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Estimation of the Chromosomal Copy Number in *Synechococcus elongatus* PCC 7942

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[Abstract] Cyanobacteria are prokaryotic organisms that perform oxygenic photosynthesis. Freshwater cyanobacteria, such as *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803, are model organisms for the study of photosynthesis, gene regulation, and biotechnological applications because they are easy to manipulate genetically. However, while studying these cyanobacteria, care has to be taken with respect to genetic heterogeneity in the establishment of gene disruptants, because these cyanobacteria contain multiple chromosomal copies per cell. Here, we describe a method for the estimation of chromosomal copy number in *Synechococcus* 7942. Using this method, we have recently observed that the chromosomal copy number of *Synechococcus* 7942 significantly changes during its growth phases. This technique is available for studying polyploidy not only in cyanobacteria, but also in other polyploid organisms.

Materials and Reagents

1. Plastic disposable dish (for BG-11 plates)
2. 1.5 ml microtubes
3. *Synechococcus elongatus* PCC 7942
4. *Escherichia coli* K-12 W3110
5. Chloramphenicol (Nacalai tesque, catalog number: 06285)
6. Glutaraldehyde solution (50% in water) (KANTO KAGAKU, catalog number: 17026-32)
7. Tween 20 (KANTO KAGAKU, catalog number: 40350-02)
8. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: D5652)
9. Liquid nitrogen
10. Trisodium citrate dihydrate (KANTO KAGAKU, catalog number: 37150-00)
11. SYTOX Green Nucleic Acid Stain (5 mM solution in DMSO) (Thermo Fisher Scientific, catalog number: S7020)
12. BG-11 liquid medium (Castenholz, 1988) (see Recipes)
13. BG-11 plates (see Recipes)
14. 50x BG-11 stock (see Recipes)
15. 1,000x Micro elements A6 (see Recipes)
16. 1,000x K_2HPO_4 (see Recipes)
17. 1,000x $CaCl_2$ (see Recipes)

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18. 1,000x Ammonium Iron(III) citrate brown (see Recipes)
19. 1,000x $Na_2S_2O_3$ (see Recipes)
20. 1 M HEPES-KOH (pH 8.2) (see Recipes)
21. M9 medium (Sambrook *et al.*, 1989) (see Recipes)
22. 10x M9 salt (see Recipes)
23. PBS buffer (see Recipes)

Equipment

1. Plant growth chamber (TOMY, model: CLE-405)
2. Growth chamber (YAMATO, model: IC602)
3. Laboratory shaker (TAITEC, model: PERSONAL11)
4. UV/Vis spectrophotometer (Shimadzu, model: UV-1800)
5. Pharmaceutical refrigerator (Panasonic, model: MPR-3120CN-PJ)
6. Rotator (TAITEC, model: RT-50)
7. FACS Calibur instrument (BD Biosciences, FACSCalibur™)
8. 100 ml test tubes (for culturing)
9. Microtube centrifuge (TOMY, model: MX-107)

Software

1. Analytical software: BD CellQuest Pro (Becton-Dickinson)

Procedure

A. Preparation of *Synechococcus* 7942 culture
   1. *Synechococcus* 7942 is streaked and pre-incubated on BG-11 agar plates at 30 °C under continuous illumination (40 µE/m^2/s) for 1 week in the plant growth chamber.
   2. Cells from the plates are suspended in approximately 1 ml of BG-11 medium with high turbidity and diluted into 80 ml of BG-11 medium at an OD_{750} of 0.05.
   3. The culture is incubated at 30 °C under continuous illumination (40 µE/m^2/s) with 2% CO_2 bubbling for 10 days.
   4. The culture is then diluted to OD_{750} = 0.2 with fresh BG-11 medium. After 18-h incubation under dark conditions at 30 °C with 2% CO_2 bubbling, the culture is transferred to light conditions (40 µE/m^2/s) to restart cell growth.
   5. A 1-ml aliquot of the cultured cells is used for the following assay (Procedure C, D and E).

