RecF and RecR Play Critical Roles in the Homologous Recombination and Single-Strand Annealing Pathways of Mycobacteria

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ABSTRACT

Mycobacteria encode three DNA double-strand break repair pathways: (i) RecA-dependent homologous recombination (HR), (ii) Ku-dependent nonhomologous end joining (NHEJ), and (iii) RecBCD-dependent single-strand annealing (SSA). Mycobacterial HR has two presynaptic pathway options that rely on the helicase-nuclease AdnAB and the strand annealing protein RecO, respectively. Ablation of adnAB or recO individually causes partial impairment of HR, but loss of adnAB and recO in combination abolishes HR. RecO, which can accelerate annealing of single-stranded DNA in vitro, also participates in the SSA pathway. The functions of RecF and RecR, which, in other model bacteria, function in concert with RecO as mediators of RecA loading, have not been examined in mycobacteria. Here, we present a genetic analysis of recf and recr in mycobacterial recombination. We find that RecF, like RecO, participates in the AdnAB-independent arm of the HR pathway and in SSA. In contrast, RecR is required for all HR in mycobacteria and for SSA. The essentiality of RecR as an agent of HR is yet another distinctive feature of mycobacterial DNA repair.

IMPORTANT

This study clarifies the molecular requirements for homologous recombination in mycobacteria. Specifically, we demonstrate that RecF and RecR play important roles in both the RecA-dependent homologous recombination and RecA-independent single-strand annealing pathways. Coupled with our previous findings (R. Gupta, M. Ryzhikov, O. Koroleva, M. Unciuleac, S. Shuman, S. Korolev, and M. S. Glickman, Nucleic Acids Res 41:2284–2295, 2013, http://dx.doi.org/10.1093/nar/gks1298), these results revise our view of mycobacterial recombination and place the RecFOR system in a central position in homology-dependent DNA repair.

 nature has evolved mechanisms to repair the structurally diverse DNA lesions that arise during chromosomal replication and from exogenous mutagens. Double-strand breaks (DSBs) are especially deleterious, insofar as a single chromosomal DSB can be lethal to a cell if not repaired prior to cell division. The major mechanisms of DSB repair are conserved, at least in their general principles, from bacteria to humans. This parallel is especially relevant for mycobacteria, which implement the same three distinct DSB repair pathways operative in eukarya: homologous recombination (HR), nonhomologous end joining (NHEJ), and single-strand annealing (SSA). Mycobacterial NHEJ relies on the end-binding protein Ku, DNA ligase LigD (a multifunctional ligase–polymerase–3'-phosphoesterase), DNA ligase LigC1, and two additional polymerases (PolD1 and PolD2) (1–9). The SSA pathway of mycobacteria repairs DSBs that arise between repeats, with consequent deletion of the DNA between the repeats. SSA is independent of the strand exchange protein RecA but requires the helicase-nuclease RecBCD and the strand-annealing protein RecO (9, 10).

The central agent of homology search in the HR pathways of all organisms is a strand exchange protein that polymerizes on the single-strand DNA (ssDNA) resulting from end resection at a DSB. In bacteria, the strand exchange protein is RecA; in eukarya, it is Rad51. RecA nucleation on ssDNA is regulated in order to prevent inappropriate nucleoprotein filament formation, which can be toxic (11). The presence of single-strand binding protein (SSB) on ssDNA prevents RecA loading unless a “mediator” protein or protein complex catalyzes the exchange of SSB for RecA to create the RecA nucleoprotein filament. In Escherichia coli, RecBCD has mediator activity (12, 13), with the RecFOR system playing a secondary role at DSBs when RecBCD is inactivated (14). However, the RecFOR system plays a major role in recombinational repair of single-strand gaps in wild-type (WT) E. coli (15, 16). In Bacillus subtilis and Deinococcus radiodurans, the RecFOR system is the principal mediator that loads RecA onto ssDNA. In B. subtilis, recr and recO are required for RecA focus formation on the chromosome in response to replication stress and exogenous DNA damage (17). Loss of RecF does not abolish RecA focus formation during damage but does delay the kinetics, including following a DSB produced by the homing endonuclease I-SceI (17). In contrast, all three components of the RecFOR system, in addition to RecA, are required for B. subtilis spore resistance to ionizing radiation (IR) and desiccation (18). D. radi-
dedicated to the SSA pathway and is not required for HR (9). Thus, the RecFOR system is an important mediator in many bacteria.

The biochemical properties of RecF, RecO, and RecR that underlie their mediator function are only partially understood. The RecF protein is an ABC-family ATPase that dimerizes upon ATP binding (20). RecF binds ssDNA or double-strand DNA (dsDNA); ATP hydrolysis causes its dissociation from the DNA (21, 22). The RecO protein has an N-terminal OB (oligonucleotide-binding) domain and can bind to ssDNA or dsDNA (23). D. radiodurans and Mycobacterium RecO have an additional tetracysteine zinc-binding domain; zinc binding has been shown to stimulate annealing of SSB-coated cDNA strands by these RecO proteins (10, 24). DNA strand annealing activity has also been demonstrated for other bacterial RecO proteins, which do not require zinc but instead rely on an interaction with the SSB C terminus, an interaction that the mycobacterial and Deinococcus RecO proteins have relinquished (10, 25, 26). In addition to strand annealing, RecO can promote RecA nucleation and filament formation on SSB-coated ssDNA, but this activity requires RecR (27, 28).

