Differential cross-complementation patterns of *Escherichia coli* and *Neisseria gonorrhoeae* RecA proteins

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The *Escherichia coli* RecA protein is one of the best-studied enzymes, but less is understood about how RecA homologues of other species are similar to or different from the *E. coli* RecA. In the Gram-negative pathogen *Neisseria gonorrhoeae* (the gonococcus; Gc), the causative agent of gonorrhoea, RecA is involved in DNA transformation, pilin antigenic variation, and DNA repair. By expressing the *recA* genes from Gc and *E. coli* under control of *lac* regulatory sequences in *E. coli*, the authors have shown that the Gc RecA fully complements an *E. coli* *recA* mutant for homologous recombination, but only partially complements for survival to DNA damage. By expressing similar constructs in Gc, it was shown that the *E. coli* RecA complements for pilin antigenic variation, partially complements for DNA transformation, but does not complement for survival to DNA damage, suggesting that species-specific interactions are important for DNA repair, but not for homologous recombination. Co-expression of the *E. coli* *recA* and *recX* genes in Gc suggests that in this heterologous system RecX modulates RecA-mediated processes.

Keywords: DNA repair, pilus antigenic variation, homologous recombination, *recX*

INTRODUCTION

Homologous recombination is an essential function in all organisms. It is central to both sexual and asexual genetic exchange, maintenance of chromosomal integrity, DNA repair and DNA replication. In *Escherichia coli*, RecA has a pivotal role in homologous recombination mediated by either the RecBCD or the RecF pathway. RecA is also integral to the repair of damaged DNA and coordinates error-prone DNA repair via SOS mutagenesis through interactions with several other proteins (Cox, 1993; Kowalczykowski *et al.*, 1994). The generation of a battery of mutant *E. coli* *recA*s in conjunction with the elucidation of the *E. coli* RecA crystal structure (Story *et al.*, 1992) has allowed the assignment of functional domains and residues to the RecA protein. Further understanding of RecA protein structure and function has been enhanced by both functional complementation assays and sequence comparisons of *recA* homologues from other organisms (Miller & Kokjohn, 1990).

The obligate pathogen *Neisseria gonorrhoeae* (Gc) expresses type IV pili, a virulence factor that mediates the initial attachment of the bacterium to the host epithelium. Pili are essential for full infectivity (Kellogg *et al.*, 1963; Swanson *et al.*, 1987) and are required for full DNA transformation competence (Seifert *et al.*, 1990; Sparling, 1966). The Gc pilus is mainly composed of the *pilE* gene product, pilin (Swanson *et al.*, 1971), and antigenic variation of the pilus occurs when the sequence of *pilE* is altered, leading to changes in the amino acid sequence of the pilin protein (Virji & Heckels, 1983). Antigenic variation of the pilus can also result in changes in pilus-dependent colony morphology (Long *et al.*, 1998). Therefore, changes in gonococcal colony morphology can be used to estimate the frequency of pilin antigenic variation (Haas & Meyer, 1986; Swanson *et al.*, 1986).

Pilus antigenic variation is a RecA-dependent process (Koomey & Falkow, 1987; Koomey *et al.*, 1987). The genome of Gc contains multiple, incomplete, silent copies of the pilin gene (*pilS*), portions of which are recombined unidirectionally into the expressed *pilE* gene (Haas & Meyer, 1986; Hagblom *et al.*, 1985).

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Abbreviation: Gc, *Neisseria gonorrhoeae*.

Molecular models for these unique recombination reactions propose a *pilE/pilS* hybrid intermediate, which has a crossover at a small region of conserved sequence (Howell-Adams *et al.*, 1996) and targets recombination with the recipient *pilE* gene (Howell-Adams & Seifert, 2000). The *recA* gene of Gc was identified by its ability to partially complement an *E. coli* *recA* mutant deficient in both homologous recombination and DNA repair functions (Fyfe & Davies, 1990; Koomey & Falkow, 1987). The Gc RecA protein is 65% identical and 81% similar to the *E. coli* RecA protein and shows significant sequence similarity to other RecA proteins. Sequence comparisons of RecA proteins from many bacteria have revealed the carboxy terminus of RecA to be the most divergent portion of the protein (Miller & Kokjohn, 1990), and it is this portion of RecA that is the most variable between the Gc and *E. coli* RecA proteins (Fyfe & Davies, 1990).

In Gc, the RecA protein is essential for DNA transformation, DNA repair and pilus antigenic variation (Koomey & Falkow, 1987; Koomey *et al.*, 1987). In Gc, genetic transfer of chromosomal markers is achieved through DNA transformation, whereas in *E. coli* transfer of chromosomal genes is accomplished through transduction or conjugation. However, in both bacteria these processes are dependent on the RecBCD pathway of homologous recombination (Kowalczykowski *et al.*, 1994; Mehr & Seifert, 1998), and DNA transformation in Gc is also affected by mutation of *recN* (Skaar *et al.*, 2002). In Gc, efficient DNA repair requires genes of the RecBCD pathway (*recB*, *recC*, *recD*) and the RecF-like pathway (*recJ*, *recN*, *recO* and *recQ*) (Hill, 2000; Mehr & Seifert, 1998; Skaar *et al.*, 2002). In contrast, mutations in *E. coli* *recD* or *recQ* do not affect DNA repair (Kowalczykowski *et al.*, 1994). Additionally, the SOS system of DNA repair appears to be absent in Gc (Black *et al.*, 1998). Thus, the mechanisms utilized for horizontal chromosomal DNA transfer in Gc and *E. coli* appear to be similar, but DNA repair mechanisms diverge more between these two bacteria.

Pilus antigenic variation is a Gc-specific, *recA*-mediated cellular process. In addition to the RecA protein, the RecF-like pathway is essential for pilus antigenic variation (Mehr & Seifert, 1998; Skaar *et al.*, 2002; Hill & Grant, 2002), and conflicting reports suggest either that the RecBCD pathway is not required for efficient antigenic variation (Mehr & Seifert, 1998; Hill & Grant, 2002), or that it plays an inhibitory role in mediating colony phenotype switches (Chaussee *et al.*, 1999). Finally, the RdgC protein is involved in pilus antigenic variation and cell morphology (Mehr *et al.*, 2000).

