0273-2289/00/84–86/0447/$11.75

Salinity-Regulated Replication of the Endogenous Plasmid pSY10 from the Marine Cyanobacterium *Synechococcus* sp.

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Abstract

The endogenous plasmid pSY10 in the marine cyanobacterium *Synechococcus* sp. NKBG042902 is maintained at a high copy number when cells are grown in seawater and at a low copy number when cultured in freshwater. The mechanism of salinity-regulated replication of this plasmid was investigated. Transcription of *repA* was depressed under freshwater, which was accompanied by a low copy number of pSY10 and the appearance of a new protein that was expressed only in cells cultured in freshwater. This protein was observed to bind to putative *repA* promoters (*Prep*1 and *Prep*2) on pSY10. Moreover, this protein was observed only in *Synechococcus* sp. NKBG042902. The data suggest that this protein(s) regulates *repA* transcription in pSY10, stress responsive and encoded by the host chromosome.

Index Entries: Plasmid pSY10; replication; marine cyanobacterium; *Synechococcus* sp.; salinity stress; *repA* gene; band shift assay; copy number.

Introduction

Organisms have responded to environmental stress by evolving physiological defense responses. For example, organisms have several strategies for adapting to osmotic stress *(1–3)*. We have screened many marine photosynthetic microorganisms from several oceanic areas and have found a unique marine cyanobacterium, *Synechococcus* sp. NKBG042902. This cyanobacterium grew under a wide range of salinity conditions (0–5% [w/v] NaCl in liquid medium) and quickly adapted to a salinity shift. This

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particular strain contained four endogenous plasmids, one of which (pSY10) responded to salinity stress *(4)*. pSY10 was maintained at a high copy number when the host was cultured in synthetic medium containing 3% NaCl (w/v), the same as seawater, and at a low copy number when cultured in freshwater (0% NaCl). Sequence analysis indicated pSY10 to be 2561 bp in length and to possess *repA (5)*.

Initiation of plasmid replication requires a specific plasmid-encoded Rep initiator protein. Rep proteins recognize specific sequences at the origin of replication to form a nucleoprotein initiation complex. This is similar to the mechanism of action of the DnaA initiator protein during bacterial chromosomal replication *(6)*. In addition, many Rep proteins can generate complexes that negatively regulate their own synthesis and the frequency of initiation *(7,8)*. The specific binding of RepA to a replication control region downstream of the *repA* gene in pSY10 was observed under seawater conditions and resulted in a high copy number of pSY10 *(9)*. In this study, we describe further mechanisms for salinity-regulated replication of pSY10 in *Synechococcus* sp. This study especially, focuses on the regulation of *repA* gene expression of pSY10 under different salinity conditions.

Materials and Methods

Bacterial Strains and Plasmid Construction

The marine cyanobacteria *Synechococcus* sp. NKBG042902 *(10)* and NKBG15041c *(11)* were aerobically cultured in BG-11 medium (ATCC, cat. no. 617) supplemented with or without NaCl (3% w/v) at 29°C under cool-white fluorescent light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$). *Escherichia coli* DH5α-MCR was used as a host for plasmid construction. Plasmid pEA was constructed by inserting an *Eco*RI-*Acc*I fragment (1974 bp, containing the replication region) from pSY10 (GenBank D14403) into the multicloning site of vector pUC18.

Cyanobacterial Transformation by Electroporation Method

Cyanobacterial transformation was performed as previously described *(12)*. Cells were electroporated at a field strength of 6.25 kV/cm and time constants of 4–5 ms using a Gene Pulser (Bio-Rad, Tokyo, Japan) equipped with a pulse controller. Cells were given a single pulse and then immediately diluted 50-fold with BG-11 medium. Cells were incubated under specific growth conditions for 12–24 h. Then transformants were selected on BG-11 marine agar plates containing ampicillin or kanamycin (25 μg/mL of medium).

Measurement of Promoter Activity

An *Acc*I-digested fragment (939 bp of the upstream region of *repA* and 296 bp of the 5' terminal of *repA*) was cloned upstream of the promoter-deficient chloramphenicol acetyltransferase (CAT) gene in pKCAT1 plasmid (12.8 kbp, Km^r). pKCAT1 is a pKT230 derivative plasmid that successfully replicates in cyanobacteria *(11)*. Promoter activity of the

inserted fragment in pKCATPrep plasmid was evaluated by measuring CAT activity of cell extracts prepared by lysing cells with acetone. CAT activity was measured using Fast Cat Green (Deoxy) Chloramphenicol Acetyltransferase Assay Kits (FluoReporter FAST CAT, Molecular Probes, OR).

