Selective Alleviation of Mitomycin C Sensitivity in lexA3 Strains of Escherichia coli Demands Allele Specificity of rif-nal Mutations: A Pivotal Role for rpoB87-gyrA87 Mutations

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Abstract

Very recently, we have reported about an unconventional mode of elicitation of Mitomycin C (MMC) specific resistance in lexA3 (SOS repair deficient) mutants due to a combination of Rif-Nal mutations (rpoB87-gyrA87). We have clearly shown that UvrB is mandatory for this unconventional MMC resistance in rpoB87-gyrA87-lexA3 strains and uvrB is expressed more even without DNA damage induction from its LexA dependent promoter despite the uncleavable LexA3 repressor. The rpoB87 allele is same as the rpoB3595 which is known to give rise to a fast moving RNA Polymerase and gyrA87 is a hitherto unreported NalR allele. Thus, it is proposed that the RNA Polymerase with higher elongation rate with the mutant DNA Gyrase is able to overcome the repressional hurdle posed by LexA3 to express uvrB. In this study we have systematically analysed the effect of three other rpo8 (rif) mutations-two known to give rise to fast moving RNAP (rpoB2 and rpoB111) and one to a slow moving RNAP (rpoB8) and four different alleles of gyrA - NalR mutations (gyrA199, gyrA247, gyrA250, gyrA259) isolated spontaneously, on elicitation of MMC resistance in lexA3 strains. Our results indicate that in order to acquire resistance to 0.5 μg/ml MMC cells require both rpoB87 and gyrA87 but resistance to 0.25 μg/ml of MMC can be brought about by either rpoB87, gyrA87, fast moving rpoB mutations or other nal mutations also. We have also depicted increased constitutive uvrB expression in strains carrying fast moving RNAP (rpoB2 and rpoB111) with gyrA87 and another nal mutation with rpoB87 and expression level in these strains is lesser than rpoB87-gyrA87 strain. These results evidently suggest an allele specific role for the rif-nal mutations to acquire MMC resistance in lexA3 strains via increased constitutive uvrB expression and a pivotal role for rpoB87-gyrA87 combination to elicit higher levels of resistance.

Introduction

Mitomycin C (MMC) is a DNA intercalating agent that gives rise to DNA interstrand crosslinks. MMC has been used as an antitumor antibiotic that is naturally derived from Streptomyces caesposus [1]. The DNA crosslinking by MMC leads to the induction of the SOS response in Escherichia coli by activating the RecA protein to its activated form called as RecA* and subsequent cleavage of the LexA repressor [2,3]. The LexA is the SOS regulon repressor controlling the expression of more than 50 genes including most of the genes involved in repair of damaged DNA occurring due to exposure of cells to a wide range of DNA damaging agents [2,4]. A mutant form of the repressor due to the mutation lexA3, renders the repressor nonreactive to the activated RecA* and thus fails to cleave itself. This mutation makes E. coli strains sensitive to all DNA damaging agents including MMC as the expression of all the SOS induced genes are turned off in lexA3 strains [5]. But an alternate path for MMC repair in SOS deficient strains (lexA3 or recA) was identified by Kumaresan and Jayaraman in 1980 and was termed as “SOS Independent Repair” or SIR. This SIR effect increased the MMC resistance of a lexA3 strain by two additional spontaneously acquired mutations – A Rif resistant mutation, named rpoB87, mapped to the rpoB gene that codes for the β subunit of RNA Polymerase and a Nal resistant mutation, named gyrA87, mapped to gyrA gene that codes for the DNA Gyrase A subunit [6]. Our recent study on this unconventional mode of elicitation of Mitomycin C (MMC) resistance revealed an increase in uvrB expression even without DNA damage. This constitutive increase in uvrB expression was seen to be from its LexA dependent promoter despite the presence of uncleavable LexA3 repressor. It was shown that mutations inactivating the uvrB gene abolished this resistance. The study revealed that the rpoB87 mutation carries a C1565⇒T1565 transition in the 522nd codon of rpoB [7]. This lesion is the same as reported to be present in rpoB3595 mutant as reported by Jin and Gross [8]. RNAP with RpoB3595 β subunit was shown to have increased termination
read through/increased elongation rates [9,10]. This allele is known to define a fast moving RNA Polymerase. The gyrA87 is a hitherto unreported NalR allele that carries a G244→A244 transition changing the 82nd codon of gyrA gene giving rise to a D82N change in the GyrA protein [7]. This defines a novel allele for Nalidic acid resistance. An allele of gyrA giving a D82G mutation, when present alone, has been shown to exhibit low level of resistance to quinolone drugs but not to nalidixic acid [11]. This D82G mutation although not resistant to nalidixic acid like D82N mutation of gyrA87, has been shown to exhibit 2–3 fold decreased supercoiling activity in vitro [12] and also alters the steady state transcriptional activity of more than 800 genes [13]. Thus it is possible that the D82N mutant of DNA Gyrase A subunit also possesses altered negative supercoiling and a combination of the fast moving RNA Polymerase coupled with altered negative supercoiling can even increase the transcription of genes posing repressional roadblocks to the transcribing RNAP.

