**Original Article**

# Growth and Division Mechanisms by Which Genetic Resisters Emerge from the Rifampicin-Surviving Population of Differentially Antibiotic-Susceptible Mycobacterial Subpopulations

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## Abstract

**Background:** We recently reported the *de novo* emergence of unusually high numbers of antibiotic resisters from the *in vitro* cultures of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* surviving in the presence of minimum bactericidal concentration (MBC) of antituberculosis antibiotics. The resisters emerged due to multiple asymmetric divisions of elongated mother cells containing multiple nucleoids and multiple septae. We had earlier found a minor subpopulation of short-sized cells (SCs) and a major subpopulation of normal-sized cells (NCs) (10% and 90%, respectively, of the whole population), with significant difference in antibiotic susceptibility and resister generation frequency, in the *in vitro* cultures of *M. tuberculosis*, *M. smegmatis*, and *Mycobacterium xenopi*, as well as in pulmonary tuberculosis patients' sputum. However, the mechanisms of growth and division promoting the emergence of antibiotic resisters from these subpopulations remained unknown. Therefore, here, we took up the first-time study to find out the mechanism of growth and division by which antibiotic resisters emerge from the antibiotic-surviving population of the two subpopulations of *M. smegmatis*. **Methods:** *M. smegmatis* SCs and NCs were fractionated from mid-log phase cultures using Percoll gradient centrifugation; their purity was checked and exposed to 10×, 2×, and 0.4× MBC of rifampicin for 120 h. The colony-forming units (CFUs) were determined on rifampicin-free plates for the total population and on rifampicin-containing plates for scoring rifampicin resisters. The phenotype and the morphology of the cells at various stages of the exposure were determined using transmission electron microscopy. The dynamic growth and division mechanisms of the cells to emerge as rifampicin resisters were monitored using live-cell time-lapse imaging. The rifampicin resisters were sequenced for mutations in the rifampicin resistance determining region of *rpoB* gene. Statistical significance was calculated using two-tailed paired *t*-test, with *$P \leq 0.05$ and **$P \leq 0.01$. **Results:** Multinucleated and multiseptated elongated cells emerged from their respective antibiotic-surviving populations. They produced a large number of sibling-daughter cells through multiple asymmetric divisions in short durations, showing abnormally high spurts in CFUs of antibiotic resisters. The CFUs were several-fold higher than that expected from the mass-doubling time of the subpopulations. Despite this commonality, the subpopulations showed specific differences in their response to different multiples of their respective MBC of rifampicin. **Conclusions:** Mycobacterial subpopulations come out of rifampicin stress by undergoing multiple nucleoid replications, multiple septation for nucleoid segregation, and acquisition of antibiotic target-specific mutations, followed by multiple asymmetric divisions to generate unusually a large number of rifampicin resisters. Because we had earlier shown that SCs and NCs are present in the pulmonary tuberculosis patients' sputum, the present findings have clinical relevance on the mechanism of emergence of antibiotic-resistant strains from mycobacterial subpopulations.

**Keywords:** Antibiotic resistance, antibiotic-surviving population, cell number spurt, minor and major subpopulations, multinucleated, multiseptated, *Mycobacterium smegmatis*, rifampicin

## INTRODUCTION

When exposed to different concentrations of antibiotics, strains that are resistant to the same and other antibiotics emerge

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from most bacteria of diverse genera such as *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Mycobacterium smegmatis*.[1-10] The genetic resisters of *E. coli*, *S. aureus*, and *M. smegmatis* to antibiotics were found to emerge from a small population that was surviving in the continued presence of lethal concentrations of the antibiotic even after major portion of the cells were killed.[5,6,8-10] However, the mode of growth and division by which antibiotic resister clones emerged from the antibiotic-surviving population remained unknown. In this regard, we found the unique mode of cell growth and division that facilitated the emergence of unusually large numbers of antibiotic resisters of *M. smegmatis* mid-log phase (MLP) cultures exposed to minimum bactericidal concentration (MBC) of rifampicin and moxifloxacin for prolonged duration.[11] Under the sustained antibiotic pressure, the nucleoids of a certain proportion of the antibiotic-surviving population replicated and segregated multiple times and developed multiple septae causing elongation of the cells. The elongated multinucleated, multiseptated mother cells divided multiple times within short durations and gave rise to unusually high number of cells that were phenomenally much higher than that could be expected from the mass-doubling time.[11] Nevertheless, all these studies reported the antibiotic response of the whole population of bacterial cultures, but not of the subpopulations.

We had earlier reported that *in vitro* cultures of *M. tuberculosis*, *M. smegmatis*, *Mycobacterium xenopi*, and tubercle bacilli from the sputum of freshly diagnosed and drug-resistant tuberculosis patients consisted of two subpopulations: the short-sized cells (SCs) and the normal-sized cells (NCs) at 10:90 ratio.[12,13] The respective enriched subpopulations, prepared from the whole population, were called the SCs-enriched fraction (SCF) and the NCs-enriched fraction (NCF).[14] The SCF, under normal conditions of growth, will grow and divide to give a normal MLP population consisting of 90% NCF and 10% SCF.[14] However, on exposure to antibiotics, oxidative stress, and nitrite stress, the SCF stopped growth and division until they gained mutations, re-grew, and divided to give a normal MLP population consisting of 90% NCs and 10% SCs.[14-16] The NCF also behaved in a similar manner. The SCF always showed significantly higher antibiotic resister generation frequency than the NCF, despite being significantly more susceptible to oxidative stress and nitrite stress.[14-16]

These observations hinted that the independent response of the subpopulations to antibiotics might differ despite being together in the whole population. It further implied that the response of the individual subpopulations to antibiotics might have remained masked during the studies on the antibiotic response of the whole populations of *M. tuberculosis* and *M. smegmatis*.[5,6,8-11] Thus, the response of the whole population measured against different kinds of stress agents might have been an average response of the whole population consisting of the subpopulations. Therefore, we took up the present study on the response of the SCF and NCF subpopulations surviving in the presence of bactericidal concentrations of rifampicin.

## METHODS

### Bacterial strains and culture conditions

*M. smegmatis* mc² 155[17] was cultured in Middlebrook 7H9 liquid medium containing 0.2% glycerol (Fisher Scientific) and 0.05% Tween 80 (Sigma) at 37°C and 170 rpm. Mycobacteria 7H11 agar plates (Difco) with and/or without rifampicin (50× MBC; 125 µg/ml; MP Biomedicals) were used for plating. All the experiments were initiated with the MLP culture of 10⁶ cells/ml in flasks, in biological triplicates.

### Fractionation of mycobacterial subpopulations

SCs and NCs of *M. smegmatis* were fractionated from the MLP culture using gradients of Percoll ranging from 64% to 80%, with each time with 2% increment, as described, which does not affect their physiological characteristics in any way.[14] The purity of the SCF and NCF was verified after every fractionation, as reported in our earlier work.[14] We have always gotten the maximum purity of ~90% for both the preparations. See Supplementary Material for details.

### Construction of growth curve for the normal-sized cell-enriched fraction and small-sized cell-enriched fraction subpopulations

The NCF and SCF were re-inoculated at 1% inoculum into fresh Middlebrook 7H9 medium, and the growth was monitored at $OD_{600\ nm}$ for 45 h. The mass-doubling time was calculated using the formula given below.[18] N1 and N2 are the number of cells at time t1 and t2, respectively.

Mass-doubling time = log10 (2) (t2−t1) ÷ (log10 [N2] − log10 [N1]).

### Transmission electron microscopy

Transmission electron microscopy (TEM) was performed, as described,[19] with minor modifications.[20] The sections were observed under transmission electron microscope (BioTwin, FEI) at 120 kV and images were procured. See Supplementary Material for details.

### Colony-forming unit determination and calculations

From the colony-forming unit (CFU) values observed on the plate, the fold change in the CFU between two consecutive time points was calculated by dividing the CFU value for a time point (considered as "A") with its earlier time point value (considered as "B"). Because the mass-doubling time of *M. smegmatis* cells is ~3 h both in *in vitro* cultures[21] and during growth and division on agarose pads during live-cell time-lapse imaging,[13] the expected fold increase in the CFU in 6-h duration is 4-fold. Accordingly, >4-fold increase in the CFU during any 6-h duration was considered as CFU spurt. The tabulations of the CFUs were made by noting the observed CFUs and the calculated CFUs expected in the 6-h durations of the samples.

### Statistical calculations

Statistical significance was calculated using two-tailed paired *t*-test, with *$P \leq 0.05$ and **$P \leq 0.01$.

### Live-cell time-lapse imaging

Agarose pad method for the live-cell time-lapse microscopy of *M. smegmatis* regrowth phase (RGP) cells was performed, as described,[22,23] with minor modifications.[13] See Supplementary Material for details.

### Genomic DNA isolation

Colonies taken from antibiotic-free or antibiotic-containing plates were resuspended in 50 ml sterilized fresh Middlebrook 7H9 (Difco) liquid media and subjected to pipetting at least 10–15 times to remove clumps. The cell suspension was reinoculated into fresh sterilized Middlebrook 7H9 (Difco) liquid medium with and/or without antibiotic (25 μg/ml rifampicin; MP Biomedicals) and cultured to $10^6$ cells/ml density. The cell pellet was used for genomic DNA isolation, as described.[5] See Supplementary Material for details.

### Polymerase chain reaction amplification of rifampicin resistance determining region sequence and DNA sequencing

Rifampicin resistance determining region (RRDR) locus was polymerase chain reaction (PCR) amplified using RRDR-specific forward and reverse primers (5'gtcgtctgcgcaccgtc3' and 5'ctcgatgaagccgaacgg3', respectively) using Phusion DNA Polymerase (Thermo Fisher Scientific, USA), as described.[6] Agarose gel (1.5%) was used to run the PCR-amplified products, and the specific amplified band was eluted using gel elution kit (GeneJET Gel Extraction Kit; Thermo Fisher Scientific, USA). Sequence determination was performed for both the strands of the DNA by Chromous Biotech, Bangalore, India.

