Electrophoretic Separation of Chromosomes in an Achlorophyllous Microalga, *Prototheca zopfii*

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Abstract

Somatic cell fusion for strain improvement has become a key technique in the field of algal and cyanobacterial biotechnology. For the first step of cell fusion, it is important to know the information on chromosomes in the cells. Chromosomes separation of *Prototheca zopfii* Krüger, a petroleum-degrading achlorophyllous micro-alga, was performed by pulsed-field gel electrophoresis. More than 9 chromosomal DNAs of this alga were fractionated, and all the sizes of these molecules were less than 7 Mega base pairs. The banding pattern was compared with that of a green alga, *Chrolella ellipsoidea* C-87.

Key words: achlorophyllous microalga, protoplast, pulsed-field gel electrophoresis, *Prototheca*

1. Itroduction

Prototheca zopfii is known as an important oil degrader in the environment (Walker et al. 1975; Suzuki et al. 1998; Yamaguchi et al. 1999a) and an agent of bovine mastitis (Costa et al. 1996; Juensen et al. 1998). The application of *P. zopfii* cells to the bioreactor system has been investigated (Yamaguchi et al. 1999b; Suzuki & Yamaya 2005). The immobilized biomass in polyuretrhane foam pieces was incorporated into a bubble-column type bioreactor and a successful result obtained (Yamaguchi et al 1999a).

This microalga was classified as a colorless, heterotrophic version of *Chlorella* sp. on the basis of morphology, physiological characteristics and the guanine (G) plus cytosine (C) ratio of nuclear DNA (Kerfin & Kessler 1977; 1978). A modified bacterial adherence to hydrocarbon assay revealed the strong hydrophobicity of the outermost surface of the algal cells (Suzuki et al. 1998). The adherence of the algal cells to the test hydrocarbons increased with an increase in the hydrophobicity of the hydrocarbons. Although high-weight genetic DNA preparations from *P. zopfii* is one of the fundamental requirements for biochemical and molecular genetic studies, isolation of intact nucleic acid from this alga was hampered by the lack of the methods to obtain the protoplast or spheroplast. The algal cells have layers of cell wall including sporopollenin, which is the most extraordinarily resistant carotenoid to enzymatic degradation (Atkinson et al. 1972). The method of mechanical cell

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homogenization with grass beads or sonication causes shearing of DNA, hence detailed genetic analysis using unshearing DNA of this alga has not yet been performed.

In the previous study, the method of protoplast formation of *P. zopfii* using Macerozyme R-200 was reported (Suzuki et al. 1998). The rate of protoplast formation was mainly affected by the incubation temperature and the age of algal cells. The optimal condition for the maximum protoplast yield (64%) was determined to be mid-logarithmic phase cells and the enzyme concentration of 4% at a temperature of 35 ℃. The purpose of the present study is to separation of chromosomes in *P. zopfii* by subjecting algal cell lysates to pulsed-filed gel electrophoresis, and the banding pattern was compared with that of *Chlorella* sp.

2. Materials and Methods

Cultivation of Alga

P. zopfii Krüger ATCC 30253 and *Chlorella ellipsoidea* Gerneck IAM C-87 (obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) were used in this study. The strain of *P. zopfii* was cultured in Sabouraud Dextrose Broth (Difco, USA) at pH 6.8-7.0. The cultivation was carried out in flasks on a reciprocal shaker at 25 ℃. Cell growth was monitored by measuring optical density at 600 nm.