In this study, we have analysed whether the fast moving RNA Pol coded by the tpoB87 RifR allele and the possible altered supercoiling due to mutant Gyrase coded by the gyrA87 NalR allele only can play a role in the elicitation of MMC resistance as seen in phenotypically SIR+ strains or any other tpoB or gyrA allele(s) can also bring about the same effect. Thus, we have studied the effect of three other known RifR mutations, tpoB2 (Fast moving RNA Pol), tpoB0 (Slow moving RNA Pol) and tpoB111 (Fast moving RNA Pol) [10,14,15] and four different alleles of spontaneously acquired Nal mutations on the MMC resistance of the SIR phenotype. The data reported here clearly imply allele specificity among the mutations affecting the β subunit of RNA Pol and mutations in the DNA gyrase A subunit in elicitation of MMC resistance of ΔlexA3 strains. We have also shown that this allele specific increase in MMC resistance of the tpoB and gyrA mutations is due to the activity of UvrB protein which arises as a result of difference in the basal level of uvrB expression in these strains.

Materials and Methods

Bacterial Strains Used

Given in Table 1 is the list of Bacterial strains used in this study. Genetic Nomenclature is according to Demerec et al, 1966 [16].

Media and Chemicals

LB and minimal media [17] with appropriate supplements were used. Cells were routinely grown in LB at 37°C unless specified otherwise. Whenever required the following chemicals/antibiotics were added to the media in the final concentrations indicated. MMC (0.5 µg/ml or 0.25 µg/ml), Rif (20 µg/ml), Nal (20 µg/ml), Tet (10 µg/ml), Kan (45 µg/ml), Amino acids (30 µg/ml). The chemicals used were purchased from Himedia, India, Sigma, USA, Qiagen, India, Invitrogen, India and Sisco laboratories, India. MMC was purchased from Biobasic. Inc. The primers used for the study were obtained from Chromous Biotech, Bangalore, India. The enzymes used were obtained from Fermentas, India.

Mobilization of rpoB82, rpoB88 and rpoB111 Mutations into DM49N (gyrA87-lexA3) and DM49 (lexA3) Strains

All P1 mediated transductions were performed as described in Miller [17,18]. argE and tpoB are linked and therefore cotransduce. Hence, using the P1 lysate made directly from the strains carrying the various tpoB mutations, the argE marker was transduced into argE recipients, DM49N and DM49 (Table 1). The Arg+ transductants obtained on selective minimal plates lacking Arginine were screened for Rif resistant colonies.

Isolation, Mapping and Sequence Analyses of gyrA NalR Mutants from DM49R (rpoB87-lexA3) Strain

Spontaneous Nalidixic acid resistant mutants were isolated by plating 100 µl of overnight cultures of the DM49R strain in LB plates containing 20 µg/ml of Nalidixic acid. 12 such NalR mutants of DM49R strain were isolated in four independent set of experiments. The obtained NalR mutants were then mapped to the gyrA gene initially by transducing zfa23:Tn10 linked to gyrA to obtain TetR mutants and then looking for the cotransduction of NalR phenotype with zfa23:Tn10. Subsequently, the 546 bp region spanning the NalR resistance of all the 12 gyrA mutants were amplified using the the forward primer nalF : ATGAGCGACCTTGGAGAGA and the reverse primer nalR : CGGGATTTGGTGGCCATAC. The obtained products were checked on 1% agarose gels along with a 1kb DNA ladder and purified using DNA extraction Kit obtained from Fermentas, India. The purified PCR products were then sequenced by Chromous Biotech, Bangalore, India. The sequence results obtained were then analysed with BLAST tool in NCBI nucleotide database and mutations/mismatches in DNA sequence of E. coli K12 substrain MG1655 obtained manually analysed.

Mitomycin C Survival Analyses

Overnight cultures were sub-cultured into fresh LB broth and grown for ~8–9 hours. The samples were then diluted in fresh 0.85% saline solution and appropriate dilutions were spotted on LB plates and on MMC containing plates containing relevant concentration of MMC for the cell titre. The % survival of the strains was calculated when required with the cfu/ml on MMC plates and the cfu/ml on LB plates.

%Survival = cfu per ml on MMC containing LB plates × 100

In other cases the plates were scanned and the growth on MMC plates were analysed qualitatively and the plates were photographed. The growth on LB plates was analysed ~12–14 hours after the spotting while the growth on MMC containing plates were analysed after ~24 hours of incubation.