### Calculation of rifampicin resister generation frequency

Resister generation frequency = Number of cells (CFU) that could survive on the particular time point on rifampicin plate/ total number of cells present in culture at that same time point. Total amount of cells present in that particular time point were measured by the CFU found on antibiotic-free plate on that particular time point.

## RESULTS

### Experimental rationale and strategy

The specific steps in the experimental strategy were presented in a flowchart [Figure S1]. We had earlier determined 2.5 μg/ml as the 1× MBC of rifampicin for $10^6$ cells/ml of the whole MLP culture.[11] Because the whole culture consisted of the NCF and SCF subpopulations, we used 2.5 μg/ml as the 1× MBC for both the subpopulations, as 2.5 μg/ml would be the concentration to which they would have been exposed when they were naturally together in the whole population. The 10×, 2×, and 0.4× MBC of rifampicin was used as *M. smegmatis*, unlike *M. tuberculosis* possesses rifampicin-inactivating mono-ADP-ribosyl transferase (Arr) activity,[24] which would reduce the levels of functional rifampicin if lower concentrations of rifampicin were used. Therefore, the use of multiples of MBC would ensure that at least one or more of the MBCs of rifampicin would remain

bactericidal for 120 h despite the Arr activity-mediated inactivation. Nevertheless, the use of 0.4× MBC of rifampicin would enable the study of the response to low antibiotic concentrations as well. The cell density of $10^6$ cells/ml was used in all the experiments to preempt the formation of natural resisters to rifampicin that arise at $10^{-8}$ frequency in the MLP cultures.[6,25]

### Growth characteristics of the normal-sized cell-enriched fraction and short-sized cell-enriched fraction cultures

The mass-doubling time of the NCF and SCF subpopulations was found to be 188 ± 3 and 215 ± 8 min, respectively, where the difference was not statistically significant [Figure S2]. The mass-doubling time of the subpopulations was comparable to the mass-doubling time of ~180 min of the whole *M. smegmatis* population in *in vitro* cultures and during live-cell time-lapse imaging on agarose pads.[13,21] The notable difference was that the SCF culture took more time to enter the log phase of growth. Nevertheless, it reached comparably same cell density as that of the NCF culture at the end of the log phase. By then, the NCs and SCs established a normal culture with 90:10 proportion. Subsequently, the cultures grew similarly, with the SCF culture showing lower cell density until stationary phase. Subsequently, the cell density of the SCF culture became higher than that of the NCF culture [Figure S2].

### Response of the normal-sized cell-enriched fraction and short-sized cell-enriched fraction subpopulations to 10× minimum bactericidal concentration rifampicin

*Higher susceptibility and lower recovery of the short-sized cell-enriched fraction subpopulation*

Aliquots of the cultures were plated on rifampicin-free and 50× MBC rifampicin-containing plates at 6-h interval during the entire 120 h of the culture to determine total CFU and the CFU of antibiotic resisters, respectively. The triphasic CFU curve showed killing phase (KP) with drastic reduction in CFU, followed by the antibiotic-surviving phase showing no apparent change in the CFU of the population and the RGP showing rise in CFU, as reported.[5,8,11] The exposure of $10^6$ cells/ml of the NCF and SCF MLP cultures to 10× MBC rifampicin showed ~3-$\log_{10}$ reduction in the CFUs from 0 to 48 h to reach a CFU of ~5 × $10^3$ cells/ml [Figure 1a, green and blue lines, respectively; *n* = 3 of each culture]. From 48 h, after a small plateau with no apparent change in CFU up to 54 h, the NCF CFU increased steeply and reached a plateau of ~5 × $10^8$ cells/ml at 102 h. From 102 to 120 h, the density of NCF ($10^8$ cells/ml) was ~2-$\log_{10}$-fold higher than that at the start of the exposure ($10^6$ cells/ml). On the contrary, the SCF CFU stayed without change from 48 to 60 h, decreased a bit till 66 h, and stayed as plateau at ~5 × $10^2$ cells/ml till 78 h. Subsequently, it started rising gradually to reach a cell density of ~5 × $10^4$ cells/ml at 120 h, which was ~2-$\log_{10}$-fold lower than the starting density of the culture. The shallow increase in the growth of the rifampicin-exposed SCF might have been probably due to the significantly higher natural
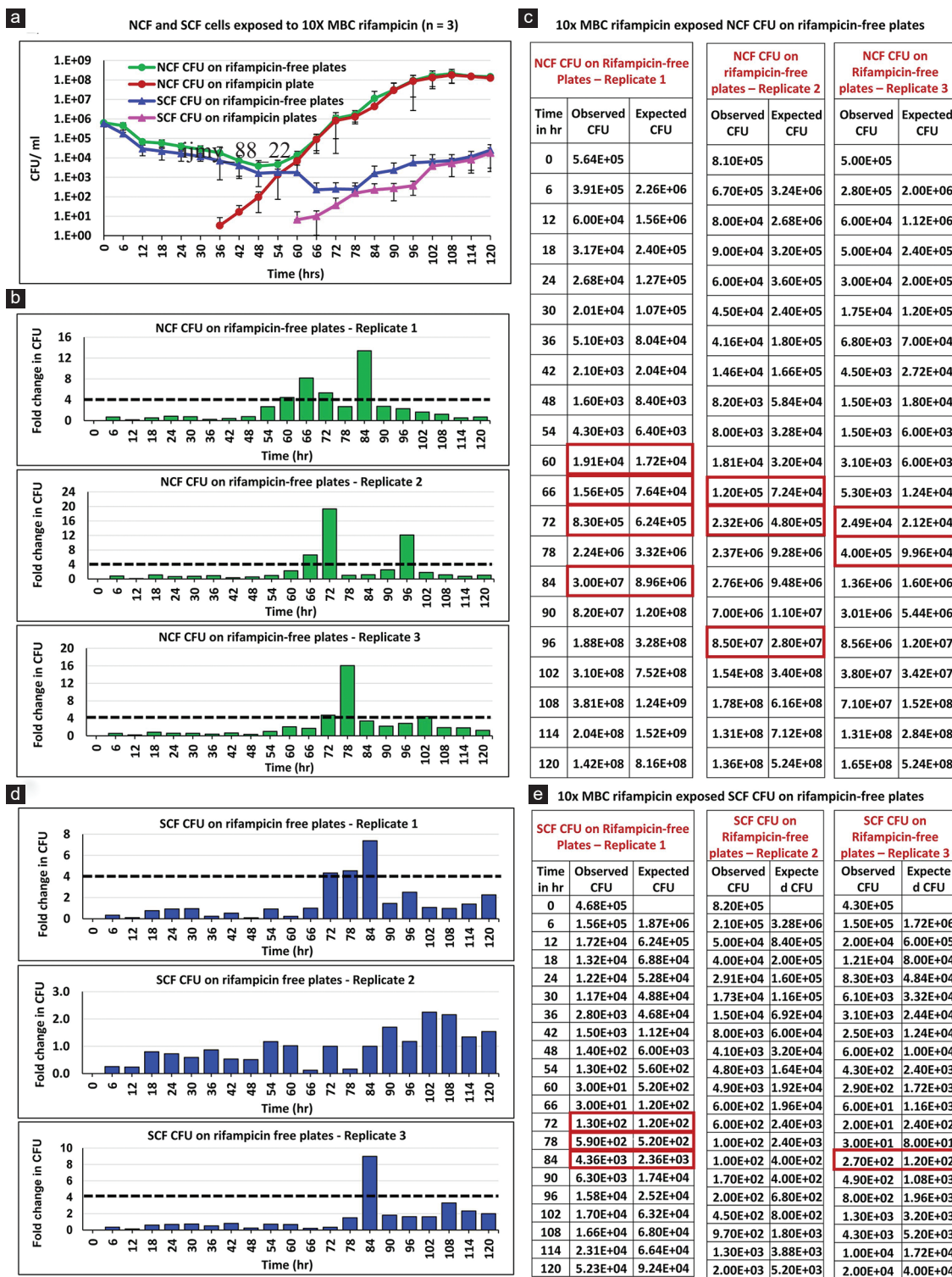
**a** NCF and SCF cells exposed to 10X MBC rifampicin (n = 3)

**b** NCF CFU on rifampicin-free plates – Replicate 1; NCF CFU on rifampicin-free plates – Replicate 2; NCF CFU on rifampicin-free plates – Replicate 3

**d** SCF CFU on rifampicin free plates – Replicate 1; SCF CFU on rifampicin free plates – Replicate 2; SCF CFU on rifampicin free plates – Replicate 3

