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Abstract

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In Vitro Replication of Cyanobacterial Plasmids from *Synechocystis* PCC 6803

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Little knowledge of DNA replication in cyanobacteria is available. In this study, we report the development and characterization of an *in vitro* system for studies of replication of the endogenous plasmids from the unicellular cyanobacterium *Synechocystis* 6803. This system (fraction III) was isolated at high salt concentrations and partially purified on a heparin–agarose column. DNA polymerases in *Synechocystis* 6803 appeared to be associated with membranes and could be released by the addition of ammonium sulfate to 20% saturation. DNA synthesis in fraction III was dependent on the addition of cyanobacterial plasmids isolated from the same strain. The *in vitro* replication products consist mostly of the supercoiled form of the plasmids. Unlike replication of many *Escherichia coli* plasmids, replication of cyanobacterial plasmids did not require added ATP, was not inhibited by omission of the ribonucleotides, and was insensitive to the RNA polymerase inhibitor rifampicin and the gyrase inhibitor novobiocin, but was inhibited by ethidium bromide. These data suggest that RNA may not be involved in the initiation of replication of cyanobacterial plasmids from *Synechocystis* 6803. In addition, intermediates of replication have been detected by two-dimensional gel electrophoresis. Density labeling experiments also indicate that cyanobacterial plasmid synthesis *in vitro* occurs by a semiconservative replication.

The *in vitro* replication of *Escherichia coli* chromosomal DNA and plasmid DNA has been well characterized in numerous studies. These investigations have enabled an examination in great detail of the biochemical mechanisms involved in the initiation and elongation of DNA chains and a characterization of the protein factors required for these processes (Kornberg and Baker, 1992; Kues and Stahl, 1989). Such *in vitro* studies have focused on replication of *E. coli* plasmids such as ColE1 (Sakakibara and Tomizawa, 1974; Staudenbauer, 1976; Conrad and Campbell, 1979), RSF1010 (Diaz and Staudenbauer, 1982), R6K (Inuzuka and Helinski, 1978a,b), Ori C plasmids (Fuller *et al.*, 1981), and R1 (Diaz *et al.*, 1981), as well as many phages (Kornberg and Baker, 1992; Wickner, 1990). *In vitro* replication has also been studied for the plasmid pT181 (Khan *et al.*, 1981) from *Staphylococcus aureus* and a yeast 2-μm plasmid (Jaswinski and Edelman, 1979). In general, these studies have been combined with *in vivo* investigations employing genetic mutations to yield considerable insight into the replication of plasmids and associated regulation (Kornberg and Baker, 1992; Kues and Stahl, 1989).

Except for ColE1-type plasmids, most plasmids encode one or more proteins as replication initiation factors necessary for their replication (Kornberg and Baker, 1992; Kues and Stahl, 1989). At present, two different initiation mechanisms for plasmid replication are known. Most *E. coli* plasmids initiate either bidirectionally or unidirectionally from an RNA primer at the origin(s) of replication (Kornberg and Baker, 1992; Kues and Stahl, 1989). As the replication proceeds, a theta-

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like structure is observed. On the other hand, a number of small plasmids such as pT181, pC194, and pUB110 from gram-positive bacteria replicate by a rolling-circle mechanism through extending the DNA strand from a specific nick created by a plasmid-encoded replication initiation protein. During the replication process, a sigma-like structure is formed and after one round of replication, a single-stranded plasmid intermediate is generated (Gruss and Ehrlich, 1989).

Cyanobacteria are a diverse group of gram-negative bacteria carrying photosynthetic constituents that are very similar to those in chloroplasts of higher plants. Many strains of cyanobacteria contain one or more phenotypically cryptic plasmids of different sizes (Houmard and Tandeau de Marsac, 1988). Little is known about replication of either chromosomal DNA or plasmids in cyanobacteria, in spite of the fact that DNA replication in *E. coli* and chloroplasts has been extensively investigated. To date, only three cyanobacterial plasmids have been sequenced (Yang and McFadden, 1993, 1994; Perkins and Barnum, 1992). The origins of replication in cyanobacterial plasmids have not been characterized. However, *E. coli* plasmids do not autonomously replicate within cyanobacteria (Kuhlemeier *et al.*, 1983), nor do cyanobacterial plasmids replicate in *E. coli* (Kuhlemeier *et al.*, 1981; Shermand and van de Putte, 1982), suggesting basic differences between the replication of *E. coli* and that of cyanobacterial plasmids. In the first report addressing the molecular mechanism of replication of cyanobacterial plasmids, we have recently shown that small plasmids pCA2.4 and pCB2.4 from *Synechocystis* 6803 replicate by a rolling-circle mechanism (Yang and McFadden, 1993, 1994). This is in contrast to the case for *E. coli* plasmids, most of which replicate by a theta-form mechanism (Kornberg and Baker, 1992). Since *E. coli* vectors cannot be used for cyanobacterial genetic engineering, endogenous cyanobacterial plasmids should prove to be very important in the construction of genetic engineering vectors for these bacteria. Numerous shuttle plasmids composed of an *E. coli* plasmid sequence and cyanobacterial chromosomal or plasmid sequence have been constructed (Buzby *et al.*, 1983; Chauvat *et al.*, 1986; Golden and Sherman, 1983). However, the lack of knowledge about the replication of cyanobacterial plasmid DNA limits the utility of these shuttle plasmids, which do not replicate efficiently within cyanobacteria. In many cases, these shuttle vectors are found to be integrated into cyanobacterial chromosomes or to recombine with resident cyanobacterial plasmids (Chauvat *et al.*, 1986; Williams and Szalay, 1983). Often they cannot be directly visualized with the ethidium bromide on agarose gels (Chauvat *et al.*, 1986) after transformation, indicating that they are stringently controlled in their replication.

