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Modulating cellular recombination potential through alterations in RecA structure and regulation

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Summary

The wild-type *Escherichia coli* RecA protein is a recombinase platform with unrealized recombination potential. We have explored the factors affecting recombination during conjugation with a quantitative assay. Regulatory proteins that affect RecA function have the capacity to increase or decrease recombination frequencies by factors up to sixfold. Autoinhibition by the RecA C-terminus can affect recombination frequency by factors up to fourfold. The greatest changes in recombination frequency measured here are brought about by point mutations in the *recA* gene. RecA variants can increase recombination frequencies by more than 50-fold. The RecA protein thus possesses an inherently broad functional range. The RecA protein of *E. coli* (EcRecA) is not optimized for recombination function. Instead, much of the recombination potential of EcRecA is structurally suppressed, probably reflecting cellular requirements. One point mutation in EcRecA with a particularly dramatic effect on recombination frequency, D112R, exhibits an enhanced capacity to load onto SSB-coated ssDNA, overcome the effects of regulatory proteins such as PsiB and RecX, and to pair homologous DNAs. Comparisons of key RecA protein mutants reveal two components to RecA recombination function – filament formation and the inherent DNA pairing activity of the formed filaments.

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Introduction

The bacterial RecA protein catalyses homologous recombination (HR), and thereby plays critical roles in DNA metabolism and especially recombinational DNA repair of stalled or collapsed replication forks (Cox *et al*., 2000; 2007a; Kowalczykowski, 2000; Lusetti and Cox, 2002). The RecA of *Escherichia coli* (EcRecA) has been carefully studied for three decades, and the repair of stalled replication forks is likely its primary function under normal growth conditions (Cox *et al*., 2000; Michel *et al*., 2007). In times of severe stress, the EcRecA protein also promotes the induction of the SOS response. In other bacterial species, the repair requirements can vary markedly. RecA-mediated recombination underlies processes ranging from the precise recombination events responsible for pilin variation in *Neisseria gonorrhoeae* (Stohl *et al*., 2002; Kline *et al*., 2003) and cell adherence in *Vibrio cholerae* (Kumar *et al*., 1994), to the massive recombinational repair required for genome reconstitution after extreme levels of radiation damage in *Deinococcus radiodurans* (Cox and Battista, 2005; Blasius *et al*., 2008) or the directed oxidative damage inflicted by macrophages on pathogens such as *Salmonella typhimurium* (Buchmeier *et al*., 1993).

RecA protein is activated for recombination by polymerization on single-stranded DNA (ssDNA) in the presence of ATP and Mg^{+2} ions to form a presynaptic filament. The filament then promotes a search for homology with a double-stranded DNA (dsDNA) partner, leading to a strand exchange reaction between the recombining DNA partners (Cox, 2007b). These consecutive steps describe the initiation of HR as it has been studied *in vitro*.

In vivo, HR comes about by means of two genetic pathways, RecBCD and RecFOR (Clark and Sandler, 1994). The former initiates recombination mainly on dsDNA breaks or dsDNA ends in the context of double strand break repair (DSBR) (Cromie and Leach, 2000). In this case, the RecBCD helicase-endonuclease loads RecA protein onto a segment of ssDNA created for that purpose at a DNA end exposed at the break to form the presynaptic filament (Arnold and Kowalczykowski, 2000;

Churchill and Kowalczykowski, 2000). The latter pathway uses mainly ssDNA gaps or nicks to stimulate the mechanism of single strand gap repair (SSGR) (Cromie and Leach, 2000), when the RecFOR proteins load RecA onto gapped DNA to accelerate DNA strand exchange (Morimatsu and Kowalczykowski, 2003; Hobbs *et al*., 2007; Sakai and Cox, 2009). Additionally, RecFOR can contribute significantly to DSBR in the absence of functional RecBCD (Cromie and Leach, 2000). According to a current view, DSBR results mainly in the formation of cross-over type recombinants while SSGR tends to produce recombinants of the conversion type (i.e. via heteroduplex formation) (Cromie *et al*., 2000). However, neither cross-overs nor conversions can be completely excluded from recombinants formed by SSGR or DSBR.

Heteroduplexes generated during recombination are DNA molecules containing base-base mismatches or small nucleotide insertion/deletion mispairs. They are targets for the methyl-directed long-patch repair system MutHLS, which corrects mismatches or mispairs to the sequence of methylated strands in heteroduplexes (Modrich, 1991). MutH, MutL and MutS initiate the repair process, in which MutS plays a role of 'mismatch recognition' protein. In principle, recombinational heteroduplexes can be corrected to either donor or recipient sequence, altering the outcome of genetic exchanges during conjugal crosses (Jones *et al*., 1987).

Although many different RecA protein mutants have been studied, there have been fewer efforts directed at investigating the range of RecA protein function that is possible *in vivo* than *in vitro*. Thus, it is not always clear how alterations of RecA, due to directed mutation, translate into changes in cellular repair and recombination capacity. It is also not clear to what extent recombination potential reflects RecA protein structure itself, the modulation of RecA protein function by other factors or the activities of entirely different recombination proteins. Therefore, to directly address the affects of these variables on the *in vivo* function of RecA, we took advantage of a quantitative genetic analysis of the linkage of donor markers following bacterial conjugation by determining the frequency of recombination exchanges per DNA unit length (FRE) (Namsaraev *et al*., 1998; Bakhlanova *et al*., 2001; Chervyakova *et al*., 2001; Lanzov, 2002; Baitin *et al*., 2003; 2006; 2008; Lanzov *et al*., 2003). These methods have shown that RecA from *E. coli* (EcRecA) has a relatively moderate recombinase activity *in vivo* (Namsaraev *et al*., 1998; Lanzov *et al*., 2003; Baitin *et al*., 2006; 2008), although the activity is presumably optimal for this bacterium. The *E. coli* FRE level provides a useful benchmark against which to measure any increase or decrease of FRE values, which we define as hyper- or hypo-recombination (Bakhlanova *et al*., 2001; Lanzov, 2002). Hyper-recombination arises as a result of the induction of the SOS response, either transiently by treating normal cells with a DNA damaging agent (Sassanfar and Roberts, 1990) or constitutively as observed with certain RecA mutant variants such as RecA E38K (*recA*730) (Lavery and Kowalczykowski, 1992). Hyper-recombination can also be SOS-independent, as observed in a set of RecA proteins including the *Pseudomonas aeruginosa* RecA (PaRecA) (Namsaraev *et al*., 1998; Baitin *et al*., 2003; 2006) and different *E. coli*/*P. aeruginosa* chimeric RecAX proteins (Bakhlanova *et al*., 2001; Baitin *et al*., 2006; 2008) expressed in *E. coli*.

RecA-like proteins are ubiquitous but their activities must be limited because their uncontrolled activity can have deleterious consequences. The biochemical and physicochemical data accumulated during the last decade show that RecA is autoregulated by its own C-terminus (Eggler *et al*., 2003; Lusetti *et al*., 2003a,b). In effect, the wild-type *E. coli* RecA protein is completely inactive for recombination activities in the presence of the 1–2 mM free Mg ion concentrations that are thought to be physiological (Lusk *et al*., 1968; Kuhn *et al*., 1983; Alatossava *et al*., 1985; Kuhn and Kellenberger, 1985; Lusetti *et al*., 2003a,b). The common use of relatively high concentrations of free Mg ion (8–10 mM) for RecA-mediated reactions *in vitro* apparently reflects a conformation change – mediated by free Mg ion – that converts RecA nucleoprotein filaments into a more open and active conformation (Haruta *et al*., 2003; Lusetti *et al*., 2003a,b). Deletion of 17 amino acid residues from the RecA C-terminus obviates the need for added Mg ion (beyond that for chelating the ATP) to activate RecA (Lusetti *et al*., 2003a,b). The *in vitro* activity of RecA protein is also regulated by the action of such regulatory proteins as RecF, RecO, RecR, DinI, RecX, UvrD and PsiB (Cox, 2007a). The RecFOR proteins function in loading RecA protein onto SSB-coated ssDNA (Morimatsu and Kowalczykowski, 2003; Cox, 2007a; Hobbs *et al*., 2007; Sakai and Cox, 2009). The DinI protein stabilizes RecA filaments (Lusetti *et al*., 2004a; Cox, 2007a), although it may inhibit some filament functions. The RecX, UvrD and PsiB proteins inhibit RecA filament function or formation in various ways (Drees *et al*., 2004; Veaute *et al*., 2005; Cox, 2007a; Petrova *et al*., 2009).

It is clear that recombination potential can vary from one bacterial species to another, and within a species due to introduced mutations. However, what factors are most important in this variation? Imposed regulation must play a role. Autoregulation and mutational variation in RecA must also play a role. In this study, we provide a broad assessment of these factors to determine which are particularly important in establishing cellular recombination potential. The results are more than a comparative exercise. They reveal a large but evolutionarily suppressed recombination potential within the structure of

most bacterial RecA proteins that affects both the formation of RecA nucleoprotein filaments and the inherent DNA pairing activity of those filaments.

