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ORIGINAL ARTICLE

TlyA Expression is Necessary for Mycobacterial Susceptibility to Capreomycin: Wild-type Strains are Naturally Resistant

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ABSTRACT

The human pathogen *Mycobacterium tuberculosis* has coevolved with humans and uses novel strategies for intracellular survival as well as for drug resistance. In this communication, we have compared the drug resistance of *Mycobacterium marinum* as well as the H37Ra, an avirulent form of the human pathogen *M. tuberculosis* against second-generation antibiotic capreomycin. Interestingly, *M. marinum* and its three mutants are naturally resistant to capreomycin, while the H37Ra showed susceptibility and also it showed surface expression for the TlyA protein since function of TlyA is necessary for susceptibility to capreomycin. It is postulated that the resistance to capreomycin can occur in wild-type strains through suppression of expression of TlyA, while the H37Ra is unable to do the same.

Keywords: Capreomycin, Drug resistance, Drug susceptibility, H37Ra, *Mycobacterium marinum*, *Mycobacterium tuberculosis*.

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Conflict of interest: Nil

INTRODUCTION

Emergence of drug-resistant strains of mycobacterial species is a serious problem, which has been highlighted by the World Health Organization.[1] Although many aspects have come to light regarding this problem, the major reasons attributed to drug resistance could be due to evolution of mutant forms and or other mechanisms. Considering the number of possibilities that can contribute to drug resistance, the following are distinct possibilities, viz.

- Drug is not experienced by the bacterium, i.e., its failure to penetrate the bacterium or effectively pumped out of the bacterium.
- The drug does not find its target.
- Processing of pre- or pro-form of the drug fails.
- Emergence of mutations that prevent the drug binding either in nucleic acid sequences or its product proteins.

Based on these broad categories, one can easily see many subcategories except for the first possibility which requires specialized efflux pumps which are yet to be delineated at the molecular level. It remains to be explored whether they can pump all drugs that are currently in use. However, the possibilities 2 to 4 have an underlying phenomenon, i.e., whether or not heterogeneity can contribute to drug resistance. For example, a given drug may not find its target if a single bacterium has evolved in such a way that it does not express or partition a particular protein during cell division as pictorially depicted in Figure 1. In such a scenario, the bacterium that retained the bulk of the protein will be susceptible while the bacterium that did not receive or make the same protein will be resistant. In this possibility, it is important to note that the genome of both bacteria is the same, while one is susceptible and the other resistant. Hence, the question, whether such bacteria in principle exist or not can be experimentally explored. In this present work, we are able to identify that *M. marinum*, a close relative of the human pathogen *M. tuberculosis*, and its three mutants appear to be resistant to the second-generation antibiotic, capreomycin, while the H37Ra, the avirulent form of the same human pathogen, is susceptible to the same drug.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains *M. marinum* and its transposon mutants M1, M2, and M3 were a generous gift from Dr Eric Brown, Genentech, USA, which were described in Gao et al.[2] The *M. tuberculosis* H37Ra was obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. All the antibiotics used in this study were of analytical grade and were obtained from Sigma-Aldrich Co., USA.

Bacterial Growth

All the bacterial strains were cultured as per protocols suggested by American Type Culture Collection (ATCC) using complete 7H9 media glycerol (0.2% v/v), albumin,