B. Preparation of *E. coli* culture as a standard
   1. *E. coli* is streaked and pre-incubated on M9 agar plates at 37 °C in the growth chamber.

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2. Cells from the plates are suspended in approximately 1 ml of M9 medium with high turbidity and then diluted into 10 ml of M9 medium at an OD_{600} of 0.2.
3. The culture is incubated at 30 °C with shaking for 4 h.
4. 10 µl of 20 mg/ml chloramphenicol is added to the culture (final concentration, 20 µg/ml).
5. After incubation with shaking for 2 h, 1 ml of cell culture is subjected to the following assay.

C. Fixation

1. To the 1-ml aliquots of cell culture, 10 µl of 0.5% Tween 20 and 20 µl of 50% glutaraldehyde are added (final conc. 0.005% and 1%, respectively), and incubated for 30 min at 4 °C with gentle agitation by a rotator.
2. The cells are centrifuged at 20,000 *x g* for 1 min and then washed with 1 ml of PBS, and the cell pellets were stored at -30 °C until further analysis.

D. DNA staining

1. Cell pellets were frozen with liquid N_2, and then thawed at room temperature.
   Note: This step is necessary for the permeation of SYTOX Green.
2. The cell pellets are resuspended in 50 µl of 10 µM SYTOX Green solution, which was diluted with 50 mM trisodium citrate (pH 8.0).
3. The samples are incubated overnight at 4 °C.

E. FACS analysis

1. The SYTOX Green-stained cells are subjected to FACS analysis. The stained cell suspension (5 µl) is diluted with 250 µl of PBS and immediately analyzed by FACS.
2. The cells could be selected by cell size and internal complexity by using forward (FSC) and side scatter (SSC) values. The SYTOX Green signal corresponds to the DNA amount, and is detected by the FL1 detector (laser: 488 nm, filter: 533/30 nm) (Figures 1 and 2).
   Note: The signal of Synechococcus 7942 cells can be identified based on chlorophyll autofluorescence using FL3 (laser: 488 nm, filter: 610/20 nm).
3. The major peak of the chloramphenicol-treated *E. coli* sample, which corresponds to 1 copy of the chromosome per *E. coli* cell, is extracted and used as a standard for estimating the chromosomal copy number.

F. Estimation of chromosomal copy number

The *Synechococcus* 7942 chromosome copy number (ca. 2.7 Mbp) is calculated based on the peak location of *E. coli* (ca. 4.7 Mbp) (Figure 3). The copy number of *Synechococcus* 7942 changes under our culture conditions (2-4 copies under dark and 3-8 copies under light conditions) (Watanabe *et al.* 2015) as reported previously (Binder and Chisholm, 1995; Griese *et al.*, 2011).

Equation: Copy number of the *Synechococcus* 7942 chromosome = $FL1\ value_{Synechococcus}$ x 4.7 (Genome size in *E. coli*)/$FL1\ value_{E.coli}$ x 2.7 (Genome size in *Synechococcus*)

