JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 7161–7164 Vol. 179, No. 22
0021-9193/97/$04.00+0

Plasmid pRQ7 from the Hyperthermophilic Bacterium *Thermotoga* Species Strain RQ7 Replicates by the Rolling-Circle Mechanism

JAE-SUNG YU AND KENNETH M. NOLL*

Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269-3125

Received 5 May 1997/Accepted 15 September 1997

The hyperthermophilic bacterium *Thermotoga* species strain RQ7 harbors an 846-bp plasmid, pRQ7, with a single open reading frame. Previously published analyses of the DNA sequence of pRQ7 suggested that it may replicate by a rolling-circle (RC) replication mechanism, and this report provides experimental evidence supporting this hypothesis. Single-stranded pRQ7 DNA accumulates in strain RQ7, as evidenced by the facts that this DNA bound to nitrocellulose membranes under nondenaturing conditions, was sensitive to S1 nuclease digestion, and hybridized to only one of two homologous DNA probes specific for each strand of the plasmid. The DNA encoding the open reading frame was cloned and expressed in *Escherichia coli* and gave a protein with a molecular mass of 26 kDa, similar to that deduced by sequence analysis. This protein bound to a fragment of pRQ7 that contains a putative double-stranded replication region in a magnesium-dependent reaction and made this fragment sensitive to S1 nuclease activity. It did not cause this same S1 nuclease sensitivity in the remainder of pRQ7. This activity on pRQ7 DNA suggests that this protein plays a role in plasmid replication.

Modern genetic analysis of prokaryotes relies on the use of vectors to introduce DNA into the organisms under investigation. Plasmids and viruses serve as mobile carriers of genetic information. The development of genetic tools for hyperthermophilic bacteria and archaea depends upon the discovery of such potentially useful replicons (18). A number of plasmids and viruses from some archaeal hyperthermophiles have been isolated in recent years, and some of these are under development as vectors (1, 26).

Two newly discovered plasmids from hyperthermophiles belong to the family of replicons that replicate by a rolling-circle (RC) mechanism. The small cryptic plasmid pRQ7 was recently discovered in *Thermotoga* sp. strain RQ7 (8). Its sequence was determined (Fig. 1), and it was shown to be the smallest natural replicon so far described (846 bp). Sequence analysis suggested that a single major open reading frame (ORF) on the plasmid could encode a 25,460-Da protein. On the basis of sequence comparisons, this putative protein was postulated to be a replication initiation (Rep) protein and the plasmid was postulated to be an RC plasmid. A similar cryptic RC plasmid was subsequently discovered in the archaeon *Pyrococcus abysii*. This plasmid, pGT5, is 3.4 kb and contains two major ORFs (4). Evidence was presented that replication of pGT5 causes single-stranded DNA intermediates to accumulate in vivo. Its larger ORF encodes a putative 75-kDa protein with similarities to RC Rep proteins.

Many plasmids from gram-positive bacteria and a few from gram-negative bacteria replicate by the RC mechanism (5, 6, 11, 14, 24, 25). During the replication of an RC plasmid, a single-stranded DNA intermediate accumulates inside the cell (6). RC plasmids replicate by using a plasmid-encoded Rep protein which binds to a double-stranded replication origin (*dso*) region and nicks one strand (the positive strand) of the plasmid DNA (3). Replication commences from the exposed 3′ end using host-encoded replication enzymes. Unit length plasmids are presumably formed through the action of the Rep protein, which in some cases remains covalently bound to the positive DNA strand. Synthesis of the second strand of this intermediate initiates at the single-stranded origin (*sso*). Although initiation of DNA synthesis at the *dso* of an RC plasmid can occur in a wide range of hosts, initiation at the *sso* is typically host strain specific (21).

