Ectopic Expression of Rv0023 Mediates Isoniazid/Ethionamide Tolerance via Altering NADH/NAD⁺ Levels in Mycobacterium smegmatis

Shailesh Kumar Gupta1, 2†, Rajendra Kumar Angara1†, Suhail Yousuf1†, Chilakala Gangi Reddy1, 3† and Akash Ranjan1*  

1 Computational and Functional Genomics Group, Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India,  
2 Graduate Studies, Manipal Academy of Higher Education, Manipal, India,  
3 Regional Centre for Biotechnology, Faridabad, India

Tuberculosis (TB) caused by Mycobacterium tuberculosis (Mtb) accounts for nearly 1.2 million deaths per annum worldwide. Due to the emergence of multidrug-resistant (MDR) Mtb strains, TB, a curable and avertable disease, remains one of the leading causes of morbidity and mortality. Isoniazid (INH) is a first-line anti-TB drug while ethionamide (ETH) is used as a second-line anti-TB drug. INH and ETH resistance develop through a network of genes involved in various biosynthetic pathways. In this study, we identified Rv0023, an Mtb protein belonging to the xenobiotic response element (XRE) family of transcription regulators, which has a role in generating higher tolerance toward INH and ETH in Mycobacterium smegmatis (Msmeg). Overexpression of Rv0023 in Msmeg leads to the development of INH- and ETH-tolerant strains. The strains expressing Rv0023 have a higher ratio of NADH/NAD⁺, and this physiological event is known to play a crucial role in the development of INH/ETH co-resistance in Msmeg. Gene expression analysis of some target genes revealed reduction in the expression of the ndh gene, but no direct interaction was observed between Rv0023 and the ndh promoter region. Rv0023 is divergently expressed to Rv0022c (whiB5) and we observed a direct interaction between the recombinant Rv0023 protein with the upstream region of Rv0022c, confirmed using reporter constructs of Msmeg. However, we found no indication that this interaction might play a role in the development of INH/ETH drug tolerance.

Keywords: XRE family of protein, whiB5, isoniazid resistance, ethionamide resistance, transcription regulation

INTRODUCTION

Tuberculosis (TB) remains a major cause of death worldwide and the leading cause by a single infectious agent (World Health Organisation, 2018). Even though the disease can be cured and managed by several multidrug regimens, the emergence of multidrug-resistant (MDR) TB is proving to be a major challenge for complete eradication of the disease. Worldwide, MDR TB constitutes 3.5% of new TB cases and 18% of previously treated cases (World Health Organisation, 2018). To overcome these challenges and to better counter resistance in Mycobacterium tuberculosis (Mtb), understanding the mechanisms and deciphering the pathways majorly responsible for generating resistance are greatly required.
Isoniazid (INH), in combination with rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA), forms the first-line therapy for TB. Cycloserine, ethionamide (ETH), and amikacin/capreomycin are used as second-line drugs. INH was first used as an anti-TB drug in 1952 and shortly the first INH-resistant Mtb (INHr) clinical isolates were reported (Bernstein et al., 1952; Fox, 1952; Middlebrook and Cohn, 1953). INH and ETH are prodrugs that are converted into their active forms by enzymes encoded by katG (catalase peroxidase KatG) and ethA (monooxygenase EthA), respectively (Johnson and Schildt, 1994; Johnsson et al., 1997; Baulard et al., 2000; DeBarber et al., 2000; Vannelli et al., 2002; Fraaije et al., 2004). Although katG and inhA (encoding an NADPH-dependent enoyl-ACP reductase) are the main genes involved in INH resistance, clinical isolates with mutations in the ndh gene have been identified in INH-resistant Mtb strains (Lee et al., 2001). While in the study by Lee et al. (2001), INH resistance mutations were observed only in the ndh gene, in other studies, ndh mutations occur simultaneously with mutations in other genes (Hazbón et al., 2006; Cardoso et al., 2007). The role of the ndh gene mutations in INH and ETH co-resistance in Mycobacterium smegmatis (Msmeg) and Mycobacterium bovis (Mbovis) has been shown (Vilcheze et al., 2005), while the role of ndh in conferring resistance in Mtb is yet to be determined. The gene ndh encodes the type II NADH dehydrogenase and its ortholog in Escherichia coli (E. coli) has been characterized. In E. coli, it exists in a monomeric state bound to the membrane, where it oxidizes NADH, reduces quinone, and catalyzes the transfer of electrons from reduced flavin to quinone (Jaworowski et al., 1981; Matsushita et al., 1987; Yagi, 1993; Gennis and Stewart, 1996; Kerscher, 2000). In Msmeg, mutations in ndh lead to an increase in NADH cellular concentration and inhibition of INH-NAD and ETH-NAD adducts formation (Vilcheze et al., 2005).

