A dual role for mycobacterial RecO in RecA-dependent homologous recombination and RecA-independent single-strand annealing

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ABSTRACT

Mycobacteria have two genetically distinct pathways for the homology-directed repair of DNA double-strand breaks: homologous recombination (HR) and single-strand annealing (SSA). HR is abolished by deletion of RecA and reduced in the absence of the AdnAB helicase/nuclease. By contrast, SSA is RecA-independent and requires RecBCD. Here we examine the function of RecO in mycobacterial DNA recombination and repair. Loss of RecO elicits hypersensitivity to DNA damaging agents similar to that caused by deletion of RecA. We show that RecO participates in RecA-dependent HR in a pathway parallel to the AdnAB pathway. We also find that RecO plays a role in the RecA-independent SSA pathway. The mycobacterial RecO protein displays a zinc-dependent DNA binding activity in vitro and accelerates the annealing of SSB-coated single-stranded DNA. These findings establish a role for RecO in two pathways of mycobacterial DNA double-strand break repair and suggest an in vivo function for the DNA annealing activity of RecO proteins, thereby underscoring their similarity to eukaryal Rad52.

INTRODUCTION

The maintenance of genome integrity is critical to all living organisms, and the systems that repair DNA damage are diverse. Double-strand breaks (DSBs) are a particularly lethal form of DNA damage that prevent chromosome replication if not repaired. In most bacteria, homologous recombination (HR) is the dominant pathway of DSB repair and is mediated by the RecA protein, which executes homology search and strand invasion of the broken ends into an intact homologous template, resulting in faithful repair. Mycobacteria elaborate two additional DSB repair pathways: non-homologous end joining (NHEJ) and single-strand annealing (SSA) (1–4). The core components of the NHEJ pathway are Ku and DNA ligase D, which cooperate to repair DNA damage in late stationary phase caused by ionizing radiation or desiccation (5,6), to seal linearized plasmids (1,7), and to rectify I-SceI-induced chromosomal DSBs (3,6). Mycobacteria lacking RecBCD cannot execute repair via the SSA pathway, whereas cells lacking the AdnAB helicase/nuclease are partially defective for HR (3). The proteins that mediate the AdnAB-independent HR pathway, which accounts for ~50% of HR events in wild type cells, are undefined.

The bacterial RecFOR machinery supports single-strand gap repair and restart of stalled replication forks (8–10). The contribution of RecFOR to the HR pathway of DSB repair in wild-type bacteria varies among taxa. In Escherichia coli, where RecBCD drives the dominant pathway of recombination by performing the end-resection and RecA loading functions required to initiate HR, RecFOR is relegated to a backup role evident when RecBCD is inactive and the bacterium acquires sbe suppressor mutations (11). By contrast, two parallel RecA-dependent HR pathways of recombination operate in Bacillus subtilis, one driven by the AddAB...
helicase/nuclease (which is homologous to mycobacterial AdnAB) and another by RecFOR. The AddAB and RecFOR pathways contribute equally to clastogen resistance in *Bacillus*, such that both pathways must be inactivated to ablate HR in vivo (12). In *Deinococcus radiodurans*, which lacks both RecBCD and AddAB, the RecFOR system constitutes the main pathway of recombinational repair of all DNA breaks, whether single-strand gaps or DSBs (13–15).

The RecO protein of *E. coli* is an essential component of the RecOR and RecFOR recombination mediator complexes that load RecA onto SSB-coated ssDNA (16–19). RecO can also anneal complementary ssDNA protected by its cognate SSB (20–22). Both the RecA loading and single-strand annealing activities of RecO depend on its interaction with the SSB C-terminal peptide (SSB-Ct) (18,22). These RecO functions resemble the activities of yeast Rad52, which mediates loading of Rad51 (the eukaryal RecA homolog) and can anneal complementary ssDNA coated with RPA (the eukaryal SSB) (23,24). In yeast, Rad52 is required for the Rad51-independent single-strand annealing pathway (25), suggesting an *in vivo* role for the annealing activity. However, the *in vivo* function of the single-strand annealing activity of RecO is less clear, as this activity should not be required for RecO to serve as a RecA mediator. Although RecET stimulated illegitimate recombination and plasmid recombination have been demonstrated to be RecO dependent in *E. coli* and appear to involve an SSA type mechanism (26,27), a bonafide SSA pathway that repairs double strand breaks flanked by repeats has not been demonstrated. One suggestion is that *E. coli* RecO might (by analogy to Rad52) promote second-end capture during HR, i.e., the annealing of the processed second DSB end to the single-strand of the D-loop created by RecA-dependent strand invasion (28).