Results

Measurement of FRE to assess elevated or depressed levels of recombination *in vivo* has been developed over the past 15 years (Namsaraev *et al*., 1998; Bakhlanova *et al*., 2001; Chervyakova *et al*., 2001; Lanzov, 2002; Baitin *et al*., 2003; 2006; 2008; Lanzov *et al*., 2003). The method has the advantage that it can detect essentially all types of genetic exchanges that might occur during conjugation (Baitin *et al*., 2008). The method is somewhat more laborious than the convenient and robust method developed by Konrad (Konrad, 1977). However, the Konrad method relies on genetic recombination between two inverted repeats in the *E. coli* chromosome. The region separating the repeats appears to be refractory to inversion (Konrad, 1977), so some kinds of recombination events may be missed. The recombinants may arise entirely through exchanges between sister chromosomes (Mahan and Roth, 1991). In addition, the recombinants appear as the cells go into stationary phase, suggesting their appearance may not be coupled to replication (Zieg and Kushner, 1977). The FRE approach is similar to approaches developed by others (Lloyd, 1978). By employing the widely used strain AB1157 as recipient, this approach also facilitates measurements in a wide variety of strain backgrounds while minimizing new strain construction.

During mating, the donor Hfr KL227 transfers markers into recipients in the order *leu*$^+$, *ara*$^+$ and *thr*$^+$. Recombination exchanges in this region of the *E. coli* map are adequately described mathematically by the Haldane formula (Lanzov *et al*., 2003). In the slightly rearranged form, $\lambda = -2l/\ln(2\mu - 1)$, this formula relates the average distance between two neighbouring genetic exchanges (in minutes) to the linkage of selected and unselected markers (μ), and the distance (l), in minutes between markers on the *E. coli* map. The distance, l, in minutes between the *thr* (0.05) and *leu* (1.75) markers is 1.7, and between the *ara* (1.47) and *leu* (1.75) markers is 0.28. Donor KL227 transfers *leu*$^+$ and *thr*$^+$ as a proximal and distal marker respectively. The frequency of recombinational exchanges is expressed as FRE, the average number of exchanges per one *E. coli* genome equivalent (100 min), and thus equals $100/\lambda$. For wild-type *E. coli*, FRE = 5.0 (Lanzov *et al*., 2003). In this study, we are particularly interested in changes in FRE, using the wild-type *E. coli* values as a benchmark called FRE_2. Therefore, as described previously (Bakhlanova *et al*., 2001; Lanzov, 2002; Baitin *et al*., 2003; 2006; 2008; Lanzov *et al*., 2003), our reported value of ΔFRE, or the ratio of FRE_1/FRE_2, indicates the measured FRE for the cross under investigation relative to FRE_2. ΔFRE can also be calculated by use of the formula: $\Delta FRE = \ln(2\mu_1 - 1)/\ln(2\mu_2 - 1)$, where μ_1 is the linkage observed with either a modified RecA or the wild-type RecA under particular conditions of HR, and μ_2 is the linkage observed with wild-type EcRecA under standard conditions (Bakhlanova *et al*., 2001).

In many genetic crosses, heteroduplex DNA intermediates are transiently created that are subject to DNA mismatch repair, which can alter the quantitative outcome of the trial. Using our conjugational assay, we have revealed two quite different effects of the mismatch repair system in *E. coli*. For most crosses, inactivation of the MutHLS complex by a *mutS215* mutation results in a two to sixfold increase in the FRE value for HR, indicating that mismatch repair normally suppresses the measured effects of many recombination events. This is true for recombination promoted by the EcRecA protein, as well as by the RecA protein from *P. aeruginosa* (PaRecA) (Bakhlanova *et al*., 2001; Baitin *et al*., 2008). This is in accord with the established effects of the MutHLS long-patch repair system (Jones *et al*., 1987). Unexpectedly, the reverse effect has also been found for another hyper-rec chimeric protein RecAX53, which contains 12 amino acids from PaRecA in the middle of the EcRecA structure (Baitin *et al*., 2008). In this case, the *mutS215* mutation leads to a 2.5-fold decrease of FRE (Baitin *et al*., 2008). These results have been interpreted as arising from different types of recombination events promoted by the various RecA protein variants (Baitin *et al*., 2008). In brief, if all the possible types of genetic exchange in this system are considered, the *mutS* alteration should increase FRE if most of the exchanges being monitored are cross-overs, and should decrease FRE if most of the exchanges being monitored are conversion events (Baitin *et al*., 2008). We do not address the mechanistic origin of *mutS215* effects in the present study. We include measurements derived from crosses using *mutS215* strains so that we can evaluate the effects of mismatch repair on our other measurements.

Effects of proteins that modulate RecA function

RecFOR proteins contribute to recombination potential. During conjugation, the RecFOR pathway is readily observed only in a *recBC sbcB sbcCD* background (Clark and Sandler, 1994). This entails the inactivation, respectively, of the RecBCD helicase-nuclease (Taylor and Smith, 2003), the (3′→5′)-directed ssDNA exonuclease I (Clark, 1971), and the ATP-dependent dsDNA exonuclease SbcCD (Connelly *et al*., 1997). The RecBCD pathway predominates in HR leading to normal *rec*$^+$ transconjugants, although the RecFOR pathway is not

Table 1. The FRE value dependence from *recF*, *recO*, *recR* and *mutS* mutations in transconjugants of AB1157 line formed after mating with donor KL227.

rec and *mut* genotype of recipients[a]	Yield of Thr$^+$Str^r recombinants (% to donors)	Linkage (μ) between selected *thr*$^+$ and unselected *leu*$^+$ markers	FRE	ΔFRE	*P*-value[a]
rec$^+$ *mut*$^+$	5.4 ± 0.5	0.935 ± 0.020 (600)	5.0 ± 0.1	1	
mutS215::Tn10	3.2 ± 0.3	0.682 ± 0.033 (600)	29.4 ± 0.3	6.0	1.36E-13
recF349Δ	4.6 ± 0.4	0.959 ± 0.021 (500)	2.6 ± 0.1	0.5	2.60E-04
recF349Δ mutS215	3.7 ± 0.3	0.778 ± 0.063 (1200)	17.2 ± 0.2	3.4	5.45E-08
recO1504::Tn5	4.9 ± 0.4	0.948 ± 0.054 (1200)	3.2 ± 0.2	0.6	3.64E-03
recO1504::Tn5 mutS215	3.7 ± 0.4	0.770 ± 0.068 (700)	18.2 ± 0.3	3.6	1.56E-08
recR252::Tn10-9	4.5 ± 0.5	0.950 ± 0.056 (1100)	3.1 ± 0.4	0.6	0.02
recR252::Tn10-9 mutS215	2.5 ± 0.3	0.839 ± 0.072 (900)	11.5 ± 0.2	2.3	2.35E-12

a. *P*-values were calculated for linkage data sets relative to *rec*$^+$ *mut*$^+$.

silent in these cells and plays an important role in recombinational DNA repair (Amundsen and Smith, 2003).

New ssDNA is constantly presented during conjugation in the donor DNA transferred into recipients. The incoming donor ssDNA is converted to a duplex form via the synthesis of Okazaki fragments, creating gapped DNA (Lawley *et al*., 2002). Additional gaps appear in recipient DNA during normal chromosome duplication. The SSGR mechanism promoted by the RecFOR pathway results in heteroduplex formation that can be corrected, at least partially, by the MutHLS complex.

There was a 1.7- to 2-fold decrease in FRE values, relative to *rec*$^+$, in the transconjugants observed in the deletion mutant *recF349*, or in the insertion mutations *recO1504* and *recR252* (Table 1). Combinations of these mutations, *recF349 recO1504* or *recF349 recR252*, did not enhance this effect (data not shown). This suggests that the proteins of the RecFOR pathway take some part in the promotion of approximately half of the recombination events that occur during conjugation, presumably by the RecBCD pathway. The *mutS215* mutation consistently increased FRE by four to sevenfold relative to the values seen in the strains lacking it. In sum, the RecFOR genes appear to play a modest role in establishing overall recombination function by this assay.

DinI and RecX proteins contribute modestly to recombinational potential. A temporal induction of SOS functions in response to DNA damage is regulated in part by two SOS-inducible proteins, DinI and RecX. These proteins compete directly as modulators of RecA functions (Lusetti *et al*., 2004b). In effect, in the presence of DinI, the RecA filament disassembly is blocked, but assembly can proceed, while in the presence of RecX, assembly is blocked, but disassembly can proceed. RecX specifically blocks the RecA filament extension (Drees *et al*., 2004) and inhibits RecA recombinase activity *in vitro* and *in vivo* (Stohl *et al*., 2003). It seems reasonable to suggest that expression of each of the proteins should have an effect on FRE.