A gene designated *recX* has been identified downstream of *recA* in many organisms, and in some instances the coding regions for the *recA* and *recX* genes overlap (De Mot *et al.*, 1994; Yang *et al.*, 2001). In mycobacteria and *Streptomyces lividans* the genes are co-transcribed (Papavinasundaram *et al.*, 1997; Vierling *et al.*, 2000). Further work has led to the hypothesis that RecX acts by downregulating RecA activity (Sano, 1993;

Papavinasundaram *et al.*, 1998; Vierling *et al.*, 2000), though this hypothesis has not been directly tested, and a *recX* mutation in *S. lividans* does not affect any RecA-mediated processes or RecA expression levels. *E. coli* contains a RecX homologue located downstream of *recA*, designated *oraA* (Zaitsev *et al.*, 1994). Due to the established nomenclature of other RecX homologues, we will refer to the *E. coli* *oraA* as *recX* in this paper. No work has to our knowledge been published detailing the role of the *E. coli* *recX* in DNA recombination and repair. However, in Gc the *recX* gene is located in a different chromosomal locus than *recA*, and a *recX* mutant exhibits decreases in the RecA-mediated phenotypes of DNA repair, DNA transformation and pilus antigenic variation (Stohl & Seifert, 2001).

If the different cellular processes catalysed by RecA require specific interactions with other proteins or involve unique biochemical properties of the RecA proteins themselves, then expression of RecA from a different species may not result in full complementation. Furthermore, since DNA repair may occur through different mechanisms in Gc and *E. coli* and since pilus antigenic variation is a process unique to Gc, it would be interesting to determine whether expression of *E. coli* *recA* in Gc could mediate each of these cellular processes. To test these hypotheses we expressed the *E. coli* and Gc *recA* genes in both organisms under the control of the *lac* regulatory system. In each organism both RecA proteins were able to act as recombinases, but they differed markedly in their ability to mediate DNA repair. Studies of three strains of Gc expressing the *E. coli* *recA* alone, an *E. coli* *recA* genomic clone that additionally contains the *E. coli* *recX* gene, or *E. coli* *recA* and overexpressed *E. coli* *recX* on separate transcripts revealed differential RecA phenotypes that were reflected by varying RecA protein levels.

METHODS

Bacterial strains and growth conditions. *E. coli* strain DH5 α (Gibco-BRL), used to propagate plasmids, and all other *E. coli* strains, were grown on Luria-Bertani (LB) broth or agar at 37 °C unless otherwise indicated. Minimal medium (M9) agar was prepared according to Miller (1992). *E. coli* strains LB1 and LB2, Δ *recA* derivatives of AB1157 (DeWitt & Adelberg, 1962), and DM49 (Mount *et al.*, 1972), were constructed by transducing AB1157 and DM49 to Tet^R with P1 phage grown on JC10289 (Csonka & Clark, 1979) to introduce the Δ (*recA*–*srl*)306::Tn10 marker. Gc strains were grown on Gc Medium Base (Difco) plus Kellogg supplements (GCB) (22.2 mM glucose, 0.68 mM glutamine, 0.45 mM co-carboxylase, 1.23 mM Fe(NO₃)₃; all from Sigma) (Kellogg *et al.*, 1963) at 37 °C in 5% CO₂, or in Gc Liquid (GCB) medium [1.5% protease peptone no. 3 (Difco), 0.4% K₂HPO₄ (Fisher), 0.1% KH₂PO₄ (Fisher), 0.1% NaCl (Fisher)] with Kellogg Supplements and 0.042% sodium bicarbonate (Sigma) at 37 °C with rotation. All gonococcal strains used in this study were derivatives of strain FA1090 1-81-S2 with matched *pilE* sequences (Seifert *et al.*, 1994) determined as described by Stohl & Seifert (2001). Antibiotics were added at the following concentrations. For *E. coli*: chloramphenicol,

Fig. 1. Maps of *recA* alleles. *lacI*^Q encodes the Lac repressor, *OPOP* represents the dual *tac* and *UV5* operator and promoter sequences, and RBS indicates the ribosome-binding site. Replacement of the *EcoRI* restriction site with a *PacI* site, and creation of a new site by *BglII/BamHI* ligation, is indicated (see Methods). The *recA6* allele was previously described (Seifert, 1997). In Gc, constructs are carried in either the Gc *recA* or the Cg8 locus, as indicated. In *E. coli*, alleles *recAX*_{Ec} and *recA6* are carried on the pACYC184 derivative pVD300 (Kookey & Falkow, 1987).

30 mg l⁻¹; erythromycin (Erm), 275 mg l⁻¹; kanamycin, 40 mg l⁻¹. For Gc: Erm, 2 mg l⁻¹; spectinomycin (Spc), 50 mg l⁻¹; nalidixic acid (Nal), 1.25 mg l⁻¹. IPTG (Diagnostic Chemicals) was supplied at either 1.5 mM (for *E. coli*) or 1 mM (for Gc) in solid agar or liquid medium.

DNA manipulations and analysis. Standard procedures were performed as described by Sambrook *et al.* (1989). Plasmid DNA was isolated from strains of *E. coli* using Qiagen plasmid kits. DNA was isolated from agarose gels using Qiaquick gel extraction kits (Qiagen). Enzymes were used according to the manufacturers' directions (Promega and New England Biolabs). *E. coli* strains were transformed by electroporation using the Gene Pulser II Electroporation System (Bio-Rad) according to the manufacturer's specifications. For Southern blot analysis, DNA was transferred to Magnagraph nylon membrane according to the manufacturer's specifications (Micro Separations). Labelling of digoxigenin-labelled dUTP probes, hybridization, and chemiluminescent blot development were performed using the Genius kit (Roche Pharmaceuticals) according to the manufacturer's instructions. Sequencing reactions were performed using the Big-Dye Terminator Cycle Sequencing kit (Perkin-Elmer) and sequencing products were separated on an ABI model 377 automated DNA sequencer. DNA sequences were aligned and compiled using Lasergene software (DNASTAR) and VectorNTI software (Informax).