In this study, we examine the function of RecO in mycobacteria. Our results indicate that RecO has dual functions in mycobacterial DSB repair: in a pathway of mycobacterial HR parallel to that mediated by AdnAB, and also in the mycobacterial SSA pathway. Consistent with these dual *in vivo* functions, the biochemical properties of mycobacterial RecO differ from those of the *E. coli* homolog. Mycobacterial RecO displays strong zinc-dependent DNA binding and anneals complementary SSB-coated DNA without interacting physically with the SSB-Ct.

**MATERIALS AND METHODS**

**recO knockout and complementation**

In-frame deletion of *Mycobacterium smegmatis* recO in the Δ*adnAB ΔrecBCD* strain was achieved by a two-step allelic exchange process that uses a suicide vector containing a hygromycin-resistance marker and the counter-selectable marker * sacB*, as described previously (1). *recO* was deleted in WT, Δ*adnAB* and Δ*recBCD* backgrounds by specialized transduction using a temperature-sensitive mycobacteriophage. The hygR marker (flanked by *loxP* sites) at the disrupted locus was subsequently excised by expressing Cre recombinase, to generate the unmarked strains Δ*recO, ΔrecO ΔadnAB* and Δ*recO ΔrecBCD*. Southern blotting was performed to confirm *recO* knockout using either 5′ or 3′ flanking DNA sequence as the probe, and *recBCD* and *adnAB* loci were verified using 5′ and 3′ flanking DNA sequences as the probes, respectively. For complementation, the *M. smegmatis* recO gene along with its 5′ untranslated region (202 bp) containing the presumed promoter was cloned in the mycobacterial integrative vector pMV306kan, to generate pRGM30. The Δ*recO* and Δ*recO ΔadnAB* strains were complemented with *recO* by integrating pRGM30 at the *attB* site of the chromosome by plasmid transformation. Rescue of the Δ*recO* mutant phenotypes was tested by assaying clastogen sensitivity. WT, Δ*recO* and Δ*recO ΔadnAB* strains harbouring pMV306kan in the chromosomal *attB* locus served as controls in these experiments.

**Chromosomal DSB repair assay**

Competent *M. smegmatis* cells of the specified genotypes harbouring the chromosomally integrated *lacZ* reporter construct for I-SceI-mediated DSB repair assay (3) were transformed separately with the same molar amount of the I-SceI plasmid and the control vector plasmid to determine the frequency of the DSB repair outcomes and net % survival as described previously (3). For each strain, the experiment was performed at least thrice using different batches of competent cells, and results are expressed as mean values. Among the blue colonies, the gene conversion (GC) and SSA events were distinguished by scoring for kanamycin resistance; kan^R^ (kanamycin-resistant) colonies represent GC and kan^S^ (kanamycin-sensitive) colonies denote SSA. Because the % survival values remained constant between different genetic backgrounds, frequencies of different repair outcomes were compared by calculating the relative repair fractions, as follows: relative GC frequency = (GC events/blue events) × (fraction blue); relative SSA frequency = (SSA events/blue events) × (fraction blue).

**Growth studies**

*M. smegmatis* strains were revived from frozen glycerol stocks by repeated sub-culturing in LB medium (supplemented with 0.5% glycerol, 0.5% dextrose and 0.1% Tween 80) to ensure that all cells had entered log phase. To study growth kinetics, the strains were re-inoculated into fresh medium to *A*₆₀₀ of 0.1, incubated at 37°C with constant shaking (150 rpm) and aliquots were removed at regular intervals to measure *A*₆₀₀ and determine colony forming units (CFUs) by serial-dilution plating. Change in absorbance or CFUs was plotted against time. Doubling times were calculated using the formula: doubling time = time (t) - time (t₀)/number of generations (G), where *G* = (log[#bacteria or *A*₆₀₀ at time(t)] - log[#bacteria or *A*₆₀₀ at time(t₀)])/0.301.

**Clastogen sensitivity assays**

Mycobacterium smegmatis cultures were grown at 37°C in LB medium supplemented with 0.5% glycerol, 0.5% dextrose and 0.1% Tween 80 to log-phase (*A*₆₀₀ of 0.1, incubated at 37°C with constant shaking (150 rpm) and aliquots were removed at regular intervals to measure *A*₆₀₀ and determine colony forming units (CFUs) by serial-dilution plating. Change in absorbance or CFUs was plotted against time. Doubling times were calculated using the formula: doubling time = time (t) - time (t₀)/number of generations (G), where *G* = (log[#bacteria or *A*₆₀₀ at time(t)] - log[#bacteria or *A*₆₀₀ at time(t₀)])/0.301.