Xenobiotic response element (XRE) family of transcription factors are one of the most frequently occurring families of regulators in bacteria. Among the well-studied members of the XRE family are the lambda and Cro repressors, from lambda bacteriophage, and the prophage repressor Xre from Bacillus subtilis. The XRE family of regulators share a conserved N-terminal helix-turn-helix (HTH) DNA binding domain, while the C-terminal regulatory region is highly variable. The XRE family of regulators control diverse metabolic functions; e.g., SinR regulates developmental process in B. subtilis (Gaur et al., 1991), ClgR regulates Streptomyces growth and controls Clp proteolytic complex (Beller and Mazodier, 2004), PuuR regulates putrescine utilization pathway in E. coli K-12 (Nemoto et al., 2012), and BzdR is involved in the anaerobic catabolism of benzoate in the denitrifying Azotobacter sp. strain CIB (Barragán et al., 2005). There are seven members of the XRE family of transcription regulators in Mtb: Rv0023, Rv0465c, Rv0474, Rv1129, Rv2017, Rv2021, and EspR (Rv3849). Except for EspR, which positively regulates the ESX-1 protein secretion system, the principal virulence determinant of Mtb (Raghavan et al., 2008), the remaining XRE transcription regulators in Mtb are uncharacterized.

Rv0023 is a regulator from the XRE family of transcriptional regulators, known to induce 488 genes and repress 404 genes (Rustad et al., 2014). Rv0023 regulon is enriched for the regulation of NAD reductases (Rustad et al., 2014). Rv0023 is transcribed in an operon together with Rv0024, a gene that codes for an NLPC/p60 family protein and is transcribed divergently from whiB5, belonging to the WhiB family of transcriptional regulators. The whiB5 gene product is a positive regulator of transcription and contributes to Mtb virulence and reactivation (Casonato et al., 2012). At present, very little is known about Rv0023 functions and its effect on Mtb physiology.

Here, we studied the effects of Rv0023 overexpression in Msmeg. The results show that ectopic expression of Rv0023 confers enhanced INH and ETH tolerance in Msmeg. Rv0023 ectopic expression downregulates the expression of the ndh gene and increases NADH/NAD⁺ levels, which are known to be mediators of INH and ETH resistance in Msmeg (Miesel et al., 1998; Vilcheze et al., 2005). We further studied the regulation of the whiB5-Rv0023 locus and identified Rv0023 as a negative regulator of whiB5. We have characterized its binding site and identified the promoters of Rv0023 and whiB5.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Condition**

A complete list of strains used in this study is mentioned in Table 1. Cloning and plasmid propagation were done using E. coli strain DH5α. For protein expression, E. coli BL21 (DE3) was used. Both strains were grown in Luria Bertani (LB) medium at 37°C. Mbovis BCG Pasteur 1173P2, Msmeg mc²155, and the recombinant strains were grown in Middlebrook 7H9 (Himedia) broth supplemented with 10% OADC (oleic albumin dextrose catalase) (Himedia), 0.2% glycerol, and 0.05% Tween80 (20% stock) or on 7H10 agar without Tween80 at 37°C. Kanamycin (50 µg/ml) (Sigma-Aldrich), ampicillin (100 µg/ml) (Sigma-Aldrich), and hygromycin (50 µg/ml) (Invitrogen) were used as and when required.

**Plasmids and DNA Manipulation**

Cloning, genomic, and plasmid DNA isolations were done as per standard molecular biology procedures (Sambrook et al., 1989). The plasmids and primers used in this study are listed in Tables 2, 3, respectively. Overlapping extension PCR was performed to generate site-directed mutations for promoter and critical residues studies. Sequences of all clones generated were confirmed by Sanger sequencing.

**Protein Expression and Purification**

pET23a-0023 plasmid containing Rv0023 ORF was used to transform E. coli BL21 (DE3) strain. Cells were grown in LB broth at 37°C till mid-log phase, and then 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside) was added to induce the expression of protein. Cells were grown for another 4 h at 37°C, after which cells were harvested and purified using Ni-NTA affinity chromatography as described earlier (Yousuf et al., 2018). The purity of recombinant protein was analyzed.
### TABLE 1 | List of strains used in this study.

| Strain | Chromosomal genotype | Source |
|--------|-----------------------|--------|
| E. coli DH5α | F- Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (k2-ΔmK1) supE44 thi1 gyrA96 relA1 | Lab repository |
| E. coli BL21 (DE3) | fhuA2 [lon] ompT gal (Δ DE3) [dcm] | Lab repository |
| M. smegmatis mc²155 | M. smegmatis mc²155 harboring pVv16 plasmid | This study |
| MsmegPVV16 | M. smegmatis mc²155 harboring pVv16 plasmid | This study |
| MsmegPVV0023 | M. smegmatis mc²155 harboring pVv0023 plasmid | This study |
| MsmegEJwhiB5WT | M. smegmatis mc²155 harboring pEJwhiB5 plasmid | This study |
| MsmegEJwhiB5MUT | M. smegmatis mc²155 harboring pEJwhiB5MUT plasmid | This study |
| MsmegEJ0023WT | M. smegmatis mc²155 harboring pEJ0023 plasmid | This study |
| MsmegEJ0023MUT | M. smegmatis mc²155 harboring pEJ0023MUT plasmid | This study |
| MsmegEJwhiB5-pPVV16 | M. smegmatis mc²155 harboring pEJwhiB5 and pVV16 plasmids | This study |
| MsmegEJwhiB5-pPVV0023 | M. smegmatis mc²155 harboring pEJwhiB5 and pVV0023 plasmids | This study |
| MsmegEJ0023-pPVV16 | M. smegmatis mc²155 harboring pEJ0023 and pVV16 plasmids | This study |
| MsmegEJ0023-pPVV0023 | M. smegmatis mc²155 harboring pEJ0023 and pVV0023 plasmids | This study |
| MsmegPVV0494 | M. smegmatis mc²155 harboring pVV0494 plasmid | Yousuf et al., 2015 |

