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The RdgC protein of *Escherichia coli* binds DNA and counters a toxic effect of RecFOR in strains lacking the replication restart protein PriA

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PriA protein provides a means to load the DnaB replicative helicase at DNA replication fork and D loop structures, and is therefore a key factor in the rescue of stalled or broken forks and subsequent replication restart. We show that the nucleoid-associated RdgC protein binds non-specifically to single-stranded (ss) DNA and double-stranded DNA. It is also essential for growth of a strain lacking PriA, indicating that it might affect replication fork progression or fork rescue. *dnaC* suppressors of *priA* overcome this inviability, especially when RecF, RecO or RecR is inactivated, indicating that RdgC avoids or counters a toxic effect of these proteins. Mutations modifying ssDNA-binding (SSB) protein also negate this toxic effect, suggesting that the toxicity reflects inappropriate loading of RecA on SSB-coated ssDNA, leading to excessive or untimely RecA activity. We suggest that binding of RdgC to DNA limits RecA loading, avoiding problems at replication forks that would otherwise require PriA to promote replication restart. Mutations in RNA polymerase also reduce the toxic effect of RecFOR, providing a further link between DNA replication, transcription and repair.

Keywords: DNA repair/RecFOR/recombination/RNA polymerase/SSB

Introduction

The *rdgC* gene of *Escherichia coli* encodes a 34 kDa protein associated with the nucleoid (Ryder *et al*., 1996; Murphy *et al*., 1999). Its deletion has no obvious effect except in a nuclease-deficient *recBC sbcBC* mutant background where it confers a recombination dependent growth phenotype (hence *rdg*). Thus, a Δ*rdgC recBC sbcBC* strain is only viable provided the RecA and RecF proteins necessary for recombination in this background are functional (Ryder *et al*., 1996). Given recombination underpins genome replication (Cox *et al*., 2000), this raises the possibility that RdgC may normally aid replication fork progression. Replication forks assembled at *oriC* are inherently processive, but the current view is that their progress is often blocked by lesions in or on the template DNA, with estimates ranging from 10 to 50% of cells requiring some form of fork reactivation (Cox *et al*., 2000; McGlynn and Lloyd, 2002b). The problem becomes acute in cells exposed to a DNA-damaging agent such as UV light and is complicated by the stalling of transcription complexes at non-pairing lesions in the template strand, creating further obstacles to fork progression (Hanawalt *et al*., 1994; McGlynn and Lloyd, 2000; van den Boom *et al*., 2002).

Recent studies in *E.coli* have suggested that stalled forks might unwind so that the parental strands reanneal and the nascent daughter strands anneal to form a Holliday junction (Seigneur *et al*., 1998). This may occur spontaneously via release of positive supercoiling ahead of the fork, but is more likely catalysed either by RecG helicase or via the strand exchange activity of RecA, aided perhaps by the RecF, RecO and RecR proteins (Robu *et al*., 2001; Singleton *et al*., 2001; McGlynn and Lloyd, 2002a,b). Once formed, the Holliday junction may be driven further from the lesion by the RuvAB branch migration complex (Seigneur *et al*., 1998). Such reactions would require the replisome complex to have dissociated, which raises the question of how DNA synthesis might subsequently resume. Furthermore, the offending lesion has to be repaired or bypassed for this renewed synthesis to continue without mishap. Fork reversal and Holliday junction formation provide possible solutions to these problems. Backing away from the block may create room for repair, although the timing of repair is still unknown and may depend on how replication is resumed. Creating a Holliday junction provides a substrate that recombination enzymes can exploit to promote restart. At least two general models have been proposed. Both rely on the primosome assembly factor PriA to load the DnaB replicative helicase (Liu and Marians, 1999; Liu *et al*., 1999; Marians, 2000; Sandler and Marians, 2000), but employ two different DNA structures for this purpose. The first relies on direct restoration of a (corrected) fork structure, the second on recombination to first form a D loop, which is then converted to a fork (Seigneur *et al*., 1998; Gregg *et al*., 2002; McGlynn and Lloyd, 2002a,b). The latter may sometimes involve fork breakage, possibly via RuvABC-mediated cleavage of a Holliday junction formed during fork reversal. However, Courcelle and Hanawalt have proposed alternative models for direct fork restoration that do not involve formation of a Holliday junction (Courcelle and Hanawalt, 1999, 2001).

Both direct and indirect models of fork rescue emphasize the critical role of PriA. This is consistent with the fact that inactivation of PriA results in reduced cell viability, defective cell division, sensitivity to DNA damage, chronic SOS induction and recombination deficiency (Nurse *et al*., 1991; Kogoma *et al*., 1996). PriA initiates assembly of a primosome at replication fork and

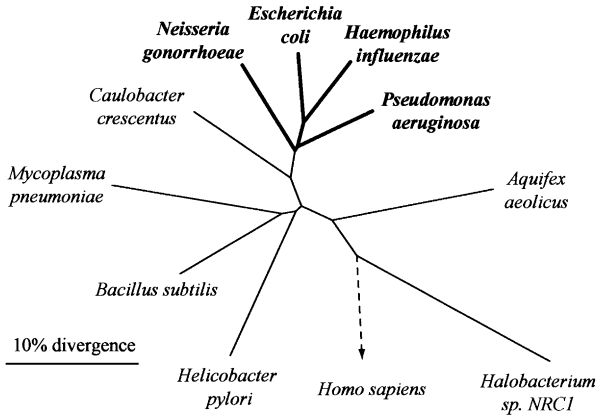

Fig. 1. Evolutionary distribution of *rdgC*. The tree was generated by alignment of 23S rRNA sequences using Clustal_X. An *rdgC*-like gene is present in the lineage and species highlighted in bold.

D loop structures via a series of defined protein–protein interactions involving PriB, DnaT and possibly PriC, culminating in transfer of DnaB from a DnaB–DnaC complex to the PriA–DNA complex and subsequent binding of DnaG primase (McGlynn *et al*., 1997; Liu and Marians, 1999; Liu *et al*., 1999). DNA polymerase III holoenzyme can then be loaded to form a fully functional replisome capable of coupled leading and lagging strand synthesis.

The interaction of PriA with the DnaB–DnaC complex is normally crucial for DnaB loading at replication fork and at D loop structures. However, certain amino acid substitutions in DnaC can circumvent this requirement, enabling DnaB to be loaded without PriA and suppressing the phenotype of *priA* null strains (Sandler *et al*., 1996; Xu and Marians, 2000; Gregg *et al*., 2002). Such *dnaC* suppressor mutations accumulate rapidly in cultures of *priA* null strains because of the very substantial improvement in cell viability (Sandler *et al*., 1996; Gregg *et al*., 2002).

In this paper, we show that RdgC is a DNA-binding protein that forms stable complexes with both single-stranded (ss) DNA and double-stranded (ds) DNA, and that it is required for growth of *priA* null strains and for the ability of *dnaC* suppressor mutations to improve their viability. We also show that the RecF, RecO and RecR proteins are responsible for the low viability of *priA dnaC rdgC* mutant strains and that this toxic effect can be eliminated by changes to the ssDNA-binding protein, SSB. The results presented provide new insight into the interactions between DNA replication, recombination and repair, and raise the possibility that nucleoid organization is important in maintaining replication fork progression in rapidly dividing cells.

