Elucidating the functional role of *Mycobacterium smegmatis recX* in stress response

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The RecX protein has attracted considerable interest because the recX mutants exhibit multiple phenotypes associated with RecA functions. To further our understanding of the functional relationship between recA and recX, the effect of different stress treatments on their expression profiles, cell yield and viability were investigated. A significant correlation was found between the expression of *Mycobacterium smegmatis recA* and recX genes at different stages of growth, and in response to different stress treatments albeit recX exhibiting lower transcript and protein abundance at the mid-log and stationary phases of the bacterial growth cycle. To ascertain their roles *in vivo*, a targeted deletion of the recX and recArcX was performed in *M. smegmatis*. The growth kinetics of these mutant strains and their sensitivity patterns to different stress treatments were assessed relative to the wild-type strain. The deletion of recA affected normal cell growth and survival, while recX deletion showed no significant effect. Interestingly, deletion of both recX and recA genes results in a phenotype that is intermediate between the phenotypes of the ΔrecA mutant and the wild-type strain. Collectively, these results reveal a previously unrecognized role for *M. smegmatis* recX and support the notion that it may regulate a subset of the yet unknown genes involved in normal cell growth and DNA-damage repair.

To maintain genomic integrity, bacteria have developed a complex network of DNA-damage response (DDR) pathways to sense, respond to, and repair different types of DNA damage⁴⁻¹⁰. In *Escherichia coli*, DNA damaging agents induce the SOS regulon - an adaptive stress response - that contributes to cell survival. Most notably, SOS induction increases the mRNA and protein levels of approximately fifty genes belonging to the SOS regulon through RecA-dependent autocatalytic cleavage of the LexA repressor¹⁻⁴,⁵. Multiple investigations have established that mutations in *E. coli* recA are pleiotropic, affecting not only homologous recombination (HR) but also DNA repair, DNA replication, mutagenesis and cell division through the SOS response⁴⁻⁶,⁷. These crucial functions are carried out by a helical RecA nucleoprotein filament through three distinct, but related biological processes: (a) recombination-based DNA repair; (b) transcriptional upregulation of SOS genes through cleavage of the LexA repressor and (c) error-prone replication by DNA polymerase IV⁴⁻⁵.

In *E. coli*, a variety of accessory proteins regulate RecA function and amongst these the single-stranded binding (SSB) protein and RecFOR abet RecA in the formation of nucleoprotein filaments on single stranded DNA (ssDNA)⁴⁻⁷,⁸. Two different types of accessory factors, referred to as RecA mediators and RecA modulators, act at different levels to regulate the formation and/or stability of the RecA-ssDNA filament⁴⁻⁻¹⁰. The RecA mediators act during the binding of RecA on SSB-coated ssDNA. In contrast, the RecA modulators regulate the function of the nucleoprotein filament during the search for homology and/or strand exchange. The well characterized RecA mediators, SSB, RecF, RecO and RecR, act in overcoming the barrier to RecA filament nucleation and stimulate the rate of its polymerization⁴⁻⁷,¹¹. Differing from these mediators, DinI and RecX proteins modulate RecA protein function but have very different effects⁴⁻¹¹. Whilst DinI stabilizes RecA nucleoprotein filaments, RecX binds to RecA in solution and also inhibits RecA nucleoprotein elongation and its biochemical functions¹²⁻¹⁵. Other known negative modulators include RdgC and PsiB: whereas RdgC acts as a negative regulator of RecA protein functions, PsiB binds to the RecA that is free in solution and the resultant complex impedes the formation of RecA nucleoprotein filaments¹⁶⁻¹⁸. Another accessory factor, RecC, a bacterial SMC (Structural Maintenance of Chromosome)-like accessory protein, stimulates the DNA strand-invasion step of...
RecA-mediated recombination-based DNA repair\textsuperscript{20}. However, the functional and mechanistic properties of RecA accessory factors in mycobacteria are not well understood.

The RecX protein has attracted much interest because the recX mutants exhibit multiple phenotypes associated with RecA functions. The Mycobacterium tuberculosis RecX was first identified for its ability to suppress DNA pairing and strand exchange, ATPase and co-protease activities of RecA\textsuperscript{14}. Surprisingly, the E. coli recX, which is far less abundant in the cell, interacts with DNA but it is not clear how such an interaction relates to its capacity to inhibit RecA activities. Furthermore, the mechanism by which RecF antagonizes the E. coli RecX promoted destabilization of RecA nucleoprotein filaments is not well understood\textsuperscript{22}. The electron microscopy reconstructions of mixed RecA-RecX filaments revealed that RecX binds within the helical groove of the RecA nucleoprotein filament\textsuperscript{23}. As a result, a repression of HR might ensue by the steric clash of RecX with the process of DNA strand exchange and impede the ATP-coupled allosteric changes in RecA\textsuperscript{14,23,24}. Studies in Bacillus subtilis suggest that RecX facilitates HR by modulating RecA activities by inhibiting RecA\textsuperscript{25} and RecX aids all RecA-related processes in Neisseria gonorrhoeae\textsuperscript{26}. Other studies have found that mechanical forces antagonize the inhibitory effects of M. tuberculosis RecX, and a partially de-polymerized RecA filament could re-polymerize in the presence of RecX\textsuperscript{27}. Although these findings shed light on the role of RecX, the molecular mechanisms that underlie the variety, magnitude and taxa specific activities are not fully understood.