In order to begin to understand replication of cyanobacterial plasmids and to contrast it with that of *E. coli* plasmids and chloroplast DNA, we now describe and characterize an *in vitro* replication system from the unicellular cyanobacterium *Synechocystis* 6803 that catalyzes the replication of exogenously added plasmids.

MATERIALS AND METHODS

Materials

[*methyl*-^{3}H]TTP (83 Ci/mmol), [α-^{32}P]-dATP, and [α-^{32}P]dTTP (3000 Ci/mmol) were purchased from NEN Nuclear. dATP, dCTP, dGTP, and dTTP were purchased from Pharmacia LKB, BrdUTP was from Sigma, and heparin–agarose from BioRad.

Strain and Growth Conditions

The cyanobacterium, *Synechocystis* 6803, was inoculated into 4 liters of BG-11 medium from a fresh culture until $OD_{730} = 0.15$ and then grown in a culture chamber with fluorescence lights and bubbling of a gas mixture of 95% N_2 and 5% CO_2 that had been filtered through a $CuSO_4$ solution and sterile

filter. Under these conditions, cells can grow up to log phase within 48 h. The log phase cells were harvested by centrifugation at 8000*g* for 5 min, washed with buffer A (see below) twice, and stored at −70°C.

Isolation of Endogenous Plasmids

Log phase cells of *Synechocystis* 6803 frozen at −70°C were thawed and then treated with 18 ml of saturated NaI solution at 37°C for 20 min. This treatment renders the cell wall susceptible to digestion by lysozyme. After washing with 250 ml of water, NaI-treated cells were resuspended in 30 ml of the lysis buffer containing 50 mM glucose, 50 mM Tris–Cl, pH 8.0 (25°C), and 10 mM EDTA. Lysozyme was added to a final concentration of 15 mg/ml. After gentle mixing, the cells were lysed at 37°C for 1 h. Then, 60 ml of 0.2 N NaOH/1% SDS was added, the mixture was set on ice for 5 min and neutralized by adding 60 ml of 3 M potassium acetate, and DNA was isolated according to Sambrook *et al.* (1989). DNA was treated with 20 μg/ml RNase for 15 min at 37°C. Plasmids were further purified by banding twice in an ethidium bromide/CsCl gradient according to Sambrook *et al.* (1989). The DNA isolated from *Synechocystis* 6803 contains two 2.4-kb plasmids (pCA2.4 and pCB2.4; Yang and McFadden, 1993, 1994), one of 5.2 kb, and one or two of >50 kb (Houmard and Tandeau de Marsac, 1988).

Preparation of in Vitro Extract

After thawing on ice, the frozen log-phase cells were resuspended in 20 ml of buffer A consisting of 20 mM Tris–Cl, pH 8.0 (25°C), 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM α-mercaptoethanol, 1 mM α-toluenesulfonylfluoride, and 20% glycerol. The resuspended cells were then lysed by passing through a French press 3–4 times at a pressure of 20,000 psi. To the lysed cells on ice, solid ammonium sulfate was added slowly within a 10-min period to 20% saturation. This step is crucial to release DNA polymerase activity. Then, the entire mixture was centrifuged at 14,500*g* at 2°C for 30 min to remove cell debris. Ammonium sulfate was added to the supernatant to 65% saturation, the mixture was stirred at 0°C for 30 min, and the precipitate was collected by centrifugation at 20,000*g* for 20 min at 2°C, dissolved in 2.0 ml of buffer A, and dialyzed against the same buffer for 4 h at 4°C. This partially purified extract, called fraction II, was frozen at −70°C.

Chromatography on a Heparin–Agarose Column

Fraction II was adjusted to 25 mM ammonium sulfate and applied to a 10-ml heparin–agarose column that had been equilibrated with buffer A containing 25 mM ammonium sulfate, and the column was washed with 50 ml of buffer A containing 25 mM ammonium sulfate and finally eluted with 0.5 M ammonium sulfate in buffer A. The DNA polymerase activity of each fraction (2.0 ml) was assayed under the following conditions: 5 μl enzyme fraction, 5 μl of 100 μM dATP/dCTP/dGTP plus 0.5 μCi [^{3}H]TTP, 15 μl activated calf thymus DNA (7.5 μg), and 25 μl 2× replication buffer (100 mM Tris, pH 8.0, 25°C, 24 mM $MgCl_2$, and 240 mM KCl). The reaction was carried out at 37°C for 30 min and stopped by adding 1.0 ml of cold 10% TCA. After setting on ice for 10 min, the mixture was passed through a GF/C glass filter, which then was washed twice with 5% TCA containing 20 mM sodium pyrophosphate and once with 70% ethanol. Filters were dried and counted in 5.0 ml of Amersham BCS fluid in a Packard liquid scintillation counter. The fractions containing active DNA polymerase were pooled and concentrated to $\frac{1}{10}$ vol by ultrafiltration with an Amicon YM10 membrane. The concentrated fractions were dialyzed against 100 vol of buffer A for 2 h and could be stored at −70°C for 6 months without significant loss of activity. The heparin–agarose column-purified fractions were called fraction III.