We measured FRE values for transconjugants bearing a deletion either in the *recX* gene (*ΔrecX*) or in *dinI* (*ΔdinI::Km*) (Table 2). In the former case, a negligible change, if any, in the FRE value was observed while in the latter, inactivation of the dinI gene resulted in a 40% increase of FRE (Table 2). These data are well supported

Table 2. The FRE value dependence from inactivation or an increased expression of *recX* and *dinI* genes in transconjugants of AB1157 line crossed with donor KL227.

Recipient	Relevant genotype of recipients	Relative amount per cell[a]		Linkage μ (*thr*$^+$–*leu*$^+$)[b]	FRE	ΔFRE	*P*-value[c]
		RecX	DinI				
AB1157	*rec*$^+$	1.0	1.2	0.922 ± 0.031 (1500)	5.0 ± 0.2	1.0	
AB1157-X	*ΔrecX*	ND	–	0.912 ± 0.084 (1300)	5.7 ± 0.5	1.1	0.21
AB1157-I	*ΔdinI::Km*	–	ND	0.872 ± 0.091 (1200)	8.7 ± 1.0	1.7	4.13E-07
AB1157/pT7	*rec*$^+$	1.0	1.0	0.923 ± 0.062 (600)	4.9 ± 0.3	1.0	0.75
AB1157/pT7/precX	*rec*$^+$/p*recX*$^{++}$	45.3	–	0.940 ± 0.084 (600)	3.8 ± 0.3	0.76	7.45E-03
AB1157/pT7/pdinI	*rec*$^+$/p*dinI*$^{++}$	–	10.3	0.989 ± 0.032 (1500)	0.7 ± 0.04	0.13	3.05E-20

a. As described in Fig. 1.
b. Yield of Thr$^+$ Str^r recombinants in crosses between donor KL227 and given recipients was as follows: AB1157 = 3.2 ± 0.3 (in % from donors), AB1157-X = 4.6 ± 0.4, AB1157-I = 1.8 ± 0.1, AB1157/precX = 5.6 ± 0.5, AB1157/pdinI = 4.6 ± 0.6, AB1157/pT7 = 5.2 ± 0.5, AB1157/pT7/precX = 2.2 ± 0.3, AB1157/pT7/pdinI = 2.1 ± 0.4. The linkage data were averaged from three repeats.
c. *P*-values for linkage data sets were calculated relative to AB1157.

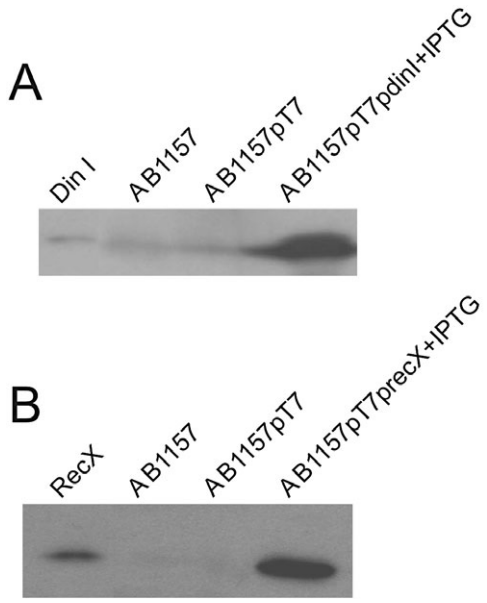

Fig. 1. Visualization and quantification of the RecX and DinI proteins by immunoblotting with polyclonal antibodies raised against RecX and DinI. For controls, amounts of the DinI (A) and RecX (B) proteins in AB1157 and AB1157pT7 strains were used. Lanes DinI and RecX served as reference points. The intracellular amounts of DinI and RecX in control and analysed (AB1157pT7pdinI and AB1157pT7precX) strains were determined similar to the procedure described earlier (Baitin *et al*., 2006), the bands were scanned by the program 'Kodak Digital Science 1D' and the data presented in Table 2 relative to the amount of a given protein in the strain AB1157pT7.

by FRE measurements under conditions of *recX* and *dinI* gene overexpression in transconjugants. A 45-fold increase of RecX amount led to a small (1.3-fold) decrease in FRE while a 10-fold increase of DinI expression resulted in a significant 7.7-fold suppression of HR activity as measured by this assay. The relative values of RecX and DinI intracellular amounts under conditions of *recX* and *dinI* overexpression were measured by immunoblotting analyses as pictured in Fig. 1. Given the strong effects of both DinI and RecX recorded *in vitro*, these measured effects on *in vivo* recombination capacity appear modest.

The data presented in this section indicate that compared with RecX, the DinI protein appears to be a stronger regulator of HR activity measured through FRE, the inhibitory role of which can be measured even under normal unstressed conditions.

Effects of autoregulation of RecA via its C-terminus

In most of the published RecA crystal structures from all bacterial species, the 25 C-terminal amino acids are disordered. They form free peptide tails that usually contain a substantial number of negatively charged amino acids (e.g. seven or six for EcRecA or PaRecA respectively). This peptide is a RecA autoregulatory flap (Story *et al*., 1992; Lusetti *et al*., 2003a,b). Removing 25 or even 17 amino acids from the C-terminal end of EcRecA makes a wide range of RecA activities more robust. These include the enhancement of DNA binding and pairing functions, a more rapid displacement of SSB on ssDNA, and a more active LexA repressor cleavage (Benedict and Kowalczykowski, 1988; Eggler *et al*., 2003; Lusetti *et al*., 2003a,b; 2004b). The addition of substantial amounts (8–10 mM) of free Mg ion to activate wild-type RecA protein *in vitro* is no longer necessary in the C-terminal deletion mutant proteins (Lusetti *et al*., 2003a,b).

It seems reasonable to expect an increase of FRE values measured in transconjugants expressing the truncated EcRecAΔC17 [17 amino acid from its C-terminus (Eggler *et al*., 2003; Lusetti *et al*., 2003a,b)] or PaRecAΔC11 (11 amino acids from its C-terminus) proteins, in which all negatively charged amino acids on their free C-terminal tails have been removed. The results of such experiments are presented in Table 3, for normal and truncated EcRecA and PaRecA proteins promoting HR in *E. coli*. As described earlier, the normal FRE values for wild-type EcRecA and the hyper-rec PaRecA appeared to be suppressed in a *mutS*-dependent fashion, exhibiting a 6-and 2.2-fold FRE increase in the *mutS215* background respectively. The truncation of 17 and 11 amino acids from EcRecA and PaRecA, respectively, resulted in a FRE value enhancement, by 4.3 and 2.9 times. However, these truncated proteins exhibited a reverse dependence on MutS, with the *mutS215* background leading to a drop of their FRE by 1.7 and 5.8 times respectively.

The inherent recombinational capacity of EcRecA protein is evolutionarily suppressed

As shown earlier, 12 amino acid substitutions in the central domain of EcRecA [RecAX53 (Bakhlanova *et al*., 2001)], four substitutions in the N-terminal domain of EcRecA [RecAX21 (Tateishi *et al*., 1992)] and one at the N-terminus of EcRecA [RecA730 (Bakhlanova *et al*., 2001; Lanzov *et al*., 2003)] resulted in nine, three and sevenfold increase of the FRE value, respectively, in an SOS-independent (RecAX53, RecAX21) and SOS-dependent [RecA E38K (RecA730)] manner (Lanzov, 2002). This indicates that even one substitution in RecA can significantly change its recombinogenic potential. In addition, there appears to be a conserved group of amino acids that are responsible for the natural recombinase activity of a given RecA. This leads to questions about the upper limits for relaxation of suppressed HR recombinase activity or FRE value increase. In searching for new hyper-rec mutations, we have become interested in mutations located near or at the RecA subunit interface. A few

Table 3. FRE values promoted by normal and truncated (from the C-terminal end) RecA proteins.

recA and *mutS* genotype of recipients[a]	Yield of Thr$^+$Str^r or Ara$^+$Str^r recombinants	Linkage μ (selected – unselected markers)		FRE	ΔFRE	*P*-value[b]
		thr$^+$–*leu*$^+$	*ara*$^+$–*leu*$^+$			
Normal proteins						
EcRecA$^+$	3.7 ± 0.8		0.986 ± 0.007 (600)	5.0 ± 0.1	1.0	
EcRecA$^+$ *mutS215*	3.1 ± 0.8		0.923 ± 0.017 (900)	30.0 ± 0.5	6.0	1.35E-04
PaRecA$^+$	4.5 ± 1.0		0.896 ± 0.032 (900)	41.6 ± 1.5	8.3	1.50E-07
PaRecA$^+$ *mutS215*	2.9 ± 0.6		0.801 ± 0.015 (1300)	90.9 ± 1.7	18.1	4.80E-11
Truncated proteins						
recAΔC17Ec	0.16 ± 0.01	0.747 ± 0.061 (1220)		21.6 ± 2.1	4.3	5.40E-16
recAΔC17Ec mutS215	0.17 ± 0.01	0.820 ± 0.005 (600)		13.2 ± 0.5	2.6	7.34E-13
recAΔC11Pa	1.8 ± 0.1		0.754 ± 0.020(1200)	119.3 ± 8.7	23.9	2.32E-17
recAΔC11Pa mutS215	4.6 ± 0.5	0.757 ± 0.067 (1450)		20.4 ± 7.5	4.1	1.39E-09

a. All recipients were of AB1157 line. Mutation *mutS215* was introduced by P1 transduction. Normal *EcRecA*$^+$ and *PaRecA*$^+$ genes (Namsaraev *et al*., 1998) as well as truncated genes *recAΔ17Ec* (Lusetti *et al*., 2003b) and *recAΔ11Pa* were located on plasmids and introduced in AB1157 *ΔrecA*. Plasmid precAΔ11Pa was constructed on the base of pEAW337 (Lusetti *et al*., 2003b) via the displacement of *recAΔ11Ec* with *recAΔ11Pa*.
b. *P*-values for linkage data sets were calculated relative to *EcRecA*$^+$.
The FRE value dependence from the integrity of mismatch repair system in transconjugants: *mutS*$^+$ relative to *mutS215*. Crosses with donor KL227.

mutations of this kind have been described and partially characterized earlier (Cox *et al*., 2006; 2008).