Moreover, very little is known about the \textit{in vivo} functional relationship between \textit{recA} and \textit{recX}, especially in intracellular bacterial pathogens. \textit{M. tuberculosis} is the causative agent of tuberculosis, which is characterized by slow growth. Therefore, \textit{M. smegmatis} is often used as a surrogate model to study the pathogenesis of mycobacterial infection and drug resistance. Further, investigations into the interaction mechanisms between \textit{M. smegmatis} recX and recA might reveal insights into the phylogenetic diversification among the mycobacterial species. To this end, \textit{M. smegmatis ΔrecX} and \textit{ΔrecAΔrecX} mutants were constructed and subjected to various stress conditions. The growth phenotypes of these mutants and recA and recX gene expression profiles were compared with those of the wild-type strain. In the absence of DNA damage, recX was found to be non-essential for \textit{M. smegmatis} growth, and \textit{ΔrecAΔrecX} double mutant strain exhibits a phenotype that is intermediate between the phenotypes of the \textit{ΔrecA} mutant and the wild-type strain. Additionally, a significant correlation was seen between the expression of the \textit{recA} and recX at different stages of growth and after exposure to DNA damaging agents, albeit recX exhibiting lower transcript and protein abundance at the mid-log and stationary phases of growth. Collectively, these results suggest a previously unrecognized role for \textit{M. smegmatis recX} and support the idea that it may regulate a subset of the yet unknown genes involved in normal cell growth and DNA damage repair.

**Methods**

**Chemicals and enzymes.** All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All restriction endonucleases, Phusion polymerase, Klenow polymerase and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Pure H\textsubscript{2}O\textsubscript{2}, methylmethane sulphonate (MMS), ciprofloxacin, kanamycin and hygromycin were purchased from Sigma-Aldrich (St. Louis, MO). RT-qPCR reagents, SYBR RT-qPCR master mix and cDNA synthesis kit were obtained from Bio-Rad Laboratories (Hercules, CA). The DNA oligonucleotides were synthesized by Sigma-Genosys (Bangalore, India) and [\textit{α-\textsuperscript{32}P}]ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India).

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this study are described in Table 1. The \textit{M. smegmatis} mc\textsubscript{155} ΔrecX and Δ\textit{recAΔrecX} strains were generated as described\textsuperscript{28}. The pJV53 vector (encoding mycobophage derived recombinases under acetamide-inducible promoter was transformed into \textit{M. smegmatis} mc\textsubscript{155}\textsuperscript{35,36}. The \textit{ΔrecAΔrecX} mutants were derived from \textit{M. smegmatis} mc\textsubscript{155} strain. These strains were grown in liquid 7H9 Middlebrook broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (ADC) (henceforth referred to as 7H9 broth) with continuous agitation (120 rpm) at 37 °C for 2 days. \textit{M. smegmatis} mc\textsubscript{155} was grown on 7H10 agar (4.7 g/L) plates supplemented with 10% ADC. The starter cultures were prepared by inoculating a single colony of \textit{M. smegmatis} mc\textsubscript{155} into 15 ml of 7H9 broth and grown at 37 °C in a shaking incubator (120 rpm), until the cultures reached an OD\textsubscript{600} of 1. Then 1.5 ml of the cell suspension was inoculated into 30 ml of 7H9 Middlebrook medium to give an OD\textsubscript{600} of 0.05. The antibiotics (kanamycin and hygromycin) were added to a final concentration of 25 µg/ml and 100 µg/ml respectively.

The \textit{M. smegmatis ΔrecA} mutant was a kind gift from Elaine O. Davis, National Institute for Medical Research, London. The characteristics of plasmids (pJV53, pVV16 and p0004S) have been described elsewhere\textsuperscript{29}. The pVV16 vector (encoding mycobophage derived recombinases under acetamide-inducible promoter was transformed into \textit{M. smegmatis} mc\textsubscript{155}\textsuperscript{35,36}. The \textit{ΔrecAΔrecX} mutants were derived from \textit{M. smegmatis} mc\textsubscript{155} strain. These strains were grown in liquid 7H9 Middlebrook broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (ADC) (henceforth referred to as 7H9 broth) with continuous agitation (120 rpm) at 37 °C for 2 days.

**Cloning of \textit{recA} and \textit{recX} genes.** \textit{M. smegmatis recA} (MSMEG\textsubscript{2723}) and \textit{recX} (MSMEG\textsubscript{2724}) ORFs were obtained from the SmaegList website (http://svitsrv8.epfl.ch/mycobrowser/smaeglist.html). The coding sequences of \textit{recA} and \textit{recX} were amplified by PCR using gene-specific forward and reverse primers (Table 2). The pVV16-vector was digested with NdeI and HindIII. The PCR products were gel purified, and ligated separately into the linear plasmid pVV16 DNA at

\textsuperscript{1}Scientific
Plasmids or strains | Relevant characteristics (genotype and phenotype) | Reference or source
---|---|---
pJV53 | Plasmid encoding phage CheK6 and 61 genes under acetamide-inducible promoter, kan’ | (48)
pVV16 | Shuttle vector (E. coli and M. smegmatis) used for expression of genes in M. smegmatis, kan’ | (48)
p0004S | Vector backbone used for the cloning of allelic exchange substrate of ΔrecA ΔrecX strains | (48)
ΔrecA | M. smegmatis mc155 disrupted for recA gene, Hyg’, retarded growth under normal growth condition, severe growth defect under DNA damaging conditions | This study
ΔrecA ΔrecX | M. smegmatis mc155 disrupted for recA and recX genes, Hyg’, growth phenotype intermediate of WT and ΔrecA strains | This study
ΔrecX | M. smegmatis mc155 disrupted for recX gene, Hyg’, no phenotype | This study
M. smegmatis mc155 recombineering strain | M. smegmatis mc155 electroporated with pJV53 vector | This study

Table 1. Bacterial strains and plasmids used in this study.

| Primers | Oligonucleotide Sequence (5’→3’) | Restriction Enzyme |
|---|---|---|
| MorecA (FP) | AGCATAAGATGCCAGCAC| NdeI |
| MorecA (RP) | GATTTAAGCTTTCAAGATCAG | HindIII |
| MoreX (FP) | TCTCTATATGACGTTCCCAGCCGCGG | NdeI |
| MoreX (RP) | AATTACAGCTTTATAGACCCGACG | HindIII |

NdeI/HindIII site. The resulting plasmids contained either full-length M. smegmatis recA or recX genes under the control of the Hsp60 constitutive promoter.

