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Rolling-circle replication of the plasmid pKYM isolated from a Gram-negative bacterium

(DNA replication/single-stranded DNA/homology of Rep proteins from plasmids)

HIROO YASUKAWA, TETSU HASE, ASAKO SAKAI, AND YUKITO MASAMUNE

Department of Microbiology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan

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ABSTRACT **Plasmid pKYM isolated from a Gram-negative bacterium encodes a Rep protein that is essential for plasmid replication. A comparison of Rep protein from pKYM to Rep proteins encoded by other plasmids shows that it has homology to Rep proteins of the pUB110 plasmid family from Gram-positive bacteria. These plasmids replicate by a rolling-circle mechanism in which a tyrosine residue in the Rep protein acts as the acceptor for the 5′ end of the single-strand break introduced by the Rep protein. A Tyr → Phe substitution in the pKYM Rep protein abolishes its activity. Strand-specific single-stranded circular plasmid DNA can be recovered from the cells carrying pKYM and thus we propose that the plasmid pKYM replicates by a rolling-circle mechanism.**

Various types of plasmids are found in Gram-positive and Gram-negative bacteria (1–5). Analyses of their multiplication suggest that the former plasmids (for example, pUB110 and pT181) replicate by a rolling-circle mechanism (1–3) whereas the latter plasmids (for example, F, pSC101, R6K, and ColE1) replicate by θ forms (3, 4). Consequently, the origin of plasmids from Gram-positive and Gram-negative bacteria may be different. These plasmids generally require the specific Rep proteins encoded by their genome for their replication (1–5). Most of the *Escherichia coli* (Gram-negative) plasmids, moreover, require DnaA protein for their replication (4, 5). DnaA is encoded in the host genome and is essential for the replication of the host genome (6). ColE1 is a unique plasmid that does not encode a Rep protein and requires DNA polymerase I (7) for its replication (4, 5).

We have been studying a tiny multicopy plasmid, pKYM, isolated from *Shigella sonnei* (Gram-negative bacterium) obtained from K. Kiritani (Hokuriku University). The plasmid named pKYM has 2083 base pairs and requires a plasmid-encoded Rep protein (Rep_{pKYM}) for its replication. It can multiply normally in *E. coli* and does not require DnaA protein or DNA polymerase I for its replication (8).

A comparison of the amino acid sequence of the pKYM Rep protein with similar proteins of Gram-positive and Gram-negative bacterial plasmids suggests that the plasmid pKYM belongs to the pUB110 plasmid family, plasmids from Gram-positive bacteria. Analysis of the DNA extracted from the cells carrying pKYM suggests that it replicates by a rolling-circle mechanism.

MATERIALS AND METHODS

Bacteria, Plasmids, and Phages. *E. coli* strains used in the experiments were HB101 (9), YS1 (10), BMH71-18 (11), MV1184 (12), JM109 (13), WA802 (14), and WA802 *polA* (15). Plasmids used are pBR322 (16), pKYM, and dl13, a derivative of pBR322 carrying the region of pKYM essential for its self-replication as described (17). pOPM3702 is a derivative of R6K carrying Rep_{pKYM} and is used to supply Rep_{pKYM} in trans (18). The following phages are M13 derivatives. For M13tv18-dl13, the *Bgl* II–*Sph* I fragment of dl13 containing the replication origin and the *rep* gene of pKYM was inserted between *Bam*HI and *Sph* I sites at the multicloning site of M13tv18 (19). For M13mp18-ori(+), the *Bgl* II–*Hin*cII fragment of dl13 containing only the replication origin of pKYM was cloned at the multicloning site of M13mp18 (20). The plasmid was used to detect the plus strand of pKYM, which corresponded to the coding strand of Rep_{pKYM} (17). For M13mp19-ori(−), the same *Bgl* II–*Hin*cII fragment of dl13 was cloned at the multicloning site of M13mp19 and used to detect the minus strand of pKYM.

