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# Plasmid replication initiator protein RepD increases the processivity of PcrA DNA helicase

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## ABSTRACT

**The replication initiator protein RepD encoded by the *Staphylococcus* chloramphenicol resistance plasmid pC221 stimulates the helicase activity of the *Bacillus stearothermophilus* PcrA DNA helicase *in vitro*. This stimulatory effect seems to be specific for PcrA and differs from the stimulatory effect of the *Escherichia coli* ribosomal protein L3. Whereas L3 stimulates the PcrA helicase activity by promoting co-operative PcrA binding onto its DNA substrate, RepD stimulates the PcrA helicase activity by increasing the processivity of the enzyme and enables PcrA to displace DNA from a nicked substrate. The implication of these results is that PcrA is the helicase recruited into the replisome by RepD during rolling circle replication of plasmids of the pT181 family.**

## INTRODUCTION

Replication of plasmids by a rolling circle mechanism is mechanistically similar to the replication of single-stranded (ss)DNA bacteriophages in *Escherichia coli* (1,2). Rolling circle plasmid replication is initiated by the introduction of a single-strand nick on the leading strand at the origin of replication. This is carried out by replication initiator proteins (Rep proteins) encoded by such plasmids. For example, many staphylococcal plasmids of the pT181 family conferring tetracycline (pT181), chloramphenicol (pC221) and streptomycin (pS194) resistance code for homologous Rep proteins (3–6). These origin-specific DNA binding proteins act as dimers, exhibiting a nicking–closing activity which results in replication initiator activity *in vivo* and topoisomerase activity *in vitro* (7–10).

Upon binding to the double-stranded origin the Rep dimer actively initiates melting, cruciform extrusion and nicking at a unique site within the second of three inverted repeats in this region (11,12). Following the introduction of the nick, Rep forms a covalent bond with the 5′-end of the nick on the leading strand via a phosphotyrosine linkage (9) and, by analogy with the replicative processes of single-stranded phage ϕX174 (13,14), replisome components including DNA polymerase, ssDNA binding protein and a helicase are thought to assemble at the origin. While the free 3′-end at the nick is used for 3′ extension during replication, the covalently bound Rep protein at the 5′-end is involved in termination of replication by facilitating the strand exchange between the new and the old DNA of the leading strand, after one round of replication.

The identity of the helicase protein recruited into the pT181 plasmid replisome is not known. Based on genetic evidence, PcrA helicase has been suggested to fulfil this role in *Staphylococcus aureus* (15–17) and in *Bacillus subtilis* (18). Despite the elucidation of the crystal structure of the *Bacillus stearothermophilus* PcrA helicase (19), its physiological role inside the cell is still not clear. In conditional pcrA mutants of *B.subtilis*, rolling circle replication of the plasmid pT181 is inhibited (18). In these mutants, analysis of the replication intermediates showed that leading strand synthesis of pT181 was prevented upon PcrA depletion. The *Staphylococcus* mutation *pcrA3* was originally found to reduce the copy number of pT181 and related plasmids (15). This mutation also leads to an increased accumulation of initiation complexes for pT181. Subsequent characterization of *pcrA3* at the nucleotide sequence level led to the identification of PcrA, a putative helicase with significant sequence identity to UvrD and Rep helicases of *E.coli*. Related sequences have since been identified in *B.subtilis* (18) and *B.stearothermophilus*. Thus PcrA is implicated in rolling circle replication of pT181 family plasmids, acting as a helicase once an initiation complex is formed.

In this study we present *in vitro* biochemical evidence for the interaction of the PcrA helicase from *B.stearothermophilus* with the RepD protein encoded by the staphylococcal chloramphenicol resistance plasmid pC221. The result of this interaction is an increase in the processivity of PcrA. The presence of RepD attached to a nicked DNA substrate also serves to direct PcrA activity specifically towards that substrate. Our results are consistent with previous *in vivo* genetic evidence implicating PcrA helicase in rolling circle plasmid replication in *S.aureus* and in *B.subtilis*.

## MATERIALS AND METHODS

### Protein purifications

Purification protocols for PcrA and RepD have been described previously (9,10,20). DnaB was a gift from H. Pan. RepD/D*

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heterodimer, carrying the oligonucleotide tail Tyr-AATAGCCGGTT-3′ covalently attached via Tyr191 to a single subunit of the protein dimer, was prepared by combining RepD dimer with the oligonucleotide 5′-CTAATAGCCGGTT-3′ in a 1:1 molar ratio as required in RepD reaction buffer (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 200 mM KCl, 10 mM $MgCl_2$, 10% v/v ethanediol) and incubation for 30 min at 30°C. RepD/D* heterodimer was separated from unreacted RepD homodimer and free oligonucleotide by ion exchange chromatography. RepD mutant R189A is a truncated, 34 kDa version of RepD (10) which also encodes alanine at position 189 instead of arginine.

### Helicase assays

Untailed and 3′ tailed DNA substrates for helicase assays were prepared as described elsewhere (20), whereas 5′, 3′+5′ tailed and untailed 'long' DNA substrates were prepared by annealing synthetic oligonucleotides (5′-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTAC-3′ for 5′ tailed, 5′-GTTATTGCATGAAAGCCCGGCTGGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGGTTATTGCATGAAAGCCCGGCTG-3′ for 3′+5′ tailed and 5′-GGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCAT-3′ for the 'long' DNA substrate) to M13mp18 ssDNA as described (20). One molecule of DNA substrate is defined as one molecule of M13mp18 ssDNA with one molecule of the appropriate oligonucleotide annealed onto it.