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Representative data

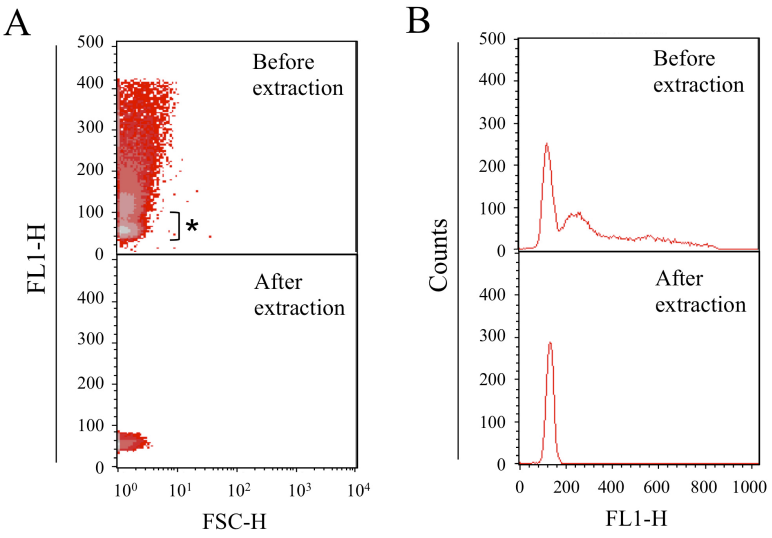

Figure 1. DNA content profiles of chloramphenicol-treated *E. coli* cells. After culturing for 2 h in M9 medium with 20 µg/ml chloramphenicol, the cells are fixed and analyzed. A. Profiles of cell size (X axis: FSC-H) and DNA content (Y axis). B. Profiles of DNA content (X axis) and cell number (Y axis). For preparing the standard peak, which corresponds to single copy chromosome in *E. coli*, the major peak (asterisk) was extracted. The bottom panel represents the profiles obtained after extraction by gating.

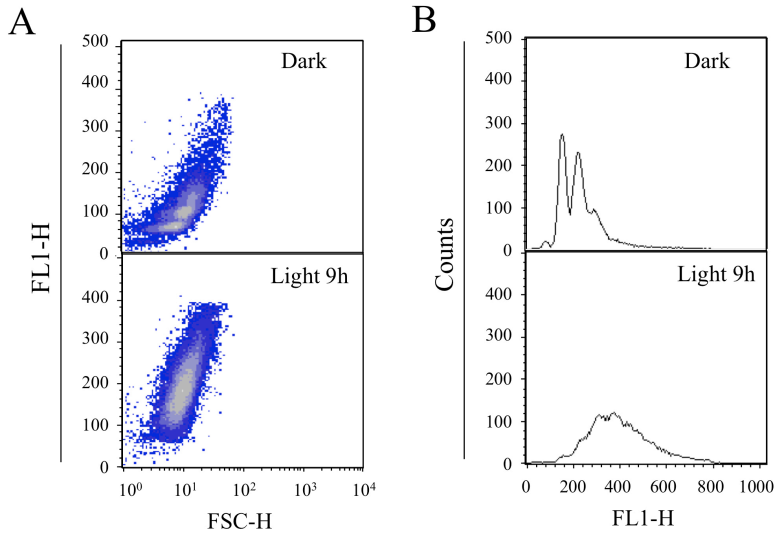

Figure 2. The DNA content profiles of cells cultured in the dark (Top) and lag-phase *Synechococcus* 7942 cells (bottom) cultured in light conditions for 9 h. A. Profiles of cell size (X axis: FSC-H) and DNA content (Y axis). B. Profiles of DNA content (X axis) and cell number (Y axis).

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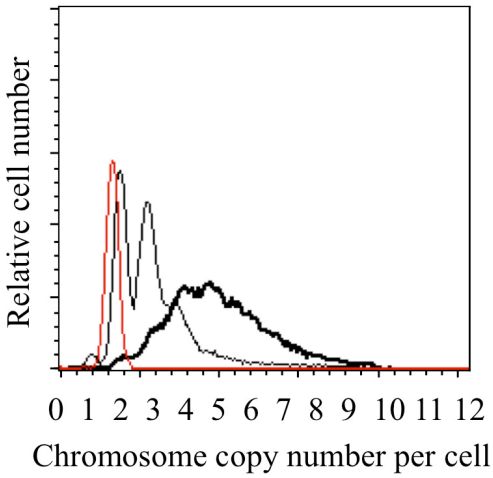

Figure 3. DNA content profiles of cells cultured in the dark (thin line), lag-phase *Synechococcus* 7942 cells cultured in light conditions for 9 h (bold line), and the chloramphenicol-treated *E. coli* culture (thin red line). The copy number of the *Synechococcus* 7942 chromosome (ca. 2.7 Mbp) was calculated based on the peak location of *E. coli* (ca. 4.7 Mbp).