### TABLE 2 | List of plasmids used in this study.

| Plasmid | Features | Source/reference |
|---------|----------|-----------------|
| pET23a0023 | pET21b carrying Rv0023 gene | This study |
| pEJ14 | km' and lacZ reporter vector | Papavinasasundaram et al., 2001 |
| pEJ0023WT | pEJ14 carrying 400bp upstream and 50bp downstream of Rv0023 start codon | This study |
| pEJ0023MUT | Derivative of pEJ0023 where TCATAG is mutated to CCAGAG | This study |
| pEJwhiB5WT | pEJ14 carrying 250 bp upstream and 50 bp downstream of whiB5 start codon | This study |
| pEJwhiB5MUT | Derivative of pEJwhiB5 where ATACGCTT is mutated to GCACGCGG | This study |
| pVW16 | Hsp60 promoter, Km' and Hyg' | Kordulakova et al., 2002 |
| pVW0023 | pVv16 carrying Rv0023 gene | This study |
| pVW0494 | pVv16 carrying Rv0494 gene | Yousuf et al., 2015 |

### TABLE 3 | List of primers used for cloning.

| Clone name | Primer | Sequence | Restriction site |
|------------|--------|----------|-----------------|
| pET0023 | 0023FP | GGGAATTCCATATGAGCCGTGAGTCGGCCGGCGCGGCC | NdeI |
| pET0023 | 0023RP | AAAACTGCAGCTGCTGCCCCTCATCCGCGTCGTG | XbaI |
| pEJ0023WT | UP0023FP | CTAGTCTAGAAGCTGTTCGCGCTTTCGGTACTGGC | XbaI |
| pEJ0023WT | UP0023RP | CCGCTCGAGCGCGAAGTGCGCGAATGGCCGC | HindIII |
| pEJwhiB5WT | UPwhiB5FP | CACAGACATGCCACGCAGTGGCTATGTGTTTCTGTTCAACAA | XbaI |
| pEJwhiB5MUT | UPwhiB5MUT | CACAGACATGCCACGCAGTGGCTATGTGTTTCTGTTCAACAA | XbaI |
| pEJwhiB5WT | UPwhiB5DF | TGCGGCACCGCGTCAGGGCGCGCGTCGCTT | XbaI |
| pEJwhiB5MUT | UPwhiB5DF | AGGCCACCGCGTCAGGGCGCGCGTCGCTT | HindIII |
| pVW0023 | pvw0023FP | GGGAATTCCATATGAGCCGTGAGTCGGCCGGCGCGGCC | NdeI |
| pVW0023 | pvw0023RP | AAAACTGCAGCTGCTGCCCCTCATCCGCGTCGTG | XbaI |
| pEJUF1 | UF1FP | GGCGTGCGCCGCCGCAAGCTGGTGGCTATGTGTTTCTGTTCAACAA | XbaI |
| pEJUF2 | UF2FP | CGCGTGCGCCGCCGCAAGCTGGTGGCTATGTGTTTCTGTTCAACAA | XbaI |

Restriction site in the primer was underlined. The point mutations incorporated in SDM primers were highlighted in bold.
by 12% SDS-PAGE and protein concentration was measured by Bradford assay.

**β-Galactosidase Assay**

*Mycobacterium smegmatis* mc²155 strain was transformed with various constructs as required and grown in 7H9 media. The cultures were grown to mid-log phase and β-galactosidase activity was measured in Miller Units (MU) and all experiments were done in triplicate (Miller, 1972).

**Electrophoretic Mobility Shift Assay**

To verify the interaction between Rv0023 and the upstream region of *whiB5*, electrophoretic mobility shift assay (EMSA) was performed. The 500-bp upsteam region and the 50-bp downstream region of *whiB5* were PCR amplified and labeled with γ-32P ATP (3000 Ci mmol⁻¹) using T4 polynucleotide kinase as per manufacturer’s instructions (New England Biolabs). Labeled DNA was purified using nucleotide purification kit (Qiagen). Purified labeled DNA was then incubated with increasing concentrations of recombinant Rv0023 protein in an EMSA reaction buffer (25 mM HEPES, pH 7.9, 0.1 mM EDTA, 10 mM MgCl₂, 20 mM KCl, and 5% glycerol) with 50 ng/µl poly(dI–dC) as a non-specific DNA competitor. The protein–DNA complex was incubated for 45 min and was resolved on a 6% non-denaturing polyacrylamide gel in 0.5 × tris-borate (TBE) buffer. The gel was run for 3–4 h to allow sufficient resolution of protein–DNA complex.

