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Molecular Mechanism of R1162 Plasmid Incompatibility Exerted by Direct Repeat in the Replicative Origin

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Abstract: In order to elucidate the molecular mechanism of plasmid incompatibility of broad host-range plasmid R1162, the plasmid-encoded replication protein RepIB was purified and tested for binding to the 20 bp direct repeat (DR) DNA sequence which is reiterated 3 and 1/2 times within the replicative origin of the plasmid. The RepIB protein specifically binds to the DR DNA. Point mutations in the DR which affect expression of plasmid incompatibility also coordinately affect binding. These results indicate that the incompatibility of broad host-range plasmid R1162 is exerted by the DR DNA by titrating the essential replication protein RepIB.

Key words: plasmid copy number control, plasmid incompatibility.

Two distinct but related plasmids (which share the same mechanisms for replication or partitioning) cannot be stably co-inherited. This phenomenom is termed plasmid incompatibility. Although in some cases incompatibility is the result of partitioning functions (Austin and Nordstrom, 1990), in most cases, incompatibility is related to plasmid copy number control, because most functions on plasmids which result in alteration of incompatibility also cause change in the copy number of the plasmid (Uhlin and Nordstrom, 1975; Tomizawa and Itoh, 1981).

R1162 is a broad host-range plasmid that replicates with a high copy number in *Escherichia coli* (Barth and Grinter, 1974; Meyer *et al.*, 1982). The replicative origin (*oriV*) region contains three and one-half 20 bp direct repeats (DRs). The DRs are responsible for expression of incompatibility, and also affect plasmid copy number (Lin and Meyer, 1986). The strength of incompatibility is a function of the additive effect of the number of the DRs present on the plasmid, indicating that each DR is a unit contributing to the expression of incompatibility (Lin *et al.*, 1987). The DRs are also required for plasmid replication (Kim *et al.*, 1987). Replication of the plasmid requires three plasmid-encoded replication proteins (RepIA, RepIB and RepII) as well as the *oriV* region (Meyer *et al.*, 1985). Overproduction of the RepI proteins raises the copy number of R1162 derivatives, and also protects these plasmids against an expression of incompatibility by the cloned DR DNA (Kim and Meyer, 1985). An additional DR cloned into R1162 lowers the copy number of the plasmids (Lin and Meyer, 1984; Lin and Meyer, 1986). These observations suggest that the copy number of R1162 is determined by the intracellular amounts of the RepI proteins. The RepIB protein is known to induce localized melting of the AT-rich region adjacent to the DRs in the origin region which probably serves as an entry point of DNA helicase (Kim and Meyer, 1991). These results indicate that the interaction between the DRs and replication proteins is responsible for plasmid incompatibility.

In the experiments described in this study, we look more closely at the role of the DRs in the expression of incompatibility. Here we present evidence that the DRs are a binding site for the RepIB protein, and that this binding is the basis of R1162 plasmid incompatibility.

Materials and Methods

Bacterial strains, media and plasmids

The *Escherchia coli* K12 strains used in this study are MV10 (*thr leu lacY thi supE44 fhuA ΔtrpE5*), a derivative of C600 (Hershfield *et al.*, 1974), and GM1 (*ara Δ[lac pro] thi/F' [lac pro lacI^q L8*) (Miller *et al.*, 1977). Bacteria were routinely grown in TYE broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented as required with ampicillin (Amp) (50 μg/ml), or tetracycline (Tc) (25 μg/ml). Plasmid pUT935 (Tc^r, *repIB*$^+$) is a pBR322 derivative in which the small *Eco*RI-*Pst*I fragment of the vector is replaced by an