P1 Transduction Mediated Construction of uvrB::kan and lon::kan Markers into the Relevant Strains

P1 lysate prepared from the relevant donor strains for each cross (Table 1) was used to transfer the relevant marker (Kanamycin resistance) into recipient strains and the transduced cells were then plated on appropriate selective plates containing Kanamycin. The plates were incubated at 37°C until transductants appeared. The obtained transductants were segregated by streaking them on appropriate antibiotic containing LB agar plates. Representative transductants in each case were purified and stored for further analyses. The relevant recipient and donor strains are also mentioned in Table 1.

RT-PCR Analyses

Reverse Transcription PCR was performed by isolating RNA from late-log phase cultures of the respective strains using the RNA isolation kit from Qiagen, India. The RNA obtained was then normalized with corresponding OD at 260nm and used as template for Reverse Transcription. First step cDNA was obtained from the RNA samples using the cDNA synthesis kit from Invitrogen, India. The PCR with first strand cDNA for uvrB was performed with uvrBRTFor 5'-CGACGCTGTGTTGAT-
Table 1. List of E. coli K12 strains used in this study, their relevant Genotype and source.

| Strain            | Relevant Genotype | Source/Reference/Construction                  |
|-------------------|-------------------|-----------------------------------------------|
| AB1157            | F- thr-1, araC14, leuB6(Am), lacI(q-proA)62, lacY1, supE44, hisG4(Oc), rpoS390(Am), rplL31(stl), argE3(Oc), thi-1 | Laboratory collection                      |
| DM49              | Same as AB1157 but lexA3 Ind  ` | M.K. Berlyn, CGSC, USA*                     |
| DM49N             | Same as DM49 but zfa | Shannmughapriya and Munavar, 2012 [11]      |
| DM49R             | Same as DM49 but argE rpo87 | Shannmughapriya and Munavar, 2012 [11]      |
| DM49RN            | Same as DM49N but argE rpo87 | Shannmughapriya and Munavar, 2012 [11]      |
| MMR1              | F- Δlon510 cspB10::lac, rpo87 | Meenakshi and Munavar (Manuscript under preparation) |
| HR318             | F-, λ-, rph-1, btl8::Tn10, rpo88 | Dr. Harinarayanan, CDFD, India*               |
| NAM1              | F-, λ-, rph-1, rpoB111 | Agarwal, Shannmughapriya and Munavar (Unpublished work) |
| DM49RN2N          | Same as DM49RN but argE rpo82 | This study, DM49N X P1/(MMR1)                |
| DM49RN8N          | Same as DM49RN but argE rpo82 | This study, DM49N X P1/(HR318)               |
| DM49RN111N        | Same as DM49N but argE rpo82 | This study, DM49N X P1/(NAM1)                |
| DM49RN9           | Same as DM49RN but zfa | This study                                   |
| DM49RN4           | Same as DM49RN but zfa | This study                                   |
| DM49RN7           | Same as DM49RN but zfa | This study                                   |
| DM49RN9           | Same as DM49RN but zfa | This study                                   |
| DM49R2            | Same as DM49R2 but argE rpo82 | This study, DM49 X P1/(MMR1)                |
| DM49R8            | Same as DM49R8 but argE rpo82 | This study, DM49 X P1/(HR318)               |
| DM49R111          | Same as DM49R111 but argE rpo811 | This study, DM49 X P1/(NAM1)                |
| DM49R3            | Same as DM49R3 but zfa | This study                                   |
| DM49R4            | Same as DM49R4 but zfa | This study                                   |
| DM49R7            | Same as DM49R7 but zfa | This study                                   |
| DM49R9            | Same as DM49R9 but zfa | This study                                   |
| JW0762-2          | F-, Δ(araD-araB)1567, Δlac24787(creB-3), ΔuvrB751::kan | M.K. Berlyn, CGSC, USA*                     |
| 49RNUB            | Same as DM49RN but ΔuvrB751::kan | Shannmughapriya and Munavar, 2012 [11]      |
| 49R2NUB           | Same as DM49R2N but ΔuvrB751::kan | This study, DM49R2N X P1/(49RNUB)           |
| 49R8NUB           | Same as DM49R8N but ΔuvrB751::kan | This study, DM49R8N X P1/(49RNUB)           |
| 49R111NUB         | Same as DM49R111N but ΔuvrB751::kan | This study, DM49R111N X P1/(49RNUB)         |
| 49R3NUB           | Same as DM49R3N but ΔuvrB751::kan | This study, DM49R3N X P1/(49RNUB)           |
| 49R4NUB           | Same as DM49R4N but ΔuvrB751::kan | This study, DM49R4N X P1/(49RNUB)           |
| 49R7NUB           | Same as DM49R7N but ΔuvrB751::kan | This study, DM49R7N X P1/(49RNUB)           |
| 49R9NUB           | Same as DM49R9N but ΔuvrB751::kan | This study, DM49R9N X P1/(49RNUB)           |
| JW0429-1          | F-, Δ(araD-araB)1567, Δlac24787(creB-3), Δlon-725::kan, j`, rph-1, Δ(rhaD-rhaB)10058, hsdR514 | M.K. Berlyn, CGSC, USA*                     |
| SM49LK            | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(UW0429-1)           |
| SM49LN            | Same as AB1157 but Δlon::kan | This study, AB1157 X P1/(SM49L)              |
| SM49L             | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(SM49L)              |
| MM2L              | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(SM49L)              |
| MM4L              | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(SM49L)              |
| MM111L            | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(SM49L)              |
| MM3L              | Same as DM49LN but Δlon::kan | This study, DM49LN X P1/(SM49L)              |