Results

Distribution of rdgC genes in bacteria

BLAST searches identified *rdgC* genes in the Beta (e.g. *Neisseria sp.*) and Gamma (e.g. *E.coli*) subdivisions of the Proteobacteria, but in no other species (Figure 1). Thus, *rdgC* appears to have arisen in the lineage leading to the Beta and Gamma Proteobacteria. It is present in all fourteen species of these groups for which a complete genome sequence is available. The encoded protein sequences show a high degree of conservation (alignments not shown), but provide no insight as to their function.

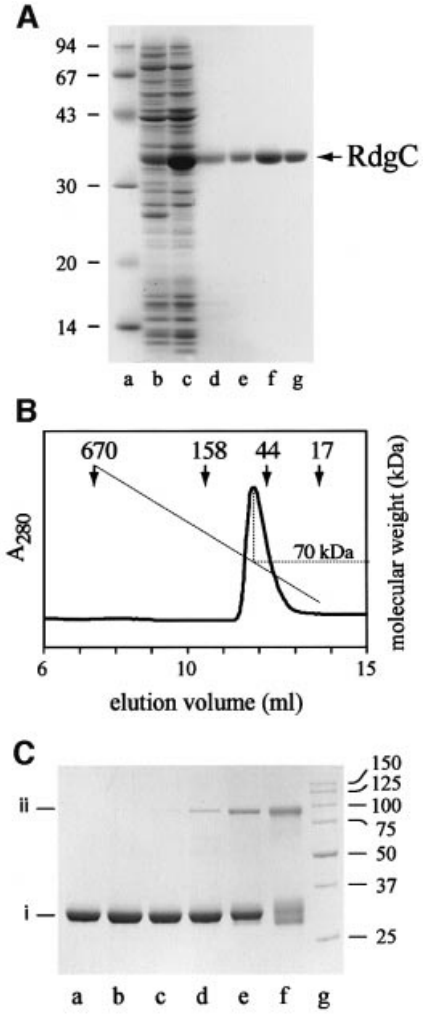

Fig. 2. Purification and physical properties of RdgC. (**A**) SDS–PAGE gel summarizing the recovery of RdgC at different stages of the purification protocol. Lane (a) molecular weight markers, (b) induced cell lysate, (c) 50–70% ammonium sulfate cut, (d–g) peak fractions from heparin, gel filtration, butyl–Sepharose and Q Sepharose columns, respectively. (**B**) Elution profile of RdgC during gel filtration. The elution volumes of molecular weight standards are indicated by arrowheads. (**C**) Glutaraldehyde cross-linking of RdgC. Reactions contained 10 μM RdgC and glutaraldehyde at 0 (a), 2 (b), 8 (c), 32 (d), 128 (e) and 512 μM (f), and were analysed by SDS–PAGE. Molecular markers are in (g). Monomeric (i) and cross-linked (ii) RdgC species are identified.

Purification and physical properties of RdgC

The 34 kDa *E.coli* wild-type RdgC protein was expressed from pGS853 and purified to homogeneity as described in Materials and methods (Figure 2A). Gel filtration indicated that the protein might form a dimer in solution (Figure 2B). Glutaraldehyde cross-linking of the purified protein followed by SDS–PAGE analysis revealed a single cross-linked species with an apparent molecular mass of nearly 100 kDa (Figure 2C). This would be consistent with a trimer of RdgC. However, the absence of any intermediate bands resulting from the cross-linking of only two monomers argues against this possibility. We therefore

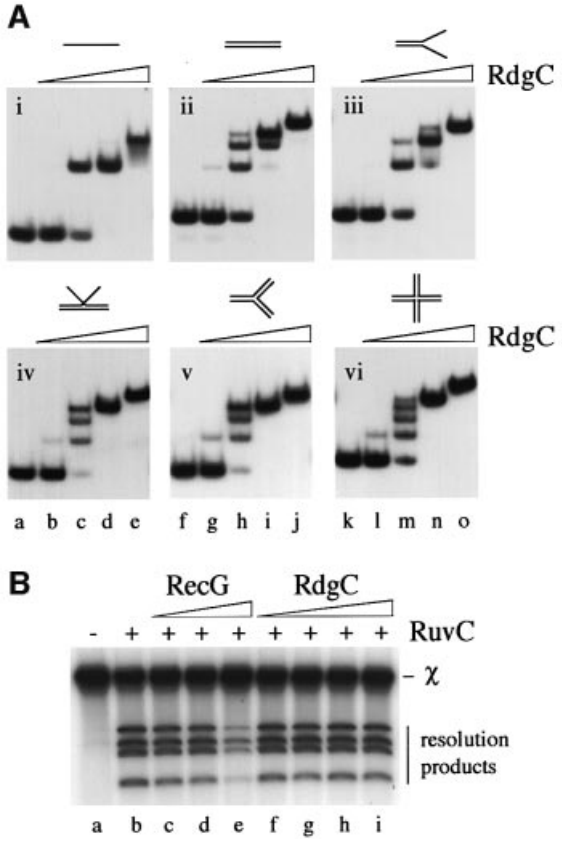

Fig. 3. DNA-binding activity of RdgC. (**A**) Gel assays showing binding of RdgC to linear and branched DNA structures. Binding reactions contained 0.1 nM DNA species, (i) 61 nucleotide ssDNA, (ii) 61 bp dsDNA, (iii) flayed duplex, (iv) three-strand junction, (v) Y-DNA, (vi) Holliday junction J12, and RdgC at 0, 0.5, 5, 50 and 500 nM in lanes a–e, f–j and k–o, respectively. (**B**) Effect of RecG and RdgC on cleavage of χ DNA by RuvC. Reactions contained 0.05 nM χ junction, 5 nM RuvC monomers (lanes b–i), 1, 10 and 100 nM RecG (lanes c–e), and 1, 10, 100 and 1000 nM RdgC dimers (lanes f–i), as indicated. The χ junction is labelled on all four arms and therefore all four possible products of junction resolution are detected (McGlynn and Lloyd, 2000).

conclude that native RdgC is a dimer with a molecular mass of 68 kDa, although we cannot exclude the possibility of a trimeric state.

RdgC binds DNA

Two pieces of evidence suggest RdgC might be a DNA-binding protein. First, it is released from *E.coli* nucleoids by digestion with DNase I (Murphy *et al*., 1999). Secondly, during attempts at purification, RdgC was found to bind heparin and dsDNA cellulose columns (data not shown). Ryder *et al*. (1996) suggested RdgC might be an exonuclease, but no nuclease activity of any kind could be detected with the purified protein using a variety of linear and circular DNA substrates under a range of conditions (data not shown). However, band-shift assays confirmed that RdgC binds DNA. Well-defined complexes were detected with linear ssDNA and dsDNA substrates and with a variety of partial duplex and branched molecules (Figure 3A, panels i–vi). Two complexes are formed with a 61 nucleotide single strand (panel i) and four with a 61 bp linear duplex (panel ii).