The RecR protein has no known enzymatic activity. Whereas E. coli RecR shows no DNA binding, D. radiodurans and B. subtilis RecR have been reported to bind DNA (29, 30). E. coli or Thermus thermophilus RecR can stimulate DNA binding activity of either RecF or RecO by physically interacting with both via the RecR TOPRIM domain. Thus, RecR can be perceived as the central protein that stabilizes the assembly of a RecFOR complex on recombination substrates (31, 32).

The presynaptic phase of mycobacterial recombination is incompletely understood. Mycobacteria encode two resection helicase-nucleases, RecBCD and AdnAB. RecBCD in mycobacteria is dedicated to the SSA pathway and is not required for HR (9). AdnAB was originally described in mycobacteria (33–36) and participates in end resection in the mycobacterial HR pathway (9). However, the HR defect in the M. smegmatis ΔadnAB strain is incomplete. The M. smegmatis ΔadnAB strain is only mildly sensitized to IR and other clastogens. In the repair of I-SceI generated DSBs, HR is reduced in the ΔadnAB strain, but a residual ΔadnAB-independent HR pathway is evident (9). This residual HR is abolished in a ΔadnAB ΔrecO strain, indicating that adnAB and recO define parallel pathways leading to recA-dependent HR. However, it is not known whether the AdnAB enzyme has intrinsic RecA loading activity (analogous to E. coli RecBCD) or whether a separate mediator complex controls RecA loading after AdnAB-catalyzed duplex unwinding and end resection. The role of RecF and RecR in the RecO arm of the mycobacterial HR pathway is unknown.

As mentioned above, mycobacterial RecO is required for the SSA pathway also. The RecO protein is a prokaryotic analog of Rad52, which is required for SSA in yeast (37). Rad52 (38–42) and RecO (10, 26) can anneal ssDNA coated by RPA (the eukaryal single-strand DNA binding protein) or SSB, respectively. However, whether RecO is the only component of the RecFOR system required for the mycobacterial SSA pathway, or whether RecF and RecR also are required, is not known.

In this study, we undertake a genetic analysis of recF and recR in mycobacteria. Our results indicate that RecR is an essential agent of HR and SSA and that RecF is required for SSA.

### MATERIALS AND METHODS

**Gene knockout and complementation experiments.** The genotypes of the *M. smegmatis* strains used in the study are listed in Table S1 in the supplemental material, and the list of primers is included in Table S2. Deletion of *recR* and *recF* was performed in *M. smegmatis* by specialized transduction using a temperature-sensitive mycobacteriophage, phAE87, as described previously (10), which yielded hygromycin-resistant transductants. The hygromycin marker (*hgy*; ranked by *loxP* sites) was removed subsequently by transiently expressing Cre recombinase. Southern blotting was performed using either 5’ or 3’ flanking DNA sequence as the probe to confirm the final unmarked ΔrecR and ΔrecF mutants. Following a similar approach, double mutants were constructed by deleting recR and recF in the ΔadnAB strain to generate ΔrecR ΔadnAB and ΔrecF ΔadnAB mutants. To obtain the ΔrecF ΔrecO double mutant, recO was deleted in the ΔrecF background and confirmed by Southern analysis. Attempts to construct the ΔrecR ΔrecO double mutant were unsuccessful despite multiple attempts, suggesting either synthetic lethality or impairment of recombination. To distinguish between these possibilities, we followed a four-step procedure wherein we first introduced the kanamycin resistance plasmid pRG109 (containing the intact *recO* gene along with its native promoter) at the chromosomal *attB* site of the ΔrecO strain, followed by deletion of *recR* by specialized transduction, exchange of the chromosomal *recO* copy at the *attB* site with a streptomycin-resistance vector pDB60 (43), and the final unmarking of the ΔrecO::hyg allele to generate the ΔrecR ΔrecO double mutant. Following this scheme, we eventually obtained the ΔrecR ΔrecO double mutant, which was confirmed by Southern blotting and showed that loss of *recR* and *recO* in *M. smegmatis* is not synthetically lethal but deleting a second gene in either of the two single mutants (ΔrecR or ΔrecO) is difficult due to severely reduced levels of recombination. To perform I-SceI assays with the ΔrecR ΔrecO double mutant, the streptomycin resistance vector at the *attB* site was replaced with the kanamycin resistance plasmid pRG110 (9).