**c** 10x MBC rifampicin exposed NCF CFU on rifampicin-free plates

| Time in hr | NCF CFU on Rifampicin-free Plates – Replicate 1 | | NCF CFU on rifampicin-free plates – Replicate 2 | | NCF CFU on Rifampicin-free plates – Replicate 3 | |
|---|---|---|---|---|---|---|
| | Observed CFU | Expected CFU | Observed CFU | Expected CFU | Observed CFU | Expected CFU |
| 0 | 5.64E+05 | | 8.10E+05 | | 5.00E+05 | |
| 6 | 3.91E+05 | 2.26E+06 | 6.70E+05 | 3.24E+06 | 2.80E+05 | 2.00E+06 |
| 12 | 6.00E+04 | 1.56E+06 | 8.00E+04 | 2.68E+06 | 6.00E+04 | 1.12E+06 |
| 18 | 3.17E+04 | 2.40E+05 | 9.00E+04 | 3.20E+05 | 5.00E+04 | 2.40E+05 |
| 24 | 2.68E+04 | 1.27E+05 | 6.00E+04 | 3.60E+05 | 3.00E+04 | 2.00E+05 |
| 30 | 2.01E+04 | 1.07E+05 | 4.50E+04 | 2.40E+05 | 1.75E+04 | 1.20E+05 |
| 36 | 5.10E+03 | 8.04E+04 | 4.16E+04 | 1.80E+05 | 6.80E+03 | 7.00E+04 |
| 42 | 2.10E+03 | 2.04E+04 | 1.46E+04 | 1.66E+05 | 4.50E+03 | 2.72E+04 |
| 48 | 1.60E+03 | 8.40E+03 | 8.20E+03 | 5.84E+04 | 1.50E+03 | 1.80E+04 |
| 54 | 4.30E+03 | 6.40E+03 | 8.00E+03 | 3.28E+04 | 1.50E+03 | 6.00E+03 |
| 60 | 1.91E+04 | 1.72E+04 | 1.81E+04 | 3.20E+04 | 3.10E+04 | 6.00E+03 |
| 66 | 1.56E+05 | 7.64E+04 | 1.20E+05 | 7.24E+04 | 5.30E+03 | 1.24E+04 |
| 72 | 8.30E+05 | 6.24E+05 | 2.32E+06 | 4.80E+05 | 2.49E+04 | 2.12E+04 |
| 78 | 2.24E+06 | 3.32E+06 | 2.37E+06 | 9.28E+06 | 4.00E+05 | 9.96E+04 |
| 84 | 3.00E+07 | 8.96E+06 | 2.76E+06 | 9.48E+06 | 1.36E+06 | 1.60E+06 |
| 90 | 8.20E+07 | 1.20E+08 | 7.00E+06 | 1.10E+07 | 3.01E+06 | 5.44E+06 |
| 96 | 1.88E+08 | 3.28E+08 | 8.50E+07 | 2.80E+07 | 8.56E+06 | 1.20E+07 |
| 102 | 3.10E+08 | 7.52E+08 | 1.54E+08 | 3.40E+08 | 3.80E+07 | 3.42E+07 |
| 108 | 3.81E+08 | 1.24E+09 | 1.78E+08 | 6.16E+08 | 7.10E+07 | 1.52E+08 |
| 114 | 2.04E+08 | 1.52E+09 | 1.31E+08 | 7.12E+08 | 1.31E+08 | 2.84E+08 |
| 120 | 1.42E+08 | 8.16E+08 | 1.36E+08 | 5.24E+08 | 1.65E+08 | 5.24E+08 |

**e** 10x MBC rifampicin exposed SCF CFU on rifampicin-free plates

| Time in hr | SCF CFU on Rifampicin-free Plates – Replicate 1 | | SCF CFU on Rifampicin-free plates – Replicate 2 | | SCF CFU on Rifampicin-free plates – Replicate 3 | |
|---|---|---|---|---|---|---|
| | Observed CFU | Expected CFU | Observed CFU | Expected CFU | Observed CFU | Expected CFU |
| 0 | 4.68E+05 | | 8.20E+05 | | 4.30E+05 | |
| 6 | 1.56E+05 | 1.87E+06 | 2.10E+05 | 3.28E+06 | 1.50E+05 | 1.72E+06 |
| 12 | 1.72E+04 | 6.24E+05 | 5.00E+04 | 8.40E+05 | 2.00E+04 | 6.00E+05 |
| 18 | 1.32E+04 | 6.88E+04 | 4.00E+04 | 2.00E+05 | 1.21E+04 | 8.00E+04 |
| 24 | 1.22E+04 | 5.28E+04 | 2.91E+04 | 1.60E+05 | 8.30E+03 | 4.84E+04 |
| 30 | 1.17E+04 | 4.88E+04 | 1.73E+04 | 1.16E+05 | 6.10E+03 | 3.32E+04 |
| 36 | 2.80E+03 | 4.68E+04 | 1.50E+04 | 6.92E+04 | 3.10E+03 | 2.44E+04 |
| 42 | 1.50E+03 | 1.12E+04 | 8.00E+03 | 6.00E+04 | 2.50E+03 | 1.24E+04 |
| 48 | 1.40E+02 | 6.00E+03 | 4.10E+03 | 3.20E+04 | 6.00E+02 | 1.00E+04 |
| 54 | 1.30E+02 | 5.60E+02 | 4.80E+03 | 1.64E+04 | 4.30E+02 | 2.40E+03 |
| 60 | 3.00E+01 | 5.20E+02 | 4.90E+03 | 1.92E+04 | 2.90E+02 | 1.72E+03 |
| 66 | 3.00E+01 | 1.20E+02 | 6.00E+02 | 1.96E+04 | 6.00E+01 | 1.16E+03 |
| 72 | 1.30E+02 | 1.20E+02 | 6.00E+02 | 2.40E+03 | 2.00E+01 | 2.40E+02 |
| 78 | 5.90E+02 | 5.20E+02 | 1.00E+02 | 2.40E+03 | 3.00E+01 | 8.00E+01 |
| 84 | 4.36E+03 | 2.36E+03 | 1.00E+02 | 4.00E+02 | 2.70E+02 | 1.20E+02 |
| 90 | 6.30E+03 | 1.74E+04 | 1.70E+02 | 4.00E+02 | 4.90E+02 | 1.08E+03 |
| 96 | 1.58E+04 | 2.52E+04 | 2.00E+02 | 6.80E+02 | 8.00E+02 | 1.96E+03 |
| 102 | 1.70E+04 | 6.32E+04 | 4.50E+02 | 8.00E+02 | 1.30E+03 | 3.20E+03 |
| 108 | 1.66E+04 | 6.80E+04 | 9.70E+02 | 1.80E+03 | 4.30E+03 | 5.20E+03 |
| 114 | 2.31E+04 | 6.64E+04 | 1.30E+03 | 3.88E+03 | 1.00E+04 | 1.72E+04 |
| 120 | 5.23E+04 | 9.24E+04 | 2.00E+03 | 5.20E+03 | 2.00E+04 | 4.00E+04 |

**Figure 1:** Rifampicin susceptibility profile and CFU fold-change of *M. smegmatis* NCF and SCF exposed to 10× MBC rifampicin for 120 h. (a) The CFU of NCF and SCF plated on rifampicin-free and rifampicin plates (50× MBC) after exposure to 10× MBC rifampicin. (b and d) Fold-change in the CFU at specific 6-h intervals of (b) NCF and (d) SCF. The expected 4-fold increase in CFU in 6 h is indicated by the horizontal dotted line. (c and e) The CFU spurts at specific 6-h intervals of (c) NCF and (e) SCF. The spurts of CFU beyond the expected 4-fold in both the samples in 6-h periods are indicated in red box. *n* = 3 in all cases. MBC: Minimum bactericidal concentration, CFU: Colony forming unit, NCF: Normal-sized cells-enriched fraction, SCF: Short-sized cells-enriched fraction

susceptibility of the SCF subpopulation to rifampicin, reported by us earlier.[14,15] Multiple independent preparations of the subpopulations also showed consistently comparable growth profile against 10× MBC rifampicin, indicating the

reproducibility of the response of SCF cells to the high concentration of rifampicin.

### Rifampicin resisters emerged at different time from the normal-sized cell-enriched fraction and short-sized cell-enriched fraction

A notable difference between the responses of the NCF and SCF cultures to 10× MBC rifampicin was the time of emergence of the resisters. While we could observe the first colony of the NCF resisters at 36 h, the SCF resister colony was visible first time only at 60 h of exposure [Figure 1a, brown and pink lines, respectively]. When we extrapolated the CFU curves of the two subpopulations with best-fit linear graphs, the lines crossed the X-axis at ~30 h and ~48 h, respectively, for the NCF and SCF resisters [Figure S3]. It meant that the first single rifampicin resister clone of the NCF and the SCF subpopulations emerged at ~30 h and ~48 h, respectively. The CFUs of the NCF and SCF resisters started increasing from ~36 h and ~60 h, respectively. Despite the late emergence of the respective resisters, the CFUs on the rifampicin plates increased and became equal to the CFUs on the rifampicin-free plates, from ~66 h and ~102 h, respectively [Figure 1a, brown and green lines, and pink and blue lines, respectively]. This implied that from these respective time points of exposure onward, every colony on the rifampicin-free and rifampicin plates would have emerged from the rifampicin resisters, where all the CFUs were from resisters. Although one could not rule out the presence of nongrowing/nondividing, live cells that may not yield CFU, nondividing cells could not be found in any of the fields during live-cell time-lapse imaging experiments (the data on live-cell time-lapse imaging). The CFU profile of the rifampicin-unexposed NCF and SCF cultures did not show triphasic response, and rifampicin resister generation, when plated on 125 µg/ml of rifampicin, was also negligible [Figure S4]. It showed that the triphasic CFU profile and the emergence of rifampicin resisters at high frequency were the features associated with exposure to MBC of rifampicin. Further, the rifampicin concentration in the medium was found to remain without any appreciable change till 102 h consistently in many similar experiments performed earlier in the laboratory.[8,11] Hence, the rifampicin levels were not monitored in the SCF and NCF cultures in the present study.

### Abrupt, high colony-forming unit spurts in the rifampicin-exposed normal-sized cell-enriched fraction and short-sized cell-enriched fraction cultures

#### Spurts in cell number detected from the colony-forming units on rifampicin-free plates

Because the mass-doubling time of the NCF and SCF was found to be ~3 h [$188 \pm 3$ min and $215 \pm 8$ min, respectively; Figure S2], two mass doubling with 4-fold increase in the CFU was expected in 6-h period. However, surprisingly, the CFUs of the 10× MBC rifampicin-exposed NCF and SCF cultures showed CFU spurts of >4-fold during several 6-h period from 54 h onward in the NCF and from 72 h in the SCF cultures [Figure 1b-e, respectively; $n = 3$ in each case]. It indicated unusually high sudden spurts in cell number that were much higher than the expected 4-fold increase in 6-h interval. The biological replicate 2 of the SCF culture did not show CFU spurt even though normal growth was observed in the flask [Figure 1e, replicate 2]. There were no cell clumping issue, which would have otherwise given spurts in CFU, as the cultures with and without mild sonication gave superimposable CFU values [Figure S5]. Contrary to the cells in the rifampicin-exposed cultures, the cells in the rifampicin-unexposed cultures did not show CFU spurts [Figures S6 and S4 for growth curve]. This control experiment confirmed that the CFU spurt occurred in response to rifampicin exposure only and not due to any other growth or stress conditions that might have been prevailing during prolonged duration of the cultures.