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TACCTG-3', uvrBRTRev 5'–CTACCAGACGTC-GAACTCA-3'. The PCR reaction was done with thirty amplification cycles with the following conditions. 15 min at 94°C, 30 sec at 94°C, 45 sec at 55°C, 30 sec at 72°C, 7 min at 72°C and maintained at 4°C. The amplicon size was verified by agarose gel electrophoresis. A PCR reaction with DNA as sample was used as a positive control reaction and one with RNA as sample was used as negative control to eliminate possibility of DNA contamination in RNA sample. Equal volumes of RT-PCR samples were loaded in each lane to get to know the urB expression level.
Filamentation Analyses

Relevant strains were grown till mid log phase under Mitomycin C induced and un-induced conditions. The un-induced cultures were directly heat fixed on glass slides, stained with saffranin red and viewed. For inductions, the mid-log phase cells were treated with 0.5 mg/ml of MMC for 2 hours before processing and about 10 µl of each culture was spotted, smeared and the samples were directly heat fixed on glass slides, stained with saffranin red and viewed. The results were then analysed by observing the stained preparations of the cells under light microscope with 40x resolution. More than 10 frames of the slides were then observed under the Nikon Eclipse Ti light microscope at 40x resolution and images were viewed and photographed using the software NIS elements D version 3.0.

Results

Effect of *rpoB2*, *rpoB8* and *rpoB111* with *gyrA87* on MMC Resistance of *lexA3* Strains

Three Rifampicin resistant mutations other than the *rpoB87*/*rpoB3595* mutation were used to study their effect on elicitation of SIR phenotype. Two of these three mutations (*rpoB2* and *rpoB111*) are known to give rise to fast moving RNA Polymerases and one mutation *rpoB8*, was known to give rise to a slow moving RNA Polymerase as mentioned in the introduction section. The *rif* alleles, base changes, amino acid changes and known relevant phenotypes of all the *rpoB* mutations are listed in Table 2. The *rpoB2*, *rpoB8* and *rpoB111* mutations were introduced into DM49N (*lexA3 gyrA87*) strain as mentioned in materials and methods. The relative survival of various strains was carried out at 37°C in this study as opposed to the 30°C assays used in the previously published data of MMC survival [7] because during the course of this investigation we found that the resistance to MMC was found to be a little better at higher temperatures compared to 30°C (Shanmughapriya and Munavar, Unpublished data). MMC survival analyses of the relevant strains clearly indicate that none of the other mutations could make the *lexA3* strain to survive on 0.5 µg/ml MMC containing plates but the *rpoB87-gyrA87-lexA3* strain could survive well at the same concentration (Fig. 1). Quantification of the MMC survival of the strains carrying respective *rif* mutation revealed that the DM49R2N and DM49R111N carrying the two fast moving RNAP alleles *rpoB2* and *rpoB8* were able to survive well on 0.5 µg/ml MMC containing plates whereas the DM49R87N carrying the slow moving allele *rpoB8* could not survive.

![Figure 1. Sequential spotting test for analyses of survival of various Rif-Nal strains on LB plates containing 0.5 µg/ml of MMC after ~24 hours incubation. Appropriate control strains (AB1157 for positive control and DM49 for Negative control) were also tested. The growth of the respective strains on LB plates without MMC after ~12–14 hours incubation is given on the right. doi:10.1371/journal.pone.0087702.g001](image-url)
Figure 2. Survival of different rif-nal strains on MMC plates. a. % survival of relevant gyrA87-RifR strains on LB plates containing 0.5 μg/ml of MMC. b. % survival of relevant rpoB87-NalR strains on LB plates containing 0.5 μg/ml of MMC. The values plotted are the average of three
and spoB111 respectively show a marginal (<5 fold) increase in MMC resistance which was not observed in the DM49RN strain with the slow moving RNAP allele spoB8 (Fig. 2a). But even this resistance with fast moving RNAP alleles was still >1000 fold less than that observed with the DM49RN strain bearing spoB87-gyrA87 alleles (Fig. 2a). The survival of all the strains on LB plates was found to be in the order of ~10^3 cfu/ml.