Novick classified RC plasmids on the bases of the organization of their functional genes and sites and on the similarities of their *dso*'s (19). This classification gives four groups of RC plasmids, represented by plasmids pT181, pC194, pE194, and pSN2. The Rep proteins from all but the pT181 group share three sequence motifs, one of which appears to be involved in catalyzing strand cleavage (9, 13). They are functionally related to proteins involved in RC replication of single- and double-stranded DNA bacteriophages and may also be related to those used to mobilize conjugative plasmids (9). If these proteins evolved from an ancestral Rep protein, examination of Rep proteins that function in organisms closely related to the common ancestor of bacteria and archaea may provide clues to their evolutionary origin. The recently discovered plasmids from hyperthermophilic organisms may provide this information about the evolution of these plasmids and the RC mode of DNA replication.

In this report, evidence is presented that demonstrates that the *Thermotoga* plasmid pRQ7 replicates by an RC mechanism. It also demonstrates that its single ORF encodes an enzyme with activity consistent with a role in plasmid replication.

Single-stranded pRQ7 DNA accumulates in vivo. During the replication of RC plasmids, single-stranded DNA intermediates often accumulate inside the cell (6). We identified single-stranded DNA extracted from cells based upon its nitrocellulose-binding properties and its sensitivity to S1 nuclease (Fig.

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, U-125, 75 N. Eagleville Rd., Storrs, CT 06269-3125. Phone: 860-486-4688. Fax: 860-486-4331. E-mail: noll@uconnvm.uconn.edu.

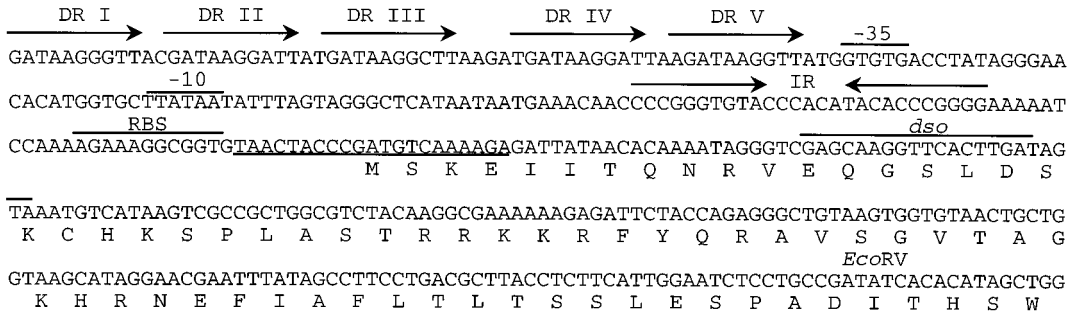

FIG. 1. Partial nucleotide sequence of pRQ7 (the full sequence can be found in reference 8). The five direct repeats (DR), promoter (−35 and −10), inverted repeat (IR), ribosome binding site (RBS), and the putative double strand origin (*dso*) are indicated as well as restriction endonuclease sites mentioned in the text. A portion of the sequence of gpORF1 is shown beneath the coding strand. The 5′ PCR primer region is indicated as an underlined nucleotide sequence. The 3′ PCR primer was identical to the final six codons of the ORF.

2). Plasmid pRQ7 was isolated from *Thermotoga* sp. strain RQ7 by a modified alkaline lysis method (15). Purification of cellular DNA without denaturation by lysis of cells using hexadecyltrimethyl ammonium bromide gave the same results, but the resolution of the resulting plasmid DNA was not as distinct as that shown in Fig. 2 (data not shown). Since double-stranded DNA does not bind to nitrocellulose without prior denaturation, the band visualized in lane 7 must be due to single-stranded pRQ7 DNA extracted from cells. This is supported by the results shown in lane 8, in which the DNA giving rise to these bands was digested by single-strand-specific S1 nuclease while double-stranded DNA was still visible following the same treatment (lane 6). Two bands of single-stranded DNA are apparent when the DNA is resolved in a Tris-borate buffer but not in a Tris-acetate buffer (data not shown). The identities of these two forms of single-stranded DNA are not known. The single-stranded DNA migrates between form II and form I species of the plasmid (the upper and lower bands, respectively, in lane 5; analysis not shown).