It may be noted that the unexpected increase in the CFUs occurred to different magnitudes at different time points in the biological triplicates of the SCF and NCF cultures. Therefore, the CFU values of each of the replicate cultures of the subpopulations were presented independently. The CFU values were not presented as averages of the triplicates with standard deviation, as averaging would have shown large variations in the standard deviations. This would have masked the phenomenon in the individual replicates and therefore would have remained underappreciated, looked inconsistent, seemingly meaningless, and erroneous. Statistical significance was calculated from the observations on the biologically independent triplicates.

### Spurts in cell number detected from the colony forming units on rifampicin plates

Parallel plating on rifampicin agar medium showed CFU spurts of >4-fold increase during several 6-h periods in both the cultures [Figure 2a-d, respectively; $n = 3$]. The SCF replicate 2, which did not show CFU spurt on rifampicin-free plates [Figure 1e], did not show CFU spurt on rifampicin plates either [Figure 2d, replicate 2]. The rifampicin resister generation frequencies of the subpopulations, which were calculated from the first time point of appearance of the resister colonies on the rifampicin plates, ranged from $10^{-4}$ to $10^{-1}$ in the case of NCF and from $10^{-2}$ to $10^{-1}$ in the case of SCF [Figure 2e and f, respectively, $n = 3$]. These frequencies were $4-7 \log_{10}$-fold and $6-7 \log_{10}$-fold higher than the natural resister generation frequencies of NCF ($10^{-8}$) and SCF ($1.5 \times 10^{-8}$) subpopulations in the MLP reported.[16] Thus, even though SCF resisters emerged ~18 h later than NCF resisters, the resister generation frequency of SCF was slightly higher than that of the NCF cells [Figures 1a and 2e, f, respectively, $n = 3$].

### Multiple nucleoids/septae in the normal-sized cell-enriched fraction and short-sized cell-enriched fraction cells before colony-forming unit spurts

The sudden unusually high increase in the CFU during the RGP could be due to multiple divisions of the mother cells,
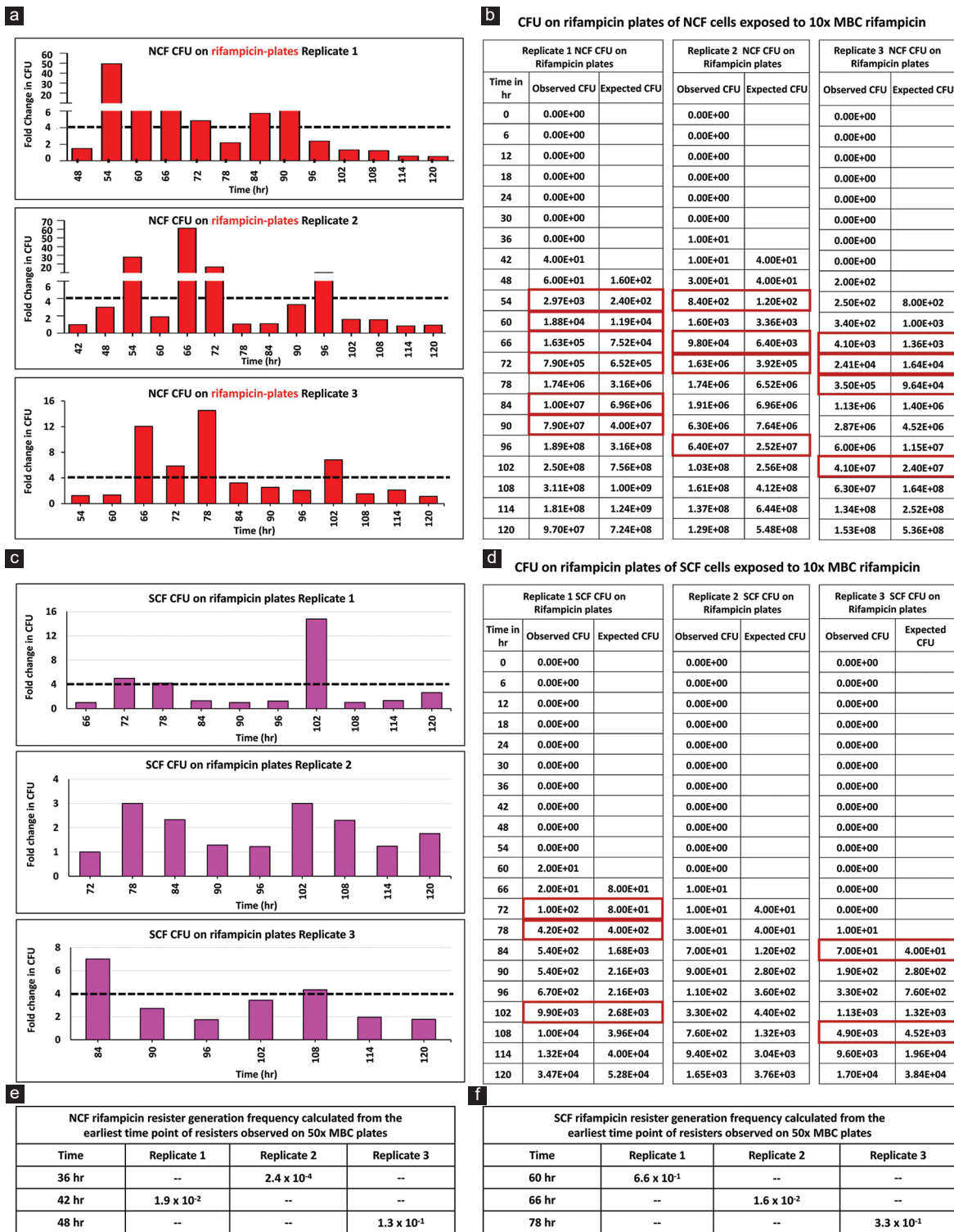
**a**

NCF CFU on rifampicin-plates Replicate 1

NCF CFU on rifampicin-plates Replicate 2

NCF CFU on rifampicin-plates Replicate 3

**b** CFU on rifampicin plates of NCF cells exposed to 10x MBC rifampicin

| Time in hr | Replicate 1 NCF CFU on Rifampicin plates | | Replicate 2 NCF CFU on Rifampicin plates | | Replicate 3 NCF CFU on Rifampicin plates | |
|---|---|---|---|---|---|---|
| | Observed CFU | Expected CFU | Observed CFU | Expected CFU | Observed CFU | Expected CFU |
| 0 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 6 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 12 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 18 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 24 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 30 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 36 | 0.00E+00 | | 1.00E+01 | | 0.00E+00 | |
| 42 | 4.00E+01 | | 1.00E+01 | 4.00E+01 | 0.00E+00 | |
| 48 | 6.00E+01 | 1.60E+02 | 3.00E+01 | 4.00E+01 | 2.00E+02 | |
| 54 | 2.97E+03 | 2.40E+02 | 8.40E+02 | 1.20E+02 | 2.50E+02 | 8.00E+02 |
| 60 | 1.88E+04 | 1.19E+04 | 1.60E+03 | 3.36E+03 | 3.40E+02 | 1.00E+03 |
| 66 | 1.63E+05 | 7.52E+04 | 9.80E+04 | 6.40E+03 | 4.10E+03 | 1.36E+03 |
| 72 | 7.90E+05 | 6.52E+05 | 1.63E+06 | 3.92E+05 | 2.41E+04 | 1.64E+04 |
| 78 | 1.74E+06 | 3.16E+06 | 1.74E+06 | 6.52E+06 | 3.50E+05 | 9.64E+04 |
| 84 | 1.00E+07 | 6.96E+06 | 1.91E+06 | 6.96E+06 | 1.13E+06 | 1.40E+06 |
| 90 | 7.90E+07 | 4.00E+07 | 6.30E+06 | 7.64E+06 | 2.87E+06 | 4.52E+06 |
| 96 | 1.89E+08 | 3.16E+08 | 6.40E+07 | 2.52E+07 | 6.00E+06 | 1.15E+07 |
| 102 | 2.50E+08 | 7.56E+08 | 1.03E+08 | 2.56E+08 | 4.10E+07 | 2.40E+07 |
| 108 | 3.11E+08 | 1.00E+09 | 1.61E+08 | 4.12E+08 | 6.30E+07 | 1.64E+08 |
| 114 | 1.81E+08 | 1.24E+09 | 1.37E+08 | 6.44E+08 | 1.34E+08 | 2.52E+08 |
| 120 | 9.70E+07 | 7.24E+08 | 1.29E+08 | 5.48E+08 | 1.53E+08 | 5.36E+08 |

**c**

SCF CFU on rifampicin plates Replicate 1

SCF CFU on rifampicin plates Replicate 2

SCF CFU on rifampicin plates Replicate 3

**d** CFU on rifampicin plates of SCF cells exposed to 10x MBC rifampicin

| Time in hr | Replicate 1 SCF CFU on Rifampicin plates | | Replicate 2 SCF CFU on Rifampicin plates | | Replicate 3 SCF CFU on Rifampicin plates | |
|---|---|---|---|---|---|---|
| | Observed CFU | Expected CFU | Observed CFU | Expected CFU | Observed CFU | Expected CFU |
| 0 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 6 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 12 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 18 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 24 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 30 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 36 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 42 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 48 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 54 | 0.00E+00 | | 0.00E+00 | | 0.00E+00 | |
| 60 | 2.00E+01 | | 0.00E+00 | | 0.00E+00 | |
| 66 | 2.00E+01 | 8.00E+01 | 1.00E+01 | | 0.00E+00 | |
| 72 | 1.00E+02 | 8.00E+01 | 1.00E+01 | 4.00E+01 | 0.00E+00 | |
| 78 | 4.20E+02 | 4.00E+02 | 3.00E+01 | 4.00E+01 | 1.00E+01 | |
| 84 | 5.40E+02 | 1.68E+03 | 7.00E+01 | 1.20E+02 | 7.00E+01 | 4.00E+01 |
| 90 | 5.40E+02 | 2.16E+03 | 9.00E+01 | 2.80E+02 | 1.90E+02 | 2.80E+02 |
| 96 | 6.70E+02 | 2.16E+03 | 1.10E+02 | 3.60E+02 | 3.30E+02 | 7.60E+02 |
| 102 | 9.90E+03 | 2.68E+03 | 3.30E+02 | 4.40E+02 | 1.13E+03 | 1.32E+03 |
| 108 | 1.00E+04 | 3.96E+04 | 7.60E+02 | 1.32E+03 | 4.90E+03 | 4.52E+03 |
| 114 | 1.32E+04 | 4.00E+04 | 9.40E+02 | 3.04E+03 | 9.60E+03 | 1.96E+04 |
| 120 | 3.47E+04 | 5.28E+04 | 1.65E+03 | 3.76E+03 | 1.70E+04 | 3.84E+04 |