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**Clastogen sensitivity assays**

*Mycobacterium smegmatis* cultures were grown at 37°C in LB medium supplemented with 0.5% glycerol, 0.5% dextrose and 0.1% Tween 80 to log-phase (*A*₆₀₀ of
0.4–0.5) and subjected to treatment with different DNA-damaging agents, i.e. UV, Methyl methanesulfonate (MMS) and IR as described previously (3). Ten-fold serial dilutions of the treated and untreated cells (derived from the same culture) were then spotted on LB agar plates or LB agar plates containing 20 µg/ml kanamycin (in case of recO complementation experiments), and % survival was calculated compared with untreated control cells.

**Purification of RecO and SSB**

His-tagged *M. smegmatis* RecO (MsRecO) protein was cloned in pMCSG7, and expressed in *E. coli*, and purified using methods described previously for the *Deinococcus radiodurans* and *E. coli* RecO proteins (22). *E. coli* SSB (EcSSB) and a C-terminal truncation SSBΔC EcSSBΔC were purified as described (29). *M. smegmatis* SSB (MsSSB) was expressed from plasmid Pei21b-smegSSB (30), which was modified to introduce a translational stop codon before the polyhistidine tag such that the expressed SSB protein has no affinity tag. *M. smegmatis* SSB was produced in *E. coli* and purified by sequential polyethyleneimine and ammonium sulfate precipitation steps, followed by heparin affinity and size exclusion chromatography steps (29). The EcSSBΔC expression plasmid was a gift from Dr M. Cox. The purity of all proteins was verified by SDS-PAGE and is shown in Supplementary Figure S2.

**DNA and SSB-Ct binding assays**

Assay components were prepared as previously described (22). Proteins were dialysed or diluted into buffer A (50 mM NaCl, 25% (v/v) glycerol, 50 mM HEPES pH 7.5, 1 mM Tris(2-carboxyethyl)phosphine (TCEP)), buffer B (100 mM NaCl, 25% glycerol, 20 mM Bis-Tris-Propane, pH 7.5, 1 mM TCEP), buffer C (50 mM NaCl, 25% glycerol, 20 mM Bis-Tris-Propane, pH 7.5, 1 mM TCEP) or buffer D (200 mM NaCl, 25% glycerol, 20 mM Bis-Tris-Propane, pH 7.5, 1 mM TCEP). Protein concentrations were determined by absorbance at 280 nm and with extinction coefficients of 24,595 M⁻¹ cm⁻¹ (EcRecO) or 30,668 M⁻¹ cm⁻¹ (MsRecO). Lyophilized fluorescein 5'-labelled (FAM) dT₁₅ and dT₃₅ oligonucleotides were solubilized with Milli-Q H₂O. Double-stranded DNA was generated by mixing FAM-labelled 15mer (5'-TATCCGCAAGTGGG) with an equimolar amount of complementary unlabelled 15mer (5'-CCAACTCTGCG GATA) and incubating at 95°C for 1 min, followed by annealing at room temperature for 30 min. FAM-labelled EcSSB-Ct peptide (WMDFDDIPF (Genscript)) was solubilized with dimethylformamide and diluted into assay buffer; peptide concentration was measured by tryptophan absorbance at 280 nm with an extinction coefficient of 5500 M⁻¹ cm⁻¹. The concentration of FAM-labelled MsSSB-Ct peptide [GADDEPF (Lifetein Inc.)] was measured by FAM absorbance at 485 nm using EcSSB-Ct FAM absorption as a reference. Fluorescence anisotropy assays were performed by serially diluting RecO in the indicated buffer with or without Zn(OAc)₂. FAM-labelled DNA (5 nM) or SSB-Ct peptide (20 nM) was added to RecO in a final volume of 100 µl and incubated for 30 min. Binding was measured with excitation/emission of 485/528 nm at room temperature using a BioTek Synergy 2 plate reader. Anisotropy values were normalized using equation: \( I = (A_r - A_i)/A_i \), where \( A_i \) and \( A_r \) are anisotropy values of free and protein-bound FAM-labelled DNA or SSB-Ct, respectively. Binding constants were calculated using BioKin DynaFit using simple binding equations \([\text{RecO}]+[\text{DNA or SSB-Ct}] \leftrightarrow [\text{RecO.DNA or SSB-Ct}]\).

