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A High-Copy-Number Plasmid Capable of Replication in Thermophilic Cyanobacteria

MASATO MIYAKE,[1] HIROSHI NAGAI,[2]
MAKOTO SHIRAI,[2] RYUICHIRO KURANE,[1] AND YASUO ASADA*,[1]

[1]National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Higashi 1-1, Tsukuba, Ibaraki 305-8566, Japan, E-mail: yasada@ccmail.nibh.go.jp; and [2]Division of Biotechnology, Ibaraki University, Ibaraki, Japan

Abstract

A 2.5 kb high-copy-number plasmid, pMA4 in thermophilic cyanobacterium *Synechococcus* sp. MA4 was isolated and characterized to develop a genetic engineering system for thermophilic cyanobacteria. The copy number of pMA4 was determined to be by densitometry about 350/cell. The pMA4 may be a type of rolling-circle plasmid, because a possible *rep* gene encoding 34 kD-protein and a consensus sequence of a double-stranded origin nick site of rolling circle plasmids were found in the pMA4 sequence. The pMA4 was electro-introduced into another thermophile, *Synechococcus* sp. MA19, which is the strongest poly-β-hydroxybutyrate (PHB) accumulator in photoautotrophic organisms. The pMA4 was incorporated and retained in MA19. These results indicate that pMA4 could be developed as a useful vector for thermophilic cyanobacteria.

Index Entries: Thermophilic cyanobacteria; poly-β-hydroxybutyrate; carbon dioxide fixation; biodegradable plastic; genetic engineering; rolling circle plasmid.

Introduction

The increase in atmospheric CO_2, which may be partly owing to combustion of tremendous amount of fossil fuel, may contribute to environmental degradation. One possible way to mitigate the problem could be the large-scale fixing of CO_2 gas through photosynthesis. Microalgae and cyanobacteria have been studied as possible biological catalysts to convert CO_2 into organic matters, such as protein, lipid, sugar, hydrocarbon, carotenoid,

*Author to whom all correspondence and reprint requests should be addressed.

and pigment *(1)*. Producing hydrogen gas, a renewable energy carrier, with cyanobacteria and algae might help solve the problem *(2)*.

Genetic engineering could be a powerful tool to extend the ability of cyanobacteria for practical application of photosynthetic material production. For example, heterogenous genes that encode larvicidal protein *(3–6)*, fish growth hormone *(7)*, and alpha-amylase *(8)* were expressed in cyanobacteria. Moreover, additional metabolic pathways such as eicosapentaenoic acid synthesis *(9)*, ethylene synthesis *(10)*, and poly-beta-hydroxybutyrate (PHB) synthesis *(11)* were expressed in cyanobacteria.

Thermophilic cyanobacteria have some advantages over mesophiles as microbial catalysts to convert CO_2 into organic matter: 1. They grow faster; 2. They are unlikely to be contaminated by pathogens; and 3. They do not require cooling *(12)*. For application, a thermophilic cyanobacterium was isolated from its natural environment and characterized as a powerful PHB accumulator that uses CO_2 as the sole carbon source *(13)*, because PHB is a raw material of biodegradable plastics that can be applied to common uses and to specialties such as medical materials *(14)*.

Recently, a thermophilic cyanobacterium, *Synechococcus elongatus*, was genetically transformed with electroporation *(15,16)* or conjugation *(16)*. However, the transformations were restricted by homologous recombination with DNA because no plasmid vectors were available for the thermophilic cyanobacterium.

Here we report characteristics of a high-copy-number plasmid isolated from a thermophilic cyanobacterium, *Synechococcus* sp. MA4, which was isolated from a hot spring in Miyake-jima island in Japan, and its replication in *Synechococcus* sp. MA19, a potent PHB accumulator *(13,17)*.

Materials and Methods

Bacterial Strains and Cultivation

Synechococcus sp. MA4 and MA19 *(13)* are stored in the culture collection of the Molecular Bioenergetics Laboratory of the National Institute of Bioscience and Human-Technology (Higashi, Tsukuba, Ibaraki, Japan). MA4 and MA19 were cultivated at 50°C as described by Miyake et al. *(13)*. *Escherichia coli* HB101 was cultivated at 37°C in LB medium containing 1% (w/v) Bactotryptone (Difco, Detroit, MI), 0.5% (w/v) yeast extract (Difco), and 1% (w/v) NaCl. The pH in the medium was adjusted to 7.2 by 2 *N* NaOH.