**e**

| NCF rifampicin resister generation frequency calculated from the earliest time point of resisters observed on 50x MBC plates | | | |
|---|---|---|---|
| Time | Replicate 1 | Replicate 2 | Replicate 3 |
| 36 hr | -- | $2.4 \times 10^{-4}$ | -- |
| 42 hr | $1.9 \times 10^{-2}$ | -- | -- |
| 48 hr | -- | -- | $1.3 \times 10^{-1}$ |

**f**

| SCF rifampicin resister generation frequency calculated from the earliest time point of resisters observed on 50x MBC plates | | | |
|---|---|---|---|
| Time | Replicate 1 | Replicate 2 | Replicate 3 |
| 60 hr | $6.6 \times 10^{-1}$ | -- | -- |
| 66 hr | -- | $1.6 \times 10^{-2}$ | -- |
| 78 hr | -- | -- | $3.3 \times 10^{-1}$ |

**Figure 2:** The CFU fold-change of rifampicin resisters of *M. smegmatis* NCF and SCF exposed to 10× MBC rifampicin for 120 h. (a and b) The fold-change in the CFU of rifampicin resisters of NCF and SCF, respectively, plated at 6-h intervals on rifampicin plates (50× MBC) after exposure to 10× MBC rifampicin. The expected 4-fold increase in CFU in 6 h is indicated by the horizontal dotted line. (c and d) The CFU spurts at specific 6-h intervals of (c) NCF and (d) SCF. The spurts of CFU beyond the expected 4-fold in both the samples in 6-h periods are indicated in red boxes. (e and f) The rifampicin resister generation frequency of: (e) NCF and (f) SCF, calculated from the first time point of emergence of the resister colonies. *n* = 3 in all cases. MBC: Minimum bactericidal concentration, CFU: Colony forming unit, NCF: Normal-sized cells-enriched fraction, SCF: Short-sized cells-enriched fraction

containing multiple nucleoids and multiple septae, producing multiple sibling-daughter cells. Another possibility could be that the mononucleoid cells in the RGP might be undergoing faster growth and division. A combination of both these phenomena

also could cause CFU spurt. TEM of the 72-h NCF culture, where CFU spurt was observed [Figure 1b and c], revealed elongated phenotype of the cells with multiple nucleoids (>2n) and/or multiple septae (>1) [Figure 3a; more images in Figure S7]. About 30% of the NCF cells ($n$ = 409) were with ≤2n nucleoids, while the remaining ~70% contained >2n nucleoids [Figure 3b]. Further, about 17% of the total septated and nonseptated NCF cells ($n$ = 409) possessed single or multiple septae [Figure 3c]. However, in the total number of septated NCF cells ($n$ = 67), ~20% of cells contained multiple septae while the remaining ~80% possessed single septum [Figure 3d]. The presence of ~70% of cells with >2n nucleoids and ~20% of cells with multiple septae indicated that multiple repeated divisions of the nucleoids had occurred and multiple septae were formed to compartmentalize them. Multiple repeated divisions of these multi-nucleated/septated NCF might have caused the sudden increase in the CFU. The elongated phenotype must have been necessitated spaciously by the presence of multiple nucleoids and septae, caused by the multiple repeated replication and segregation of the nucleoids and multiple septation without division.[26,27] The parallel culture of the rifampicin-unexposed control sample showed unelongated cells without multiple

nucleoids/septae [Figure S8]. It confirmed that the elongated phenotype with multiple nucleoids/septae was formed in response to the exposure to rifampicin.

Live-cell time-lapse imaging of the $10 \times$ MBC rifampicin-exposed NCF samples from the 60 h (RGP) showed cells with multiple constrictions (a postseptation event), thereby confirming multiple septae beneath the constrictions[26,27] [Figure 4a and Video S1]. Tracking the lineage of the growth and divisions of a single NCF mother cell and its progeny cells for several consecutive generations revealed multiple divisions within short periods [Figure 4b; image taken from the Video S1]. Two consecutive divisions (1st and 2nd divisions) occurred within a span of 10 min [Figure 4c; time calculated from Figure 4b]. Similarly, the 10× MBC rifampicin-exposed SCF samples from the 84 h (RGP) also showed cells with multiple constrictions [Figure 4d and Video S2]. Tracking the lineage of the growth and divisions of a single SCF mother cell and its progeny cells for several consecutive generations showed cells with multiple constrictions and divisions at the constrictions [Figure 4e; image taken from the Video S2]. Two consecutive divisions (3rd and 4th divisions) occurred within a span of 20 min [Figure 4f; time calculated from Figure 4e].
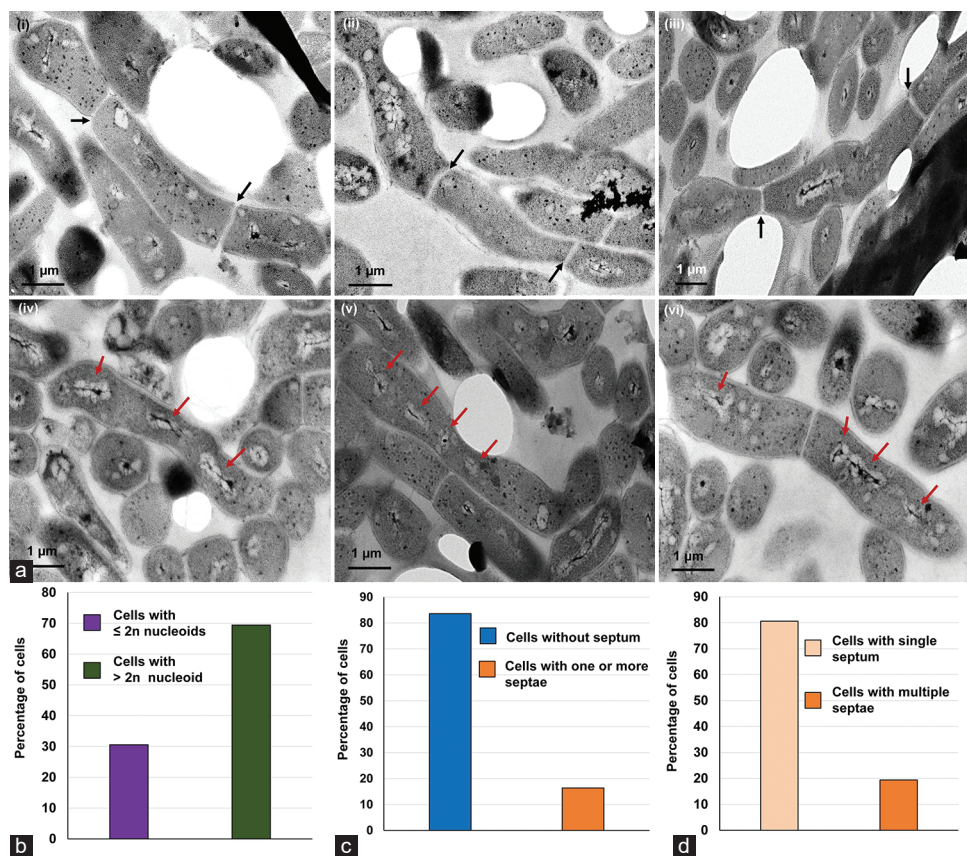


**Figure 3:** Transmission electron micrographs of NCF cells exposed to 10× MBC of rifampicin. (a) Transmission electron micrographs of *M. smegmatis* NCF cells exposed to 25 μg/ml of rifampicin for 72 h. (i-iii) Longitudinal sections of *M. smegmatis* NCF cells with multiple septae (black arrows). (iv-vi) Longitudinal sections of *M. smegmatis* NCF cells with multiple nucleoids (red arrows). (b-d) Quantitation of the TEM data of the NCF cells with multiple nucleoids, multiple septae or nil septum. Percentage of NCF cells: (b) with single or multiple nucleoids ($n$ = 409); (c) with single or multiple septae ($n$ = 67); (d) with or without septum/septae out of the total number of septated and nonseptated cells ($n$ = 409 cells), MBC: Minimum bactericidal concentration, NCF: Normal-sized cells-enriched fraction, TEM: Transmission electron microscopy

Thus, the data obtained on rifampicin surviving cells using two different methodologies, from TEM (using fixed cells) and live-cell time-lapse microscopy (using live cells), showed the presence of a proportion of the cells with multiple nucleoids and/or multiple septae. These cells showed multiple constrictions/divisions within short durations, resulting in the generation of multiple progeny cells. This must have caused the unusually high spurt in cell number/CFU. We did not find nondividing cells in any of the fields during live-cell time-lapse imaging.

To compare the regrowth and division features of rifampicin-exposed NCF and SCF with those of the rifampicin-unexposed NCF and SCF (control samples), we performed live-cell time-lapse imaging of the NCF and SCF enriched from the MLP cultures grown in the absence of rifampicin for 120 h. The NCF and SCF from the 60 h and 84 h of exposure, respectively, were used for live-cell time-lapse imaging. These time points were same as the time points of the rifampicin-exposed NCF and SCF used of live-cell time-lapse imaging [Figure 4]. The images showed normal binary

bacterial division, in comparable duration, and not multiple divisions in short duration [Figures S9 and S10, respectively; Videos S3 and S4, respectively]. The normal single binary division of the rifampicin-unexposed NCF and SCF confirmed that the multinucleated and/or multiseptated elongated phenotype was formed in response to rifampicin exposure. From all the data obtained so far, we made a head-to-head comparison and contrast of several parameters pertaining to the response of the NCF and SCF subpopulations to 10× MBC rifampicin compared to those of the respective samples unexposed to rifampicin [Table 1]. The differences in these parameters showed the striking difference between the response of the NCF and SCF subpopulations to rifampicin.

