A Specific Region in the N Terminus of a Replication Initiation Protein of Plasmid RK2 Is Required for Recruitment of Pseudomonas aeruginosa DnaB Helicase to the Plasmid Origin*

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A critical step in the initiation of DNA replication is the recruitment, loading, and activation of the replicative helicase. Helicase activity is not only necessary for progression of the replication fork but, as studies with the Escherichia coli chromosomal oriC have revealed, the DnaB helicase makes essential contacts with other proteins in the replication complex. Recruitment of the helicase in E. coli requires association with the host-encoded DnaC protein to recruit DnaB to the initiation complex (17, 18). With phage P2, the phage-encoded B protein appears to be required for DnaB recruitment for lytic replication, although not replication of P2 as a plasmid (19).

Broad host range plasmids are able to replicate in diverse bacteria. As such, they provide the means to examine replication of a specific replicon in different host backgrounds. RK2 is a promiscuous plasmid belonging to the IncP group. It is noted for its ability to transfer into and be stably maintained in a wide variety of Gram-negative bacteria. Only two regions of an IncP plasmid are essential for broad host range replication, the cis acting origin for DNA replication (oriV) and the trfA gene that encodes two forms of a trans-acting replication initiation protein. The host bacterium provides all other proteins essential for replication. The simplicity of this system has allowed for a direct comparison of the mechanism for DNA replication initiation in different bacterial species (20, 21).

IncP plasmids can be divided into two subgroups based on sequence differences: IncPa, to which RK2 belongs, and IncPB, which is represented by the plasmid R751. Both RK2 and R751 can replicate in E. coli and Pseudomonas aeruginosa. Earlier studies had shown that the smaller form of the RK2 initiation protein, TrfA-33, was sufficient for stable replication of an RK2 mini-replicon in E. coli or Pseudomonas aeruginosa. Earlier studies had shown that the larger form of the protein, TrfA-44, was required for plasmid replication in P. aeruginosa (22–24). Recently, it has been shown that TrfA-44 is unique among plasmid initiation proteins in that it can load and activate the DnaB helicase of P. aeruginosa or P. putida on the RK2 origin in vitro in the absence of the DnaA protein (25). By contrast, the TrfA-33 protein requires DnaA protein to load and activate the helicase.
N-terminal TrfA-44 Mutants Altered in Helicase Loading

of *P. putida* and requires DnaA plus DnaC to load the helicase of *E. coli*. Consistent with the earlier in *vitro* studies, TrfA-33 did not function in *vitro* with the DnaB helicase of *P. aeruginosa* either in the presence or in the absence of *P. aeruginosa* DnaA (25).

Since it has been shown that the open complex formed on a supercoiled oriV template is indistinguishable when either TrfA-33 or TrfA-44 proteins were used with the DnaA proteins of *E. coli*, *P. putida*, or *P. aeruginosa* (20), the observed differences in activity of the two forms of the TrfA initiation protein in *P. aeruginosa* are likely due to a difference in their ability to interact with the *P. aeruginosa* DnaB helicase. This suggested a role for the N-terminal 97 amino acids, which are unique to TrfA-44, in DnaB recruitment in *Pseudomonas*. We therefore undertook a mutational analysis of the first 97 amino acids of this protein in an attempt to identify those regions within the N terminus that are important in the loading of the *Pseudomonas* helicases at the RK2 replication origin.

**EXPERIMENTAL PROCEDURES**

**Construction of TrfA Mutations—**Plasmid pGC1 (20) was used to express His6TrfA-33/M98L/G254D/S267L, a variant of TrfA-33 that has histidinyl residues inserted between the first amino acid (Met) and the second amino acid (Asn) of the native protein to allow for full purification of the M98L amino acid substitution that replaces the native methionine start of TrfA-33 with a leucine and thus eliminates TrfA-33 expression, and the G254D and S267L changes that result in a primarily monomeric form of the protein. A previous study had shown that although wild-type TrfA protein is primarily a dimer in solution, it primarily monomeric form of the protein. A previous study had shown that although wild-type TrfA protein is primarily a dimer in solution, it

To change amino acid residue 22 in the *trfA* gene of *P. putida* G35 (GAA) to Ala (GCG), and coincidentally introduce a SacII site, primer pZZ29, for expression of TrfA-44 deletion protein, which contained either one or two additional amino acids at the site of the deletion. The resulting plasmids were pZZ22, for expression of TrfA-44, pZZ22, for expression of TrfA-44, and pZZ23, for expression of TrfA-44.