In Vitro Replication of Cyanobacterial Plasmids

Replication of cyanobacterial plasmids was tested in 100 μl of reaction mixture containing the following components: 40 mM Tris–Cl, 10 mM $MgCl_2$, 90 mM KCl, 1 mM DTT, 300 μM creatine phosphate, 20 μM each dATP, dCTP, dGTP, and 1 μCi of [^{3}H]TTP, 0.5 μg of isolated cyanobacterial plasmids (mixture of endogenous plasmids), and 20 μl of fraction III. The reaction was allowed to proceed at 37°C for 30–40 min. Then the reaction was terminated by adding 0.5 ml of cold 10% TCA and 20 μg of tRNA as a carrier for DNA precipitation. The acid-insoluble materials were collected and assayed for [^{3}H]-TTP incorporation as described in the previous section.

Analysis of Replication Products by Agarose Gel Electrophoresis

To examine the products of *in vitro* replication, [^{3}H]TTP was replaced by 1 μM [^{32}P]-TTP (10 μCi) in the standard incubation mixture. After 30 min reaction, DNA was extracted twice with 1 vol of phenol/chloroform and once with 1 vol of chloroform and then precipitated with 0.5 vol of 7.5 M ammonium acetate containing 10 μg of tRNA (as a carrier) and 2 vol of cold ethanol. The precipitated DNA was centrifuged and washed twice with cold 70% ethanol. The DNA was resuspended in TE buffer containing 10 μg/ml RNase A and incubated at 37°C for 15 min before loading onto an 0.8% agarose gel. The gel electrophoresis was performed according to Sambrook *et al.* (1989). The gel was fixed with 7% TCA, dried, and exposed to an X-ray film (X-Omat-AR from Kodak) at −70°C.

For two-dimensional electrophoresis, the ^{32}P-labeled replication products were digested by 10 units of *Hpa*I or *Xba*I/*Acc*I at 37°C for 4 h and then subjected to separation in a 0.4% agarose gel in 1X TBE (89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA, pH 8.0) at 1 V/cm for 15 h. The lane was excised, recast at 90°C on the top of the second agarose gel (1%), and subjected to electrophoresis at 5 V/cm in 1X TBE containing 0.3 μg/ml ethidium bromide for 4 h. The gel was fixed, dried, and autoradiographed as above.

Equilibrium Centrifugal Analysis of in Vitro Replication Products

Two micrograms of cyanobacterial plasmids was incubated for 40 min with [^{32}P]-dATP in 400 μl of reaction mixture under standard conditions using fraction III with the exception that TTP was replaced with 20 μM BrdUTP, and dATP with [^{32}P]dATP. DNA was isolated as above and subjected to ultracentrifugation together with the DNA labeled exactly as the ^{32}P-labeled DNA but with [^{3}H]TTP and without BrdUTP on a neutral CsCl density gradient based on the method of Inuzuka and Helinski (1978a). After centrifugation at 60,000 rpm in a VTi 65 rotor for 15 h at 20°C, the gradient was fractionated from the bottom of the tube and the flow was controlled by adjusting a syringe inserted into the top of the tube. The radioactivity of each fraction was determined.

RESULTS

In Vitro DNA Replication

Many extracts that catalyze DNA replication have been developed from *E. coli* and other organisms using a wide variety of plasmids. To prepare these extracts, cells have been quickly frozen and thawed and then treated with lysozyme at 0°C. Finally, treated cells have been gently disrupted by a cycle of freezing and thawing or by adding a detergent such as Brij-58. However, this approach has been unsuccessful in preparing active extract from the cyanobacterium *Synechocystis* 6803, since this strain, like most cyanobacteria, is highly resistant to treatment by lysozyme. Thus, in the present work *Synechocystis* cells have been subjected to rupture by the French press. After passing through the French press three times, most cells are lysed

TABLE 1

MEMBRANE-ASSOCIATION OF THE CYANOBACTERIAL DNA POLYMERASE[a]

Fractions	DNA polymerase activity (units)	Percentage
Lysate	566.5	100
Supernatant	39.9	7
Pellet/$(NH_4)_2SO_4$	498.7	88

[a] The DNA polymerase activity was assayed as described under Methods and Materials. One unit of enzyme activity is defined as the amount of enzyme catalyzing incorporation of 1 pmol of [^{3}H]TTP into the activated calf thymus DNA at 37°C in 30 min. The lysate was obtained by lysing cells with a French press, and the supernatant obtained by centrifugation of the lysate at 15,000*g* for 30 min. The pellet was resuspended in buffer A and treated with solid $(NH_4)_2SO_4$ to 20% saturation. The mixture was centrifuged and the supernatant was assayed for the DNA polymerase activity (fraction: pellet/$(NH_4)_2SO_4$).

efficiently. However, surprisingly, an extract with DNA polymerase activity could not be obtained in the supernatant after high-speed centrifugation of the lysed cells. In contrast to *E. coli,* most DNA polymerase activity was cosedimented into the pellet with cell debris after centrifugation of the lysate at 14,500*g,* but it could be released with ammonium sulfate treatment (Table 1), suggesting that DNA polymerase or the DNA replication system in *Synechocystis* may be membrane-associated. Alternatively, the membrane-associated activity could be released in lysates with ammonium sulfate addition to 20% saturation (equivalent to 0.8 M) before centrifugation. Other salts such as KCl and NaCl facilitated the release of activity to some extent, but not as efficiently as ammonium sulfate. A nonionic detergent, Triton X-100 at 0.5 and 2.5%, did not dissociate the DNA polymerase from the membrane, and a combination of ammonium sulfate and Triton did not increase the release (data not shown).