**DNA annealing assay**

RecO-mediated DNA annealing was assayed as described previously (22). SSB concentrations were measured by absorbance at 280 nm and with extinction coefficients of 18,975 M⁻¹ cm⁻¹ (EcSSB), 18,010 M⁻¹ cm⁻¹ (EcSSBΔC) or 17,401 M⁻¹ cm⁻¹ (MsSSB). 49mer 5'-FAM-labelled and complementary 3'-Dabcyl-labelled oligonucleotides at 20 nM were incubated separately with 200 nM of SSB for 30 min at room temperature in buffer D. Complementary oligo(SSB complexes were mixed, and RecO was added and incubated for 30 min at room temperature in the presence or absence of 50 µM Zn(OAc)₂. Annealing was measured by a decrease of FAM emission (excitation/emission of 485/528 nm) as a function of time for a total of 21 min. Final annealing amplitude was normalized using equation: \( I = -\frac{1}{I_0} \times \left( I_0 - I_t \right) \), where \( I_0 \) and \( I_t \) are intensity values of ssDNA and annealed dsDNA.

**RESULTS**

*recO is not required for M. smegmatis viability*

Early bioinformatic surveys of the predicted mycobacterial proteome failed to identify RecO (31,32), but later analyses did (33). An alignment of the primary structure of the putative *M. smegmatis* RecO protein (a 280 aa polypeptide encoded by MSMEG_4491) with the RecO proteins from *D. radiodurans*, *Bacillus subtilis*, *Helicobacter pylori*, *Neisseria gonorrhoeae* and *E. coli* is shown in Supplementary Figure S1. *M. smegmatis* RecO is 24% identical/38% similar to *E. coli* RecO, 26% identical/46% similar to *B. subtilis* RecO, and 31% identical/47% similar to *D. radiodurans* RecO. Mycobacterial RecO contains a tetracysteine motif (see green boxes in Supplementary Figure S1) that is also found in the RecO proteins from *D. radiodurans* and *B. subtilis*, but not in *E. coli* RecO. *M. smegmatis* RecO is highly conserved among mycobacteria—with homologs in *M. marium* (83% identical), *M. tuberculosis* (81% identical), *M. intracellulare* (82% identical), *M. leprae* (82% identical), *M. gilvum* (84% identical), *M. avium* (80% identical), *M. abscessus* (83% identical) and *M. bovis* (79% identical). RecO is also conserved in other *Actinomycetales* such as *Rhodococcus equi* (78% identical) and *Nocardia abscessus* (75% identical). Despite the limited amino acid sequence conservation between the *E. coli* and *D. radiodurans* RecO proteins (30% identity), the secondary structural elements of the two proteins, indicated above and below the alignment in Supplementary Figure S1, are well conserved.

To interrogate the role of RecO in mycobacterial DSB repair, we deleted the recO gene (MSMEG_4491) from the
Figure 1. Deletion of recO in Mycobacterium smegmatis. (A) Generation of the ΔrecBCD ΔadnAB ΔrecO strain by two-step allelic exchange. Map of the wild-type and ΔrecO loci with NcoI restriction sites, probe location and predicted fragment sizes for WT (11683 bp) and ΔrecO (2065 bp). Below the restriction maps is a Southern hybridization of chromosomal DNA isolated from ΔrecBCD ΔadnAB M. smegmatis (labelled WT with regard to the recO locus), seven sucrose-resistant, hygromycin-sensitive recombinants, and the parental recO duplication strain (recO dup) which contains the wild-type allele of recO and the ΔrecO deletion allele at the 3′-end of the recO locus, separated by the hsg and sucB markers. The lane marked with the asterisk denotes the strain used for further studies. (B) Deletion of recO by specialized transduction. Map of the wild-type and ΔrecO::loxP-hyg-loxP loci with SmaI restriction sites, probe location, and predicted fragment sizes for WT (2245 bp) and ΔrecO::loxP-hyg-loxP (7012 bp) after SmaI restriction of chromosomal DNA. Below the restriction maps is a Southern hybridization of chromosomal DNA isolated from "M. smegmatis" (WT), ΔrecO::hyg (lanes 2, 5, 8 in the indicated strain backgrounds) or ΔrecO::loxP (after Cre recombinase-mediated excision of the hyg marker, lanes 3, 4, 6, 7).