Construction of plasmids pVD300*recAX*_{Ec} and pEAS3/*recA*_{Ec} and expression from the Gc *recA* locus. The *E. coli recA/recX* genomic region was amplified from plasmid pERA (R. D. Porter, unpublished) by PCR using primers M13REV (5'-CAGGAAACAGCTATGACCATGATTAC-3') and ECRECANOTFOR (5'-GGCGGCCGAGGAGTAAAAATGGCTATC-3'), which introduces a *NotI* site (underlined), digested with *BamHI* and *NotI*, and cloned between the *NotI* and *BglII* sites of pVD300NL1 (Seifert, 1997) to replace the Gc *recA* gene. The insert was sequenced to verify that no errors had been introduced by PCR. A *NotI* fragment from plasmid pHSX-*tetM-lacIOP* containing the tetracycline-resistance gene (*tetM*) and *lac* regulatory elements was inserted into the single *NotI* site of the construct created above to yield pVD300*recAX*_{Ec} (Fig. 1).

Plasmid pEAS3 was constructed to allow simple, one-step, directional cloning of genes under control of *lac* regulatory elements for recombination into the *recA* locus of Gc. Plasmid pHSX-*tetM-lacIOP* was digested with *EcoRI*, and the ends were blunted with Klenow and ligated to a *PacI* linker that was digested with *PacI* and religated to create vector pEAS1. pEAS1 was subsequently digested with *BglII*, the ends were

treated with mung bean nuclease, and the vector was religated to destroy the *BglII* site, yielding construct pEAS2. pEAS3 was created by inserting the *NotI* fragment from pEAS2, which contains the tetracycline-resistance gene (*tetM*) and *lac* regulatory elements, into the single *NotI* site of pVD300NL1.

The *E. coli recA* gene was amplified from plasmid pERA using primers ECRECAFORPAC (5'-GACTTTAATTAAGGAGTAAAAATGGCTATC-3'), which introduces a *PacI* site (underlined), and ECRECAREVBGL (5'-AGTGAGATCTGATTCTGTCATGGCATATCC-3'), which introduces a *BglII* site (underlined) by PCR using *Pfu* polymerase. To construct pBLUNT/*EcrecA*, the 1.1 kb PCR fragment was gel purified and cloned into pCR-Blunt (Invitrogen), and sequenced to verify that no errors had been introduced by PCR. To construct pEAS3/*recA*_{Ec} the 1.1 kb *EcrecA*-containing fragment was released from pBLUNT/*EcrecA* with a *PacI/BglII* double digest, gel purified, and ligated to *PacI/BglII*-digested and gel-purified pEAS3, replacing the Gc *recA* with the *E. coli recA*. Therefore, apart from the insert, the only difference between plasmids pVD300*recAX*_{Ec} and pEAS3/*recA*_{Ec} is the multiple cloning site into which the insert is cloned (Fig. 1).

Plasmid constructs pVD300*recAX*_{Ec} and pEAS3/*recA*_{Ec} were recombined into the Gc chromosome at the *recA* locus, replacing the wild-type allele with *E. coli recA* gene alone or the *recA/recX* locus, both under control of *lac* regulatory elements (Fig. 1). Gc strain FA1090 1-81-S2 was transformed with each plasmid by co-culture of plasmid DNA and Gc on solid media (spot transformation). Briefly, FA1090 was plated onto GCB and 20–40 µg of plasmid DNA was spotted onto individual areas of the plate. After co-incubation for 18–22 h, colonies from the spotted areas were collected with a Dacron swab, resuspended in GCB-L and plated on GCB-Tet to select for transformants. Transformants were screened by Southern analysis of *ClaI*-digested chromosomal DNA with *recA*- and *tet*-specific probes (data not shown).

Construction of plasmid pGCC4/*EcrecX* and expression from the gonococcal Cg8 locus. The *E. coli recX* gene was amplified from plasmid pERA using primers ECRECXFORPAC (5'-GTAGGTTAATTAAGTTGTAAGGATATGCCATGAC-3'), which introduces a *PacI* site (underlined), and ECRECXREV (5'-AGTCGCTAGCAATACCGTATGCGTTCAGTCG-3') by PCR using *Pfu* polymerase. pGCC4/*EcrecX* was constructed by gel purifying the 0.5 kb *recX* PCR product and cloning it into *PacI/PmeI*-digested pGCC4. A single clone was sequenced to verify that no errors had been introduced by PCR. Strain FA1090*recA*_{Ec} was transformed with pGCC4/*EcrecX* by spot transformation as

described above. Transformants were selected on GCB-Erm and screened by PCR using primers LACPFOR (5'-GAGCGGATAACAATTTCACA-3') and ECRECXREV to demonstrate insertion in the genome. To verify insertion into the proper chromosomal locus, a Southern blot analysis of *Clal*-digested genomic DNA isolated from FA1090*recA*_{Ec} and FA1090 *recA*_{Ec}/*recX*_{Ec} was performed using *recX*- and *erm*-specific probes (data not shown).

Genetic transfer in *E. coli* and *Gc*. P1 transduction and Hfr conjugation were performed as described by Miller (1992). For transduction, recipient strains of LB1, carrying one of the indicated plasmids, were grown for 4 h with 1.5 mM IPTG induction before adding P1 phage grown on Hfr Cavalli (Bachmann, 1972). *Leu*⁺ or *Pro*⁺ transductants were selected in 2.5 ml top agar on glucose minimal medium. For conjugation, overnight cultures of recipient strain LB1 carrying one of the indicated plasmids were diluted 1:10, the donor strain (Hfr Cavalli) was diluted 1:50, and both were grown for 4 h with 1.5 mM IPTG. After normalizing by OD₆₀₀, mating was performed at 1:5 donor to recipient for 3.5 h. After washing with PBS, *Leu*⁺ or *Pro*⁺ transconjugant colonies were selected on glucose minimal agar. Donor c.f.u. were selected on glucose minimal agar with methionine. Recipient colonies were selected on LB agar with 50 µg streptomycin ml⁻¹.