Characterization of the in Vitro Replication

Although both the ammonium sulfate-precipitated fraction (fraction II) and fraction III had high replication activity as shown by [^{3}H]-TTP incorporation into the acid-precipitable material, fraction II was less specific for the cyanobacterial plasmids as templates, presumably because this fraction contains nuclease activity which nicks the template randomly, rendering it a nonspecific template for DNA polymerases. For similar reasons, analogous crude extracts from chloroplasts have been further purified by a heparin–agarose column to remove endonuclease activities (Gold *et al.,* 1987; Carillo and Bogorad, 1988). Therefore, the cyanobacterial fraction II was further purified by chromatography on a heparin–agarose column. In the eluate containing fraction III, the endogenous DNA had been removed and DNA synthesis was dependent on the addition of exogenous cyanobacterial plasmids (Fig. 1). The added *Synechocystis* plasmids were efficient templates for DNA synthesis *in vitro,* whereas *E. coli* plasmid, pUC19, did not support significant [^{3}H]TTP incorporation in the presence of fraction III, suggesting high specificity of that fraction. Moreover, the relatively low template activity of pUC19 suggests that repair of nicked plasmids by fraction III was minimal.

From the time course shown in Fig. 1, it is clear that [^{3}H]TTP incorporation was biphasic, leveling off at about 15 min. It is noteworthy that a lag period was not observed, as seen for *in vitro* replication of many *E. coli* plasmids. The lack of a lag period may indicate that the initiation of plasmid replication in fraction III from *Synechocystis* does not depend upon synthesis of RNA as a primer.

The dependence of [^{3}H]TTP incorporation on the plasmid concentration and the protein concentration in fraction III is presented in Figs. 2A and 2B. Optimal incorporation was reached at about 10 μg/ml plasmid DNA and 800 μg/ml proteins.

Table 2 demonstrates the requirements for DNA synthesis *in vitro.* Like other replication systems, fraction III required Mg^{2+} for activity. However, it was not dependent on added ATP, but was rather appreciably inhib-

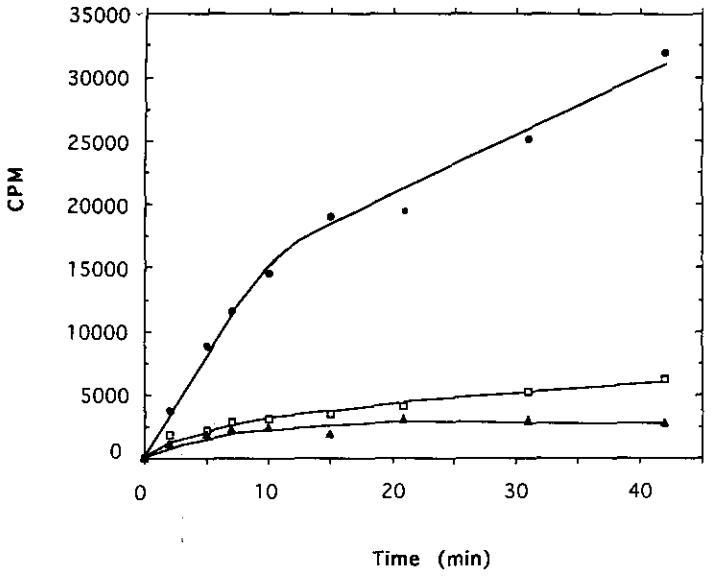

FIG. 1. Time course of *in vitro* cyanobacterial plasmid replication catalyzed by fraction III. Under standard conditions (see Methods), 200 μl of reaction mixture was incubated with 1.0 μg of added *Synechocystis* plasmid DNA (●), pUC19 DNA (□), or no exogenous DNA (▲). An aliquot of 25 μl of reaction mixture was taken at desired time points and measured for [^{3}H]TTP incorporation.

ited by added ATP, in contrast to many reports that ATP is required for *in vitro* replication of plasmids (Staudenbauer, 1976; Conrad and Campbell, 1979; Diaz *et al.*, 1981). It is noteworthy that the apparent lack of ATP dependence in DNA synthesis has also been observed for chloroplast extracts with which plasmids containing chloroplast replication origins were able to replicate in the absence of added ATP (Gold *et al.*, 1987; Carillo and Bogorad, 1988). In addition, synthesis of DNA was slightly reduced in the absence of ribonucleotides (Table 2) or in the presence of the RNA polymerase inhibitor, rifampicin, at 10 μg/ml, suggesting that RNA synthesis may not be required for initiation of replication of cyanobacterial plasmids *in vitro*. The possibility that the added templates had been preprimed seems unlikely, since the plasmids isolated had been treated with alkali. Omission of dNTPs from the reaction mixture reduced incorporation by 90% (data not shown). The DNA gyrase inhibitor, novobiocin, and protein synthesis inhibitor, chloramphenicol, were slightly inhibitory of DNA synthesis (Table 2). Cyanobacterial DNA polymerase was also highly resistant to chain termination by ddTTP. Only 40% inhibition of synthesis was observed even at a 100:1 ratio of ddTTP/TTP (data not shown), at which *E. coli* DNA polymerase would be completely inhibited (Fuller *et al.*, 1981). The high resistance to chain termination by ddTTP has also been observed for a DNA polymerase isolated from *Anacystis nidulans* (Lin *et al.*, 1990). However, ethidium bromide, a DNA intercalater, completely blocked plasmid [^{3}H]TTP incorporation by fraction III. The inhibition of DNA synthesis by ethidium bromide has been observed with *in vitro* chloroplast systems (Wu *et al.*, 1986; de Haas *et al.*, 1987).