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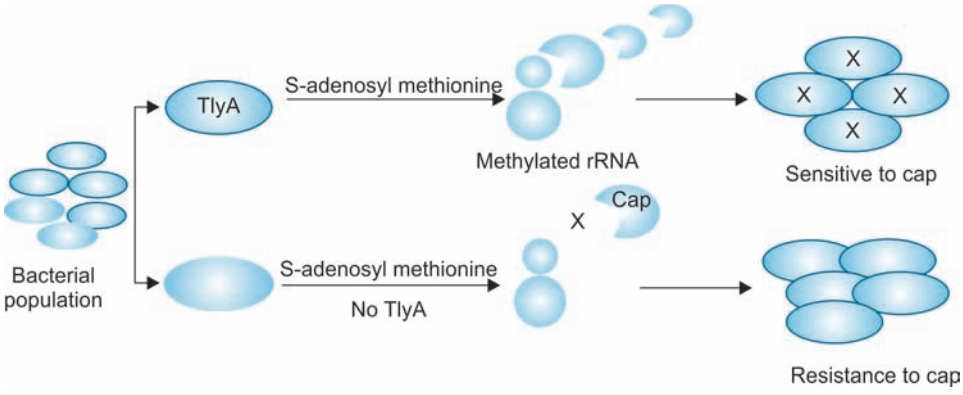


Fig. 1: Illustration of TlyA expression-dependent susceptibility. The bacteria that express the TlyA protein are susceptible to capreomycin due to methylation of 16S and 23S rRNA, while the bacteria that do not express the protein will be resistant in the absence of methylation. It is known in literature that the TlyA is not essential for *in vitro* growth of mycobacteria (based on transposon mutagenesis) and hence, the possibility of lack of its expression

dextrose and catalase (ADC), and Tween-80 (0.05% v/v). Each of the strains (WT, M1, M2 and M3) was enumerated (taken from a log phase culture) and inoculated in flasks with equal volume of medium with an inoculum ratio at 1:100 with or without antibiotics. The growth curve was monitored by taking A_{600} of each sample for every 6 hours for indicated time intervals.

Antibiotic Disk Susceptibility Assay

The bacterial culture described earlier was washed thrice washed in phosphate-buffered saline containing Tween-80 (0.05%) and passed through 26-gauge syringe to dissociate clumps. All cultures having 10^6 cells/mL were spread onto complete 7H10 agar plates containing glycerol (0.2% v/v), oleic acid, albumin, dextrose and catalase (OADC), and Tween-80 (0.05% v/v). The antibiotics were applied with Whatmann filter paper No.1 disc that were saturated with desired antibiotic concentration. The plates were incubated at 30°C or 37°C and observed after 21 days. A plain 7H10 agar plate was also kept as negative control. The antibiotics tested were ampicillin (100 and 50 µg/mL), hygromycin B (50 µg/mL), and capreomycin with concentrations of 100, 80, 50, 40, 10, 5, 1, and 0.1 µg/mL.