**Complementation assay.** The complementation assay was performed using M. smegmatis mc155 ΔrecA, ΔrecA ΔrecX and ΔrecX mutant strains. The plasmid complemented strains bearing wild-type copies of M. smegmatis recA or recX ORFs were created by electroporation. These were grown in liquid 7H9 broth to mid-log phase, until cultures reached an OD_{600} of 0.6. The cultures were centrifuged and pellets were resuspended to an OD_{abs} of 0.8 in sterile Milli-Q water. An aliquot from each culture was serially diluted and spotted on 7H10 agar plate containing 100 µg/ml of hygromycin B. The cells were exposed to 10 J/m² of 254 nm UV light and incubated at 37 °C in the dark for 3 days. The images were acquired in the digitization mode using a Quant LAS 4000 imaging system (GE Healthcare, Piscataway, NJ).

**Construction of recX and recA-recX gene replacement mutants in M. smegmatis.** The DNA manipulations were carried out using standard protocols (49). Plasmid DNA and DNA fragments were isolated from the gels using Qiagen miniprep kit (Qiagen India, New Delhi) according to the manufacturer’s instructions. The genomic DNA of M. smegmatis was PCR-amplified as described (31). The electrocompetent M. smegmatis mc155 cells were prepared and stored as described (32). The construction of allelic exchange substrate (AES) and its subsequent delivery to the recombineering strain of M. smegmatis mc155 was carried out as described (38). Table 3 shows the sequences of upstream and downstream primers corresponding to the M. smegmatis recA-recX locus. For recX gene deletion, the upstream and downstream flanking regions of M. smegmatis mc155 recX were PCR-amplified from the genomic DNA using specific primers that contained the restriction sites in the counter-selectable vector p0004S. The upstream and downstream flank fragments were digested with PIIIMI and directionally cloned on either side of the Hyg resistance gene to generate the recX-AES. To generate allelic exchange substrates, the upstream and downstream flanking sequences of recA-recX locus were PCR-amplified using genomic DNA as the template and specific primers. The amplified products were digested with PIIIMI and directionally cloned on either side of the Hyg resistance gene. The resulting constructs were independently transformed into E. coli DH5α and plated on LB agar containing 100 µg/ml of hygromycin. The positive clones were identified by restriction analysis of the isolated plasmid using EcoRV to generate the linear blunt-ended recombineering-proficient (containing upstream region−hygr−downstream region) DNA fragments.

**Preparation of M. smegmatis mc155 recombineering strain and construction of ΔrecX and ΔrecA ΔrecX strains.** The M. smegmatis mc155 derivative harbouring the pJV53 vector was used in recombineering (38). The AES constructs generated for disruption of recX and recA were linearized using EcoRV and then isolated by electroelution. The linearized AES constructs (100 ng each), were independently electroporated into the M. smegmatis mc155 recombineering strain. The transformed cells were cultured with shaking at 37 °C for 4h. The transformants were plated on 7H10 agar plates, supplemented with 100 µg/ml hygromycin B and 25 µg/ml kanamycin. The transformants were selected and their genomic DNA was isolated and analyzed by PCR to ascertain recombination in the M. smegmatis mc155 genome at the allelic site.
Southern blot hybridization. The genomic DNA from the M. smegmatis mc^2155 wild-type, ΔrecA and ΔrecAΔrecX strains was isolated to confirm deletion of the recX and recArcX genes. The probe (548 bp) corresponding to the upstream region of recA was PCR-amplified using forward (5′ GCTCACCTCCATCGACAGGATCCT 3′) and reverse primers (5′ GTGTGCTCTCAGGATGCTCTCCT 3′) and reverse primers (5′ GGTGCCTCTCCGAGTAGTCGTGTC 3′). The PCR amplified product (labelled probe) was prepared using [α-32P]ATP, random primers (hexamer) and Klenow polymerase as described (30). The radiolabeled probe was heat denatured at 95 °C for 10 min followed by snap-chill prior to use. Approximately 10 μg of genomic DNA was digested using NdeI and SalI (10 U each) at 37 °C overnight and the DNA fragments were separated by 0.8% agarose gel electrophoresis. The gel was soaked in 0.25 N HCl for 20 min followed by washing twice with 40 ml of sterile water. Subsequently, the gel was submerged in a solution containing 0.5 N NaOH and 0.6 M NaCl for 15 min each to denature the DNA, followed by neutralization of the gel pH in a buffer containing 1.5 M NaCl and 0.5 M Tris–HCl (pH 7.5) for 30 min, and finally in 10X SSC (pH 7). The DNA was transferred by capillary action to a nylon membrane and was fixed to dry membranes by exposure to UV light at 254 nm using a Stratalinker (Stratagene Corporation, La Jolla, CA). The nylon membrane was soaked in a pre-hybridization buffer (0.5 M phosphate buffer pH 7, 7% SDS, 1 mM EDTA) by gentle shaking for 1 h at 65 °C and then hybridized using the 32P-labelled probe at 65 °C for 16 h. Following hybridization, the membrane was washed with 1X SSC and 0.1% SDS to remove the unbound probe. The bands were visualized using the Fuji FLA 9000 phosphorimager.

Analysis of M. smegmatis recA and recX gene expression under hypoxic conditions. Wayne and Hayes dormancy culture system was used to study the hypoxic response of M. smegmatis recA and recX expression as described (35,36). Briefly, M. smegmatis mc^2155 strain was grown as described above until the culture reached an OD_{600} of 0.6. An aliquot of the culture was diluted in Middlebrook 7H9 broth to an OD_{600} of 0.05. Equal volumes (5 ml) of diluted culture were placed in each of 100 ml screw-cap flasks having a headspace ratio of ~ 0.5 (35 ml of head space air vol. to 65 ml of liquid medium) containing a stir bar. The cultures were gently shaken (120 rpm) at 37 °C. To ascertain hypoxic culture conditions, we used a standard color assay (viz., methylene blue reduction) (35). Methylene blue (1.5 µg/ml) was added to cultures, and reduction was monitored visually (by decolorization). To avoid aeration of cultures during sampling, separate flasks were used for each time point in these experiments. A small portion of the culture from each flask was used for Western blot analysis, while the rest was used for total RNA extraction and RT-qPCR as described below.