Electrophoretic mobility shift assay with commercially synthesized overlapping oligonucleotide and motifs was performed to identify the exact binding region of Rv0023.

**Primer Extension**

To map the (+1) transcription start site (TSS), primer extension was performed as described earlier and in Cold Spring Harbor protocols (Carey et al., 2013; Angara et al., 2018). As the ORFs and intergenic region of *whiB5*-Rv0023 locus are 100% conserved between *Mtb* and *Mbovis*, we used *Mbovis* RNA for primer extension studies. RNA was isolated with the Qiagen RNeasy kit. SuperScript III reverse transcriptase (Invitrogen) was used to generate cDNA using random hexamers. Real-time PCR was carried on the BioRad CFX96 system using gene-specific primers (Table 5) and EvaGreen qPCR mastermix (Applied Biological Materials Inc.) as per standard protocol. The fold change in expression relative to *M. smegmatis* pVV16 (vector control) was calculated after normalizing to *sigA*. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression (Livak and Schmittgen, 2001).

**Stress Assay**

To check the sensitivity of *M. smegmatis* toward various stress conditions, *M. smegmatis* WT, *M. smegmatis* pVV16, and *M. smegmatis* pVV0023 cells were grown in complete 7H9 media (supplemented with 10% OADC, 0.2% glycerol, and 0.05% Tween80) at 37°C overnight. Then, the cells were centrifuged and washed with PBS. The cells were then suspended in 7H9 media and the cell concentration was adjusted to 0.02 OD at 600 nm. The sensitivity of bacterial cultures toward 0.1% SDS (Sigma Aldrich) and 5 mM hydrogen peroxide (Merck) was measured at 37°C for 6 h. Sensitivity to 250 µg/ml lysozyme (Sigma Aldrich) was measured at 37°C for 24 h. Susceptibility of *M. smegmatis* toward the antibiotics (Sigma Aldrich) INH (10 µg/ml, MIC of 5 µg/ml), RIF (10 µg/ml, MIC of 1 µg/ml), and ETH (2.5 µg/ml, MIC of 10 µg/ml) was determined at 37°C for 24 h. For determining CFU numbers for each stress condition and antibiotics, the cells were serially diluted (10-fold) and plated onto 7H10 agar plates.

Further, the strains *M. smegmatis* WT, *M. smegmatis* pVV16, and *M. smegmatis* pVV0023 were serially diluted (10-fold) and spotted on the 7H10 agar plates containing increasing concentrations of INH (0, 5, 10, and 15 µg/ml) and ETH (0, 2.5, and 5 µg/ml). The plates were incubated at 37°C for 48 h.

**qRT-PCR**

Total RNA from *M. smegmatis* pVV16 (vector control) and *M. smegmatis* pVV0023 (Rv0023 protein overexpressed) cultures were isolated using Qiagen RNeasy kit. SuperScript III reverse transcriptase (Invitrogen) was used to generate cDNA using random hexamers. Real-time PCR was carried on the BioRad CFX96 system using gene-specific primers (Table 5) and EvaGreen qPCR mastermix (Applied Biological Materials Inc.) as per standard protocol. The fold change in expression relative to *M. smegmatis* pVV16 (vector control) was calculated after normalizing to *sigA*. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression (Livak and Schmittgen, 2001).

**NADH/NAD⁺ Cellular Concentration**

To measure the cellular concentration of NADH and NAD⁺, *M. smegmatis* pVV16 and *M. smegmatis* pVV0023 cells were grown to an OD₆₀₀ of 0.8–1.2. Cells were collected (1 ml) by centrifugation (11,000 rpm for 2 min). The supernatant was removed and 300 µl of 0.2 M HCl (for NAD⁺ extraction) or 0.2 M NaOH (for NADH extraction) was added to the cells. The cells were resuspended and incubated at 50°C for 10 min after which extracts were cooled to 0°C. The bacterial suspensions were neutralized by adding 0.1 M HCl (for NADH extraction) or

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**TABLE 4** | List of primers used in primer extension.

| Primer | Sequence |
|--------|----------|
| 0023PE1 | CTCACGGCTCAGCCAGGCTCCG |
| 0023PE2 | CGGGAAAGTGCGCGAATGCGCG |

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**TABLE 5** | List of primers used in real-time PCR.

| Primer | Sequence |
|--------|----------|
| inhARTFP | GTAGGGGCACAAACAGATCCGAC |
| inhARTFP | GTACAGATATACGGCGAGATGTT |
| rhdRFTFP | CGGCTTCAAGACGAGATCG |
| rhdRFTP | CTTCTCGTGTTCTGAC |
| katGFP | CCAAATGGACACACAGCTTC |
| katGFPR | QAATCOCGAGTCAAGATCTG |
| ethAFP | CGGGCAAAAGGACGACAAAT |
| ethARP | AATGCTTCTGCAAGCTGAAA |
0.1 M NaOH (for NAD<sup>+</sup> extraction) dropwise while vortexing at high speed. Centrifugation was done to remove the cell debris and supernatant was transferred to a new tube and used immediately. NADH and NAD<sup>+</sup> concentrations were obtained by spectrophotometrically measuring the rate of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 4.2 mM] (Amresco), reduction by yeast alcohol dehydrogenase II (Sigma Aldrich) in the presence of PES (phenazine ethosulfate, 16.6 mM) (Sigma Aldrich) at 570 nm (Leonardo et al., 1996; San et al., 2002; Vilcheze et al., 2005). The concentration of nucleotide (NADH/NAD<sup>+</sup>) is proportional to the rate of MTT reduction.