Gonococcal transformation assays were performed as described previously (Stohl & Seifert, 2001), and transformation efficiency was expressed as the number of *Spc*^R transformants per c.f.u.

UV and nalidixic acid resistance assays. To measure UV resistance in *E. coli*, overnight cultures of each strain were grown for 4 h with 1.5 mM IPTG, serially diluted, and spot-dilutions were plated onto LB agar with 1.5 mM IPTG. Plates were exposed to 1, 2.5, 5.0, 7.5 and 10 J m⁻² UV light. Relative survival at each dose was calculated by comparing the number of c.f.u. in the irradiated sample to the number of c.f.u. in the non-irradiated sample. UV resistance of *Gc* was measured as previously described (Stohl & Seifert, 2001).

Nalidixic acid causes DNA double-strand breaks (Drlica & Zhao, 1997), and resistance to nalidixic acid exposure can be used to assess defects in DNA repair (McDaniel *et al.*, 1978). To measure nalidixic acid resistance in *E. coli*, 1:10 dilutions of overnight cultures of each *E. coli* strain were grown for 4 h with 1.5 mM IPTG and plated on LB agar with 15 µg ml⁻¹ nalidixic acid. Total c.f.u. were calculated by plating spot dilutions of the cultures onto LB agar. Colonies were counted after 36 h growth, and resistance to nalidixic acid was expressed as Nal^R c.f.u. per total c.f.u. for each strain. Resistance to nalidixic acid in *Gc* was assayed as described by Stohl & Seifert (2001). This assay is not a measure of mutation frequency (see Results).

Quantitative colony phase variation assay. Colony phase variation was assessed as described by Stohl & Seifert (2001) by streaking four piliated (P⁺) colonies heavily onto GCB with 1 mM IPTG, growing cells for 24 h, collecting cells and plating on GCB. After 18–20 h growth, colonies with a domed colony morphology and defined edge were scored as piliated (P⁺), whereas colonies with a flat morphology and a less-defined edge were scored as non-piliated (P⁻). Colony phase variation was expressed as the percentage of colonies exhibiting a non-piliated morphology.

Western blot analysis. Total *Gc* lysate was run on SDS-PAGE gels as described by Stohl & Seifert (2001). Protein concentration was determined using the BCA protein assay (Pierce Chemicals). Gels were blotted to PVDF membrane (Millipore)

as described by Stohl & Seifert (2001) and developed according to the ECL Western blotting protocol (Amersham Life Science) with the modifications described by Stohl & Seifert (2001). Primary anti-RecA antibodies (a gift of Michael Cox, University of Wisconsin-Madison) and secondary goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Roche Pharmaceuticals) were diluted 1:5000 in blocking solution. Primary RecX anti-peptide antibodies, raised in rabbits against peptide DDSRFVARFIASRSRKG conjugated to MAP resin (Research Genetics), were diluted 1:2500 in blocking solution. For quantitative Western blot analyses, equal amounts of total protein from strains FA1090*recAX*_{Ec}, FA1090*recA*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec} were separated on SDS-PAGE gels and probed with anti-*E. coli* RecA or anti-*E. coli* RecX peptide antiserum. We used whole-band densitometry to compare protein levels of the strains as follows: dilutions of four independent samples of each strain were run on duplicate gels, the gels were scanned, and signals were quantified using ImageQuant software (Molecular Dynamics).

RESULTS

Rationale

We tested the ability of the *Gc recA* gene to complement an *E. coli recA* mutant and the ability of the *E. coli recA* gene to complement a *Gc recA* mutant. In both species, the heterologous *recA* was compared to the homologous *recA* expressed from identical, tandem *lac*-derived promoters and operators (*tac* and UV5) to prevent differences in transcriptional initiation or regulation from influencing expression levels. In *E. coli*, comparisons were also made to a vector-carrying *recA* mutant strain. We have previously shown that expression of *Gc recA* with this *lac*-derived regulatory system results in RecA-dependent phenotypes being dependent on IPTG in the growth medium (Seifert, 1997).

Analysis of RecA-dependent phenotypes in *Gc*

To examine the ability of the *E. coli recA* to mediate recombination processes in *Gc*, we introduced the *E. coli recA* locus and the associated gene *recX* (Fig. 1), under control of *lac* regulatory signals, into the *recA* locus of *Gc* FA1090 variant 1-81-S2 by natural transformation (FA1090*recAX*_{Ec}). We compared the ability of FA1090*recAX*_{Ec} to mediate the *recA*-dependent phenotypes of DNA transformation, DNA repair and pilus antigenic variation to strain FA1090*recA*6, which has the *Gc recA* under control of the identical *lac* regulatory sequences, and to strain FA1090*recA*9, a *recA* null mutant that has *recA* disrupted by an *ermC* resistance cassette (Seifert, 1997) (Table 1). The DNA transformation efficiency of FA1090*recAX*_{Ec} was significantly decreased relative to FA1090*recA*6, but much higher than FA1090*recA*9, suggesting that the recombinase activity of the *E. coli* RecA functions in *Gc* transformation, albeit not at wild-type levels (Fig. 2a).

DNA repair ability was assessed by measuring resistance to UV irradiation, which causes lesions in DNA, and resistance to nalidixic acid exposure, which introduces double-strand breaks into DNA (Drlica & Zhao, 1997)

Table 1. Complementation of RecA-mediated phenotypes

+, +/–, – and – – refer to full complementation, intermediate complementation, no complementation and lower than the null allele, respectively.

