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Plasmids from Two Morphologically Distinct Cyanobacterial Strains Share a Novel Replication Origin†

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A 2.9-kbp replication origin from a plasmid endogenous to the filamentous cyanobacterium *Fremyella diplosiphon* UTEX 481 was genetically characterized and sequenced. Deletion analysis of the 2.9-kbp DNA fragment delimited the minimum region necessary for replication in *F. diplosiphon* Fd33 to approximately 2.5 kbp. DNA sequence analysis revealed that the *F. diplosiphon* plasmid replication origin is structurally very similar to and shares significant identity with the 1.75-kbp replication origin reported for plasmid pDU1, isolated from the morphologically distinct cyanobacterium *Nostoc* sp. strain PCC 7524. Each cyanobacterial plasmid replication origin includes a large open reading frame that predicts a conserved protein of unknown function; the predicted proteins of the replication origins are of similar sizes and 30% identical in amino acid sequence. Each cyanobacterial plasmid replication origin also possesses a region of dyad symmetry approximately 300 bp upstream of the conserved open reading frame.

Cyanobacteria are a widely distributed and diverse group of prokaryotes characterized by the ability to perform oxygenic photosynthesis. Many cyanobacterial strains, both unicellular and filamentous, possess one or several (up to eight) phenotypically cryptic plasmids (10–12, 15, 19, 20). On the basis of plasmid size, restriction endonuclease digestion patterns, and DNA homology, several geographically distinct but genetically related cyanobacterial strains possess identical plasmids (6, 11, 12, 22). More interestingly, similar analyses show that some genetically unrelated strains appear to harbor identical or nearly identical plasmids (6, 12, 22). These studies, in conjunction with those that demonstrate the widespread distribution of the strains, suggest possible interspecific or intergeneric transmission of some cyanobacterial plasmids in nature. A number of physiological roles for cyanobacterial plasmids have been hypothesized (reviewed in reference 20); however, direct evidence for any of these roles remains to be established. Currently, little is known about these plasmids with respect to molecular mechanisms of replication, compatibility, transmission, and copy number control.

Plasmid DNA has been utilized to construct shuttle vectors capable of replicating in both *Escherichia coli* and the cyanobacteria (20). Sequences on cyanobacterial plasmids provide the genetic information necessary for replication in the cyanobacterial host. Because cyanobacterial plasmids do not replicate in *E. coli* (9) and *E. coli* cloning vectors are limited in their ability to replicate in a cyanobacterium (8), shuttle vectors that can replicate in both organisms have proven valuable in molecular genetic studies of various cyanobacteria. We recently reported the construction and characterization of the 5.6-kbp shuttle vector pPL2.7 that can be introduced into cells of strain Fd33 (5) of the filamentous cyanobacterium *Fremyella diplosiphon* UTEX 481 by either conjugation or electroporation (2). This vector includes a 2.9-kbp DNA fragment that contains the cyanobacterial replication origin from a plasmid recently identified as pFdA (5), which is endogenous to wild-type *F. diplosiphon* (7).

Few origins of replication on cyanobacterial plasmids have been genetically characterized. The best characterized is that of the 6.28-kbp plasmid pDU1 (14) isolated from the filamentous cyanobacterium *Nostoc* sp. strain PCC 7524. The replication origin of plasmid pDU1, which has been used in the construction of a series of shuttle vectors designed for conjugal transfer of DNA from *E. coli* to certain filamentous cyanobacteria (24), was localized to a 1.75-kbp DNA fragment (18). DNA sequence analysis of the pDU1 replication origin revealed regions of dyad symmetry and a central large open reading frame (ORF), ORF1, that predicts a 373-amino-acid polypeptide hypothesized to be involved in replication (23). Here, we report genetic characterization and DNA sequence analysis of the 2.9-kbp replication origin from *F. diplosiphon* plasmid pFdA and show that the pFdA replication origin is structurally, and probably functionally, similar to that of *Nostoc* sp. strain PCC 7524 plasmid pDU1.

Strains and growth conditions. Short-filament mutant strain Fd33 (5) of the filamentous cyanobacterium *F. diplosiphon* UTEX 481 (hereafter referred to as *F. diplosiphon* unless otherwise specified; similar to *Calothrix* sp. strain PCC 7601) was used for these studies. Cells were grown in liquid or on solid BG-11 medium (1) as previously described (2). Transformants of strain Fd33 were cultured in the presence of kanamycin sulfate at 25 $\mu g \cdot ml^{-1}$.

All *E. coli* host and conjugal strains used in this work have been described previously (2, 16) and were cultured in liquid or on solid Luria-Bertani medium with antibiotics at standard concentrations (16). For production of single-stranded DNA sequencing templates using helper phage M13K07, plasmid subclones were maintained in *E. coli* XL1-Blue which was propagated in 2× YT medium (16).