Bioinformatics

Rv0023 protein sequence (UniProt P9WM13) was used as a query in the SynTax web server<sup>2</sup> for synteny analysis within the Mycobacteriaceae family (Oberto, 2013); a conserved domain database search was performed to identify HTH_3 and XRE domains (Marchler-Bauer et al., 2015). Orthologous sequences of the *whiB5–Rv0023* intergenic region from different mycobacterial species were retrieved from KEGG genome database and aligned using CLUSTAL OMEGA (Sievers et al., 2011).

<sup>2</sup>http://archaea.u-psud.fr/SyntTax

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**FIGURE 1** | Survivability of Rv0023 expressing *Msmeg* in stress conditions. (A) The *MsmegWT*, *MsmegpVV16*, and *MsmegpVV0023* were subjected to 0.1% SDS for 6 h, 250 µg of lysozyme for 24 h, 5 mM hydrogen peroxide for 6 h, INH (10 µg/ml), RIF (10 µg/ml), and ETH (5 µg/ml) for 24 h. Cells were plated on 7H10 agar plates and bacterial CFU were counted. Data are presented as the mean ± SD from three biological replicates. (B) *MsmegpVV16* and *MsmegpVV0023* cells were serially diluted and spotted on plates containing increasing concentrations of INH. (C) *MsmegpVV16* and *MsmegpVV0023* cells were serially diluted and spotted on plates containing increasing concentrations of ETH.
Statistical Analysis
Data were presented as mean ± SD. Student’s t-test and one-way ANOVA were used to determine the statistical significance between groups and values with \( p < 0.05 \) were considered to be significant. GraphPad Prism software version 5.02 was used for the statistical analysis.

RESULTS
Overexpression of Rv0023 Confers Increased INH and ETH Tolerance in Msmeg
Rv0023 is a non-essential gene in Mtb, yet it regulates a high number of genes in the genome (Sassetti et al., 2003; Rustad et al., 2014). Msmeg genome does not harbor the ortholog of Rv0023 and hence serves as a good model to study the function of Rv0023. To ascertain the role of Rv0023, we constructed an overexpressing strain of Rv0023 in Msmeg, MsmegpVV0023. The wild-type MsmegWT, vector control MsmegpVV16, and MsmegpVV0023 strains were subjected to acid fast staining to rule out the effect of vector insertion on cellular integrity. The cells were found to be intact and acid fast (Supplementary Figure 1). To further study the role of Rv0023 in cellular physiology, MsmegWT, MsmegpVV16, and MsmegpVV0023 were subjected to different stress conditions as depicted in Figure 1A, and tolerance was measured in terms of CFU. The CFU was counted for all conditions and we observed that overexpression of Rv0023 leads to increased tolerance with regard to INH and ETH, and no effect was observed with any other stress conditions. Tolerance to INH and ETH was not seen either on the wild type or in vector control strains (Figure 1A).

The role of Rv0023 in generating tolerant strains was further confirmed via spotting the wild type, vector control, and Rv0023 overexpressed strains in the presence of increasing concentrations of INH and ETH. MsmegWT, MsmegpVV16, and MsmegpVV0023 cells were serially diluted and spotted on plates containing 5, 10, and 15 \( \mu \text{g/ml} \) of INH and 2.5 and 5 \( \mu \text{g/ml} \) of ETH. Even at higher concentrations, MsmegpVV0023 showed tolerance toward INH and ETH (Figures 1B,C and Supplementary Figure 2). The results indicate that Rv0023 expression specifically contributes toward the higher tolerance of INH and ETH in Msmeg.

Rv0023 Expression in Msmeg Alters the NADH/NAD\(^+\) Levels
Isoniazid and ethionamide are both prodrugs and they are activated by the protein catalase-peroxidase KatG and the NADPH-specific flavin adenine dinucleotide-containing monooxygenase, EthA, respectively. After activation, they react with NAD\(^+\) to form INH-NAD and ETH-NAD adduct. This species then inhibits the enoyl ACP reductase InhA, which leads to the inhibition of mycolic acid biosynthesis and eventual mycobacterial cell death (Figure 2A). The major common genes to both pathways are the \( ndh \) (maintains NADH/NAD\(^+\) ratio) and \( inhA \). Msmeg orthologs of these and other important genes

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** | \( ndh \) gene regulation by Rv0023. (A) Schematic representation of mechanisms of action of INH and ETH. (B) Expression levels of genes involved in INH and ETH resistance pathway in MsmegpVV16 and MsmegpVV0023 cells. Fold change was calculated relative to the vector control pVV16. Data are presented as the mean ± SD from three biological replicates. One-way ANOVA was applied and the \( p \)-value obtained was \( p < 0.0001 \). (C) EMSA studies with radiolabeled 300 bp of upstream region of \( ndh \). Lane 1: probe without any protein. Lanes 2–4: probe with increasing concentration of purified Rv0023 protein.
TABLE 6 | Cellular concentration of NADH and NAD\(^+\).