Induction of Protoplasts

Preparations of protoplasts of *P.zopfii* and *C. ellipsoidea* IAM C-87 were reported previously (Suzuki et al. 1998; Yamada & Sakaguchi 1981). For *P.zopfii*, the cells in the logarithmic growth phase were harvested by centrifugation at 1000 × *g* for 5 min, washed and re-suspended in the solution consisting of either 0.85M NaCl or 0.6M sorbitol as the osmoticum (osmotic stabilizer) with one or more of the following polysaccharide-degrading enzymes: cellulose Onozuka RS (derived from *Trichoderma viride*, Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan), Macerozyme R-10 and R-200 (from *Rhizopus* sp., Yakult, Japan), funcelase (from *Trichoderma viride*, Yakult, Japan), and pectinase (from *Rhizopus* sp., Sigma, USA). The cell suspension was incubated with gentle shaking at 25 to 35 ℃. For *C. ellipsoidea*, the cell suspension in 25 mM-phosphate buffer (pH 6.0) containing 0.6 M-sorbitol/mannitol (1:1), 4% (w/v) cellulose Onozuka RS, 2% (w/v) Macerozyme R-10, and 1 % (w/v) pectinase was incubated at 25 ℃ in a shaker water bath with gentle shaking.

The formation of protoplast was monitored by adding 0.1 ml of the culture to 0.9 ml of water. The protoplast yield (the rate of protoplast formation) which is expressed in the ratio of the number of osmotically labile cells to the number of original cells was measured by counting disrupted cells with a hemocytometer by microscopy.

Chromosomes separation

Protoplasts were washed twice and resuspended in 1 mL solution containing 1.7M Sorbitol as a osmotic stabilizer, 60mM EDTA (pH 8.0), 0.1M Trisodium citrate and 10mM 2-Mercaptoethanol, and mixed with 1 mL 1% low gelling temperature agarose (Type VII,

Sigma) containing 0.125M EDTA and 1.7M Sorbitol at 45 ℃, and then solidified in the dish at room temperature.

To the agarose blocks, 2 mL 1mg/mL Proteinase K (from *Tritirachium* album, Sigma) containing 0.5M EDTA (pH 8.0), 10mM Tris-HCl (pH 8.0) and 1% N-Lauroylsarcosine was added and incubated at 37 ℃ for 12h (protoplasts or spheroplasts of *P.zopfii* set in agarose block were lysed). Then the agarose block was loaded on a 0.6% pulsed field certified agarose gel (BIO-RAD) in 0.5×TBE and subjected to electrophoresis on a counter-clamped homogeneous field electrophoresis (CHEF) apparatus (CHDF-DR Ⅲ, BIO-RAD) at 14 ℃. Run-time was 72h at 2V/cm with a 20 minute switch time ramp at an included angle of 106°.

3. Results and Discussion

The banding patterns of *P. zopfii* and *C. ellipsoidea* C-87 chromosomal DNA molecules by pulse-field gel electrophoresis are shown in Fig.1. The pattern of *P. zopfii* indicated that there were at least 9 chromosomes, one of 3.5 to 4.6 Mb and the others under 3.5 Mb. On the other hand, the banding pattern of *C. ellipsoidea* C-87 indicated the presence of at least 7 chromosomes, one of 5.7Mb, four of 4.6 to 3.5 Mb and the others under 3.5 Mb.

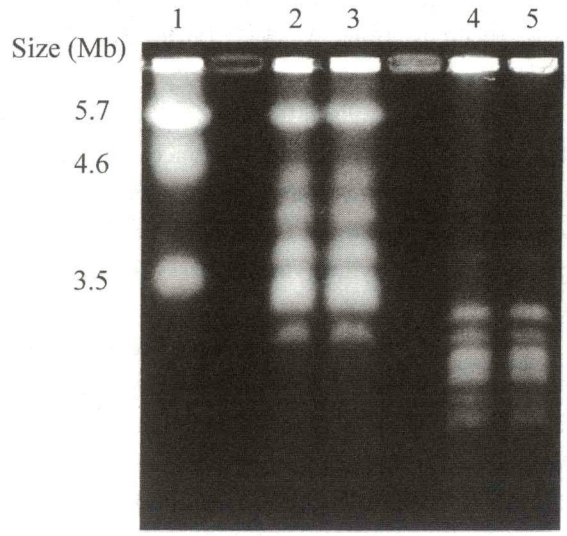

Figure 1. Pulsed-field gel electrophoresis patterns of *Prototheca zopfii* Krüger ATCC 30253 and *Chlorella ellipsoidea* C-87 DNA: 1- *Schizosaccharomyces pombe* (as Mr marker); 2 and 3 - DNA from *C. ellipsoidea* C-87; 4 and 5 - DNA from *P. zopfii*