PcrA helicase reactions were carried out as described previously (20), using 1 nM DNA substrate, 50 nM PcrA and 600 nM RepD dimer, unless stated otherwise. DnaB helicase reactions were performed as described for PcrA but using 250 nM DnaB monomer instead of PcrA. Quantitative analysis of gels was done using a PhosphorImager and Molecular Dynamics software.

### ATPase assays

ATPase assays were carried out by linking ATP hydrolysis to NADH oxidation and measuring it spectrophotometrically as described previously (20). The effect of RepD on the ATPase activity of PcrA was examined by performing ATPase reactions at 24 nM PcrA, 96 nM RepD dimer, 1.2 µM poly$(dT)_{16}$ and varying the ATP concentration (Fig. 6a). The effect of RepD on DNA binding was also examined indirectly, by performing ATPase reactions at 24 nM PcrA, 96 nM RepD dimer, 2 mM ATP and varying the concentration of the DNA cofactor, poly$(dT)_{16}$ (Fig. 6b).

### Substrate plasmid

Substrate plasmid pCER*oriD* carries the *cer* monomer resolution site of plasmid ColE1 as a 377 bp *Hpa*II fragment inserted via the *Nar*I site of pUC19, into which the double-stranded replication origin of pC221 was created using the oligonucleotides 5′-AGCTTTAGACAATTTTTCTAAAACCGGCTACTCTAATAGCCGGTTAAGTGGTAATTTTTTTACCAC-3′ and 5′-CCGGGTGGTAAAAAAATTACCACTTAACCGGCTATTAGAGTAGCCGGTTTTAGAAAAATTGTCTAA-3′, annealed and inserted via the *Hin*dIII and *Xma*I sites. Negatively supercoiled pCER*oriD* was purified by density gradient centrifugation in caesium chloride/ethidium bromide.

### Initiation complex experiments

*Preparation of helicase substrate*. A covalently linked RepD–DNA helicase substrate was prepared using RepD mutant R189A and pCER*oriD*. Protein was attached to DNA by reaction of 3.5 µg of RepD R189A with 40 µg of pCER*oriD* in a final volume of 150 µl of RepD reaction buffer for 30 min at 30°C. The reaction mixture was then loaded on a Nick-Spin column (Pharmacia) pre-equilibrated with restriction buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$) and collected in the flow-through after spinning at 500 *g* for 4 min at 21°C. DNA concentration in the flow-through was determined spectrophotometrically by absorbance at 260 nm. Two units each of *Eco*RI and *Hin*fI/µg of DNA were added and the volume was adjusted with restriction buffer to a final DNA concentration of 0.1 µg/µl. The reaction mixture (referred to as 'restriction digest mixture' hereafter) was then incubated at 37°C overnight.

*Preparation of protease-treated helicase substrate*. Where required, protease treatment of this restriction digest mixture consisted of incubation in the presence of 0.1 mg/ml pronase (Sigma) at 37°C for 2 h, followed by phenol extraction and ethanol precipitation. Treated material was resuspended once more in restriction buffer and adjusted to 0.1 µg/µl.

*Helicase reactions and exonuclease VII treatment*. The helicase reaction involved incubation of 1 µg of restriction digest mixture (with or without prior protease treatment) with 0.46 µg PcrA in a final volume of 18 µl (adjusted to 30 mM Tris–HCl pH 8.0, 63 mM NaCl, 15 mM $MgCl_2$, 1.5 mM DTT, 2.5 mM ATP) at 37°C for 30 min. Controls optionally omitted ATP and/or PcrA from the reaction mixture. Exonuclease digestion following the helicase reaction consisted of the addition of 0.2 U exonuclease VII (Amersham) and incubation at 37°C for a further 30 min.

All reactions were terminated by addition of 2 µl of 0.25 M EDTA, pH 8.0, 50% (v/v) glycerol, 0.1 mg/ml bromophenol blue, and products were separated by electrophoresis through 1.5% (w/v) agarose gels in TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH 8.3) at 10 V/cm for 2 h. DNA was visualized by staining in ethidium bromide at 1 µg/ml for 30 min followed by UV illumination at 300 nm.

## RESULTS

### RepD stimulates the helicase activity of PcrA

RepD enhances the ability of PcrA to displace short oligonucleotides annealed onto M13mp18 ssDNA. In the presence of ATP, PcrA at optimal concentrations can displace short oligonucleotides, with either 3′, 5′, 3′+5′ tails or untailed, annealed onto M13mp18 ssDNA. However, in the presence of RepD the ability of PcrA to displace these oligonucleotides is enhanced significantly even at suboptimal concentrations (Fig. 1). The level of this stimulatory effect is not affected either by premixing PcrA and RepD before adding to the helicase reaction mix nor by premixing RepD and DNA substrate in the helicase reaction mixture before adding PcrA helicase (data not shown). Furthermore, the heterodimer RepD/D* (consisting of the RepD homodimer with an 11 base single-stranded oligonucleotide covalently attached via its 5′-end to Tyr191 of one of the protein monomers) also stimulates the PcrA helicase in a similar manner to RepD homodimer (data not shown).

