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Molecular Interactions of a Replication Initiator Protein, RepA, with the Replication Origin of the Enterococcal Plasmid p703/5

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Abstract We previously identified the origin of replication of p703/5, a small cryptic plasmid from the KBL703 strain of *Enterococcus faecalis*. The origin of replication contains putative regulatory cis-elements required for replication and a replication initiator (RepA) gene. The replicon of p703/5 is similar in its structural organization to theta-type plasmids, and RepA is homologous to a family of Rep proteins identified in several plasmids from Gram-positive bacteria. Here, we report molecular interactions between RepA and the replication origin of p703/5. DNase I footprinting using recombinant RepA together with electrophoretic mobility shift assays confirmed the binding of RepA to the replication origin of p703/5 *via* iterons and an inverted repeat. We also demonstrated the formation of RepA dimers and the different binding of RepA to the iteron and the inverted repeat using gel filtration chromatographic analysis, a chemical crosslinking assay, and electrophoretic mobility shift assays in the presence of guanidine hydrochloride. Our results suggest that RepA plays a regulatory role in the replication of the enterococcal plasmid p703/5 *via* mechanisms similar to those of typical iteron-carrying theta-type plasmids.

Keywords: *Enterococcus faecalis*, replication, replication initiator, RepA

Replication origins of theta replicating plasmids comprise multiple cis-acting elements including iterons, AT-rich regions, dnaA boxes, inverted repeats (IR), and replication initiator (Rep)-encoding gene(s) [7]. In the theta family of plasmids, the iteron, a tandem array of repeated sequences, is essential for both initiation of plasmid replication and restriction of plasmid copy number [11, 20]. AT-rich regions function as a melting point during initiation of replication and regulate replication by interacting with Rep proteins [21, 23]. Rep proteins play central roles in the initiation of plasmid replication by interacting with cis-elements of the replication origin including iterons and AT-rich regions [12]. In some cases, Rep proteins also bind to IRs serving as an operator of *rep* genes, leading to repression of *rep* gene expression [6, 15]. The distinct interaction of Rep proteins with iterons and IRs is regulated by conformational and configurational changes [9, 16]. Although the mechanisms of plasmid replication in Gram-negative bacteria have been extensively studied [2, 5, 22], there are relatively few studies on the molecular mechanisms underlying replication of plasmids from Gram-positive bacteria, and most of those studies have focused on rolling circle replicating plasmids [17, 18] or large conjugative plasmids [13, 29].

The plasmid p703/5 is a small low-copy-number 4.7-kb plasmid isolated from *Enterococcus faecalis* KBL703 [8], and its 1.2-kb-long replication origin contains a putative AT-rich region, seven iterons, an IR, and an open reading frame encoding a putative replication initiator [28] (Fig. 1). Independent to our studies, a theta-replicating plasmid from *E. faecalis*, pS86, was reported to be highly homologous to p703/5 [25]. The DNA sequence of the replicons of the

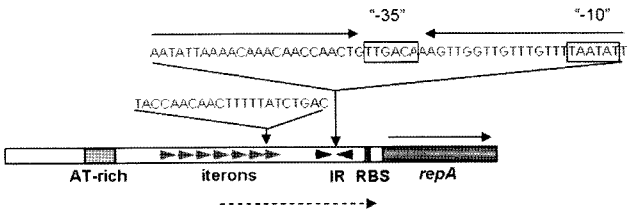

Fig. 1. Structure of the replication origin of p703/5 from *Enterococcus faecalis* KBL703.
An AT-rich region, seven direct repeats (iterons), a ribosome binding site (RBS), an inverted repeat (IR), and a replication initiator (RepA) gene are shown. A putative "−35" box and "−10" (TATA) box in the *repA* promoter region are indicated. The dot arrow indicates the region analyzed by DNase I footprinting.

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two plasmids is identical, with the exception that p703/5 contains seven direct repeats, whereas pS86 contains five and a half direct repeats. The amino acid sequence of the two Rep proteins is also identical. RepA proteins from p703/5 and pS86 are both composed of 240 amino acids and are homologous to a family of replication initiator proteins from pLRC255 [25] of *Streptococcus gallolyticus* (85.4% identity), from pCD3.4 [3] of *Carnobacterium divergens* (65.8% identity), from pLA103 [19] of *Lactobacillus acidophilus* (33.1% identity), and from pUCL287 [4] of *Tetragenococcus halophila* (34.2% identity). The plasmids pCD3.4, pUCL287, and pS86 have replicons consisting of multiple regulatory elements found in typical iteron-containing theta-replicating plasmids, and all replicate in a theta-mode. The molecular mechanism underlying the function of Rep proteins in this family of plasmid origins is unknown. In the present study, we investigated the molecular interactions between RepA and the replication origin of p703/5 and found that RepA binds to iterons and IR *via* different mechanisms that translate into a complex regulatory mechanism controlling replication of the enterococcal plasmid p703/5.