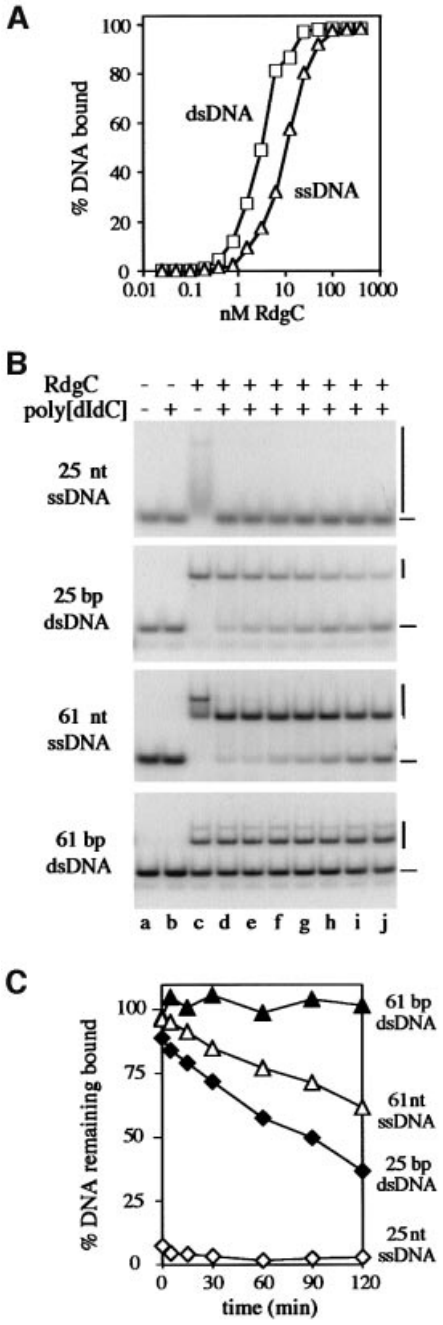

Fig. 4. Affinity of RdgC for ssDNA and dsDNA. (**A**) Binding isotherm showing relative affinity of RdgC for a 61 bp linear duplex DNA and a 61 nucleotide single strand. Binding reactions contained 0.1 nM labelled DNA and RdgC at 0.025–410 nM. Data are means of two experiments. (**B**) Competition binding assays. Reactions contained 0.1 nM of the labelled DNA indicated. RdgC was present at 250 nM (25 nucleotide ssDNA), 25 nM (25 bp dsDNA), 20 nM (61 nucleotide ssDNA) and 2 nM (61 bp dsDNA). The RdgC concentration used in each case was the amount needed to achieve a significant bandshift without formation of a substantial fraction of higher order complexes. After 10 min on ice, 50 ng unlabelled poly[dIdC] competitor DNA was added as indicated and the reactions were kept on ice for a further 0, 5, 10, 30, 60, 90 or 120 min (lanes d–j) before electrophoresis, as described in Materials and methods. Horizontal and vertical lines identify substrate DNA and RdgC–DNA complexes, respectively. (**C**) Relative stability of RdgC complexes with ssDNA and dsDNA. The data from gels of the type shown in (B) were quantified and the complexes detected in the presence of poly[dIdC] expressed as the percentage of the DNA bound in the absence of competitor (B, lane c in each case). Data are averages of two or more experiments.

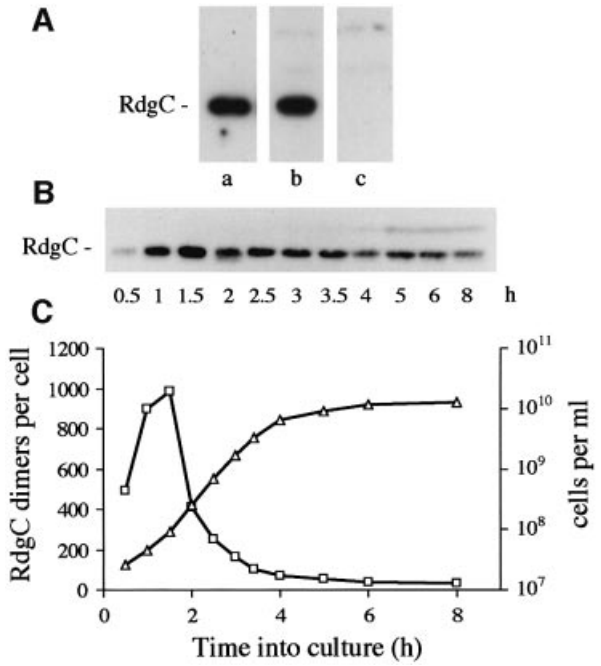

Fig. 5. Immunodetection of RdgC and effect of growth phase on expression. (**A**) Western blots of purified RdgC (6 ng, lane a) and cells extracts from wild-type strain MG1655 (lane b) and Δ*rdgC* strain N4586 (lane c) probed with anti-RdgC polyclonal antibodies. (**B** and **C**) Growth phase expression of RdgC. Strain MG1655 was grown in LB broth for 8 h from an initial A_{650} of 0.02. Samples were taken at intervals and assayed by western blotting for RdgC levels in total cell extracts and by microscopy for total cell numbers. (B) Western blots showing immunodetection of RdgC at the intervals shown. (C) Growth-dependent expression of RdgC. The data from blots of the type shown in (B) were quantified by reference to standard concentrations of pure RdgC. Squares indicate the amount of RdgC (dimers) per cell and triangles the total cell number. Data are averages of two experiments.

Although, we do not know the precise stoichiometry of these complexes, the results are consistent with the binding of two and four dimers of RdgC, respectively. Complexes formed with the branched substrates vary in number with length of ssDNA and dsDNA available (panels iii–vi). With linear duplexes, the number of complexes increased in proportion to DNA length, indicating that RdgC binds at any point along the DNA and not just to DNA ends (data not shown). The five distinct complexes detected with the Holliday junction substrate, J12, supports this conclusion. A distributive mode of binding is also supported by the fact that RdgC binds circular plasmid DNA and uniformly protects linear duplex DNA from attack by hydroxyl radicals (data not shown).

The binding data suggested that RdgC does not bind with higher affinity to branch points in DNA as opposed to linear duplex DNA (Figure 3A; data not shown). To confirm this finding, we investigated whether RdgC could interfere with the cleavage by RuvC resolvase of a Holliday junction structure in which the branch point is flanked by long duplex arms (Figure 3B, lane b). RecG protein, which binds with a high affinity to the branch point, clearly interferes with junction resolution (Figure 3B, lanes c–e). RdgC does not, even when in 400-fold molar excess over RuvC (Figure 3B, lanes f–i), indicating that it does not bind the branch point specifically.

RdgC has a higher apparent binding affinity for dsDNA than for ssDNA (Figure 4A). This is particularly noticeable with short substrates. RdgC binds a 25 nucleotide single strand (Figure 4B, lane c), but the complex is unstable and dissociates on electrophoresis. It has a much higher affinity for a 25 bp linear duplex, forming a single, sharply-defined complex (Figure 4B, lane c). Competition binding studies confirmed that RdgC forms an unstable complex with the 25 nucleotide single strand. Hardly any retarded complex could be detected following the addition of poly[dIdC] (Figure 4B, lanes d–j and C). They also showed that the complexes formed with a 25 bp duplex and a 61 nucleotide single strand were much more stable (Figure 4B, lanes d–j and C). Complexes formed with the 61 bp linear duplex proved very stable, with no detectable dissociation during 2 h on ice.

RdgC is expressed at a high level in dividing cells

Previous studies indicated that RdgC might be a fairly abundant, nucleoid-associated protein (Murphy *et al.*, 1999). Western blots revealed that RdgC can be detected in extracts of wild-type *E.coli* cells (Figure 5A, lane b). The polyclonal antibodies used are highly specific for RdgC as no signal is detected in extracts from a Δ*rdgC* strain (Figure 5A, lane c). This enabled us to accurately measure RdgC in cell extracts at different phases of growth (Figure 5B). RdgC was at its highest level during exponential phase, reaching at its maximum ~1000 dimers per cell. Its level decreased sharply to ~50 dimers per cell in stationary phase (Figure 5C). This profile suggests RdgC might function during the period of DNA replication.