Enzymes. Restriction enzymes were purchased from Toyoba (Osaka) and Takara Shuzo (Kyoto). S1 endonuclease, calf intestine alkaline phosphatase, T4 DNA polymerase, T4 DNA ligase, *E. coli* DNA ligase, and the "Random primer DNA labeling" kit were purchased from Takara Shuzo. Sequenase, deoxynucleoside triphosphates, and dideoxynucleoside triphosphates were purchased from Toyoba. T4 polynucleotide kinase was purchased from New England Biolabs. Exonuclease VII was purchased from Bethesda Research Laboratories Life Technologies. All enzymes were used by following the instructions of the suppliers.

Nucleotides. ATP was purchased from Boehringer Mannheim Biochemica. [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Amersham. [*methyl*-^{3}H]Thymidine (42 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. The oligonucleotide whose genetic code changed from tyrosine to phenylalanine (oligo Y237F: 5′-TTCCGGTTTAACG-GAAAACTTAAGCGTTTCAGCAACCGC) was synthesized using an Applied Biosystems DNA synthesizer. The original sequence of the underlined region was 5′-CTGAAATAC. In addition to this change of the genetic code of tyrosine (TAC) to phenylalanine (TTT), 5′-CTGAAA was also changed to 5′-CTTAAG to create an *Afl* II site without changing the code.

Site-Directed Mutagenesis of Rep Protein. The Tyr-237 → Phe substitution in Rep_{pKYM} (the number indicates the amino acid position in the protein numbered from N-terminal residue as number 1) (17) was made using oligo Y237F as primer and M13tv18-dl13 as template, by the method of Kramer *et al.* (11, 12). The intended mutant M13mp18-Y237F was isolated by its susceptibility to *Afl* II and was confirmed by nucleotide sequencing. dl13Y237F was constructed by recloning the *Sma* I–*Sph* I fragment of M13mp18-Y237F that contained the replication origin and *rep* gene of pKYM into *Pvu* II–*Sph* I site of pBR322.

Nitrocellulose Filter Binding Assay of Rep Protein. The syntheses of Rep_{pKYM} and the mutant protein were assayed by nitrocellulose filter binding of [^{3}H]thymidine-labeled dl13 DNA by the method described by Sugiura *et al.* (21). The filter was the product of Schleicher & Schuell. DNAs from dl13 and pBR322 labeled with [^{3}H]thymidine were prepared by the method of Sugiura *et al.* (21). The specific activities of

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^{3}H-labeled dl13 and pBR322 DNAs were 6.8×10^5 cpm/pmol and 6.0×10^5 cpm/pmol, respectively. Fifty nanograms of DNA was used for an assay. The binding was examined after a 20-min incubation at 20°C with various quantities of an extract prepared from *E. coli* HB101 carrying dl13 or dl13Y237F. Cultures were grown to 5×10^8 cells per ml in the medium containing 10 g of Polypeptone (Wako Pure Chemical, Osaka), 2.5 g of NaCl, and Extract Ehrlich (Wako Pure Chemical) per liter of H_2O and the extract was prepared by freezing and thawing cells after digestion with lysozyme.

Southern Blot Hybridization. Plasmid DNA was extracted from *E. coli* YS1 carrying pKYM by the method of te Riele *et al.* (2, 22). The structure of the extracted DNA was analyzed by Southern blot hybridization after agarose gel electrophoresis. Anti-M13mp18-ori(+) and anti-M13mp19-ori(−) DNAs labeled with ^{32}P (the label was in the opposite strand) were prepared using the "Random primer DNA labeling" kit (23) by following the instruction of the supplier and were used as probes.

Other Methods. Extraction and purification of plasmid DNA, agarose gel electrophoresis analysis, nucleotide sequence analysis, construction of the recombinant plasmids, and transformation of cells with these plasmids were performed as described (15, 17).