DNA damage-sensitivity assays. The M. smegmatis mc^2155 wild-type, ΔrecA, ΔrecX and ΔrecAΔrecX strains were grown in 7H9 broth to mid-log phase to a cell density of 0.6. After 3 h of incubation with different concentrations of ciprofloxacin, MMS or hydrogen peroxide, the cells were collected by centrifugation and resuspended in 1 ml sterile Milli-Q water at a cell density of 0.8. A portion of the resuspended cells were serially diluted and spotted on 7H10 agar plates containing 100 µg/ml hygromycin B. Similarly, the serial dilutions of wild-type and mutant strains were plated on 7H10 agar and irradiated under 254 nm UV light. The plates were incubated at 37 °C in the dark. The images were acquired in digitization mode using the Quant LAS-4000 chemidocumentation system (GE Healthcare Life Sciences, Pittsburgh). In parallel experiments, serial dilutions of cell cultures, treated with the indicated concentrations of MMS, H_{2}O_{2}, or ciprofloxacin, were spread on 7H10 agar plates containing 100 µg/ml hygromycin. The survival was determined by counting viable cells.

RNA isolation and quantitative real-time PCR. The expression of recA and recX in M. smegmatis mc^2155 cells, grown under aerobic or hypoxic conditions, were determined by quantitative real-time PCR using specific primers (Table 4). The cultures were harvested at the time points corresponding to early-log, exponential and stationary phases. The cell pellets were suspended in 5 ml of freshly prepared protoplast buffer (15 mM Tris–HCl, pH 8, 0.45 M sucrose, 8 mM EDTA and 4 mg/ml lysozyme) and incubated at 37 °C for 1 h. The suspension was centrifuged at 4000 rpm for 10 min at 4 °C, and the resulting pellet was resuspended in 1050 µl of RLT buffer (Qiagen India, New Delhi). The total RNA was isolated from the whole cell lysates using the Qiagen RNeasy-kit as per the manufacturer's instructions. The RNA preparations were denatured at 65 °C for 10 min and were used as a negative control.

| Sl. no. | Primers | Oligonucleotide sequence (5′→3′) |
|--------|---------|----------------------------------|
| 1      | recA 5′ flank FP | CACCTTTTCCATAAAATGGGATATGCCGGCCCGCGGGATGCCGGC |
| 2      | recA 5′ flank RP | TTTTTTTTCACTTTTTGGGCTTGCGCACTCTTTGCGG |
| 3      | recX 5′ flank FP | CACCTTTTCACAAATTGCGATACCTCAGAGATGGCGCCAG |
| 4      | recX 5′ flank RP | TTTTTTTTCATCTTTTTGGGATATAAGCCGGCCGCGGGG |
| 5      | recX 3′ flank FP | TTTTTTTTCACCTTTTTGGGATATCCGGCCGCGGGG |
| 6      | recX 3′ flank RP | TTTTTTTTCACCTTTTTGGGATATCCGGCCGCGGGG |

Table 3. The sequences of oligonucleotides used for the construction of AES. (RP and FP stand for reverse and forward primers respectively). Note: the sequences shown in bold are the recognition sites of PlmI and those underlined are for EcoRV. Primer pairs 1, 2, 3 and 4 were used for the amplification of upstream region of recA and recX genes, respectively. Primers 5 and 6 were used to amplify downstream region of recX gene.
levels of MSMEG_2725, MSMEG_2726 and MSMEG_2720 ORFs in M.
cation, a melting curve analysis was performed to verify the authenticity of the amplified product. Similarly, the real time PCR detection system. The cycling program comprised initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 sec, primer annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product. Similarly, the levels of MSMEG_2725, MSMEG_2726 and MSMEG_2720 ORFs in M. smegmatis mc²155 wild-type, ∆recA
∆recX and ∆recX mutant cells were determined by RT-qPCR.

Western blot analysis. The whole cell lysates of M. smegmatis mc²155 cells, grown under aerobic or hypoxic conditions, were prepared as described above (see under RNA isolation and RT-qPCR). The antibody against GroEL was purchased from Sigma-Aldrich (St. Louis, MO). The polyclonal antibodies against M. tuberculosis RecA and RecX were raised in rabbits and characterized as described 14. The whole cell lysate (30 µg of protein) was boiled in SDS/PAGE buffer for 10 min, and the proteins were separated by SDS-PAGE using 10% (wt/vol) polyacrylamide gels as described 36. After gel electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with either anti-RecA, anti-RecX or anti-GroEL antibodies at a dilution of 1:15000, 1:1000 and 1:80000 respectively as described 47. The membranes were blocked with 3% BSA in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) and washed with TBST (50 mM Tris-HCl, pH 8, 150 mM NaCl and 0.1% Tween 20). Subsequently, the membranes were incubated with peroxidase-conjugated (Sigma-Aldrich, St. Louis, MO) secondary antibody. The blots were washed with TBST (50 mM Tris-HCl, pH 8, 150 mM NaCl and 0.1% Tween 20). Subsequently, the membranes were incubated with peroxidase-conjugated (Sigma-Aldrich, St. Louis, MO) secondary antibody. The blots were developed using luminol and hydrogen peroxide. The digital images of Western blot bands were acquired using the Quant LAS-4000 chemidocumentation system (GE Healthcare Life Sciences, Pittsburgh, PA), and quantified using the ImageJ software.

Statistics and data presentation. Statistical significance was determined using the unpaired t-test. P-value (p) less than 0.05 indicate significant difference and p > 0.05 is not significant (ns). The symbols *, ** and *** correspond to p < 0.05, p < 0.01 and p < 0.001 respectively.

