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