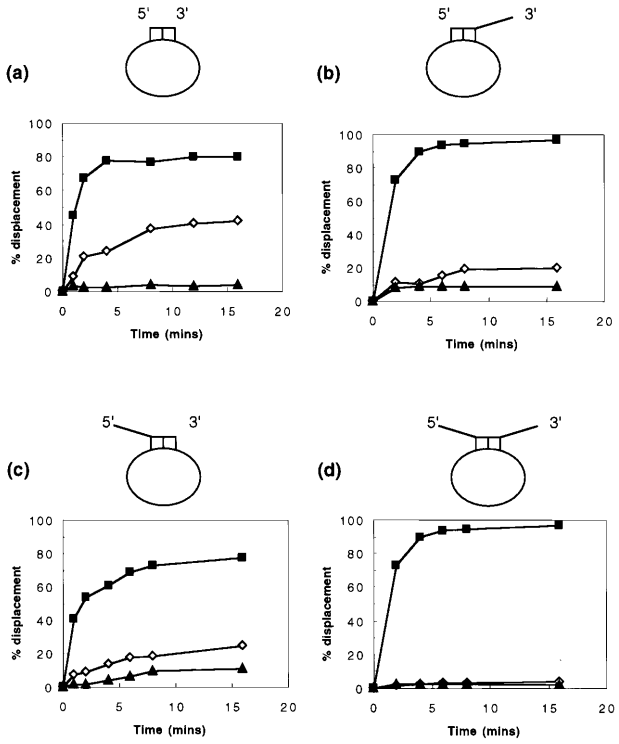


**Figure 1.** Time course helicase reactions showing the stimulatory effect of RepD on the ability of PcrA helicase to displace short oligonucleotides annealed onto M13mp18 ssDNA. Helicase reactions using DNA substrates with no tail (**a**), 3′ tail (**b**), 5′ tail (**c**) and 3′+5′ tail (**d**) are shown. DNA substrates are also depicted schematically at the top of each graph for clarity. Reactions were carried out in the presence of RepD alone (▲), PcrA alone (◇) or RepD plus PcrA together (■). Experimental conditions are as described in Materials and Methods.

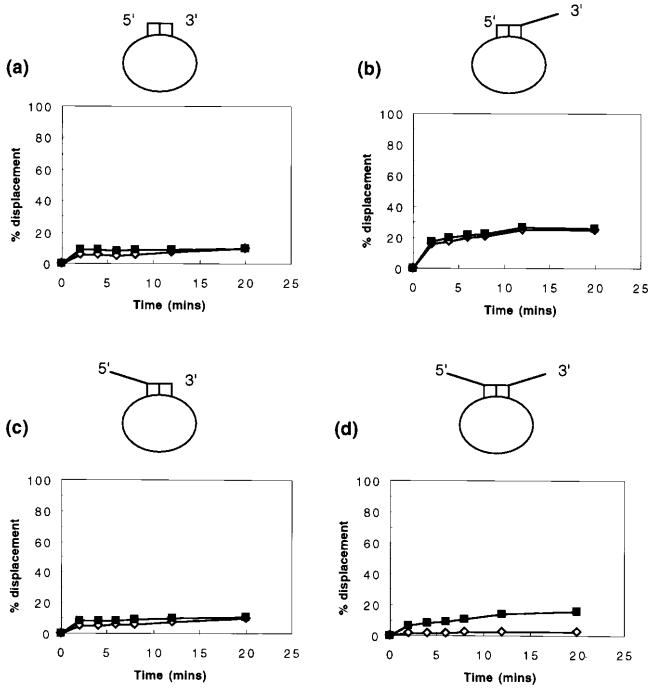


**Figure 2.** Time course reactions showing that RepD does not stimulate the ability of DnaB helicase to displace short oligonucleotides annealed onto M13mp18 ssDNA. Helicase reactions using DNA substrates with no tail (**a**), 3′ tail (**b**), 5′ tail (**c**) and 3′+5′ tail (**d**) are shown. DNA substrates are also depicted schematically at the top of each graph for clarity. Reactions were carried out in the presence of RepD alone (▲), DnaB alone (◇) or RepD plus DnaB together (■). Experimental conditions are described in Materials and Methods.

RepD does not displace short oligonucleotides annealed onto M13mp18 ssDNA either in the presence (Figs 1 and 2) or absence of ATP (data not shown).

## The stimulatory effect of RepD is specific for PcrA

RepD failed to enhance significantly the ability of the hexameric helicase DnaB (from *B.stearothermophilus)* to displace short oligonucleotides, with either 3′, 5′, 3′+5′ tails or untailed, annealed onto M13mp18 ssDNA (Fig. 2). Therefore, it seems that RepD shows specificity for PcrA DNA helicase.

## RepD increases the processivity of PcrA

Although PcrA can displace short oligonucleotides annealed onto M13mp18 ssDNA, it is not processive and fails to displace a ‘long’ untailed oligonucleotide (81mer) annealed onto M13mp18 ssDNA even at high concentrations where all the DNA binding sites on the ssDNA have been saturated with PcrA molecules. Figure 3a shows that increasing the concentration of PcrA in the reaction mixture from 0.5 to 5 μM does not result in effective displacement of the annealed 81mer. Since all the reactions were done at 1 nM DNA substrate concentration, 0.5, 1.5 and 5 μM PcrA concentrations are equivalent to 14.5, 4.8 and 1.45 nt of M13mp18 ssDNA per PcrA molecule, respectively. Therefore, PcrA is not a processive DNA helicase *in vitro*. However, in the presence of RepD it becomes processive and readily displaces the annealed 81mer oligonucleotide (Fig. 3c).

The stimulatory effect of RepD on PcrA helicase differs from that of the ribosomal protein L3. The *E.coli* ribosomal protein L3 was shown previously to stimulate the ability of PcrA to displace short oligonucleotides annealed onto M13mp18 ssDNA, by promoting co-operative binding of PcrA to its DNA substrate (20). L3 failed to affect the ability of PcrA to displace the annealed untailed 81mer oligonucleotide. Even in the presence of L3, PcrA failed to show any significant helicase activity on this DNA substrate (Fig. 3b), suggesting that L3 does not affect the processivity of PcrA helicase. RepD is a more potent stimulator of helicase activity than L3 and enables PcrA to displace long oligonucleotides annealed onto M13mp18 ssDNA (Figs 3c and 4).