Table 4 summarizes comparative FRE and SOS (a relative level of SOS regulon derepression) characteristics for 11 recombination-proficient mutations located at positions 6, 9, 28, 89, 112, 113 and 139 of the EcRecA structure. Taking into account that constitutive SOS derepression for RecA E38K is quite strong [ΔSOS = 17 (Bakhlanova *et al*., 2001)], we can characterize additional RecA mutants that provide moderate (N113A; ΔSOS = 5.4), weak (D112R, K6A, K6D, R28D; ΔSOS = 2.1–2.8) or an absence of SOS induction (all other; ΔSOS = 0.9–1.5). With respect to FRE, we found a wide range of values relative to normal EcRecA, with ΔFRE changes of 1.4–52.6. Three mutations resulted in a high degree of hyper-recombination in an SOS-independent manner, with ΔFRE values of 52, 38 and 27 for D112R, R28D and R28A respectively. The combination of R28D + D112R did not further increase the

Table 4. FRE and SOS effects of amino acid substitutions in the interface of subunit interactions in EcRecA filament.

Amino acid substitutions in the EcRecA protein of recipient[a]	Yield of Ara$^+$Str^r or Thr$^+$Str^r recombinants (% to donors)	Linkage (μ) *ara*$^+$–*leu*$^+$	Linkage (μ) *thr*$^+$–*leu*$^+$	FRE[b]	ΔFRE	SOS	ΔSOS	*P*-value[c]
wt	4.9 ± 0.4	0.986 ± 0.013 (900)	0.924 ± 0.016 (900)	5.0 ± 0.1	1	30.3 ± 3.1	1	
R28A	3.3 ± 0.3	0.734 ± 0.063 (1000)	–	135.1 ± 11.3	27.0	26.3 ± 2.8	0.9	7.25E-13
D112R	2.4 ± 0.2	0.616 ± 0.054 (700)	–	263.1 ± 27.3	52.6	71.0 ± 11.2	2.3	6.82E-10
N113A	2.3 ± 0.3	0.758 ± 0.076 (1200)	–	117.6 ± 12.4	23.5	164.8 ± 20.1	5.4	3.70E-08
K6A	4.8 ± 0.5	–	0.721 ± 0.055 (900)	23.8 ± 2.1	4.8	65.0 ± 7.1	2.1	1.16E-11
K6D	5.2 ± 0.5	–	0.805 ± 0.072 (700)	14.5 ± 1.3	2.9	84.5 ± 9.2	2.8	2.34E-07
R28N	5.6 ± 0.5	–	0.892 ± 0.077 (900)	7.1 ± 0.6	1.4	34.8 ± 3.5	1.1	6.13E-03
R28D	0.8 ± 0.1	0.673 ± 0.061 (400)	–	188.7 ± 17.4	37.8	66.6 ± 6.8	2.2	6.94E-07
T89V	2.0 ± 0.2	0.978 ± 0.012 (900)	–	8.2 ± 0.3	1.6	35.0 ± 3.4	1.2	0.17
T89A	1.8 ± 0.2	0.934 ± 0.083 (900)	–	25.0 ± 2.3	5.0	34.6 ± 3.3	1.1	9.80E-04
D139A	5.3 ± 0.6	–	0.878 ± 0.076 (900)	8.3 ± 0.7	1.7	36.8 ± 3.7	1.2	2.03E-07
D139K	5.5 ± 0.6	–	0.868 ± 0.070 (800)	9.0 ± 0.8	1.8	45.3 ± 3.9	1.5	4.45E-04
K6D/D139K	1.7 ± 0.8	–	0.897 ± 0.096 (300)	6.8 ± 0.8	1.4	39.5 ± 4.2	1.3	0.37
R28D/D112R	1.3 ± 0.8	0.661 ± 0.057 (700)	–	204.1 ± 18.5	40.8	38.5 ± 3.6	1.3	1.90E-07
R28A *mutS215*	3.7 ± 0.3	0.786 ± 0.062 (600)	–	100.0 ± 9.3	20.0	–	–	4.24E-06
D112R *mutS215*	3.9 ± 0.5	0.728 ± 0.050 (800)	–	140.8 ± 11.2	28.2	–	–	2.67E-09
N113A *mutS215*	4.5 ± 0.4	0.825 ± 0.073 (600)	–	76.9 ± 6.4	15.3	–	–	3.90E-07

a. All recipients were of AB1157 line. Mutation *mutS215* was introduced by P1 transduction. Normal *EcRecA*$^+$ gene as well as *EcRecA* genes with appropriate modifications (Eldin *et al*., 2000) were located on plasmids and introduced in AB1157 *ΔrecA*.
b. FRE values were determined in recombinants after crosses of recipients carrying normal and modified *EcRecA* genes with donor KL227.
c. *P*-values for linkage data sets were calculated relative to the appropriate wt *ara*$^+$–*leu*$^+$ or *thr*$^+$–*leu*$^+$ cross.
Sign '–' means that the data were not determined.

FRE value, providing indirect evidence that a ΔFRE = 41–50 may be the maximum possible increase of FRE that is still compatible with cell survival (or that can be documented with this assay). Alternatively, the R28D and D112R mutations may disrupt the same cross–subunit interaction in the active RecA filament (Story *et al*., 1992; Eldin *et al*., 2000; Chen *et al*., 2008) and therefore their effect would not be additive.

The lower part of Table 4 analyses the *mutS*-dependence of hyper-rec events observed for three RecA proteins, bearing R28A, D112R and N113A mutations. In these cases, all of which produce a rather high ΔFRE, the presence of *mutS215* leads to a decrease in their FRE values.

The data indicate that EcRecA, as is probably the case with other RecA proteins, has a number of critical amino acid residues, which modulate the HR recombinogenic activity and optimize it for the conditions prevalent in *E. coli*. The data may also suggest the existence of limits for the level of hyper-rec changes that are tolerated *in vivo*. Most important, the data show that recombinational potential has not been evolutionarily optimized in EcRecA. Small changes in amino acid sequence uncover a high level of recombination function that is normally suppressed, even beyond the limits imposed by regulatory mechanisms.

Biochemical properties of a RecA variant that exhibits a hyper-rec phenotype

The EcRecA D112R mutant protein produces the highest FRE levels observed in our studies to date by a protein that did not also produce a constitutive SOS response. The biochemical properties of this protein are thus of interest. In some or all of the studies below, RecA D112R is compared with wild-type RecA, and with two other mutant RecA proteins that have been previously characterized. One is the RecA ΔC17 protein, a variant that removes the C-terminal 17 amino acids from the protein. These C-terminal amino acids constitute an autoregulatory flap that suppresses many RecA functions (Eggler *et al*., 2003; Lusetti *et al*., 2003a,b; Baitin *et al*., 2006). As already noted, their removal has a substantial effect on FRE (Table 3). The second is the RecA E38K mutant protein (RecA730). Previous characterization has indicated that this variant is particularly robust in its capacity to displace SSB and to bind to both ssDNA and dsDNA (Lavery and Kowalczykowski, 1992). The constitutive SOS response is one result, and the expression of RecA E38K in an *E. coli* cell produces an increase of FRE of approximately sevenfold (Bakhlanova *et al*., 2001).

In addition, the conjugational F plasmid encodes an alternative SSB protein (Jones *et al*., 1992) that may play a role in modulating RecA access to the ssDNA during conjugation. The gene for this SSB variant is transferred to the recipient cell and expressed very early in conjugation. The F plasmid SSB, derived from the plasmid JM101 (monomer M_r = 19 505), functions as a tetramer (M.M. Cox, unpubl. data) as does its *E. coli* counterpart (EcSSB). We have incorporated this SSB protein into our study.