To identify which strand of pRQ7 DNA accumulates during replication, hybridization probes were synthesized that are specific for each strand of pRQ7. Digoxygenin-labelled hybridization probes were synthesized from the T7 and T3 promoters of plasmid pOH19 (which flank the cloning site into which pRQ7 was inserted) using Klenow polymerase and primers complementary to each promoter. Plasmid pOH19 contains pRQ7 cloned into the *Eco*RV site of plasmid pBluescript KS+ (Stratagene, La Jolla, Calif.) (Fig. 1 and reference 8). The primer specific to the T7 promoter of pBluescript directed synthesis of a probe complementary to the sequence shown in Fig. 1. A primer specific to the T3 promoter directed synthesis of the complementary sequence. Extracted plasmid DNA bound to nitrocellulose hybridized only to the probe synthesized using the T7 primer (compare lanes 3 and 7, Fig. 2). This DNA was also sensitive to S1 nuclease (Fig. 2, lane 8). Since that strand (the one shown in Fig. 1) accumulates during replication, it is by definition the plus strand.

ORF1 encodes a 26-kDa polypeptide. Plasmid pRQ7 has only one significant ORF (Fig. 1). This ORF could encode a protein of 25,460 Da with a calculated pI of 9.9 (8). Since plasmids must direct the host's DNA replication apparatus to the plasmid replication origin, this ORF would likely encode a protein involved in replication. To test this hypothesis, the ORF was subcloned into the *Escherichia coli* expression vectors pET-11a and pET-15b by PCR using primers directed to the sites illustrated in Fig. 1 and that introduced restriction sites for cloning. After isopropyl-β-D-thiogalactopyranoside (IPTG) induction of the T7 *lac* promoter of pET-11a, a heat-stable polypeptide, gpORF1, accumulated, as shown in Fig. 3, lane 4. This protein was not present in the strain carrying the vector (Fig. 3, lanes 1 and 2), nor was it produced without induction by IPTG (Fig. 3, lane 3). The protein has a mass of

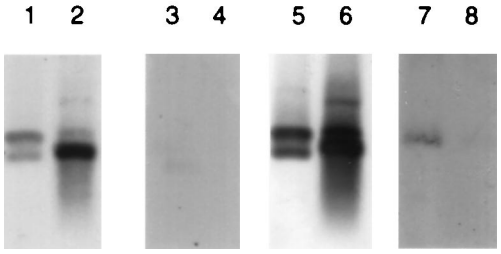

FIG. 2. Single-stranded pRQ7 DNA accumulates in vivo and identification of the plus strand. Plasmid pRQ7 DNA was treated (lanes 2, 4, 6, and 8) or not treated (lanes 1, 3, 5, and 7) with S1 nuclease. S1 nuclease digestion of single-stranded DNA was carried out with 0.8 μg of DNA in 30 μl of S1 nuclease reaction mixture (30 mM sodium acetate [pH 4.6], 0.5 mM $ZnCl_2$, 250 mM NaCl) for 30 min at 37°C in the presence of 150 U of S1 nuclease per ml. The resulting DNA was subjected to electrophoresis at 4.16 V/cm through gels of 1% agarose in 40 mM Tris-acetate buffer. The DNA was transferred from the gel to a nitrocellulose membrane with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) denaturation. The DNA was hybridized to digoxygenin-labeled T3-based probes (lanes 1, 2, 3, and 4) or T7-based probes (lanes 5, 6, 7, and 8). The Genius system (Boehringer Mannheim, Indianapolis, Ind.) was used to label and visualize hybridization probes.

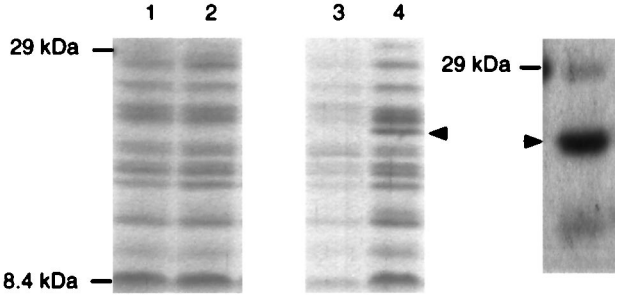

FIG. 3. Resolution of gpORF1 expressed in *E. coli* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The migrations of the molecular mass markers are shown. The arrowheads indicate gpORF1. Proteins resolved in lanes 1 and 2 were from cell extracts harboring the pET-11a vector alone. Proteins resolved in lanes 3 and 4 were from subcloned PCR products in pET-11a. Proteins resolved in lanes 1 and 3 were extracted prior to induction, while proteins in lanes 2 and 4 were from 3 h after induction. Purified proteins, gpORF1 expressed in the pET-15b vector, were resolved in lane 5.