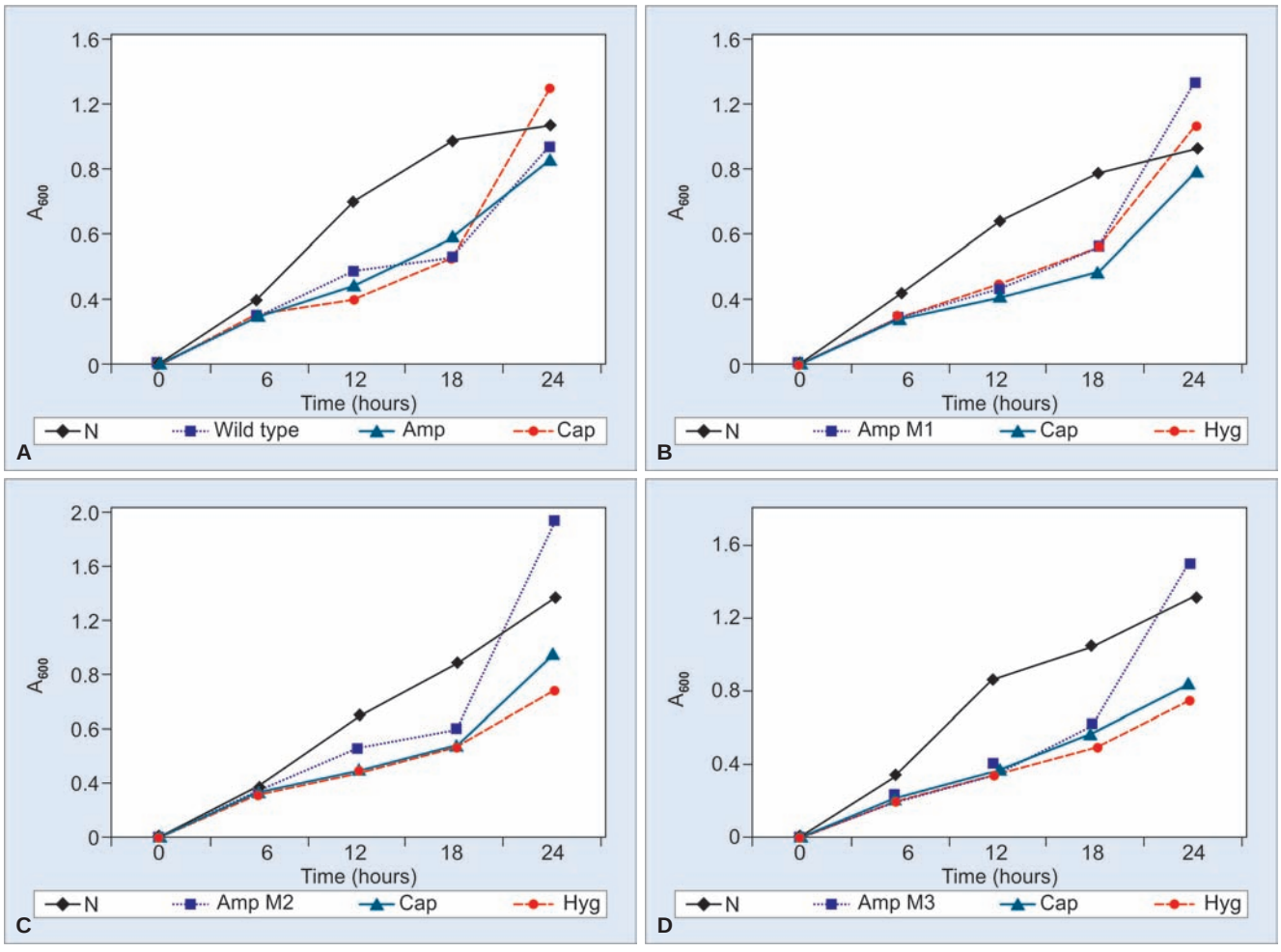
Confocal Staining

Surface staining of H37Ra was carried out as per earlier report using same reagents.[3]

RESULTS AND DISCUSSION

The genus *Mycobacterium* has many members that are pathogenic to variety of species and in humans, it is known by the tuberculosis disease. The *M. marinum* causes systemic tuberculosis like disease in fish and frogs and also localized skin suppurations in immunocompromised humans.[4] Both the human and animal infections are marked by the presence of a granulomatous host response, the hallmark of *M. tuberculosis*. *Mycobacterium marinum* is genetically closely related to *M. tuberculosis* and has been used increasingly as a model for understanding the pathogenesis of tuberculosis. *Mycobacterium tuberculosis* and *M. marinum* share >90% sequence homology based on 16S ribosomal ribonucleic acid (rRNA) sequences.[5] The virulence of nonpathogenic strains of mycobacterial species can be restored with the counterparts of the *M. tuberculosis* which is well demonstrated in the literature.[2,6,7] Detailed study of *M. marinum* can greatly help in developing screening methods for antimycobacterial agents since: (i) It has phylogenetically close relationship with *M. tuberculosis;* (ii) it has a relatively rapid doubling time; (iii) it shows similar drug susceptibilities to *M. tuberculosis;* and (iv) it is less expensive for biosafety level environment and its evolutionary aspects can be studied safely.