MATERIALS AND METHODS

Plasmid Construction

To construct pET14b-RepA, RepA full-length DNA (720 bp) was PCR-amplified from pS3.6, which was previously constructed by inserting a 3.6 kb SalI fragment containing the p703/5 origin of replication into pUC18 [28], using the forward primer 5'-GCATATGAGTGATAAATCGGAATT-AGCTG-3' and the reverse primer 5'-CGGGATCCTTATT-CTCCGTTTACCCAATCGTGCAT-3'. The start and stop codons of the *rep* structural gene located in the forward and reverse primers are underlined. The PCR product was cloned into pT7 blue (Novagen, WI, U.S.A.), from which the NdeI-BamHI fragment was excised and inserted between the NdeI and BamHI sites of pET-14b (Novagen) to generate an N-terminally hexahistidine-tagged RepA in *Escherichia coli*.

Purification of RepA from *Escherichia coli*

Escherichia coli (*E. coli*) BL21 (DE3) was used as an expression host, and hexahistidine-tagged RepA (His-RepA) was purified using nickel-chelating resin. From a 0.5-l culture, cells were harvested, resuspended in 50 ml of extraction buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1 mM PMSF) containing 10 mM imidazole and 0.1 mg/ml lysozyme, and sonicated. After centrifugation, the supernatant was incubated with 0.5 ml of nickel-chelating resin at 4°C for 2 h. The beads were washed extensively with extraction buffer containing 20 mM imidazole, and His-RepA was eluted with 3 ml of extraction buffer containing 300 mM imidazole. RepA was obtained by removing the hexahistidine tag from His-RepA using thrombin. His-RepA bound to nickel-chelating resin was incubated with thrombin in cleavage buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) at room temperature for 2 h with agitation. Following centrifugation, the thrombin remaining in the RepA-containing supernatant was inactivated with 1 mM PMSF and 5 mM benzamidine. The purified His-RepA and RepA were dialyzed against storage buffer [100 mM $(NH_4)_2SO_4$, 20 mM Mes-NH_4, pH 6.0, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 10% glycerol] and the purity of RepA and His-RepA was monitored by 10% SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.

DNase I Footprinting

DNase I footprinting was performed essentially as previously described [14]. The probe DNA (392 bp) containing the region from the AT-rich region to the RBS was produced by PCR using the forward primer (5'-ATATCGCGGACCTGTTTTG-3') and [^{32}P]-labeled reverse primer (5'-TCACTCATTCC-TTTCGCC-3'). RepA (1–28 μg) and the asymmetrically end-labeled DNA probe (50,000 cpm) were incubated for 15 min at room temperature in 50 μl of a reaction buffer (20 mM Tris-HCl, pH 7.0, 6 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 50 μg/ml BSA, 200 mM $NaCl_2$, 20 μg/ml calf thymus DNA), followed by incubation with 2 μg of DNase I for 1 min at room temperature. The reactions were discontinued by the addition of 75 μl of DNase I stop solution (20 mM EDTA, pH 8.0, 1% SDS, 200 mM NaCl, 125 μg/ml yeast tRNA), followed by phenol extraction and ethanol precipitation. The DNA was resuspended in 5 μl of formamide loading buffer (80% [w/v] deionized formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene-cyanol FF, and 1 mg/ml bromophenol blue) and electrophoresed on a 7 M urea-containing 8% polyacrylamide gel, followed by autoradiography. The protected areas were determined by comparison with the position of G bases determined by chemical sequencing.