| Strain         | NADH (\(\mu\)M) | NAD\(^+\) (\(\mu\)M) | NADH/NAD\(^+\) |
|----------------|-----------------|-----------------------|----------------|
| MsmegpVV16     | 8.29            | 9.93                  | 0.83           |
| MsmegpVV0023   | 11.27           | 9.44                  | 1.19           |

involved in INH and ETH resistance were subjected to qRT-PCR quantification. Total RNA was isolated from MsmegpVV16 and MsmegpVV0023 and the expression levels compared between the two strains. We found that the expression level of ndh (MSMEG_3621) in MsmegpVV0023 was approximately twofold lower than that of MsmegpVV16. No changes in the expression levels were observed in inhA (MSMEG_3151), katG (MSMEG_6384), and ethA (MSMEG_6440) between both strains (Figure 2B). As ndh encodes NdhII, which oxidizes NADH to NAD\(^+\), we reasoned that the ratio of NADH/NAD\(^+\) might be altered in MsmegpVV0023 strain. So, we measured the cellular ratio of NADH and NAD\(^+\) in MsmegpVV16 and MsmegpVV0023 strains and found that the ratio of NADH/NAD\(^+\) is increased in MsmegpVV0023 (Table 6). These results indicate that Rv0023 expression alters both the transcript levels of ndh gene and NADH/NAD\(^+\) levels. To find out whether these effects are directly mediated by Rv0023 or through some indirect means, we studied the interaction of recombinant Rv0023 with the upstream region of ndh (MSMEG_3261) gene. However, under the experimental conditions mentioned, we did not observe any such positive interaction (Figure 2C).  

Rv0023 Negatively Regulates whiB5

Rv0023 is in operon with Rv0024 and is transcribed divergently from whiB5 (Figure 3A). Rv0023 and whiB5 are absent from the non-pathogenic, fast-growing Msmeg but are present in Mtb. So, we tried to find the co-occurrence of these two genes in other mycobacterial species. Synteny analysis of Rv0023 and whiB5 was performed for important species of the Mycobacterium genus using the “SynTax” web server (Figure 3B). It was observed that whiB5 is in synteny with Rv0023 and that both are present only in pathogenic species of mycobacteria (Supplementary Table 1).

The syntenic relationship of the two regulators prompted us to probe the regulation of the whiB5-Rv0023 locus. To identify the regulatory elements at this locus, upstream regions of whiB5 and Rv0023 were cloned in the promoter-less vector pEJ414 and named pEJwhiB5 and pEJ0023, respectively. The coding region of Rv0023 was cloned in the pVV16 vector to obtain pVV0023. Msmeg was transformed with pEJwhiB5 or pEJ0023. The strains were again transformed with pVV023.

FIGURE 3 | Rv0023 exists in a syntenic relationship with whiB5 and negatively regulates whiB5 expression. (A) Genomic organization of whiB5 and Rv0023 locus. (B) Schematic representation showing synteny of Rv0023 and whiB5 (within rectangular boxes) from seven mycobacterial species strains. (C) β-Galactosidase activity of whiB5 promoter with ectopic expression of Rv0023 protein and Rv0494 (non-specific protein for negative control). (D) β-Galactosidase activity of Rv0023 promoter in the presence of Rv0023 and Rv0494 overexpression.
to obtain *MsmegpEJwhiB5*-pVV0023 and *MsmegpEJ0023*-pVV0023 double transformants. Promoter activities were measured in double transformants (*MsmegpEJwhiB5*-pVV0023, *MsmegpEJwhiB5*-pVV16, *MsmegpEJ0023*-pVV0023, and *MsmegpEJ0023*-pVV16). Overexpression of Rv0023 caused approximately fivefold decrease in whiB5 promoter activity (Figure 3C), whereas no significant change in the promoter activity of Rv0023 was noted (Figure 3D). Rv0494, a FadR transcriptional regulator, was used as a negative control, which did not affect the promoter activities of any of these genes. These data suggest that Rv0023 negatively regulates the expression of whiB5, but it is not auto-regulatory.

**Rv0023 Binds to whiB5 in vitro**

As we have observed that Rv0023 expression represses the promoter activity of whiB5 in *Msmeg*, it prompted us to further test whether Rv0023 interacts with whiB5 promoter or not. Five hundred base pairs upstream and 50 bp downstream of whiB5 gene containing the promoter were radiolabeled and EMSA was performed with the recombinant Rv0023.
The radiolabeled upstream region of *whiB5* interacted with the purified recombinant Rv0023 in a dose-dependent manner. No interaction of Rv0023 was observed with non-specific DNA (Figure 4A).