For genetic complementation of the ΔrecR strain, the *M. smegmatis* recR gene along with the 332-bp upstream sequence (which presumably contains its native promoter overlapping the coding sequence of the gene encoding the hypothetical protein MSMEG_6280) was cloned in the mycobacterial integrative vector pMV306Kan to generate pRGM41. Because *M. smegmatis* recR seems to be in an operon, with *dnaN* being the first gene, the 489-bp sequence encompassing the presumed *dnaN* promoter was fused to the *recF* coding sequence in the pMV306Kan vector backbone to create the complementation clone pRGM42. The complementation clones were integrated into the chromosome of the knockout strains by plasmid transformation. The phenotypes of complemented strains were studied in different clastogen sensitivity assays alongside other control strains that harbor the chromosomally integrated vector pMV306Kan and have different genetic backgrounds, such as WT, ΔrecR, ΔrecF, and ΔadnAB backgrounds.

**Growth studies.** *M. smegmatis* strains were grown at 37°C in LB medium supplemented with 0.5% glycerol, 0.5% dextrose, and 0.1% Tween 80. For determination of doubling times, the strains were inoculated into fresh medium to an *A*₅₆₀ of ~0.1 and incubated at 37°C with constant shaking (150 rpm). Once log phase was achieved, the cultures were repeatedly diluted into fresh medium at regular intervals to maintain growth in log phase. Culture aliquots were removed at these time points to measure the *A*₅₆₀ and determine viable counts by serial dilution plating. A change in absorbance or colony counts was plotted against time, and doubling time was calculated using the following formula: doubling time = (log₂[A]₀/Aₜ]) / tₑ, where *tₑ* is the hour at which an aliquot from a growing culture was removed and *tᵢ* is time zero, the hour at which the culture was reinfused into fresh medium to begin the growth curve, and where G (the number of generations) = (log[number of bacteria or *A*₅₆₀ at *tᵢ] − log[number of bacteria or *A*₅₆₀ at *tₑ]])/0.301.

**Clastogen sensitivity.** *M. smegmatis* strains grown at 37°C to log phase (*A*₅₆₀ of 0.3 to 0.4) in LB medium supplemented with 0.5% glycerol, 0.5% dextrose, and 0.1% Tween 80 were subjected to treatment with increasing...
doses of three different DNA damaging agents, UV, methyl methane sulfonate (MMS), and IR, as described previously (10). Tenfold serial dilutions of the treated and untreated cells (for each strain) were spotted on LB agar plates or LB agar plates containing 20 μg/ml kanamycin (in the case of recR and recF complementation experiments), and percent survival was calculated compared to the viable counts of untreated control cells once visible colonies emerged on the plates.

To study the phenotype of the ΔrecRO double mutant, which harbors the streptomycin resistance empty vector pDB60 at the atbB site, strains with a chromosomally integrated copy of pDB60 were derived for other genetic backgrounds, such as WT, ΔrecF, ΔrecO, ΔrecA, and ΔadnAB backgrounds. Treatment with UV and IR was conducted as described above, except that serial dilutions of the treated and untreated cells were spotted on LB agar plates containing 20 μg/ml streptomycin for determination of the percent survival. Each clastogen treatment was performed twice with each experiment containing biologic duplicates, and the mean value of percent survival (with standard errors of the means [SEM]) for every strain is plotted in the figures shown. Mean survival differences between strains were compared using the unpaired Student’s t test, and the calculated P values are mentioned in the text with P < 0.05 as the threshold for significance.

I-SceI-mediated chromosomal recombination assay. M. smegmatis wild-type and mutant strains harboring the chromosomally integrated loxZ reporter construct, pRGm10, were subjected to the plasmid transformation-based I-SceI DSB repair assay as described previously (9). Briefly, the same molar amount of the I-SceI plasmid and the control vector plasmid were transformed to determine the frequencies of HR and SSA among the blue colonies by scoring for kanamycin resistance, whereby the frequency is the number of SSA events divided by the number of blue events times the frequency is the number of HR events divided by the number of blue survivors. Therefore, the frequency of SSA among most known species (both pathogenic and nonpathogenic) of the genus Mycobacterium (including M. smegmatis, M. tuberculosis, M. marinum, M. leprae, M. ulcerans, M. kansasii, M. bovis, M. avium, M. abscessus, M. gilvum, M. fortuitum, and M. vanbaalenii), with the RecF and RecR proteins sharing 70 to 88% and 86 to 98% amino acid identities, respectively. Homologs also are found in other Actinomycetales, such as Streptomyces coelicolor, Corynebacterium diphtheriae, Nocardia farcinica, and Rhodococcus equi, ranging in amino acid identity from 51 to 61% for RecF and 59 to 86% for RecR. Primary structure alignments of RecF and RecR from M. smegmatis, M. tuberculosis, E. coli, D. radiodurans, and B. subtilis are shown in Fig. S1 and S2 in the supplemental material. As we observed previously for mycobacterial RecO (10), M. smegmatis RecF and RecR are more similar to their homologs in D. radiodurans (36% identical for RecF; 51% identical for RecR) and B. subtilis (29% identical for RecF; 50% identical for RecR) than to the E. coli counterparts (26% identical for RecF; 43% identical for RecR).