RESULTS

Amino Acid Sequence Homology Among Rep Proteins. The amino acid sequence of Rep_{pKYM} was deduced from the nucleotide sequence. The protein consists of 321 amino acids and the calculated molecular weight is 37 kDa (17). The amino acid sequence homology between Rep_{pKYM} and similar proteins of other plasmids was examined by dot-plot analysis (24). The results show that the Rep_{pKYM} has significant homology to proteins encoded by the pUB110 plasmid family (25), such as pUB110, pC194, pFTB14, pBAA1, and pLP1 (Fig. 1). Plasmids of the pUB110 family are the parasites of Gram-positive bacteria. Rep_{pKYM} does not show any significant homology to Rep proteins of plasmids from Gram-negative bacteria, such as F, pSC101, R6K, and R1, by the same analysis.

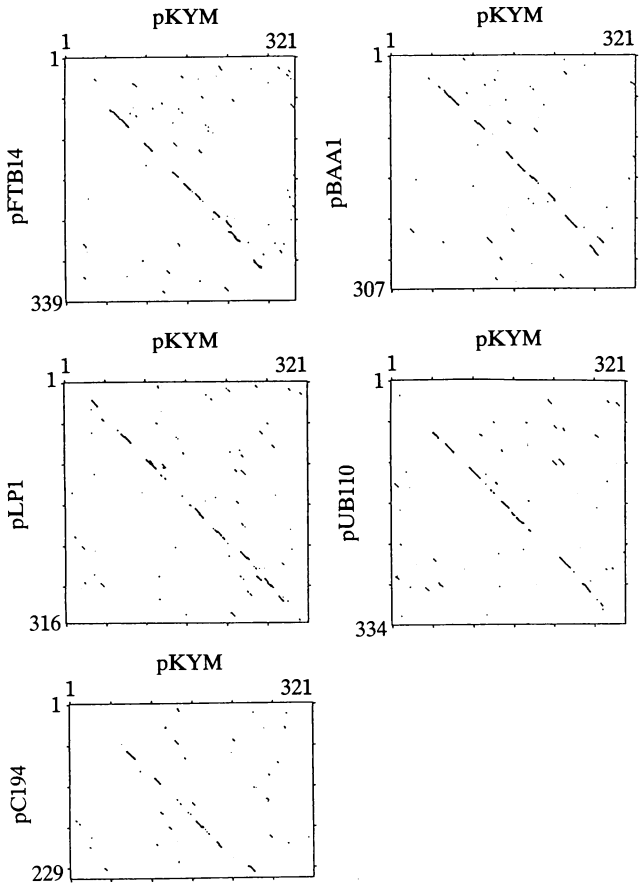

FIG. 1. Comparison of the amino acid sequence of Rep_{pKYM} with sequences of Rep proteins from the pUB110 plasmid family. The DNASIS program (Hitachi Software Engineering) was set to score five perfect matches in a window of nine bases. Rep_{pKYM} is plotted on the horizontal axis against the various other Rep proteins as indicated on the vertical axis.

Conservation of the Amino Acid Sequence Around Tyr-237 in Rep Proteins. The tyrosine residues, corresponding to Tyr-237 of Rep_{pKYM}, in Rep proteins of plasmids from Gram-positive bacteria are believed to be important for their activity (25). Amino acid sequence conservation around these tyrosines is significant in the pUB110 plasmid family and in pKYM (Fig. 2). Conservation of the sequence Lys-Tyr is also observed in the pT181 plasmid family, another plasmid family of Gram-positive bacteria (26) including pT181, pC221, pS194, and pC223, and even in the gene A proteins of coliphages ϕX174 and G4 (27). There was, however, no significant homology when the entire sequence of Rep_{pKYM} was compared with sequences of proteins encoded by the pT181 plasmid family and phages.

Plasmids of Gram-positive bacteria and phages ϕX174 and G4 replicate by a rolling-circle mechanism and the tyrosines of some Rep and gene A proteins accept the 5′ end of the cleaved DNA at the replication origin (27, 28). These results suggest that plasmid pKYM replicates by a rolling-circle mechanism and that Tyr-237 of Rep_{pKYM} plays an important role in its function.