The RepD stimulatory effect on PcrA helicase can also be seen at very low PcrA concentrations. Even at molar ratios of PcrA versus DNA of ≤1, RepD can still stimulate the PcrA and helicase activity is clearly detectable (Fig. 4). At 1 nM PcrA concentration, which corresponds to a 1:1 PcrA:DNA molar ratio, RepD stimulates the helicase activity of PcrA and ~60% of the annealed oligonucleotide is displaced from the 3′+5′ tailed DNA substrate in 30 min (Fig. 4). This is particularly impressive since a 1:1 (PcrA:DNA) molar ratio corresponds to 7250 nt of M13mp18 ssDNA per PcrA molecule. Even when the concentration of PcrA was reduced to 133 pM (equivalent to 1 molecule of PcrA per 7.5 molecules of DNA substrate), helicase activity was still clearly

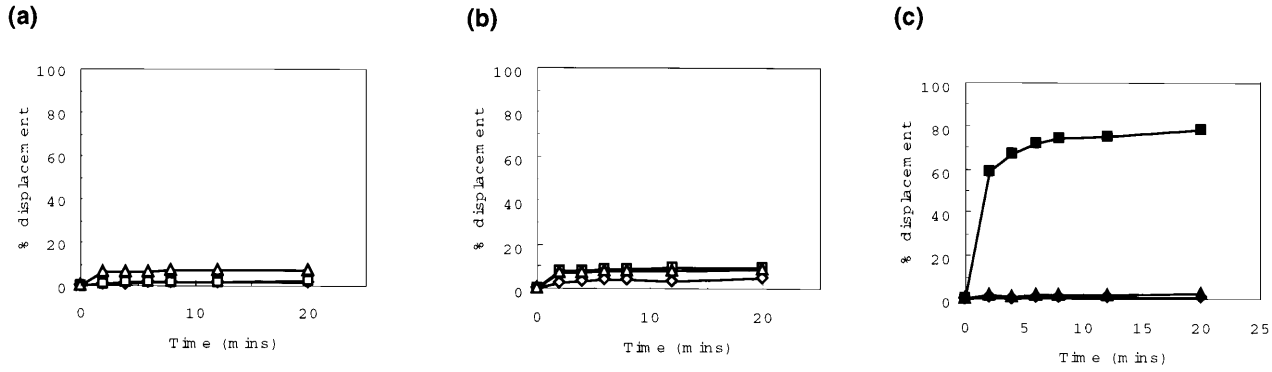


**Figure 3.** (**a**) Time course helicase reactions in which PcrA helicase fails to displace a long oligonucleotide (untailed 81mer) annealed onto M13mp18 ssDNA. Reactions were carried out at increasing PcrA concentrations of 0.5, 1.5 and 5 μM corresponding on the graph to diamonds (◇), squares (□) and triangles (Δ), respectively. The concentration of the DNA substrate was 1 nM and all other experimental parameters are as described in Materials and Methods. (**b**) Similar time course helicase reactions carried out in the presence of 750 nM L3. (**c**) The effect of RepD on the ability of PcrA helicase to displace the long untailed oligonucleotide annealed onto M13mp18 ssDNA. Reactions were carried out in the presence of RepD (600 nM dimer) alone (▲), PcrA (50 nM) alone (◇) or RepD (600 nM dimer) plus PcrA (50 nM) together (■). The graphs representing reactions carried out in the presence of RepD alone and PcrA alone are superimposed on each other and are not easy to separate on this graph.

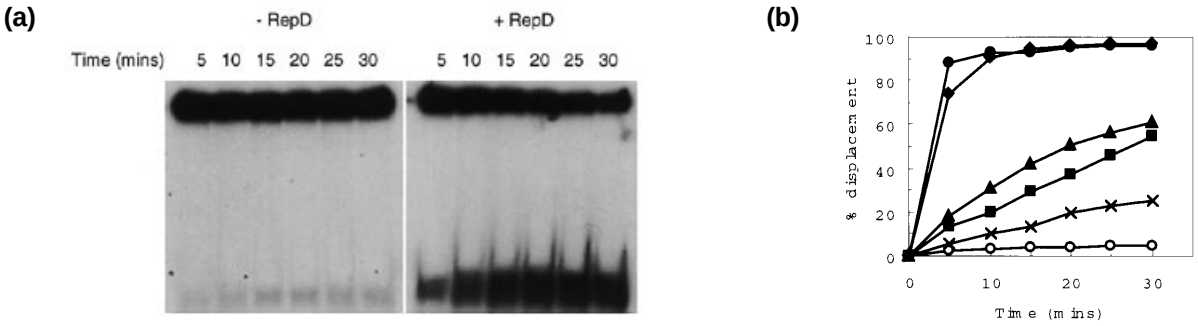


**Figure 4.** Time course helicase reactions, in the presence or absence of RepD, using 1:1 molar ratio of PcrA versus 3′+5′ tailed DNA substrate are shown in (**a**). The reactions were performed at 600 nM RepD dimer, 1 nM PcrA and DNA concentrations. Quantitative analysis of helicase assays performed at 600 nM RepD dimer, 1 nM 3′+5′ DNA substrate and varying the concentration of PcrA is shown in (**b**). Helicase reactions were carried out at 133 pM (×) and 1 (■), 2 (▲), 4 (◆) and 8 nM (●) PcrA. Open circles (○) represent reactions carried out at 10 nM PcrA in the absence of RepD.

detectable. After 30 min ~25% of the annealed oligonucleotide was displaced (Fig. 4, symbol ×). Compare this with the failure of PcrA to demonstrate any significant helicase activity in the absence of RepD, even when a molar ratio of 10:1 PcrA versus DNA substrate was used in the assay mixture (Fig. 4, symbol ○).