Results and Discussion

Hypoxia causes an increase in the expression of M. smegmatis recA and recX genes. During the course of infection and proliferation, M. tuberculosis is exposed to physiological stress conditions such as hypoxia and acidic pH stress, which could compromise its survival 38–40. For instance, acute hypoxic stress arrests DNA replication and triggers robust DNA damage 41–43. An increase in the expression of genes of the SOS pathway, SOS response) was found to be toxic in the absence of RecX in certain bacterial species including Pseudomonas aeruginosa 11, S. lividans 48, M. smegmatis 52, and Xanthomonas oryzae 53. These findings are consistent with the notion that RecX acts as an anti-recombinase to quell inappropriate recombination events promoted by RecA 14. In contrast, an overexpression of RecA in the E. coli ∆recX strain is not deleterious and the deletion of recX does not affect recA expression 54. The recX transcription in E. coli is downregulated compared to recA under

| Primers | Oligonucleotide sequence (5′ → 3′) |
|---------|----------------------------------|
| recA FP | CTGAAGTTCTACGCTCGGT |
| recA RP | AGACCTGTCTCTGACGACCT |
| recC FP | GGTGGTGCGCGCAAGTT |
| recX RP | CATCGACTGTTGATTCGCC |
| sigH2 FP | CTTCAACACACTGTCGAC |
| sigH2 RP | TCTTCAACACACTGTCGAC |
| sigH7 FP | CTTGTACTGCTGTCGTC |
| sigH7 RP | GCTGTCATTACGCTGTAAT |
| 16S rRNA FP | CTTGATACGGCCCAAGCTC |
| 16S rRNA RP | CTGTGATACGGCCCAAGCTC |
| MSMEG_2725 RP | ATCGGCTACCAGATCAGTT |
| MSMEG_2725 FP | CGCAAAAGTCCAGCTGATCA |
| MSMEG_2726 FP | CAACCATCAAACATGACGGTG |
| MSMEG_2726 RP | TCCCCATCATGTTACATCACC |
| MSMEG_2720 FP | TTTCCTGCGCGTTGCTTCA |
| MSMEG_2720 RP | CGCACAATACCCTTCGTTC |

Table 4. The sequences of primers used for RT-qPCR. (RP and FP stand for reverse and forward primers respectively).
normal growth conditions because of the existence of a transcription termination site between the recA and recX coding sequences. However, a recA-recX mRNA transcript resulting from transcriptional read-through accumulates in the range of 5–10% of the total recA mRNA content. The recX transcript was hardly detectable during the vegetative growth of E. coli, but robust expression of recX occurs following treatment with DNA damaging agents.

The genomes of both M. smegmatis and M. tuberculosis contain a single copy each of recA and recX in a single operon. To investigate the expression and regulation of these genes under aerobic and hypoxic growth in M. smegmatis, a commonly used surrogate model for M. tuberculosis, the relative abundance of mRNA at various growth phases was measured by a RT-qPCR assay and normalized to that of the constitutively expressed 16S rRNA gene. The cycle threshold (Ct) values for recA and recX mRNA were normalized against the Ct value of the 16S rRNA gene. In contrast to E. coli, significant amount of recA mRNA transcripts were seen at early-log phase under both aerobic and hypoxic culture conditions (Fig. 1A). Interestingly, hypoxic conditions enhanced the accumulation of recX mRNA by 1.5-fold at mid-log phase, and decreased thereafter as the cells entered the stationary phase. A similar pattern was observed with recX mRNA abundance under both aerobic and hypoxic conditions, albeit at reduced levels (Fig. 1B). These observations support the idea that the two genes are co-transcribed and coordinately regulated under aerobic and hypoxic growth conditions. Intriguingly, no difference was seen in the amounts of mRNA under aerobic and hypoxic growth conditions at the early-log phase. The transcriptional profiles of M. smegmatis growth-phase-specific marker genes (sigH2 and sigH27) was used to ascertain the times of the early-log, the mid-log and the stationary phases (Fig. 1C). In multi-drug resistant clinical strains of M. tuberculosis, the levels of both recA and recX mRNA were found to be higher than in the drug-susceptible strains and the levels of recX mRNA increased concomitantly with a rise in recA mRNA.

However, it should be noted that the mRNA levels cannot be used as surrogates for the corresponding protein levels. Therefore, the abundance of RecA and RecX proteins in M. smegmatis was measured under aerobic and hypoxic conditions by a Western blot assay using anti-RecA and anti-RecX antibodies. GroEL was probed as a protein-loading control. The densitometric analysis of immunoblots revealed that cells accumulated higher levels of RecA under hypoxic growth conditions compared to aerobic conditions (Fig. 2A,B). A comparative analysis showed that the cells consistently accumulated 2-fold higher levels of RecA at mid-log phases relative to the early-log phase under aerobic growth conditions, which decreased at the stationary phase to levels seen at the early-log phase (Fig. 2A,B). A similar pattern was observed for RecX, albeit the levels were less pronounced than that of RecA (Fig. 2A,C). Although the mRNA and protein expression patterns were comparable, important differences were noted. First, the RecX protein was hardly detectable at early-log phase during hypoxic conditions. Second, the abundance of RecA mRNA and protein were higher compared to RecX.

Although, these results suggest that M. smegmatis recA and recX genes are induced under hypoxic conditions, there is no correlation between the amount of recX mRNA and corresponding protein in the early-log phase. Both M. tuberculosis and M. smegmatis have been shown to stabilize their mRNA transcripts under growth-inhibiting conditions. Among possible mechanisms, tRNA reprogramming and selective codon-biased translation have been shown to play a role in mycobacteria in response to hypoxia. One possible explanation for the lack of correlation between the recX mRNA versus protein level in the early log phase could be due translational repression of recX mRNA.

Construction of ΔrecX and ΔrecA ΔrecX mutant strains. The difference in the RecA and RecX protein levels in M. smegmatis indicate possible regulation of gene expression at the level of transcript. In many bacteria studied so far, the recX gene is located on the same coding strand downstream of recA and the two genes are co-transcribed. However, in the lexA-recA-recX locus of Xanthomonas pathovars, each gene is expressed from its own promoter. Furthermore, in D. radiodurans, B. subtilis and N. gonorrhoeae, the recA and recX genes are separated by several hundred kb in length and transcribed from their own promoters.