The Tyr-237 → Phe Substitution. Plasmid dl13Y237F whose Rep_{pKYM} Tyr-237 was changed to Phe was constructed by site-directed mutagenesis. The substitution was confirmed by nucleotide sequence analysis (Fig. 3). The mutant could not multiply in *E. coli* WA802 *polA* but could multiply when Rep_{pKYM} was supplied in trans by plasmid pOPM3702 (Table 1).

Synthesis of the Mutant Protein. The impact of the mutation on synthesis of the Rep protein was examined by a nitrocel-

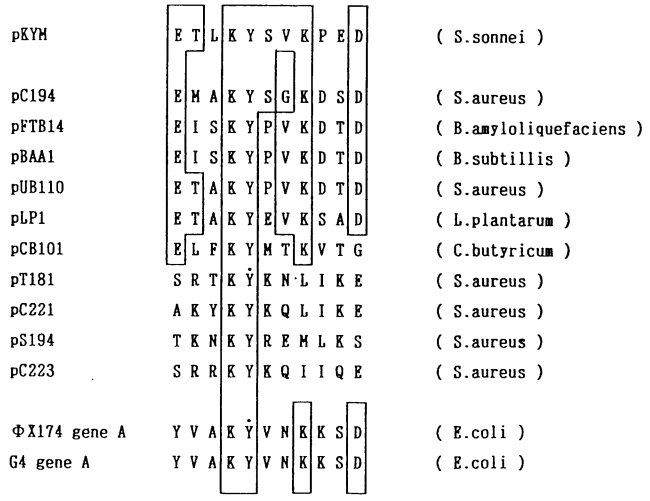

FIG. 2. Conserved region of Rep proteins from pKYM, the pUB110 family, and the pT181 plasmid family and gene A proteins of coliphages ϕX174 and G4. Amino acids common to Rep_{pKYM} are boxed. The tyrosine residues marked with asterisks are described as the linkage sites to the DNA when nicking occurs at the origin. *S. aureus; Staphylococcus aureus; B. amyloliquefaciens, Bacillus amyloliquefaciens; B. subtillis, Bacillus subtilis; L. plantarum, Lactobacillus plantarum; C. butyricum, Clostridium butyricum.*

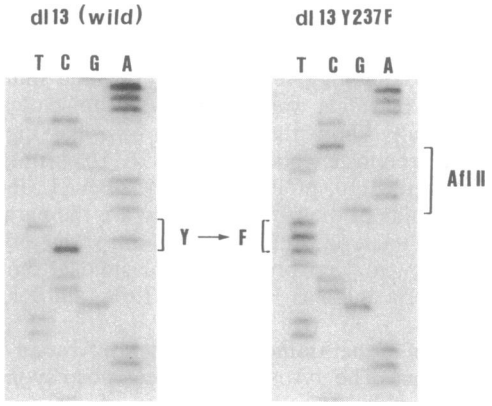

FIG. 3. DNA sequence of the Tyr → Phe substitution mutant. Only the region of interest is shown. The autoradiogram shown on the left is the sequence of the wild-type Rep_{pKYM} and that on the right is the mutated Rep_{pKYM}. The sequence TAC coding for tyrosine is changed to TTT for phenylalanine. The nucleotide sequence 5′-CTGAAA-3′ was changed to 5′-CTTAAG-3′ to create an *Afl* II site without changing the amino acid.

