Short Communication

Replication of Filamentous Cyanobacterial Plasmids, pPF1 from *Phormidium foveolarum* and pPB1 from *Plectonema boryanum*

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Single-stranded plasmid DNA of pPF1 from *Phormidium foveolarum* that was specifically degraded by S1 nuclease was detected by Southern hybridization. This is also the case of the homologous plasmid pPB1 from *Plectonema boryanum*. These observations suggest that such small cryptic plasmids as pPF1 and pPB1, both from Gram-negative and filamentous cyanobacteria, replicate by a rolling circle mechanism in their living cells.

Cyanobacteria, unicellular or filamentous, mostly harbor cryptic plasmid DNAs. Their dynamic functions in the cells are still unknown. Here we wish to propose that the pPF1 plasmid (1509-bp) from the filamentous cyanobacterium, *Phormidium foveolarum*, and pPB1 (1.5-kbp) from *Plectonema boryanum* replicate through the rolling circle replication (RCR) mechanism. Most recently Yang and Mcheddah demonstrated that a small plasmid, pCA2.4, from a unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 replicated also by the RCR mechanism.1)

Numerous plasmids from Gram-positive bacteria that replicate by the RCR mechanism accumulate single stranded DNA molecules as replication intermediates.2) Cyanobacteria, unicellular or filamentous, are Gram-negative organisms in general. Recent discoveries that a plasmid pKYM from the Gram-negative bacterium *Shigella somenii*3) and the plasmid pCA2.4 from *Synechocystis* sp. strain 68031) replicate by the rolling circle mechanisms appeared to be rather exceptional. This situation prompted us to examine if pPF1 and pPB1, both from Gram-negative and filamentous cyanobacteria, replicate by the RCR systems. If pPF1 replicates by the RCR mechanism, we would predict that pPF1 would accumulate strand-specific single strand DNA (ssDNA) in *P. foveolarum* cells. Thus, the cells harboring pPF1 were tested for the presence of the plasmid ssDNA.

*P. foveolarum* (M-43) was given us by the Research Institute of Applied Microbiology, The University of Tokyo, and *P. boryanum* by Prof. Asada, Research Institute for Food Science, Kyoto University. Both strains are cyanobacteria belonging to the LPP group, which comprises the genera *Lyngbya*, *Plectonema*, and *Phormidium*. The complete nucleotide sequence of pPF1 (1509 bp) from *Phormidium foveolarum* was presented by us4) (DDBJ Accession No. D10842) and that of the pPB1 from *Plectonema boryanum* by Barnum5) and the Walton group,6) respectively. The sequences of pPF1 and pPB1 had 98% identity over their total lengths.7)

Extraction and purification of plasmid DNA, agarose gel electrophoresis analysis, construction of the recombinant plasmids, and transformation of cells with these plasmids were done as described previously.8) *P. foveolarum* cells of a 10-day culture were treated with a solution containing EDTA and then lysozyme. Most of the filamentous cells were reduced to unicellular protoplast-like cells, which were then treated by the routine procedure of “lysis by alkali.” Electrophoretic analysis of the pPF1 plasmid showed that ccDNA moved faster than acDNA as described previously.9) Linear double strand pPF1 DNA that was cut out of the cloned hybrid DNA, pPF1-Bluescript KS,1) was used as the DNA source for the probe preparation. The DNA probes were labeled with χ-32P-dCTP by using the “Random primer DNA labeling kit” according to the instruction of the supplier (Takara Research Laboratories, Japan). Southern hybridization was done with 50% formamide at 37°C10) by using the labeled probe. The filters were washed in 1 × SSC (0.15 m NaCl plus 0.015 m sodium citrate, pH 7.0, containing 0.1% SDS) at 37°C for 0.5 h with two wash changes and fluorographed at −70°C by using RX medical X-ray film (Fuji Photo Film, Japan).