Recipes

1. BG-11 liquid medium (pH 8.2)/1 L
   50x BG-11 stock 20 ml
   1,000x Micro elements A6 1 ml
   1,000x K_2HPO_4 1 ml
   1,000x $CaCl_2$ 1 ml
   1,000x Ammonium Iron(III) citrate brown 1 ml
   1 M HEPES-KOH (pH 8.2) 20 ml
   H_2O up to 1 L
   Autoclave (A. C.) (120 °C, 20 min)
2. BG-11 plates (pH 8.2)/1 L
   a. 2x BG-11 stock
      50x BG-11 stock 20 ml
      1,000x Micro elements A6 1 ml
      1,000x K_2HPO_4 1 ml
      1,000x $CaCl_2$ 1 ml
      1,000x Ammonium Iron(III) citrate brown 1 ml
      1,000x $Na_2S_2O_3$ 1 ml
      1 M HEPES-KOH (pH 8.2) 20 ml
      H_2O up to 500 ml
      A. C. (120 °C, 20 min)

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b. 2x Agar stock

Bacto agar (Nissui) 15 g

H_2O 500 ml

A. C. (120 °C, 20 min)

After dissolve Recipe 2b, mix with Recipe 2a.

3. 50x BG-11 stock, 1 L

$NaNO_3$ 75 g

$MgSO_4 \cdot 7H_2O$ 3.75 g

Citric acid 0.33 g

EDTA-3Na Salt 0.05 g

Na_2CO_3 1.0 g

H_2O up to 1 L

A. C. (120 °C, 20 min)

4. 1,000x Micro elements A6, 1 L

H_3BO_3 2.86 g

$MnCl_2 \cdot 4H_2O$ 1.81 g

$ZnSO_4 \cdot 7H_2O$ 0.222 g

$Na_2MoO_4 \cdot 2H_2O$ 0.391 g

$Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g

$CuSO_4 \cdot 5H_2O$ 0.08 g

H_2O up to 1 L

A. C. (120 °C, 20 min)

5. 1,000x K_2HPO_4, 100 ml

K_2HPO_4 3.0 g

H_2O up to 100 ml

A. C. (120 °C, 20 min)

6. 1,000x $CaCl_2$, 100 ml

$CaCl_2 \cdot 2H_2O$ 3.6 g

H_2O up to 100 ml

A. C. (120 °C, 20 min)

7. 1,000x Ammonium Iron(III) citrate brown, 100 ml

Ammonium Iron(III) citrate brown 0.6 g

H_2O up to 100 ml

A. C. (120 °C, 20 min)

8. 1,000x $Na_2S_2O_3$, 100 ml

$Na_2S_2O_3$ 15.81 g

H_2O up to 100 ml

A. C. (120 °C, 20 min)

9. 1 M HEPES-KOH (pH 8.2), 1 L

HEPES 238.3 g
pH adjusted to 8.2 with KOH
A. C. (120 °C, 20 min)

10. M9 medium, 1 L
    H_2O (A. C.) 895 ml
    10x M9 salt 100 ml
    1 M $MgSO_4$ (A. C.) 1 ml
    40% Glucose (A. C., 120 °C, 15 min) 5 ml
    1% Thiamine-HCl (A. C.)1 ml
    1M $CaCl_2$ (A. C.) 100 µl
11. 10x M9 salt, 1 L
    Na_2HPO_4 60 g
    KH_2PO_4 30 g
    NaCl 5 g
    NH_4Cl 10 g
    H_2O up to 1 L
    A. C. (120 °C, 20 min)
12. PBS buffer
    PBS powder 9.6 g
    H_2O up to 1 L
    A. C. (120 °C, 20 min)

Acknowledgments

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