Both EMSA and β-galactosidase assays confirmed that Rv0023 interacts with *whiB5* upstream and negatively regulates *whiB5* expression. In order to find the exact binding site of Rv0023, the 550 bp upstream of *whiB5* was divided into two fragments: UF1 of 340 bp and UF2 of 250 bp (Figure 4B). We observed that only the UF2 fragment binds to Rv0023 (Supplementary Figure 3). The UF2 fragment was further divided into six overlapping fragments, F1–F6 (Figure 4B and Table 7). Purified Rv0023 binds to fragments F1 and F2, whereas no binding was observed with other fragments (Figure 4C). So, we looked for a binding site at the overlapping region of F1 and F2. We generated new oligonucleotides (OL1–OL5) covering the F1–F2 overlapping region (Figure 5A and Table 8). EMSA with these radiolabeled fragments showed that Rv0023 binds to OL1 and OL3 fragments whereas no binding was observed with other fragments (Figure 5B). Close examination of these fragments revealed the presence of an imperfect palindrome present in both sequences, TATAGcTTTT in OL1 and TATACgcTTTT in OL3 (Figure 5C). To confirm the binding sites identified, we mutated TATAGcTTTT to TGTGcTTTT in OL1 and TATACgcTTTT to TGTGcgcTTTT in OL3 to obtain OL1M and OL3M, respectively (Table 8). Binding studies have shown that Rv0023 binds to the native OL1 and OL3 fragments, but not to the mutant fragments, OL1M and OL3M (Figure 5C). We conclude that there are two binding sites (TATAGcTTTT and TATACgcTTTT) of Rv0023 in the upstream region of *whiB5* comprising an 11-bp imperfect palindromic sequence with a 3-bp spacer region.

**Analysis of Rv0023 Binding Site**

The importance of each residue within the Rv0023 binding site was determined by generating 39 bp probes (M0–M12)
containing specific point mutations on each half of the imperfect palindrome and in the spacer region (Figure 6A). The probes were radiolabeled and were used in binding studies with recombinant Rv0023. The binding was observed with probes M0 (unaltered), M1, M4, and M5, and significant loss of binding was observed with M2, M3, M6, M7, and M8 fragments, suggesting the importance of these residues in Rv0023 binding. Changing the residues in the spacer region (M9–M10) did not affect Rv0023 binding; however, changing the length of spacer region by one base lead to significant loss of Rv0023 binding (M11–M12) (Figure 6B). As mentioned earlier, the organization of Rv0023 and whiB5 is conserved across pathogenic mycobacterial species. Therefore, we looked into the conservation of identified Rv0023 binding sites across the conserved genomes. For this, we aligned the upstream region of whiB5 orthologs from different pathogenic mycobacterial species strains and found that the two binding sites of Rv0023 are conserved across the analyzed mycobacterial species (Figure 6C). These results highlight the important residues for Rv0023 binding and its conservation across mycobacterial species.

TABLE 8 | List of overlapping fragments used in Figure 5.

| Overlapping fragment | Sequence |
|----------------------|----------|
| OL1                  | CACCCCTTATGTATATACGTTTTTATCGCGATTCTCTTGC |
| OL2                  | TTTTATCGCGATTCTCTTGCAGAGCCCGCCACAGACATAT |
| OL3                  | AGAGCCCGCCACAGACATATACGCTTTTGCCTATGTTTCG |
| OL4                  | ACGCTTTTGCCTATGTTTCGTTCAACAAGGAGGCCGGCAC |
| OL5                  | ATTCTCTTGCAGAGCCCGCCACAGACATATACGCTTTTGC |
| OL1M                 | CACCCCTTATGTAGAGACGTGTGTATCGCGATTCTCTTGC |
| OL3M                 | AGAGCCCGCCACAGACAGAGACGCGTGTGCCTATGTTTCG |

Rv0023 Binding Site Overlaps With the whiB5 Promoter Region

In order to understand the mechanism of Rv0023-mediated repression of whiB5, the TSSs of whiB5 and Rv0023 were mapped. The putative −10 region of whiB5 was identified earlier by RACE (Casonato et al., 2012) (Figure 7A). To test the functionality of the putative −10 region, we cloned the 250 bp upstream of whiB5 into lacZ reporter vector (pEJwhiB5WT). The promoter activity was measured using the
β-galactosidase assay in Msmeq. Mutations in the putative −10 region of whiB5 (atacgctt to gcacgcgg) abolished the promoter activity, confirming the −10 region of whiB5 (Figure 7B). To identify the promoter region of Rv0023, primer extension was performed. We identified the TSS at 228 bp upstream of Rv0023 start codon (Figure 7C). A −10-like hexamer sequence TCATAG was identified upstream of TSS. To validate the functionality of the −10 region of Rv0023, 400 bp upstream of Rv0023 was cloned into a lacZ reporter vector (pEJ0023WT). β-Galactosidase assay with WT promoter and mutated promoter (pEJ0023MUT) indicated significant decrease in the promoter activity of the mutant strain compared to WT (Figure 7D). Mapping the promoters of whiB5 and Rv0023 with Rv0023 binding site revealed that Rv0023 binding site overlaps with
the promoter of whiB5 but not of the Rv0023 promoter region (Figure 7E).

