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Characterization of DNA restriction-modification systems in *Spirulina platensis* strain pacifica

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Abstract

Four unique restriction enzymes were identified in the soluble protein fraction of *Spirulina platensis* strain pacifica, a commercially important strain of marine cyanobacterium that is used as a supplement in a human diets. These are *Spa*I, *Spa*II, *Spa*III and *Spa*IV, which are isoschizomers of *Tth*111I, *Pvu*I, *Pvu*II and *Hin*dIII, respectively. The recognition sites of each of these four enzymes were identified by restriction digests of different plasmid DNAs of known sequence and determining the cleavage sites by sequencing. *Spa*I is the most predominant restriction enzyme present in *S. platensis* strain pacifica. It shows high activity at 37 °C compared to 65 °C for its isoschizomer *Tth*111I.

Introduction

Spirulina is a commercially important filamentous cyanobacterium that is grown in large scale and processed industrially (Dillon *et al.*, 1995). It is now produced by several companies and sold in many health food stores around the world. It is used as feed for fish, poultry and farm animals and as a supplement in human diets. It is a rich source of protein, minerals, vitamin B12, β-carotene and essential fatty acids like γ-linolenic acid (Belay *et al.*, 1993). Furthermore, due to the absence of cellulose cell wall, *Spirulina* does not require chemical or physical processing steps in order to become digestible. Removal of moisture by simple sun-drying is sufficient as postharvesting treatment (Dillon *et al.*, 1995). It has been demonstrated that *Spirulina* has therapeutic effects against hyperlipidemia (Nakaya *et al.*, 1988). Therefore, there is a potential for future pharmaceutical use of *Spirulina* to produce many health related products. It may be also possible to further enhance the production of certain compounds such as β-carotene in *Spirulina* through genetic engineering. It may become a suitable organism to be used as a biological factory for producing certain kinds of proteins. Unfortunately, the unavailability of a gene transfer system in *Spirulina* has blocked the development of new and improved strains of *Spirulina* through genetic engineering. One of the requirements for the development of a gene transfer system in *Spirulina* is the characterization of its DNA restriction-modification system. Previously, three restriction enzymes have been identified in *Spirulina platensis* subspecies siamese (Kawamura *et al.*, 1986). In this report, we describe the identification of four restriction enzymes from commercially-grown strain of *Spirulina platensis*.

Materials and methods

Strain and culture condition

The *Spirulina platensis* strain pacifica was a kind gift of Cyanotech Inc., Hawaii. This strain, originating from the UTEX strain collection (UTEX, 1926), has been grown in continuous culture in Hawaii since 1984. It

is commercially cultivated by Cyanotech Inc. under the trade name '*Spirulina pacifica*'. An axenic culture of this strain was established by selecting single filaments under the microscope to grow new cultures. Cultures were grown in 0.5 × Zarrouk's medium (Zarrouk, 1966) under continuous light with shaking for 72 to 96 h. Cells were harvested using a reusable filter device (Nalgene, Inc.) containing a nylon net and stored at −20 °C. The axenic culture condition was verified by microscopic examinations everytime a large culture was grown for isolation of restriction enzymes.