Effect of Spontaneous NalR Mutants with spoB87 on MMC Resistance of lexA3 Strains

12 spontaneous Nalidixic acid resistant mutants were isolated and were mapped to the gyrA gene as described in materials and methods section. Sequencing of the NalR region of these alleles indicated that the 12 mutations defined four different alleles of gyrA gene and these alleles were named gyrA199, gyrA250, gyrA259 and gyrA247. Although these nal alleles have been previously reported by different groups in various contexts, this is the first study where their roles have been analysed in relation with DNA repair. The gyrA allele, base change and codon/ amino acid change in each of these four different alleles together with spoB87 were then studied. Survival of these strains on LB plates containing 0.5 g/ml of MMC indicates that none of these strains could survive as efficiently as like the DM49RN strain carrying the spoB87-gyrA87-lexA3 strain (Fig. 2b). Quantification of this survival indicated that the gyrA alleles of different groups when present along with spoB87 increased the resistance of the strains up to 50 fold, this resistance was still >50–100 fold less than that observed in with spoB87-gyrA87-lexA3 strain DM49RN (Fig. 2b).

Reduced 'SIR' Effect due to rif and nal Alleles Exclusive of the Other

While the level of resistance to 0.5 μg/ml of MMC observed in spoB87-gyrA87-lexA3 strains could not be matched with resistance level of any other rif/nal allelic combination, it is still tenable that some of these other mutations can display increased resistance to lower concentrations of MMC in combination or in isolation. Thus, the survival of these strains was checked on LB plates containing 0.25 μg/ml of MMC. At this decreased concentration of MMC while the lexA3 strain DM49 was still unable to grow, the spoB87-lexA3 strain DM49R and the gyrA87-lexA3 strain DM49N also grew well foregoing the need for both the mutations to be present. This effect can be called the reduced 'SIR' effect and either the spoB87 or gyrA87 mutation alone could elicit this effect. Therefore, other Rif<sup>R</sup>-gyrA87-lexA3 strains and the Nal<sup>R</sup>-spoB87-lexA3 strains were also growing well on 0.25 μg/ml MMC. Thus, the relevant rif or nal alleles were alone transduced into the lexA3 strain DM49 without the gyrA87 or spoB87 allele (as described in Materials and methods) to analyse their reduced 'SIR' effect. It was seen that the spoB2 and spoB111 alleles are able to alleviate the MMC sensitivity of the DM49 strain at 0.25 μg/ml MMC (Fig. 3). The gyrA199, gyrA250, gyrA259 and the gyrA247 alleles were also able to increase the resistance of the DM49 strain. But this reduced 'SIR' effect was not seen only with the spoB8 slow moving RNAP allele (Fig. 3).

Table 2. spoB alleles used in this study and their relevant characteristics.

| spoB allele | Base pair change/Codon or amino acid change | Type of RNAP produced | Relative Termination read through | Source/Comments |
|-------------|--------------------------------------------|------------------------|---------------------------------|----------------|
| spoB87/spoB3955 | C1560 → T1560/TCT → TTT (5222F) | Fast moving | 2.5–3.5 | Kumaressan and Jayaraman, 1988, Shannughapriya and Munavar, 2012, Jin and Gross, 1998 |
| spoB82 | C1570 → T1570/CAC → TAC (H5280Y) | Fast moving | 1.3–3.3 | Meenakshi and Munavar, Unpublished data |
| spoB88 | A1518 → C1538/CAG → CCG (Q513P) | Slow moving | 0.3–0.6 | R. Harinarayanavan, CDFD, Hyderabad, India |
| spoB8111 | C1609 → T1609/CCT → CTT (P564L) | Fast moving | 1.5–2.5 | This study |

*Values given as fold increase in termination read through are from Jin et al, 1988. doi:10.1371/journal.pone.0087702.t002

Pivotal Role for UvrB in Increased MMC Resistance Seen in Strains with Different rif-nal Alleles Tested

As could be seen from the above results, it is evident that there exists some level of allele specificity among the rif and nal mutations in eliciting resistance to even low level of MMC. It was shown in our previous study that the increased MMC resistance of the spoB87-gyrA87-lexA3 strain was due to the increase in constitutive/basal level of expression of the gene uvrB. Other studies have shown that out of all the Nucleotide Excision Repair functions (UvrA, UvrB and UvrC/Cho) that take part in repair of MMC-induced DNA damage only UvrB is mandatory [19]. Our results which showed that increase in expression of the uvrB gene in a lexA3 strain could increase the MMC resistance correlated well with the above observation [7]. This places UvrB in a prominent role in SIR mediated MMC resistance observed in SOS deficient strains. Thus, it is quite possible that it is the increased expression/activity of UvrB that is also causing the little increase in MMC resistance observed in the other rif-nal alleles tested in this study.