DNA Preparation

Total DNA was isolated from *E. coli* transformants and the cyanobacteria according to the method of Mac and Ho *(18)*. Plasmids from cultures of *E. coli*, *Synechococcus* sp. MA4 and MA19 were isolated and purified according to the alkaline lysis method described by Sambrook et al. *(19)*.

Electrotransformation

The plasmids were incorporated into the cyanobacteria via the electrotransformation method described by Miyake and Asada *(15)*. The electroporation mixture (100 µL) contained 1.0×10^7 cells, 10 µg DNA, 272 m*M* sucrose, 8 m*M* HEPES, pH 7.4. The cells were then incubated in 1 mg/mL deoxyribonuclease I (Sigma, St. Louis, MO) solution (in TE buffer containing 10 m*M* $MgCl_2$, pH 8.0) at 37°C for 1 h to remove excess plasmid outside the cells. The cells were resuspended into 1 mL of BG-11 *(20)* medium and incubated at 50°C overnight in dark conditions.

DNA Sequencing

Plasmid pMA4 was isolated and purified from *Synechococcus* sp. MA4, then digested by *Bam*HI. Three fragments (0.5 kb, 0.6 kb, and 1.4 kb) were obtained. The *Bam*HI-fragments were cloned into a plasmid vector, pBluescript II KS+, for further DNA sequencing. DNA was sequenced using an automatic sequencer (Applied Biosystems, model 373A-18). Sequencing reagents (dye primer cycle sequencing kit) were purchased from Perkin-Elmer. Purified double-stranded plasmids were used as the templates of dideoxy-mediated chain termination sequencing. The DNA sequence was determined on both strands and was analyzed by DNASIS software (Hitachi Software Engineering Co.).

Results and Discussion

Copy Number of pMA4

Figure 1 shows electrophoresis of total DNA from *E. coli* and cyanobacteria harboring plasmids. The DNA samples were prepared from 1.6×10^9 cells. The strong and smear bands of more than 11.5 kb were fragmentated chromosomal DNA. A novel plasmid of high-copy-number designated as pMA4 was found in a thermophilic cyanobacterium, *Synechococcus* sp. MA4 (Fig. 1, lane 2). The bands c and d in lane 2 (Fig. 1) were covalently closed circular (ccc) and open circular (oc) forms of the pMA4, respectively. The bands of the pMA4 were strong, as were those of ccc (A) and oc (B) of pUC18 *(21)* in *E. coli* transformants (Fig. 1, lane 1). The weak bands e in lane 3 and f in lane 4 were the low copy-number plasmids, pMW219 *(22)* (five copies in *E. coli*) and pECAN8 *(23)* (10 copies in *Synechococcus* PCC7942).

The copy number of the pMA4 was estimated from the amount of plasmid DNA extracted from *Synechococcus* sp. MA4 (1.6×10^9 cells) determined from the density of bands c and d according to Sambrook et al. *(19)*. The copy number of pUC18 in *E. coli* was 690/cell according to this method and the number agreed to Sambrook et al. and Yannisch-Perron et al. *(19,21)*. From the triplicate experiments, the copy number of pMA4 was determined to be 346 ± 8/cell.