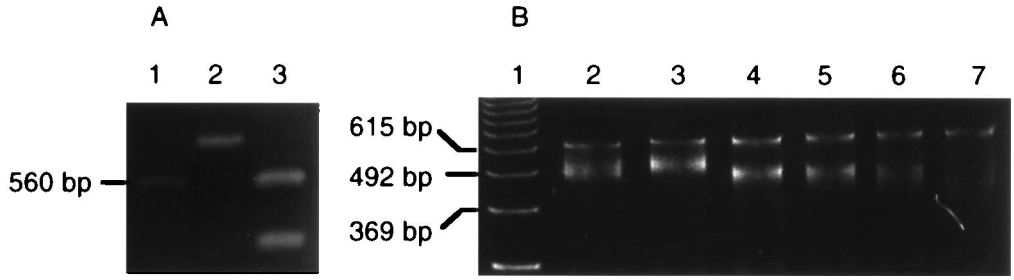

FIG. 4. gpORF1 activity measured by S1 nuclease sensitivity. The migration of molecular weight standards are shown (lane 1 in panels A and B). (A) Agarose gel electrophoresis of substrate DNA fragments. pRQ7 DNA was excised from pOH19 by *Eco*RV digestion and purified (lane 2). This *Eco*RV fragment was digested with *Sma*I, yielding two fragments (lane 3). The smaller DNA fragment contains the putative pRQ7 *dso* region (Fig. 1). (B) Activity of purified gpORF1 on the *Eco*RV/*Sma*I fragments of pRQ7. Incubation of DNA fragments was either with (lanes 3 to 7) or without (lane 2) highly purified gpORF1. The reaction was also carried out either with (lanes 2 and 4 to 7) or without (lane 3) $MgCl_2$. The concentration of highly purified gpORF1 used to treat each DNA sample was 0, 3, 0.5, 1, 2, and 3 μg in lanes 2 to 7, respectively.

approximately 26 kDa, similar to that predicted from the sequence data. gpORF1 expressed from plasmid pET-11a was relatively heat labile since it was lost from extracts following heating at 82°C for 2 h. It was also expressed in amounts insufficient to allow substantial purification, so expression in pET-15b was used to provide an N-terminal His-tagged fusion protein for purification, using a chelated nickel resin (HisBind resin; Novagen). Using this procedure, a highly purified protein was obtained (Fig. 3, lane 5).

gpORF1 binds to a specific fragment of pRQ7. This highly purified gpORF1 was used to test for Rep protein activity. Rep protein activity is typically measured by protein-DNA binding assays (23) and site-specific nuclease activity assays (12). Although protein-DNA binding has been measured using a DNA-binding protein from a hyperthermophilic methanogen (22), no such assays using hyperthermophilic enzymes have been described. Repeated attempts to measure this activity with gpORF1 were unsuccessful. We attempted to identify the pRQ7 *dso* to provide a template for a detailed study of its activity. When Rep proteins bind to their corresponding *dso* region, they stabilize a secondary structure in that region and introduce a nick in one strand of the DNA. For example, the pT181 initiator protein enhances cruciform structure extrusion at the origin (17). We reasoned, then, that treatment with gpORF1 should make the template DNA susceptible to S1 nuclease activity if the nuclease was used at a concentration low enough to digest nicked or single-stranded DNA but insufficient to digest double-stranded DNA (20). This is less than the amount used to digest single-stranded DNA in the experiment described above (5 versus 1.35 U/μl).