### Response of the normal-sized cell-enriched fraction and short-sized cell-enriched fraction subpopulations to 2× minimum bactericidal concentration rifampicin

The response of the subpopulations to 2× MBC rifampicin also showed the SCF cultures to be more susceptible than the
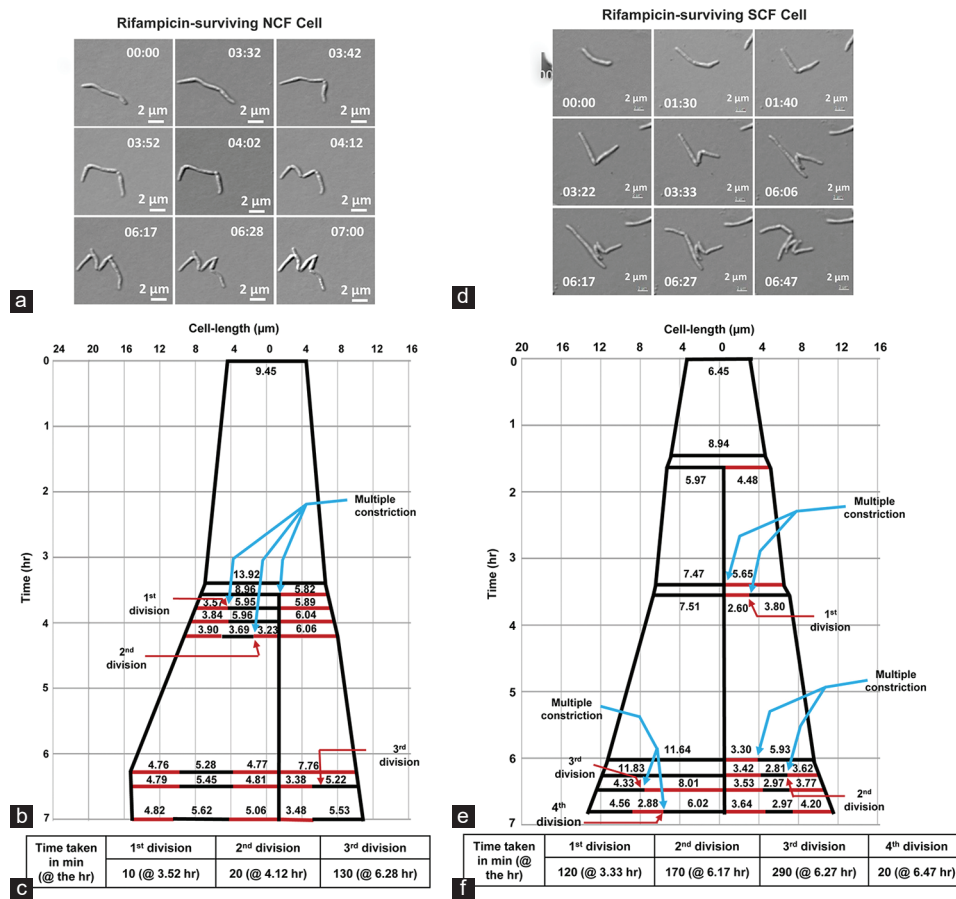


**Figure 4:** The lineage of consecutive divisions of *M. smegmatis* NCF and SCF cells from the 10× MBC rifampicin exposed culture. The NCF and SCF cells were sampled for live-cell time-lapse imaging from the 60-h and 84-h of the exposure shown in Figure 1a. (a and d) The DIC images of the: (a) NCF and (d) SCF cells taken at different time points. (b and e) The lineage tracked from the live-cell time-lapse images of Supplementary Video S1 (b) on NCF cells and Supplementary Video S2 (e) on SCF cells. The zero-time point does not correlate with the birth of the starting mother cell. Blue arrows indicate multiple constrictions. Red arrows indicate specific divisions. Black-and-red-colored lines indicate different sibling-daughter cells. The cell-length was not drawn to scale. (c and f) The division duration of the individual: (c) NCF cells and (f) SCF cells between consecutive divisions, calculated by measuring the time taken from its birth to the next division. One video image each of the three biological replicates of the NCF and SCF subpopulations is shown. NCF: Normal-sized cell-enriched fraction, SCF: Short-sized cell-enriched fraction, DIC: Differential Interference Contrast

NCF [Figure S11a, blue line and green line, respectively]. Nevertheless, the SCF culture started growing from 48 h and reached a cell density close to that of the NCF culture at 66 h and then attained a plateau. Like from the 10× MBC rifampicin-exposed cultures, the NCF and SCF rifampicin resisters against 2× MBC rifampicin also emerged at different times. The first colony of the NCF and SCF resisters could be observed at ~24 h and 42 h of exposure, respectively [Figure S11a, brown and pink lines, respectively]. The first single resister cell clone of the NCF and the SCF subpopulations at ~16 h and ~36 h, respectively [Figure S12]. Unlike in exposure to 10× MBC rifampicin, the resister CFUs on the rifampicin plates did not become equal to the CFUs on the rifampicin-free plates at any point during the exposure [Figure S11a, brown and green lines, and pink and blue lines, respectively]. This implied that the NCF and SCF cultures contained populations of rifampicin resisters and nonresisters. The CFUs of the NCF and SCF cultures on rifampicin-free plates and rifampicin plates showed cell number spurts of >4-fold during several 6-h period during the RGP, from 60 h and 72 h onward, respectively [Figures S11b-e and S13a-d, respectively]. The rifampicin resister generation frequency ranged from $10^{-5}$ to $10^{-6}$ for the NCF and $10^{-3}$ to $10^{-4}$ for the SCF, respectively [Figure S13e and f, respectively; $n = 3$ in each case]. These resister frequencies were higher by ~2–3 $\log_{10}$-fold and ~4–5 $\log_{10}$-fold, respectively, than their natural resister generation frequency.[16]

### Response of the normal-sized cell-enriched fraction and short-sized cell-enriched fraction subpopulations to 0.4× minimum bactericidal concentration rifampicin

The CFU profile of SCF and NCF against 0.4× MBC rifampicin showed a lag phase, instead of KP, up to 24 h [Figure S14a, blue and green lines, respectively]. The CFU profiles of the subpopulations on the rifampicin plates never-overlapped with the CFUs on rifampicin-free plates, unlike their response to 10× and 2× MBC rifampicin [compare with Figures 1 and S14, respectively]. The first resister colonies of both NCF and SCF cultures emerged at 24 h of exposure [Figure S14a, brown line and pink line, respectively]. The first resister cell clones of the SCF and NCF emerged at around ~9 and ~12 h

of exposure, respectively [Figure S15]. Their CFUs on the rifampicin plates were ~1–3-$\log_{10}$ lower than the CFUs on the rifampicin-free plates at any point during the exposure [Figure S14a, brown and green lines, and pink and blue lines, respectively]. It indicated that besides rifampicin resisters, rifampicin nonresisters were also present in their surviving populations, like in the response to 2× MBC, but unlike to 10× MBC [Figures S14a and 1a]. Only one of the triplicate cultures of NCF and two of the triplicate cultures of the SCF showed unusual spurts in cell number on rifampicin-free plates [Figure S14b-e, respectively]. This was unlike their response to 10× and 2× MBC rifampicin [compared with Figures 1b, c and S14b, c, respectively]. On the contrary, the CFUs on rifampicin plates indicated several cell number spurts of the resisters of both the subpopulations [Figure S16a-d, respectively], like in the case against 10× MBC and 2× MBC rifampicin [compared with Figures 2 and S16, respectively]. The rifampicin resister generation frequency was in the range of $10^{-5}$ to $10^{-6}$ [Figure S16e, f, respectively; $n = 3$ in each case], which were ~2–3-$\log_{10}$-fold higher than their natural resister generation frequency.[16] Thus, the resister generation frequencies of the subpopulations against ×0.4 MBC were comparable unlike their 2-$\log_{10}$-fold difference against 2× and 10× MBC rifampicin [compare Figure S16e and f, respectively, with Figures S16e, f, and 2e, f, respectively].

### Detection of rifampicin resistance determining region mutations in the normal-sized cell-enriched fraction and short-sized cell-enriched fraction resisters

The clones randomly selected from the rifampicin plates of the NCF and SCF subpopulations exposed to 0.4×, 2×, and 10× MBC rifampicin showed mutations in the RRDR of the *rpoB* gene [Figure S17]. These mutations were identical to and at identical positions as found in the RRDR of the *rpoB* gene of *M. tuberculosis* and *M. smegmatis* cells of the rifampicin-surviving population from *in vitro* cultures[5,6,8] and from clinical samples.[28-32] We had earlier reported that the even the antibiotic-unexposed SCF cells have naturally higher oxidative stress than the NCF cells and that their resisters, with C → T and A → G mutations indicative of oxidative stress,

**Table 1: Comparison of some parameters of the rifampicin-exposed and rifampicin-unexposed normal-sized cell-enriched fraction and short-sized cell-enriched fraction samples**

| Features | NCF exposed | SCF exposed | NCF unexposed | SCF unexposed |
|---|---|---|---|---|
| Resisters' emergence (at the hour of exposure) | 36 | 60 | 30 | 30 |
| Cell length | Up to 20 μm (due to multinucleation/multiseptation) | Up to 15 μm (due to multinucleation/multiseptation) | 4-5 μm (normal length as reported)[14] | 2-3 μm (normal length as reported)[14] |
| Growth profile | Triphasic (KP, ASP, RGP) | Triphasic (KP, ASP, RGP) | Not triphasic | Not triphasic |
| Resister generation frequency | $10^{-4}$-$10^{-1}$ | $10^{-2}$-$10^{-1}$ | $10^{-8}$, as reported[16] | $10^{-8}$, as reported[16] |
| Nucleoid status | Multiple nucleoids | Multiple nucleoids | n/2n nucleoid | n/2n nucleoid |
| Septation status | Multiple septae | Multiple septae | Single septum | Single septum |
| Cell division frequency | Multiple times per mother cell | Multiple times per mother cell | Single time per mother cell | Single time per mother cell |

KP: Killing phase; ASP: Antibiotic-surviving phase; RGP: Regrowth phase, NCs: Normal-sized cells, NCF: NCs-enriched fraction, SCs: Short-sized cells, SCF: SCs-enriched fraction

can be selected against rifampicin and moxifloxacin.[16] Hence, we did not sequence the RRDR of rifampicin-unexposed SCF and NCF samples in the present study.