Electrophoretic Mobility Shift Assay

Oligonucleotides used for electrophoretic mobility shift assays (EMSAs) were as follows: iteron 5'-TACCAA-CAACAACTTTTTATCTGACT-3'; IR 5'-ATTAAAACAA-ACAACCAATGTTGACAAAGTTGGTTGTTTGTTTTAAT-3'. Oligonucleotides were end-labeled with 20 μCi [γ-^{32}P] ATP (3,000 Ci/mmol) using T4-polynucleotide kinase and annealed with complementary oligonucleotides. Labeled probes were incubated with the indicated amounts of RepA in binding buffer (20 mM mes-NH_4, pH 6.0, 0.5 mM DTT, 0.1 mM EDTA, 50 μg/ml BSA, 200 mM NaCl, 5% glycerol, 2 μg of poly[dI-dC]) for 15 min at room temperature. For competition assays, the indicated amount of unlabeled DNA was included in the reaction mixture. At

the end of the incubation, the reaction mixtures were electrophoresed on a 5% polyacrylamide gel in 0.5×TBE buffer and analyzed by autoradiography.

Gel Filtration Chromatography

Gel filtration chromatography was performed on Sephacryl-300 HR (Amersham Biosciences, Buckinghamshire, England) using storage buffer, and proteins were monitored at A_{280}. The gel filtration standard (Bio-Rad, CA, U.S.A.) was used for calibration of the column.

Protein Crosslinking

For RepA crosslinking, 0.1 μg of His-RepA protein was incubated with or without the indicated concentrations of guanidine-hydrochloride (Gu-HCl) in 50 μl of reaction buffer (20 mM HEPES-NaOH, pH 7.5, 250 mM NaCl) for 30 min at room temperature. Chemical crosslinking was initiated by adding freshly prepared ethylene glycol-bis-succinic N-hydroxy-succinimide ester (EGS) to 1 mM and continuing the incubation for 10 min. The reaction was terminated by adding 5 μl of 1.5 M Tris-HCl (pH 7.5) and desalted before Western blot analysis.

Western Blotting

Western blot analysis was performed using rabbit anti-polyhistidine antibody and horseradish peroxidase-conjugated anti-rabbit antibody. The signal was visualized using enhanced chemiluminescent reagent.

RESULTS AND DISCUSSION

Recombinant RepA was expressed in *E. coli* and purified as an N-terminally hexahistidine-tagged protein (His-RepA) using nickel-chelating resin. RepA was obtained by removing the N-terminal tag from His-RepA using

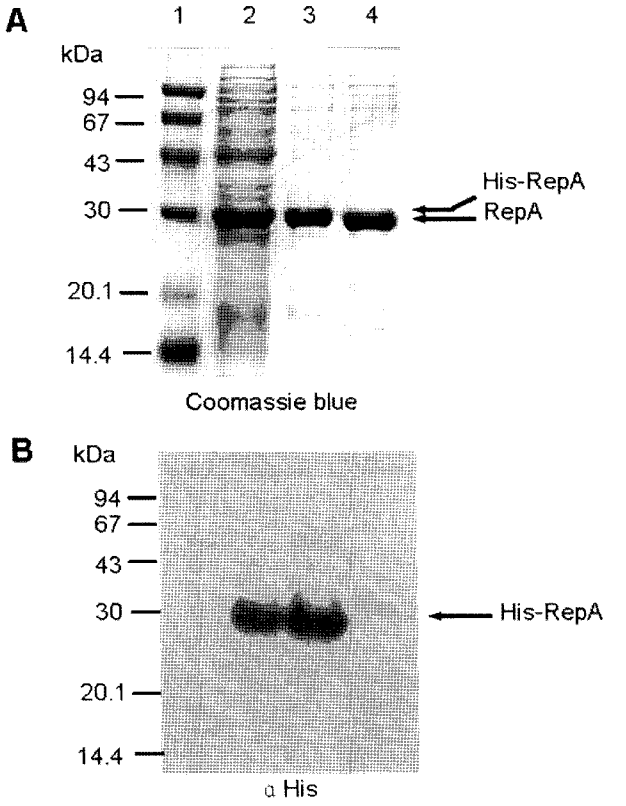

Fig. 2. Production of recombinant RepA from *Escherichia coli*.
N-Terminally hexahistidine-tagged RepA (His-RepA) and RepA were produced from *E. coli* and analyzed by 10% SDS-PAGE and Coomassie blue staining (**A**) or anti-polyhistidine Western blotting (**B**). Lane 1, protein size marker; lane 2, total lysate; lane 3, 1 μg of His-RepA; lane 4, 1 μg of RepA (hexahistidine tag removed by thrombin).