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Gene transfer and plasmid rescue. Introduction of shuttle vector pPL2.7 and related deletion clones into cells of strain Fd33 by conjugation or electroporation was performed as described previously (2). To retrieve plasmids from individual exconjugants or electroporants, total DNA was isolated from axenic cultures and used to transform *E. coli* DH5α to Km^r (2). Minipreparations of plasmid DNA were isolated from the Km^r colonies and mapped by restriction analysis.

DNA methods. DNA restriction endonucleases and modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Pharmacia Biochemicals (Piscataway, N.J.), and International Biotechnologies, Inc. (New Haven, Conn.). Deoxyadenosine 5′-[α-^{35}S]thio-triphosphate was purchased from Dupont/NEN (Boston, Mass.). DNA manipulations including digestion, ligation, and production of single-stranded DNA sequencing templates were done as described by Sambrook et al. (16). Double-stranded DNA sequencing templates were purified by using a kit from Promega Biotec (Madison, Wis.).

Construction of shuttle vector pPL2.7 deletion clones. The shuttle vector pPL2.7 (2) includes a 2.9-kbp DNA fragment from plasmid pJCF22:H51 that contains the genetic information necessary for replication in *F. diplosiphon* Fd33 (4). Plasmid pJCF22:H51 harbors a 7.5-kbp DNA fragment from plasmid pFdA (5), which is endogenous to wild-type *F. diplosiphon*. To localize and delimit the replication origin, a series of pPL2.7-based shuttle vectors deleted for regions within the 2.9-kbp DNA fragment were generated (Fig. 1A). Briefly, plasmids pPL2.4, pGCMS58, pGCMS48, pGCMS49, and pGCMS51 were generated by digesting plasmid pPL2.7 with *Bam*HI and *Eco*RI, *Bam*HI and *Bss*HII, *Hin*cII, *Bal*I, and *Spe*I, respectively, blunting the cut ends when necessary, diluting the digestion mixture, and religating the truncated fragments. Plasmid pPL2.1 is the equivalent of plasmid pPL2.7 without the region between the *Kpn*I and *Cla*I sites on the 2.9-kbp replication origin; pPL2.1 was generated by ligating the blunt-ended 2.4-kbp *Kpn*I-to-*Xmn*I fragment of plasmid pJCF22:H51 into *Hin*cII-linearized plasmid pKm-2 (2).

DNA sequencing. The 3.1-kbp *Bam*HI-to-*Pst*I DNA fragment from shuttle vector pPL2.7 (2) was subcloned into plasmid pUC118, creating plasmid pGCMS21. This 3.1-kbp DNA fragment contains the 2.9-kbp replication origin from *F. diplosiphon* plasmid pFdA (Fig. 1A). Single- and double-stranded sequencing of the pGCMS21 insert was done by the dideoxynucleotide chain-termination method (17) using Sequenase version 2.0 modified T7 DNA polymerase purchased from United States Biochemical (Cleveland, Ohio). Sequencing reactions were primed with M13 universal primers or with oligonucleotides synthesized on a Biosearch model 8600 oligonucleotide synthesizer. DNA and protein sequences were analyzed by using the Pustell sequence analysis program of International Biotechnologies, Inc., and compared with sequences in the GenBank data base.

Deletion analysis of the 2.9-kbp replication origin. Shuttle vector pPL2.7, which includes a 2.9-kbp DNA fragment that contains the cyanobacterial replication origin from endogenous plasmid pFdA, was designed and constructed for introducing recombinant DNA into cells of *F. diplosiphon* Fd33 by either conjugation or electroporation (2). Unlike wild-type *F. diplosiphon*, strain Fd33 does not harbor the endogenous plasmid pFdA (data not shown). During complementation of strain Fd33 regulatory mutants (3) with libraries of genomic DNA subcloned into vector pPL2.7, we observed a decrease in transformation frequency that correlated with an increase in plasmid size. To help construct a smaller shuttle vector capable of transforming strain Fd33 at a higher frequency, we set out to localize the cyanobacterial replication origin on the 2.9-kbp fragment. A series of pPL2.7-based shuttle vectors deleted for regions within the 2.9-kbp DNA fragment were generated and introduced into cells of strain Fd33 by electroporation and conjugation. Stable replication was realized by extraction of intact plasmids from Km^r, transformed colonies. Of the shuttle vectors depicted in Fig. 1, only pPL2.4 was capable of replicating in strain Fd33; introduction of deletion clones pGCMS58, pGCMS48, pGCMS49, pGCMS51, and pPL2.1 failed to yield stable transformants. These results indicate that the 356-bp region (*Bam*HI to *Eco*RI) (Fig. 1A) at the 5′ end of the 2.9-kbp fragment is not essential for replication in strain Fd33 and that nearly the entire remaining 2.5-kbp fragment (*Eco*RI to *Cla*I) (Fig. 1A) is required.