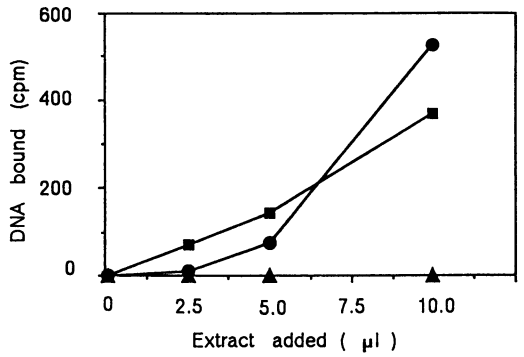

FIG. 4. Binding activities of Rep_{pKYM} and RepY237F to dl13 DNA. Extracts (1 μg of protein per μl) of *E. coli* HB101 carrying dl13 (■), dl13Y237F (●), or pBR322 (▲) were incubated at 20°C with 50 ng of ^{3}H-labeled dl13 DNA and 18 μg of sonicated calf thymus DNA in 400 μl of reaction buffer. After a 20-min incubation, the reaction mixtures were filtered through nitrocellulose membrane filters. The filters were washed and dried. The retained radioactivity was counted in a liquid scintillation counter. When ^{3}H-labeled pBR322 DNA was used as a probe, it did not bind to the membrane filter.

lulose filter binding assay. Fig. 4 clearly shows that the mutation did not affect synthesis of the mutant Rep protein. The amount of ^{3}H-labeled dl13 DNA bound to the nitrocellulose blot of the extract of *E. coli* carrying dl13Y237F was almost the same as that bound with the extract of *E. coli* carrying dl13. Since ^{3}H-labeled plasmid pBR322 DNA did not bind to the blot and the extract prepared from the cells carrying pBR322 did not bind ^{3}H-labeled dl13 DNA, this assay specifically detected the Rep protein. These results suggest that the Tyr-237 → Phe substitution abolished the activity of Rep_{pKYM} as the initiator of DNA replication. Thomas *et al.* (28) reported that the Tyr → Phe substitution in the Rep protein of pT181 did not affect the binding of the protein to pT181 DNA but destroyed the ability to make single-strand breaks.

Conservation of the Nucleotide Sequence at the Replication Origin. Comparison of the nucleotide sequences of the origins of pKYM and the pUB110 plasmid family (17, 25) shows significant conservation (Fig. 5). The homologous sequences were also found in the origins of coliphages φX174 and G4 (27). For φX174, cleavage at 5′-G ↓ ATA in this sequence by gene A protein initiates rolling-circle DNA replication (26). pKYM and the pUB110 plasmid family probably initiate replication by using their Rep proteins to introduce a single-strand break in this region.

Southern Blot Analysis. If pKYM replicates by a rolling-circle mechanism, a specific single-stranded circular DNA would be generated as an intermediate (1–3). The presence of such DNA would support the hypothesis that plasmids of Gram-positive bacteria replicate by a rolling-circle mechanism (1–3). Plasmid DNA extracted from *E. coli* YS1 carrying pKYM was analyzed by Southern blot hybridization. The DNA was digested with S1 endonuclease or exonuclease VII and electrophoresed through an agarose gel to analyze the structure of the DNA. The DNA was transferred to nitrocellulose filters directly or after alkaline denaturation. If single-stranded DNA was present, it would be trapped by the filter without denaturation (2, 22). The DNAs were hybridized with ^{32}P-labeled anti-M13mp18-ori(+) or anti-M13mp19-ori(−) DNA. As shown in Fig. 6, the anti-M13mp18-ori(+) DNA clearly hybridized with the fastest-moving DNA and this DNA was also trapped by the filter without denaturation (Fig. 6*A*, bands ss). This single-stranded DNA was not detected after digestion with S1 endonuclease, which attacks single-stranded DNA (lanes 2 and 7) (29) but was present after digestion with exonuclease VII, which degrades single-stranded linear DNAs but not circular DNAs (lanes 5 and 10) (30). Lane M indicates the position of single-stranded pKYM DNA. Open circular ^{32}P-labeled pKYM DNAs were heat-denatured and electrophoresed in parallel with the samples. The result suggests that the single-stranded DNA recovered from the cell is the unit length of pKYM. Anti-M13mp19-ori(−) DNA hybridized slightly with the single-stranded DNA (Fig. 6*B*). However, since anti-M13mp19 DNA also hybridized with the single-stranded DNA to the same extent (data not shown), the hybridization with anti-M13mp19-ori(−) appears to be nonspecific. The result suggests that the fastest-moving DNA is the single-stranded circular plus strand of pKYM. The amount of single-stranded DNA in total plasmid DNA was assayed using the image analyzer (Bio-