## Stoichiometry of PcrA–RepD interaction

Helicase reactions were carried out at constant PcrA concentration (50 nM) and varying the concentration of RepD (Fig. 5). Conditions were chosen such that the maximal rate was not limited by substrate availability. Using both the 3′+5′ tailed (data not shown) and the 'long' untailed (Fig. 5) substrates, we observed maximal stimulation at 100 nM RepD monomer. This suggests that under our experimental conditions, the optimal PcrA:RepD molar ratio for stimulation of PcrA is 1:2, implying that one RepD dimer interacts with one PcrA monomer.

## RepD does not affect the ATPase activity of PcrA

RepD is not an ATPase and thus has no detectable ATPase activity *in vitro* (data not shown). Furthermore, it does not affect the ATPase activity of PcrA *in vitro*. In the presence of a 4-fold molar excess of RepD over PcrA the $k_{cat}$ and $K_m$ values for ATP hydrolysis are not affected (Fig. 6a). The concentrations of PcrA and RepD used in our ATPase assays were comparable with those used in the helicase assays. While in the helicase assays there was a clear stimulatory effect on helicase activity, in the ATPase assays there was no detectable effect. Furthermore, we examined the effect of RepD on binding of PcrA to the DNA cofactor. RepD did not affect the apparent $K_m$ value for poly(dT)$_{16}$ (Fig. 6b).

## PcrA interacts with RepD at the replication origin

Since it has been proposed that in both *S.aureus* (15–17) and *B.subtilis* (18), PcrA helicase is involved in the replication of pT181 and related plasmids, we initially tested the effect of *B.stearothermophilus* PcrA on the nicking–closing (topoisomerase) activity of wild-type RepD *in vitro*. Such activity (Fig. 7) is thought to represent the events of initiation and termination of rolling circle replication *in vivo* (10). However, no change was observed in the reaction parameters for nicking or religation in the presence of PcrA (data not shown).

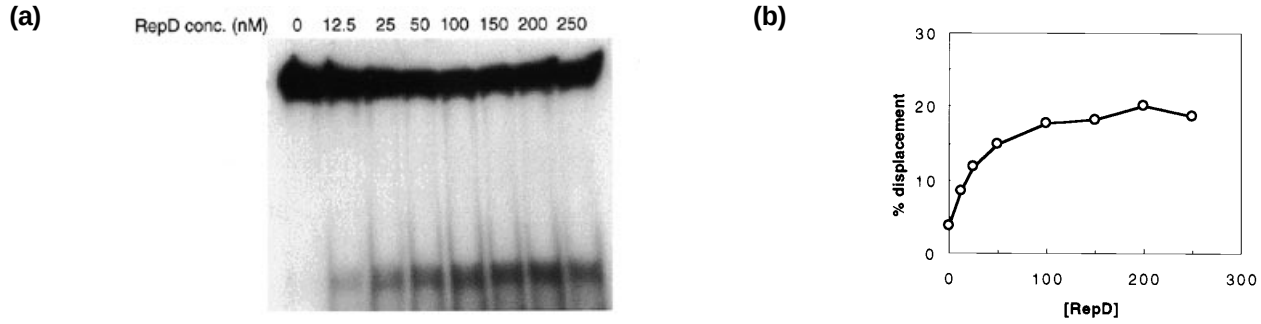


**Figure 5.** (**a**) A gel with PcrA helicase assays carried out at 50 nM PcrA concentration in the presence of increasing concentrations of RepD. The 'long' untailed DNA substrate (1 nM) was used and all reactions were terminated after 90 s. (**b**) The quantitative analysis is shown where the percentage displacement of the annealed oligonucleotide has been plotted against the concentration of RepD in the reaction mixture. All concentrations of RepD in this figure refer to the RepD monomer.

In case this observation was due to the transient nature of the covalent intermediate, further studies were conducted using a RepD mutant (R189A). R189A retains the ability to nick and covalently attach to the DNA but is unable to religate the nick, hence representing a stable form of the intermediate. The nicked complex formed between R189A and plasmid pCER*oriD* was isolated, then digested with restriction enzymes *Hin*fI and *Eco*RI to release the origin of replication as a 275 bp fragment with RepD covalently attached. This fragment was then tested as a helicase substrate (Figs 8 and 9).

The presence of RepD mutant R189A covalently attached to the origin fragment results in retardation of this band on electrophoresis (Fig. 8a). No further shift, indicative of a stable multiprotein complex, was witnessed on the addition of PcrA; in fact the addition of PcrA and ATP together resulted in the apparent restoration of the shifted band to its normal position. In a separate experiment, no effect was noted when PcrA was replaced with DnaB (data not shown).

The interpretation of these results is given in Figure 9a and b. Rather than causing hydrolysis of the RepD–DNA phosphodiester linkage, ATP-dependent PcrA activity more probably serves as a helicase to separate the final 48 bp between the nick site and the proximal end of the DNA fragment. This releases RepD covalently attached to a 48mer single-stranded oligonucleotide and leaves a partially duplex DNA fragment, with a single-stranded extension, migrating near the position of the original 275 bp fragment.

This interpretation is confirmed by subsequent treatment with exonuclease VII. Exonuclease VII digests strictly ssDNA substrates. Upon such digestion, the semi-duplex product of the helicase reaction is indeed shortened to the expected size (Figs 8b and 9c).

These effects were shown to be associated with the presence of the covalently attached RepD protein and not solely due to the presence of a nick in the substrate. Pronase digestion effectively removes the attached RepD from the DNA, restoring this nicked molecule to its normal position on gel electrophoresis (Fig. 8c and 9d). However, the nicked DNA alone is not a substrate for PcrA activity, as evidenced by subsequent exonuclease VII treatment (Fig. 8d, compare tracks 16 and 18). We therefore conclude that PcrA does interact with the R189A mutant RepD protein.