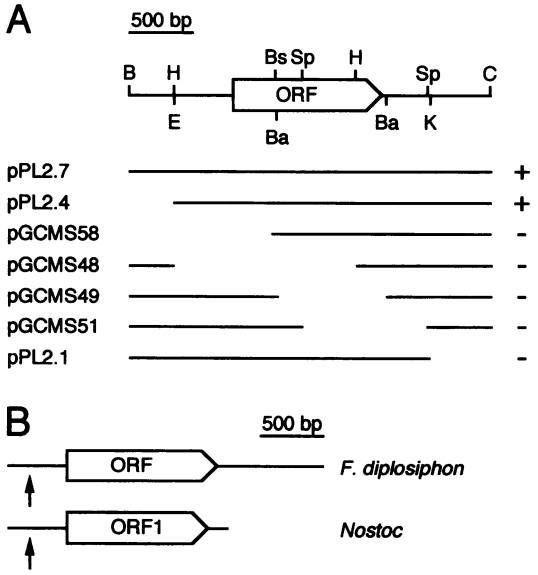

FIG. 1. (A) Physical map of the 2.9-kbp DNA fragment containing the replication origin from *F. diplosiphon* plasmid pFdA. The 1,185-bp ORF (open box) was determined by sequence analysis. Restriction sites are shown for enzymes used in cloning. The horizontal bars below the map indicate the corresponding regions present in the shuttle vector pPL2.7 deletion clones, with the names of the modified vectors at the left and with symbols at the right denoting replication (+) or nonreplication (−) of the clones in *F. diplosiphon* Fd33. Restriction endonuclease sites: B, *Bam*HI; Ba, *Bal*I; Bs, *Bss*HII; C, *Cla*I; E, *Eco*RI; H, *Hin*cII; K, *Kpn*I; Sp, *Spe*I. (B) Structural comparison of the replication origins from plasmid pFdA of *F. diplosiphon* and from *Nostoc* sp. strain PCC 7524 plasmid pDU1. The minimum regions necessary for replication in the respective strains (bars), conserved ORFs on the *F. diplosiphon* and *Nostoc* sp. strain PCC 7524 (23) plasmid replication origins (open boxes), and the location of an upstream region of dyad symmetry determined in this study for *F. diplosiphon* and reported for the *Nostoc* sp. strain PCC 7524 (23) (arrows) are indicated.

DNA and protein sequence analysis. The nucleotide sequence of the 2.9-kbp fragment containing the replication origin from *F. diplosiphon* plasmid pFdA is presented in Fig. 2A. This sequence corresponds to the *Bam*HI-to-*Cla*I fragment shown in Fig. 1. A search of the GenBank DNA data base revealed that the *F. diplosiphon* plasmid replication origin (nucleotide positions 328 to 2086) shares 52% DNA sequence identity with the 1.75-kbp replication origin re-