To study the function of RecF and RecR in mycobacteria, we deleted these genes from the M. smegmatis chromosome, both in the wild-type background and in strains lacking other recombination factors, including adnAB and recO (see Table S1 for a complete strain list and Materials and Methods for details of strain construction). Figure 1A illustrates the strategy for genotyping and confirming the strains by Southern blotting for the ΔrecR::loxP-hyg-loxP allele and the ΔrecF::loxP allele after expression of Cre recombinase. Figure 1B shows the confirmation of ΔrecF::loxP-hyg-loxP and ΔrecF::loxP alleles. Finally, a double mutant of recO and recF was constructed by introducing the ΔrecO allele in the ΔrecF::loxP strain using specialized transduction, and genotypes were confirmed by Southern hybridization as previously described for recO (10) and as described above for recF (Fig. 1C).

Loss of recF or recR slows growth. We first characterized the growth of ΔrecR and ΔrecF strains alongside the WT and other recombination mutants by monitoring both the cell density measurements and the number of viable bacteria with time. The doubling time of the ΔrecR strain calculated from A660 cell density measurements was 219 min, and that of the ΔrecF strain was 191 min, compared to 174 min for the WT, 193 min for the ΔadnAB strain, 212 min for the ΔrecO strain, and 226 min for the ΔrecA strain (10). Doubling times determined by quantitation of viable bacteria were similar: 220, 187, and 188 min for ΔrecR, ΔrecF, and ΔadnAB strains, respectively. We also determined the doubling times of the combination mutants of recF and recR. Adding either ΔadnAB or ΔrecO to the single-mutant backgrounds resulted in slightly further reduced growth rates, as exhibited by the doubling times of 235 min for the ΔrecR ΔadnAB strain, 221 min for the ΔrecO strain, and 254 min for the ΔrecA strain (10). Doubling times determined by quantitation of viable bacteria were similar: 227 min for ΔrecR ΔadnAB, 216 min for ΔrecF ΔadnAB, 249 min for ΔrecR ΔrecO, and 238 min for ΔrecF ΔrecO strains. The slower growth of the recombination-defective mutants is reflected in their respective colony sizes on agar media (Fig. 2C, untreated).

Roles of RecF and RecR in resistance to UV-induced DNA damage. Studies on RecF and RecR in E. coli (44, 45), B. subtilis (17, 46), and D. radiodurans (19, 47) have led to the proposal that these two proteins function in concert with RecO as RecA mediators in the repair of DNA breaks and gaps. The M. smegmatis ΔrecO mutant is extremely sensitive to DNA damage and participates in two different pathways of recombination, recA-dependent HR and recA-independent SSA (10), implying that the function of RecO in the SSA pathway is distinct from its classic RecA mediator function. To determine if RecF and RecR are similarly involved in DNA repair in mycobacteria, we irradiated mutants...
FIG 1 Deletion of recF and recR from M. smegmatis. (A) Deletion of recR by specialized transduction. Map of the wild-type (WT) and recR::loxP-hyg-loxP loci with DraIII and EcoNI restriction sites, probe location, and predicted fragment sizes for the WT (8,703 bp with DraIII; 982 bp and 3,546 bp with EcoNI) and recR::loxP-hyg-loxP (4,042 bp with DraIII; 2,085 bp and 3,546 bp with EcoNI) after restriction digestion of chromosomal DNA. For the final recR::loxP mutant (after loss of the hygromycin marker), predicted fragment sizes are 8,200 bp with DraIII and 327 bp and 3,546 bp with EcoNI. Next to the restriction maps is the Southern hybridization of chromosomal DNA from WT M. smegmatis (lane 1), the recR::hyg mutant (lanes 2 and 5 in the indicated strain backgrounds), or the recR::loxP mutant (after Cre recombinase-mediated excision of the hygromycin marker; lanes 3, 4, 6, and 7). (B) Deletion of recF by specialized transduction. Map of the wild-type and recF::loxP-hyg-loxP loci with DraIII and AatII restriction sites, probe location, and predicted fragment sizes for the WT (3,161 bp with DraIII, 2,624 bp with AatII) and recF::loxP-hyg-loxP (1,819 bp with DraIII, 1,381 bp with AatII) after restriction digestion of chromosomal DNA. For the final recF::loxP mutant (after loss of the hygromycin marker), predicted fragment sizes are 2,161 bp with DraIII and 1,624 bp with AatII. Below the restriction maps is the Southern hybridization of chromosomal DNA from wild-type M. smegmatis, the recF::hyg strain (lanes 4 and 5 in the first two panels and lanes 2 and 9 in the third panel with the adnAB deletion background), and the recF::loxP strain (after Cre recombinase-mediated excision of the hygromycin marker; lanes 1 and 2 in the first two panels and lanes 3, 4, 6, 7, 10, 11, 13, and 14 in the third panel). (C) Deletion of recO in the ΔrecF strain by specialized transduction. Shown is the Southern hybridization for recO and recF loci of chromosomal DNA from WT M. smegmatis (lane 1), the recF::loxP recO::hyg strain (lanes 2 and 3, and the recF::loxP recO::loxP strain (after Cre recombinase-mediated excision of the hygromycin marker from the recO locus; lanes 4 to 12).
lacking these genes with increasing doses of UV light and compared their survival with that of ΔrecO, ΔadnAB, and ΔrecA strains. Loss of recR led to severe sensitivity to UV-induced DNA damage, equivalent to that of ΔrecO and ΔrecA strains (P = 0.367 for comparison of survival of the ΔrecR strain to that of the ΔrecO strain at 20 mJ/cm²) (Fig. 2A and C). In contrast, the ΔrecF strain showed a sensitivity phenotype intermediate between that of ΔadnAB and ΔrecO mutants (P = 0.002 for comparison of survival of the ΔrecF strain to that of the ΔadnAB strain at 20 mJ/cm² and P = 0.023 for comparison of survival of the ΔrecF strain to that of the ΔrecO strain at 20 mJ/cm²) (Fig. 2A and C).