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**Source of Proteins—**The N-terminal His-tagged G254D/S267L form of TrfA-33 (27), TrfA-44 (20), and the TrfA-44 N-terminal point and deletion mutants were used throughout this study. Plasmids expressing the TrfA-44 N-terminal mutants were transferred into E. coli strain JP131 to overexpress and purify the proteins as described previously for TrfA-44 (20).

C-terminal His-tagged DnaA proteins of *E. coli*, *P. putida*, and *P. aeruginosa* (21) and C-terminal His-tagged DnaB proteins of *E. coli*, *P. putida*, and *P. aeruginosa* (20) were purified as described. The modified proteins have been found to behave similarly to the native *E. coli* proteins in several in *vitro* assays (28, 29). *E. coli* DnaC and *E. coli* DNA gyrase were purified from strain RSOS80 and AN1459(pPS62), respectively, kindly provided along with purification protocols by Dr. Nick Dixon. HU was a generous gift from Dr. Roger McMacken. Commercially available proteins were SSB (Promega), creatine kinase and bovine serum albumin (Fraction V) (Sigma), and DNA restriction and modification enzymes from various commercial sources.

**Helicase Unwinding Assay—**Helicase unwinding assays (17) forma-

1 The abbreviation used is: SSB, single-stranded DNA binding protein.
TrfA-44, which encompassed this helical region. The analysis of the in vitro activity of this deletion mutant, designated as TrfA-44Δ, led to the construction of deletions TrfA-44N (amino acid residues 37–55 inclusive deleted) and TrfA-44G (amino acid residues 21–32 inclusive deleted), which removed, separately, two of the three remaining predicted helical regions.

**In Vitro Helicase Loading Activity of TrfA-44 Deletion Mutants**—Loading and activation of DnaB helicase at a replication origin on a supercoiled plasmid template can be examined in vitro using the FI* assay (2, 29). The basis of this assay is that helicase unwinding of a supercoiled template in the presence of DNA gyrase and SSB produces a highly unwound form of the DNA, termed FI*, that can be distinguished electrophoretically from the template FI form.

The FI* assay was used to test the functionality of the mutant TrfA proteins in the recruitment, loading, and activation of DnaB at the RK2 origin. To ensure that slight differences in the activity of the mutants would be seen, the amount of DnaB helicase added to the standard assay was titrated to determine the lowest level of DnaB protein needed to get full activity of this deletion mutant, designated as TrfA-44Δ. The TrfA-44Δ protein, 50 ng for DnaB from *P. aeruginosa* (lanes 1–6) or 100 ng of *P. putida* DnaB (lanes 7–12), the homologous DnaA protein (0, 200, or 600 ng indicated as −, +, or ++), and 300 ng of TrfA-33 or TrfA-44G as indicated. The positions of the FI* (covalently closed highly underwound), FI (covalently closed supercoiled), FII (open circular), and FIII (linear) forms of the template DNA are noted by arrows.

In the TrfA-44Δ mutant, activity with DnaB from *P. aeruginosa* was less than reported previously (25), activity with TrfA-44G showed that the observed defects in the TrfA-44G sequence.

**Index of the N terminus of the TrfA-44 protein of plasmid RK2 (amino acid residues 1–90) with the N terminus of TrfA1 of plasmid R751 (amino acid residues 1–120) using ClustalW (*, identical residues; :, strongly similar residues; ., weakly similar residues) is as shown. The methionine residue that is the start of the smaller TrfA proteins (wt lanes 1, 3, 5, and 7, and compare Fig. 2, lane 5 or 6, with lane 4, and compare lane 11 or 12 with lane 10). We then confirmed that TrfA-33 was able to load this lower level of *P. putida* helicase, but only in the presence of *P. putida* DnaA (Fig. 2, lanes 7–9), and that even with DnaA present, *P. aeruginosa* DnaB, at this lower concentration, could not be loaded by TrfA-33 (Fig. 2, lanes 2 and 3).

The ability of the three TrfA-44 deletion mutant proteins to load *P. aeruginosa* or *P. putida* helicase onto RK2 orvI was then tested. As shown in Fig. 3, TrfA-44Δ (lanes 5) was fully functional with DnaB from *P. aeruginosa* (A) or *P. putida* (B). TrfA-44Δ (lanes 4) had reduced activity, particularly with *P. putida* DnaB, whereas TrfA-44G (lanes 3) was not functional with either helicase. Activity with TrfA-44Δ was not restored by the addition of *P. aeruginosa* DnaA to reactions containing *P. aeruginosa* DnaB but was restored by the addition of *P. putida* DnaA to reactions containing *P. putida* DnaB protein (data not shown).

