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Identification of novel loci associated with mycobacterial isoniazid resistance

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SUMMARY

Despite the known association of several genes to clinical Isoniazid (INH) resistance, its molecular basis remains unknown in ~16% of clinical isolates of *Mycobacterium tuberculosis* (*M. tb*). While screening a set of *Mycobacterium smegmatis* (*M. smegmatis*) transposon mutants with altered colony morphology for differential susceptibility to INH, we found six resistant mutants and mapped their transposon insertion sites. The disrupted genes in six INH resistant mutants were homologs of *M. tb ctaE, rplY, tatA, csd* and *tatB* with one insertion mapping to the promoter region of *M. smegmatis ctaE*. MIC measurements indicated a wide spectrum of INH resistance in these mutants, with complementation analyses of four selected mutants with the cognate *M. smegmatis* genes and their *M. tb* homologs confirming the association of the disrupted genes with INH resistance. Our discovery of novel genes associated with INH resistance could lead to the identification of novel INH resistance mechanisms and possibly new diagnostic modalities as well.

1. Introduction

Isonaizid (INH), a pro-drug, is an integral part of first line TB treatment in conjunction with rifampicin, pyrazinamide and ethambutol [1]. Upon conversion into the NAD adduct by the catalase-peroxidase KatG, INH-NAD inhibits the enoyl-ACP reductase InhA and blocks mycolic acid biosynthesis, leading to bacillary killing [2]. It is also believed that the $NO^{\bullet}$ released during INH activation is partially responsible for the killing by targeting respiratory enzymes in *M. tb* [3]. Resistance to INH is frequently associated with mutations in *katG* [4], *inhA* promoter, and *ndhII* which encodes NADH dehydrogenase [5]. Mutations in other genes such as *ahpC*, *Rv0340-0343*, *fadE24, efpA* and *kasA* are also associated with clinical INH resistance, but since they either occur in conjunction with mutations in *katG* and/or *inhA* promoter or are present in INH sensitive isolates of *M. tb*, their roles in resistance remain ambiguous [5]. Current drug susceptibility tests for INH involve phenotypic methods including monitoring bacillary growth, enzyme assays and microscopic observation of drug susceptibility. Since the turnaround time for these assays is long, more rapid genotypic methods like MTBDR and MTBDR-Plus which test for mutations in *katG* and *inhA* apart from *rpoB*, have recently come into practice [6]. However, these tests exhibit a lower sensitivity for detecting INH resistance since these mutations are absent in approximately 16% of INH resistant clinical isolates [7]. Consequently, there is a need to identify new loci linked to INH resistance to improve the sensitivity of the above genotypic approaches.

M. smegmatis transposon mutant screens have been previously used to establish the role of several mycobacterial genes including *katG*, *nudC* (involved in hydrolysis and deactivation of INH-NAD adduct), *arr* (involved in inactivation of rifampin) and *roxY* (involved in host antimicrobial peptide resistance) in conferring differential susceptibility to TB drugs and stress causing agents [8–11]. In this study, from a set of *M. smegmatis* transposon mutants with altered colony morphologies [12], we isolated six INH resistant mutants carrying insertions in genes not documented to be associated with INH resistance thus far. MIC determination and complementation studies with the corresponding *M. smegmatis* and *M. tb* genes validated their association with INH resistance. We believe that our findings will lead to new insights into the mechanism of INH resistance and may help in developing more sensitive diagnostic tests for resistance detection.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

M. smegmatis mc^26 and *Escherichia coli* (*E. coli*) DH5α were cultured as described [13]. The following antibiotics were added

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when necessary - Kanamycin (15 μg/ml for *M. smegmatis*), Hygromycin (200 μg/ml for *E. coli* and 50 μg/ml for *M. smegmatis*).

2.2. Whole genome transposon mutagenesis and isolation of INH resistant strains

The *Himar1* based transposon mutant library of *M. smegmatis* was generated as described earlier [14]. To phenotype INH resistance, 1 μl of exponential phase cultures of WT and mutant strains were spotted on Middlebrook 7H10 agar plates containing INH. As a control for growth, all cultures were spotted onto plates lacking INH.

2.3. Transposon insertion site mapping

A modified genome walking protocol was followed to identify transposon insertion sites. The sequences obtained were aligned using the BLAST program (NCBI) and the point of insertion (POI) of the transposon for each mutant was determined to be 1 bp before the 5′ coordinate of insertion, as described earlier [14].