Enzyme isolation

Restriction enzymes were separated according to Kawamura (1986) with the following modifications: After resuspension of the pellet from ammonium sulfate precipitation of soluble proteins from 20 g *Spirulina* cells in phosphate buffer [10 mM KH_2PO_4, 10 mM mercaptoethanol, 0.1 mM EDTA, pH 7.0] and overnight dialysis in the same buffer at 4 °C, the solution was loaded on a 1.0 × 15 cm Heparin-agarose culumn (BIO-RAD) equilibrated in phosphate buffer. The column was then washed sequentially with 250 ml of phosphate buffer and 50 ml of phosphate buffer containing 20 mM NaCl. One large fraction (fraction A) was eluted with 40 ml of 100 mM NaCl. The enzymes left in the column were eluted in 50 ml of 100–1000 mM NaCl using a linear gradient former (Bethesda Research Laboratories, MD). Two fractions that showed enzymatic activity were identified: fraction A, which contained a single activity, and fraction B [samples eluted with 360–800 mM NaCl], which contained three overlapping activities. Each of these two fractions was dialyzed against Tris buffer (10 mM Tris HCl pH 7.5, 10 mM Mercaptoethanol, 0.1 mM EDTA and 10% glycerol) and applied to a 1.0 × 8.0 cm DEAE column equilibrated with Tris buffer. The column containing fraction B was washed sequentially with 5 ml Tris buffer, 3 ml Tris buffer containing 60 mM NaCl and 3 ml Tris buffer containing 100 mM NaCl. The enzymes were eluted with 100 mM to 300 mM NaCl in a step gradient of 20 mM steps. The rest of the enzymes was eluted from the column with 10 ml of 500 mM NaCl. Two fractions that showed enzymatic activity were identified: (1) fraction C, which contained a single activity [samples eluted with 280–500 mM NaCl], and fraction D [samples eluted with 100–220 mM NaCl], which contained two overlapping activities. Fraction D was dialized against phosphate buffer and reapplied to a 1.0 × 8.0 cm Heparin-agarose column equilibrated with phosphate buffer. The column was washed with 10 ml of phosphate buffer and 5 ml of phosphate buffer containing 100 mM NaCl. The enzymes were eluted in a step gradient from 100 to 810 mM NaCL in 15 mM steps. Two fractions that showed enzymatic activity were identified: (1) fraction E [samples eluted with 480–560 mM NaCl], which contained a single activity and fraction F [samples eluted with 600–680 mM NaCl], which contained a single activity. When fraction A was further purified through DEAE chromatography, a single enzyme activity was identified in samples eluted with 100 mM NaCl.

Restriction enzymes assay

Restriction enzyme assays were done in 30 μl volume containing 1 μg of DNA, 5 μl of a fraction, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris HCl pH 7.5, 2 mM mercaptoethanol and 0.3 μg BSA. DNA used for digestion were Lambda, pBR322, pUC18 and pEB1, a plasmid constructed in our laboratory which is 5445-bp in size with known sequence and contains restriction sites such as *Tth*111I, *Pvu*I, *Pvu*II, *Eco*RI, *Hin*dIII etc. The digested DNA fragments were separated in 1% agarose gels. The amount of NaCl in the digest was adjusted after considering the concentrations of NaCl in the enzyme fraction. The assay conditions were optimized by changing the pH to 7.0 and replacing NaCl with KCl in some cases.

Determination of cleavage site

Purified enzyme fractions and their commercial isoschizomers were used to digest DNA. After digestion, DNA was precipitated and resuspended in water. Digested DNAs were used as sequencing templates in sequencing reactions using fluorescent-dye terminators. Primers were chosen to position restriction site 100–200 bp downstream of oligonucleotide binding site. Samples were run on an ABI 373 DNA Sequencer. Cleavage site was identified at position where sequence signal intensity greatly diminished.

Results and discussion

Four unique restriction enzyme activities were identified in the soluble protein fraction of *S. platensis* strain pacifica. The recognition sites of each of these four activities was identified by analysis of restric-