To interrogate epistasis, we examined the phenotypes of ΔadnAB ΔrecF, ΔadnAB ΔrecR, and ΔrecO ΔrecF double mutants. The ΔadnAB ΔrecF mutant was more sensitive to UV than either the ΔadnAB or ΔrecF strain (P = 0.036 for comparison of survival of the ΔadnAB ΔrecF strain to that of the ΔrecF strain at 20 mJ/cm², P = 0.002 for comparison of survival of the ΔadnAB ΔrecF strain to that of the ΔadnAB strain at 20 mJ/cm²) (Fig. 2B), indicating that these two genes act in different pathways of UV resistance. However, the ΔadnAB ΔrecF strain did not phenocopy the sensitivity of the ΔrecO strain. The ΔrecF ΔrecO double mutant was slightly more sensitive than the ΔrecO strain (Fig. 2B and C) (P = ~0.05 [0.047] for comparison of survival of the ΔrecF ΔrecO strain to that of the ΔrecO strain at 20 mJ/cm²). Epistasis analysis of recF indicated that the ΔrecF ΔadnAB strain was significantly more sensitive than the ΔadnAB strain but no more sensitive than the ΔrecR strain, which itself is highly sensitive and phenocopies the ΔrecA strain (Fig. 2A) (P = 0.002 for comparison of survival of the ΔrecR ΔadnAB strain to that of ΔadnAB strain at 20 mJ/cm², P = 0.188 for comparison of survival of the ΔrecR ΔadnAB strain to that of the ΔrecR strain at 20 mJ/cm²).

**Effects of recF and recR deletion on susceptibility to DNA alkylation.** Involvement of recF and recR in survival after DNA damage was investigated further by measuring the sensitivity of the different mutant strains to methyl methane sulfonate (MMS), which methylates DNA bases. The repair of methylated DNA bases involves creation of abasic intermediates and DNA strand breaks, which are repaired by the base excision machinery or channeled into recombinational repair (48). We observed that the ΔrecR mutant was highly sensitive to MMS, to a degree almost as severe as that of the ΔrecA and ΔrecO mutants (Fig. 3A and C) (P = 0.233 for comparison of survival of the ΔrecR mutant to that of the ΔrecO mutant at 60 min of exposure). The ΔrecF strain displayed an intermediate sensitivity, between that of the WT and the ΔrecA, ΔrecO, and ΔrecR strains, which was similar to that of the ΔadnAB mutant (Fig. 3A) (P = 0.099 for comparison of survival of the ΔrecR mutant to that of the ΔadnAB mutant at 60 min of exposure, P = 0.015 for comparison of survival of the ΔrecF mutant to that of the ΔrecO mutant at 60 min of exposure). Epistasis testing between adnAB and recF showed that the ΔadnAB ΔrecF strain was more sensitive than either single mutant, suggesting parallel pathways (Fig. 3B and C) (P = 0.019 for comparison of survival of the ΔadnAB ΔrecF mutant to that of the ΔrecF mutant at 60 min of exposure, P = 0.001 for comparison of survival of the ΔadnAB ΔrecF mutant to that of the ΔrecF mutant at 60 min of exposure). In contrast, loss of adnAB in the ΔrecR background slightly sensitized the ΔrecR strain at short exposure times, but the survival was indistinguishable at the final time point of MMS exposure (Fig. 3A and C) (P = 0.001 for comparison of survival of the ΔrecR ΔadnAB mutant to that of the ΔrecR mutant at 60 min of exposure). In addition, the ΔrecO strain was not further sensitized by the loss of recF (Fig. 3B and C).
(P = 0.182 for comparison of survival of the ΔrecF ΔrecO mutant to that of the ΔrecO mutant at 60 min of exposure), indicating that almost all resistance to MMS is recO and recR dependent and that only a fraction of this resistance requires recF and/or adnAB.