In order to characterize the products of replication, plasmids were isolated after ^{32}P incorporation in the reaction mixture, precipitated with ethanol, and subjected to electrophoresis on an agarose gel followed by autoradiography. Figure 3 shows the ethidium bromide-stained gel and autoradiogram of the reisolated plasmids. Five ethi-

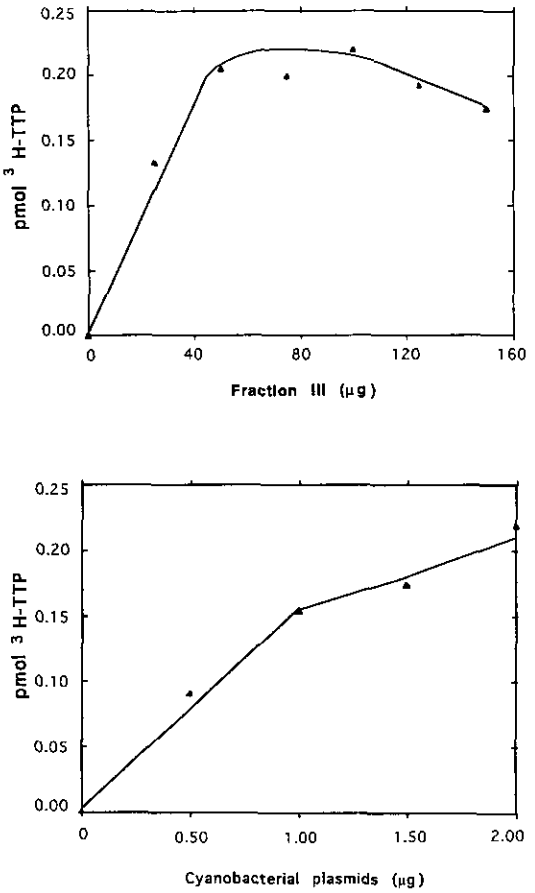

FIG. 2. Effects of protein (top) and DNA template (bottom) concentrations on the [^{3}H]TTP incorporation into cyanobacterial plasmids *in vitro*. Standard mixtures (100 μl) containing different amounts of fraction III or *Synechocystis* plasmids as shown were incubated at 37°C for 30 min.

dium bromide-stained bands were observed (Fig. 3A). Plasmids pCA2.4 and pCB2.4 and the 5.2-kb plasmid are the major plasmids in the isolated mixture. The lowest band on the gel corresponds to supercoiled forms (2.4 kb) of pCA2.4 and pCB2.4 (sc 2.4). Above it is the relaxed form (r 2.4) of these plasmids. Bands noted sc 5.2 and r 5.2 represent the 5.2-kb plasmid, and the top band represents the large, l, plasmid (50 kb). As shown in Fig. 3B, after 7 min reaction, about 50% of [^{32}P]-TTP incorporation was found in the relaxed and supercoiled forms of the 2.4-kb plasmids and 5.2-kb plasmid. As the reaction proceeded, more of the relaxed forms of these plasmids were converted into supercoiled plasmids implying function of a DNA gyrase in fraction III. The supercoiled form produced after 75 min of reaction contained about 70% of the total label in 2.4-kb plasmids. From the autoradiogram (Fig. 3B), it is obvious that the small plasmids of about 2.4 kb replicated more actively than the larger plasmid of 5.2 kb, whereas the amounts of both 2.4- and 5.2-kb plasmids were similar as indicated on the ethidium bromide-stained gel. This suggests that DNA synthesis catalyzed by fraction III is somewhat specific with respect to the endogenous templates.

In these experiments (Fig. 3B), replication intermediates of short half-life and low abundance, as is the case for pCA2.4 and pCB2.4 (Yang and McFadden, 1993, 1994), would not have been detected (Nawotka and Huberman, 1988).

TABLE 2

REQUIREMENTS OF DNA SYNTHESIS WITH CYANOBACTERIAL PLASMIDS AS TEMPLATES BY FRACTION III OF *Synechocystis* 6803

Conditions	[^{3}H]TTP incorporation (picomoles)	Percentage
Complete	0.105	100
−ATP	0.371	353
−$MgCl_2$	0.039	37
−Ribonucleotides	0.069	65
+Rifampicin (10 μg/ml)	0.067	64
+Novobiocin (1.5 μg/ml)	0.87	83
+Chloramphenicol (100 μg/ml)	0.80	76
+Ethidium bromide (10 μg/ml)	0.00	0

Note. The replication assay was done in 100 μl volume as described in the complete reaction conditions as follows: 40 mM Tris–Cl, pH 8.0, 20 μM dATP, dCTP, dGTP, 0.5 μCi of [^{3}H]TTP, 10 mM $MgCl_2$, 90 mM KCl, 250 μM each CTP, GTP, UTP, 2 mM ATP, 300 μM creatine phosphate, 1 mM DTT, 1 μg of cyanobacterial plasmids, and 20 μl of fraction III. When $MgCl_2$ was omitted from the reaction mixture, Mg^{2+} present in fraction III had not been removed.