One barrier to RecA protein binding to ssDNA is the SSB protein. On ssDNA, and if added prior to any SSB protein, the D112R mutant protein exhibits about the same ATPase activity as wild-type RecA protein, or slightly less in some experiments (Fig. 2A). When bound to poly(dT) in the absence of any SSB, the RecA D112R protein exhibits a slightly decreased ATPase activity (Fig. 2B). In the presence of poly(dT), RecA filaments are in a dynamic equilibrium in which new RecA filaments are constantly being formed, and existing ones are disassembling (Lusetti and Cox, 2002; Cox, 2004; Cox *et al*., 2008). Thus, the observed ATP hydrolysis often does not reflect complete binding of the DNA substrate. The slight diminution of ATP hydrolysis seen with RecA D112R may reflect a somewhat less persistent DNA binding by this mutant protein, perhaps due to faster dissociation. The effect is relatively small.

The RecA D112R mutant exhibited an enhanced capacity to bind to regions of secondary structure in ssDNA relative to the wild-type protein (Fig. 2C). On M13 ssDNA, the wild-type RecA protein exhibits a level of ATP hydrolysis that is only about 40% of that seen when SSB is later added, as shown in a normalized plot (Fig. 2C). Addition of more wild-type protein has little effect. In contrast, addition of higher amounts of RecA D112R protein produced levels of ATP hydrolysis that were eventually comparable to those observed in the presence of SSB, indicating that the mutant protein was binding to and melting out the regions of secondary structure on its own.

The RecA D112R protein exhibited an enhanced capacity to displace both the EcSSB protein, and the purified SSB protein encoded by the conjugational F plasmid, from the ssDNA (Fig. 2D and E). In both cases, the RecA D112R protein was able to bind the ssDNA faster than the wild-type EcRecA protein or (when tested) the RecA ΔC17 C-terminal truncation mutant. In the case of EcSSB, the advantage of the mutant protein over the wild-type RecA protein is enhanced when the concentration of Mg ion is reduced. This indicates that the D112R mutation may be stabilizing a RecA structural form with an enhanced DNA binding capacity. Notably, the RecA E38K protein bound to ssDNA and displaced the F plasmid SSB protein faster than RecA D112R (Fig. 2E). A similar result has been obtained with EcSSB (data not shown).

Autocatalytic cleavage of LexA protein was only slightly affected by the D112R mutation. If either SSB protein was

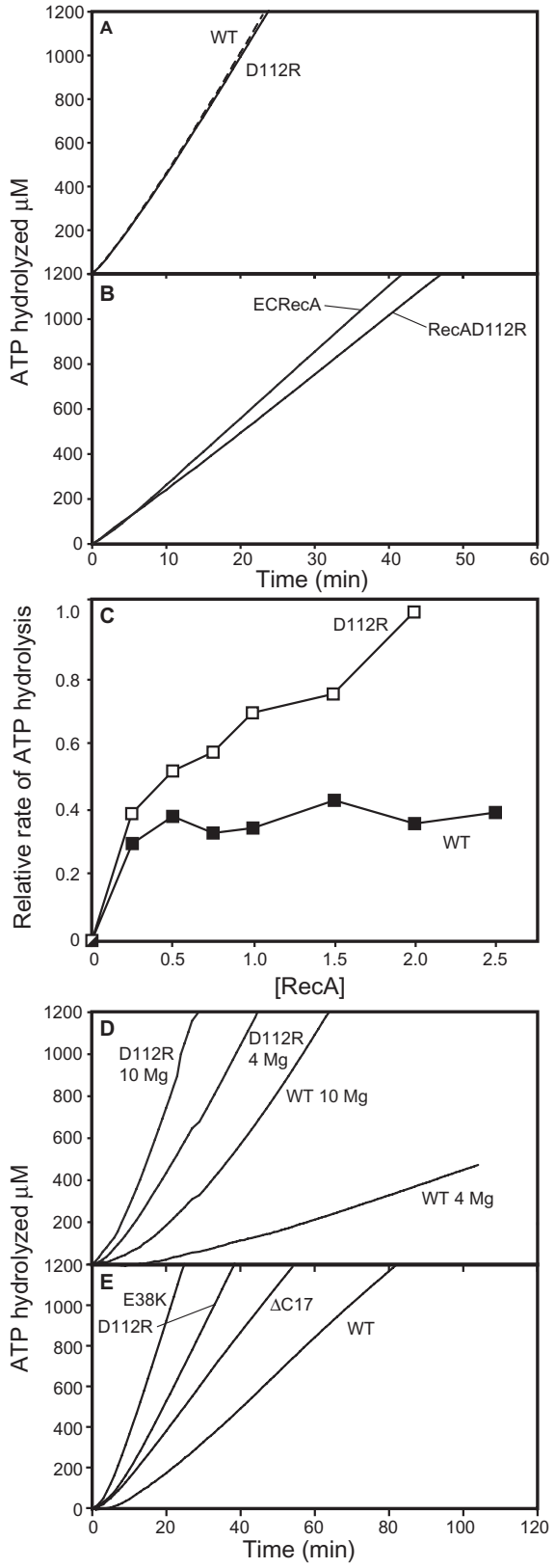

Fig. 2. ATPase activity and effects of SSB. Reactions were carried out as described in *Experimental procedures*.
A. The wild-type and D112R RecA proteins were incubated with M13mp18 ssDNA for 10 min prior to the addition of SSB.
B. ATPase activity of the wild-type and D112R RecA proteins in the presence of 7 μM poly(dT). The wild-type and D112R RecA protein concentrations were 1 μM.
C. ATP hydrolytic activity in the presence of 5 μM M13mp18 ssDNA.
D. The *E. coli* SSB protein (0.5 μM) was incubated with 5 μM M13mp18 ssDNA for 10 min prior to the addition of 3 μM of the indicated RecA protein in the presence of either 4 or 10 mM Mg ion.
E. The SSB protein encoded by the F plasmid (0.5 μM) was incubated with 5 μM M13mp18 ssDNA for 10 min prior to the addition of 3 μM of the indicated RecA protein.

added to the ssDNA prior to addition of a RecA protein, the D112R mutant protein exhibited faster rates of LexA autocatalytic cleavage than the wild-type RecA protein (Fig. 3). This is probably due in large measure to the more rapid binding of the mutant protein to the SSB-coated ssDNA. When RecA was added prior to the SSB, such that full RecA filaments were reliably present prior to LexA addition, the D112R mutant protein cleaved the LexA protein slightly faster than the wild-type protein. However, the effect was modest (Fig. 3). This result indicates that the inherent capacity to enhance LexA cleavage is intact in the D112R mutant protein.

We explored the effect of two negative regulators of RecA protein. In addition to the SSB homologue, the conjugational F plasmid encodes a RecA inhibitor called PsiB, also transferred to the recipient cell very early in conjugation. PsiB binds to free RecA protein and inhibits nucleation onto SSB-coated ssDNA (Petrova *et al.*, 2009). The RecA D112R mutant was less affected by PsiB than

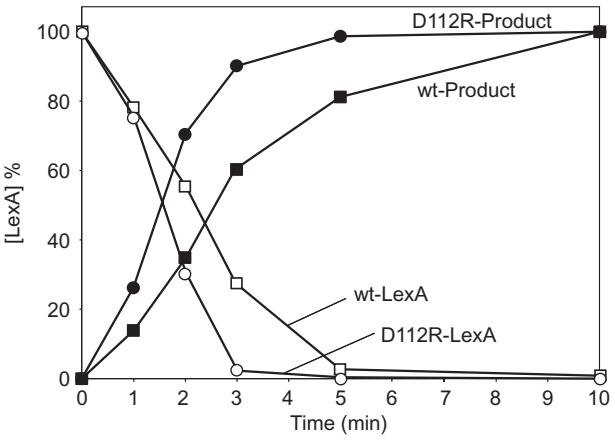

Fig. 3. Autocatalytic LexA cleavage in the presence of RecA D112R. Reactions were carried out under the conditions described in *Experimental procedures*. In the experiment shown, the indicated RecA protein (1 μM) was added to 2 μM M13mp18 ssDNA and incubated at 37°C for 5 min. The *E. coli* SSB (0.2 μM) was then added, followed by incubation for another 5 min. The LexA protein (6 μM) was then added to initiate the reaction.