The strains designated with M1, M2, and M3 respectively, represent the transposon insertion strains of *M. marinum* that disrupt Mh3866, Mh3867, and Mh3868, which are homologous to Rv3866, Rv3867, and Rv3868 of *M. tuberculosis* respectively. Based on the literature, these genes are necessary for hemolysis exhibited by *M. marinum*.[2] We have earlier shown that the *M. marinum* expresses the *tlyA* gene product which has been independently shown to possess both the activities, i.e., hemolysis and S-adenosyl-L-methionine-dependent rRNA methylation activities.[3,8] Susceptibility to capreomycin by a bacterium is dependent upon the expression of *tlyA* gene product, which methylates the nucleotides C1409 and C1920 of 16S and 23S rRNA respectively. Methylation of rRNA results in reduced translational ability, as the methylation of the ribosomes facilitates the binding of capreomycin.[9] Hence, bacteria that do not carry the *tlyA* gene are naturally resistant to capreomycin, e.g., *Escherichia coli* has no natural homologue and is resistant to capreomycin which, upon expression of the *tlyA* gene, shows susceptibility.[10]



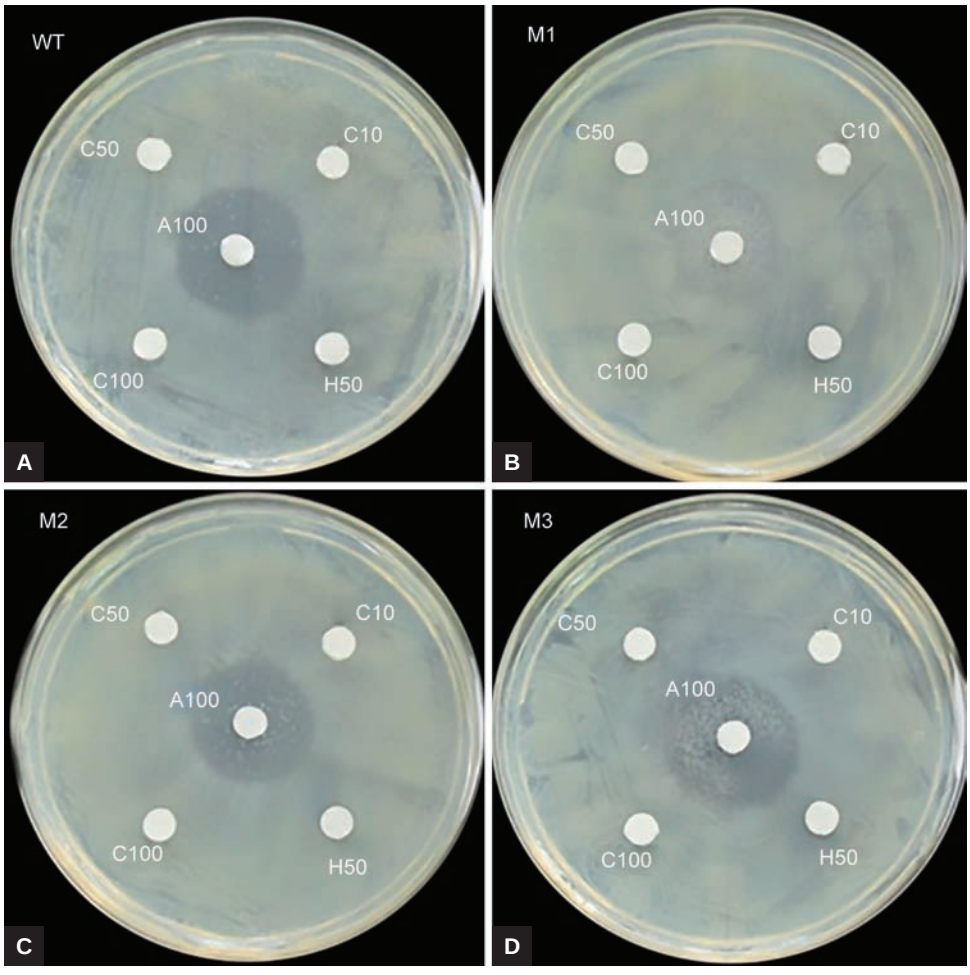
Graphs 1A to D: *In vitro* culture of *M. marinum* wild type, M1, M2, and M3 in the presence of indicated antibiotics: all the strains were cultured as described in Methods section in the presence of ampicillin (100 µg/mL), hygromycin B (50 µg/mL), and capreomycin (100, 50, and 10 µg/mL) and their growth was monitored for 24 hours