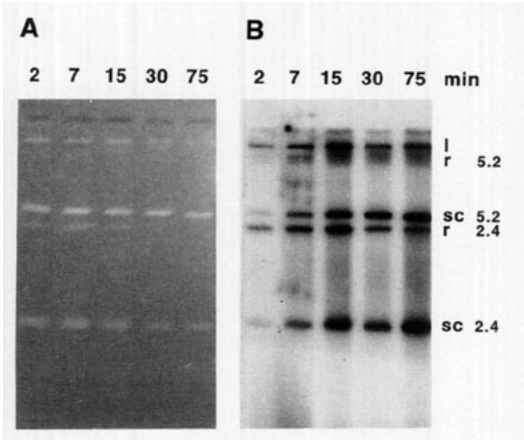

FIG. 3. Electrophoretic analysis of the *in vitro* replication products of the *Synechocystis* plasmids after different times of reaction as indicated on the top of the photographs. (A) Ethidium-stained gel. (B) Autoradiogram. The *in vitro* products were isolated by phenol/chloroform extraction and then separated on an 0.8% agarose gel. The gel was dried and exposed to an X-ray film. Bands are marked for B and correspond to the supercoiled and relaxed forms of 2.4-kb plasmids, sc 2.4 and r 2.4; supercoiled and relaxed 5.2-kb plasmid, sc 5.2 and r 5.2; and l, the large plasmid (about 50 kb).

In order to demonstrate that the ^{32}P incorporation into the cyanobacterial plasmids represents true replication, two-dimensional agarose gel electrophoresis was carried out to probe for replication intermediates during incorporation catalyzed by fraction III. The ^{32}P-labeled plasmids were purified and digested with *Hpa*I or *Acc*I/*Xba*I. *Hpa*I cuts the plasmid pCA2.4 into 1.7-, 0.34-, and 0.25-kb fragments; *Acc*I cuts pCB2.4 uniquely, and *Xba*I digests pCA2.4 into 2.33- and 0.017-kb fragments. The technique of 2-D gel electrophoresis has been used to map the replication origins of yeast plasmids (Brewer and Fangman, 1987; Huberman *et al.*, 1987) and chromosomal DNA (Huberman *et al.*, 1988; Linskens and Huberman, 1988). Recently, this approach has also been employed to analyze DNA replication of plasmids *in vitro* (Mahbubani *et al.*, 1992; Hyrien and Mechali, 1992). Specifically, the restriction fragments from the replicating DNA are separated in the first dimension on an agarose gel of low percentage (0.4% agarose), on which fragments migrate at a rate depending on their molecular weights. For second-dimensional electrophoresis, the lane of the first dimension is excised and embedded perpendicularly to the initial direction on the top of the second agarose gel of higher percentage (1.0%). In the second gel, DNA molecules migrate not only according to their molecular weights but also depending on their shapes or secondary structures. Nonlinear molecules consisting of the replicative intermediates will migrate slower than the linear molecules of the same size. Thus, linear molecules will migrate at positions along the diagonal of the gel, whereas intermediates having replication branches (such as Y or double Y molecules) or bubbles will run off the diagonal on the gel, appearing as a small arc (Brewer and Fangman, 1987) as illustrated in Fig. 4A. Figures 4B and 4C show heavily exposed autoradiograms of the 2-D agarose gels of restriction-digested replication products. In addition to the spots migrating on the diagonal in the gel, lighter arcs off the diagonal from the 2.4- (Figs. 4B and 4C) and 3.7-kb (Fig. 4B) fragments generated by the *Hpa*I or *Xba*I/*Acc*I digestion (Figs. 4B and 4C) were observed, indicating that intermediates of nonlinear structure and of low abundance were synthesized *in vitro*. This low abundance of intermediates in DNA replication has been noted in similar studies (Huberman *et al.*, 1987, 1988; Hyrien and Machali, 1992). However, the typical patterns of the arc for the single or double Y or bubble molecules were not clearly seen. We suggest that the observed DNA molecules with nonlinear structure (Figs. 4B and 4C) were double-stranded linear molecules with a single-stranded branch at a certain position. These structures may be generated from the sigma-like molecules that are known intermediates of rolling-circle replication, which has been recently described as the mechanism for replication of the *Synechocystis* plasmids pCA2.4 and pCB2.4 (Yang and McFadden, 1993, 1994). Since repair processes only generate short patches of DNA which will not cause