Preparation of Cyanobacterial Cell Extract and Band Shift Experiments

Approximately 5×10^{11} cyanobacterial cells were suspended in 20 mL of 10 m*M* Tris-HCl, pH 7.5, and were disrupted by two cycles of French Press treatment (1500 kgf/cm^2). Crude cell extract was centrifuged (150,000*g*, 2 h, 4°C), and then the supernatant was filtered (membrane type: 0.2-μm pore filter) and used as the cell extract.

Fluorescein isothiocyanate (FITC)-labeled target DNA fragments of pSY10 as DNA probes in band shift assay were prepared by polymerase chain reaction (PCR) amplification. Binding reactions between DNA probes and protein were performed at 20°C for 30 min in a 10-μL vol containing 6 μL of cell extract; 100 ng of poly (dI-dC) · poly (dI-dC); and approx 10 ng of FITC-labeled target DNA fragment of pSY10 in 10 m*M* Tris-HCl (pH 8), 11 m*M* EDTA, 50 μg/mL of bovine serum albumin, 0.1 m*M* dithiothreitol, 25 m*M* $MgCl_2$, and 0.2 *M* NaCl. The DNA-protein complexes were detected by electrophoresis on 7.5% acrylamide gels (20 mA, 45 min). Developed gels were visualized by fluorescence using a FluorImager 575 (Molecular Dynamics, CA).

Results

Effect of Salinity on the Activity of repA *Promoter*

Sequence analysis of pSY10 reveals two promoter sequences (*Prep*1 and *Prep*2) for *repA*, located 692 and 180 nucleotides upstream of the translation initiation codon, respectively. Putative -35 and -10 hexamers were similar to the –10 and –35 consensus regions of *E. coli* σ^{70} (Table 1). *RepA* promoter activities were monitored by measuring CAT activity in *Synechococcus* sp. NKBG042902 harboring pKCATPrep plasmid incubated for 9 h under different salinity conditions. Figure 1 shows the change in *repA* promoter activity with salinity shift from freshwater to seawater, and vice versa. CAT activity was increased as NaCl concentration was raised from 0 to 3% (w/v) in the medium. Reciprocally, it was decreased as salinity dropped from 3 to 0% (w/v). These results indicate that the activity of the *repA* promoter is depressed under freshwater conditions.

Binding of Protein(s) on repA *Promoter Region Under Different Salinity Conditions*

To elucidate the mechanism of depression in activity of the *repA* promoter in freshwater conditions, band shift assays were performed using cyanobacterial crude extracts and several DNA fragments (comprising *repA*

Table 1
Comparison of Promoter Sequence

Promoter	–35		–10	
E. coli σ^{70}[a]	TTGACA	- - - 16 to 18 - - -	TATAAT	
lac[b]	AGATCAAAGGATCTTC	TTGAGA	TCCTTTTTTTTCTGCGCG	TAATCT	GCTGCTTGCAA
trc[b]	AATATTCTGAAATGAGCTG	TTGACA	ATTAATCATCCGGCTCG	TATAAT	GTGTGGAATT
psbA[c]	ATGGGTTTTTTAGTC	TAGTAA	ATTTGCGTGAATTCATG	TAAATT	TTATGAGACAGGC
rep1[d]	TTGCTGAGATAGAACCTAAA	TTGACC	TGTTAGAAAATAGCGGT	TATTAT	TGAAGCAATAC
rep2[d]	ACACAAGCGTTTCAAGTTTA	TTGACA	GCGTAGAAGCCCCCTGAC	TATGAT	GGGAGTTACCACA

[a]*E. coli* consensus σ^{70} promoter *(13)*.
[b]Promoter of *E. coli lac* operon (GenBank b1:L09138, b2:U13872).
[c]Promoter of *psbA* coding D1 polypeptide in photosystem II of *Anabaena* sp. PCC7120 (GenBank U21331).
[d]*repA* promoters derived from pSY10.