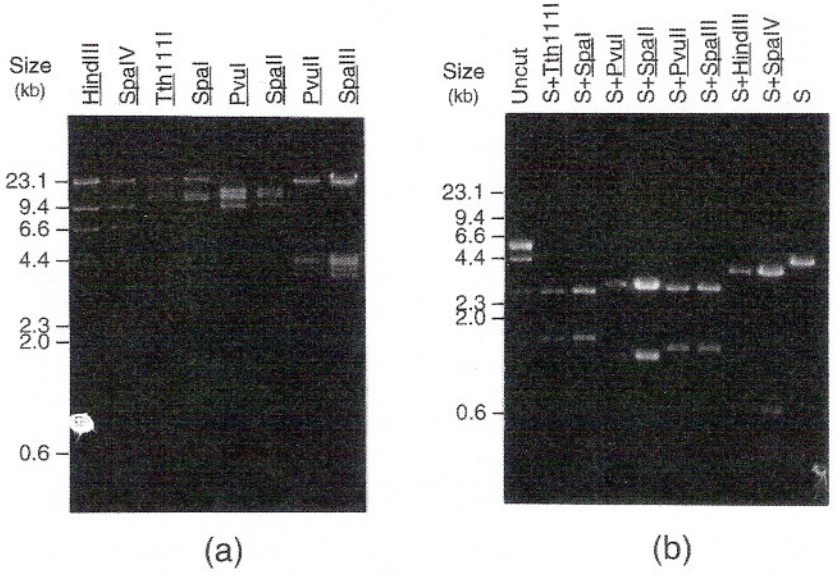

Fig. 1. (a) Lambda DNA digested with the four restriction enzymes purified from *S. platensis* strain pacifica: *Spa*I, *Spa*II, *Spa*III, *Spa*IV and their four isoschizomers *Tth*111I, *Pvu*I, *Pvu*II and *Hin*dIII, respectively. (b) pBR322 DNA double-digested with *Sal*I (S) and one of the four restriction enzymes purified from *S. platensis* strain pacifica or their commercially available isoschizomers. 0.5 μg Lambda or plasmid DNA was digested for 3 h at 37 °C with restriction enzymes as described in Materials and Methods.

tion digests of different plasmid DNAs of known sequence (see Fig. 1). Fraction A (*Spa*I) contains an isoschizomer of *Tth*111I. Fraction C (*Spa*II) contains an isoschizomer of *Pvu*I. Fraction E (*Spa*III) contains an isoschizomer of *Pvu*II. Fraction F (*Spa*IV) contains an isoschizomer of *Hin*dIII. Only one of these four enzyme activities (*Tth*111I) is found in *Spirulina platensis* subspecies siamese (Kawamura *et al.*, 1986). *Spa*I is the most predominant restriction enzyme present in the cells of *S. platensis* strain pacifica.

Assay conditions for the enzymes isolated from *S. platensis* strain pacifica were evaluated and compared with those for their commercial isoschizomers. *Spa*I, the isoschizomer of *Tth*111I from *S. platensis* shows high activity at 37 °C compared to 65 °C for *Tth*111I from *Thermus thermophilus* 111 (New England Biolab, MA). *Spa*I is inactivated quickly at 65 °C. This enzyme shows high activity in 100 mM NaCl and a pH of 7.0. *Spa*II, *Spa*III and *Spa*IV showed higher activity when the NaCl in the reaction is replaced with 100 mM KCl (data not shown). These three enzymes showed maximum activity at pH 7.5 (all reactions contained 10 mM Tris HCl pH 7.5 or pH 7.0, 10 mM $MgCl_2$, 2 mM mercaptoethanol and 0.3 μg BSA).

These purified enzyme fractions were used to determine the exact site of DNA cleavage within the restriction site for three of these *Spirulina* enzymes. The cleavage sites of *Spa*I, *Spa*II, and *Spa*III have been confirmed by automatic DNA sequencing. Two commercially available DNAs, pGEM-9zf(-) and M13 mp19 RF, were used to determine the cleavage sites for the three enzymes. These DNAs were chosen because restriction sites for the *Spirulina* enzymes are located near the universal primer binding sites in these vectors. The M13 mp19 RF DNA has sites for *Pvu*I and *Pvu*II. After separate digestions with *Spa*II and *Spa*III and their two commercial isoschizomers, each of the digested products was precipitated and resuspended in water. Automated sequencing was performed using the M13 reverse sequencing primer. The results showed that *Spa*II and *Spa*III had the same cleavage sites as *Pvu*I and *Pvu*II, respectively. The pGEM-9zf(-) plasmid has a *Tth*111I site. Following the same procedure described for the M13 DNA, the automated sequencing results showed that the *Spa*I also had the same cleavage site as *Tth*111I.

With these results, the first step in developing a gene transfer system in *S. platensis* strain pacifica has been completed. With the identification of the restriction enzymes present in this strain, modification enzymes corresponding to these restriction enzymes can be identified and used to modify DNA that will be introduced into *Spirulina*. This will prevent genetically engineered DNA constructs from being destroyed

by the *Spirulina* restriction system when we introduce these constructs into *Spirulina* cells. These modifications will make the introduced DNA resistant to cleavage inside the *Spirulina* cell, thereby increasing DNA transformation efficiencies. We have already isolated a DNA fragment that contains the modification gene for *Spa*I (unpublished results) using a variation of the genomic DNA library enrichment strategy (Renbaum *et al.*, 1990) that was used to isolate the CpG methylase gene from *Spiroplasma*. We are currently investigating other requirements for a *S. platensis* gene transfer system, which include: (1) establishing conditions to reproducibly regenerate *Spirulina* filaments from fragments containing only a few cells, (2) developing DNA vectors for use in *Spirulina*, and (3) developing physical or biological methods for introducing foreign DNA into *Spirulina* cells.

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