Therefore, we ventured to study the effect of inactivation of the uvrB gene on the MMC resistance of the lexA3 strains carrying the different rif-nal allelic combinations. For this purpose, we introduced the ΔuvrB:kan allele from relevant donor strain (see Table 1) into all the rif-nal-lexA3 strains analysed in this study. This
was done using P1 mediated transduction as given in the materials and methods section. The relevant genotypes of the uvrB inactivated derivatives of all the rif-nal-lexA3 strains analysed in this study are given in Table 1. The growth of the relevant ΔuvrB:kan derivatives along with respective parental controls were checked on LB plates containing 0.5 μg/ml MMC. It was seen that inactivation of uvrB gene in all of the rif-nal-lexA3 strains led to loss of the very little MMC resistance (if any) observed in the

Table 3. Sequence changes in various gyrA alleles used in the study.

| gyrA allele | Base pair change/Codon and amino acid change | Source/comments          |
|-------------|---------------------------------------------|--------------------------|
| gyrA87      | G244→A244/GAC→AAC (D82N)                   | Kumaresan and Jayaraman, 1988, Shanmughapriya and Munavar, 2012 |
| gyrA199     | G199→T199/GCC→TCC (A67S)                   | Isolated and sequenced in this study |
| gyrA250     | G250→T250/GCG→TCG (A84S)                   | Isolated and sequenced in this study |
| gyrA259     | G259→A259/GAC→AAC (D87N)                   | Isolated and sequenced in this study |
| gyrA247     | T247→G247/TCG→GCG (S83A)                   | Isolated and Sequenced in this study |

Figure 3. Sequential spotting test for analyses of survival of various Rif-Nal strains on LB plates containing 0.25 μg/ml of MMC after ~24 hours incubation. Appropriate control strains (AB1157 for positive control and DM49 for Negative control were also tested). The growth of the respective strains on LB plates without MMC after ~12–14 hours incubation is given on the right. doi:10.1371/journal.pone.0087702.g003
The resistance of these mutants to 0.25 μg/ml MMC was also checked and it was observed that the uvrB inactivated mutants were also totally sensitive to 0.25 μg/ml MMC. It was observed that the ΔuvrB::kan lexA derivative of parent strain, AB1157 was not completely sensitive to 0.25 μg/ml MMC but displayed a very sick growth. However, this strain was completely sensitive to 0.5 μg/ml MMC. This may be possibly due to the fact that in AB1157 all the other SOS repair functions are fully active and thus might be capable of giving rise to a much diminished survival to very low concentrations of MMC. The phenotypic effects of the relevant strains on survival to MMC is summarised in Table 4.

From this observation, it is clear that the increased MMC resistance of the gyrA87-lexA3 strains with rpoB2 or rpoB111 is indeed a result of uvrB gene function. Likewise, in an rpoB87-lexA3 strain carrying gyrA199, gyrA250, gyrA259 or gyrA247, increased MMC resistance is due to the function of UvrB.

**Table 4. Level of MMC survival in relevant strains and its implications.**

| Strain   | Relevant Genotype | MMC survival at 0.5 μg/ml/(R/S) | MMC survival at 0.25 μg/ml/(R/S) | Possible explanation/Reasons/comments |
|----------|-------------------|---------------------------------|----------------------------------|--------------------------------------|
| AB1157   | lexA⁺ rpoB⁻ gyrA⁺ | R (+++)                         | R (+++)                          | Completely resistant to DNA damaging agents being lexA⁺ |
| DM49     | lexA₃ rpoB⁺ gyrA⁺ | S (−)                           | S (−)                            | Sensitive to all DNA damaging agents being lexA₃, SOS uninducible strain |
| DM49RN   | lexA₃ rpoB87 gyrA⁸⁷ | R (++)                          | R (++)                           | Selective suppression of only Mitomycin C sensitive phenotype of lexA₂ by rpoB87 gyrA⁸⁷ mutations due to expression of uvrB. |
| DM49R    | lexA₃ rpoB87 gyrA⁺ | S (−)                           | R(++)                            | Reduced SIR effect due to only rpoB87 fast moving RNAP producing allele |
| DM49N    | lexA₃ gyrA⁸⁷ rpoB⁺ | S (−)                           | R(++)                            | Reduced SIR effect due to only gyrA⁸⁷ |
| DM49R2N  | lexA₃ rpoB2 gyrA⁸⁷ | S (−)                           | R (++)                           | Reduced SIR effect due to only gyrA⁸⁷ and perhaps rpoB2 |
| DM49R8N  | lexA₃ rpoB8 gyrA⁸⁷ | S (−)                           | R (++)                           | Reduced SIR effect due to only gyrA⁸⁷ |
| DM49R11N | lexA₃ rpoB111 gyrA⁸⁷ | S (−)                           | R (++)                           | Reduced SIR effect due to only gyrA⁸⁷ and perhaps rpoB111 |
| DM49RN3  | lexA₃ rpoB87 gyrA⁹⁹⁹ | S (−)                           | R (++)                           | Reduced SIR effect due to only rpoB87 fast moving RNAP producing allele |
| DM49RN4  | lexA₃ rpoB87 gyrA²⁵⁰ | S (−)                           | R (++)                           | Reduced SIR effect due to only rpoB87 fast moving RNAP producing allele |
| DM49RN7  | lexA₃ rpoB87 gyrA²⁵⁷ | S (−)                           | R (++)                           | Reduced SIR effect due to only rpoB87 fast moving RNAP producing allele |
| DM49RN9  | lexA₃ rpoB87 gyrA²⁴⁷ | S (−)                           | R (++)                           | Reduced SIR effect due to only rpoB87 fast moving RNAP producing allele |
| 49RNUB   | lexA₃ rpoB87 gyrA⁸⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49R2NUB  | lexA₃ rpoB2 gyrA⁸⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49R8NUB  | lexA₃ rpoB8 gyrA⁸⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49R11NUB | lexA₃ rpoB111 gyrA⁸⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49RN3UB  | lexA₃ rpoB87 gyrA⁹⁹⁹ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49RN4UB  | lexA₃ rpoB87 gyrA²⁵⁰ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49RN7UB  | lexA₃ rpoB87 gyrA²⁵⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |
| 49RN9UB  | lexA₃ rpoB87 gyrA²⁴⁷ ΔuvrB::kan | S (−) | S (−) | Complete loss of MMC resistance due to loss of uvrB function |