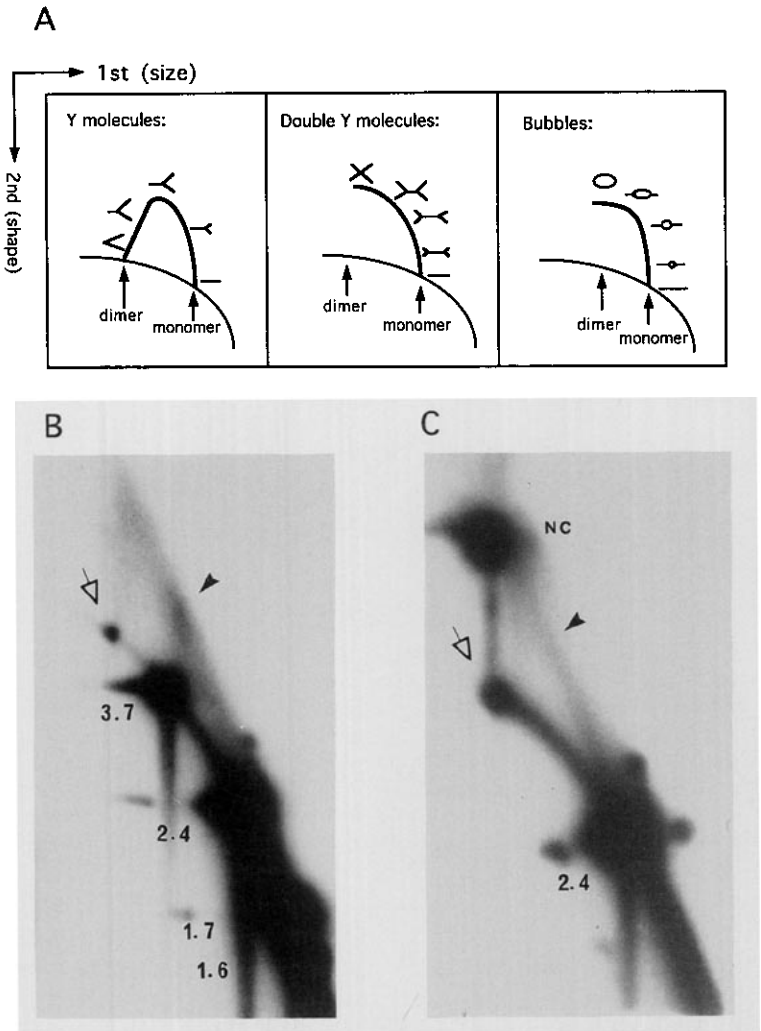

FIG. 4. Two-dimensional electrophoresis and autoradiography of the *in vitro* products. A, redrawn from Mahbubani *et al.* (1992) by permission of Oxford University Press, shows the typical 2-D profiles of replication intermediates such as Y, double Y, and bubble molecules. These branched intermediates migrate off the diagonal of the 2-D gel, depending on the degree of replication and on their structures. The photographs (corresponding to the monomer region only in A) show the electrophoretic analysis of *in vitro* products digested by *Hpa*I (B) or *Xba*I/*Acc*I (C). The open arrows point to the diagonal line formed by linear labeled molecules such as fragments from completed unbranched plasmids. The solid arrows indicate the replicative intermediates consisting of nonlinear molecules that migrate off the diagonal on the gel. If incorporation occurred by a DNA repair process, only the diagonal line would be observed. The sizes of individual restriction fragments of plasmids are also marked. The downward lines from each spot of linear fragments are due to breaking of DNA by UV light during photography of the first-dimensional gel. NC indicates the nicked circle of the 5.2-kb plasmid.

migration off the diagonal on the 2-D gel, the above observation suggests that the ^{32}P incorporation *in vitro* represents the replication of plasmids.

To test further for replication *in vitro*, cyanobacterial plasmids were incubated in a standard reaction mixture for 40 min with BrdUTP and [^{32}P]dATP added in place of

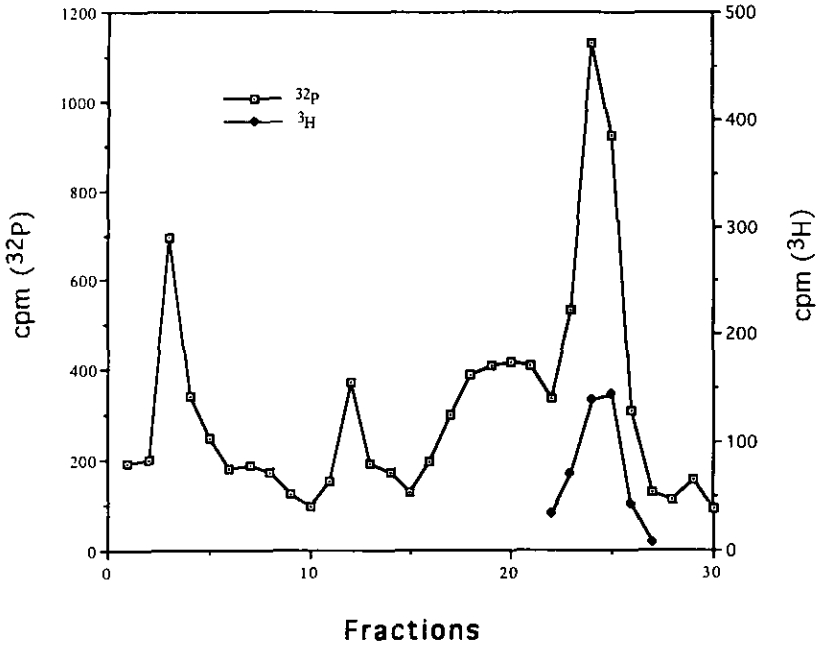

FIG. 5. CsCl equilibrium density shift analysis of the *in vitro* products. Cyanobacterial plasmids were labeled *in vitro* as described with [^{32}P]dATP in the presence of BrdUTP, isolated by phenol/chloroform extraction, and then subjected to centrifugation in 1.72 g/ml of CsCl at 60,000 rpm in a Beckman VTi 65 rotor overnight. ^{3}H-labeled cyanobacterial plasmids were added as density standards. Fractions were collected from the bottom of the tube, and radioactivity of each fraction was determined. In the ^{32}P profile more dense peak (fractions 15–22) corresponds to hybrid molecules consisting of one BrdUMP-labeled strand and one parental strand, and the least dense peak (fractions 22–27) consists of two parental strands. This latter peak corresponds in density to the peak shown in the ^{3}H profile.