## DISCUSSION

RepD strongly stimulates the *in vitro* helicase activity of PcrA. It enhances the ability of PcrA to displace short oligonucleotides annealed onto M13mp18 ssDNA and forming a variety of helicase substrates with 3′, 5′, 3′+5′ tails or no tails at all (Fig. 1). This stimulatory effect seems to be specific for PcrA, since RepD failed to stimulate the *in vitro* helicase activity of the *B.stearothermophilus* DnaB helicase on the same DNA substrates (Fig. 2).

Although PcrA can displace short oligonucleotides annealed onto M13mp18 ssDNA quite effectively *in vitro*, it cannot do so with longer oligonucleotides. We used an 81mer synthetic oligonucleotide to anneal onto M13mp18 ssDNA and produced a 'long' untailed DNA substrate. When we used this substrate in our helicase reactions we could not demonstrate any helicase activity with PcrA (Fig. 3a). Even when the concentration of PcrA was increased to be equivalent to one molecule of PcrA for every 1.5 bases of M13mp18 ssDNA, still we could not detect any significant helicase activity (Fig. 3a). The *E.coli* ribosomal protein L3, which has been previously shown to enhance the ability of PcrA to displace short oligonucleotides annealed onto M13mp18 ssDNA, by promoting cooperative binding of PcrA to its DNA substrate (20), failed to stimulate the helicase activity on the 'long' untailed DNA substrate (Fig. 3b). In contrast, in the presence of RepD, PcrA helicase activity was stimulated and the long 81mer was readily displaced from the M13mp18 ssDNA (Fig. 3c). It is therefore clear that the stimulatory effect of RepD is very different to that of the ribosomal protein L3. Instead of promoting cooperative binding of PcrA to its DNA substrate, RepD seems to increase the processivity of the enzyme. It could achieve this effect in either of two ways, by enabling the enzyme to stay longer on the DNA or by increasing its translocation speed along the DNA while it is carrying out its strand separation reaction. RepD is a site-specific DNA binding protein which binds to *oriD* with an apparent dissociation constant of ~1 nM (10). By comparison, the affinity for non-specific DNA sequences is 1700 times lower. It is therefore unlikely that the stimulatory effect on PcrA helicase is DNA substrate dependent. The simplest explanation is that RepD interacts directly with PcrA via a protein–protein interaction.

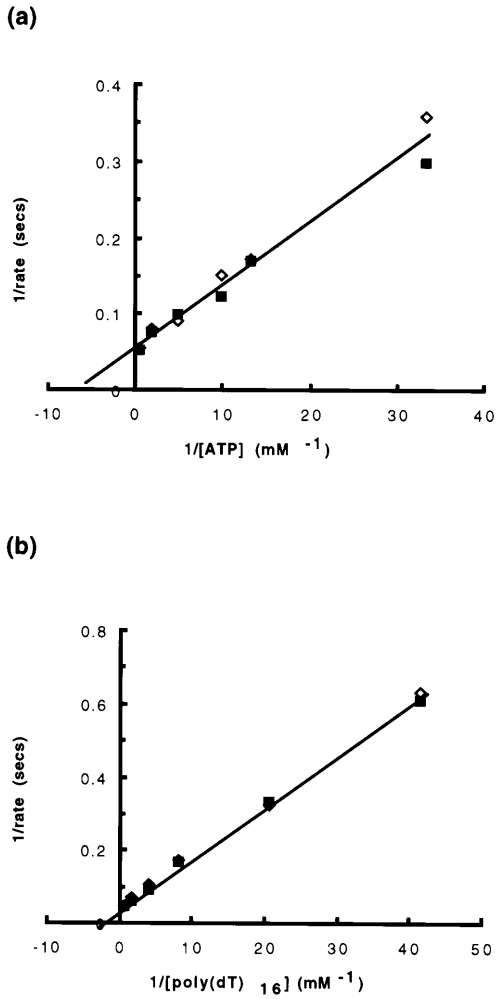


**Figure 6.** (**a**) A double reciprocal Lineweaver–Burk plot for the ATPase activity of PcrA in the presence (◆) or absence (■) of RepD. ATPase reactions were also done at saturating ATP concentration (2 mM), varying the concentration of the DNA cofactor, in the presence (◇) or absence (■) of RepD (**b**). Experimental details are described in Materials and Methods.

By keeping the PcrA concentration constant at 50 nM and varying the concentration of RepD, we were able to show that maximal stimulation was achieved with a molar ratio of 2:1 RepD (monomer) versus PcrA (Fig. 5). This suggests that one molecule of PcrA interacts with two molecules of RepD which is compatible with the oligomerization state of both proteins in solution. Whereas PcrA exists in solution as a monomer (21), RepD exists in solution as a dimer (9).

We were able to detect indirectly the PcrA–RepD interaction by using the RepD mutant protein R189A to mimic an initiation complex normally formed transiently during *in vivo* rolling circle plasmid replication (Figs 7–9). The presence of the covalently attached R189A mutant protein on the 5′-end of the nick was essential for PcrA helicase activity whereas the removal of the covalently linked R189A mutant protein from the nicked DNA substrate by proteolysis resulted in the abolition of PcrA helicase activity. This is indirect evidence for a RepD:PcrA protein–protein interaction which results in the stimulation of the PcrA helicase activity. In similar studies of the CisA:Rep helicase interactions during ϕX174 replication, a CisA:DNA complex was used as a substrate for the helicase reaction (22).