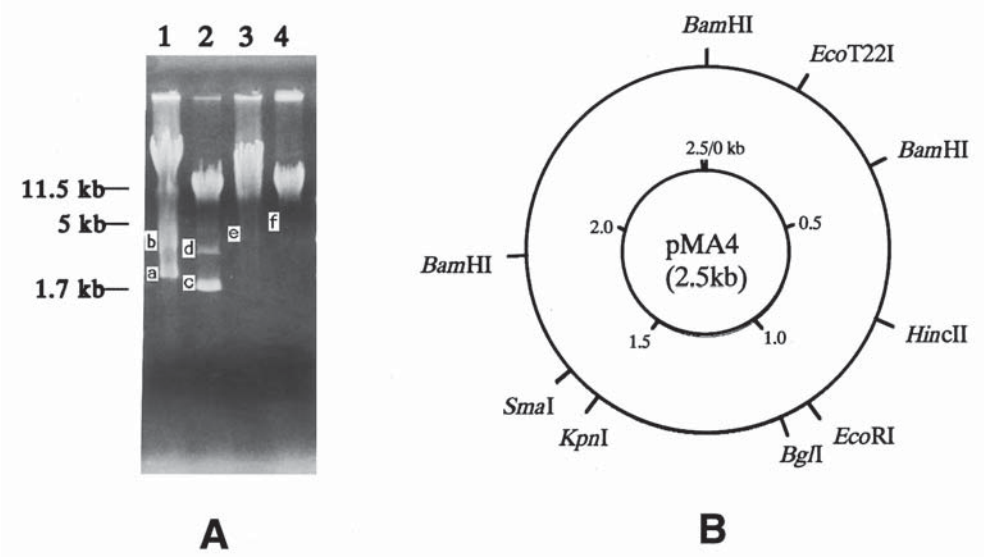

Fig. 1. The plasmid, pMA4, from *Synechococcus* sp. MA4. **(A)** Agarose electrophoresis of total DNA. Lane 1, *E. coli* (pUC18); lane 2, *Synechococcus* sp. MA4 (pMA4); lane 3, *E. coli* (pMW219); lane 4, *Synechococcus* sp. PCC7942 (pECAN8). **(B)** A restriction map of the pMA4.

DNA Sequence of pMA4

Figure 2 shows the sequence of pMA4. The size of pMA4 was 2,514 bp and the G + C content was 48.2%. The restriction sites of *Apa*I (1,432 bp); *Hae*II (250 bp, 401 bp, 1,706 bp, and 1,982 bp); *Xba*I (1,960 bp); *Bgl*I (1,087 bp and 1,926 bp); and *Hae*III (33 bp, 318 bp, 411 bp, 546 bp, 1,433 bp, 1934 bp, 2,164 bp, 2,453 bp, and 2,507 bp) found in the sequence were insensitive to the restriction endonucleases. These sites were probably modified by methylation.

A repeat sequence 'TACTCCCTATTAGGGATTGAAAC' was found in the 2,139 bp and 2192 bp regions. This sequence showed high homology (87%) to a cyanobacterial repetitive sequence *(24)*. An inverted repeat sequence was found from 2,235–2,275 bp. The consensus sequence of a double-stranded origin nick site, 'TTGATA', which is found in pC194 group plasmids *(25)*, was located in the A + T-rich (71%) region (1991–2043 bp).

Five open reading frames (ORFs) encoding more than 100 amino acids were found. The largest ORF1 (Fig. 2) encoded a putative 34kD-protein. The estimated pI of this putative protein was 10.4. The N-terminal domain (28 aa-64 aa) and the C-terminal domain (175 aa-198 aa) were very similar to those of a replication-associated protein from a cyanobacterium, *Plectonema boryanum (26)* (Fig. 3A).

The C-terminal domain included a consensus motif at the active site in Rep proteins of the pC194 group of rolling-circle plasmids, which have also been found from some cyanobacteria such as *Nostoc* sp. *(25)*, *Microcystis aeruginosa (25)*, *Plectonema (27)*, and *Synechocystis* sp. *(28)* (Fig. 3B). The