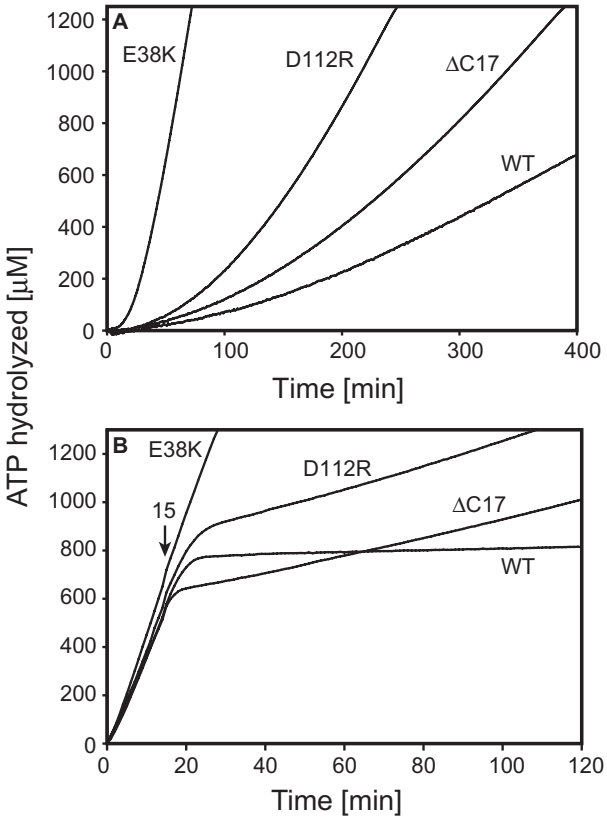

Fig. 4. Effects of RecA protein inhibitors on RecA protein variants.
A. The effects of the PsiB protein. M13mp18 ssDNA (3 μM) was preincubated with EcSSB (0.5 μM) for 5 min at 37°C. PsiB protein (10 μM) was added and the incubation continued for 10 min. The subsequent addition of 2 μM of the indicated RecA variant defines $t = 0$ min on the plot.
B. The effects of RecX protein. In each reaction, the indicated RecA variant protein (2.4 μM) was incubated at 37°C with 4 μM M13mp18 ssDNA for 10 min, followed by addition of 0.4 μM EcSSB and incubation for another 15 min. At $t = 16$ min, each reaction was challenged with 0.1 μM RecX protein. Details of reactions and reaction conditions are provided in *Experimental procedures*.

was the wild-type RecA or the RecA ΔC17 deletion mutant (Fig. 4A). Another RecA regulatory protein, RecX, is encoded on the *E. coli* chromosome immediately downstream of the *recA* gene. RecX acts to halt RecA filament extension (Drees *et al*., 2004), resulting in a slow decline in observed ATPase activity as the filaments dissociate from the uncapped end. The RecA D112R mutant protein was again less affected by the RecX protein than were the wild-type RecA or the RecA ΔC17 deletion mutant (Fig. 4B). In all cases, the capacity of the D112R mutant to resist the effects of the regulators was substantial, but less than that observed with the RecA E38K mutant.

Although RecA D112R displaces SSB and resists inhibition by RecX and PsiB less well than RecE38K, expression of the former protein in *E. coli* produces a FRE value (52.6) that is much greater than that seen in strains expressing the latter (FRE = 7). Acknowledging that this comparison is complicated by the constitutive SOS induction produced by RecA E38K, we nevertheless sought properties that might provide an explanation for the high FRE seen with RecA D112R. We thus directly examined the DNA pairing properties of this mutant protein, employing a Fluorescence Resonance Energy Transfer (FRET) assay to monitor the association of homologous single-stranded and duplex DNA oligonucleotides (see *Experimental procedures*). The work was carried out at 27°C instead of 37°C in order to slow the kinetics sufficiently for convenient measurement. As shown in Fig. 5, the RecA

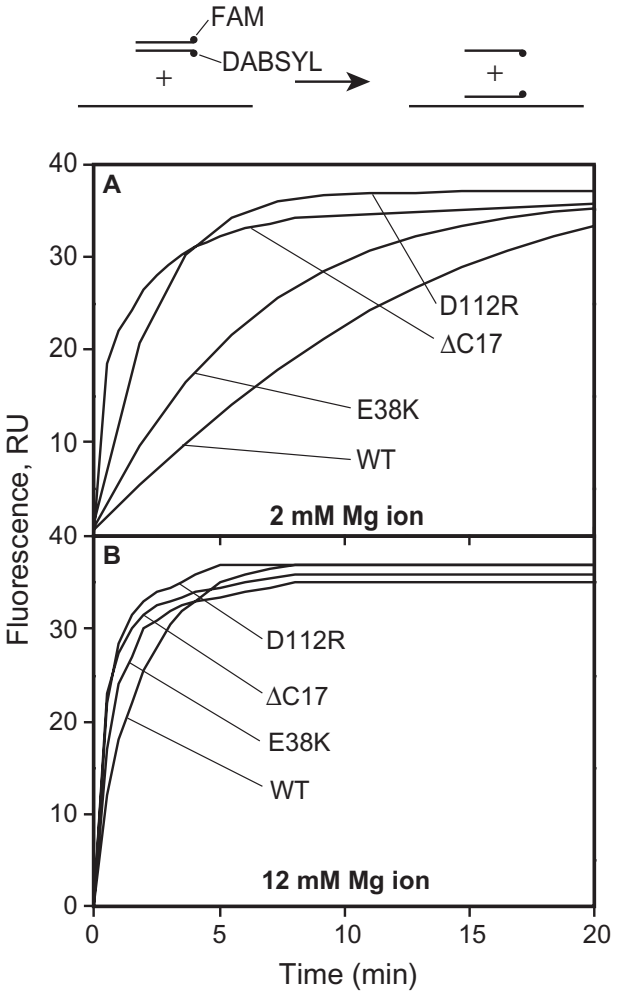

Fig. 5. Enhanced DNA pairing by RecA D112R.
A. Reaction schematic. The DNA substrates are a single strand oligonucleotide (102 nucleotides in length) and a duplex oligonucleotide with the indicated labels, 34 bp in length.
B and C. FRET analysis of the DNA pairing reaction promoted by the indicated RecA protein variant. The ssDNA (3 μM) and the indicated RecA protein variant (1.2 μM) were preincubated at 27°C with 0.3 μM SSB and 0.7 mM ATPγS for 5 min. The DNA pairing reaction was initiated by addition of the labelled duplex oligonucleotide substrate (1.5 μM) at $t = 0$ min. Details of the reactions and reaction conditions are provided in *Experimental procedures*.

D112R protein pairs the oligonucleotides substantially faster than the wild-type RecA protein. The effect is much more prominent at lower concentrations of Mg ion. As already noted, unphysiological levels (8–10 mM) of free Mg ion are generally required in wild-type RecA protein strand exchange reactions *in vitro* in order to produce an efficient reaction (Cox and Lehman, 1981; Lusetti *et al*., 2003a). This requirement reflects the constraining effects of the RecA C-terminus (Lusetti *et al*., 2003a). Such high levels of free Mg ion are unlikely to be encountered *in vivo*, and it is presumed that the activity of RecA in cells is somehow activated as needed by regulatory proteins. The results of Fig. 5 indicate that the RecA D112R protein is more proficient at DNA pairing at significantly lower levels of free Mg ion. As the RecA E38K mutant displaces SSB from ssDNA faster than does RecA D112R or RecAΔC17, but the latter two proteins are more proficient at DNA pairing, we infer that RecA filament formation is not the sole determinant of RecA recombination activity. Instead, substantial increases in the inherent DNA pairing activity of RecA protein are possible.

Discussion

This work leads to two major conclusions. Our first and primary conclusion is that the RecA protein can be viewed as a recombinase platform with unrealized recombination potential. The overall recombinational potential of bacterial cells is affected by many factors. Among these, the recombination function inherent to the RecA protein itself is particularly important. Individual point mutations in RecA can have a profound effect on observed recombination frequencies *in vivo*, substantially greater than those revealed by releasing RecA from the constraints imposed by any known regulatory mechanism. Evolution can increase or decrease this potential over a large range according to the recombinational DNA repair requirements of the bacterial species in question. Inasmuch as many of the amino acid residues examined in this study are highly conserved in bacterial RecA proteins, the expression of an optimized RecA recombination potential may be deleterious in most bacterial species. Recombination potential can be suppressed by inactivating RecA or any of several other recombination functions that enable RecA, such as RecBCD or RecFOR. We have not yet assessed the effects of RecBCD mutations in this system. However, it is clear that substantial increases in cellular recombination that may be desired for biotechnology can be realized with alterations in RecA. The use of appropriate RecA mutant proteins may complement or provide an alternative approach to some classical methods for stimulating recombination, such as the introduction of a directed double strand break.

A second conclusion is that the recombination potential of RecA has at least two main facets. First, the protein must form nucleoprotein filaments on the ssDNA, and recombination can be enhanced if filament formation is rendered more efficient. However, it is not enough to simply get RecA filaments onto the DNA. The second facet is the inherent capacity of the nucleoprotein filament to pair homologous DNA molecules and promote DNA strand exchange – particularly at low concentrations of Mg ion, which can be enhanced by C-terminal deletions and by particular point mutations. The RecA D112R mutant RecA protein provides an example of increased DNA pairing capacity that may be relevant to recombination potential in an *in vivo* environment.