Inviability of ΔrdgC priA and its suppression by dnaC mutations

The high expression of RdgC during the period of very active DNA synthesis and its ability to bind DNA are consistent with the previous suggestion by Ryder *et al.* (1996) that RdgC may have a role in promoting chromosome replication. To investigate whether RdgC promotes replication fork progression, we tried to introduce the *priA2* null mutation into a Δ*rdgC* strain. P1 phage grown on the *priA2*::Km strain, AG181, was used to transduce strain AB1157 and its Δ*rdgC* derivative, DIM037, selecting resistance to kanamycin. Although a high number of transductants were obtained with AB1157, none were obtained with DIM037, suggesting that a strain lacking both PriA and RdgC is inviable. Attempts to combine *priA2* and Δ*rdgC* in the MG1655 background also failed.

Given that mutations in *dnaC* such as *dnaC212* or *dnaC810* suppress *priA* (Sandler *et al.*, 1996; Gregg *et al.*, 2002), we tried to transduce Δ*rdgC*::Tm from strain N4586 to the *priA2 dnaC212* strain AG181 and the *priA2 dnaC810* strain DIM215, this time selecting resistance to trimethoprim. Transductant colonies were obtained in both cases, although they took several days to appear. However, they could be subcultured, indicating that a *priA dnaC* Δ*rdgC* construct is viable. One such clone, DIM063, was kept for further analysis.

Strain DIM063 grows slowly in LB broth. The cells are highly filamentous and have grossly distorted nucleoids (Figure 6E), and only 10–20% are able to form colonies on

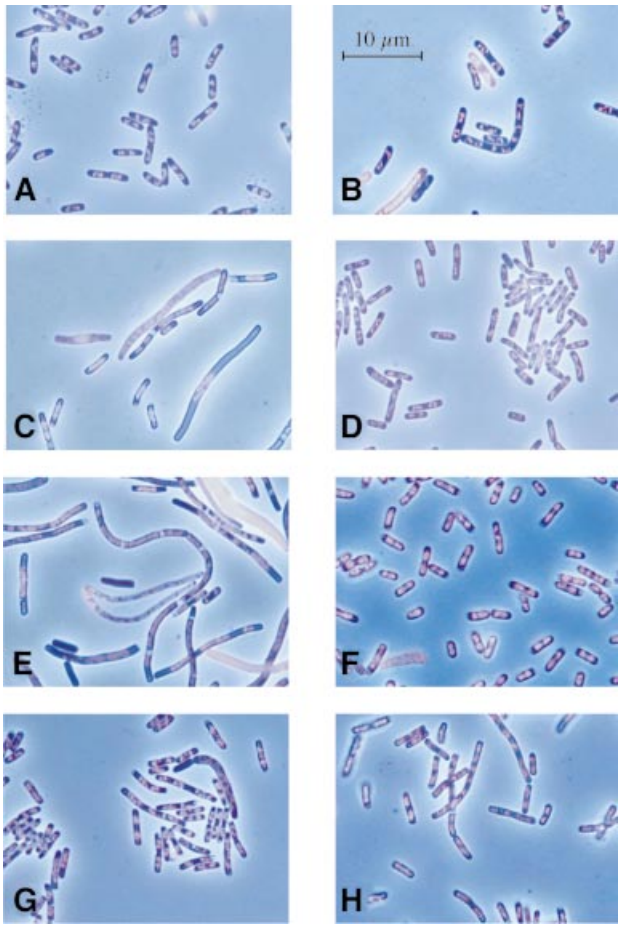

Fig. 6. Filamentous cell morphology of a Δ*rdgC priA2 dnaC212* strain and suppression of filamentation by *fgv* mutations. Phase-contrast and DAPI images are merged to show nucleoid organization within the cell. The strains shown are (**A**) AB1157 (*priA*$^+$ *dnaC*$^+$ *rdgC*$^+$), (**B**) DIM037 (Δ*rdgC*), (**C**) DIM070 (*priA2*), (**D**) AG181 (*priA2 dnaC212*), (**E**) DIM063 (*priA2 dnaC212* Δ*rdgC*), (**F**) DIM061 (*priA2 dnaC212* Δ*rdgC rpoB*), (**G**) DIM060 (*priA2 dnaC212* Δ*rdgC ssb*) and (**H**) DIM064 (*priA2 dnaC212* Δ*rdgC recO*). Details of the *rpoB*, *ssb* and *recO* suppressor mutations are in Table I.

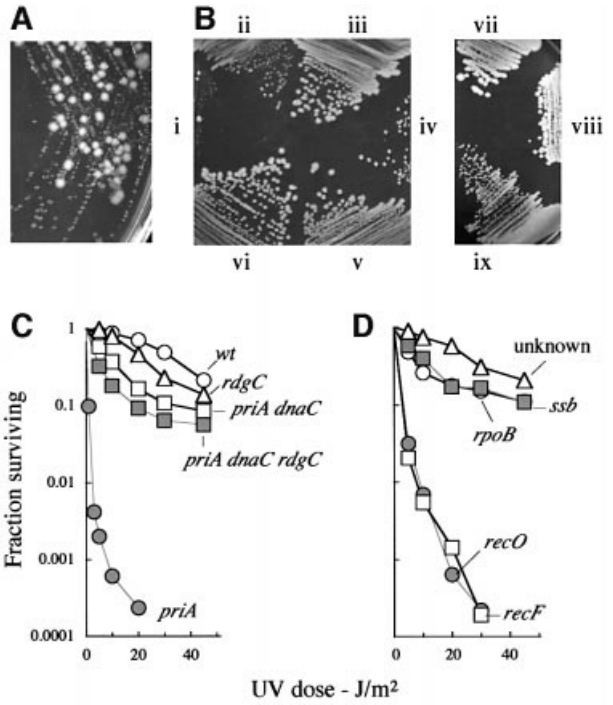

Fig. 7. Suppressors of the slow growth and poor viability of a Δ*rdgC priA dnaC* strain. (**A**) Photograph of an LB agar plate streaked with Δ*rdgC priA dnaC* strain DIM063 and incubated for 60 h at 37°C. Colonies of fast-growing variants are readily visible against the background of small colonies of the parent strain. (**B**) Colony morphology of purified fast-growing variants of DIM063 incubated for 48 h on LB agar. The strains shown (all Δ*rdgC priA2 dnaC212*, except AG181) are (i) DIM063 (Δ*rdgC priA dnaC* parent strain), (ii) DIM057 (unidentified *fgv*), (iii) DIM060 (*ssb*), (iv) DIM061 (*rpoB*), (v) DIM064 (*recO*), (vi) AG181 (*priA dnaC* control strain), (vii) DIM089 (*recF*), (viii) DIM122 (*recF143*) and (ix) DIM123 (*lexA3*). Details of the *ssb*, *rpoB, recF* and *recO* suppressor mutations are in Table I. (**C**) Effect of *priA*, *dnaC* and *rdgC* mutations on sensitivity to UV light. The strains are AB1157 (*wt*), DIM037 (*rdgC*), AG181 (*priA dnaC*), DIM063 (*priA dnaC rdgC*) and DIM070 (*priA*). (**D**) UV sensitivity of derivates of *priA dnaC rdgC* strain DIM063 carrying suppressors of the slow-growth phenotype. The strains are HP126 (*rpoB*), DIM062 (*ssb*), DIM057 (unidentified *fgv*), HP125 (*recO*) and DIM089 (*recF*). Details of the *rpoB*, *ssb*, *recO* and *recF* mutations are in Table I.