In a number of mycobacterial species, as well as in a few other bacteria, the 5′ region of the recX coding sequence overlaps the 3′ region of the recA gene. The overlap is 32 bp long in M. smegmatis, while in M. tuberculosis and M. leprae the overlap is 35 bp. The effect of recX deletion on the phenotypic characteristics has been studied in various organisms. The knockout mutants of recX show a range of phenotypes associated with RecA functions: the inactivation of B. subtilis recX rendered the cells sensitive to MMS and H2O2, the S. lividans recX mutants displayed decreased resistance to UV damage and the N. gonorrhoeae recX mutant showed a small decrease in its ability to survive DNA damage that was caused by double-strand breaks. However, the impact of deletion of both recA and recX genes on the growth characteristics and DNA damage repair has not been investigated in any organism. Moreover, a full understanding of the in vivo role of recX in mycobacteria is not fully understood.

Previous studies have demonstrated that the M. smegmatis ΔrecA strain exhibited sensitivity to UV-induced DNA damage and fails to promote HR. We combined the recA mutation with the deletion of recX to investigate the effects of the double mutations. For this purpose, M. smegmatis mc^155 recX single and recXΔrecX double mutant strains were generated. As a control, the effect of recA deletion in M. smegmatis was re-evaluated for direct comparison. Using the recombineering method, which is based on a protocol developed for M. tuberculosis and M. bovis BCG, the M. smegmatis mc^155 ΔrecA and ΔrecAΔrecX mutants were generated using 3.279 kb and 3.302 kb linear ΔrecA::hgy and ΔrecAΔrecX::hgy AES constructs, respectively. Approximately 100 ng of linear AES DNA fragments were generated by restriction digestion of the respective plasmids with EcoRV (Fig. 3A). These were transformed into competent M. smegmatis mc^155:pJVS3 recombineering cells. After 4–5 days of incubation, 8–10 Hgy-resistant colonies were found for the ΔrecX and ΔrecAΔrecX mutants. The putative
mutant strains were screened using PCR to determine if the recX and recArecX genes were deleted from the chromosome (data not shown). The correct mutants were grown in 7H10 Middlebrook agar medium for 5–8 generations to allow the loss of pJV53.

Genotypic and phenotypic analysis of M. smegmatis ΔrecX and ΔrecA ΔrecX mutants. After screening by PCR, 2 each of the ΔrecX and ΔrecA ΔrecX knockout mutants of M. smegmatis mc²155 were obtained. The ΔrecX and ΔrecA ΔrecX mutants were characterized by restriction enzyme mapping and Southern blot hybridization (Fig. 3). Upon hybridization with appropriate radiolabeled probes, a 4.1 kb fragment was seen in the case of wild-type M. smegmatis mc²155 cells. The predicted 3.14 kb and 2.09 kb fragments were observed in the ΔrecX and ΔrecA ΔrecX mutants respectively (Fig. 3B,C). Both these bands are smaller than 4.1 kb, indicating that the recX and recArecX genes have been deleted by allelic replacement. The frequency of allelic exchange with respect to recX and recArecX was in the range of 70% and 80% respectively.

One caveat of this analysis is that the observed effects of ΔrecX and ΔrecA ΔrecX mutations could result from the polar effects of hygromycin-resistance gene insertion. In general, genetic alterations around the recA-recX locus could lead to polar effects on the expression of genes downstream of recA-recX locus. Two independent experiments were carried out to investigate the potential polar effects. First, the ΔrecX and ΔrecA ΔrecX mutants were complemented with the functional copies of the recA and recX genes. The transformants were evaluated for their ability to grow in a standard culture medium and protect mutant cells against UV irradiation. Ten-fold serial dilutions were spotted on 7H10 agar plates and analyzed. As shown in Fig. 4A,B, the wild-type recA partially complemented the ΔrecA and ΔrecA ΔrecX mutant strains, as deduced from their ability to support growth and provide protection against UV irradiation. However, wild-type recX failed to rescue the phenotype of ΔrecA ΔrecX mutant strains observed upon DNA damage induction with UV irradiation. Since, deletion of recX

Figure 1. (A,B) Relative levels of expression of M. smegmatis mc²155 recA and recX genes at the early log, mid-log and stationary phases under aerobic and hypoxic growth conditions. (C) Relative levels of expression of sigH2 and sigH7 genes at the early log, mid-log and stationary phases. The signal intensities were determined as described in the Methods section. The histograms represent the mean values of three independent experiments. The expression levels were determined and normalised to 16S ribosomal RNA expression and induction ratios calculated relative to the amount of transcript in the aerobic early log phase culture. The error bars represent the standard error of the mean calculated from 3 independent replicates.
had no discernible effect on its growth under normal and DNA damaging conditions, ∆recX and its corresponding complemented strain showed similar growth as that of wild-type.

Second, we assessed the expression of two *M. smegmatis* mc²155 genes, *gldD* (MSMEG_2725) and *glnH* (MSMEG_2726), located downstream of the recA recX locus in the ∆recA ∆recX and ∆recX mutant strains in comparison with the wild-type strain. The ∆*M. smegmatis hyd2* (MSMEG_2720) gene located upstream of the recA-recX locus was used as a positive control. The total RNA was prepared from the wild-type, ∆recA ∆recX and ∆recX mutant strains. The relative abundances of the gene transcripts were determined by quantitative RT-qPCR and normalized to that of the constitutively expressed chromosomal 16S rRNA gene. The expression levels were measured from cells grown in the late exponential growth phase. In all cases, the gene expression profiles of both the upstream and downstream ORFs were similar for the wild-type and mutant strains (Fig. 4C). Taken together, these results exclude the possibility that insertion of the hygromycin-resistance gene causes polar effects.