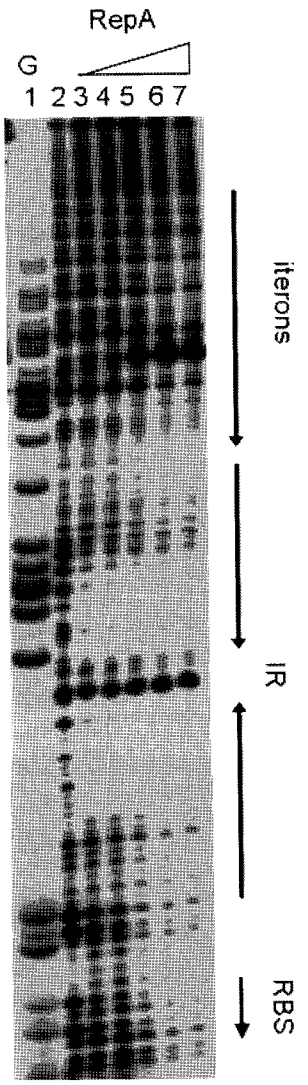

Fig. 3. DNase I footprinting of the origin of replication of p703/5.
DNase I footprinting was performed using 2 pmol of asymmetrically labeled DNA and increasing amounts of RepA (0, 0.5, 1, 2, 4, 8 μg in lanes 2–7). G (lane 1) indicates guanidine bases by chemical sequencing. Protected areas are indicated by arrows.

thrombin. SDS-polyacrylamide gel electrophoresis revealed His-RepA and RepA as 31 kDa and 28 kDa, respectively (Fig. 2A). The successful removal of the hexahistidine tag was confirmed by Western blot analysis using the anti-polyhistidine antibody (Fig. 2B). The genetic elements bound to by RepA in the p703/5 plasmid origin of replication were examined by DNase I footprinting. Asymmetrically end-labeled DNA fragments containing the p703/5 plasmid origin of replication were incubated with or without increasing levels of RepA, followed by limited digestion with DNase I. Fig. 3 shows several regions protected by RepA in the p703/5 plasmid origin of replication including the IR (the most significantly protected region), iterons, and a ribosome binding site (RBS). The interaction between RepA and iterons or IRs is known to be crucial for regulation of plasmid replication [6, 11]. For this reason, we further confirmed the binding of RepA to the iteron and IR using the electrophoretic mobility shift assay (EMSA). Consistent with the DNase I footprinting analysis, RepA bound to a single iteron (Figs. 4A and 4B) and IR (Figs. 4C and 4D). The binding of RepA was specific, since the formation of both the RepA-iteron and RepA-IR complexes was unaffected by competition with 10 to 100-fold molar excess of unrelated DNA, whereas the formation of RepA-iteron and RepA-IR complexes was inhibited by unlabeled specific DNA. The EMSA revealed three interesting features. First, the mobility of the RepA-IR complex was lower than that of the RepA-iteron complex. The Rf (distance traveled by RepA-probe/distance traveled by free probe) values of the RepA-iteron and RepA-IR complexes were 0.5 and 0.36, respectively. Second, an additional minor band with retarded mobility was observed in the EMSA between RepA and IR when the gel was overexposed. Third, the IR probe was saturated with as low as 0.35 μg of RepA, but detectable levels of the RepA-iteron complex could be formed only in the presence of higher levels of RepA. These results suggest that RepA binds to the iteron and the IR *via* distinct mechanisms.

Rep proteins are known to form monomers or dimers, each with a different affinity to iterons and IRs [15, 24,