Thus, taken together, the NCF and SCF subpopulations formed antibiotic-surviving populations on continued exposure to different folds of MBC of rifampicin. The antibiotic-surviving cells became multinucleated and multiseptated and produced multiple daughter cells through multiple asymmetric divisions showing unusually high spurts in cell number. The cells, which had acquired mutation at the RRDR locus of *rpoB*, got selected in the continued presence of rifampicin and emerged as rifampicin resisters at high frequency from this population.

## The model for the growth and division behind the emergence of rifampicin resisters

The data showed that both the NCF and SCF subpopulations surviving in the presence of rifampicin went through unusually high spurts in the CFU. During the late time points of the rifampicin-surviving phase (RSP), the CFUs on the rifampicin-free and rifampicin-containing plates were found to be comparable. It indicated that from then on, all the cells that gave CFU were antibiotic resistant. Earlier studies, where we patch plated the rifampicin exposed whole *M. smegmatis* populations from the antibiotic-free to antibiotic-containing plates, showed every single colony from the late time points of RSP to be resistant.[11] The antibiotic-surviving population would be highly heterogeneous and would contain diverse phenotypes of cells, including classical persisters, slow-growing/dividing persisters, and antibiotic-tolerant cells, surviving by multiple mechanisms.[33-38] The cells of many of those phenotypes, which might be present at different densities, would not give CFUs in the continued presence of the antibiotic. Such phenotypes would be present as long as the antibiotic is present in the cultures, even during the RGP of the antibiotic resisters. Among all the cells in the rifampicin-surviving population, those that had acquired mutation would proliferate and emerge out of the antibiotic stress giving a high spurt in the CFU readout.

The specific features of the sequential cellular events in the response of NCF and SCF to rifampicin were depicted in a model [Figure 5]. The model shows that the exposure to 10× MBC rifampicin led to the killing of the majority of NCF and SCF populations. The respective cells, which survived the killing and hence present in the RSP, were called the rifampicin-surviving cells. Because the original composition of the NCF: SCF was 90:10,[14] the proportion of the cells surviving in the RSP were also correspondingly low. At about 60th h, the NCF started elongating due to multinucleation and multiseptation, and by 80th h, the elongated cells started undergoing multiple divisions to give spurts in CFU. Similarly, at about 90th h, the SCF started elongating due to multinucleation and multiseptation, and by 100th h, the elongated cells started undergoing multiple divisions to give spurts in CFU. The cell divisions would have taken place once the multiple nucleoids in the respective elongated cells acquired *rpoB* mutations. The respective rifampicin-resistant strains grew and proliferated to yield respective rifampicin-resistant population.
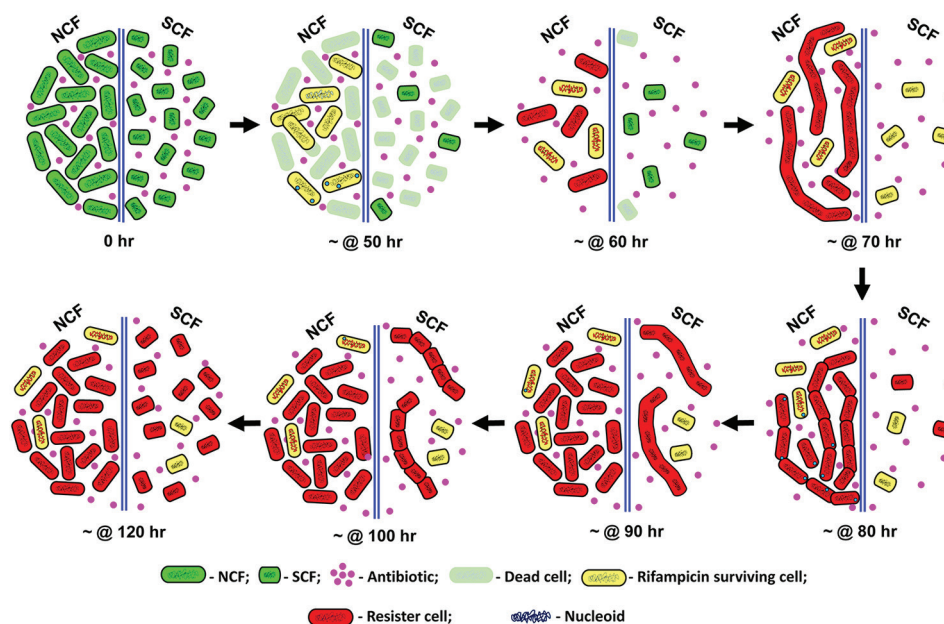


**Figure 5:** The model depicting the cellular events involved in the emergence of rifampicin resisters from the antibiotic surviving phase cells formed upon exposure of the NCF and SCF cells to ×10 MBC rifampicin for prolonged duration. The NCF cells began elongation, multi-nucleation and multi-septation at 70 h, while the SCF cells began the same at 90 h. The images are not drawn to scale. NCF: Normal-sized cell-enriched fraction, SCF: Short-sized cell-enriched fraction, MBC: Minimum bactericidal concentration. The long red coloured cells are elongated multi-nucleated cells prior to multiple divisions at the multiple septae

## DISCUSSION

### Commonality/differences between the response of normal-sized cell-enriched fraction/short-sized cell-enriched fraction to rifampicin

In the present study, we saw both the rifampicin-surviving populations of NCF and SCF undergoing elongation with multinucleation and/or multiseptation, followed by multiple divisions in short durations. This tells that the strategy to overcome rifampicin stress and proliferate back to form a population is same for both the subpopulations. It is also natural to expect such a commonality between the two subpopulations. Nevertheless, despite this commonality, we saw several differences between the responses of the two subpopulations to rifampicin. (i) The SCF subpopulation grew much slower than the NCF subpopulation after the RSP, as revealed by the CFU on rifampicin-free plates [please see the green and blue lines, respectively, in Figure 1a]. (ii). The formation of multinucleated and/or multiseptated phenotype occurred with a time delay of 12 and 18 h between the NCF and SCF cells, with the NCF forming the phenotype 12–18 h earlier than the SCF. This was revealed by the CFU on the rifampicin-free and rifampicin-containing plates [please see the brown and pink lines in Figure 1a; the tables in Figure 1c and e, respectively; and the tables in Figure 2c and 2d, respectively]. (iii) Every single NCF arising from the RSP was a resister as shown by the overlap of the growth curves of the cells in the rifampicin-free and rifampicin-containing plates [please see the merging of the green and brown lines in Figure 1a]. On the contrary, it was not the case for the SCF until 102 h of the exposure [please see the blue and pink lines in Figure 1a]. (iv) Both the nonresistant and the rifampicin-resistant strains of SCF grew slower than the corresponding strains of the NCF.

### Sudden unusually high spurts in cell number

The most striking feature of the response of the NCF and SCF subpopulations to different fold MBC of rifampicin was sudden unusually high spurts in cell number at different time points and to different magnitudes among rifampicin resisters and nonresisters in the antibiotic-surviving population in the biological triplicate cultures in the liquid medium. The multiple cell number spurts of the nonresisters found against $10 \times$ and $2 \times$ MBC rifampicin were not observed among the nonresisters of the samples against $0.4 \times$ MBC rifampicin [Figure S14]. This might be indicating the requirement for the presence of a certain threshold level of functional rifampicin necessary to induce the phenomenon among nonresisters as the rifampicin inactivating Arr activity might have reduced the functional rifampicin concentration further down from $0.4 \times$ MBC.[24] Even with this possibility, one can still explain the high cell number spurts of the resisters against $0.4 \times$ MBC rifampicin [Figure S16] as even very low concentrations of antibiotics can induce *de novo* resistance mutations that get rapidly enriched and selected.[2] Therefore, it might imply that a certain threshold level of functional antibiotic stress might be necessary to induce cell number spurts through multinucleation

and multiseptation leading to multiple asymmetric divisions but might not be for the infliction of mutations. This possibility is supported by CFU spurts even on rifampicin-free plates at periods wherein there was no overlap of the CFU curves from the rifampicin-free and rifampicin plates. The normal binary mode of cell division of the rifampicin-unexposed cultures, without multinucleation and multi-septation, confirmed that the phenomenon occurred in response to rifampicin exposure. It seemed to be an adaptive strategy of the rifampicin-surviving NCF and SCF to come out of the antibiotic stress.