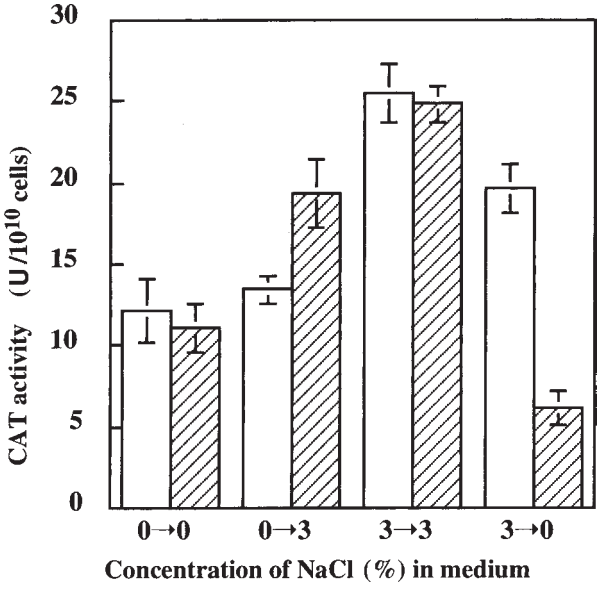

Fig. 1. CAT activity in *Synechococcus* sp. NKBG15041c harboring pKCATPrep incubated under different salinity conditions. □, Incubated after 0 h; ▨, incubated after 9 h.

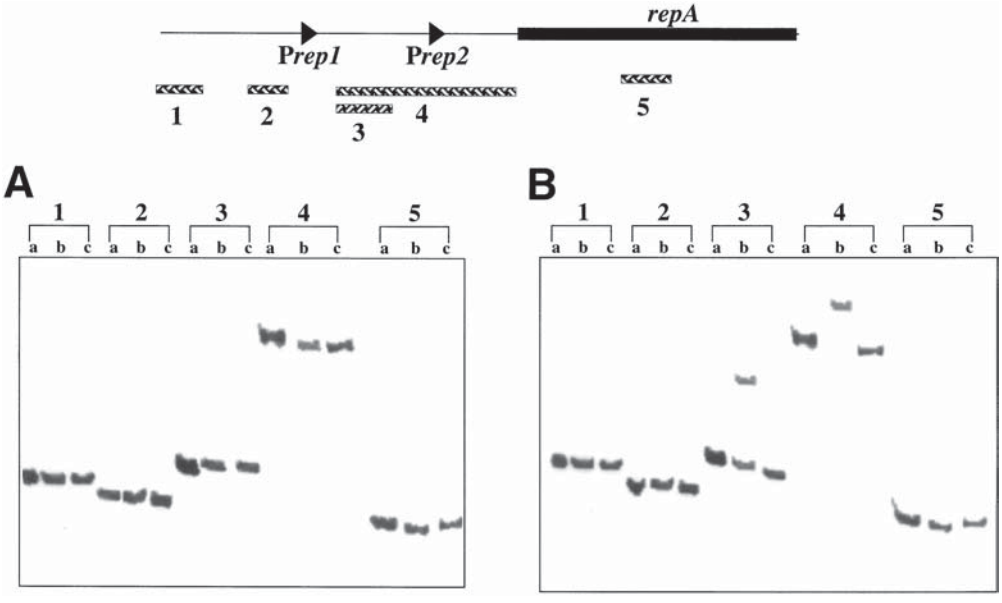

Fig. 2. Band shift assay of cell extracts from *Synechococcus* sp. NKBG15041c harboring pEA **(A)** and *Synechococcus* sp. NKBG042902 harboring endogenous pSY10 **(B)**. Lane a, DNA fragment; lane b, DNA fragment + extract of cells grown under fresh water conditions; lane c, DNA fragment + extract of cells grown under seawater conditions. FITC-labeled target DNA fragments (numbers 1–5) of pSY10 prepared by PCR amplification were used as DNA probes in the band shift assay. The number of DNA fragments corresponds with that appearing above the gel.