LB agar, suggesting they have a major problem with chromosome replication and segregation. Filamentation is more extensive than in a *priA2* single mutant (Figure 6C). By comparison cells of the *priA dnaC212* parent, AG181, and of the Δ*rdgC* strain DIM037 have close to 100% viability. They generally resemble wild-type cells, although Δ*rdgC* cells appear a little more elongated and some form short filaments (Figure 6A, B and D). Thus, elimination of RdgC reverses the ability of *dnaC212* to suppress the low viability of *priA2* cells. Indeed, it exacerbates defects in cell division, which may explain the inability to construct a *priA2* Δ*rdgC* strain. However, it does not restore sensitivity to DNA-damaging agents. Strain DIM063 is hardly more sensitive to UV than the *priA2 dnaC212* parent and is certainly much more resistant than a *priA2* single mutant (Figure 7C). It grows very weakly on LB agar containing mitomycin C (MC) at 0.5 μg/ml, but this can be attributed to slow growth and reduced cell viability (data not shown).

Previous studies revealed that the SOS response is chronically induced in *priA2* strains (Nurse *et al*., 1991), and that this phenotype is alleviated by *dnaC* suppressors (Sandler, 1996). The highly filamentous morphology of DIM063 cells is indicative of chronic SOS induction, consistent with Δ*rdgC* reversing the effect of *dnaC212* in a *priA* null background. To investigate this directly, we made constructs carrying *lacZ* fused to the SOS-inducible *sfiA* gene and tested SOS expression by measuring β-galactosidase activity. In cultures grown in LB broth to an A_{650} of 0.2, we detected 333 ± 24 units of enzyme activity in the *priA2* construct, DIM173, compared with only 39 ± 1 units in the wild type, N5170. Activity was reduced to 83 ± 7 units in a *priA2 dnaC212* construct, DIM175, consistent with the suppression of *priA*, but adding Δ*rdgC* partially reversed this effect, increasing activity in strain DIM177 to 164 ± 8 units. However, it is significant that this level is <50% of the activity in the *priA2* construct. Taken together, these data indicate that chronic SOS induction is a feature of the *priA2 dnaC212* Δ*rdgC* strain DIM063. However, this chronic induction may not be the only factor responsible for the severe growth defects.

Table I. Isolation and characterization of fast-growth variants (*fgv*) of strain DIM063[a]

Strain	Selection	*fgv* allele	Gene mutation	Protein alteration	UV survival[b]
DIM063					0.064
DIM089	LB	*fgv-010*	$recF_{T451A}$	$RecF_{W151R}$	0.000087
DIM064	LB	*fgv-007*	$recO_{\Delta 173-362}$[c]	$RecO_{\Delta 58\text{-end}}$	0.00014
HP125	LB	*fgv-037*	$recO_{\Delta T332}$[d]	$RecO_{\Delta 110\text{-end}}$	0.00022
HP101	LB	*fgv-013*	$recR_{\Delta 59-150}$[e]	$RecR_{\Delta 19\text{-end}}$	0.00014
DIM060	LB + MC	*fgv-004*	ssb_{C289T}	SSB_{R97C}	0.1
DIM062	LB + MC	*fgv-006*	$ssb_{\Delta 345-434}$[f]	$SSB_{\Delta 115-144}$	0.16
DIM061	LB + MC	*fgv-005*	$rpoB_{C1565A}$	$RpoB_{S522Y}$	0.084
DIM104	LB + Rif	*fgv-011*	$rpoB_{C1578A}$	$RpoB_{H526Q}$	0.11
DIM105	LB + Rif	*fgv-012*	$rpoB_{A1547G}$	$RpoB_{D516G}$	0.039
HP118	LB	*fgv-030*	$rpoB_{C604T}$	$RpoB_{R202C}$	0.27
HP126	LB	*fgv-038*	$rpoB_{G2542A}$	$RpoB_{E848K}$	0.15
DIM057	LB + MC	*fgv-001*	Unknown	–	0.31
DIM058	LB + MC	*fgv-002*	Unknown	–	0.23
DIM059	LB + MC	*fgv-003*	Unknown	–	0.28

[a]Samples from independent cultures of DIM063 (*priA dnaC* Δ*rdgC*) grown from independent inocula were spread on the indicated plates (MC at 0.5 μg/ml, Rif at 20 μg/ml), and after 48–60 h at 37°C, colonies of fast-growing variants were purified.
[b]Fraction surviving 30 J/m^2 UV relative to unirradiated cells. Survival of wild-type strain AB1157 was 0.5. Data are means from at least two experiments.
[c]Deletion of a sequence flanked by tctcttgc direct repeats and one of the repeats.
[d]Deletion from a run of 7 T residues.
[e]Deletion of a sequence flanked by ccgggcg direct repeats and one of the repeats.
[f]Deletion of a sequence flanked by gggtgg direct repeats and one of the repeats.

Cultures of a priA2 dnaC212 ΔrdgC strain accumulate fast-growing variants

Strain DIM063 forms small colonies on LB agar and these are quickly overtaken by faster-growing variants. After 3 days incubation, these variants stand out against a background of small colonies (Figure 7A). They are stable and retain their fast-growth phenotype on subculture (Figure 7B). Such variants can also be selected directly by plating DIM063 on LB agar containing MC at 0.5 μg/ml, or rifampicin at 20 μg/ml. We isolated 39 independent isolates of these fast-growth variants (*fgv*). The gross filamentous phenotype of the parent (Figure 6E) is suppressed in all cases. Typical examples are shown in Figure 6F–H. However, phenotypic and genetic analyses indicated that they fall into at least four different classes. Table I lists examples that we have studied in some detail. All retain the *priA2*, *dnaC212* and Δ*rdgC* mutations (data not shown), indicating the presence in each case of an additional suppressor mutation. The high frequency with which these suppressors arise suggests that a single suppressor mutation is responsible. This is supported by our mapping of the *fgv* mutations, which in each case showed that the slow growth phenotype of the parental strain could be fully restored by introducing the wild-type allele for the mutated gene identified.

RecFOR are toxic to ΔrdgC priA dnaC strains

Of the 39 *fgv* isolates analysed, 16 proved quite sensitive to UV, and remained sensitive to MC. Genetic analyses with 14 of these indicated the presence of an additional mutation in *recF* (four isolates), *recO* (two isolates) or *recR* (eight isolates). Four were sequenced, two in *recO* and one each in *recF* and *recR*. The changes found indicated that protein function is most likely inactivated in each case (Table I). This is consistent with the observed sensitivity to UV (Figure 7B; Table I; data not shown). The *recO* mutation in strain DIM064 was transferred to wild-type strain AB1157 by exploiting its linkage to *pheA*. The resulting construct had the same sensitivity to UV as a *recO* null strain (Mahdi and Lloyd, 1989; data not shown). We also constructed a *priA2 dnaC212* Δ*rdgC* strain carrying the well-characterized *recF143* allele. This construct has the same fast-growth, UV-sensitive and non-filamentous cell morphology phenotype as the *recF* isolate, DIM089 (Figure 7Bviii; data not shown). Taken together, these data demonstrate that the RecF, RecO and RecR proteins are toxic to *priA2 dnaC212* Δ*rdgC*. Furthermore, all three proteins have to be active in order to achieve this effect, suggesting a common basis for the toxicity.