The high cell number spurts among the nonresisters indicated that these cells multiplied in the unusual mode probably even before acquiring *rpoB* mutation. Subsequently, these daughter cells might have acquired mutation and got selected on rifampicin plates. The merging of the CFUs on rifampicin-free plates with those on rifampicin plates for the cultures from the same points of exposure indicated this possibility. The subsequent continued overlapping of the CFUs on the rifampicin-free and rifampicin plates suggested that all the cells in the culture from that time point of exposure might have been genetic resisters to rifampicin, as evident from the RRDR mutations [Figure S17]. In fact, in our study on the whole population of *M. smegmatis* against $10 \times$ MBC rifampicin, patch plating of the colonies pertaining to the CFU overlapping periods from rifampicin-free plates into rifampicin plates showed that all the colonies on the rifampicin-free plates also gave CFU on rifampicin plates.[11] However, such overlapping of the CFUs on the rifampicin-free and rifampicin plates was not observed in the NCF and SCF cultures against $0.4 \times$ MBC rifampicin, probably due to the low functional concentration of the antibiotic influencing the extent of emergence of the resisters.[39]

Clumping of samples inconsistently before plating could be attributed as a possibility for CFU spurt at different times. However, clumping of the samples did not occur in the samples due to the following reasons. (i) The cultured *M. smegmatis* cells in biological triplicates were mildly sonicated or syringed in the same growth medium containing Tween 80 and plated to find out whether the CFUs were consistently and reproducibly comparable for each time point with statistical significance. This exercise was repeated multiple times, and the whole process was rigorously standardized to get consistent and reproducible CFUs in the experiments. The superimposable CFU profiles from the cultures with and without sonication clearly showed that there was no clumping issue [Figure S5]. In addition, as standardized by us, the CFUs of the biological triplicates in Figures 1c, e, and 2c, d, showed reproducible and consistent spurt across the same time point. (ii) The CFUs arising from clumping would have been erratic and varying from time point to time point of the samples. However, they were not so as per the data in Figures 1c, e, and 2c, d. This also ruled out clumping possibility. (iii) The NCF and SCF samples used for the live cell imaging experiments did not show any clumping [Videos S1-S4]. The cultures unexposed to rifampicin (control), which had very high cell density due to

uninterrupted growth and division in the absence of rifampicin, should have shown very low CFU values if there had been clumping of the cells. On the contrary, the lack of very low CFU values in the high-density control cultures technically ruled out any clumping problem in the samples.

Furthermore, we did not use tyloxapol, another nonionic detergent in place of Tween 80, especially in the presence of rifampicin, for the following reasons: (i) Rifampicin, in the presence of tyloxapol, was found to be two-fold more potent against *M. smegmatis* mc² 155 in *in vitro* cultures than in its absence.[40] It clearly showed that tyloxapol would have favorably affected the membrane permeability of rifampicin as tyloxapol is suspected to mitigate the rifampicin-induced remodeling of *M. smegmatis* mycolic acid layer. As such, the cells in the antibiotic-surviving phase were stressed in the continued presence of MBC of rifampicin. Hence, we did not find it prudent to use tyloxapol, as an anticlumping agent in culture. (ii) Some workers have resuspended the cultured *M. smegmatis* cells in tyloxapol and then plated.[41] However, we did not attempt it as the resuspension would have quickly changed the metabolic status of the cells enabling them to come out of the stress with altered phenotype such as cell growth and division which would have changed CFU.

## Multinucleation/multiseptation/mutation/multiple asymmetric divisions

The basis for the high spurts in cell number was the formation of multiple nucleoids and septae in the cells of the antibiotic-surviving population. The data from CFU, TEM, and live-cell time-lapse imaging confirmed the occurrence of this phenomenon. Although we did not analyze the populations surviving under 0.4× (and 2×) MBC rifampicin for the presence of multinucleated/multiseptated cells, the high cell number spurts suggested the occurrence of multiple divisions of multinucleated/multiseptated cells. Multinucleation/septation occurs when bacterial cells suffer from DNA damage due to oxidative stress or when get exposed to stress conditions.[41-45] In such situation, the physical division *per se* of the cell remains blocked until the multiplication of nucleoids and formation of multiple septae have been completed. The elongated phenotype of the multinucleated, multiseptated *M. smegmatis* cells could be compared to the elongated *E. coli* cells having multiple nucleoids and septae formed due to repeated nucleoid replication and segregation without or with septation but without constriction for division under conditions of cell division arrest due to exposure to various stress agents.[26,27,41-45] Reduced levels of expression of cell division *fts* proteins, which is induced by diverse stress agents, have been found to induce such phenotype in many bacterial systems.[44] For instance, the cells with multiple ridges from a 1.1 OD$_{600\,nm}$ cultures (in early stationary phase and hence under nutrient stress) of *M. tuberculosis* have been found to undergo multiple asymmetric divisions from the cell tips.[46] Thus, it is possible that unusual cell division regulatory mechanisms might be operational in the antibiotic-stressed-and-surviving cells and nutrient-stressed-and-surviving cells to undergo

multinucleation/septation flowed by multiple divisions. The slow growth and division of *M. smegmatis* persisters surviving in the presence of isoniazid (Wakamoto *et al.*, 2013) implied that different modes of cell division mechanisms are operational in antibiotic survivors of diverse types.

## When did the antibiotic-surviving cells acquire rifampicin resistance determining region mutation?

It was not clear from our present study as to whether the acquisition of RRDR mutation occurred before or after multinucleation/multiseptation. The occurrence of high cell number spurts among the rifampicin resisters and the nonresisters in the liquid culture indicated that the acquisition of mutation might not be necessary for the multinucleation/multiseptation. Nevertheless, the infliction of mutation through SOS-driven mechanisms while being mononucleoid and subsequent replication and segregation of the mutant nucleoid to become multinucleated cell is conceivable. However, it is also possible that after multinucleation, one of the multiple nucleoids acquired mutation, followed by the acquisition of the same mutation by all the other sister nucleoids in the same elongated cell through allelic recombination between chromosomes. This possibility was suggested in the generation of resisters from the antibiotic-exposed filamented *E. coli* cells.[47] In this study, the *E. coli* cells in culture, exposed to subminimal concentration of ciprofloxacin, first formed multinucleated filaments, which then underwent division either upon withdrawal of the antibiotic or upon acquisition of mutation. On withdrawal of the antibiotic, the elongated/filamented *E. coli* cells underwent continuous repeated asymmetric division from the filament tips. On the contrary, on continuous maintenance of the antibiotic, single cell resisters budded from the filaments.[47] Thus, the elongated phenotype of the cells underwent two different types of cellular changes in the absence or the presence of the antibiotic to produce multiple sibling-daughter cells or single cell resisters, respectively.

In another study, *E. coli* stationary phase persister cells exposed to high concentrations of ofloxacin formed multinucleated filaments, which upon recovery gave rise to large number of genetic resisters to ofloxacin and other antibiotics, implying genome-wide mutagenesis during the recovery period.[48] These studies seemed to suggest that the acquisition of mutation conferring genetic resistance to rifampicin in the mycobacterial subpopulations might have occurred before, after, or during multi-nucleation/septation process. Further, the elongated phenotype of the cells, with multiple nucleoids and septae, and some with elongated nucleoids, in the present study, was like the phenotype of the bacterial cells that underwent nucleoid replication and segregation multiple times without proceeding to septation or to constriction even after septation.[26,27] Thus, the cellular changes accompanying response to antibiotic stress in the mycobacterial subpopulations in the present study, in the earlier study on the *M. smegmatis* whole population,[11] and in *E. coli*,[47,48] looked similar. This commonality seemed to suggest that multi-nucleation/septation followed by multiple repeated asymmetric divisions may be a common strategy

of bacterial cells of diverse genera to survive and overcome antibiotic stress.

## Clinical significance of multi-nucleation/-septation of antibiotic-surviving cells

The SCs and NCs of *M. tuberculosis* were found to be present not only in *in vitro* cultures but also in the sputum of freshly diagnosed pulmonary tuberculosis patients and among the multidrug-resistant strains isolated from tuberculosis patients.[12] In the patients, the tubercle bacilli are continuously exposed to antituberculosis antibiotics for a prolonged duration as designed experimentally in the present study and earlier study.[11] Hence, there is a very good possibility that the antibiotic-resistant strains might be arising through the mechanism discovered in the present study. During the rifampicin exposure, very single resister that emerged from the NCF subpopulation was a resister. This would be advantageous for the propagation of the population as resisters. Meanwhile, the SCF grew at a slower rate throughout the rifampicin exposure period even after gaining rifampicin-resistant mutations. The slower growth of SCF, either rifampicin-nonresistant or rifampicin-resistant, will ensure that it would take prolonged duration to kill SCF. We had earlier reported that due to genome-wide mutations inflicted by the DNA-nonspecific mutagen, hydroxyl radical, the resisters of *M. tuberculosis* and *M. smegmatis* selected against rifampicin and arising out of the rifampicin surviving population were resistant to other antituberculosis antibiotics also.[5,8] Thus, the genome-wide mutations and the slower growth of the SCF cells (both rifampicin-nonresistant and rifampicin-resistant) will be advantageous for their survival during antibiotic combination therapy in the tuberculosis treatment regimen. It will ensure survival of the population even if majority of the fast-growing NCF cells get killed. This can cause the emergence of antibiotic-resistant strains.

Although *M. smegmatis* is generally called a saprophyte, it is an opportunistic human pathogen also.[49-51] Further, all the RRDR mutations found in the rifampicin-resistant strains isolated in this study have been found at identical positions in the rifampicin-resistant *M. tuberculosis* clinical strains isolated from tuberculosis patients as well.[28,32] Thus, the findings in the present study bear clinical significance in light of the commonality in the behavior of *M. smegmatis* and *M. tuberculosis* to antituberculosis antibiotics for prolonged periods.[5,6,8,10,11,52] Such an antibiotic response occurring in *E. coli*[9,47,48] would have relevance in antibiotic resistance in its pathogenic strains as well.

The striking commonality in the unique features of the phenotype, growth, and division of the cells in the antibiotic-surviving populations of bacteria of genera as diverse as mycobacteria and *E. coli* in the presence of antibiotic stress seemed to indicate the existence of a common mechanism to survive and multiply in the continued presence of antibiotics.

## Limitation of the Study

The limitation of the study was that it was performed on a saprophytic-cum-opportunistic pathogenic *Mycobacterium* in *in vitro* culture system but not in animal models or human patients. However, the mutations acquired were identical to and at identical positions as found in the clinical rifampicin-resistant strains of *M. tuberculosis*. Nevertheless, the presence of these subpopulations in the sputum of pulmonary tuberculosis patients, and therefore, the possibility of their response to rifampicin stress by the growth and division mechanism reported, here makes this study worthwhile.

## Ethical clearance

Ethical clearance was not required for the current study as there was no use of human patients or pathogenic organisms.

## Conflicts of interest

There are no conflicts of interest.

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