

Determination of Rifampicin-resistance Mutation Frequency and Analysis of Mutation Spectra in Mycobacteria

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[Abstract] Understanding the genetic safeguarding mechanism of *Mycobacterium tuberculosis* (Mtb) may help us to explain i), how Mtb survive the genetic assaults elicited by both reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by host macrophages and ii), why some strains of Mtb, e.g., Mtb strains from East Asian lineage and Beijing sublineage, exhibit high mutation rate and are more likely to acquire drug resistant mutations (e.g., rifampicin-resistance mutation) during infection. Mutation frequency analysis is a basic methods to study the genetic safeguarding mechanism. Moreover, to study the molecular mechanism of mutation, it is necessary to analyse the mutation spectrum (for example, oxidized cytosine may induce CG to TA mutation). This protocol describes a method to determine the mutation frequency and understand the mutation spectrum in both *Mycobacterium smegmatis* (Msm) and Mtb.

Materials and Reagents

1. *Mycobacterium smegmatis* mc² 155 or *Mycobacterium tuberculosis* H37Rv
2. Middlebrook 7H9 broth (BD Biosciences, catalog number: 271310)
3. Middlebrook 7H11 agar (BD Biosciences, catalog number: 283810)
4. Middlebrook OADC (BD Biosciences, catalog number: 212351)
5. Glycerol (Sigma-Aldrich, catalog number: G5516)
6. Tween 80 (Sigma-Aldrich, catalog number: P1754)
7. Rifampicin (Sigma-Aldrich, catalog number: R3501)
8. 7H9 medium (see Recipes)
9. 7H9OADC medium (see Recipes)
10. 7H11 agar (see Recipes)
11. 7H11OADC agar (see Recipes)
12. LBG agar (see Recipes)
13. PBST (see Recipes)
14. TE buffer (see Recipes)

Equipment

1. Incubation shaker
2. Centrifuge
3. 37 °C incubator
4. 100-ml flask
5. Sterile glass beads
6. 96-well flat bottom plate
7. Adhesive film
8. 96-well PCR plate
9. PCR instrument
10. Petri dish (70 mm diameter)

Software

1. BLAST or ClustalW

Procedure

A. Determination of rifampicin-resistance mutation frequency

1. Streak *Mycobacterium smegmatis* culture on 7H11 agar plate (does not contain rifampicin) and culture at 37 °C for 3 days until colonies are visible.
For Mtb, streak it on 7H11OADC agar plate and culture at 37 °C for 4 weeks.
2. Inoculate a single colony in 5 ml 7H9 medium (for Mtb, use 7H9OADC medium) and grow at 37 °C with shaking (150 rpm) until the culture is saturated ($OD_{600} \sim 2.0$).
3. The culture is sub-cultured (1% v/v inoculation) into 20 ml 7H9 medium (for Mtb, use 7H9OADC medium) in a 100-ml flask and grown at 37 °C with shaking to exponential phase ($OD_{600} = 0.4 \sim 0.5$).

Note: Culture at this growth stage can be used for stress induction, e.g., oxidative stress (H_2O_2 treatment) or starvation.

4. For CFU determination, the culture is serially diluted 1: 100,000 in PBST. 50 μ l of the 10^{-5} dilution is plated on LBG agar plate (for Mtb, use 7H11OADC agar plate). Incubate the plates at 37 °C for 4 days. $CFU/ml = (\text{the number of colonies on each plate}) \times 2 \times 10^6$.
Note: At least three independent dilutions are required for each sample.
5. 3 ml of the cell culture from step 3 is centrifuged at $3,000 \times g$ for 5 min and the cell pellet is resuspended in 100 μ l PBST (The bacilli may form clumps in liquids without Tween 80.). To isolate rifampicin-resistant mutants, the resuspended pellet is spread with sterile

glass beads on LBG agar plates containing 250 µg/ml rifampicin and incubated at 37 °C for 4 days.

For Mtb, the resuspended cell pellet is spread on 7H11OADC agar plate containing 10 µg/ml rifampicin and incubate at 37 °C for 4 weeks.