Modifications to SSB protein improve growth of ΔrdgC priA dnaC strains

The remaining mutants grow well on LB agar containing MC. Indeed, several were selected on this basis (Table I). Two were shown by sequencing to carry mutations in *ssb*, which encodes the SSB protein (Table I, strains DIM060 and DIM062). One has a single nucleotide change encoding an arginine to cysteine substitution at position 97. The other has an in-frame deletion of 90 bp, eliminating 30 amino acid residues from the C-terminal half of SSB. A further three strains were tentatively identified as carrying *fgv* mutations in *ssb* on the basis of linkage to *malE* and the phenotypic similarity to strains DIM060 and DIM062 (data not shown). Given SSB is an essential protein playing crucial roles in DNA replication and repair (Kuzminov, 1999), it is most unlikely that the *ssb* alleles in these five strains lead to a substantial loss of SSB activity. This conclusion is supported by the fact that the *fgv* isolates carrying these mutations have a fairly normal cell morphology (Figure 6G), grow very well on LB agar (Figure 7Biii), are resistant to MC and are no more sensitive to UV light than the *priA2 dnaC212 rdgC* parent (Figure 7D).

RNA polymerase mutations improve viability

Five *fgv* isolates were shown to carry mutations in the β-subunit (RpoB) of RNA polymerase. These were identified because of their resistance to MC (DIM061) or to rifampicin (DIM104 and DIM105), or simply faster growth on LB agar (HP118 and HP126). The latter are also resistant to MC, but not to rifampicin (data not shown). All five reduce cell filamentation (Figure 6F) and substantially improve viability (data not shown), but have little or no effect on sensitivity to UV light (Figure 7D). However, despite the increased viability, the *fgv* isolates carrying these mutations tend to form colonies that are generally smaller than those carrying *recF*, *O*, *R* or *ssb* mutations (Figure 7B; data not shown).

Other fgv suppressors

The final group of *fgv* isolates are resistant to MC and form smaller colonies, but unlike the RpoB class, they are slightly more resistant to UV light than the parent (Figure 7B and D; Table I; data not shown). Based on the absence of linkage to *argE*, we can exclude mutations in *rpoB* or *rpoC*. We can also exclude *recF*, *recO* and *recR* and we failed to detect linkage of the *fgv* allele to *malE*, thereby excluding *lexA* or *ssb* (data not shown).

Given the filamentous phenotype of the parent strain, and the chronic SOS induction, we considered the possibility that these strains might carry mutations inactivating the SOS-regulated SulA (SfiA) division inhibitor. We therefore examined the viability and morphology of the *sfiA priA2 dnaC212* Δ*rdgC* construct, DIM177. Although the *sfiA* mutation reduced filamentation a little, the strain grew just as poorly as the *sfiA*$^+$ control and fast-growing variants appeared just as frequently (data not shown). Thus, *sfiA* mutations are unlikely to account for this group of *fgv* isolates.

To investigate whether inactivation of some other SOS-inducible factor might be responsible, we made a *lexA3 priA2 dnaC212* Δ*rdgC* strain. *lexA3* encodes a non-cleavable LexA protein, the repressor of SOS, and therefore prevents induction of SOS genes. Figure 7B shows that this *lexA3* construct (streak ix) grows better than the *lexA*$^+$ parent, DIM063 (streak i), but not as strongly as derivatives of DIM063 carrying *recFOR* or *ssb* suppressors. It is also very UV sensitive (data not shown), as might be expected from its inability to induce SOS. Nevertheless, this observation allows for the possibility that the unidentified *fgv* mutations might be alleles of some SOS-inducible gene, or genes. However, the UV-resistant phenotype of these isolates would make it unlikely that they carry mutations inactivating *recA*, *ruvAB*, *uvrA* or *uvrB*.

Discussion

We have shown that the RdgC protein of *E.coli* binds DNA and is essential for growth of a strain lacking PriA, providing a strong indication that this nucleoid-associated factor might affect replication fork progression or fork rescue. We have also shown that *dnaC* suppressors of *priA* overcome this inviability, especially when either RecF, RecO or RecR is inactivated, indicating that RdgC avoids or counters a toxic effect of the RecFOR proteins. Furthermore, we have demonstrated that mutations modifying SSB protein can negate this toxic effect, raising the possibility that binding of RdgC to DNA avoids creating problems at replication forks that would otherwise require PriA to promote replication restart. Mutations in RNAP have a similar effect, providing a connection to transcription.

Native RdgC protein is a homodimer of 34 kDa subunits. Its high expression during early exponential phase encourages the view that it is associated with replication of the chromosome. Its ability to bind dsDNA in a non-specific manner and its association with the nucleoid might suggest a structural role. However, it also forms stable complexes with ssDNA and might bind single strands unwound during replication fork progression. The maximal level of RdgC indicates it would be among the least abundant of nucleoid proteins. Others, such as HU, H-NS, IHF, Fis and StpA are expressed at 5- to 25-fold higher levels (Azam *et al*., 1999). Fis has a similar expression profile to RdgC and is thought to control transcription and DNA replication during periods of rapid growth and to help maintain chromosome structure (Finkel and Johnson, 1992; Schneider *et al*., 2001).

Our inability to make a *priA2* Δ*rdgC* double mutant suggests RdgC may have a role that normally reduces problems with replication fork progression, or that RdgC helps to stabilize or repair damaged forks. However, unlike *priA* null mutants, Δ*rdgC* strains are resistant to UV, indicating that they have little or no difficulty in maintaining replication fork progression. Furthermore Δ*rdgC recB* and Δ*rdgC recA* strains are viable, suggesting that the absence of RdgC does not increase replication fork breakage (Ryder *et al*., 1996). Either DNA repair and the maintenance of replication fork progression do not require RdgC or these processes can be managed perfectly well by other proteins.

We could make strains lacking PriA and RdgC only if they carried a *dnaC212* or *dnaC810* suppressor of the *priA2* allele used to knock out PriA, but these constructs grow slowly, have low viability and, from their cellular morphology, appear to have more serious defects in chromosome segregation and cell division than a *priA*-null single mutant. The mutant DnaC proteins in these strains allow PriA-independent loading of DnaB (Xu and Marians, 2000). Because of the absence of PriA helicase activity to unwind a lagging strand at a fork, such DnaB loading is presumably restricted to D loops and forks without a lagging strand at the branch point (Jones and Nakai, 2001; Gregg *et al*., 2002). So, do strains lacking PriA and RdgC have a specific problem with damaged forks that have a lagging strand obstructing DnaB loading? This is unlikely as a Δ*rdgC* strain carrying *priA300*, which encodes a helicase-defective PriA K230R protein (Zavitz and Marians, 1992), is indistinguishable from the single mutants (data not shown).

A more likely explanation stems from our discovery that an activity dependent upon RecF, RecO and RecR is toxic to *priA dnaC* Δ*rdgC* strains, and that this toxic effect can be avoided by mutations that modify SSB protein. Derivatives of a *priA dnaC* Δ*rdgC* strain carrying these *ssb* mutations, or mutations that inactivate *recF*, *recO* or *recR*, are very healthy. Although the precise function of the RecF, RecO and RecR proteins remains elusive, the current view is that they act as accessory proteins to

facilitate loading of RecA on ssDNA coated with SSB and to help maintain stable RecA–nucleoprotein filaments (Kuzminov, 1999). Thus, the toxicity of RecFOR may reflect some deleterious effect of the RecA filament formed on ssDNA.