The *M. smegmatis* ∆recA and ∆recA ∆recX mutant strains show impaired growth and reduced cell yield relative to the wild-type strain. To characterize the *M. smegmatis* ∆recX and ∆recA ∆recX mutant strains, the growth profiles of the wild-type and knockout strains were measured in 7H9 Middlebrook broth at 37 °C (Fig. 5). The recX deletion had no discernible effect (compared to the wild-type strain) on the growth of *M. smegmatis*. Under similar conditions, recA deletion markedly
Figure 3. Isolation of *M. smegmatis* mc^2^155 ∆recX and ∆recA ΔrecX mutant strains. (A) physical map of the *M. smegmatis* recA recX, ∆recAΔrecX and ∆recX regions. The horizontal lines with arrowheads on both ends represent the size of the genomic DNA fragment between NdeI and SalI restriction sites corresponding to the wild-type, ∆recA ΔrecX and ∆recX strains. The lightning bolt symbols indicate recognition sites for the indicated restriction enzymes. The little black boxes (adjacent to the NdeI recognition site) indicate hybridization probes used in Southern blotting experiments. (B) Southern blot analysis of genomic DNA from the wild-type and ∆recX strains. Lane 1 and 4, molecular weight markers; 2, genomic DNA from the wild-type strain; 3, genomic DNA of the ∆recX strain. (C) Southern blot analysis of genomic DNA of the wild-type and ∆recAΔrecX strains. Lane 1, molecular weight markers; 2, genomic DNA of the wild-type strain; 3, genomic DNA of the ∆recAΔrecX double mutant strain.

Figure 4. Insertion of a hygromycin resistance gene into *M. smegmatis* mc^2^155 recA-recX locus exerts no polar effect. The precA and precX denote pVV16-vector bearing one functional copy of either recA or recX gene under the control of the Hsp60 constitutive promoter. EV denotes pVV16 empty vector. precA and precX indicate plasmids bearing wild-type copies of *M. smegmatis* recA and recX genes respectively. These have been transformed into the indicated wild-type and mutant strains. Complementation of *M. smegmatis* mc^2^155ΔrecA ΔrecX and ΔrecX mutant strains for aerobic growth (panel A) and sensitivity against UV irradiation (panel B) with plasmids bearing wild-type alleles of *M. smegmatis* recA or recX. (C), quantitative real-time PCR analysis of genes around the recA-recX locus of *M. smegmatis* mc^2^155 wild-type and mutant strains. The error bars represent the standard error of the mean calculated from 3 independent replicates. Significant differences are indicated by ns, not significant.
increased the length of the lag phase and concomitantly reduced the exponential growth phase, suggesting that recA plays a role in the growth and viability of M. smegmatis. Furthermore, these results are consistent with the known function of RecA in the rescue of stalled or collapsed replication forks. Because the recA and recX genes form a single operon, the impact of their deletion on M. smegmatis growth was investigated. The ΔrecA ΔrecX knockout strain exhibited a growth phenotype that was intermediate between the ΔrecA mutant and the wild-type strain; however the growth at late log and stationary phases was similar to that of ΔrecA and wild-type strains.

**Deletion of recA, but not recX, renders M. smegmatis more susceptible to DNA damaging agents.** Similar to other eubacteria, the mycobacterial RecA protein plays a crucial role in regulating the SOS response upon DNA damage. To test whether the absence of recA and recX affect the ability of M. smegmatis to effectively repair damaged DNA, the mutants were exposed to a range of agents with varied mechanisms of DNA damage. In these experiments, stress was induced by exposing the ΔrecA, ΔrecX and ΔrecA ΔrecX mutant strains to UV-irradiation, MMS, H2O2 or ciprofloxacin during the exponential phase (OD600 of 0.6) of the bacterial growth. After 3 h incubation, the cells were washed and their viability was assayed by spotting ten-fold serial dilutions of the cultures on Middlebrook 7H10 agar plates containing hygromycin (100 μg/ml). The most appropriate concentration/dose of the DNA-damaging agents was determined after evaluating the differences in cell viability between the strains using plate assays. In the absence of DNA-damaging agents, all the strains exhibited similar levels of cell viability (Fig. 6). In contrast, in the presence of DNA-damaging agents, ΔrecA strain showed a pronounced growth defect accompanied with 100-fold decrease in plating efficiency relative to the wild-type strain. This phenotype is consistent with the available literature. In contrast, the lethal effect of UV, MMS or ciprofloxacin, but not H2O2, was partially blocked by deletion of the recX gene in the ΔrecA strain. Although the basis for lack of H2O2 effect is not clear, the concentration used was probably not high enough to affect growth. Interestingly, the ΔrecX strain exhibited a slightly more resistant phenotype against all four DNA-damaging agents than the ΔrecA ΔrecX strain, indicating a possible gain-of-function due to loss of recX gene. Given these results, it is apparent that recX plays an active role in the SOS response and that the sensitivity of DNA damaging agents is slightly suppressed in ΔrecX strain.

**Survival after treatment with different concentrations of DNA-damaging agents.** Mycobacteria experience a number of adverse stress conditions, such as oxidative, nutritional and drug-induced stresses. To further explore cell viability differences, experiments were carried out in which the cell viability was measured by colony forming units (CFUs). First, the viability of M. smegmatis mc²155 ΔrecA, ΔrecX and ΔrecA ΔrecX mutants were tested in comparison with that of the wild-type by challenging them with increasing concentrations of MMS. The strains were grown in the presence of indicated concentrations of MMS, until the cultures reached an OD600 of 0.6. The cell viability was assayed by plating predetermined number of cells on 7H10 agar plates. The cells were judged alive if they were able to divide and form colonies. The mutants, unlike the wild-type strain, showed increased sensitivity to MMS in a concentration-dependent manner. The quantitative analysis indicated that the ΔrecA mutant exhibited relatively higher sensitivity to MMS, which increased with increasing concentrations of MMS (Fig. 7A). In the case of ΔrecX, the cells were ~2-fold less sensitive to MMS compared to the ΔrecA cells. On the other hand, the ΔrecA ΔrecX mutant showed more sensitivity to MMS in contrast to the ΔrecX mutant. The sensitivity of the ΔrecX mutant to MMS indicate that recX may have yet unidentified targets in addition to RecA. This assumption needs further investigation.