Total DNA prepared from *P. foveolarum* cells, after separation on an agarose gel, was transferred to a nylon transfer membrane (MagnaGraph, Micron Separations Inc., U.S.A.) directly or after alkaline denaturation. If ssDNA was present in the preparations, it would be trapped by the membrane without previous denaturation.11) To analyze the structure of the DNA, the DNA was digested with S1 nuclease or exonuclease VII and electrophoresed through an agarose gel. The DNA, transferred to the nylon transfer filters, was hybridized with 32P-labeled pPF1. As shown in Fig. 1, the cccpPF1 moved on the agarose gel almost comitatively with the predicted ssDNA. The 32P-pPF1 clearly hybridized with this fastest-moving DNA (lane, A-3) and this DNA was also trapped by the filter without denaturation (lane, B-3). This single stranded DNA was not detected after digestion with S1 nuclease, which attacks ssDNA (lane, B-4). However, the ssDNA did not disappear after digestion with exonuclease VII, which degrades ss-linear DNAs but not circular DNAs (lane, B-5).

Lane 2 indicates the position of circular single-stranded DNA (cs-pPF1) that was obtained by the heat-denaturation of the double stranded pPF1 (ds-pPF1) from intact *P. foveolarum* cells. The ds-pPF1 was shown on the lane A-1 as a control, which thus does not appear on panel B of ‘without prior denaturation’ (lane, B-1). They were electrophoresed in parallel with samples and hybridized. It may be difficult to understand the result of the A-4 lane. However, it could be possible that S1 nuclease

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Fig. 1. Synthesis of the ss Plasmid Intermediate of pPF1 in Phormidium flexuolatum Cells

DNA prepared from P. flexuolatum cells was electrophoresed and transferred to a nylon transfer membrane with or without prior denaturation and then hybridized to α-32P-dCTP-labeled pPF1. Panel A, with denaturation; Panel B, without denaturation. Lanes 1, linear double strand pPF1 DNA (ds-pPF1) that was cut out of the cloned pPF1-BlueScript KS+ vector; Lanes 2, heat-treated, thus circular single-stranded pPF1 (ss-pPF1); Lanes 3, Total DNA from P. flexuolatum cells; Lanes 4, DNA treated with S1 nuclease; Lanes 5, DNA treated with exonuclease VII.

is able to hydrolyse a single-stranded portion such as a hairpin loop of the cccDNA. Thus, in the presence of S1 nuclease, the cccDNA often gives ocDNA, which apparently does not disappear after the action of S1 nuclease (lane, A-4). Such circumstances have been shown in the experiments to show the RCR systems of the plasmid pKYM from Shigella sonnei and the pLAS1000 from Lactobacillus hilgardii.3,12

The results obtained here show that circular ssDNA was generated from pPF1 plasmid in P. flexuolatum cells. This circumstance was absolutely true of the pPB1 replication (data not shown). These observations suggest that pPF1 and pPB1, both from Gram-negative and filamentous cyanobacteria, replicated through the RCR mechanisms in their living cells. Recently, M. Espinosa announced “a wall falls” in his review article stating that rolling circle replicating plasmids are found not only in the Gram-positive but also in the Gram-negative bacteria.13 He described also how the generation of ssDNA is considered to be the hallmark of any RCR plasmid.

Upon a DNAsis computer search, a sequence of 5'-CTTATAAT-3', which is the nicking site of the bacteriophage f1 plus strand,14,15 was also found in the pPF1 strands. Figure 2 shows partial sequences of the pPF1 strands in which the possible nicking cassette occurs. Nicking might occur between T and A residues of the sequence CTTTATAAT in the initiation of replication of the pPF1. A big hairpin structure as well as possible small inverted motifs are shown with inverted arrows. The homologous plasmid, pPB1, has also the same sequence at almost the same positions in the strands. Novick et al. described how, in such a plasmid family as pT181, the leading strands replication origin often consists of two adjacent inverted repeat elements, which are involved in origin recognition by the initiator protein.15

These circumstances suggest that the plasmids pPF1 and pPB1 replicate by a fd bacteriophage type RCR mechanism. Moreover, some strains of the filamentous cyanobacteria of the LPP group carry plasmid DNAs of such small and similar sizes as 1.5 kbp.7 Thus we suppose that at least the small promiscuous plasmids of the LPP group cyanobacteria may also replicate by the common RCR mechanisms. Researches on the detailed mechanism of the RCR system are now in progress.

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References