Genetic studies indicate that RecF, RecO and RecR function as a complex, and this is supported by evidence of interaction between these proteins *in vitro* (Umezu *et al*., 1993; Umezu and Kolodner, 1994; Webb *et al*., 1997; Bork *et al*., 2001). Inactivation of any one of the three proteins might therefore limit the loading of RecA at single-strand gaps in DNA. Efficient RecA loading might be restricted largely to situations in which RecBCD enzyme acts to facilitate binding of RecA to ssDNA (Anderson and Kowalczykowski, 1997). That is, RecA might be loaded only at DNA ends processed by RecBCD enzyme. RecO also interacts with SSB, and both RecO and RecR bind SSB-coated ssDNA (Umezu and Kolodner, 1994). Such interactions are crucial for replacing SSB with RecA. The *ssb* suppressors we identified might prevent these interactions by removing the residues in SSB interacting with Rec(F)OR, or possibly by increasing the affinity of SSB for ssDNA. The former possibility is likely in strain DIM062, which carries a C-terminal deletion of SSB (Table I). This deletion affects a region of SSB implicated in several protein–protein interactions, leaving the N-terminal DNA-binding region unaffected. Whether one might expect such *ssb* mutations to confer sensitivity to UV, which these particular alleles do not, is an intriguing question. It also begs the question why the toxic effect of RecFOR occurs in a *priA2 dnaC212* strain only when RdgC is missing. One tantalizing possibility is that, like SSB, RdgC binds ssDNA exposed during replication, thereby limiting the loading of RecA. SSB is limited to ~800 tetramers per cell (S.Kowalczykowski, personal communication), whereas our studies indicate that up to 1000 dimers of RdgC may be available during the period of rapid DNA replication. Alternatively, by binding to dsDNA, RdgC may directly or indirectly reduce the amount of ssDNA exposed.

The inactivation of RecFOR would be expected to interfere with many RecA-dependent processes, including post-replication repair of gaps in newly synthesized DNA, SOS induction, replication fork repair and replisome reactivation (Kuzminov, 1999; Courcelle and Hanawalt, 2001; Rangarajan *et al*., 2002). Which of these RecA-dependent reactions might be toxic in *priA2 dnaC212* Δ*rdgC* cells is unclear, although our studies indicate that it cannot be attributed entirely to SOS induction. Excessive RecA-mediated strand exchange is a distinct possibility. Petit and Ehrlich (2002) found that the low viability of a *rep uvrD* strain can be suppressed by *recFOR* mutations. This would be consistent with the hyper-rec phenotype of *uvrD* strains and the idea that UvrD helicase might normally eliminate recombination intermediates (Petit and Ehrlich, 2002). Inactivation of RecFOR also increases the recovery of recombinants in crosses with *recG ruv* strains (Ryder *et al*., 1994), which could be explained if, in the absence of RecG and RuvAB, 'excessive' RecFOR-mediated exchanges at gaps interfered with the viability of recombinants generated by RecBCD. Similarly, inactivation of RecF suppresses the low viability of a *rep ruv dif* strain (Michel *et al*., 2000), which would otherwise have difficulty eliminating chromosome dimers generated by recombination.

Our work has established a link between RdgC and the ability of RecFOR to stimulate homologous recombination. Therefore, it may be significant that the *Neisseria gonorrhoeae* homologue of RdgC, along with RecA and RecO proteins, is required for the locus-specific recombination associated with pilin antigenic variation (Mehr *et al*., 2000).

Why RNA polymerase mutations should alleviate growth defects of a *priA dnaC rdgC* strain is a mystery. McGlynn and Lloyd (2000) found that certain RNAP mutations can alleviate the UV sensitivity of *ruv* mutants. The available evidence indicates that these changes to RNAP may reduce transcriptional roadblocks to replication fork progression. Whether this has any relationship to the toxicity of RecFOR remains to be seen, but the observations reported here provide yet another link between DNA replication, transcription and repair. It remains to be seen how they relate to the original observation that RecA is essential for the growth of a *recBC sbcBC* strain lacking RdgC (Ryder *et al*., 1996).

Materials and methods

Strains and plasmids

E.coli K-12 strains are listed in Tables I and II. The *sfiA*::*lacZ* fusion in strain N5170 is from strain DM4000 (Sandler, 1996). *Escherichia coli* B strain BL21 DE3 pLysS was used for overexpression of RdgC. pGS853 is an *rdgC*$^+$ derivative of pT7-7 made by PCR amplification of *rdgC* from pGS830 (Ryder *et al*., 1996). Primers 5′-TGGAAATTCATATGC-TGTGG-3′ and 5′-GGCATTAAAGCTTAATCAGC-3′ were designed to amplify the coding region and incorporated *Nde*I and *Hin*dIII sites (underlined) for cloning the PCR product in pT7-7.

Media and general methods

LB broth and 56/2 salts media have been described previously (Lloyd *et al*., 1974) and contained 20 μg chloramphenicol (Cm), 40 μg ampicillin (Ap), 25 μg kanamycin (Km), 20 μg tetracycline (Tc), 20 μg trimethoprim (Tm) and 10–100 μg rifampicin (Rif) per ml, as required. MC was incorporated into LB agar at 0.2 and 0.5 μg/ml for plate sensitivity tests. Strain construction by transduction with phage P1*vir* and methods for determining sensitivity to UV light and MC followed the procedures cited (Lloyd and Buckman, 1991). Suppressors (*fgv*) of the slow-growth phenotype of *priA2 dnaC212* Δ*rdgC* strain DIM063 were located by P1 transductional crosses. Transductants of *fgv* strains inheriting markers linked to candidate genes were tested for segregation of the slow-growth phenotype. β-galactosidase activity in *sfiA*::*lacZ* fusion strains was measured as described previously (Miller, 1972) and expressed in Miller units. Cell viability was determined by measuring the ratio of colony forming units to total cells detected microscopically in LB broth cultures grown to an A_{650} of 0.4. For DAPI staining, cells were concentrated in TBS buffer (20 mM Tris–HCl pH 7.6, 137 mM NaCl), mixed with an equal volume of DAPI (10 μg/ml in TBS) and spread on polysine-coated slides (BDH). Phase-contrast and DAPI fluorescence images were captured at 1000× magnification.

Enzymes and protein analysis

RecG and RuvC were purified as cited (McGlynn and Lloyd, 2000). RdgC was purified from strain BL21 DE3 cells carrying pGS853 and pLysS following induction with IPTG. Induced cells (9 g wet weight) were lysed on ice by sonication in buffer A [50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT)] before adding NaCl to 1 M and collecting soluble proteins by centrifugation. RdgC was extracted using a 50–70% ammonium sulfate cut and the precipitate resuspended in buffer A + 0.1 M NaCl, dialysed against the same buffer and applied to a 40 ml heparin–Sepharose 6 fast flow column. Bound proteins were eluted with a linear gradient of 0.1–1.0 M NaCl in buffer A and peak RdgC fractions eluting between 0.6 and 0.8 M NaCl were pooled, precipitated with 80% ammonium sulfate, resuspended in buffer A + 0.1 M NaCl and applied to a 2.5 × 83 cm Sephacryl S200HR gel filtration column