<i>recA</i> allele	RecA-mediated phenotypes in <i>Gc</i>			RecA-mediated phenotypes in <i>E. coli</i>	
	DNA transformation	DNA repair	Pilus antigenic variation	Genetic transfer*	DNA repair
<i>Gc</i> †	+	+	+	+	+/–
<i>E. coli</i> ‡	+/–	– –	+	+	+
Null§	–	–	–	–	–

* As measured by P1 transduction and Hfr conjugation.

† Strain carries either *recA6* or pVD300*recA6*.

‡ Strain carries either *recAX_{Ec}* or pVD300*recAX_{Ec}*.

§ Strain carries either *recA9* or pACYC184.

Fig. 2. RecA-mediated phenotypes of *recA* alleles expressed in *Gc*. (a) Efficiency of transformation to spectinomycin resistance (*Spc^R*) by *Spc^R* genomic DNA. Error bars represent the SEM of three experiments done in triplicate. *, $P < 0.05$ compared to FA1090*recA6* by Student's *t*-test; #, $P < 0.05$ compared to FA1090*recA_{Ec}/recX_{Ec}* by Student's *t*-test. The transformation efficiency for FA1090*recA9* was 8.0×10^{-7} . (b) Relative survival after UV irradiation. Error bars represent the SEM of at least four experiments. At the $4 J m^{-2}$ dose the differences between all strains are statistically significant with $P < 0.05$ by Student's *t*-test. (c) Relative survival on nalidixic acid. Error bars represent the SEM of two experiments done in duplicate. *, $P < 0.05$ compared to FA1090*recA6* by Student's *t*-test. (d) Percentage colony phase variation. Error bars represent the SEM of at least four independent experiments. *, $P < 0.05$ compared to FA1090*recAX_{Ec}* by Student's *t*-test; #, $P < 0.05$ compared to FA1090*recA_{Ec}* by Student's *t*-test.

(Fig. 2b, c). Strain FA1090*recAX_{Ec}* showed a level of UV resistance significantly lower than both FA1090*recA6* and FA1090*recA9*, and the three strains showed a similar trend in nalidixic acid resistance. To verify that we were not simply measuring the rate of spontaneous mutation to nalidixic acid resistance, the effect of the different RecA proteins on frequency of spontaneous mutation to

rifampin or spectinomycin resistance was determined. No significant difference in mutation rate was observed in strains carrying the *E. coli* or *Gc* *recAs* (data not shown).

Because of the decrease in resistance to UV light and nalidixic acid, we assumed that strain FA1090*recAX_{Ec}*

would also be deficient in pilin antigenic variation. Instead, it showed a slight, albeit not statistically significant, increase relative to FA1090*recA*6. To determine whether the increase in P⁻ colony phase variants was due to an alteration of the mechanism of pilin antigenic variation, we analysed P⁻ colony variants from FA1090*recA*6 and FA1090*recAX*_{Ec}. Analysis of individually derived P⁻ colony variants from strains FA1090*recAX*_{Ec} and FA1090*recA*6 showed a similar percentage resulting from *pilE* deletions (29% in FA1090*recAX*_{Ec} vs 22% in FA1090*recA*6). Sequence analysis of the *pilE* genes of 10 independently derived, undeleted P⁻ colony variants of the FA1090*recAX*_{Ec} or FA1090*recA*6 strains showed nearly identical repertoires of *pilS* copies as donors in these *pilE* variants (data not shown). Therefore, the increase in P⁻ colony phase variants was not due to an alteration of the mechanism of pilin antigenic variation.

RecA-dependent phenotypes mediated by *E. coli recA* alone and with *recX* in Gc

To determine the possible contribution of *E. coli recX* to the phenotypes we had observed, we constructed two additional strains of Gc. Strain FA1090*recA*_{Ec} has the *E. coli recA* gene alone under *lac* regulatory signals introduced into the *recA* locus of FA1090 by natural DNA transformation, and strain FA1090*recA*_{Ec}/*recX*_{Ec} is strain FA1090*recA*_{Ec} with the *E. coli recX* introduced into an unlinked chromosomal locus (Cg8) by natural transformation, also under control of *lac* regulatory sequences. The Cg8 locus is a transcriptionally inactive region of the FA1090 chromosome determined to not play a role in recombination (Mehr *et al.*, 2000). In all strains of Gc used in these analyses there is a chromosomal copy of the Gc *recX*; however, since it is present in all strains, it does not affect the interpretation of data.

There was a consistent trend in the efficiencies of RecA-mediated processes among the Gc strains containing *E. coli recA* genes (Fig. 2). Strain FA1090*recAX*_{Ec} showed the highest levels of DNA transformation and colony phase variation, while FA1090*recA*_{Ec}/*recX*_{Ec} showed a statistically significant decrease relative to FA1090*recAX*_{Ec} in both DNA transformation and colony phase variation. Strain FA1090*recA*_{Ec} showed an intermediate level of DNA transformation ability, albeit statistically indistinguishable from FA1090*recAX*_{Ec}, and an intermediate level of colony phase variation, statistically distinct from both FA1090*recAX*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec}. The differences in UV resistance followed exactly the opposite trend, with FA1090*recAX*_{Ec} showing the lowest level of UV resistance and FA1090*recA*_{Ec}/*recX*_{Ec} showing the highest UV resistance. Additionally, all the Gc strains containing *E. coli recA* genes showed slightly lower, but statistically the same, levels of resistance to nalidixic acid than the *recA* mutant strain, FA1090*recA*9.

Fig. 3. Representative Western blot analyses of RecA or RecX protein levels. (a) SDS-PAGE gels containing 0.1 µg total protein per lane of strains FA1090*recAX*_{Ec}, FA1090*recA*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec} (lanes 1, 2 and 3, respectively) were run and transferred to PVDF membrane, with subsequent Western blot analysis using anti-RecA antisera for detection with the ECL detection kit. (b) SDS-PAGE gels containing 1 µg total protein per lane of strains FA1090*recAX*_{Ec}, FA1090*recA*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec} (lanes 1, 2 and 3, respectively), or 0.1 or 0.02 µg of FA1090*recA*_{Ec}/*recX*_{Ec} (lanes 4 and 5) were run and transferred to PVDF membrane, with subsequent Western blot analysis using anti-*E. coli* RecX peptide antisera for detection with the ECL detection kit. The arrow indicates the mobility of the RecX protein.