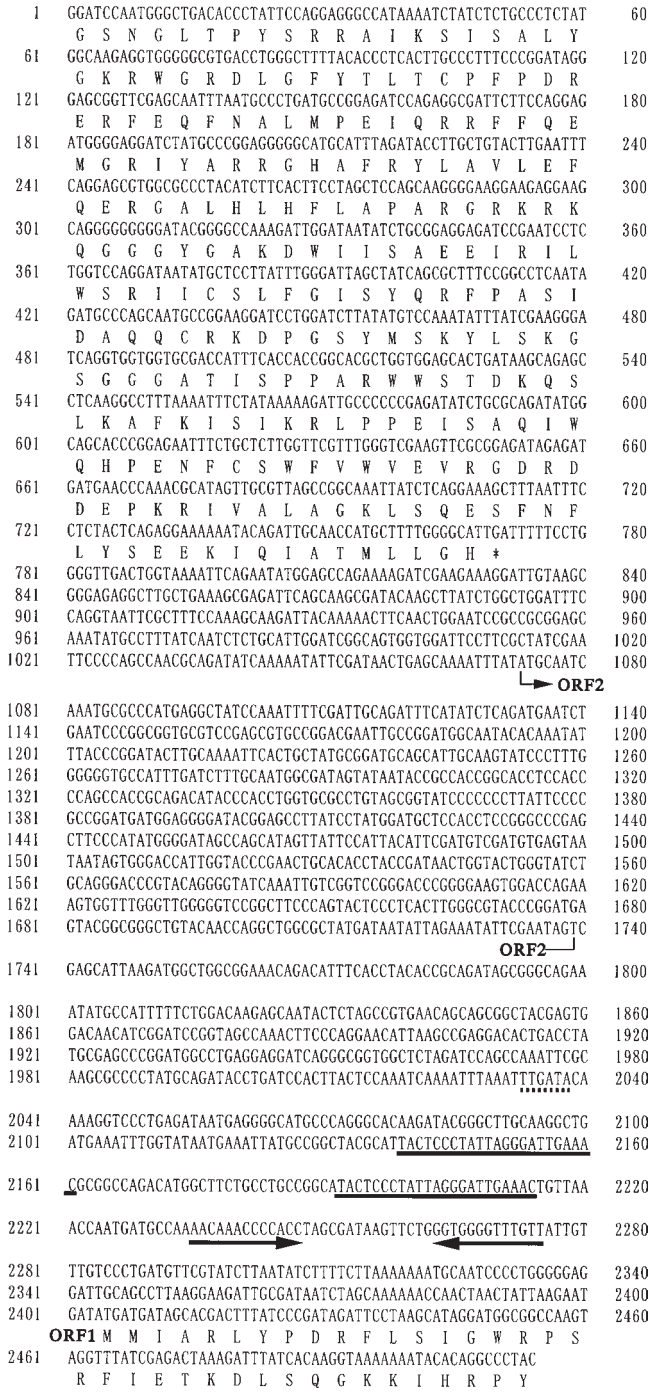

Fig. 2. Complete nucleotide sequence of the pMA4 and amino acid sequence of open reading frame 1. The nucleotide sequence is linearized at a *Bam*HI site at position 1. Direct repeats are underlined. Inverted repeats are marked by arrows. Double-stranded origin nick site is marked by a dot line. The sequence data will be submitted to DDBJ.

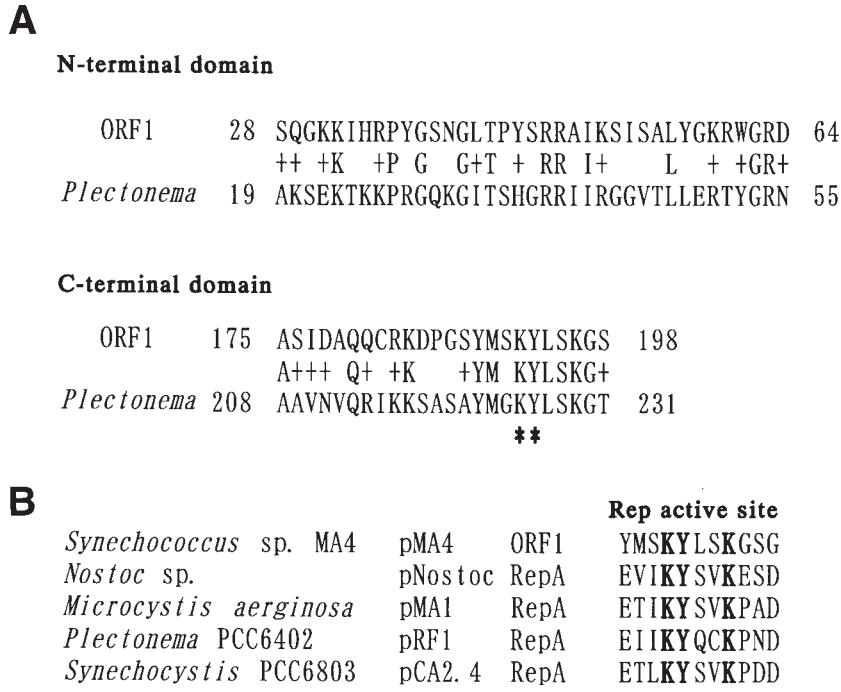

Fig. 3. Alignment of open reading frame 1 in pMA4. **(A)** Alignment of amino-acid sequence of the homologous region. **(B)** Alignment of the amino-acid sequence at the active site of the Rep protein. The consensus amino acids are indicated by bold characters.

ORF1 was a possible Rep protein of pMA4. ORF2 (Fig. 2) encoded a proline-rich putative protein with 23.5 kD. The estimated pI of ORF2 was 4.01. Many proline-rich proteins interact with DNA *(29–31)*. The ORF2 could also have an important role, although further investigation is necessary.

Electroporation of pMA4

The pMA4 was characterized as a backbone plasmid to develop a vector for thermophilic cyanobacteria, including *Synechococcus* sp. MA19, which is the powerful PHB accumulator mentioned by Miyake et al. *(13,17)*. We reported an efficient transformation of a thermophilic cyanobacterium by electroporation *(15)*, which was applied to introduce pMA4 into the strain MA19 (Fig. 4). pMA4 was efficiently incorporated from electroporation at an electric field strength of more than 9 kV/cm (Fig. 4): weak signal at 0 (lane 2), 3 (lane 3) and 6 kV/cm (lane 4), strong signals at 9 (lane 5) and 12 kV/cm (lane 6).