Table 1. Transformation with dl13Y237F

Plasmid	Transformation frequency		
	WA802	WA802 polA	WA802 polA (pOPM3702)
dl13	3.0×10^5	3.0×10^5	—
dl13Y237F	1.0×10^5	$<1.0 \times 10^2$	1.0×10^5
pBR322	3.0×10^5	$<1.0 \times 10^2$	$<1.0 \times 10^2$

Transformation frequency of dl13Y237F was examined using *E. coli* WA802, WA802 polA, and WA802 polA carrying pOPM3702. Transformation frequency is the number of transformants per μg of DNA.

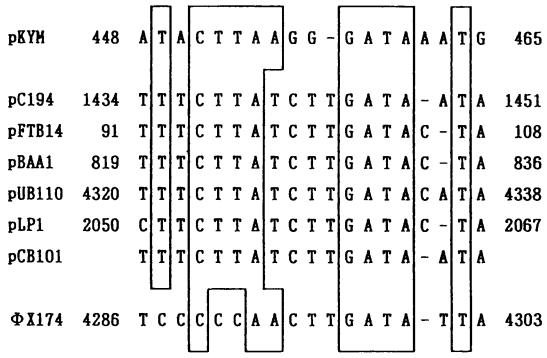

FIG. 5. Consensus sequences (5′ → 3′) within the plus origins of pUB110 family plasmids, pKYM, and coliphage φX174. Only the stretches of origin sequences showing strong homologies are presented. Nucleotide positions of sequences are given according to published maps (3). Nucleotides common to pKYM are boxed.

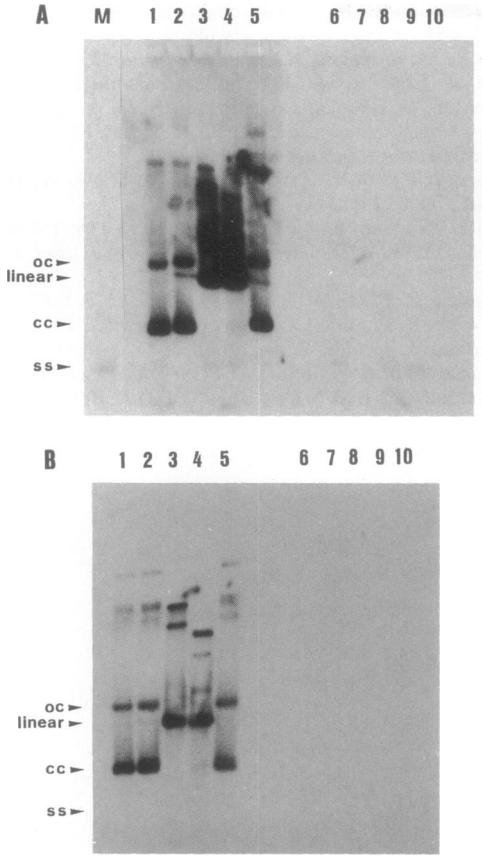

FIG. 6. Synthesis of strand-specific single-stranded DNA in *E. coli*. Lysate prepared from *E. coli* YS1 harboring pKYM was electrophoresed and transferred to nitrocellulose. The blots were then hybridized to the appropriate probes. (*A*) Anti-M13mp18-ori(+) DNA probe. (*B*) Anti-M13mp19-ori(−) DNA probe. DNA was transferred to nitrocellulose with (lanes 1–5) or without (lanes 6–10) prior denaturation. Lanes: M, single-stranded pKYM prepared by heat denaturation of open circular ^{32}P-labeled pKYM DNA; 1 and 6, intact DNA; lanes 2 and 7, DNA treated with S1 nuclease; lanes 3 and 8, DNA digested with *Eco*RI; lanes 4 and 9, DNA digested with *Cla* I; lanes 5 and 10, DNA treated with exonuclease VII. Bands: oc, open circular DNA; linear, linear plasmid DNA; cc, closed circular DNA; ss, single-stranded plasmid DNA.