*M. smegmatis* chromosome by allelic exchange. To interrogate potential epistatic relationships between recO and the adnAB-dependent HR and recBCD-dependent SSA pathways, the ΔrecO allele was also introduced into the ΔadnAB, ΔrecBCD, and ΔadnAB Δ recBCD strains. The strategy for genotyping by Southern blotting depicted in Figure 1A demonstrates the deletion of recO in the ΔadnAB Δ recBCD strain background, leaving an unmarked ΔrecO allele. Specialized transduction of a ΔrecO::loxP-hyg-loxP allele was used to delete recO in the three other strain backgrounds (Figure 1B). The hygromycin-resistance cassette was then excised using the Cre recombinase to yield unmarked ΔrecO, ΔrecO ΔadnAB, and ΔrecO ΔrecBCD mutants (Figure 1B). The presence of the ΔrecBCD and ΔadnAB alleles was confirmed by diagnostic Southern blotting (data not shown).

**ΔrecO strains have diminished growth rate**

We examined the growth characteristics of ΔrecO strains by measuring A600 in logarithmic cultures and comparing these cell density measurements to the number of viable bacteria. The apparent doubling times of the ΔrecO mutants were determined after repeated serial dilution of logarithmic phase cultures. In comparison to wild type (doubling time 174 min), the ΔrecO (212 min) and ΔrecO ΔadnAB (231 min) strains grew slower and their growth rates were similar to that observed for the ΔrecA strain (226 min). This difference in apparent doubling time was not the result of altered cell shape, as the doubling times of ΔrecO (215 min), ΔrecO ΔadnAB (222 min) and ΔrecA (213 min) calculated from determination of viable bacteria were greater than that of the wild type strain (155 min). The slow growth phenotype of the mutant strains is also manifested as small colony size when cultured on agar media (Figure 2B).

**RecO is required for survival upon DNA damage**

Our previous results indicated that the *M. smegmatis* ΔadnAB strain is sensitized to killing by diverse clastogens, whereas ΔrecBCD cells are not (3). However, the ΔadnAB strain is less sensitive to DNA damage than a ΔrecA strain, indicating that there are AdnAB-independent pathways of homology-directed repair. To investigate if RecO is involved in DNA repair in mycobacteria, we studied the survival of the ΔrecO strains after exposure to UV, IR and the alkylating agent MMS. The deletion of recO conferred severe sensitivity to UV radiation (Figure 2A), ionizing radiation (Figure 2B) and MMS (Figure 2C). The clastogen sensitivity of the ΔrecO strain was indistinguishable from ΔrecA (Figures 2A–C). Note that the ΔrecO ΔadnAB, ΔrecO ΔrecBCD and ΔrecO ΔadnAB ΔrecBCD strains were no more sensitive to UV, IR or MMS than the ΔrecO strain (Figure 2, Supplementary Figure S2). These results suggest that RecO and RecA function in the same pathway of DNA repair, conceivably by loading RecA onto single-stranded DNA at damage sites.
In the *M. smegmatis* chromosome, the *recO* gene appears to be in an operon with three other genes, namely MSMEG_4490 (*uppS*, encoding undecaprenyl diphosphate synthase), MSMEG_4489 (encoding a conserved hypothetical protein) and MSMEG_4488 (encoding a nudix family hydrolase) (Figure 1). The predicted translational start codon of *uppS* overlaps the 3' end of *recO* by 53 nucleotides, raising the possibility that the *recO* deletion in our strains might disrupt *uppS* expression. Although we were careful to leave the coding sequence of *uppS* intact, we performed a complementation experiment to confirm that the clastogen sensitivity of our Δ*recO* strain was due to loss of RecO function. Complementation of the deletion mutant with a single *recO* copy at the phage L5 integration site in the chromosome restored the clastogen resistance of the Δ*recO* strain to wild-type levels (Figure 3A and B). Complementation of the Δ*recO* Δ*adnAB* strain with *recO* restored the clastogen resistance to the level of the Δ*adnAB* strain (Figure 3). Together, these results verify that the phenotype of the *recO* deletion is due to loss of *recO* and not due to a polar effect in the *recO* operon or a spontaneous mutation elsewhere in the chromosome.