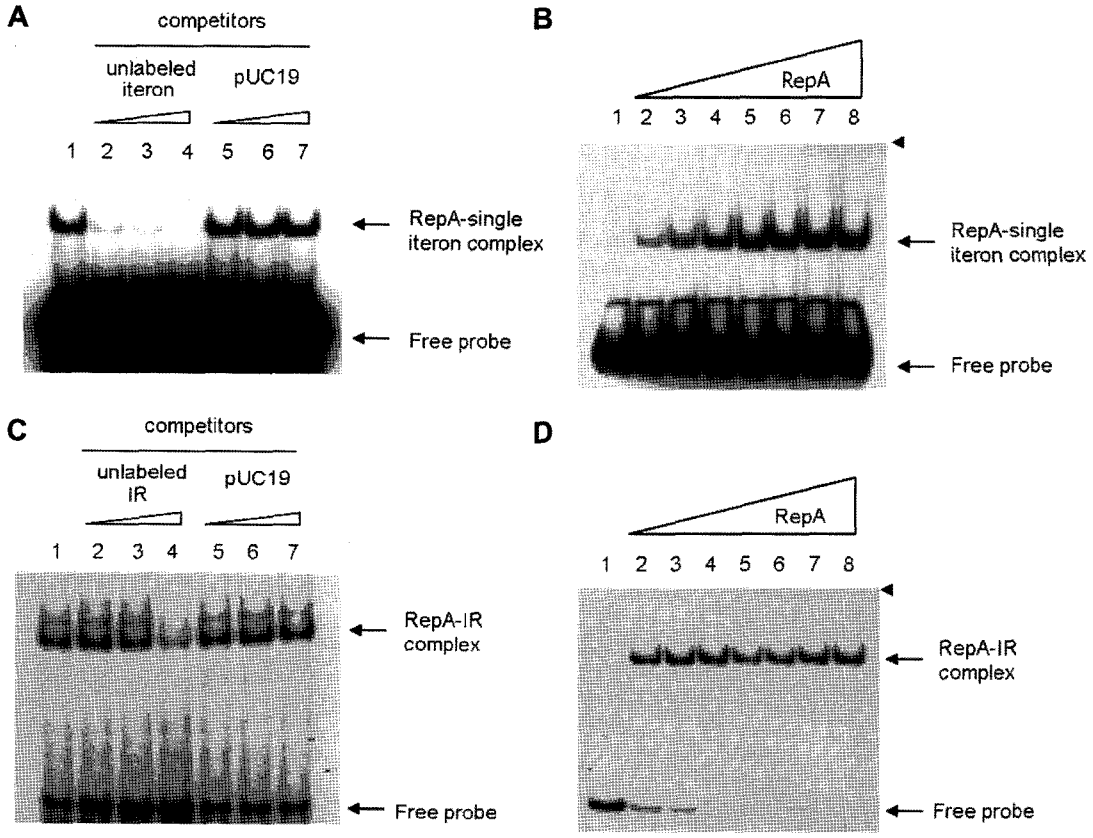

Fig. 4. Electrophoretic mobility shift assay (EMSA).

A, B. RepA binds to a single iteron. Eight μg of RepA and 2 pmol of ^{32}P-labeled single iteron were incubated in the presence of 25 (lanes 2 and 5), 50 (lanes 3 and 6), 100 (lanes 4 and 7) fold molar excess levels of unlabeled iteron or linearized pUC19 (**A**). Increasing levels of RepA (0, 4, 8, 16, 20, 22, 24 μg) were incubated with 2 pmol of ^{32}P-labeled single iteron. An arrowhead indicates the top end of the gel (**B**). **C, D.** RepA binds to inverted repeat (IR). The 0.06 μg of RepA and 2 pmol of ^{32}P-labeled IR were incubated in the presence of 10 (lanes 2 and 5), 25 (lanes 3 and 6), 75 (lanes 4 and 7) fold molar excess levels of unlabeled IR or linearized pUC19 (**C**). Increasing levels of RepA (0, 0.06, 0.12, 0.36, 0.6, 1.2, 1.8, 3.6 μg) were incubated with 2 pmol of ^{32}P-labeled IR. An arrowhead indicates the top end of the gel (**D**).