Note: Glass or metal spreader is not recommended in this step because the mycobacterial cell may adsorb and clump onto the spreader during plating, which may affect the accuracy of the result.

- The spontaneous rifampicin-resistant mutation frequency is calculated by dividing the number of rifampicin-resistant colonies on each plate by the total viable cell count per plate (Figure 1).

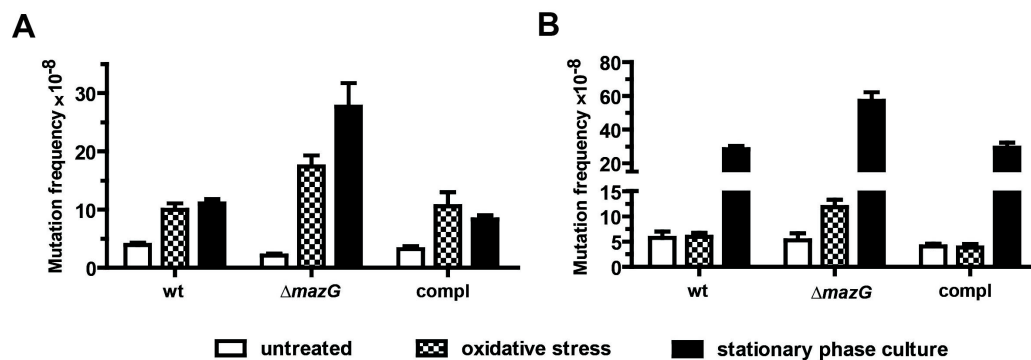


Figure 1. The antimutator role of MazG in Msm (A) and Mtb (B). The frequencies conferring resistance to rifampicin in wild-type (wt), *mazG*-null ($\Delta mazG$) and the complemented mutant (compl) strains were determined in exponential phase ($OD_{600} \sim 0.5$) with or without oxidative stress and in the stationary growth phase. Oxidative stress was induced by treating exponential phase cultures with 10 mM H_2O_2 for 5 h (Msm) or 24 h (Mtb). Stationary phase was at the 5th-day or 28th-day of culture for Msm or Mtb, respectively. The numbers shown are mean \pm S.E. of 3 independent experiments totalling 15 cultures of Msm and 6 of Mtb.

B. Mutation spectrum analysis

- A single rifampicin-resistant Msm colony is inoculated into 1 ml 7H9 media containing 100 µg/ml rifampicin in a 96-well flat bottom plate. The plate is then sealed with an adhesive film and incubated at 37 °C for 7 days. Note that at least 30 colonies are required per bacterial sample for statistically significant results.

To inoculate rifampicin-resistant Mtb colony, use 7H9OADC with 4 µg/ml rifampicin, incubate at 37 °C for 3 weeks.

- Cells are then pelleted (4,000 \times g, 5 min) and suspended in 50 µl TE buffer and the cell suspension is transferred to a 96-well PCR plate.

3. The plate is heated in a PCR instrument at 95 °C for 20 min.
4. The PCR plate is then centrifuged at 12,000 x g for 5 min and the supernatant is transferred to a new 96-well PCR plate.
5. A region of the genome sequence containing the cluster I region of *rpoB* is amplified using primers Rpo-forward (5'-CGACCACTTCGGCAACCG-3') and Rpo-reverse (5'-CGATCAGACCGATGTTGG-3').

For *Mtb*, using primer RpoTB-forward (5'-ATCACACCGCAGACGTTG-3') and RpoTB-reverse (5'-TGCATCACAGTGATGTAGTCG-3').

PCR mix: 2 µl DNA template, 0.4 ul of each primer (10 µM), 1 U *Pfu* DNA polymerase, 0.6 µl DMSO, 0.4 µl dNTPs (10 mM each), H₂O to 20 µl.

PCR cycling: 95 °C 60 s, 30 cycles of 95 °C 20 s, 58 °C 10 s and 72 °C 40 s.

Note: Most of the rifampicin-resistance mutations are occurred in the cluster I region of rpoB. If no mutation is detected in this region, it may need to amplify the whole rpoB gene (~3.5 Kbp) for sequencing.