A

B

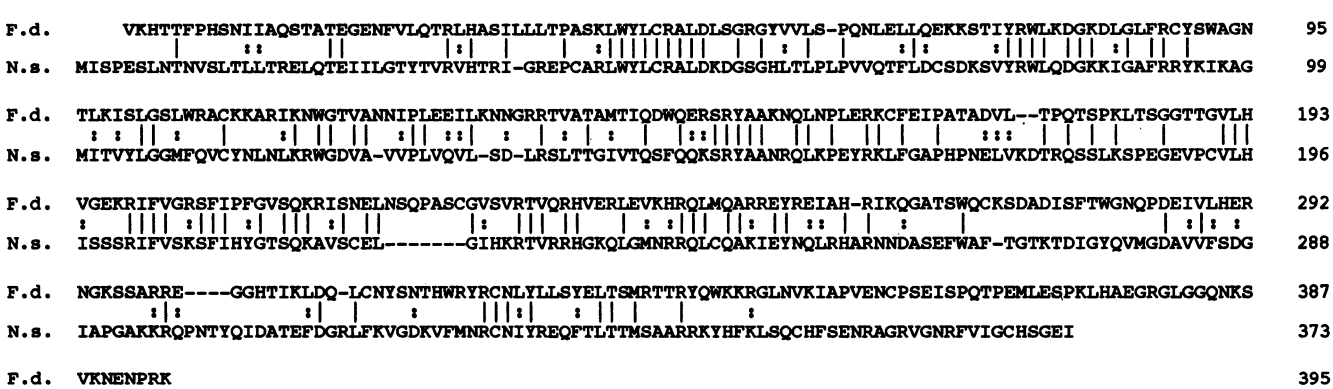

FIG. 2. (A) Nucleotide sequence of the 2.9-kbp DNA fragment containing the replication origin from *F. diplosiphon* plasmid pFdA. The first 14 nucleotides represent polylinker DNA. The predicted amino acid sequence of the 1,185-bp ORF is shown below the DNA sequence. The two regions of dyad symmetry upstream of the ORF are double underlined. Putative promoter elements are underlined. (B) Comparison of the amino acid sequence predicted by the ORF on the replication origin of the 2.9-kbp fragment from pFdA of *F. diplosiphon* (F.d.) to that on the replication origin of the 1.75-kbp fragment from pDU1 of *Nostoc* sp. strain PCC 7524 (N.s.). Amino acids that are identical (|) and conserved (:) between the two sequences are indicated. Gaps were introduced in both sequences for maximum alignment.

ported for the plasmid pDU1 from *Nostoc* sp. strain PCC 7524 (23). No other significant DNA sequence similarities were found.

The 2.9-kbp *F. diplosiphon* plasmid replication origin contains a 1,185-bp ORF (nucleotide positions 835 to 2020) predicted to encode a protein of 395 amino acids with a molecular mass of 44.9 kDa (Fig. 2A). The location within the replication origin region and predicted amino acid sequence of the *F. diplosiphon* ORF are very similar to those of the 42.5-kDa ORF1 reported for the replication origin of plasmid pDU1 (Fig. 1B). An alignment of the amino acid sequences of the *F. diplosiphon* and *Nostoc* sp. strain PCC 7524 plasmid replication origin proteins is presented in Fig. 2B. The amino acid sequence of the *F. diplosiphon* protein is

30% identical (42% conserved) to that of the 373-residue *Nostoc* sp. strain PCC 7524 protein. A search of the GenBank data base with the *F. diplosiphon* protein sequence revealed no other sequence with significant similarity. Extensive efforts in our laboratory to identify a transcript corresponding to the ORF on the pFdA replication origin have been inconclusive.

The replication origin for plasmid pDU1 is reported to have a region of extensive dyad symmetry located approximately 300 bp upstream of ORF1 (23). DNA sequence analysis of the corresponding region for the replication origin from plasmid pFdA revealed two regions of dyad symmetry similarly located upstream of the ORF (Fig. 1B) at nucleotide positions 469 to 493 and 529 to 550 (Fig. 2A). Unlike ORF1 on plasmid pDU1, plasmid pFdA does not appear to possess an obvious rho-independent transcriptional termination signal downstream of the ORF.

We have genetically characterized and sequenced the 2.9-kbp *F. diplosiphon* plasmid replication origin from plasmid pFdA and conclude that it is structurally, and probably functionally, similar to the replication origin reported for plasmid pDU1 isolated from the morphologically distinct cyanobacterium *Nostoc* sp. strain PCC 7524. The region of the 2.9-kbp fragment necessary for replication in *F. diplosiphon* Fd33 was approximately 2.5 kbp, which is slightly larger than the 1.75-kbp minimum replication origin reported for plasmid pDU1 (18).

The replication origins from plasmids of both *F. diplosiphon* and the *Nostoc* sp. strain PCC 7524 each have a conserved ORF and an upstream sequence of dyad symmetry (Fig. 1B). The finding that neither replication origin is functional without this intact ORF (reference 18 and this study) supports a role for this conserved protein in plasmid replication.

The functional significance of the region of dyad symmetry upstream of the ORFs of the cyanobacterial plasmid replication origins has not been established. Schmetterer and Wolk established a correlation between the region of dyad symmetry and the ability of pDU1 derivatives to replicate in different but morphologically related filamentous cyanobacterial strains (18). This question was not addressed in our study.

The cyanobacterial plasmid replication origins discussed above are novel in that origins with similar structures have not been reported for other bacterial strains. Several small plasmids from unicellular and filamentous cyanobacteria have been completely sequenced; however, they share DNA sequence homology with plasmids from other bacteria (13, 21). Since cyanobacteria may harbor several different plasmids (7, 10), it is possible that both *Nostoc* sp. strain PCC 7524 and *F. diplosiphon* also possess plasmids homologous to those present in other bacteria. Whether the similar structure and strong sequence identity of the *F. diplosiphon* and *Nostoc* sp. strain PCC 7524 plasmid replication origins allow for transmission of the plasmids between the two cyanobacterial strains remains to be determined.

Nucleotide sequence accession number. The DNA sequence reported in this paper has been deposited in the GenBank data base (accession no. L17027).

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