All three mutant proteins functioned as well as the wild type protein in the loading and activation of *E. coli* DnaB in the presence of *E. coli* DnaA and DnaC (Fig. 4). These results showed that the observed defects in the TrfA-44Δ and TrfA-44Δ mutants were specific to the recruitment and loading of DnaB from *Pseudomonas* in the absence of DnaA.

**Point Mutation in TrfA-44 with Altered Helicase Loading Activity**—The TrfA-44Δ mutant protein was deleted for 12
amino acids. To determine whether a specific amino acid within the deleted region was required for helicase loading, we constructed a specific point mutant. Reasoning that a charged and/or polar amino acid residue might be involved in the stabilization of a DnaB/TrfA-44 interaction, we replaced the glutamate residue at position 22 with alanine, yielding TrfA-44E22A. This change alters the charge and polarity at this position but not the helical nature of this region as predicted using PredictProtein.

The TrfA-44E22A mutant protein was tested for helicase loading activity. This protein, when added at 300 ng/assay, had slightly reduced activity when compared with wild type TrfA protein using PredictProtein. The plasmid was determined as described under Procedures.

In all cases, the peak of TrfA protein was in the void fraction between TrfA and P. aeruginosa DnaB. As shown in Fig. 6, the template DNA was present in the void, as are any proteins that are stably associated with the DNA fragment containing RK2 oriV (data not shown). A filter binding assay was used to confirm that the reduced activity of the TrfA-44E22A point mutant was due to reduced DNA binding activity of the mutant as compared with the wild type protein (data not shown). The three TrfA-44 deletion mutants were also not defective in binding to a DNA fragment containing RK2 oriV (data not shown).

Association of P. aeruginosa DnaB with Wild Type and Mutant TrfA-44 Proteins—Gel exclusion chromatography was used to confirm that the differences observed in the conversion of the RK2 mini-plasmid from the FI to the FI* form was due to differences in the ability of the DnaB helicase of P. aeruginosa to physically associate with certain of the TrfA-44 mutant proteins. Wild type and mutant TrfA-44 proteins were incubated with P. aeruginosa DnaB and the RK2 mini-replicon pTJS42. The reaction was then run through a Sepharose CL4-B column, and the eluted fractions were analyzed for the presence of DnaB protein. On this column, the template DNA is present in the void fraction between TrfA-44 wild type protein bound to the template DNA and was therefore present in the void fractions (B) but did not form a stable complex with the TrfA-44 wild type protein bound to the template DNA and was therefore present in the void fractions (B) as has been shown previously (25). P. aeruginosa DnaB also did not stably associate with the TrfA-44Δ2 mutant (C) and showed reduced association with the TrfA-44E22A mutant protein (D) and, to a lesser extent, the TrfA-44Δ3 mutant (E). Under the conditions used, the TrfA-44 wild type or mutant proteins could not be detected after Western blotting. However, TrfA protein could be detected if column fractions were directly applied to a nitrocellulose membrane that was then processed for detection as described under “Experimental Procedures.”

In Vivo Behavior of Mutants in P. aeruginosa—To determine
whether the three TrfA-44 deletion mutants and the TrfA-44E22A point mutant were functional in vivo in P. aeruginosa, the four mutated genes along with the wild type parent were transferred to the RK2 mini-replicon pRR10, yielding plasmids pRR10-98His, pRR10-98HisΔ2, pRR10-98HisΔ3, pRR10-98HisΔ4, and pRR10-98HisΔ22A. The new constructs were transferred into P. aeruginosa by electroporation. The number of transformants obtained was similar for all plasmids except pRR10-98HisΔ2, which had an ~5-fold lower frequency.

The stability of these plasmids in P. aeruginosa was then examined. As shown in Fig. 7, plasmids pRR10-98HisΔ3, pRR10-98HisΔ4, and pRR10-98HisΔ22A were almost as stable as pRR10-98His with less then 20% loss after ~84 generations of growth. Plasmid pRR10-98HisΔ2, however, was extremely unstable. Only 6% of the P. aeruginosa cells maintained the plasmid after ~28 generations of growth.

**DISCUSSION**

Plasmid RK2 can replicate in a wide range of Gram-negative bacteria, and recent studies indicate that it does so by employing a variety of strategies to recruit the necessary host proteins. Early studies showed that RK2 mini-replicons expressing only the smaller replication initiation protein, TrfA-33, were unstable in P. aeruginosa (22–24). Recently, in vitro studies revealed that TrfA-33 was unable to load the DnaB helicase of P. aeruginosa onto the RK2 origin in the presence or absence of DnaA, although this form of the replication initiation protein was fully functional when the DnaA and DnaB proteins from P. putida or the DnaA, DnaB, and DnaC proteins from E. coli were provided (20). The experiments presented here demonstrate that specific regions within the first 97 amino acids of TrfA-44 are required for the recruitment of the P. aeruginosa DnaB helicase to the RK2 replication origin.