6. The PCR products are then bi-directionally sequenced and the mutation spectrum of the sequenced region is identified by BLAST or ClustalW.
7. Calculate the mutation frequency of each specific mutation spectrum (Table 1).

Table 1. *mazG*-null *Msm* exhibited elevated CG to TA mutation under oxidative stress conditions and in stationary phase. Spontaneous rifampicin-resistant colonies were collected from 3 independent experiments . Cluster I region of *rpoB* were PCR-amplified using *Pfu* DNA polymerase and sequenced bi-directionally. All of the sequenced colonies contain single non-synonymous mutations. wt, wild-type *Msm*; *ΔmazG*, *mazG*-null *Msm*.

Growth phase	Strain (n)	Mutation frequency ×10 ⁻⁸ (n)							
		CG→TA	CG→AT	CG→GC	GC→CG	GC→TA	AT→GC	AT→CG	AT→TA
Exponential	wt (30)	2.2 (17)	(0)	(0)	(0)	0.5 (4)	1.2 (9)	(0)	(0)
	wt+H ₂ O ₂ (40)	6.8 (27)	(0)	(0)	(0)	1.2 (5)	0.8 (3)	1.2 (5)	(0)
	<i>ΔmazG</i> (43)	0.8 (18)	0.05 (1)	(0)	0.05 (1)	0.09 (2)	0.7 (14)	0.3 (7)	(0)
	<i>ΔmazG</i> +H ₂ O ₂ (69)	14.3 (57)	(0)	(0)	(0)	(0)	2.0 (8)	1.0 (4)	(0)
Stationary	wt (47)	1.9 (8)	(0)	3 (13)	(0)	5.1 (22)	0.2 (1)	(0)	0.7 (3)
	<i>ΔmazG</i> (45)	20.5 (34)	1.9 (3)	4.7 (8)	(0)	0.6 (1)	(0)	(0)	(0)

Mutation frequency of a given mutation spectrum = (the proportion of the mutation spectrum in strain A) x (the mutation frequency of strain A). For example, the mutation frequency of Msm is 10×10^{-8} , the proportion of CG-TA mutation in Msm derived rifampicin-resistant mutants is 80%, therefore, the CG-TA mutation frequency will be $0.8 \times 10 \times 10^{-8} = 8 \times 10^{-8}$.

Recipes

1. 7H9 medium (100 ml)

Dissolve 0.47 g of 7H9 broth in 80 ml dH₂O

Add 0.4 ml 50% glycerol and 0.5 ml 10% Tween 80
dH₂O to 100 ml

Sterilized by autoclaving

Stored at 4 °C

2. 7H9OADC medium (100 ml)

Dissolve 0.47 g of 7H9 broth in 80 ml dH₂O

Add 0.4 ml 50% glycerol and 0.5 ml 10% Tween 80
dH₂O to 90 ml

Sterilized by autoclaving

Cool to 50-55 °C and add 10 ml OADC

Stored at 4 °C

3. 7H11 agar (100 ml)

Dissolve 2.1 g of 7H11 agar with 99 ml dH₂O

Add 1 ml 50% glycerol

Sterilized by autoclaving

Stored at 4 °C

4. 7H11OADC agar (100 ml)

Dissolve 2.1 g of 7H11 agar with 89 ml dH₂O

Add 1 ml 50% glycerol

Sterilize by autoclaving

Cool to 50-55 °C and add 10 ml OADC

Stored at 4 °C

5. LBG agar (100 ml)

Dissolve 1 g tryptone, 0.5 g yeast extract, 1 g NaCl and 1.5 g agar with 99 ml dH₂O

Add 1 ml 50% glycerol

Sterilized by autoclaving

Stored at 4 °C

6. PBST

Dissolve the following in 800 ml of dH₂O

8 g of NaCl

0.2 g of KCl

1.44 g of Na₂HPO₄

0.24 g of KH₂PO₄

5 ml of 10% Tween 80

Adjust pH to 7.2

Adjust volume to 1 L with dH₂O

Sterilized by autoclaving

7. TE buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA

Acknowledgments

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