TTP and dATP, respectively. After isolation, the reaction products were subjected to an equilibrium centrifugation on a neutral CsCl gradient. After fractionating the gradient, 4 major peaks containing ^{32}P-labeled DNA with different densities were identified (Fig. 5). The lightest peak banded at the same density, 1.69 g/ml, as that of the peak for the ^{3}H-labeled plasmid population. A higher density ^{32}P-labeled peak banded at approximately 1.73 g/ml and corresponds exactly to the hybrid molecules consisting of the light parental strand and newly synthesized BrdUMP-labeled heavy strand of DNA (Khan *et al.*, 1981). In addition, two denser peaks were also seen in the ^{32}P profile. We are not sure what these two peaks represent but they may be BrdUMP-labeled single-stranded intermediates and/or duplex molecules which are BrdUMP labeled on both strands. Nonetheless, the observed shift to heavier density of the *in vitro* products suggests the semiconservative replication of cyanobacterial plasmids.

DISCUSSION

To study the replication of cyanobacterial plasmids, we have established an *in vitro* plasmid DNA-dependent replication system from the unicellular cyanobacterium *Synechocystis* 6803. The plasmid synthesis in this system does not require added ATP as does that catalyzed by cell-free extracts from *E. coli*. However, the lack of requirement for added ATP has also been seen for *in vitro* DNA synthesis by extracts purified from chloroplasts (Gold *et al.*, 1987). In the pres-

ent work, the major form of synthesized plasmids is supercoiled. Furthermore, DNA synthesis in the *in vitro* system is specific for added *Synechocystis* plasmids. In addition, unlike that for plasmids ColE1 and R6K (Conrad and Campbell, 1979; Inuzuka and Helinski, 1978a), the present *in vitro Synechocystis* plasmid-dependent DNA synthesis is not markedly inhibited by the DNA gyrase inhibitor novobiocin or the *E. coli* RNA polymerase inhibitor, rifampicin. Omission of ribonucleotides only slightly reduces the observed DNA synthesis. These latter observations are very similar to those observed for replication *in vitro* of pT181 and chloroplast DNA and indicate that replication of cyanobacterial plasmids may not require synthesis of RNA as a primer for the initiation of replication. In turn, this is consistent with our recent detection of a single-stranded intermediate in the replication of plasmids pCA2.4 and pCB2.4 (Yang and McFadden, 1993, 1994) in *Synechocystis* 6803 cells, which is characteristic for plasmids replicating via a rolling-circle mechanism. Therefore, the observed profile in 2-D-gel electrophoresis for restriction fragments of the *in vitro* products includes arcs that may represent branched molecules generated from sigma-like intermediates arising from rolling-circle replication. In rolling-circle replication, initiation occurs by the introduction of a nick by the plasmid-encoded Rep protein at the origin of replication. The free 3-OH end thus generated is used as a primer for the displacement synthesis of DNA (Gruss and Ehrlich, 1989). The parental strand displaced is cleaved by the same Rep protein and both ends are ligated to generate a single-stranded circular plasmid. The single-stranded plasmid is converted into double-stranded plasmids by host factors.

The replication of cyanobacterial plasmids may require plasmid-encoded proteins, as does that of many *E. coli* plasmids. We have found that one of the 2.4-kb plasmids from *Synechocystis* 6803, pCA2.4, encodes a 39.2-kDa protein called RepA that is significantly homologous to the Rep proteins of plasmids from gram-positive bacteria, which have been shown to replicate by a rolling-circle mechanism. RepA has been expressed in *E. coli* and partially characterized (Yang and McFadden, 1993). These observations and detection of single-stranded DNA for pCA2.4 and pCB2.4 in cells further suggest that the presently observed *in vitro* replication of isolated *Synechocystis* plasmids may proceed by a rolling-circle mechanism, but this must be further tested. In any case, the availability of an *in vitro* replication system for cyanobacterial plasmids, and at least one replication protein, will enable the characterization of origin sequences and mechanisms to regulate copy numbers of these plasmids. Recently, we have localized the origin sequence of the small plasmid pCA2.4 from *Synechocystis* to within a 0.38-kb *Hin*dIII DNA fragment by using the previously described *in vitro* system (Yang *et al.*, 1994).

Unlike the case of *E. coli*, studies of DNA replication of cyanobacterial plasmids are hampered by the lack of plasmid-minus mutants as well as genetic mutants whose replication is impaired. We have not carried out *in vivo* detailed studies of plasmid replication in cyanobacteria because of the complication that the recombinant chimeric plasmids consisting of *E. coli* plasmid and cyanobacterial plasmid DNA would tend to rearrange or recombine with endogenous plasmids. Nevertheless, the presently described *in vitro* plasmid synthesizing system may open a powerful new approach to study plasmid replication among the cyanobacteria.

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