From these patterns, all the chromosomes appeared to be fairly large. The chromosomes of *C. ellipsoidea* C-87 were relatively larger than those of *P. zopfii* and the banding patterns were quite different with that of *P.zopfii* that as the apochlorotic equivalent of *Chrorella* sp.

Pulse-field gel electrophoresis can only be performed using unsheared DNA, therefore requires a gentle and efficient DNA isolation method. Protoplasting or spheroplasting of the alga prior to DNA isolation is probably a crucial step in obtaining as much unsheared DNA

as possible. The successful protoplasting and separation of chromosomal DNA of *P. zopfii* in this study would be useful for the future biochemical and molecular genetic analyses on outbreak of bovine mastitis due to *P.zopfii*, ability to degrade petroleum and taxonomy of this alga.

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References

Atkinson, A. W., Gunning, B. E. S., John, P.C. L., 1972. Sporopollenin in the Cell Wall of Chlorella and Other Algae: Ultrastructure, Chemistry, and Incorporation of ^{14}C-Acetate, Studies in Synchronous Cultures. *Planta (Berl.)* **107**, 1-32.

Costa, E. O., Ribeiro, A. R., Melville, P.A., Prada, M. S., Carciofi, A. C., Watanabe, E.T., 1996. Bovine Mastitis due to Algae of the *Genus Prototheca. Mycopathologia* **133**, 85-88.

Juensen, H.E., Aalbaek, B., Bloch, B., Huda, A., 1998. Bovine Mammary Protothecosis due to *Prototheca. Medical Mycology*, **36**, 89-95.

Kerfin, W., Kessler, E., 1977. Physiological and Biochemical Contributions to the Taxonomy of the Genus Prototheca: I. Hydrogenaze, Acid Tolerance, Salt Tolerance, Thermophily and Liquefaction of Gelatin. *Archives Microbiology* **113**, 139-141.

Kerfin, W., Kessler, E., 1978. Physiological and Biochemical Contributions to the Taxonomy of the Genus *Prototheca*: II. Starch Hydrolysis and Base Composition of DNA. *Archives Microbiology* **116**, 105-107.

Suzuki, T., Yamaguchi, T., Ishida, M., 1998. Effects of Some Factors on Protoplast Formation of a Microalga, *Prototheca zopfii. World J. Microbiology Biotechnology* **13**, 355-356.

Suzuki, T., Yamaguchi, T., Ishida, M., 1998. Immobilization of *Prototheca zopfii* in Calcium-Alginate Beads for the Degradation of Hydrocarbons. *Process Biochemistry* **33**, 541-546.

Suzuki, T., Yamaya, S., 2005. Removal of Hydrocarbons in a Rotating Biological Contactor with Biodrum, *Process Biochemistry* 40, 3429-3433.

Walker, J. D., Colwell, R.R., Vaituzis, Z., Meyer, S.A., 1975. Petroleum Degrading Achlorophyllous Alga *Prototheca zopfii. Nature (London)* **254**, 423-424.

Yamada, T., Sakaguchi, K., 1981. Protoplast Induction in Chlorella Species. *Agriculture Biolog. Chem.*, **45**, 1905-1909.

Yamaguchi, T., Suzuki, T., Ishida, M., 1999a. An Immobilized Cell System in Polyurethane Foam for the Lipophilic Micro-Alga *Prototheca zopfii. Process Biochemistry* **34**, 167-171.

Yamaguchi, T., Ishida, M., Suzuki, T., 1999b. Biodegradation of Hydrocarbons by *Prototheca zopfii* in Rotating Biological Contactors. *Process Biochemistry* **35**, 403-409.