**RecO participates in a parallel pathway to AdnAB in RecA-dependent HR**

Because UV, IR and MMS produce diverse types of DNA damage, the clastogen sensitivity of the *recO* mutant does not suffice for conclusions about the function of *recO* in specific pathways of DNA repair. To interrogate the participation of RecO in DSB repair, we used an I-SceI recombination system developed previously (3). In this system, a recombination substrate is integrated into the chromosome of *M. smegmatis* at the L5 phage attachment site. This recombination substrate contains two defective lacZ alleles, *lacZ*-I-SceI and *lacZ*-ΔN, which are separated by a kanamycin-resistance cassette. *lacZ*-I-SceI is interrupted by two recognition sites for the homing endonuclease I-SceI such that cleavage by I-SceI
produces a chromosomal break with incompatible 3’ overhangs. Repair of this break can be achieved by three mechanisms: NHEJ, gene conversion (GC) or SSA (Figure 4A). NHEJ (which requires *ku* and *ligD*) ligates the broken ends after varying degrees of end-resection and end-healing, and therefore does not reconstitute an active *lacZ* gene. Thus, repair via NHEJ yields a white colony that is kanamycin-resistant in most cases, unless an especially long end-resection invades the kanamycin-resistance gene. GC (which is *recA*-dependent) uses the downstream *lacZ*-ΔN as a homologous template to repair the DSB. This mechanism of repair reconstitutes a functional *lacZ* gene while leaving the kanamycin-resistance cassette intact, yielding a blue, kanamycin-resistant colony. Finally, repair by SSA (which is *recBCD*-dependent) results from bidirectional single-strand resection from the I-SceI sites, revealing complementary single strands that can anneal and reconstitute an intact *lacZ* allele with a deletion that eliminates the kanamycin-resistance marker. Thus, all three repair outcomes can be scored on agar plates containing X-gal and counterscreening for kanamycin resistance, as depicted in Figure 4A. Repair by SSA and GC is then confirmed by diagnostic PCR amplification (3).

We applied this system to understand the function of RecO in DSB repair. Induction of a DSB by transformation of an I-SceI encoding plasmid in wild-type cells yields an approximate 50:50 mixture of white and blue colonies, as reported previously (3). Of these blue colonies, 80% are GC (40% of the total outcomes) and 20% are SSA (10% of the total) (Figure 4B–E). In the *recO* strain, we observed a 5-fold decrement in the yield of blue colonies after I-SceI transformation (Figure 4B–E). This result indicates a role for RecO in DSB repair.
Figure 4. RecO is required for *adnAB*-independent GC and SSA. (A) Schematic of DSB repair outcomes in the I-SceI system, adapted from (3). An I-SceI-induced DSB is initiated by transformation of a plasmid encoding I-SceI into a strain bearing a previously described I-SceI recombination substrate. Repair of this incompatible DSB yields either blue or white colonies. White colonies can result either from NHEJ-mediated repair, or inactivation of the I-SceI enzyme through mutation. Blue colonies indicate that the defective *lacZ* coding sequence has been restored either through SSA or GC. DNA resection that occurs during SSA results in deletion of the kanamycin marker, whereas GC does not. (B) Each panel shows *M. smegmatis* of the indicated genotype transformed with the I-SceI encoding plasmid and cultured on agar plates containing hygromycin and X-gal. (C) Graph of GC frequency according to strain genotype. For each strain, the relative GC frequency is calculated from the genotyping algorithm in (A) and the raw data presented in the table in (E). The graphed *ΔadnAB* data is from (3). (D) SSA frequency for the strains indicated. The graphed *ΔadnAB* data is from (3). (E) Survival, % blue and pathway outcome for each strain. #blue is the number of blue colonies genotyped to give the GC and SSA numbers. Relative GC frequency is given as (%blue) (#GC/#blue). SSA frequency is given as (%blue) (#SSA/#blue).
genotyping the blue colonies indicated that gene conversion outcomes were 4-fold less frequent than in wild-type cells (relative GC frequency in WT versus \(\Delta recO\), 37% versus 9.5%), compared with the 2-fold decrement observed in the \(\Delta adnAB\) strain in our prior study (3).

We then examined the GC frequency in the \(\Delta adnAB\ \Delta recO\) double mutant and observed a near complete abolition of GC outcomes (relative GC frequency in \(\Delta adnAB\ \Delta recO = 0.077\%\)). These data indicate that \(adnAB\) and \(recO\) define parallel pathways of \(recA\)-dependent gene conversion.

**Requirement for RecO in the RecBCD-dependent SSA pathway**

We next examined the role of RecO in the mycobacterial SSA pathway. In wild-type cells, SSA outcomes accounted for 26% of the blue colonies and 12.9% of the total outcomes (Figure 4D and E). In the \(\Delta recO\) strain, we observed a 15-fold decrement in SSA outcomes (relative SSA frequency in \(\Delta recO = 0.86\%\)), compared with the complete loss (0/120) of SSA previously observed in the \(\Delta recBCD\) strain. As predicted, there were no blue colonies in the \(\Delta recO\ \Delta recBCD\ \Delta adnAB\) strain, consistent with the loss of both GC and SSA pathways (Figure 4E). These assays reveal a genetic requirement for the RecO protein in the mycobacterial SSA pathway.