In view of the above observations, all the four strains were examined for growth in the presence of ampicillin, hygromycin B, and capreomycin as shown in Graph 1. The growth curves of all the four strains in the absence of the antibiotic show rapid growth, while in the presence of these three antibiotics, growth only retarded for about 18 hours, after which the growth has dominated that of the wild-type bacteria. Consistent with this observation, the antibiotic disc diffusion assay also showed no inhibition of growth as seen in Figure 2 in which we could see some inhibition only in ampicillin, but no significant inhibition in case of hygromycin B while we could not see any kind of inhibition in case of capreomycin.

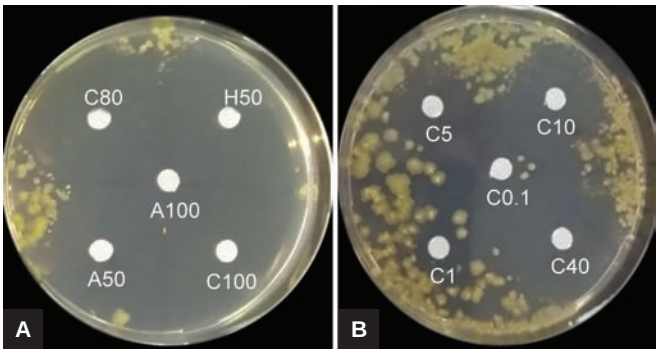
Attenuated Strain H37Ra is Susceptible to Capreomycin and exhibits Surface Expression of TlyA

Mycobacterium tuberculosis strain H37Ra, commonly used in studies with the virulent H37Rv, was originally derived from virulent strain H37.[11] Several studies have attempted to determine the genomic and proteomic differences leading to the basis of virulence attenuation of H37Ra, which appears to possess insertions, deletions, and mutations in some transcription factors.[12-14] However, detailed studies on H37Ra can have implications both in understanding the pathogenesis of virulent counterpart and the development of new vaccines and therapeutic agents. We, therefore, sought to examine H37Ra and its susceptibility to capreomycin in context of *tlyA*.

In contrast to the observations described above, the H37Ra did not grow at all in the presence of various concentrations of the capreomycin in liquid media, while the agar plate showed dramatic loss of growth in the presence of capreomycin as seen in Figure 3 and Table 1. The colony morphology of H37Ra is consistent with literature pictures as well as the ATCC cultures examined in the laboratory. We have also examined for the presence of TlyA protein on the surface of H37Ra, which showed an unambiguous presence, whereas the expression of TlyA in M1, M2, and M3 is unobservable, while the wild type is noisy (unpublished observations). As seen in Figure 4, the confocal microscopic visualization



Figs 2A to D: Disc diffusion assay of *M. marinum* wild type, M1, M2, and M3: disc diffusion visualization was carried out as described in Methods section. The C100, C50, C10, A100, and H50, respectively, represent capreomycin 100, 50, and 10 µg/mL, ampicillin 100 µg/mL, and hygromycin B 50 µg/mL respectively