**DISCUSSION**

In this study, Rv0023, a member of the XRE family of transcriptional regulators, has been characterized, and its role in INH and ETH drug tolerance has been explored. The first part of our study concerns the role of Rv0023 in the physiology of mycobacteria. For our study, we used Msmeq as a surrogate model to study the effects of various stress conditions on wild type and Rv0023-expressing strains. It was observed that overexpression of Rv0023 alters the NADH/NAD\(^+\) ratio, thereby increasing drug tolerance. In the second part of the manuscript, we identified Rv0023 as a transcriptional regulator and studied the regulatory effect at whiB5-Rv0023 locus and observed that Rv0023 negatively regulates whiB5 expression but is not auto-regulatory. Rv0023 regulates whiB5 expression by binding to specific sequences in the upstream region of whiB5. Our data indicate that there are two binding sites for Rv0023 upstream of whiB5 and one of the sites overlaps with the whiB5 promoter, thereby possibly occluding the binding of RNA polymerase. The binding site of Rv0023 is conserved across mycobacterial species as confirmed by multiple sequence alignment. Apart from this, we have also characterized the binding site of Rv0023 and found important bases for binding. While analyzing the spacer region, it was found that the length of the spacer region is important for proper binding.

Isoniazid, isonicotinic acid hydrazide, is a synthetic drug and the occurrence of INH-resistant strains is significantly more frequent than other drug-resistant Mtb clinical strains (Nachega and Chaissin, 2003). ETH, 2-ethylthioisonicotinamide, is a structural analog of INH and was first synthesized in 1956 (Grumbach et al., 1956). Many genes have been found to be associated with INH and ETH resistance in clinical isolates, but the role of transcription factors regulating INH and ETH co-resistance is poorly understood. Rv0023, a transcriptional regulator, conferring higher tolerance toward INH and ETH in Msmeq, provided us a chance to explore the role of transcription factors in INH and ETH co-resistance in Mtb. To elucidate the mechanism by which Rv0023 confers drug tolerance, we investigated two main genes known to be involved in INH and ETH co-resistance, inhA (enzyme in the synthesis of cell wall mycolic acid) (Banerjee et al., 1994) and ndh (NADH dehydrogenase maintains the NADH/NAD\(^+\) ratio) (Miesel et al., 1998). We checked their expression levels in the vector control and Rv0023-expressing Msmeq strains and differential expression was only observed in the ndh gene. The ndh gene oxidizes NADH to NAD\(^+\), which maintains the ratio of NADH/NAD\(^+\) in mycobacterial cells (Jaworowski et al., 1981). Loss of ndh function leads to higher levels of NADH in the cytosol. Previous studies have shown that the higher NADH/NAD\(^+\) levels interfere with activation of INH/ETH drugs by competitive inhibition with the formation of INH-NAD and ETH-NAD and thus confer resistance to INH/ETH in Msmeq and BCG (Miesel et al., 1998; Vilcheze et al., 2005). So, we further measured the NADH/NAD\(^+\) ratio in vector control and Rv0023-expressing Msmeq strains. The NADH/NAD\(^+\) ratio was found to be increased in the Rv0023-overexpressed Msmeq strain, suggesting that negative regulation of ndh gene by Rv0023 is probably the mechanism by which Rv0023 confers INH and ETH tolerance in Msmeq. Our results were supported by previous studies where it was shown that Rv0023 regulon is enriched for NAD reductases (Rustad et al., 2014). Rv0023 did not bind to the upstream region of ndh, indicating that the regulation occurs possibly through indirect means, which may involve multiple intermediate gene products. Further studies are required to completely decipher this mechanism.

Synteny analysis has shown that whiB5-Rv0023 locus is present mainly in pathogenic species of mycobacteria. The gene whiB5 belongs to the WhiB family of transcriptional regulators, which are exclusive to actinomycetes, such as Mycobacterium and Streptomycetes spp. (Soliveri et al., 2000). Earlier studies have shown that whiB5 is a global transcriptional regulator, regulating 58 genes of diverse functions, including sigM and genes encoding for Type VII secretion systems such as exs-2 and exs-4. It was shown that whiB5 has a role in Mtb virulence and reactivation (Casonato et al., 2012). As the whole locus of whiB5-Rv0023-Rv0024 is absent in Msmeq, it is difficult to gauge the true effect of Rv0023 in the physiology of Mtb. Further investigation in Mtb may reveal important aspects of Rv0023 regulation and its effects. Hence, taking into account the earlier studies and our current data, we surmise that Rv0023 plays a significant role in modulating genes associated with NADH/NAD\(^+\) levels, which further influences mycobacterial physiology.

In conclusion, our work shows, for the first time, that Rv0023 has a role in conferring INH and ETH tolerance in Msmeq. Understanding the role of transcriptional factors in the development of drug resistance will open new avenues in the field of drug discovery and may provide important insights into Mtb physiology.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**AUTHOR CONTRIBUTIONS**

AR, SG, RA, and SY conceived the hypothesis and rationale of the study, analyzed the results, and wrote the manuscript. CR contributed in cloning of constructs and recombinant protein purification.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00003/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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