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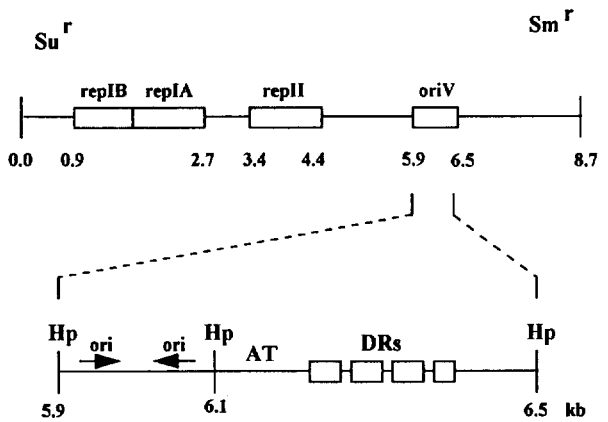

Fig. 1. Simplified map of R1162 showing location of *oriV* and *repIA*, *repIB* and *repII* region. Distances are in kb of DNA from the unique *Eco*RI cleavage site (R). In the bottom part of the figure, the map is enlarged to show the two *Hpa*II (Hp) DNA fragments containing *oriV*, and the locations of DRs and AT-rich region. The arrows indicate the orientation of initiation of DNA synthesis.

*Eco*RI-*Bam*HI fragment containing the *tac* promoter (Amann *et al.*, 1983) fused to a *Bam*HI-*Pst*I fragment containing R1162 DNA between 2.83 and 0.9 kb.

Preparation of [^{32}P]-labeled DNA

DNA fragments to be labeled were obtained by electroelution from a polyacrylamide gel. The reaction mixture (75 μl) consisted of 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, dGTP, dCTP and dTTP (0.0375 mM each), 0.1 μg of DNA fragment, 30 μCi of [α-^{32}P]dATP (3000 Ci/mmol) and 2 units of Klenow fragment. The reaction was carried out in a 30℃ water bath for 30 min. The labeled DNA was washed with ethanol, and resuspended in 100 μl TE buffer (10 mM Tris-HCl, pH 7.8, 1mM EDTA).

Purification of the RepIB protein

In order to purify the RepIB protein, RepIB-overproducing plasmid pUT935 was constructed by replacing a small *Eco*RI-*Pst*I fragment of pBR322 with an *Eco*RI-*Pst*I fragment of pUT238 (Kim and Meyer, 1985) containing *ptac* fused to a *Bam*HI-*Pst*I fragment containing R1162 DNA from 2.83 to 0.9 kb. The structure of pUT935 is shown in Fig. 2.

GM1 cells containing pUT935 were grown at 37℃ to $OD_{590}\times0.5$ in 1.5 l TYE broth containing 25 μg/ml Tc, and isopropyl-β-D-thiogalactoside was then added to 1 mM. After allowing the culture to grow for an additional 3 h, the cells (about 3 g wet weight) were harvested by centrifugation. The cells were washed in 20 ml buffer A (50 mM Tris-HCl, pH 7.7, 0.1 mM dithiothreitol, 1 mM EDTA), resuspended in the same buffer containing 1 mM phenylmethylsulfonyl chloride,