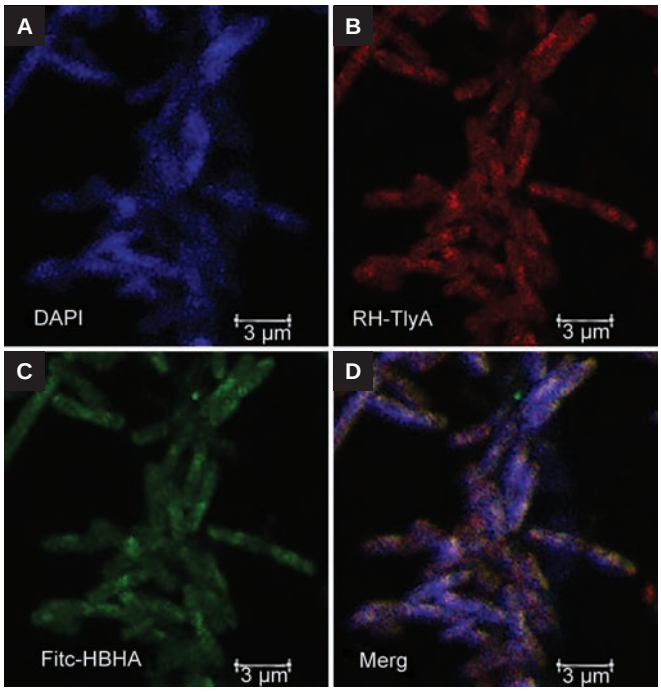
Figs 3A and B: Disc diffusion assay of H37Ra in the presence of capreomycin: Disc diffusion assay for H37Ra was carried out as described for *M. marinum* in the presence of capreomycin and other antibiotics. The C 0.01, C1, C5, C10, C40, C80, C100, A50, and H50, respectively, represent capreomycin, ampicillin, and hygromycin B with respective concentrations

Table 1: Antibiotic susceptibility of H37Ra and *M. marinum*

Antibiotic	*Conc, µg/mL*	*H37Ra*	*M. marinum*
Hygromycin B	40	s	r
	100	s	s-r
	50	s	r
Kanamycin	30	s	8
Capreomycin	100	s	r
	80	s	r
	50	s	r
	40	s	r
	10	s	r
	5	s	r
	1	s	r
	0.1	s	r

of H37Ra also showed positive surface staining for TlyA (Rh-Rhodamine channel) and heparin-binding hemagglutinin (HBHA), a well-known surface protein of H37Rv (FITC-HBHA). The observations shown in Graph 1, Fig. 2 and Table 1 are also consistent with a published literature in which many mycobacterial strains seem to be naturally resistant to capreomycin and isoniazid.[15]

It is interesting to note here that the wild-type and mutant strains of *M. marinum* are resistant to capreomycin for lowest to highest usable concentrations,



Figs 4A to D: Confocal visualization of H37Ra for TlyA expression: Surface staining of H37Ra was carried out as described earlier for *M. marinum* (3). The panels labeled with 4',6-diamidino-2-phenylindole (DAPI), rhodamine, fluorescein isothiocyanate (FITC) respectively, represent staining for deoxyribonucleic acid, TlyA staining with rhodamine-antirabbit-immunoglobulin G (IgG) and HBHA detected with FITC-antimouse-IgG

while the H37Ra is susceptible to it. Based on Figure 1, it is important to understand the expression profile of TlyA for evolution of possible heterogeneity or noise in mycobacterial species in *in vitro* culture conditions and its significance for intracellular survival.[16] In addition, the high prevalence of TlyA on the surface of H37Ra is very important for further studies, as H37Ra has the ability to survive in humans and mice, but does not cause the classical disease and also has not been shown to form granuloma. It is relevant to mention here that TlyA expression can aid in intracellular survival of even nonpathogenic versions upon expression of the same.[17] The H37Ra has been shown to contain mutations in PhoP regulon and it is necessary to focus the future studies on PhoP regulon and its role for evolution of susceptibility or resistance to second-generation antibiotic, such as capreomycin, which is not often used for treatment of tuberculosis, to enable its usage.[18] It is also not surprising that the possibility discussed in Figure 1 is found to be true, as many clinical isolates with wild-type *rrs* and *tlyA* genes have minimum inhibitory concentration values well above 0.5 to 2 mg/mL.[19] Hence, it is very important to study the evolution of this phenotype for combating the drug resistance problem posed by tuberculosis disease and such studies are currently underway.

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