E. coli RecA protein levels in strains of Gc correlate with RecA-mediated phenotypes

The three Gc strains containing *E. coli recA* genes exhibited differential RecA-mediated phenotypes in Gc, and the *E. coli recX* gene is present in two of these strains, FA1090*recAX*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec}. Studies of *recX* in other organisms have suggested that *recX* may play a role in downregulating *recA* expression (Sano, 1993; Vierling *et al.*, 2000). We therefore wanted to determine whether RecA protein levels were influenced by the presence of *recX* and whether the level of RecA protein reflected the abilities of the strains to carry out recombination-associated processes. Quantitative Western blot analysis was performed to determine levels of RecA protein (see Methods). These results show that the levels of RecA protein are highest in strain FA1090*recAX*_{Ec} and lowest in FA1090*recA*_{Ec}/*recX*_{Ec}, with FA1090*recA*_{Ec} showing an intermediate level of *E. coli* RecA protein (Fig. 3a). Values obtained were used to express the level of RecA as a percentage of the strain expressing the highest level of RecA, FA1090*recAX*_{Ec}. Strain FA1090*recA*_{Ec} expressed 76% (± 0.04 standard error) the RecA protein of FA1090*recAX*_{Ec}, and FA1090*recA*_{Ec}/*recX*_{Ec} expressed 54% (± 0.05 standard error) the RecA protein of FA1090*recAX*_{Ec}. Therefore, the level of RecA protein in these strains correlates directly with the ability of the strains to carry out DNA transformation and colony phase variation, and inversely with resistance to DNA-damaging agents.

Fig. 4. RecA-mediated phenotypes of *recA* alleles expressed in *E. coli*. Assays were done with strain LB1, a Δ *recA* derivative of AB1157, except when noted. Error bars represent the SEM of three independent experiments. (a) Hfr conjugation of proline or leucine prototrophy from strain Hfr Cavalli to strain LB1 carrying the indicated plasmids. (b) P1 transduction of proline or leucine prototrophy from strain Hfr Cavalli to strain LB1 carrying the indicated plasmids. (c) Relative survival after UV irradiation of strains LB1 or LB2, a Δ *recA* derivative of DM49 (AB1157/*lexA3*), carrying the indicated plasmids: ■, LB1(pVD300*recAX*_{Ec}); □, LB2(pVD300*recAX*_{Ec}); ●, LB1(pVD300*recA6*); ○, LB2(pVD300*recA6*); ▲, LB1(pACYC184); △, LB2(pACYC184). (d) Relative survival on nalidixic acid of strain LB1 carrying the indicated plasmids.

Overexpression of *E. coli recX* downregulates *E. coli RecA* in Gc

Since RecA protein levels may be influenced by *recX*, we wanted to determine the relative level of *E. coli recX* expression in strains FA1090*recAX*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec}. In strain FA1090*recAX*_{Ec}, *recX* is found directly downstream of *recA* on the same DNA fragment, and in strain FA1090*recA*_{Ec}/*recX*_{Ec}, *recX* is expressed from a different locus of the Gc chromosome, but in both strains *recX* is under control of *lac* regulatory signals. Western blot analysis was performed as described above, except using an anti-*E. coli* RecX peptide antibody, with strains FA1090*recAX*_{Ec}, FA1090*recA*_{Ec}/*recX*_{Ec} and FA1090*recA*_{Ec} (a negative control that does not contain the *E. coli recX*). Strain FA1090*recA*_{Ec}/*recX*_{Ec} was found to have RecX levels over 50-fold higher than FA1090*recAX*_{Ec} (Fig. 3b). Similarly, *recX* transcript levels were found to be approximately 10-fold higher in FA1090*recA*_{Ec}/*recX*_{Ec} than in FA1090*recAX*_{Ec} (data not shown), demonstrating that overexpression of *recX* results in decreased RecA levels and thereby affects RecA-mediated phenotypes.

Analysis of RecA-dependent phenotypes in *E. coli*

We were intrigued by the differential recombination phenotypes observed when the *E. coli recA* was expressed in Gc, so we tested the ability of the Gc RecA to mediate recombination processes in *E. coli* (Table 1). Δ *recA* derivatives of AB1157 (DeWitt & Adelberg, 1962), LB1 and LB2, were made by P1 transduction (see Methods). LB2 additionally contains the *lexA3* mutation (Mount *et al.*, 1972). A low-copy-number derivative of pACYC184 (Chang & Cohen, 1978), with an estimated copy number of 10, was used to express the Gc *recA* gene under IPTG control (pVD300*recA6*) in *E. coli*. This plasmid was compared to an identical plasmid carrying the *E. coli recA/recX* operon under IPTG control (pVD300*recAX*_{Ec}), where 76 bp separates the stop codon of *recA* from the start codon of *recX*. In this construct, both *recA* and *recX* are present at the same copy number and under control of the same *lac* promoter as the Gc *recA*.

The chromosomal copy of *E. coli recX* is still present in strains LB1 and LB2; however, two lines of evidence suggest that the level of expression of chromosomal

recX is negligible and should not alter the interpretation of subsequent assays. First, we were not able to detect RecX protein expression by Western blot analysis in either strain AB1157 or strain JC10289, the parental strains of LB1 and LB2 (data not shown). Second, repeated RT-PCR experiments on strain AB1157 showed a barely detectable level of *recX* RT-PCR product (data not shown). Thus, comparison of the RecA-mediated phenotypes of DNA repair and homologous recombination in the $\Delta recA$ strains transformed with the three low-copy plasmids pACYC184, pVD300*recA6* and pVD300*recAX*_{Ec} is not likely to be affected by the chromosomal copy of *recX*.