Additional evidence for a RepD:PcrA protein–protein interaction comes from helicase reactions carried out with a molar ratio ≤1 of PcrA versus DNA substrate (Fig. 4). One molecule of PcrA for every molecule of DNA substrate in the reaction mixture corresponds to one molecule of PcrA per 7250 bases of M13mp18 ssDNA. In this case a molecule of PcrA binds to M13mp18 ssDNA and then migrates to the annealed oligonucleotide in order to displace it. In the absence of RepD it is not able to do so (Fig. 4). Presumably under these conditions, PcrA undergoes repeated non-productive cycles of binding, short translocation and dissociation from the DNA. However, in the presence of RepD the annealed oligo is displaced implying that PcrA can now translocate greater distances before dissociating from the DNA, thus targeting PcrA to the annealed oligo. However, the role of RepD cannot be simply to target PcrA to the annealed oligo. Even at high concentrations of PcrA, when all the binding sites on the ssM13 DNA are saturated, the enzyme fails to displace the 81mer in the absence of RepD (Fig. 3a and b). Therefore, it is clear that RepD makes an instant contribution to the displacement activity of PcrA on long substrates.

Furthermore, a direct comparison of the helicase reactions shown in Figure 1a for the untailed DNA substrate (22mer annealed onto M13mp18 ssDNA) and Figure 3c for the ‘long’ untailed DNA substrate (81mer annealed onto M13mp18 ssDNA) reveal that the reduction in the rate with the long DNA substrate is not directly proportional to the increase in the size of the double-stranded region. If the stimulation was due entirely to a stimulation of helicase activity *per se*, then the reduction in the rate of the helicase reaction should have been ~4-fold. However, PcrA fails to demonstrate any significant helicase activity on the longer DNA substrate (Fig. 3a and b). Since processivity relates entirely to the length of the DNA displaced, we can conclude that in the absence of RepD, PcrA is a non-processive helicase whereas in the presence of RepD it becomes a much more processive enzyme.

RepD has no effect on the ATPase activity of PcrA *in vitro*. In the presence of a 4-fold molar excess of RepD over PcrA the $k_{cat}$ and $K_m$ values for ATP hydrolysis remain unchanged (Fig. 6a). Furthermore, at saturating ATP concentration (2 mM), varying the concentration of the DNA cofactor, in the presence or absence of RepD, did not affect the apparent $K_m$ of PcrA for DNA (Fig. 6b).

During rolling circle plasmid (pT181) replication, RepC (homologous to RepD) is inactivated by the addition of an oligonucleotide, giving rise to a new form of the protein designated RepC* (23), which has reduced binding affinity for and can no longer melt DNA at the double-strand origin (24). This addition involves linkage of an oligonucleotide via a covalent phosphotyrosine bond to the active site tyrosine on a single monomer of the RepC dimer. We examined the effect of a comparable RepD/D* heterodimer on the helicase activity of PcrA. The RepD/D* heterodimer is likewise similar to the RepD homodimer with an 11 base single-stranded oligonucleotide covalently attached via its 5′-end to the active site Tyr191 of one of the subunits of the homodimer. The RepD/D* heterodimer stimulates the PcrA helicase in an identical manner to the RepD homodimer (data not shown), suggesting that although addition of the oligonucleotide affects the activity of RepD (25), it does not affect its interaction with PcrA.

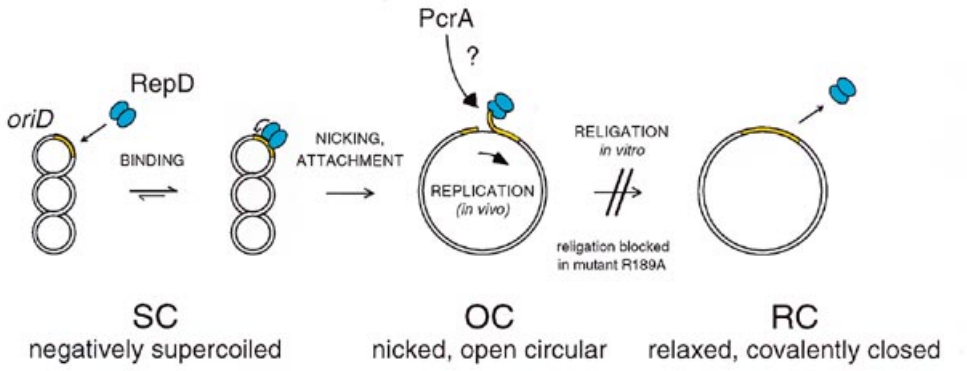


**Figure 7.** Nicking–closing activity of RepD with negatively supercoiled substrate. RepD binds at the replication origin, *oriD*, nicks the DNA and becomes covalently attached via a 5′-phosphotyrosyl ester linkage. This is observed as a transition from negatively supercoiled to nicked, open circular substrates *in vitro*. Replication may then follow the addition of a helicase to the complex *in vivo*; religation of the nick by RepD results in a relaxed, covalently closed product *in vitro*. RepD mutant R189A is incapable of religation and remains covalently attached to the nicked, open circular DNA.