As substrate, we purified the pRQ7 DNA from plasmid pOH19, the *E. coli* plasmid clone of pRQ7 (8). This linearized pRQ7 DNA (Fig. 4A, lane 2) was digested with *Sma*I to give two fragments, one of which contains the putative *dso* (the smaller fragment in Fig. 4A, lane 3). The other larger fragment served as a control for nonspecific activity of gpORF1. The substrate DNA was treated with gpORF1 in a reaction volume of 60 μl with 1 μg of gpORF1 and 0.66 μg of substrate DNA for 2 h at 65°C in a buffer containing 25 mM Tris-HCl, pH 7.5, 2 mM spermidine, 0.05 μg of bovine serum albumin per μl, 1 mM dithiothreitol, 50 mM KCl, 33 mM NaCl, 5% glycerol, 2 mM ATP, and 1 mM $MgCl_2$. Following the reaction, treated DNA was precipitated with ethanol and subjected to S1 nuclease treatment. The DNA was added to 20 μl of S1 nuclease reaction buffer, and 27 U of S1 nuclease was added. After 30 min at 37°C, EDTA was added to 1 mM along with 2 μl of DNA gel loading buffer. After treatment, DNA fragments were analyzed by nondenaturing polyacrylamide gel electrophoresis in 45 mM Tris-borate buffer, pH 7.5. When these two substrate DNA fragments were incubated with gpORF1, the resulting DNA containing the putative *dso* was sensitive to S1 nuclease (lower band, Fig. 4B, lanes 4 to 7). This conversion to a labile form required magnesium (Fig. 4B, lane 3) and was not a result of incubation in the reaction buffer at 65°C (Fig. 4B, lane 2). The control DNA was not converted to an S1-nuclease-susceptible form (upper band, Fig. 4B, lanes 4 to 7). This demonstrates that the highly purified recombinant gpORF1 is specific for the fragment of pRQ7 DNA containing the putative *dso* and is consistent with a role in plasmid replication. gpORF1 may induce S1 nuclease sensitivity by either nicking the DNA or stabilizing single-stranded regions of the DNA. Repeated attempts to experimentally identify the precise location of any nicking site on this pRQ7 restriction fragment have proven unsuccessful.

TABLE 1. Conserved amino acids motifs in gpORF1 of pRQ7 and Rep proteins of the pC194 plasmid family[a]

Replicon	Motif 1	Motif 2	Motif 3	Organism
pRQ7	FLTLTSSL	WVHMHILF	MMKYLSKEME	*Thermotoga* sp. strain RQ7
pKYM	FLTLTVRN	HPHFHCLL	TLKYSVKPED	*Shigella sonnei*
pUB110	FLTLTVKN	NQHMHVLV	MAKYSGKDSD	*Staphylococcus aureus*
pC194	FLTLTTPN	NPHFHVLI	MAKYSGKDSD	*Staphylococcus aureus*
pBAA1	FLTLTVRN	HPHFHVLI	ISKYPVKDTD	*Bacillus subtilis*
pLP1	FLTLTVKN	NQHLHVLL	TAKYEVKSAD	*Lactobacillus plantarum*
pFTB14	FLTLTVRN	HPHFHVLL	ISKYPVKDTD	*Bacillus amyloliquefaciens*
Consensus[b]	FLTLTvrn	xpHuHvlu	uxKYuvKdxd	

[a] The putative amino acid sequence from pRQ7 gpORF1 was aligned with those published in reference 9.

[b] The consensus line includes amino acid residues conserved in all RC replication initiation (Rep) proteins (uppercase) or in at least one-half of them (lowercase). u, bulky hydrophobic residues (I, L, V, M, F, Y, or W); x, fewer than one-half the residues are conserved.