RecF and RecR involvement in repair of IR-induced DNA damage. We next tested the sensitivity of these strains to IR. Besides causing base damage in the DNA, IR also directly induces DNA strand breaks. M. smegmatis lacking recR is approximately 5-fold more sensitive to IR than the ΔrecO mutant (Fig. 4A and C) (P = 0.025 for comparison of survival of the ΔrecR mutant to that of the ΔrecO mutant at 448 Gy). Loss of recF conferred more severe sensitivity than loss of adnAB but not as severe as the loss of recR (Fig. 4A) (P = 0.012 for comparison of survival of the ΔrecF mutant to that of the ΔadnAB mutant at 448 Gy, P = 0.002 for comparison of survival of the ΔrecF mutant to that of the ΔrecR mutant at 448 Gy). We observed epistasis between recF and recO in this assay (Fig. 4B) (P = 0.201 for comparison of survival of the ΔrecF ΔrecO mutant to that of the ΔrecO mutant at 448 Gy), indicating that these genes function in the same pathway of IR resistance. To our surprise, when we examined the ΔadnAB ΔrecF or ΔadnAB ΔrecR strains, we observed IR dose-specific effects on survival. At low IR doses, the double mutants phenocopied or were less sensitive than the recF or recR single mutants (Fig. 4A and B) (P = 0.199 for comparison of survival of the ΔadnAB ΔrecF mutant to that of the ΔrecF mutant at 112 Gy, P = 0.001 for comparison of survival of the ΔadnAB ΔrecR mutant to that of the ΔrecR mutant at 112 Gy). However, at higher doses of IR, loss of adnAB ameliorated the lethality seen in both recF and recR mutants (Fig. 4A and B) (P = 0.010 for comparison of survival of the ΔadnAB ΔrecF mutant to that of the ΔrecF mutant at 728 Gy, P = 0.008 for comparison of survival of the ΔadnAB ΔrecR mutant to that of the ΔrecR mutant at 728 Gy). These results indicate that AdnAB exacerbates the effects of high-dose IR in the absence of the RecFOR system, possibly due to unrestrained resection at unrepaired DSBs (discussed further below). In addition, the epistasis analysis of adnAB and recR from all three clastogen assays suggests that the adnAB pathway of DNA repair requires recR.

Genetic complementation of the ΔrecF and ΔrecR clastogen sensitivity phenotypes. The recF gene (MSMEG_003) appears to be in an operon that includes dnaN (encoding the β subunit of DNA polymerase III), MSMEG_002 (encoding a 6-phosphogluconate dehydrogenase), recF, and MSMEG_004 (encoding a hypothetical protein). The annotated translational initiation codon of the recF gene (MSMEG_6279) overlaps the 3′ end of MSMEG_6280 (which encodes a conserved hypothetical protein) by 35 nucleotides. recR is upstream of MSMEG_6278 in a possible operon arrangement, and MSMEG_6280 is immediately followed by the divergently transcribed N-acetylmuramoyl-t-alanine amidase gene, MSMEG_6281 (Fig. 1). Thus, the genomic organization of the recF and recR loci raise the possibility of polar effects from our deletion alleles. To verify that the observed phenotypes of the ΔrecF and ΔrecR strains are due to loss of the deleted genes and not to polar effects or spontaneous mutations elsewhere in the chromosome, we performed a complementation test. Providing a copy of recF or recR at the phase L5 attachment site in the chromosome of the ΔrecF or ΔrecR mutant, respectively, resulted in complete reversal of the UV and IR sensitivity phenotypes (Fig. 5A and C). Complementation of ΔrecF ΔadnAB or ΔrecR ΔadnAB strains with recF and recR, respectively, restored the UV and IR sensitivity to the level of the ΔadnAB strain in both cases (Fig. 5B and D). These results confirm that the phenotypes of the ΔrecF and ΔrecR strains are due to loss of RecF and RecR function.

Effects of loss of recF and recR on mycobacterial HR and SSA pathways. UV, MMS, and IR produce a wide spectrum of DNA lesions, including base damage and single-strand breaks, which
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We observed a complete loss of HR/gene conversion in the ΔrecR strain, whereas the ΔrecO strain had a partial defect (Fig. 6A and C). This result indicates that RecR is required for all HR in M. smegmatis, but only a subset of HR requires RecO. A 2-fold decrement in HR was observed in the ΔrecF mutant (relative HR frequency of 22.5% in the ΔrecF strain versus 39.4% in the WT) (Fig. 6A). The mild decrement in HR in the ΔrecF strain is in agreement with the results of the clastogen experiments in which the ΔrecF strain is only mildly sensitized compared to the ΔrecR strain.