**DNA binding by mycobacterial RecO is stimulated by zinc**

The genetic data indicate a role for mycobacterial RecO in RecA-dependent GC and in SSA, dual functions not reported previously for RecO proteins. To correlate these \(in vivo\) roles with the biochemical properties of the mycobacterial RecO protein, we produced His-tagged \textit{M. smegmatis} RecO in \textit{E. coli} and purified it by nickel affinity chromatography (Supplementary Figure S3). We first examined the interaction of RecO with single-stranded DNA under equilibrium conditions by fluorescent anisotropy. Initial experiments revealed a strong dependence of the MsRecO interaction with ssDNA on the presence of zinc. In buffer A with 50 mM NaCl, MsRecO interacted weakly with ssDNA with an apparent dissociation constant \((K_d)\) of \(>5\) \(\mu\)M (Figure 5A). \textit{E. coli} RecO (EcRecO) had a noticeably stronger affinity to ssDNA with an apparent \(K_d\) of 0.45 (±0.11) \(\mu\)M (Figure 5A). However, addition of 10 \(\mu\)M Zn(OAc)\(_2\) significantly stimulated MsRecO binding to ssDNA with a \(K_d\) of MsRecO was 0.036 (± 0.004) \(\mu\)M. In contrast, zinc had no or a slightly inhibitory effect on binding of EcRecO to ssDNA (Figure 5A). Previously, we demonstrated a strong inhibitory effect of increased salt concentration on DNA binding by EcRecO (22). Consistent with these prior results, EcRecO did not appreciably bind ssDNA at 100 mM NaCl, whereas MsRecO...
Mycobacterial RecO does not interact with C-terminal tail of SSB

SSB exhibits one of the highest affinities for ssDNA of any protein and is critical for protecting it from nucleases and for mediating interactions with other DNA-binding proteins (35). The majority of such interactions are mediated by a conserved C-terminal tail of SSB (SSB-Ct), which is essential for the viability of E. coli (36). Interaction of EcRecO with the conserved hydrophobic residues of SSB-Ct is important for binding of RecO to SSB-coated ssDNA and DNA annealing at moderate salt concentrations (22) as well as for loading of RecA by RecOR (18). However, this SSB-Ct binding is not conserved among all RecO proteins. For example, the SSB-Ct binding site is not conserved in DrRecO, which also contains a 4xCys zinc-binding motif, similar to MsRecO. We therefore sought to determine whether MsRecO interacts with either E. coli SSB-Ct or mycobacterial SSB-Ct and to interrogate the requirement for the RecO/SSB-Ct interaction for RecO function. An alignment of the SSB C-termini from E. coli and M. smegmatis is shown in Figure 6A. In comparison to the E. coli peptide, the M. smegmatis peptide is two amino acids shorter and differs by substitution of a proline in place of isoleucine in third position from the C-terminus and glutamate substituting aspartate in the adjacent position.

We measured the strength of the RecO/SSB-Ct interaction by anisotropy of a fluorescein-labelled peptide composed of the C-terminal amino acids of E. coli SSB (FAM-WMDFDDIPF) or M. smegmatis SSB (FAM-GADDEPPF). MsRecO did not bind SSB-Ct from E. coli or M. smegmatis (Kd > 10 μM, Figure 6B) regardless of whether zinc was present. EcRecO interacted with both peptides, albeit with lower affinity for MsSSB-Ct (Kd of 3.0 μM) compared with EcSSB-Ct (Kd of 0.12 μM) (Figure 6B). The lack of interaction between MsRecO and MsSSB-Ct raises questions about whether and how mycobacterial RecO can bind ssDNA when SSB is present.

Mycobacterial RecO can anneal SSBCA-coated ssDNA

The ΔrecO M. smegmatis strain is defective for DSB repair via the SSA pathway (Figure 4), which suggests that MsRecO may aid in annealing SSB-coated DNA at resected DSB ends. In E. coli, annealing of ssDNA by RecO requires the hydrophobic interaction between RecO and the SSB-Ct at moderate salt concentration (22). We investigated the ability of EcRecO and MsRecO to anneal SSB-coated complementary single-strand DNAs using a fluorescence quenching annealing MsSSB-coated ssDNA (Figure 7). This annealing activity was abolished when truncated EcSSBAC was included in the reaction in lieu of full length SSB (Figure 7A and B), indicating that the annealing activity of EcRecO depends on interaction with the SSB-Ct. MsRecO also promoted annealing of ssDNA coated with either EcSSB or MsSSB when Zn2+ was present in the annealing reaction. However, in contrast to EcRecO, the annealing activity of MsRecO was unaffected by deletion of the SSB-Ct (Figure 7). Therefore, mycobacterial RecO is able to anneal complementary SSB-coated DNA strands independent of an interaction with the SSB-Ct.
DISCUSSION