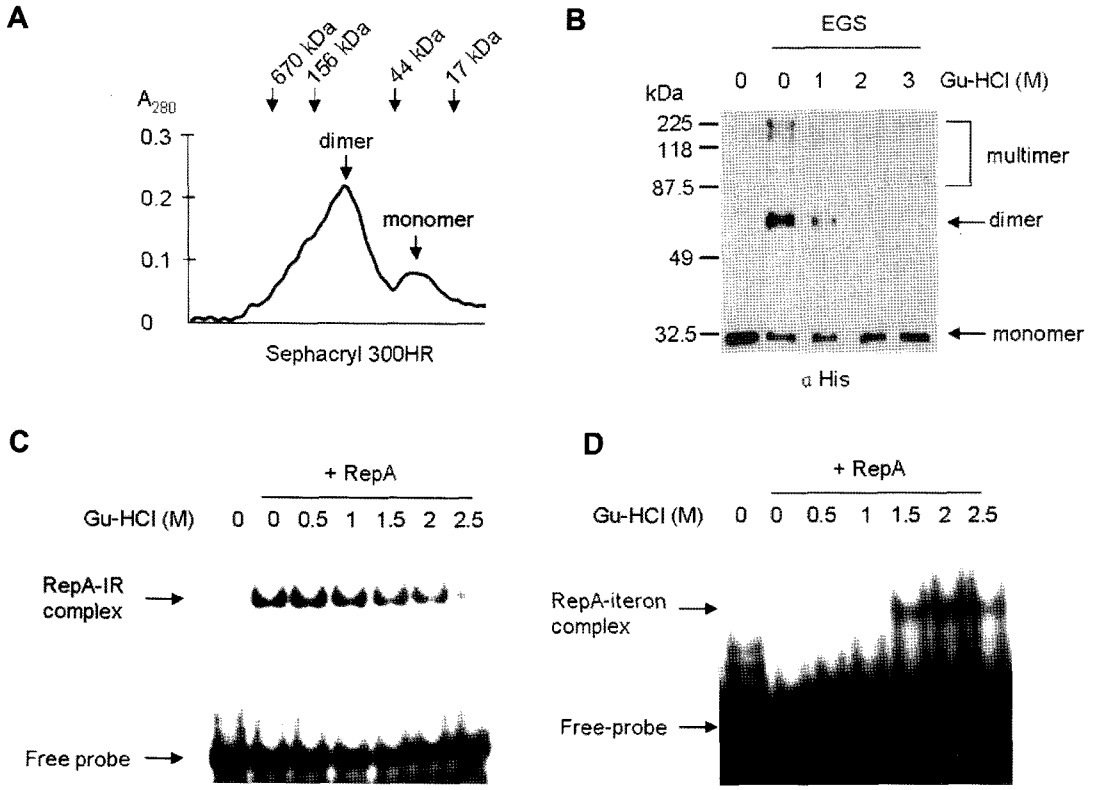

Fig. 5. RepA binds to iterons and IRs *via* distinct mechanisms.
A, B. Dimerization of RepA. Gel filtration analysis of RepA on Sephacryl 300HR (**A**). His-RepA was crosslinked using 1 mM of ethylene glycol-bis-succinic acid N-hydroxy-succinimide ester (EGS) in the absence or presence of indicated concentrations of guanidine-hydrochloride (Gu-HCl), followed by anti-polyhistidine Western blotting (**B**). **C, D.** EMSAs in the presence of Gu-HCl. EMSAs were performed in the absence or presence of increasing concentrations of Gu-HCl. Two pmol of ^{32}P-labeled IR (**C**) or iteron (**D**) were incubated with 0.05 μg or 2 μg of RepA, respectively.

30]. Gel filtration chromatography and protein chemical crosslinking were performed to examine the dimerization of RepA. As shown in Fig. 5A, the majority of RepA was eluted following Sephacryl 300HR chromatography at approximately 60 kDa with a smaller portion of RepA eluted around 30 kDa, suggesting that RepA exists mainly as a dimer in solution. The RepA dimer peak was not symmetrical and appeared to comprise multiple peaks of higher molecular mass, suggesting the existence of higher order complexes of RepA. To further establish the significance of RepA dimerization, we performed protein chemical crosslinking of His-RepA using a bifunctional crosslinker, ethylene glycol-bis-succinic N-hydroxy-succinimide ester (EGS), in the absence or presence of Gu-HCl and analyzed crosslinked proteins by Western blotting using anti-polyhistidine antibody. EGS covalently crosslinks closely positioned proteins [1], and Gu-HCl is frequently used to determine protein dimerization because nondenaturing levels of Gu-HCl destabilize protein dimers into monomers [21, 30]. Fig. 5B shows formation of the RepA dimer and multimerized RepA and that incubation of His-RepA with Gu-HCl prior to EGS abolished the crosslinked species of His-RepA, suggesting that Gu-HCl dissociates His-RepA complexes into monomers. The preferential binding of dimeric RepA to the IR may explain the EMSA findings. The higher level of RepA required for binding to the iteron than to IR may be explained by the observation that the majority of RepA exists in a dimeric form. This may also explain the slower migration of RepA-IR compared with RepA-iteron. It is possible that the lower-mobility band observed in the EMSA with RepA and the IR is a multimeric RepA-IR complex. To examine the preferential binding of dimeric RepA to the IR, the EMSA was performed in the presence of Gu-HCl. Figs. 5C and 5D show that binding of RepA to the IR is inhibited by Gu-HCl, whereas the binding of RepA to the iteron element is facilitated by Gu-HCl. Taken together, these results imply that monomeric RepA binds preferentially to a single iteron, whereas dimeric RepA binds preferentially to an IR and may reflect an important regulatory role of RepA in the replication of p703/5.