We observed that ΔrecF and ΔrecR mutants both were impaired for SSA. Among 105 blue ΔrecF mutant outcomes, we did not score a single SSA event, i.e., all of the blue colonies resulted from HR (Fig. 6C). For the ΔrecR mutant, only one blue colony was obtained among 500 total survivors, and this was an SSA event. The relative frequencies of SSA in the ΔrecF, ΔrecR, and ΔrecO strains were 0%, 0.2%, and 0.86%, respectively, versus 9.9% in the WT. This result indicates that all three proteins, RecF, RecO, and RecR, participate in the SSA pathway, with recF being strictly required for SSA repair.


dependent DSB repair pathways, we have adapted the homing endonuclease I-SceI to interrogate DSB repair pathway choice in mycobacteria (1, 9, 10). This system utilizes a recombination reporter substrate integrated into the chromosome and allows scoring of three DSB repair pathway outcomes: HR, NHEJ, and SSA. Our previous studies demonstrated that all HR events are recA dependent, but there are two parallel presynaptic pathways defined by RecO and AdnAB (10). RecO has an additional role in the recA-independent SSA pathway (10). To analyze the function of RecF and RecR in homology-dependent DSB repair, we used this I-SceI-based chromosomal recombination assay.

We observed that all ΔrecF and ΔrecR strains are not visibly, they are within the symbol. No viable counts were obtained for the ΔrecO, ΔrecF, ΔrecR, and ΔrecF ΔadnAB strains. (C) Tenfold dilutions of the indicated strains, either untreated or IR treated (112 Gy), are shown. Each graphed point represents the mean from biological duplicates, and error bars are SEM; when they are not visible, they are within the symbol. No viable counts were obtained for the ΔrecR strain in panel A and ΔrecF ΔrecO strain in panel B at the highest dose of IR exposure. Because 0% survival cannot be plotted on the logarithmic y axis, these data points are not visible.

FIG 4 Ionizing radiation sensitivity of ΔrecF and ΔrecR strains. (A) Survival curves of WT M. smegmatis and ΔadnAB, ΔrecO, ΔrecF, ΔrecR, and ΔrecR ΔadnAB strains exposed to the dose of ionizing radiation indicated on the x axis. Survival is plotted on a log scale and is calculated compared to an unexposed control for each strain. (B) Survival curves of WT M. smegmatis and ΔadnAB, ΔrecO, ΔrecF, ΔrecF ΔrecO, and ΔrecF ΔadnAB strains. (C) Tenfold dilutions of the indicated strains, either untreated or IR treated (112 Gy), are shown. Each graphed point represents the mean from biological duplicates, and error bars are SEM; when they are not visible, they are within the symbol. No viable counts were obtained for the ΔrecR strain in panel A and ΔrecF ΔrecO strain in panel B at the highest dose of IR exposure. Because 0% survival cannot be plotted on the logarithmic y axis, these data points are not visible.
mutant to that of the ΔrecR mutant at 728 Gy). In the I-SceI-based DSB repair assay, loss of recO in the ΔrecR background did not reverse the HR phenotype of the ΔrecR mutant (Fig. 7C). From these results, we conclude that RecR is required for both AdnAB- and RecO-dependent pathways of recombinational repair.

To determine if the recF and adnAB pathways of HR are parallel, we compared ΔrecF ΔrecO and ΔrecF ΔadnAB strains. Loss of recF and adnAB in combination abolished HR, consistent with these two genes defining parallel pathways of recombination (Fig. 7C). Loss of recO and recF in combination also abolished HR (Fig. 7C). The implications of these results are discussed below.

**DISCUSSION**

We have conducted a genetic analysis of the role of RecF and RecR in mycobacterial recombination. Coupled with our previous study of RecO (10), the present results prompt us to revise our model of the involvement of the RecFOR system in mycobacterial recombination.

**Homologous recombination.** Our prior studies indicated that loss of RecO resulted in a partial loss of HR at I-SceI-induced DSBs. Loss of RecO and AdnAB in combination abolished HR events, phenocopying loss of RecA. Here we find that the ΔrecF strain is clastogen sensitive, but it is less so than the ΔrecA strain. In the I-SceI assay system, the ΔrecF strain causes only a mild decrement in HR efficiency (approximately 50% of the WT level), similar to that of both the ΔrecO and ΔadnAB strains. The effacement of HR in the ΔrecF ΔadnAB strain supports an assignment of RecF to an AdnAB-independent arm of the HR pathway, previously defined by RecO (10). These results are consistent with the role of RecF in recombination in *E. coli* and *B. subtilis* as an accessory factor in the RecFOR system, in which RecF is thought to recognize the ssDNA-dsDNA junction generated after end resection.

In both of these bacteria, RecF stimulates RecOR-mediated RecA loading, but RecOR also is competent for RecA loading without RecF (17, 45).

In contrast to the accessory role played by RecF, we find that all mycobacterial HR requires RecR. This finding implicates RecR as a component of the core recombination machinery and indicates that RecR is required for both subpathways leading to RecA-dependent HR: RecFO and AdnAB. This result, coupled with the imputed role of the RecFOR complex as a mediator that loads