Sequence features suggest similarity with RC plasmid replication proteins. RC plasmids have been classified into four groups (represented by plasmids pSN2, pC194, pT181, and pE194) based upon the organization of their functional components (19). At the level of functional organization, pRQ7 is most similar to the pSN2 family because of its small size and single protein-encoding gene. The sequence of a putative *dso* of pRQ7 (Fig. 1) most closely resembles those of the pC194 plasmid family. The location of this putative *dso* within the ORF, however, is typical of members of the pT181 family (8). Three motifs in gpORF1 are like those found in the replication proteins of the pC194 plasmid family (Table 1). These motifs are well conserved among the initiator proteins for all RC plasmids except the pT181 family (9, 13). The motif 1 region may recognize the *dso* region (3). The two His residues of motif 2 are highly conserved in Rep proteins of RC plasmids. These histidine residues may be ligands binding metals such as magnesium or manganese (9). We have shown that gpORF1 activity requires magnesium ions. In motif 3, the putative Rep protein of pRQ7 has sequence identity with the highly conserved motif 3 of the pC194 plasmid family, which contains two lysine and one tyrosine residue (Table 1). Motif 3 of some Rep proteins (superfamily I of Ilyina and Koonin, which includes phage ϕX174) contains two invariant tyrosine residues involved in the nicking activity which are separated by partially conserved amino acids (13). The amino acid sequence YM MKYLSK in pRQ7 is very similar to this superfamily I motif 3 consensus. However, as noted previously, its sequence is also similar to representatives of superfamily II (which includes pC194), which has only one active tyrosine residue (13). It is not known if the activity of gpORF1 requires one or two tyrosine residues. Plasmid pGT5 from the hyperthermophilic archaeon *P. abysii*, like pRQ7, has direct and inverted repeat sequences and its *dso* is located within its ORF1 (4). The pGT5 ORF1 encodes a putative polypeptide of 75 kDa, and it was assigned to superfamily II (4).

The data reported here demonstrate that plasmid pRQ7 belongs to the class of plasmids that replicate by a single-stranded DNA intermediate, the RC plasmids. Its only ORF was cloned into and expressed in *E. coli*, producing a moderately heat-stable polypeptide of 26 kDa, a size similar to that deduced by sequence analysis. The initiator proteins of RC plasmids may enhance formation of secondary structures in the *dso* and also catalyze nicking activity. Either activity would render the origin region sensitive to S1 nuclease. We found that treatment of a DNA fragment containing the putative pRQ7 *dso* with gpORF1 caused sensitivity to S1 nuclease digestion and this activity depended upon magnesium, as found for plasmid Rep proteins. We were not able to demonstrate that this activity includes nicking the plasmid at the *dso*, so it cannot be ruled out that gpORF1 may act in concert with host-encoded factors to catalyze nicking of the replication origin. gpORF1 is smaller than RC plasmid Rep proteins and so could lack activities typically associated with Rep proteins. The demonstration that this plasmid replicates by an RC mechanism has important implications for developing it as a cloning vector. RC plasmids have been successfully developed as vectors for gram-positive organisms (2, 10, 16), although replication can be compromised when DNA is cloned into some of these plasmids (7). Those vectors can serve as models for the development of this plasmid as a vector.

We thank P. Forterre for helpful advice.

This work was supported by a grant from the National Science Foundation (MCB-9418197).