Image analyzer model BA100; Fuji Photo Film) and was found to be about 5%.

DISCUSSION

The Rep protein encoded by plasmid pKYM, isolated from the Gram-negative bacterium *S. sonnei*, shows significant homology with Rep proteins of the pUB110 plasmid family, isolated from Gram-positive bacteria (Fig. 1). It does not exhibit homology to Rep proteins encoded by plasmids from Gram-negative bacteria, including F, pSC101, R6K, and R1.

The genetic map of pKYM around the replication origin is *ori–inc*(*cop*)*–rep* (17). The organization is almost the same as that of pC194 (31), a member of the pUB110 family. pKYM may be a plasmid of the pUB110 family that was transferred accidentally to a Gram-negative bacterium. pKYM, however, could not transform *B. subtilis* (Gram-positive) under conditions in which pUB110 could transform *B. subtilis*.

A tyrosine residue in the Rep proteins of the plasmids that replicate by a rolling-circle mechanism is thought to be important for their function (25, 26). Conservation of the sequence Lys-Tyr in Rep proteins is significant as shown in Fig. 2. The tyrosine of pT181 and the gene A protein of ϕX174, in fact, accepts the 5′ end of the single-strand break introduced by these proteins (27, 28). The importance of the Rep_{pKYM} Tyr-237 was confirmed by the Tyr-237 → Phe substitution, an alternation that inhibited multiplication of pKYM. The consensus nucleotide sequence of the origins of the pT181 family is 5′-TACTCT↓AATAGCCGGTT [the arrow indicates the single-strand break site (28)] and is completely different from those of the pUB110 family and pKYM (5′-CTTANNNNGATANNT) (Fig. 5). The nucleotide sequences recognized by these proteins are different, even though Lys-Tyr is conserved in the Rep proteins of both plasmid families. Lys-Tyr does not participate for the recognition of the replication origin of the plasmids. Since the Rep proteins of the pUB110 plasmid family are supposed to introduce the single-strand break at 5′-G↓A of GATA (32), Rep_{pKYM} may also cut at the same site. The single-stranded circular DNA that corresponds to the coding strand of Rep_{pKYM} (plus strand) was recovered from the cells carrying pKYM (Fig. 6). The percentage of the single-stranded DNA to total plasmid DNA was about 5%. This ratio was comparable to that of pUB110 (33).

These results are consistent with a rolling-circle mechanism of replication where synthesis is initiated by introducing a single-strand break in the origin sequence 5′-CTTAAGGGATAAAT. Although we have looked intensively *in vivo* using two-dimensional gel electrophoresis (34) to determine if pKYM can replicate through a θ structure, we have not obtained results that suggest the presence of θ structure (data not shown). These results support our conclusion that pKYM replicates by a rolling-circle mechanism.

The chromosomes of *E. coli* and *B. subtilis* replicate by θ forms (35); in fact, all bacterial chromosomes are supposed to replicate by θ forms. On the other hand, bacteriophages replicate several mechanisms: θ forms, a rolling-circle mechanism, and protein priming (36). Parasitic genomes seem to use various methods to replicate. Most plasmids from Gram-positive bacteria seem to replicate by a rolling-circle mechanism and most plasmids from Gram-negative bacteria replicate by θ forms. Replication by θ forms seems to have some advantage in Gram-negative bacteria. Although plasmid pKYM replicates by a rolling-circle mechanism, detailed examination of the nucleotide sequence of replication origin of pKYM suggests that the origin has an intermediate structure between the plasmids of Gram-positive bacteria and of Gram-negative bacteria (unpublished data). pKYM may evolve to replicate by θ forms.

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