**FIG 5** Complementation of ΔrecF and ΔrecR strains. (A and C) Survival curves of WT *M. smegmatis* and ΔrecF, ΔrecR, ΔrecF recF, ΔrecR recR, and ΔrecR recR strains exposed to the dose of UV (A) or ionizing radiation (C) indicated on the x axis. Survival is plotted on a log scale and is calculated compared to an unexposed control for each strain. Agar media with 10-fold serial dilutions of the indicated strains are shown to the right of each graph. (B and D) Survival curves of ΔadnAB, ΔrecR ΔadnAB, ΔrecF ΔadnAB, ΔrecR ΔadnAB recF, and ΔrecF ΔadnAB recR strains exposed to the dose of UV (B) or ionizing radiation (D) indicated on the x axis. Survival is plotted on a log scale and is calculated compared to an unexposed control for each strain. Agar media with 10-fold serial dilutions of the indicated strains are shown to the right of each graph. Each graphed point represents the mean from biological duplicates. Error bars are SEM and when not visible, are within the symbol. No viable counts were obtained for the ΔrecR strain in panel C, the ΔrecR ΔadnAB strain in panel D, and the ΔrecF ΔadnAB strain in panel D at the highest dose of IR.

**FIG 6** Role of RecF and RecR in HR and SSA pathways of DSB repair. An I-SceI-induced DSB was used as previously described (9, 10). Repair of this 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 single-strand annealing (SSA) or gene conversion/homologous recombination (GC/HR). DNA resection that occurs during SSA results in deletion of the kanamycin marker, whereas GC does not. (A) Graph of HR frequency according to strain genotype. For each strain, the relative GC frequency is calculated from the genotyping algorithm presented in Materials and Methods, and the raw data are presented in the table in panel C. (B) SSA frequency for the strains indicated. For panels A and B, the data graphed for the RecO strain were reproduced from reference 10. (C) Percent survival, percent blue, and pathway outcome for each strain. # blue, the number of blue colonies genotyped to give the HR and SSA numbers. Relative HR frequency is given as percent blue events multiplied by the ratio of the HR number to the total blue number. SSA frequency is given as percent blue events multiplied by the ratio of the SSA number to the total blue number.
RecA onto SSB-coated DNA, suggests that RecR is required for all mediator function in the mycobacterial cell. The requirement for RecR in the AdnAB pathway implies that AdnAB does not have intrinsic mediator activity, in contrast to *E. coli* RecBCD, which does have such activity. This finding is similar to recent data from *B. subtilis* showing that RecO and possibly RecR, are required for RecA loading and strand exchange, even in the presence of AddAB (49). However, our data also indicate that the combination of ΔrecO and ΔrecF abolishes HR, thus phenocopying loss of RecR. This finding suggests that RecF and/or RecO are required for the RecR mediator function, and that these two proteins are redundant for this function to an extent. There is some evidence in the literature that RecFR or RecOR complex each can function as mediators for RecA loading, but RecOR has been shown to be more efficient than RecFR in recruiting RecA to DNA (45, 50).

**Role of RecFOR in the SSA pathway.** Mycobacterial SSA is a RecA-independent pathway that mediates repair of DSBs arising between repeats. Repair via SSA results in a deletion between the repeats and retention of one repeat. Although SSA is a well-characterized pathway in yeast, its existence in bacteria was not recognized until the demonstration that mycobacteria can execute SSA (9). As in yeast, the SSA mechanism does not require strand invasion and is independent of RecA in mycobacteria (and Rad51 in yeast) (9, 37). Our prior studies demonstrated that mycobacterial SSA requires RecBCD and RecO (9, 10). The involvement of RecO in SSA makes sense insofar as the RecO protein can anneal complementary SSB-coated ssDNA in vitro (10, 24), as can the eukaryal Rad52 protein (38–42), which is required for SSA. Here, we show that RecF and RecR also are required for SSA in mycobacteria. *M. smegmatis* lacking recF or recR is unable to execute SSA, despite the partial preservation of the HR pathway in the ΔrecF strain. This finding implies that all three components of the RecFOR system are required for SSA mechanism and supports a function for RecFOR that is independent of its commonly invoked...
function as a RecA mediator. The biochemical activities of these proteins are not completely defined, but some may be rationally linked to the SSA mechanism. As mentioned, RecO can anneal SSB-coated dsDNA, a central biochemical activity for SSA. RecF, or the RecFR complex, has been reported to recognize the ssDNA-dsDNA junction and can nucleate RecOR (in the case of RecF) or RecO (in the case of RecFR) onto DNA once it is bound itself. An ssDNA-dsDNA junction would be present after single-strand resection and before annealing; therefore, we envision that RecF is required for the SSA pathway through binding and recognition of the single-stranded intermediate that is the substrate for RecO-catalyzed annealing. In addition, RecF is a structural homolog of the Rad50 protein (20), which is also required for SSA in yeast (37).

Our findings raise interesting regulatory questions about recombination pathway choice in mycobacteria. Our demonstration that RecFOR participates in both HR and SSA indicates that the fate of a 3′ single-stranded DNA arising from resection at a DSB can be determined by whether the RecFOR system engages in its annealing function or its RecA loading function. If RecA is loaded to form a nucleoprotein filament, then this intermediate may be funneled into the HR pathway. If RecA is not loaded and a complementary single strand is available for annealing, the RecFOR complex may catalyze SSA. However, the determinants of this pathway choice are unknown at present and will require further investigation.

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REFERENCES


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