2.4. Mutant complementation

To generate complementation constructs, ORFs of $ctaE_{Ms}$, $rplY_{Ms}$, and $rplY_{M.\ tb}$ were PCR amplified with ~300bp of their putative promoter using gene specific primers (Table S1) and cloned into pMV306h, an integrating shuttle vector encoding hygromycin resistance. ORFs corresponding to $ctaE_{M.\ tb}$, csd_{Ms}, $csd_{M.\ tb}$, $tatB_{Ms}$ and $tatB_{M.\ tb}$ were amplified and cloned downstream of an *hsp60* promoter in pMV261h, an episomal plasmid encoding hygromycin resistance. Wild type and mutant strains were transformed with the cognate recombinant plasmids or empty vectors and selected on Middlebrook 7H10 agar containing Kanamycin and/or Hygromycin.

2.5. Resazurin based microplate assay

For MIC determination a resazurin based microplate assay was performed in 96-well plates as described earlier [15] with the following modifications – after adding cells to the antibiotic containing wells, the plates were incubated with the corresponding antibiotic for 60 h. After addition of 30 μl resazurin and 12.5 μl of 20% Tween-80 per well, the plates were incubated for 24 h and the colours of all wells were recorded. Blue was interpreted as no growth, and pink or pinkish purple was scored as growth. The MIC was defined as the lowest drug concentration which prevented a colour change from blue to pink or pinkish purple.

2.6. E-test assay

The INH E-test (Biomerieux) assay was performed according to the manufacturer's instructions with the following modifications - 200 μl of cultures with A_{600} of 1.0 were spread on Middlebrook 7H10 agar plates and dried. After applying the E-test strips the ellipse of intersection was measured after 2 days of incubation.

2.7. Microscopy

To document colony phenotypes, *M. smegmatis* strains were grown to stationary phase and streaked on Middlebrook 7H10 agar containing the appropriate antibiotics. Colony phenotypes of the strains were visualized using a Zeiss Axioplan-2 imaging microscope at 50× magnification.

2.8. NAD cycling assay

The intracellular concentrations of NADH and NAD^+ and their ratios were calculated using a NAD cycling assay which was performed as described earlier [16]with following modifications. The reaction mixtures were incubated at 30 °C for 15 min and the concentration of NAD^+ and NADH was obtained by measuring spectrophotometrically the extent of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction by the yeast type II alcohol dehydrogenase at 570 nm in 96-well plates. The assay was calibrated using NAD^+ and NADH standards.

2.9. Genotypic analysis of INH resistant clinical strains of M. tb

The genomes of 741 INH resistant clinical isolates from the GMTV database [17] (http://mtb.dobzhanskycenter.org) were screened for the presence of mutations in the genes identified in our study. A quality threshold of 20 (which indicates a probability of 1 in 100 that the observed mutation is incorrect) was set to filter out unreliable mutations that may arise due to errors in sequencing. The clinical isolates showing mutations in the genes identified in this study were further checked for the presence of mutations already reported to be associated with INH resistance [4,5].

2.10. In vitro growth kinetics

To profile their growth characteristics, *M. smegmatis* strains grown to late exponential phase were diluted to an A_{600} of 0.2 and cultured in Middlebrook 7H9 broth. Growth curves were generated by plotting A_{600} measurement against time.

2.11. Real-time PCR analysis

To determine the relative fold difference in $ctaE_{Ms}$ transcript levels in *M. smegmatis* TR67 [$P_{MSMEG\_4260\ (ctaE_{Ms})}$::Tn] *vs* the WT strain, cells were harvested at the A_{600} value of 1 and total RNA isolated from each culture using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. Following treatment with RNAse free DNAse I, cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad) and subsequently used as a template for SYBR green based PCR amplification using $ctaE_{Ms}$ *gene* specific primers (Table S1) to generate 200 bp amplicons. Gene specific transcript levels were normalised to the *M. smegmatis sigA* transcript in each sample before calculating the fold change. At least 2 biological and 6 technical replicates were performed.

3. Results and discussion

3.1. Isolation of novel INH resistant mutants of M. smegmatis

In order to identify novel mycobacterial loci associated with INH resistance we observed the growth profiles of 69 colony morphotype mutants of *M. smegmatis* on plates containing 10 mg/L INH, a concentration higher than the reported MIC for *M. smegmatis* [18]. These were originally isolated in a colony morphology screen from a library of 5000 transposon mutants [12]. Since changes in colony morphologies were likely to result from variations in cell envelope composition, we initially hypothesised that these mutants may show differential susceptibilities to INH by virtue of their altered permeability. We obtained six mutants resistant to INH (Figure 1A) and mapped the Tn insertion sites to identify the disrupted genes in these mutants (Figure 1B). All the mutants contained ORF disrupting insertions except TR67, where the Tn was inserted in the promoter of *MSMEG_4260*. None of the *M. tb* homologues of the