Iterons are elements required for the initiation of replication, and IR sequences are involved in the regulation of transcription, and both of these processes are exerted through interactions with Rep proteins [15, 27]. Consistent with these findings, the results of the present

study showed that RepA extensively bound to the origin of p703/5, with preferential binding to iteron and IR elements. Many studies have shown that binding of monomeric Rep to an iteron results in initiation of replication, whereas binding of dimeric Rep to an IR leads to repression of *rep* transcription. These are considered common features of iteron-carrying theta replicating plasmids [15, 26]. The present results also reveal that monomeric RepA preferentially binds to an iteron, whereas dimeric RepA preferentially binds to an IR. Even though the possibility of autogenous repression of *repA* transcription by RepA was not addressed in the present study, the binding of RepA to an IR might repress transcription of *repA*, since essential regions, such as those including the −35 box and −10 box for *repA* transcription initiation, overlap with the IR, as exemplified in other systems [15]. The replicon of p703/5 from *E. faecalis* has features that are distinct from well-studied enterococcal plasmids such as pAD1 [13], but are similar to a family of small theta replicating plasmids from other Gram-negative and Gram-positive bacteria [3, 15, 24]. The RepA-origin interactions of the enterococcal p703/5 are more consistent with those of iteron-containing plasmids from Gram-negative bacteria. Thus, RepA from enterococcal p703/5 may exert regulatory functions through interactions with iterons and IRs *via* mechanisms similar to those found in a family of typical iteron-carrying theta-type plasmids.

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References

1. Abdellar, R. M., P. K. Smith, and G. P. Royer. 1979. A new cleavable reagent for crosslinking and reversible immobilization of protein. *Biochem. Biophys. Res. Commun.* **87:** 734–742.
2. Aoki, K., M. Shinohara, and T. Itoh. 2007. Distinct functions of the two specificity determinants in replication initiation of plasmids ColE2-P9 and ColE3-CA38. *J. Bacetriol.* **189:** 2392–2400.
3. Belkum, M. J. and M. E. Stiles. 2006. Characterization of the theta-type plasmid pCD3.4 from *Carbobacterium divergens* and modulation of its host range by RepA mutation. *Microbiology* **152:** 171–178.
4. Benachour, A., J. Frere, and G. Novel. 1995. pUCL287 plasmid from *Tetragenococcus halophila* (*Pediococcus halophilus*) ATCC 33315 represents a new theta-type replicon family of lactic acid bacteria. *FEMS Microbiol. Lett.* **128:** 167–176.
5. Bowers, L. M., R. Kruger, and M. Filutowicz. 2007. Mechanism of origin activation by monomers of R6K-encoded pi protein. *J. Mol. Biol.* **368:** 928–938.
6. Chattoraj, D. K., R. J. Mason, and S. H. Wichner. 1988. Mini-P1 plasmid replication; The autoregulation-sequestration paradox. *Cell* **52:** 551–557.
7. Chattoraj, D. K. 2000. Control of plasmid DNA replication by iterons: No longer paradoxical. *Mol. Microbiol.* **37:** 467–476.
8. Cheon, Y. W. and H. I. Chang. 1994. Cloning of replication origin from Enterococcal plasmid p703/5. *Kor. J. Appl. Microbiol. Biotechnol.* **22:** 18–22.
9. Diaz-Lopez, T., M. Lages-Gonzalo, A. Serrano-Lopez, C. Alfonso, G. Rivas, R. Diaz Orejas, and R. Giraldo. 2003. Structural changes in RepA, a plasmid replication initiator, upon binding to origin DNA. *J. Biol. Chem.* **278:** 18606–18616.
10. Fang, L., M. J. Davey, and M. O'Donnell. 1999. Replisome assembly at oriC, the replication origin of *E. coli*, reveals an explanation for initiation sites outside an origin. *Mol. Cell* **4:** 541–553.
11. Filutowicz, M., M. J. McEachern, and D. R. Helinski. 1986. Positive and negative roles of and initiator protein at an origin of replication. *Proc. Natl. Acad. Sci. USA* **83:** 9645–9649.
12. Filutowicz, M. and S. A. Rakowski. 1998. Regulatory implications of protein assemblies at the gamma origin of plasmid R6K. *Gene* **223:** 195–204.
13. Francia, M. V., S. Fujimoto, P. Tille, K. E. Weaver, and D. B. Clewell. 2004. Replication of *Enterococcus faecalis* pheromone-responding plasmid pAD1: Location of the minimal replicon and oriV site and RepA. *J. Bacteriol.* **186:** 5003–5016.
14. Galas, D. J. and A. Schmitz. 1978. DNase footprinting: Asimple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5:** 3157–3170.
15. García de Viedma, D., R. Giraldo, M. J. Ruiz-Echevarria, R. Lurz, and R. Díaz-Orejas. 1995. Transcription of repA, the gene of the initiation protein of the *Pseudomonas* plasmid pPS10, is autoregulated by interactions of the RepA protein at a symmetrical operator. *J. Mol. Biol.* **247:** 211–223.
16. Giraldo, R., J. M. Andreu, and R. Diaz-Orejas. 1998. Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. *EMBO J.* **17:** 4511–4526.
17. Gros, M. F., H. te Riele, and S. D. Ehrlich. 1987. Rolling circle replication of single stranded DNA plasmid pC194. *EMBO J.* **6:** 3863–3869.
18. Jin, R., M. E. Fernandez-Beros, and R. P. Novick. 1997. Why is the initiation nick site of an AT-rich rolling circle plasmid at the tip of a GC-rich cruciform? *EMBO J.* **16:** 4456–4466.
19. Kanatani, K., T. Tahara, O. Masao, K. Sano, and C. Umezawa. 1995. Identification of the replication region of *Lactobacillus acidophilus* plasmid pLA103. *FEMS Microbiol. Lett.* **133:** 127–130.
20. Kim, K. and R. J. Meyer. 1985. Copy number of the broad host-range plasmid R1162 is determined by the amounts of essential plasmid-encoded proteins. *J. Mol. Biol.* **185:** 755–767.
21. Kruger, R. and M. Filutowicz. 2000. Dimers of pi protein bind the A+T-rich region of the R6K gamma origin near the