The deletion of amino acid residues 21–32 in TrfA-44 results in a mutant protein, TrfA-44Δ2, which fails to stably interact with P. aeruginosa DnaB in vitro (Fig. 6C) and is not able to recruit and activate the helicase at the RK2 origin as detected by the FI* assay (Fig. 3A). That this effect is due to the loss of the specific amino acid residues in the second helix predicted in the N terminus (Fig. 1) and not simply due to a shortening in the length of the protein is clear from the observations that deletion of the 18 amino acid residues in the fourth predicted helix (positions 71–88) had no discernible effect on helicase recruitment and loading in vitro (Fig. 3) or protein activity in vivo (Fig. 7). Furthermore, the TrfA-44Δ2 protein was fully functional in FI* assays in which the TrfA-33 version of the protein is active, i.e. with E. coli DnaA, DnaB and DnaC proteins (Fig. 4) or with P. putida DnaA and DnaB proteins (data not shown).

The defect of TrfA-44Δ2 is also apparent in vivo (Fig. 7). The unstable replication of a plasmid expressing this mutant protein in P. aeruginosa is similar to that of an RK2 replicon expressing only TrfA-33 (22–24). The results presented in this study support the conclusion that RK2 mini-replicons which express only TrfA-33 are unstable in P. aeruginosa because this protein is incapable of loading the DnaB helicase, even in the presence of DnaA protein. In fact, it is not clear that the DnaA protein plays any role in RK2 replication in P. aeruginosa as a mini-replicon deleted for all four DnaA bindings sites in oriV was fully functional in P. aeruginosa (38).

Helicase loading in E. coli requires DnaA and DnaC and utilizes either form of the TrfA protein. In this bacterium, it is likely that the additional 97 amino acids on the N terminus of TrfA-44 play no role in the recruitment and activation of DnaB. Indeed, all four TrfA-44 mutant proteins tested in this study were as active as wild type protein in the FI* assay using the E. coli replication proteins (Fig. 4 and data not shown). Interestingly, an earlier study found that a mini-replicon expressing only TrfA-33 was more stable in E. coli than a mini-replicon expressing only TrfA-44 (23).

P. putida helicase can be loaded in vitro by either of two mechanisms (25). One utilizes TrfA-33 and requires DnaA, similar to E. coli, and the other utilizes TrfA-44 and is DnaA-independent, similar to P. aeruginosa. It is clear from the results shown in Fig. 2 (lanes 4–6), that when both DnaA and TrfA-44 are present, the various possible protein interactions can interfere with helicase loading.

As shown in this study, the two Pseudomonas helicases differ somewhat in their interaction with the N terminus of TrfA-44. Although TrfA-44Δ2 was defective in vitro with both Pseudomonas helicases, the TrfA-44E22A substitution mutant and the TrfA-44Δ3 deletion mutant showed significantly reduced activity with P. putida DnaB (Figs. 3B and 5). In vivo, P. putida, like E. coli, is able to use TrfA-33 and DnaA to recruit DnaB, and as such, RK2 plasmid derivatives carrying trfA-44Δ2, trfA-44Δ3, or trfA-44E22A were able to stably replicate in this host. 2

The interaction of helicase with a replication initiation protein is not unique to TrfA-44. The replication initiation proteins of plasmids R6K (p protein) and pSC101 (RepA) as well as the chromosomal initiator protein DnaA have all been shown to interact with E. coli DnaB (5, 14, 16). The regions of DnaB that interact with these three proteins are not identical, although there is some overlap. Unlike the TrfA-44 protein, however, both the p and RepA proteins require the E. coli DnaA protein for loading and activation of E. coli DnaB helicase. The role of the TrfA-44 replication initiation protein as being solely responsible for helicase loading and activation at the RK2 origin, therefore, is unique among plasmid replications. This is possibly because most of the well studied replication systems are of narrow host range plasmids of E. coli and DnaA/DnaC interactions with DnaB may predominate in this organism but not in other bacteria. In the case of plasmid RK2, the fact that expression of the larger TrfA-44 initiation protein in Pseudomonas species obviates the need for DnaA and DnaC proteins in the recruitment and translocation of DnaB to the replication origin may be an important factor in extending the host-range of this plasmid.

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