R (++) – ~100% survival.
S (−) – ~0.01–0.001% survival.
S (-) – Complete loss of survival.

**Differential Basal Level of uvrB Expression in the Different rif-nal-lexA3 Combinations**

It is evident from the above results that UvrB once again plays the central role in MMC resistance observed in the various rif-nal-lexA3 strains like seen with rpoB87-gyrA87-lexA3 strain. It was observed in our previous study that this increase in UvrB activity in rpoB87-gyrA87-lexA3 strain stems from an increased uvrB gene expression.
expression. We thus analysed whether the role of UvrB in the other rif-nal-lexA3 strains also corresponds with increase in the constitutive/basal level of expression of uvrB gene as was seen in the tpoB87-gyrA87-lexA3 strain. Thus, semi-quantitative RT-PCR analyses were performed to analyse the level of uvrB transcript in several of these rif-nal-lexA3 strains (DM49RN, DM49R2N, DM49R8N, DM49R111N and DM49RN3) along with relevant parental controls (AB1157 and DM49). As was expected, it was seen that the basal level of uvrB transcript was much higher in the DM49RN (tpoB87, gyrA87, lexA3) strain compared with that of the other strains (Fig. 4). It was also seen that the basal uvrB transcript level of the DM49 (lexA3) strain was decreased as compared to its lexA+ parent strain AB1157. This may be perhaps due to the fact that LexA3 is an inducible repressor and it might possibly possess decreased dissociation constant from its DNA binding region: A view which is conjectural. This may be the cause for the diminished transcript levels from DM49 compared to AB1157. Our results clearly indicate that DM49R2N (tpoB2, gyrA87, lexA3), DM49R111N (tpoB111, gyrA87, lexA3), DM49RN3 (tpoB87, gyrA199, lexA3) strains were slightly increased compared to the DM49 (lexA3) strain (Fig 4). It was also seen that the basal uvrB transcript level of the DM49 RN strain carrying the slow moving RNAP is further decreased (Fig 4). This indicates that uvrB expression is central in giving increased resistance to MMC in a lexA3 strain and that only a fast moving RNAP can bring about this effect. Also this shows that the elongation rates of the RNAP present in the strain plays a major role in increasing uvrB expression along with gyrA87.

Analyses of Extent of Filamentation Observed in Δlon Derivatives of Different rif-nal-lexA3 Combinations

The hallmark of the SIR phenotype as seen from our previous results is the differential expression of genes in the SOS regulon in a lexA3 strains. It has been shown that in the DM49RN strain, while the expression of uvrB gene is increased, the expression or activity of another SOS gene, sulA, is unaffected. Thus, the effect of tpoB87 and gyrA87 mutations seemed to be limited to expressing only one (or possibly few) of the SOS induced genes in a lexA3 strain. Thus we believe that the same or similar mechanism is in action in elicitation of MMC resistance seen in the other rif-nal mutations where only uvrB is expressed but not sulA. We thus ventured to confirm whether the same is true in few other selected rif-nal allelic combinations in a lexA3 strain. For this purpose we tested whether the activity of the SulA, cell division inhibitor, is increased in the strains carrying the fast moving RNAP producing allele with gyrA87. SulA has been shown to specifically bind the cell division protein FtsZ and stall cell division during DNA repair [20]. After repair, the SulA protein is degraded by the Lon protease and thus enables progression of the cell division. It has been known that in absence of Lon protease expression of sulA by DNA damage would lead to irreversible stalling of cell division and thus the cells will be seen to give rise to filamentation [21,22]. Thus, the expression/activity of the SulA protein can be tested by checking the extent of filamentation of a strain lacking Lon protease.