leading-strand synthesis start sites: Regulatory implications. *J. Bacteriol.* **182:** 2461–2467.
22. Lee, K.-E., D.-K. Park, C.-H. Baek, W. H., and K.-S. Kim. 2006. *repABC*-type replicator region of megaplasmid pAtC58 in *Agrobacterium tumefaciens* C58. *J. Microbiol. Biotechnol.* **16:** 118–125.
23. Levchenko, I. and M. Filutowicz. 1996. Initiator protein pi can bind independently to two domains of the gamma origin core of plasmid R6K: The direct repeats and the A+T-rich segment. *Nucleic Acid Res.* **24:** 1936–1942.
24. Manen, D., L. Upegui-Gonzales, and L. Caro. 1992. Monomers and dimers of the RepA protein in plasmid pSC101 replication: Domains in RepA. *Proc. Natl. Acad. Sci. USA* **89:** 8923–8927.
25. Martinez-Bureno, M., E. Valdivia, A. Galvez, and M. Maqueda. 2000. pS86, a new theta-replicating plasmid from *Enterococcus faecalis*. *Curr. Microbiol.* **41:** 257–261.
26. Murotsu, T., H. Tsutsui, and K. Matsubara. 1984. Identification of the minimal essential region from the replication origin of miniF plasmid. *Mol. Gen. Genet.* **196:** 373–378.
27. Perris, S. and D. R. Helinski. 1993. DNA sequence requirements for interaction of the RK2 replication initiation protein with plasmid origin repeats. *J. Biol. Chem.* **268:** 3662–3669.
28. Song, J. S. and H. I. Chang. 1999. Characterization of the replication region of the *Enterococcus faecalis* plasmid p703/5. *J. Microbiol. Biotechnol.* **9:** 91–97.
29. Tanaka, T., H. Ishida, and T. Maehara. 2005. Characterization of the replication region of plasmid pLS32 from the Natto strain of *Bacillus subtilis*. *J. Bacteriol.* **187:** 4315–4326.
30. Urh, M., J. Wu, K. Forest, R. B. Inman, and M. Flutowicz. 1998. Assemblies of replication initiator protein on symmetric and asymmetric DNA sequences depend on multiple protein oligomerization surfaces. *J. Mol. Biol.* **283:** 619–631.