Table II. Strains used

Strain	Relevant genotype[a]	Source or reference
DIM167	*priA2*::Km *dnaC810 zji202*::Tn*10*	P1.AG127 × JC19009 to Tc^r
JC12334	*tna-300*::Tn*10 recF143*	A.J.Clark
JC19009	*priA2 dnaC810*	Sandler *et al*. (1996)
MG1655	F$^-$, wild type	Bachmann (1996)
N2247	*malE*::Tn*10 lexA3*	Laboratory strain
N4586	Δ*rdgC*::Tm	P1.JP947 × MG1655 to Tm^r
PN109	*priA2 sulA*	Nurse *et al*. (1991)
W3110	Wild type	Bachmann (1996)
AB1157 background[b]		
AB1157	F$^-$ *priA*$^+$ *rdgC*$^+$ *rec*$^+$	Bachmann (1996)
AG127	*zji202*::Tn*10*	A.V.Gregg
AG181	*priA2 dnaC212*	Gregg *et al*. (2002)
AG291	*priA2 dnaC212 zji-202*	A.V.Gregg
DIM037	Δ*rdgC*	P1.N4586 × AB1157 to Tm^r
DIM063	*priA2 dnaC212* Δ*rdgC*	P1.N4586 × AG181 to Tm^r
DIM070	*priA2*	P1.PN109 × AB1157 to Km^r
DIM095	*argE*$^+$ *dnaC212* Δ*rdgC*	P1.W3110 × DIM061 to Arg$^+$, and Rif^s and Km^s
DIM112	*argE*$^+$ *dnaC212* Δ*rdgC tna recF143*	P1.JC12334 × DIM095 to Tc^r
DIM113	*argE*$^+$ *dnaC212* Δ*rdgC malE lexA3*	P1.N2247 × DIM095 to Tc^r
DIM122	*priA2 dnaC212* Δ*rdgC tna recF143*	P1.AG181 × DIM112 to Km^r
DIM123	*priA2 dnaC212* Δ*rdgC malE lexA3*	P1.AG181 × DIM113 to Km^r
DIM152	*sfiA*::*lacZ* Δ*rdgC*[c]	P1.N4586 × N5170 to Tm^r
DIM156	*sfiA*::*lacZ dnaC212 zji-202*[c]	P1.AG291 × N5170 to Tc^r
DIM158	*sfiA*::*lacZ* Δ*rdgC dnaC212 zji-202*[c]	P1.AG291 × DIM152 to Tc^r
DIM173	*sfiA*::*lacZ priA2*[c]	P1.AG291 × N5170 to Km^r
DIM175	*sfiA*::*lacZ priA2 dnaC212 zji-202*[c]	P1. AG291 × DIM156 to Km^r
DIM177	*sfiA*::*lacZ priA2 dnaC212 zji-202* Δ*rdgC*[c]	P1. AG291 × DIM158 to Km^r
DIM211	*dnaC810 zji202*	P1.DIM167 × AB1157 to Tc^r
DIM215	*priA2 dnaC810 zji202*	P1.AG181 × DIM211 to Km^r
JP947	Δ*rdgC*::Tm	J.Peters
N5170	*sfiA*::*lacZ*[c]	This work

[a]After the first full listing, insertion and deletion/insertion mutations are referred to by the allele designation only.
[b]Also *thi-1 his-4* Δ*(gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfbD1(?) ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 supE44 rpsL31*, except as indicated.
[c]Also *thr+ leu+* Δ*(pro-lac)*$_{\chi 111}$ and *sfiA::lacZ* = *sfiA*::Mu-d(Ap, *lac*, B::Tn9).

equilibrated in the same buffer. The RdgC peak fractions, eluting at a position corresponding to 70 kDa, were pooled and applied to a 4 ml dsDNA cellulose column equilibrated in buffer A + 0.1 M NaCl. The column was washed with buffer A + 0.1 M NaCl and then with buffer A + 1.0 M NaCl. RdgC eluted in the flow through and the high salt wash. Both fractions were therefore pooled, dialysed against buffer A + 1.5 M ammonium sulfate, applied to a 10 ml butyl–Sepharose 4B column and eluted with a linear 1.5–0 M gradient of ammonium sulfate in buffer A. RdgC eluted as a single peak between 0.9 and 0.65 M ammonium sulfate. These fractions were pooled, dialysed into buffer B (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 mM DTT) + 0.1M NaCl, and applied to a 3.0 ml Q Sepharose fast flow column. The column was washed with 30 ml buffer B + 1 M NaCl. RdgC remains bound under these conditions. The bound protein was eluted with buffer A + 2 M NaCl, dialysed into storage buffer [buffer A + 0.1 M NaCl, 50% (v/v) glycerol] and stored in aliquots at –80°C. N-terminal sequencing of the first 10 residues confirmed the purified protein to be RdgC. Protein concentrations were determined using a modified Bradford assay (Bio-Rad), and are expressed as moles of the native dimer for RdgC, or of the monomeric species for RecG and RuvC. Protein cross-linking was conducted as described (Chan *et al*., 1997), using a final concentration of 10 μM RdgC and glutaraldehyde as indicated, before analysis of the cross-linked samples by SDS–PAGE. Estimation of the molecular weight of RdgC was made by passing 40 μg of the protein, in a volume of 50 μl, through a Superose 12 column (Amersham Pharmacia Biotech. Ltd) equilibrated in buffer A + 0.2 M NaCl. Molecular weight standards were thyroglobulin, γ-globulin, ovalbumin and myoglobulin.

Immunodetection of RdgC

Polyclonal, RdgC antibodies were obtained from rabbits inoculated with RdgC protein and were purified using an RdgC affinity column. Cellular RdgC was detected by SDS–PAGE analysis of lysed cell extracts and western blotting using purified RdgC antibodies, anti-rabbit-IgG:HRP conjugate (Bio-Rad) and ECL detection reagents (Amersham). RdgC levels were quantified using a LAS-1000 fluorescence image analyser (Fujifilm) and normalized to cell numbers detected microscopically.

DNA substrates

χ DNA, a branched DNA structure containing a Holliday junction within a 300 bp core of homology flanked by duplex arms from 0.8 to 1.6 kb in length, was made as described previously (McGlynn and Lloyd, 2000). Other DNA structures were made by annealing combinations of oligonucleotides 1–4, 6 and 7 listed by McGlynn *et al*. (1997), 8 (5′-GGGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAAGGCAC-TGGTAGAATTCGGACAGCGTC-3′), 9 (5′-AACGTCATAGACGATT-ACATTGCTA-3′) and 10 (5′-TAGCAATGTAATCGTCTATGAC-GTT-3′), one of which was ^{32}P-labeled at the 5′ end before annealing. J12 was made from oligonucleotides 1–4, the three-strand junction from 1+2+4, the Y structure from 1+4+6 and the flayed-duplex from 1+2. All these structures were made of oligonucleotides of 49–51 residues. Sixty-one base pair and 25 bp linear duplexes were made from oligonucleotides 7+8 and 9+10, respectively. The 25 and 61 nucleotide ssDNA substrates were oligonucleotides 7 and 9, respectively. Poly[dIdC] was used as competitor DNA.

Enzyme assays

Resolution of χ DNA by RuvC was measured as described previously (McGlynn and Lloyd, 2000). Reaction mixtures (10 μl) were incubated for 30 min at 37°C before deproteinization and electrophoresis through 0.8% agarose gels. For DNA-binding assays, reactions (20 μl) contained 0.1 nM ^{32}P-labelled DNA substrate mixed with the indicated amount of protein in 50 mM Tris–HCl pH 8.0, 1 mM DTT, 100 μg/ml BSA, 6% (v/v)

glycerol and 2 mM $MgCl_2$. After 10 min on ice, samples were loaded on a chilled (4°C) 4% polyacrylamide gel in 6.7 mM Tris–HCl pH 8.0, 3.3 mM sodium acetate, 2 mM $MgCl_2$. Gels were run at 160 V for 90 min, dried and analysed by autoradiography and phosphoimaging. To measure the stability of RdgC bound to ssDNA or dsDNA, binding reactions containing 0.1 nM ^{32}P-labelled DNA and sufficient RdgC to bind ~20–50% of the substrate were mixed on ice for 10 min before adding 50 ng poly[dIdC] and incubating for the times indicated before gel analysis of bound complexes.

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