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 these 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 initiation [28] (Fig. 1). Independent of 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 other plasmids.

Fig. 1. Structure of the replication origin of p/03/5 from Enterococcus faecalis KBL703. An AT-rich region, seven direct repeats (iterons), a ribosome binding site (RBS), an inverted repeat (IR), and a replication initiation (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.
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 plasmid p703/5. regulatory mechanism controlling replication of the enterococcal IR origin of p703/5 and found that RepA binds to iterons and molecular interactions between RepA and the replication is unknown. In the present study, we investigated the function of Rep proteins in this family of plasmid origins theta-mode. The molecular mechanism underlying the 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'-GCATATGAGTAAATCGGAATT- AGCTG-3' and the reverse primer 5'-CTCCGTTTACCAATCGGTAC-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 pET17b (Novagen, WI, U.S.A.), from which the Ndel-BamHI fragment was excised and inserted between 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 0.5-1.0 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 (NH₄)₂SO₄, 20 mM Mes-NH₄, 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'-AATCCGGACTTTGGAATTCGCTGAG-3') and [³²P]-labeled reverse primer (5'-TCAGCTATTTGCATTTCGTTTCTCC-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 MgCl₂, 0.1 mM EDTA, 2 mM DTT, 50 µg/ml BSA, 200 mM NaCl, 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 RNA), 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'-TACCACAACCATTTTTATCTGACT-3'; IR 5'-ATTTAACCACACACAAGTCGACGACAGTGGGTTTGGCCATCC-3'. Oligonucleotides were end-labeled with 20 µCi [³²P]-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-NH₄, 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...
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-
histidine tag 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,
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 acid 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]. 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 acid N-hydroxy-succinimide ester (EGS), in the absence or presence of Gu-HCl and analyzed crosslinked proteins by Western blotting using antipolyhistidine 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 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

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