The *lac*-regulated, plasmid-expressed Gc and *E. coli* RecAs mediated nearly identical levels of homologous recombination in LB1 as measured by P1 phage-mediated transduction and Hfr conjugation, that were significantly higher than the LB1 vector control (Fig. 4a, b). These results indicate that these RecA homologues mediate homologous recombination with similar efficiencies in *E. coli*.

Resistance to UV light and nalidixic acid, which causes double-strand breaks and is used to assess defects in DNA repair (McDaniel *et al.*, 1978), were used to measure DNA repair capabilities of the RecA proteins in strain LB1 (Fig. 4c, d), which is able to activate the SOS response. The UV resistance of the LB1 pVD300*recA6* strain was 2000–3000-fold lower than that of the LB1 pVD300*recAX*_{Ec} strain, but both strains showed higher levels of resistance than the vector control. Similar differences between the LB1 pVD300*recA6* and pVD300*recAX*_{Ec} strains were also observed when resistance to nalidixic acid was measured by direct plating (Fig. 4d). An additional assay was performed to assess resistance to nalidixic acid in which LB1 cells carrying the three plasmids were grown in liquid culture, exposed to nalidixic acid for increasing amounts of time, and subsequently plated on LB without the antibiotic. We noted the same trend in survival as observed with the direct-plating method, with LB1 pVD300*recAX*_{Ec} showing slightly higher survival than the same strain carrying pVD300*recA6*, and pACYC184 showing much lower survival than the two *recA*-containing strains. Subsequent analysis of 100 of the surviving colonies from each strain revealed that all the colonies that had been exposed to nalidixic acid remained Nal^s, demonstrating that this assay is not a measure of mutation frequency (data not shown).

Repair of UV-induced DNA lesions in *E. coli* involves induction of the SOS response. Since Gc does not have an SOS system (Black *et al.*, 1998), we wanted to test the hypothesis that the inability of the Gc RecA to induce the SOS response was responsible for the inferred inefficient DNA repair. To do this, we used two experimental approaches. First, we measured the UV resistance of strain LB2, a *lexA3* mutant with an uncleavable repressor unable to induce the SOS response, carrying plasmids pVD300*recA6* or pVD300*recAX*_{Ec} (Fig. 4c). The difference in survival between LB2 pVD300*recA6* and LB2 pVD300*recAX*_{Ec}

was reduced compared to that seen in the LB1 strains; however, the pVD300*recA6* strain was still reduced in survival relative to the pVD300*recAX*_{Ec} strain (1–2 log values lower). Second, strain LB1 carrying a chromosomal *dinD1::Mud::lacZ* (Kenyon & Walker, 1980) fusion was transformed with plasmids pVD300*recAX*_{Ec} or pVD300*recA6*. The LB1 strain carrying pVD300*recA6* showed minimal induction of *dinD1* expression relative to the LB1 strain carrying pVD300*recAX*_{Ec} after exposure to mitomycin C, an SOS-inducing signal (data not shown). Therefore, the Gc RecA inefficiently mediates DNA repair in *E. coli*, and this is partly due to an inability to induce the SOS response.

DISCUSSION

We tested the abilities of the Gc and *E. coli* RecAs to mediate the major forms of genetic transfer in *E. coli* (transduction and conjugation) and Gc (DNA transformation). In both bacteria these genetic processes share a dependency upon the RecBCD pathway of homologous recombination, although the RecBCD dependency of conjugation in *E. coli* (Kushner *et al.*, 1971; Lloyd & Thomas, 1983) is more stringent than the RecBCD dependency of DNA transformation in Gc (Mehr & Seifert, 1998). The RecA and RecBCD proteins from *E. coli* have been shown to interact *in vitro* (Amundsen *et al.*, 2000; Churchill *et al.*, 1999). Previous work by De Vries & Wackernagel (1992) suggests that RecA and RecBCD proteins from different species of bacteria do not interact as efficiently as proteins from the same species of bacteria *in vivo*. In these studies, researchers measured the ability of the RecA proteins from *Serratia marcescens* and *Proteus mirabilis*, both enterobacteria closely related to *E. coli*, to mediate conjugal recombination in *E. coli*. Although the *S. marcescens* RecA was able to catalyse recombination levels equal to the *E. coli* RecA, the *P. mirabilis* RecA was only 50% as effective, suggesting that the protein from *P. mirabilis* did not interact as efficiently with the RecBCD enzyme from *E. coli*. To further study the interactions of the RecA and RecBCD proteins from these bacteria, all possible combinations of *recA* and *recBCD* genes from *P. mirabilis*, *S. marcescens* and *E. coli* were expressed in *E. coli* and tested for recombination. Several of the interspecies combinations failed to reach the level of the intraspecies combinations, further suggesting that decreases in recombination were due to inefficient interactions of the RecA and RecBCD enzymes. In our experiments expressing the Gc and *E. coli* *recA* genes in *E. coli*, DNA transduction and conjugation were mediated with equal efficiency by both RecA proteins, suggesting that the proteins shared similar recombination activities and that the Gc RecA was able to interact as efficiently as the *E. coli* RecA with the *E. coli* RecBCD enzyme. The major form of genetic transfer in Gc (DNA transformation) was mediated with decreased efficiency by all the strains carrying *E. coli* *recA*, independent of *recX*. These results imply that the *E. coli* RecA and the Gc RecBCD interact with decreased efficiency. Alternatively, since *recBCD* mu-

Fig. 5. Relative UV survival of FA1090recA_{Ec} expressing varying levels of RecA. Error bars represent the SEM of at least two experiments. Strain *recA4* is a *recA* null allele identical to strain FA1090recA₉, except that it carries a *tet* rather than an *erm* resistance cassette (Seifert, 1997).

tants in Gc still show substantial transformation ability (Mehr & Seifert, 1998), there is likely to be RecBCD-independent recombination that functions in natural DNA transformation, and the *E. coli* RecA may be deficient in mediating recombination via this second pathway.