Additional but generally smaller changes can be brought about by altering the interaction of RecA with regulators or altering the regulators themselves. The study of proteins that regulate intracellular recombinase activity is still in its early stages. Genetic data obtained earlier (Namsaraev *et al*., 1998; Eldin *et al*., 2000; Chervyakova *et al*., 2001; Lanzov *et al*., 2003; Baitin *et al*., 2008) and those presented here highlight the complexity of the network of proteins that regulate HR activity. First, the *mutS*-dependent heteroduplex correction system at least partially alters the genetic consequences of real HR, lowering (in the EcRecA or PaRecA types of proteins) or increasing (in some strains) the natural FRE values, which we use to measure the *in vivo* recombinase activity of given cells (Baitin *et al*., 2008). Second, the RecFOR complex clearly has an effect on recombination events that are otherwise handled by the RecBCD pathway of recombination (Table 1). Third, even under normal unstressed conditions, the DinI protein appears to be a moderate suppressor (60%) of HR activity *in vivo*. When overexpressed, DinI inhibits FRE significantly (about eight times, Table 2). We could not detect a similar effect for the RecX protein. The DinI protein binds within the RecA filament groove, and – while stabilizing RecA filaments – it also constrains the reactions that can be carried out by those filaments (Lusetti *et al*., 2004a). Fourth, as expected from previous observations (Eggler *et al*., 2003; Lusetti *et al*., 2003a,b), the C-terminus of RecA limits the HR activity measured through FRE values. As is the case with RecA D112R, the deletion of the RecA C-terminus reduces the requirement of the protein for free Mg ion to produce efficient DNA strand exchange *in vitro* (Lusetti *et al*., 2003a). Fifth, the cellular recombination potential (as measured by the FRE assay) can be increased up to ~50-fold by a single mutation in a set of highly conserved amino acid residues in RecA protein. One group of these residues is located at the interface of subunit interactions in the RecA filament (Table 4). Together with the negatively

charged C-terminus, these critical residues provide a RecA protein structure that is apparently optimized for its host.

There are some other proteins that have a role in HR regulation, but are less studied. These include the helicase UvrD that can disrupt RecA filaments (Mendonca *et al*., 1993; Flores *et al*., 2005; Veaute *et al*., 2005; Centore and Sandler, 2007), and the PsiB (Bailone *et al*., 1988; Bagdasarian *et al*., 1992; Petrova *et al*., 2009) and RdgC (Moore *et al*., 2003; Drees *et al*., 2006) proteins. Additional regulatory proteins, acting not only on RecA but on other aspects of HR, may remain to be discovered.

The RecA D112R mutant produces a hyper-rec phenotype *in vivo*. Certain mutations on the same interface as D112R are known to resist inhibition by the UmuD′$_2$C complex *in vivo* (UmuR mutants) (Sommer *et al*., 1993), offering one potential explanation for the phenotype. However, the *recA D112R* mutant displays only a limited tendency to induce the SOS response under normal growth conditions. Thus, it is unlikely that increased resistance to UmuD′$_2$C-mediated inhibition of recombination contributes to the hyper-rec phenotype, given that other UmuR mutants found in this area of the RecA protein arose in the presence of elevated levels of the UmuD′$_2$C complex that would normally only be present after SOS induction (Sommer *et al*., 1993). The D112R mutant protein binds to ssDNA and displaces SSB proteins with more facility than the wild-type RecA protein, but we do not believe this accounts for the elevated recombination potential we observe. There are clearly RecA mutant proteins, notably RecA E38K, that bind to DNA and displace SSB better than the D112R mutant, but that produce a more modest effect on FRE. Based on the work reported here, we hypothesize that an enhanced DNA pairing capacity in this mutant protein is the most important contributing factor. When the wild-type RecA protein is bound to ssDNA in the presence of ATP, it forms an active filament with a state we have labelled the A state. Two different forms of the A state can be defined functionally. When there is no Mg ion present in excess of that required to chelate the ATP, a form of the A state exists (called Ac, or closed), which has much reduced competency for DNA pairing (Haruta *et al*., 2003). For many decades, an artificial addition of 8–10 mM free Mg ion has been used *in vitro* to generate a more active filament form that promotes efficient DNA strand exchange (Cox and Lehman, 1981; Shan *et al*., 1996; Lusetti *et al*., 2003a). We call the state produced with high Mg ion the Ao or the open form of the A state (Haruta *et al*., 2003). Even this filament state is only intermediate in its pairing potential, as a more robust pairing state we have defined as the P state can be detected (Haruta *et al*., 2003). All of these states have been defined functionally, and the structural changes underlying them – possibly subtle – have not been described. We speculate that an arginine residue at position 112 in the RecA backbone may stabilize a RecA filament form that is closer to the Ao or P-like states than can be achieved by the wild-type protein under *in vivo* conditions. The advantage in DNA pairing observed with this mutant protein is especially significant in the presence of Mg ion concentrations likely to be representative of those found *in vivo*.

Residue 112 is in a loop between the D and E α-helices in the RecA structure (Story *et al*., 1992; Chen *et al*., 2008). This residue is not highly conserved among bacterial RecA proteins (Roca and Cox, 1990; Karlin and Brocchieri, 1996; Brendel *et al*., 1997), and other mutations at this position are tolerated in EcRecA protein without eliminating recombination function (McGrew and Knight, 2003). This short loop may be one region of the structure where evolutionary adjustments in recombination potential are possible without compromising the structure of the core RecA domain.

It is intriguing that some mutations that facilitate RecA filament formation in the presence of SSB [e.g. E38K (Lavery and Kowalczykowski, 1992)] while others do not (RecA D112R). There may be a threshold of RecA filament formation that is required for SOS induction (Gruenig *et al*., 2008). Alternatively, SOS induction may be affected by mutations that affect the interaction of other proteins that bind to the RecA filament groove (Cox, 2007a), potentially blocking access by LexA.

The well-studied EcRecA protein has been used as a reagent in a wide range of applications in biotechnology [e.g. see (Ferrin and Camerini-Otero, 1991; 1998; Szybalski, 1997; Zhumabayeva *et al*., 1999; 2001; Wang *et al*., 2006)]. Only a few RecA-based technologies are widely used, and many attempts to use RecA for genomic engineering in various species have had limited success. As noted above, the recombination activity of RecA in wild-type *E. coli* cells may be quite dependent on the function of augmenting regulatory proteins that are not easily supplied in a heterospecific genomic alteration trial. New attempts to harness RecA in biotechnology might productively explore the untapped potential represented by a range of RecA point mutants that enhance one or more RecA functions.

Experimental procedures

Strains and plasmids

Donor KL227 (HfrP4x *metB*) and recipients: AB1157 (*thr-1 leuB6 ara14 proA2 hisG4 argE3 thi-1 supE44 rpsL31*) and recombination-deficient JC10289 (as AB1157 but *Δ[recA-srlR306]::Tn10* = *ΔrecA306*) were from A.J. Clark's collection.

rec- and *mut*-deficient strains were constructed by P1 transduction to transfer *recO1504::Tn5*, *recR252*::*Tn10-9*, *recF349*(del) and *mutS215::Tn10* mutant genes into AB1157 as described previously (Baitin *et al*., 2006; 2008). Strains AB1157-X (Stohl *et al*., 2003) and AB1157-I (Yasuda *et al*., 1998) were constructed and kindly supplied by E.A. Stohl. Plasmids precX [original name pEAW224 (Drees *et al*., 2004)] and pdinI [original name pEAW334 (Drees *et al*., 2004)] were constructed and kindly supplied by E.A. Wood. These plasmids contain *recX* and *dinI* genes under a promoter controlled with the T7 RNA polymerase. Plasmid pT7 [original name pT7POL26 (Mertens *et al*., 1995)] codes for T7 RNA polymerase under the control of a *lac* promoter. This plasmid was used to overproduce the wild-type RecX or DinI proteins under conditions of *lac* promoter induction by IPTG (0.2 mM). Normal *EcRecA* and *PaRecA* genes (Namsaraev *et al*., 1998) as well as truncated genes *recAΔ17Ec* (Lusetti *et al*., 2003b) and recA*Δ11Pa* were located on plasmids and introduced into strain AB1157 Δ*recA*. Plasmid precA*Δ11Pa* was constructed on the base of pEAW337(39) via the displacement of *recAΔ11Ec* with *recAΔ11Pa*. Plasmids with amino acid substitutions in the interface of subunit interactions in the EcRecA filament were constructed in K.L. Knight's laboratory (Eldin *et al*., 2000). Plasmid p200F′-lac was used to standardize conjugation abilities of recipient strains.

All of the plasmids expressing RecA protein or RecA protein variants use the same pTRecA430 plasmid and its *tac* promoter for expression (Eldin *et al*., 2000). RecA protein levels in all of the strains used in Table 2 were measured by Western blot analysis and found to be identical within experimental error (data not shown). RecA protein levels in all of the strains used in Table 4 were measured previously (Skiba and Knight, 1994) and found to be identical.