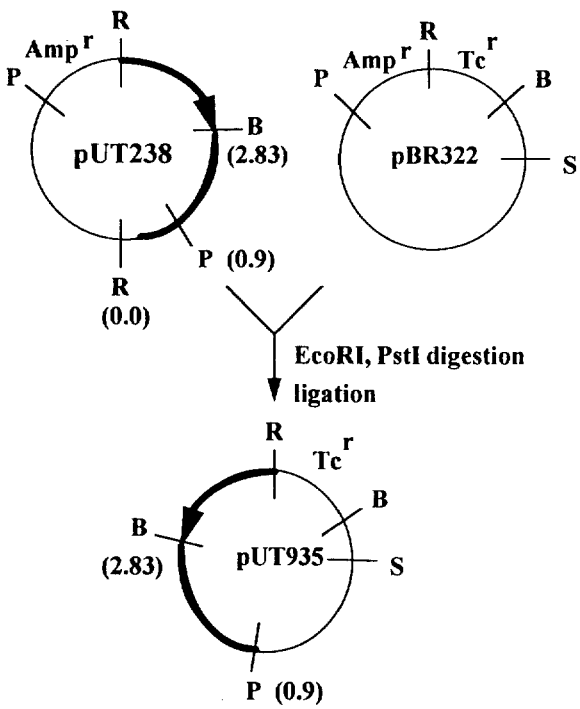

Fig. 2. Schematic representation of construction of plasmid pUT 935. plasmid pUT935 was constructed by replacing a small *Eco*RI-*Pst*I fragment of pBR322 with an *Eco*RI-*Pst*I fragment of pUT238 (Kim and Meyer, 1985), containing ptac fused to a *Bam*HI-*Pst*I fragment containing R1162 DNA from 2.83 to 0.9 kb. Thick arrow represents tac promoter. The abbreviations used are R, *Eco*RI; B, *Bam*HI; S, *Sal*I; P, *Pst*I.

and lysed by two passages through a French pressure cell. Cellular debris was removed by centrifugation at 100,000×*g* for 30 min. Streptomycin sulfate was added to the supernatant to a final concentration of 1.5%, the mixture stirred on ice for 30 min, and then centrifuged at 12,000×*g* for 10 min. In addition to the nucleic acids, the precipitate contained most of the RepIB protein. The precipitate was respended in 5 ml buffer A, and passed several times through an 18-1/2G hypodermic needle by means of a 10 ml syringe. The sheared material was then mixed with a 10 g wet DEAE-Sephacel. The RepIB protein was eluted from the DEAE-Sephacel by washing five times with 5 ml buffer A, and the protein solution was then dialyzed against 1 l buffer B (50 mM Tris-HCl, pH 7.7, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1% Brij 58, 10% glycerol) at 4℃ for 4 h.

NaCl was added to the dialyzed solution to a final concentration of 0.6 M. The solution was then applied to a heparin-Sepharose column (40.2×1 cm, bed volume 31.5 ml) pre-equilibrated with buffer B containing 0.6 M NaCl. The column was washed with buffer until the optical density of the flow-through was reduced to background level (OD_{280} less than 0.01). RepIB protein was eluted by washing with buffer B containing

1.5 M NaCl (Scherzinger *et al.*, 1984). Peak fractions were pooled, concentrated by means of an Amicon concentrator (YM 10 membrane), dialyzed against 500 ml Buffer A containing 50% glycerol for 4 h at 4℃, and stored at −20℃. Purification of the RepIB protein displayed by SDS-polyacrylamide gel electrophoresis is shown in Fig. 3.

Gel retardation assay

Assays were carried out by the method of Garner and Revzin (1981), with minor modification. The reaction mixture consisted of purified RepIB protein and end-labeled DNA, in amounts indicated for each experiment, in 20 μl reaction buffer (10 mM HEPES, pH 8.0, 5 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 20% glycerol). The mixture was incubated at 30℃ for 15 min, then 200 ng poly [dI:dC] was added and incubation continued for 5 min at room temperature. Samples were loaded onto a 5% polyacrylamide gel and DNA bands were detected by autoradiography.

Test fragments for binding contained one copy of the 20 bp DR found in the origin (Fig. 1), with either the normal sequence or with one of the previously generated point mutations, C1, G2 or G8 (Lin *et al.*, 1987). The DR sequence in the test DNA was bound on one side by a 12 bp *Eco*RI-*Cla*I fragment (Lin *et al.*, 1987). The DNA at the other side of the DR was joined to a 156 bp *Hin*dIII-*Eco*RV fragment from pBR 322 by means of a 21 bp *Hin*CII-*Hin*dIII linker fragment from M13mp19 (Norrander *et al.*, 1983). The total size of the fragment was 209 bp. A control fragment (213 bp) consisted of the 57 bp *Eco*RI-*Hin*dIII fragment from M13mp19 joined to the *Hin*dIII-*Eco*RV pBR322 fragment.