Generation of pMA4 in MA19 during the growth was tested (Fig. 5). The MA19 electroporated with pMA4 was cultivated in a newly prepared BG-11 with 0.5% inoculum. After the cell concentration reached 1.0×10^8 /mL, total DNA was extracted from the 100-µL culture and applied to an agarose gel electrophoresis (lane 3). The pMA4, which was cut by *Kpn*I and religated,

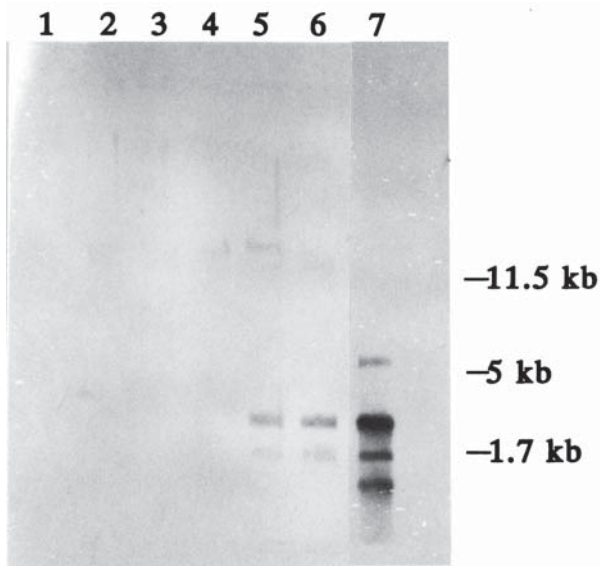

Fig. 4. Optimization of pMA4 incorporation into *Synechococcus* sp. MA19 by electroporation. MA19 was treated with pMA4 by an electroporation as follows: Lane 1, wild-type MA19; lanes 2–6, MA19 + pMA4; lane 7, pMA4. The electric strength fields in lanes 2–6 were 0, 3, 6, 9, and 12 kV/cm. 0.5 kb-*Bam*HI-fragment of pMA4 was used as a probe. Total DNA of 0.2 µg was applied to each lane.

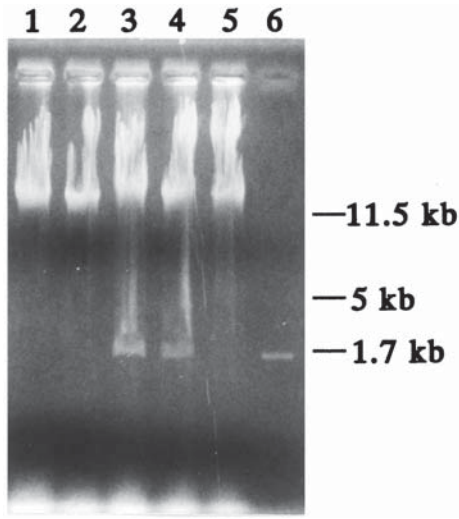

Fig. 5. Generation of pMA4 in the cells of *Synechococcus* sp. MA19 grown in the BG-11. Lane 1, wild-type MA19; lane 2, MA19 + pMA4 without electroporation; lane 3, MA19 + pMA4; lane 4, MA19 + the cut-and-religated pMA4; lane 5, MA19 + the deletion mutant of pMA4; lane 6, pMA4. Lanes 3–5, with electroporation (9 kV/cm).

was also recovered from the cultivated cells (lane 4). MA19 (wild type) had no plasmids (lanes 1, 2). These data show that pMA4 was generated and retained in MA19. The pMA4 was digested by *Kpn*I and *Sma*I and the large

fragment (2.4 kb) was ligated after the sticky end at the *Kpn*I site was deleted. The defective plasmid of the *Sma*I-*Kpn*I 100 bp ORF1 region was not generated in MA19 (lane 5). The region was necessary for plasmid replication.

Conclusions

This is the first report of a high-copy-number plasmid in thermophilic cyanobacteria. The plasmid pMA4 was small (2.5 kb). The sequencing analysis of the plasmid suggests that it may belong to a rolling-circle plasmid group (pC194 group), which includes some cyanobacterial plasmids. Finally, we demonstrated that the pMA4 is transferred and retained in MA19, which is a strong PHB accumulator. These results indicate that pMA4 may be a backbone plasmid to develop a vector system for thermophilic cyanobacteria.

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