For this purpose, lon inactivated derivatives of all the rif-gyrA87-lexA3 strains analysed in this study were constructed as described in materials and methods. The relevant genotype of the Δlon derivatives of the strains are given in the Table 1. The filamentation levels of each of these derivatives were tested as given in materials and methods. It was seen from the results observed that there was no filamentation seen with any of the rif-nal-lexA3 strains tested (Fig. 5). AB1157 was used as a positive control for observation of filamentation while DM49 was used as a negative control. This result shows that the effect of the fast moving rif and other nal alleles is very selective and increases only uvrB expression and they apparently do not affect the expression or activity of the sulA gene and its product as is the case with tpoB87-gyrA87-lexA3 strain.

Discussion

The process of transcription has been linked to DNA repair mechanisms for a long time now. This was termed as Transcription Coupled Repair which is a subpathway of the Nucleotide Excision Repair mechanisms involving the UvrABC proteins [23] and mutations in RNA Polymerase β subunit have been shown to be defective in transcription coupled DNA repair mechanisms [24,25]. The role of these tpoB mutations in DNA repair were not directly affecting the expression of genes involved in repair mechanism but implicated in processing and removal of lesions. But tpoB3395 mutation has been shown to be indirectly involved with the DNA repair processes. It was shown that the tpoB3395 mutation increases the expression of SOS genes in a strain that is constitutive for SOS [26]. In our previous study, we have shown that this increase in expression of uvrB is seen even in lexA3 strains when the tpoB87/ tpoB3395 mutation is coupled with the gyrA87 mutation [7].

The results that we have reported herein pertaining to the MMC survival patterns of different Rifampicin and Nalidixic Acid resistant mutations clearly reveal that the SIR effect observed in tpoB87 and gyrA87 mutations are highly specific to those mutations. Survival of all the other gyrA87 coupled Rif mutations other than tpoB87 and tpoB87 coupled Nal mutations other than gyrA87 was highly impaired on continuous exposure to 0.5 μg/ml of MMC. When the concentration of MMC was decreased to 0.25 μg/ml other fast moving RNA Polymerases alone and some Nal resistant mutations also were able to resist it and give rise to survivors. Our results presented here clearly
Figure 5. Extent of filamentation in MMC untreated and 0.5 μg/ml MMC treated samples of indicated strains as observed under light microscope. Relevant genotypes of the strains are mentioned wherever appropriate.

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implicate the central role for UvrB in MMC resistance of lexA3 strains carrying fast moving RNAP producing gyrA87 and other nal alleles with rpoB87. It is also seen that this role of UvrB arises from the increased expression of basal level of uvrB in gyrA87-lexA3 strains carrying any fast moving RNAP producing allele (rpoB33595, rpoB33596, or in rpoB33595-lexA3 strain with gyrA199). Even among the strains with fast moving RNAP, rpoB87/rpoB33595 gives rise to the highest uvrB expression while those carrying rpoB2 and rpoB111 gave a lesser increase in uvrB expression. It was seen that rpoB87 with other Nal mutation gyrA199 also gives a slight increase in uvrB expression as seen with gyrA87 mutants with rpoB2 and rpoB111. It is also evident that it is indeed the UvrB protein that causes the increased expression with the other alternate Nal alleles tested because inactivation of the uvrB gene gives rise to loss of MMC resistance in all these strains. But, the lexA3 strain carrying rpoB87 mutation also needs only gyrA87 to give rise to the highest uvrB expression and consequently the highest MMC resistance.

Global relaxation of DNA supercoiling has also been shown to have a great impact on gene expression and it has been shown that this can lead to decrease in expression of some genes while the expression of number of other genes is increased [27]. Also, as mentioned previously, the D82G mutation affecting the same position as that of the D82N mutation in gyrA87, was seen to affect the transcriptional levels of more than 800 genes [13]. Thus, the rpoB87 and gyrA87 mutations can be perceived to affect the expression of genes like uvrB when combined together. As we have reported here, the increased elongation rate of the RNAP along with the possible altered DNA gyrase activity caused by rpoB87 and gyrA87 mutations could be one of the major reasons for this increased expression of uvrB in lexA3 strains carrying the various nal and nal alleles. Although this increase in expression of uvrB gene can be attributed to the higher elongation rate of the RNAP produced, the effect need not be solely due to that. It may be even be specific to the nal allele present as our results given here indicate and probably needs the faster RNAP such as that from rpoB87/rpoB33595 along with gyrA87 mutation affecting specifically the codon 82 to give the best MMC resistance. The results pertaining to MMC resistance in spontaneously acquired mutations in rpoB and gyrA imply high relevance in the study of resistance to MMC in E. coli and may pave way for the better understanding of the mode of resistance of E. coli to MMC. Owing to the fact that we have shown clearly that an SOS function (uvrB) is needed for elicitation of SIR phenotype, we retained the same acronym SIR but referring to SOS Interdependent Repair instead of originally named SOS Independent Repair.

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Author Contributions

Conceived and designed the experiments: VS SM HM. Performed the experiments: VS SM. Analyzed the data: VS SM HM. Contributed reagents/materials/analysis tools: HM. Wrote the paper: VS HM.

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