Results and Discussion

Purified RepIB protein binds to the DR DNA in the *oriV*

Replicative origins containing directly-repeated DNA sequences (iterons) adjacent to AT-rich DNA are characreristic of a large class of Gram-negative bacterial replicons. These iterons are bound by replicon-specific proteins that are thought to distort the helix locally, and to enable the entry of proteins required for the initiation of DNA synthesis (Bramhill and Kornberg, 1988; Schnos *et al.*, 1988). For R1162, it was known that the RepIB protein causes localized melting of DNA at a site within the AT-rich region in the origin (Kim and Meyer, 1991). Therefore, the RepIB protein presumably binds to the DR DNA adjacent to the AT-rich region first to induce localized opening of the AT-rich

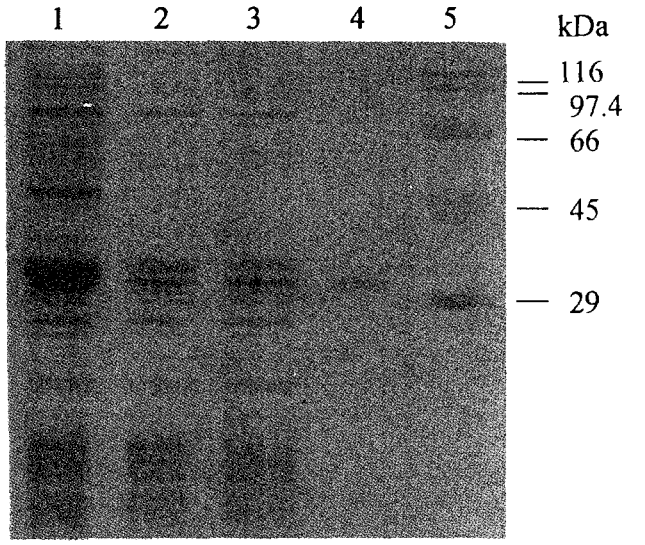

Fig. 3. Purification of RepIB protein displayed by SDS-polyacrylamide gel electrophoresis. (1) total cell extract (100 μg), (2) streptomycin sulfate precipitate (50 μg), (3) elutant from DEAE-Sephacel (40 μg), (4) pooled active fractions from the Heparin-Sepharose column (2 μg), Molecular weight markers (5) are β-galactosidase (116 kda), phosphorylase B (97.4 kda), bovine serume albumin (66 kda), egg albumin (45 kda), and carbonic anhydrase (29 kda). Gel (12% polyacrylamide, 0.31% bis-acrylamide) was prepared according to Laemmli (1970).

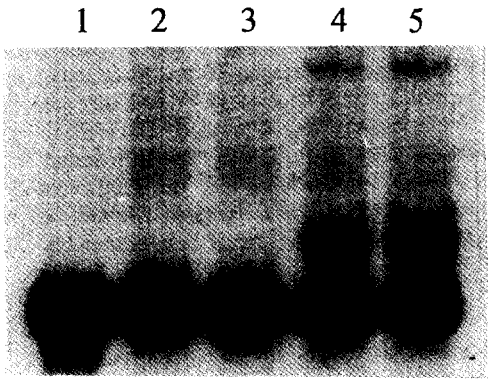

Fig. 4. Gel retardation assay for binding of purified RepIB protein to a DNA fragment containing 20 bp DR or to a control DNA. Each lane contained 1 ng of labeled DNA and indicated amount of purified RepIB protein, lane 1 : DR+no RepIB; lanes 2 : control DNA+10 ng RepIB; lane 3 : control DNA+20 ng RepIB; lane 4 : DR DNA+10 ng RepIB; lane 5 : DR DNA+20 ng RepIB.

DNA. To prove this idea, the purified RepIB protein was tested for DR DNA binding. The gel retardation assay result shows that the purified protein bound to a 209 bp DNA fragment containing the DR sequence in the *oriV* region of R1162, retarding its mobility during polyacrylamide gel electrophoresis (Fig. 4, lanes 4 and 5). There was no binding to a 213 bp control DNA fragment lacking the DR sequence (Fig. 4, lanes 2 and 3). Thus this result indicates that the RepIB protein binds to the DR DNA.