The F′SSB protein was obtained from plasmid JM101. The gene was amplified by PCR using an upstream primer consisting of a NdeI site and the first 25 bases of the F′SSB gene. The ATG bases in the NdeI site are the first bases of the F′SSB gene. The downstream primer consisted of a EcoRI site followed by the last 18 bases of F′SSB. The PCR product was digested with NdeI and EcoRI and inserted into the overproduction vector pET21A (Novagen) cut with the same restriction enzymes. The resulting F′SSB expression plasmid was designated pEAW422. The presence of wt F′SSB was confirmed by direct sequencing.

Proteins

The wild-type *E. coli* RecA (Shan *et al*., 1996; Petrova *et al*., 2009), RecA ΔC17 (Lusetti *et al*., 2003b), RecA E38K (Gruenig *et al*., 2008) and SSB (Hobbs *et al*., 2007) proteins were purified as previously described. Their concentrations were determined using native extinction coefficients: $\varepsilon_{280} = 2.23 \times 10^4$ M^{-1} cm^{-1} for all RecA protein variants (Craig and Roberts, 1981), and $\varepsilon_{280} = 2.38 \times 10^4$ M^{-1} cm^{-1} for SSB protein (Lohman *et al*., 1986). Antibodies raised against the purified RecX and DinI proteins were from 'Genetel Lab' (Madison, Wisconsin, USA).

Purification of RecA D112R. Escherichia coli cells expressing RecA D112R, STL2669 pEAW 551 (DE3), were grown to an OD_{600} of 0.5–1.0. IPTG was added to a final concentration of 0.4 mM. The cells were harvested and lysed in a 25% w/v sucrose solution with 2.5 mg ml^{-1} lysozyme and 10 mM EDTA with 5 rounds of 60% output sonication. Cell debris was removed and separated from the crude extract by centrifugation at 38 4000 *g* for 1 h. DNA and DNA-bound proteins were subsequently precipitated from the crude extract by adding 0.111 ml of 50% (w/v) polyethyleneimine to every millilitre of lysis supernatant. After precipitation by ammonium sulphate to 50% saturation, the RecA mutant protein was purified by chromatography on DEAE sepharose, Q sepharose, ceramic hydroxyapatite and Sephacryl S-300 gel filtration columns. The final purified RecA D112R was greater than 95% pure and free of detectable nuclease activity. The extinction coefficient used to calculate the concentration of the RecA D112R was $\varepsilon_{280} = 2.23 \times 10^4$ M^{-1} cm^{-1}.

Purification of the F′SSB protein. Escherichia coli cells expressing the F′SSB protein, BL21(DE3)/pT7pol26, were grown to an OD_{600} of 0.5–1.0. IPTG was added to a final concentration of 0.4 mM. The cells were harvest after 3 h and lysed in 10% w/v sucrose solution with 0.24 mg ml^{-1} lysozyme, 1.0 mM EDTA, 200 mM NaCl, 15 mM spermidine trichloride with thorough sonication. Cell debris was removed and separated from the crude extract by centrifugation at 38 400 *g* for 1 h. The cleared lysate was precipitated with 0.15 mg ml^{-1} saturation with ammonium sulphate and the protein was found in the pellet. The F′SSB protein was purified further by chromatography on heparin sepharose, and ceramic hydroxyapatite columns. Final purified F′SSB was greater than 95% pure and free of detectable nuclease activity. The extinction coefficient used to calculate the concentration of F′SSB, determined as described (Drees *et al*., 2006), was $\varepsilon_{280} = 2.22 \times 10^4$ M^{-1} cm^{-1}.

ATP hydrolysis (ATPase) assays

A coupled enzyme, spectrophotomeric assay (Morrical *et al*., 1986; Lindsley and Cox, 1990) was used to measure RecA-mediated ATP hydrolysis. The ADP generated by hydrolysis was converted back to ATP by a regeneration system of pyruvate kinase and phosphoenolpyruvate (PEP). The resultant pyruvate was converted to lactate by lactate dehydrogenase using NADH as a reducing agent. The conversion of NADH to NAD^+ was monitored as a decrease in absorbance at 380 nm. The amount of ATP hydrolysed over time was calculated using the NADH extinction coefficient $\varepsilon_{380} = 1.21$ mM^{-1} cm^{-1}. The assays were carried out in a Varian Cary 300 dual beam spectrometer, with a temperature controller and a 12-position cell changer. The path length was 0.5 or 1 cm, the band pass was 2 nm. All ATPase assays contained a reaction solution of 25 mM Tris.OAc (pH 7.5, 88% cation), 10 mM MgOAc (except where noted), 3 mM potassium glutamate, 5% w/v glycerol, 1 mM dithiothreitol, 3 mM PEP, 10 U ml^{-1} pyruvate kinase, 10 U ml^{-1} lactate dehydrogenase, 4.5 mM NADH and 5 μM M13mp18 cssDNA.

DNA pairing assay using FRET

A linear dsDNA-oligo, 34 bp in length, was labelled on opposing strand ends with a fluorescein and dabsyl pair. The oligo-

nucleotide sequence was FAM-TCACCAATGAAACCATC GATAGCAGCACCGTAAT and ATTACGGTGCTGCTATCGA TGGTTTCATTGGTGA-Dabsyl. This was reacted with a 102 nucleotide linear unlabelled ssDNA, with a sequence equivalent to three tandem repeats of the dabsyl-labelled strand of the duplex oligonucleotide. Reactions were carried out at 27°C in a 0.3 ml cuvette, and contained 25 mM Tris HCl (pH 7.5), the indicated concentration of $MgCl_2$, 3 μM ssDNA oligonucleotide, 1.2 μM of the indicated RecA protein variant, 0.3 μM SSB, 0.7 mM ATPγS, and 1.5 μM labelled duplex oligonucleotide. RecA protein filaments were formed on the ssDNA during a 5 min preincubation in the presence of the ATPγS and SSB protein. Pairing was then initiated by adding the labelled duplex DNA. Fluorescence changes were monitored with a Hitachi F-4000 fluorometer.

FRE measurement

Conjugation was carried out essentially as described (Lanzov *et al.*, 2003). Both Hfr and F^- strains were grown, crossed and selected for recombinants at 37°C in mineral salts 56/2 medium supplied with all necessary growth factors at pH 7.5. The ratio between donors and recipients in the mating mixture was 1:10, $2–4 \times 10^7$ donors and $2–4 \times 10^8$ recipients per 1 ml. The yield of Thr^+Str^r and Ara^+Str^r recombinants in all independent crosses (5–7% relative to donors) was normalized according to the mating ability of each recipient used. The latter was determined by the yield of transconjugants F′-*lac*$^+$ in crosses between the recipients and donor P200 F′-lac.

FRE value calculations were carried out as described (Lanzov *et al.*, 2003). Alterations in FRE (ΔFRE) promoted by the normal and truncated *PaRecA* gene or by the *EcRecA* gene with appropriate modifications relative to the FRE value promoted by the wild-type *EcRecA* gene were calculated using the following formula: $\Delta FRE = \ln(2\mu_1 - 1)/(2\mu_2 - 1)$, where μ_1 is the linkage of selected *thr*$^+$ or *ara*$^+$ and unselected *leu*$^+$ markers in a cross using wild-type *E. coli* strain AB1157 and μ_2 is the similar linkage in the cross being analysed. Calculations of uncertainty of relative FRE values were determined as deviations from the average values by making use of the program Excel-97 with formula [= 2*STDEV] and by inputting the values from independent repeats of three experiments. Student's *t*-tests (two-tailed, type 3) were used to calculate *P*-values comparing the indicated linkage data sets in each Table.

Determination of intracellular RecX and DinI amounts

These were done in both the control and experimental (AB1157pT7pdinI and AB1157 pT7precX) strains. *E. coli* cells were grown up to mid-log phase in Luria–Bertani medium at 37°C.

A cell pellet containing 5×10^7 cells was lysed by boiling with sodium dodecyl sulphate, electrophoresed through sodium dodecyl sulphate-10% polyacrylamide gels. The RecX and DinI amounts were detected by immunoblotting using polyclonal chicken antibodies to these proteins from 'Genetel Lab' (Madison, Wisconsin, USA) in a standard procedure described earlier (Baitin *et al.*, 2006). Primary antibody binding was visualized with secondary antibodies coupled to horseradish peroxidase (Genetel Lab). The bands were scanned by the program 'Kodak Digital 1D' and the amount of proteins were documented by the use of program TotalLab. The data of two independent experiments were averaged.

Miscellaneous

Spontaneous SOS gene expression was measured with β-galactosidase assay as described earlier (Bakhlanova *et al.*, 2001) using the strain GY7109 *sfiA::lacZ ΔrecA* carrying appropriate plasmids (Eldin *et al.*, 2000).

LexA cleavage was measured as described (Gruenig *et al.*, 2008).

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