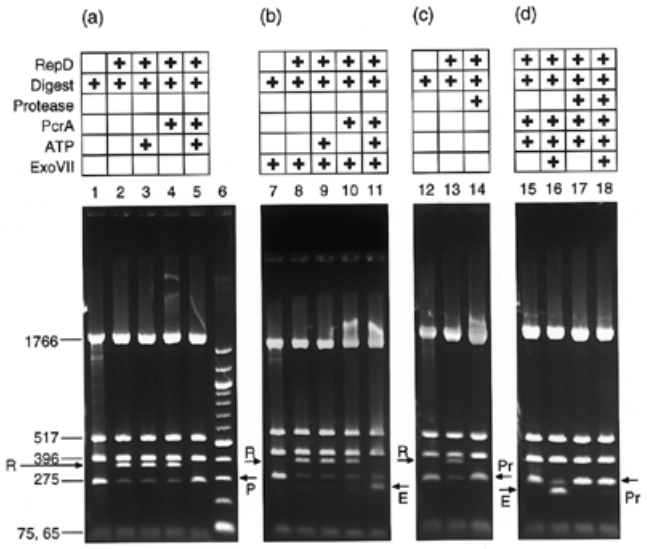


**Figure 8.** Helicase activity in the presence and absence of RepD mutant protein R189A on a nicked helicase substrate. (**a**) Helicase activity in the presence of RepD mutant protein R189A attached to the helicase substrate. Lane 1, whole pCER*oriD* plasmid digested in the absence of RepD mutant R189A; lane 2, nicked plasmid digest with RepD mutant R189A covalently attached; lane 3, as in lane 2 plus ATP; lane 4, as in lane 2 plus PcrA; lane 5, as in lane 2 plus ATP and PcrA; lane 6, DNA size markers, 100 bp intervals. DNA fragment sizes (in bp) resulting from *Hin*fI/*Eco*RI digestion are shown on the left. R indicates the position of the retarded covalent complex between RepD R189A and the 275 bp *oriD* fragment. P indicates the position of the product following treatment by the helicase (lane 5). (**b**) Exonuclease VII treatment following the helicase reaction. Lanes 7–11, as lanes 1–5 in (a), with the addition of exonuclease VII following the helicase reaction. E indicates the position of the exonuclease VII digestion product. (**c**) Removal of RepD mutant protein R189A from the helicase substrate by proteolysis. Lanes 12 and 13, as lanes 1 and 2 in (a); lane 14, as lane 13 followed by protease treatment. Pr indicates the position of the protease-treated DNA fragment. (**d**) Testing helicase activity following the removal of RepD mutant protein R189A from the helicase substrate by proteolysis. Lane 15, helicase reaction in the presence of RepD mutant R189A covalently attached to the helicase substrate; lane 16, as lane 15 followed by exonuclease VII treatment; lane 17, helicase reaction of nicked plasmid after removal of RepD mutant protein R189A due to protease treatment; lane 18, as lane 17 followed by exonuclease VII treatment.

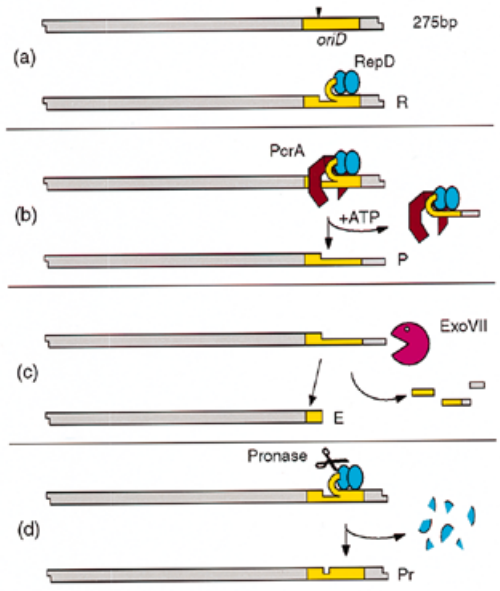


**Figure 9.** Schematic representation of the major DNA species and reactions of Figure 8. (**a**) The 275 bp *Hin*fI–*Eco*RI fragment containing *oriD* released from restriction digestion of pCER*oriD* in the absence of RepD R189A and the complex (R) following digestion of the plasmid with RepD R189A covalently attached. (**b**) Complex (R) serves as a helicase substrate for PcrA in the presence of ATP, releasing a RepD/D*-like molecule and semi-duplex product (P). (**c**) Treatment of the helicase product (P) with exonuclease VII (Exo VII) results in the shortened, double-stranded exonuclease product (E). (**d**) Removal of RepD mutant protein R189A from the helicase substrate by proteolysis results in a nicked DNA fragment with no protein attached (Pr).

Both (natural) RepC* and (synthetic) RepD/D* are also formally analogous to the covalent complex between RepD and the displaced (+) strand present during replication of pT181 family plasmids, in that all possess a covalent protein–DNA linkage. Thus we would expect this modified protein, as for the RepD/D* moiety tested above, to remain associated with PcrA

helicase at the replication fork during replication as observed for the CisA:Rep complex during ϕX174 replication (22). This interaction would both serve to maintain the elevated processivity of PcrA sufficient for replication to occur and would also serve as a mechanism whereby RepD could 'track' the displaced (+) strand until a suitable termination site is encountered, explaining how termination *in cis* could occur when tandem copies of a replication origin are present within the same plasmid (26,27).

In terms of mechanism, the requirement for RepD in the initiation complex in order for unwinding to take place could reflect an increase in the affinity of PcrA for the initiation complex and/or an enhancement of its processivity. Furthermore, the work presented above does not unambiguously differentiate between a direct PcrA:RepD interaction and a RepD:DNA:PcrA interaction, as in the latter case RepD could change the DNA conformation upon binding, making the initiation complex a more favourable substrate for PcrA. However, genetic studies do support the model for a direct protein–protein interaction between PcrA and RepD (18). The fact that a *B.stearothermophilus* helicase (PcrA) interacts with a protein encoded by a staphylococcal plasmid (RepD) is not surprising considering that the *B.stearothermophilus* and staphylococcal PcrA proteins are very closely related sharing 62% sequence identity (19,28).

Despite the genetic and biochemical evidence for the RepD–PcrA interaction, structural information in the form of a high resolution crystal structure of a RepD–PcrA complex will be essential to identify the protein–protein interface and the mechanism by which PcrA is activated by RepD.

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