DR DNA and mutations :

wt 5' - CGTGACAGTTATTGCAGGGG - 3'

C1 5' - TGTGACAGTTATTGCAGGGG - 3'

G2 5' - CATGACAGTTATTGCAGGGG - 3'

G8 5' - CGTGACAATTATTGCAGGGG - 3'

Fig. 5. Base sequence of the DR and mutated DRs. The location of each mutation is underlined. These mutations were previously generated by Lin *et al.*, 1987.

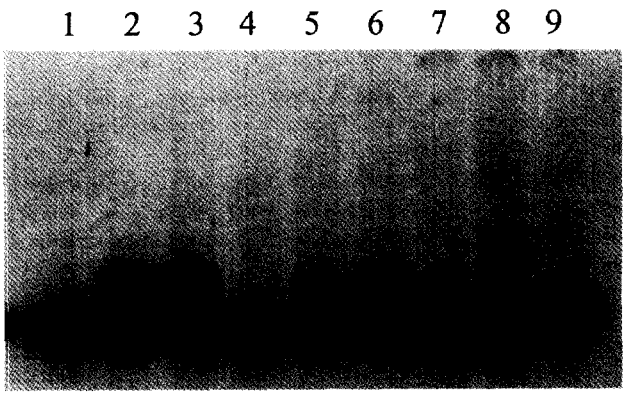

Fig. 6. Gel retardation assay for binding of purified RepIB protein to DNA fragment containing 20 bp DR or mutated DR. Each lane contained 1 ng of labeled DNA and indicated amount of purified RepIB protein, lane 1 : mutated DR C1 + no RepIB; lane 2 : mutated DR C1 + 10 ng RepIB; lane 3 : mutated DR C1 + 20 ng RepIB; lane 4 : mutated DR G8 + no RepIB; lane 5 : mutated DR G8 + 10ng RepIB; lane 6 : mutated DR G8 + 20 ng RepIB; lane 7 : mutated DR G2 + no RepIB; lane 8 : mutated DR G2 + 10 ng RepIB; lane 9 : mutated DR G2 + 20 ng RepIB.

Mutations in the DRs affecting expression of incompatibility also affect the binding of RepIB protein to the DR

DR mutation reducing incompatibility *in vivo* coordinately reduced DR-dependent inhibition of R1162 replication *in vitro* (Lin *et al.*, 1987). In order to address the question whether these mutations also decreased binding of the RepIB protein to the DR DNA, three DNA fragments containing either the C1, G2 or G8 mutation (Fig. 5) were tested for RepIB binding. The result of a gel retardation assay, shown in Fig. 6, demonstrated that the RepIB protein effectively binds to the DR containing the C1 mutation (lanes 2 and 3), whereas the RepIB protein does not bind to the DR DNA containing the G2 mutation (lanes, 8 and 9). The DR DNA having the G8 mutation shows intermediate binding (lanes, 5 and 6). Lin *et al.* (1987) demonstrated that the DR DNA containing the C1 mutation exhibited substantial inhibition of R1162 replication *in vitro* and measurable incompatibility, and that the DR DNA having the G2 mutation, which results in no incompatibility, shows little inhibition of R1162 DNA replication *in vitro*. The DR DNA containing the G8 mutation has an intermediate effect on both inhibition of replication and incompatibility. Thus the degree of RepIB binding to the DRs was related to the degree to which the mutation affected the expression of incompatibility and inhibition of R1162 DNA replication by the DRs *in vitro*. Therefore, we conclude that binding of RepIB to the DR DNA under the conditions used in these experiments reflects a biologically significant interaction, and that incompatibility expressed by cloned DR DNA *in vivo* is due to titration of essential, limiting RepIB protein, resulting in inhibition of plasmid DNA replication.

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