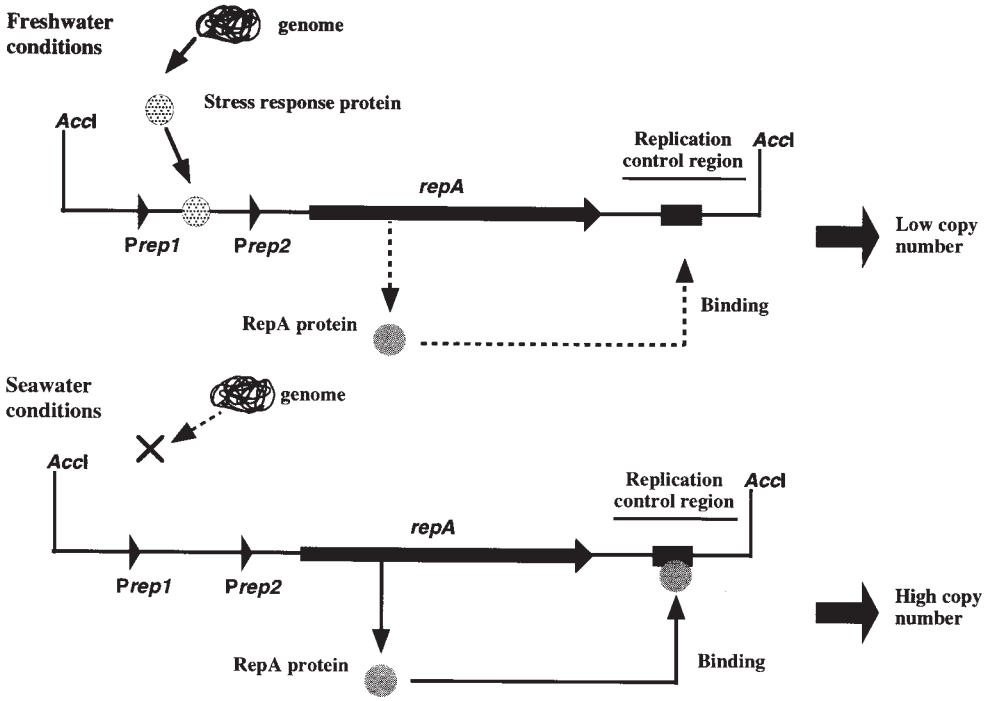

Fig. 3. Postulated control mechanisms of pSY10 replication in the marine cyanobacterium *Synechococcus* sp. NKBG042902.

promoter regions) amplified by PCR (Fig. 2). Crude extracts prepared from *Synechococcus* sp. NKBG15041c harboring pEA grown under different salinity conditions exhibited no interaction with the DNA probes prepared (Fig. 2A). By contrast, shifts were observed with DNA probes 3 and 4 when treated with the crude extract prepared from *Synechococcus* sp. NKBG042902 grown under freshwater conditions (Fig. 2B). These results suggest that a repressor protein(s) is expressed by *Synechococcus* sp. NKBG042902 only when cells are cultured under freshwater conditions, and that it binds to the promoter region, resulting in downregulation of *repA* transcription.

Discussion

In previous work, it was demonstrated that RepA protein might work to stabilize or enhance replication by binding to the putative replication control region downstream of *repA (9)*. Binding of RepA to the putative replication control region might depend in part on the amount of RepA expressed and therefore on its concentration in the cell. The expression level of RepA appears to be a function of the extracellular salinity. Consequently, replication of pSY10 (the copy number) becomes a function of the extracellular salinity. RepA does not act as a negative regulator of pSY10 replication, nor as a repressor for *repA* transcription.

Figure 3 shows a hypothetical mechanism controlling pSY10 replication in the marine cyanobacterium *Synechococcus* sp. NKBG042902. Host factors are known to regulate replication of several plasmids. Initiation of several plasmid replications requires the DnaA host replication initiator and IHF protein in addition to RepA *(14–16)*. Host chromosomal factors, which bind to the replication enhancer and stimulate activity of the replication origin for DNA replication, have been isolated from *Staphylococcus* aureus *(17)*. Replication control, and therefore copy number control, for pSY10 mostly depends on the level of RepA expression. Furthermore, *repA* promoter activity appears to be repressed by a DNA binding protein(s) encoded by the host chromosome. This is the first observation of a host chromosome–encoded repressor controlling transcription of plasmid-encoded *repA*, in which the chromosome-encoded factor is expressed only under freshwater conditions. This novel regulatory mechanism for plasmid replication will be further elucidated on identification of the repressor protein encoded by the host chromosome.

Acknowledgment

This work was funded in part by Grant-in-Aid for Scientific Research (C) no. 08833008 and no. 11650813 from the Ministry of Education, Science, Sports and Culture of Japan.

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