Next, the sensitivities of the M. smegmatis mc²155 ΔrecA, ΔrecX and ΔrecA ΔrecX mutant strains relative to the wild-type strain were determined by exposing them to increasing concentrations of H2O2. The results show that the ΔrecA mutant was less sensitive to this treatment (Fig. 7B). The deletion of recX alone, or deletion of the recA-recX locus, had only a minor effect on the viability and proliferation of mutant cells in response to H2O2 treatment. Notably, the ΔrecA and ΔrecA ΔrecX mutant strains exhibited a sensitive growth phenotype.

**Figure 5.** The kinetics of growth of M. smegmatis mc²155 wild-type, ΔrecA and ΔrecA ΔrecX and ΔrecX strains under normal growth conditions. Each data point is the mean of three independent experiments and the error bars represent standard deviations of the mean values.
to UV irradiation and ciprofloxacin that is significantly much more severe than to MMS or H$_2$O$_2$ (Fig. 7C,D). Furthermore, ΔrecA strain displayed greater sensitivity to UV irradiation and ciprofloxacin than the ΔrecA ΔrecX double mutant strain.

The expression of recA and recX genes are induced by DNA damage. The regulatory elements upstream of the recA gene are not conserved in all mycobacterial species. For example, the M. tuberculosis recA gene is transcribed from two promoters: both are DNA damage-inducible, albeit through different mechanisms. The promoter P1 of M. tuberculosis recA (proximal to the start codon) can be induced following DNA damage independent of the LexA and RecA proteins. In contrast, the promoter P2 (located away from the recA start codon) is regulated by LexA, which is functionally analogous to the E. coli recA promoter.$^{83-85}$ Interestingly, the mechanism of DNA damage induction in M. tuberculosis is not fully conserved in M. smegmatis$^{46,75}$. These findings emphasize the need to understand the expression and regulation of the M. smegmatis recA and recX genes and their roles in response to DNA-damaging agents.

As described above, hypoxia caused an increase in the expression of M. smegmatis recA and recX genes (Fig. 1). To further corroborate the notion that the expression of M. smegmatis recA and recX genes is damage-inducible,
the kinetics of induction with and without DNA damage was determined using RT-qPCR. Total RNA samples were prepared from *M. smegmatis* cells harvested at 0, 3, 6, 9 and 12 h after exposure to UV light as well as from untreated cultures and analysed as described in the Methods section. The results revealed no significant differences between the *recA* and *recX* transcript levels in untreated cells. In contrast, a marked increase in the *recA* and *recX* mRNA transcripts was observed 3 h after exposure to UV radiation, and then decreased slightly thereafter. A comparison of the RT-qPCR data indicates important differences between the *recA* and *recX* transcript levels. Exposure to UV radiation led to a ~8-fold increase in *recA* mRNA over control, while *recX* by ~3-fold (Fig. 8A,B).

Thus, although *recA* and *recX* mRNA exist in the same transcriptional unit, these results support the idea that the production and/or stability of *recX* mRNA transcript is subject to an additional posttranscriptional regulatory mechanism.

These results led us to perform additional experiments to determine the kinetics of accumulation of RecA and RecX proteins in *M. smegmatis* cells with or without DNA damage. Western blotting assays were performed on whole cell lysates using polyclonal antibodies raised against RecA and RecX (Fig. 8C). The quantification of Western blots showed that the cells contained significant quantities of both RecA and RecX proteins in uninduced cells (Fig. 8D,E). By comparison, a 2-fold increase in the abundance of both RecA and RecX (over control) was seen in cells 3 h after exposure to UV radiation and decreased thereafter. Importantly, the induction of RecA and RecX proteins exhibited a pattern reminiscent of that seen in cells under hypoxic conditions (Fig. 2), although the mechanisms by which they damage DNA are likely to be different.

**Concluding remarks.** Numerous studies have demonstrated that *recA* and *recX* perform a broad range of functions related to DNA repair and recombination\(^7,10\). To our knowledge, the stimuli that activate the expression of *M. smegmatis* *recA* and *recX* genes have not been identified. In this study, the expression levels of these genes were assessed in cells at aerobic growth and in response to various stress conditions. In *M. smegmatis*, similar to many bacterial species, *recA* and *recX* belong to the same operon, and the *recX* gene is located immediately downstream of the *recA* gene, and share overlapping coding regions\(^8,9\). It was found that DNA-damaging agents induced the expression of both genes to different extents; however, the expression ratios follow a similar pattern. Interestingly, the levels of both RecA and RecX remain high under stress conditions compared to aerobic growth conditions.

Several studies have demonstrated alternative roles for *recX* in recombinational DNA repair promoted by RecA. While RecX protein physically interacts with RecA, and functions as a potent inhibitor of all known functions of the latter in many bacterial species\(^14,46,48\), it potentiates homologous recombination in *N. gonorrhoeae* and *B. subtilis\(^25,28\). To gain insights into the role of *recX* in stress response in mycobacteria, knockout mutants of *M. smegmatis* *recX* and *recArecX* were constructed. Interestingly, the deletion of *recX* in *M. smegmatis* resulted in a slightly more resistant phenotype against all four DNA-damaging agents, indicating a possible gain-of-function due to loss of *recX* gene. The molecular basis of this effect is not clear, which seems worthwhile to explore in future studies. Whilst our analysis was mainly focused on the genetic interaction between *recA* and *recX* in...
recombinational DNA repair pathway, interestingly, recX seems to play an important role in bacterial growth. In summary, these findings are consistent with the idea that *M. smegmatis* recX plays an important role in DNA repair/recombination processes under adverse conditions, perhaps by regulating the detrimental effects of a subset of the yet unknown genes.

Abbreviations used are.  DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; kb, kilobase; MMS, methylmethane sulfonate; ODN, oligonucleotide; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride membrane; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SDS, sodium dodecyl sulphate; ssDNA, single-stranded DNA; SSC, 0.15 M NaCl-0.015 M sodium citrate (pH 7.0) buffer.

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Author Contributions
D.P. and K.M. conceived the study and wrote the manuscript. V.K.N. provided laboratory facilities and guided D.P. and D.A. in the construction of knockout mutants. D.P. performed all of the experimental work. K.M., D.P., D.A. and V.K.N. analyzed the data. All the authors reviewed the results and approved the final version of the manuscript.

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