REFERENCES

1. **Aagaard, C., I. Leviev, R. N. Aravalli, P. Forterre, D. Prieur, and R. A. Garrett.** 1996. General vectors for archaeal hyperthermophiles: strategies based on a mobile intron and a plasmid. FEMS Microbiol. Rev. **18:**93–104.
2. **Cocconcelli, P. S., M. J. Gasson, L. Morelli, and V. Bottazzi.** 1991. Single-stranded DNA plasmid, vector construction and cloning of *Bacillus stearothermophilus* α-amilase in *Lactobacillus*. Res. Microbiol. **142:**643–652.
3. **del Solar, G., M. Moscoso, and M. Espinosa.** 1993. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. Mol. Microbiol. **8:**789–796.
4. **Erauso, G., S. Marsin, N. Benbouzid-Rollet, M.-F. Baucher, T. Barbeyron, Y. Zivannovic, D. Prieur, and P. Forterre.** 1996. Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: evidence for rolling-circle replication in a hyperthermophile. J. Bacteriol. **178:**3232–3237.
5. **Galli, D., and D. J. LeBlanc.** 1994. Characterization of pVT736-1, a rolling-circle plasmid from the gram-negative bacterium *Actinobacillus actinomycetemcomitans*. Plasmid **31:**148–157.
6. **Gruss, A., and S. D. Ehrlich.** 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol. Rev. **53:**231–241.
7. **Gruss, A., and S. D. Ehrlich.** 1988. Insertion of foreign DNA into plasmids from gram-positive bacteria induces formation of high-molecular-weight plasmid multimers. J. Bacteriol. **170:**1183–1190.
8. **Harriott, O. T., R. Huber, K. O. Stetter, P. W. Betts, and K. M. Noll.** 1994. A cryptic miniplasmid from the hyperthermophilic bacterium *Thermotoga* sp. strain RQ7. J. Bacteriol. **176:**2759–2762.
9. **Ilyina, T. V., and E. V. Koonin.** 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic Acids Res. **20:**3279–3285.
10. **Jannière, L., A. Gruss, and S. D. Ehrlich.** 1993. Plasmids, p. 625–644. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
11. **Kleanthous, H., C. L. Clayton, and S. Tabaqchali.** 1991. Characterization of a plasmid from *Helicobacter pylori* encoding a replication protein common to plasmids in gram-positive bacteria. Mol. Microbiol. **5:**2377–2389.
12. **Koepsel, R. R., R. W. Murray, W. D. Rosenblum, and S. A. Khan.** 1985. The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase-like activities. Proc. Natl. Acad. Sci. USA **82:**6845–6849.
13. **Koonin, E. V., and T. V. Ilyina.** 1993. Computer-assisted dissection of rolling circle DNA replication. BioSystems **30:**241–268.
14. **Kues, U., and U. Stahl.** 1989. Replication of plasmids in gram-negative bacteria. Microbiol. Rev. **53:**491–516.
15. **Lee, S. H.-Y., and S. Rasheed.** 1990. A simple procedure for maximum yield of high-quality plasmid DNA. BioTechniques **9:**676–679.
16. **Minton, N. P., T.-J. Swinfield, J. K. Brehm, S. M. Whelan, and J. D. Oultram.** 1993. Vectors for use in *Clostridium acetobutylicum*, p. 120–140. *In* M. Sebald (ed.), Genetics and molecular biology of anaerobic bacteria. Springer-Verlag, New York.
17. **Noirot, P., J. Bargonetti, and R. P. Novick.** 1990. Initiation of rolling-circle replication in pT181 plasmid: initiator protein enhances cruciform extrusion at the origin. Proc. Natl. Acad. Sci. USA **87:**8560–8564.
18. **Noll, K. M., and M. Vargas.** 1997. Recent advances in genetic analyses of hyperthermophilic archaea and bacteria. Arch. Microbiol. **168:**73–80.
19. **Novick, R. P.** 1989. Staphylococcal plasmids and their replication. Annu. Rev. Microbiol. **43:**537–565.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Seery, L. T., N. C. Nolan, P. M. Sharp, and K. M. Devine.** 1993. Comparative analysis of the pC194 group of rolling circle plasmids. Plasmid **30:**185–196.
22. **Tabassum, R., K. M. Sandman, and J. N. Reeve.** 1992. HMt, a histone-related protein from *Methanobacterium thermoautotrophicum* ΔH. J. Bacteriol. **174:**7890–7895.
23. **Thomas, C. D., T. T. Nikiforov, B. A. Connolly, and W. V. Shaw.** 1995. Determination of sequence specificity between a plasmid replication initiator protein and the origin of replication. J. Mol. Biol. **254:**381–391.
24. **Yasukawa, H., T. Hase, A. Sakai, and Y. Masamune.** 1991. Rolling-circle replication of the plasmid pKYM isolated from a gram-negative bacterium. Proc. Natl. Acad. Sci. USA **88:**10282–10286.
25. **Zhang, N., and J. D. Brooker.** 1993. Characterization, sequence, and replication of a small cryptic plasmid from *Selenomonas ruminantium* subspecies *lactilytica*. Plasmid **29:**125–134.
26. **Zillig, W., D. Prangishvilli, C. Schleper, M. Elferink, I. Holz, S. Albers, D. Janekovic, and D. Götz.** 1996. Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic *Archaea*. FEMS Microbiol. Rev. **18:**225–236.