We have examined the genetic and biochemical functions of mycobacterial RecO and found dual in vivo functions in the HR and SSA pathways. The critical role of RecO in the RecA-dependent recombination pathway is clearly distinct from the function of RecO in E. coli, but similar to B. subtilis, in which the RecFOR system has an important role in recombination in wild-type cells. Our studies also clearly indicate a RecO-independent, AdnAB-dependent HR pathway. The genetic requirement for RecO in the mycobacterial HR pathway may reflect a RecA loading function of RecO in association with RecF and/or RecR. This model is based on the function of the RecFOR complex in E. coli as a RecA mediator (16). Alternatively, the RecO protein may be required not for RecA loading, but rather for second-end capture via its annealing activity, as documented for yeast Rad52 (28).

Our studies indicate that mycobacterial RecO differs from E. coli RecO in its interaction with SSB. Mycobacterial RecO does not interact directly with the SSB C-terminal tail. It has been reported that the mycobacterial SSB C-terminal tail interacts directly with RecA both in vitro and in vivo (37), an interaction not described for other bacterial RecA proteins (35,38). This direct interaction between SSB-Ct and RecA may suggest that RecO is not needed for recruitment of RecA to SSB-coated ssDNA. We predict that if RecO functions as a mediator for mycobacterial RecA, then the role of RecO would not be to recruit RecA to SSB-coated DNA, but rather to facilitate RecA/SSB exchange. Alternatively, the direct recruitment of RecA by the SSB-Ct may reflect a fundamentally different mechanism of RecA loading in mycobacteria that may not require RecO.

Multiple other components of the two parallel pathways of recombination in mycobacteria remain to be identified. For the RecO pathway, the nuclease that resects the DSB before RecO action is unknown. In the RecFOR systems of other bacteria, RecJ performs this function (16,39). However, no RecJ exonuclease has been identified in...
mycobacteria, leaving the identity of the resection nuclease in the mycobacterial RecO pathway obscure.

Our prior results indicated that mycobacteria have an SSA pathway that depends on RecBCD (3). In this work we implicate the RecO protein in the SSA pathway. RecO proteins are known to exhibit single-strand annealing activity. However, the \textit{in vivo} function of this annealing activity has not been demonstrated. It has been suggested that this annealing activity may catalyse the annealing of the second resected end onto the displaced D-loop created by strand invasion during RecA-dependent HR (28). However, this model was based on bacteria that do not apparently encode an SSA pathway of DSB repair. Our data suggest an additional \textit{in vivo} function for the DNA annealing activity of RecO in the annealing of single-stranded DNA during repair by the SSA pathway.

This function of RecO in mycobacteria echoes that of yeast Rad52, which also anneals single-stranded DNA and is genetically required for both the gene conversion and SSA pathways (25). Our biochemical characterization of mycobacterial RecO indicates that the protein is adept at DNA annealing. Mycobacterial RecO binds single-stranded DNA more avidly than \textit{E. coli} RecO. This high-affinity DNA binding might allow mycobacterial RecO to anneal SSB-coated single-stranded DNA without interacting with the SSB C-terminal tail, an interaction that is required for the annealing activity of \textit{E. coli} RecO. MsRecO DNA binding and annealing activities are dependent on added Zn\textsuperscript{2+}, a requirement that is likely due to the tetracysteine zinc-binding motif found in some RecO orthologs but not \textit{E. coli} RecO. It is tempting to speculate that there is a correlation between the presence of a tetracysteine zinc-binding motif in RecO and a lack of binding to the SSB-Ct, because MsRecO and DrRecO share these features. \textit{B. subtilis} RecO possesses a similar 4xCys motif, can compete for DNA binding with SsbA and SsbB (40), and can anneal ssDNA in the presence of SsbA (41), which contains a conserved SSB-Ct. However, the requirement for the SsbA C-terminus in this process has not been examined (41,42). An alternate possibility is that mycobacterial RecO may interact with a region of SSB distinct from the C-terminus, such that its DNA annealing activity still relies on interaction with SSB but via a distinct mechanism.

In summary, our data document a dual function of mycobacterial RecO in two pathways of homology-directed DNA repair. Further biochemical characterization of the RecO-SSB interaction and investigation of the ability of RecO to load RecA onto ssDNA will elucidate the biochemical function of RecO during HR. In addition, separation of function mutations that ablate RecO annealing activity will be useful to understand the novel requirement for RecO in the SSA pathway documented here.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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