Expression of the heterologous RecA protein from either bacterium did not confer as high a resistance to DNA-damaging agents as expression of the homologous RecA protein. In *E. coli*, the Gc RecA only partially complemented for survival to DNA-damaging agents; this may be due to a reduced ability of this RecA protein to induce the SOS response. Since there is no SOS-like system in Gc (Black *et al.*, 1998), the Gc *recA* may not be adapted to induce a full SOS response. The pVD300recA6 construct also lacks a LexA-binding site in the promoter region (Fyfe & Davies, 1990), and there should be no further induction of the *lac*-driven promoters during SOS. Furthermore, one outcome of SOS induction in *E. coli* is RecA-assisted cleavage of UmuD, a protein that functions in UV-induced mutagenesis (Walker, 1996). Since there is no UmuD homologue in Gc (Roe *et al.*, 1997), the Gc RecA may not be able to activate this cellular response, resulting in decreased resistance to DNA damage. *recA* genes from many bacteria have been shown to only partially complement *E. coli* *recA* mutants for UV resistance (Miller & Kokjohn, 1990). Specifically, expression of the *recA* gene from *Bacteriodes fragilis* in an *E. coli* *recA* mutant revealed the same inefficient complementation for UV resistance as we observed. Interestingly, *B. fragilis* also lacks an error-prone DNA repair system (Goodman *et al.*, 1987). A far more dramatic difference in DNA repair was observed in Gc. Strains of Gc carrying *E. coli* *recA* were actually less resistant to the DNA-damaging agents UV light and nalidixic acid than a *recA* null mutant. This markedly decreased resistance was influenced by levels of *E. coli* RecA protein, but was not simply a result of overexpression of the *E. coli* RecA. In the three Gc strains containing *E. coli* *recA* genes, the strain with the

highest level of *E. coli* RecA protein, FA1090recAX_{Ec}, was least resistant to UV irradiation, whereas the strain with the lowest amount of RecA protein, FA1090recA_{Ec}/recX, was the most resistant. In a series of UV resistance assays using strain FA1090recA_{Ec}, which has *recA* under tight control of *lac* regulatory signals, increasing the level of IPTG in the medium, thereby increasing RecA levels, resulted in decreased UV resistance (Fig. 5). However, even when levels of IPTG were titrated to zero, strain FA1090recA_{Ec} was still less resistant to UV irradiation than a *recA* mutant strain (Fig. 5). It seems possible that, even when present in small quantities in Gc, the *E. coli* RecA inhibits some protein involved in DNA repair or interacts with damaged DNA in such a way that it interferes with DNA repair. Although expression of the *recA* gene from *Acholeplasma laidlawii*, a mycoplasma, is lethal in *E. coli* (Dybvig & Zhao, 1997), as far as we are aware this is the first published example of a specific hypersensitivity to UV light conferred by expression of a heterologous *recA* gene.

Since the Gc and *E. coli* RecA proteins differed in their abilities to mediate various RecA-dependent processes in the heterologous host, and since the carboxy-terminal portion of the two RecA proteins is the most variable, we wanted to determine whether we could correlate the phenotypic differences with specific regions of the two RecA proteins. To do this, we created tripartite hybrid RecA molecules of the *E. coli* and Gc RecA proteins where the N-terminal, central and C-terminal portions of the two proteins were arranged in all possible combinations, and assayed these proteins for the ability to carry out RecA-mediated processes in *E. coli* and Gc. We found that activity of the RecA proteins did not segregate to a particular domain, suggesting that many regions of the RecA protein are important for activity (L. Blount & H. S. Seifert, unpublished data).

recX genes have been identified downstream of *recA* in many bacteria by sequence analysis (De Mot *et al.*, 1994). Work in *Pseudomonas aeruginosa*, *Mycobacterium smegmatis* and *Streptomyces lividans* has demonstrated that overexpression of *recA* from a plasmid construct is deleterious or lethal in the absence of *recX* (Papavinasasundaram *et al.*, 1997; Sano, 1993; Vierling *et al.*, 2000), leading to the hypothesis that RecX acts by downregulating expression of *recA* or activity of the RecA protein. However, this hypothesis has not been rigorously tested, and a *recX* mutation of *S. lividans* does not affect any RecA-mediated processes or RecA levels (Vierling *et al.*, 2000). Gc also contains a *recX* homologue; however, it is not located downstream of *recA*. Furthermore, inactivation of *recX* in Gc impairs the RecA-mediated processes of antigenic variation, DNA transformation and DNA repair, but does not affect RecA protein levels (Stohl & Seifert, 2001), demonstrating that RecX functions differently in certain species of bacteria. All the strains of Gc used in these experiments contain a functional Gc *recX*; however, since it is present in all strains, it is not responsible for

the differences in observed phenotypes, and we do not know if it can interact with *E. coli* RecA.

Our current data demonstrate that the *E. coli* RecX influences RecA-mediated processes; however, *E. coli* RecX acts by modulating *E. coli* RecA levels in a complex manner. Comparing RecA protein levels in strains FA1090*recA*_{Ec} and FA1090*recA*_{Ec}/*recX*_{Ec} demonstrated that overexpressing RecX results in decreased RecA levels and corresponding effects on RecA-related phenotypes. However, strain FA1090*recAX*_{Ec} shows the highest RecA protein levels and RecA-related phenotypes relative to FA1090*recA*_{Ec}, but expresses about 50 times less RecX than FA1090*recA*_{Ec}/*recX*_{Ec}. These data suggest that RecX (or *recX*) may perform an additional function of either stabilizing the *recA* transcript, stabilizing the RecA protein, or somehow enhancing RecA activity when present either at low levels or when present on the same transcript as *recA*. Therefore, the apparent biological role of *E. coli* RecX may be dependent upon the level of the RecX present in a cell, with lower levels of RecX activating or positively regulating RecA, but higher levels of the RecX protein inhibiting or downregulating RecA. Studies of the *E. coli recX* in Gc have stimulated thoughts about the function of RecX, and we are currently conducting experiments to determine the role of this protein in DNA recombination and repair in *E. coli*.

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