Infection and Drug Resistance

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SHORT REPORT

Mutation $EthA_{W21R}$ confers co-resistance to prothionamide and ethionamide in both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv

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Abstract: Ethionamide (ETA) and prothionamide (PRO) are interchangeably used in tuberculosis (TB) chemotherapy regimens. Subtle discrepancies between biochemical and genetic information on the modes of sensitivity and resistance of isoniazid (INH) and ETA warrants further studies. We report a new mutation – $EthA_{W21R}$ – in *Mycobacterium bovis* Bacillus Calmette-Guérin that corresponds with co-resistance to both PRO and ETA, which to the best of our knowledge has not been reported before. Our findings suggest that mutation $EthA_{W21R}$ could be used as a marker site for testing PRO and ETA cross-resistance.

Keywords: mutation, $EthA_{W21R}$, isoniazid, co-resistance, thioamides, molecular marker

The thioamide, ethionamide (ETA), and its propyl-analog prothionamide (PRO) are interchangeably used in tuberculosis (TB) chemotherapy regimens to treat multidrug-resistant TB (MDR-TB),[1–3] drug-susceptible TB meningitis (TBM), and miliary TB in some settings, due to their good cerebrospinal fluid (CSF) penetration ability.[4]

PRO is associated with better tolerance compared with ETA in the treatment of MDR-TB, and both are structurally similar to isoniazid (INH).[2,5,6] The only notable distinction in their mechanism(s) of action is the lack of cross-resistance to INH.[7,8]

Both ETA and PRO are prodrugs whose enzymatic activation by *Mycobacterium tuberculosis*' EthA inhibits InhA, which subsequently inhibits the *M. tuberculosis*' mycolic acid synthesis (Figure 1).[2,9] Mutations in the *ethA* gene often underlie ETA and PRO monoresistance.[2] Hanoulle et al[10] postulated that both are further transformed by EthA enzyme to a metabolite that accumulates intracellularly and acts as the final toxic compound. As illustrated in Figure 1,[11] activated ETA and PRO form adducts with nicotinamide adenine dinucleotide (NAD), which is the inhibitor of the InhA enzyme in *M. tuberculosis*.[1,12,13] Thee et al[2] suggested that the correlation between mutations conferring ETA resistance and the MIC warrants further studies because of the subtle discrepancies between biochemical and genetic information on the modes of sensitivity and resistance in the cases of INH and ETA.

Here, we report a new mutation – $EthA_{W21R}$ – in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) that corresponds with co-resistance to PRO and ETA, which to the best of our knowledge has not been reported before.

We screened wild-type *M. bovis* BCG Tice on high PRO concentrations and obtained one drug-resistant colony at 30 μg/mL PRO-containing 7H11 plate. To confirm the

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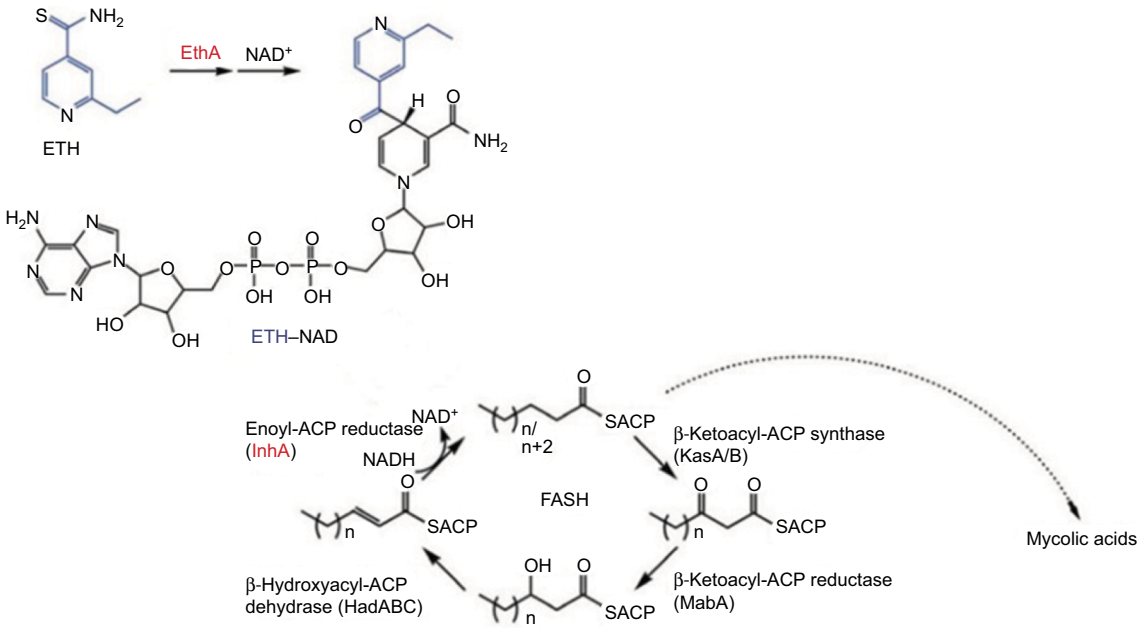

Figure 1 The mechanism of action for ETA.
Notes: ETA is activated by monooxygenase EthA to form a reactive species that binds to NAD^+. The resulting ETH–NAD adduct inhibits the enoyl-ACP reductase InhA of the FASII system, resulting in mycolic acid biosynthesis inhibition. ©2014 American Society for Microbiology. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology.[11]
Abbreviations: ETA, ethionamide; NAD, nicotinamide adenine dinucleotide.

phenotypic resistance of the single colony, we similarly re-tested it on 30 and 40 μg/mL PRO-containing 7H11 plate. We sequenced the six reported genes (e*thR*, *ethA*, *inhA katG*, *ndh*, and *ahpC*; Table 1; BGI, Shenzhen, China) associated with ETA and PRO resistance and found a single-nucleotide mutation in *ethA* gene leading to W21R mutation while the other five genes had no mutation(s).

We then overexpressed this mutated 1.4kb *ethA*^{W21R} and the *M. bovis ethA*^{wt} genes by cloning them at the *Nde*I and *Hin*dIII sites of extrachromosomal p60LuxN plasmid bearing the *M. tuberculosis hsp*60 promoter (Figure 2).[14] Recombinant plasmids p60*ethA*^{W21R} and p60*ethA*^{wt} constructs were verified by enzyme digestion and sequencing (BGI). Wild-type *M. bovis* BCG Tice and *M. tuberculosis* H37Rv strains were transformed with the plasmids p60*ethA*^{W21R} and p60*ethA*^{wt} through electroporation as described previously with some modifications.[15] Positive selection was confirmed by PCR amplification of the hygromycin resistance marker gene (*hyg*) in p60*ethA*^{W21R} and p60*ethA*^{wt} using primers hyg-r and hyg-f (Table 1).

We then evaluated the MICs of PRO and ETA against the recombinant and parental strains (control) using the classical agar plate method.[16] We show that after overexpressing the mutated *ethA*^{W21R} in wild-type BCG and *M. tuberculosis* H37Rv, both PRO and ETA MIC rose by 256- and 128-fold, respectively (Table 2). Additionally, no observable differences were noted in the MICs of the overexpressed *ethA*^{wt} recombinants and the parent strains (MIC =0.25 and 0.5 μg/mL; Table 2). Our findings suggest that the mutation *ethA*^{W21R} could be used as a marker site for testing PRO and ETA cross-resistance.

Acknowledgments

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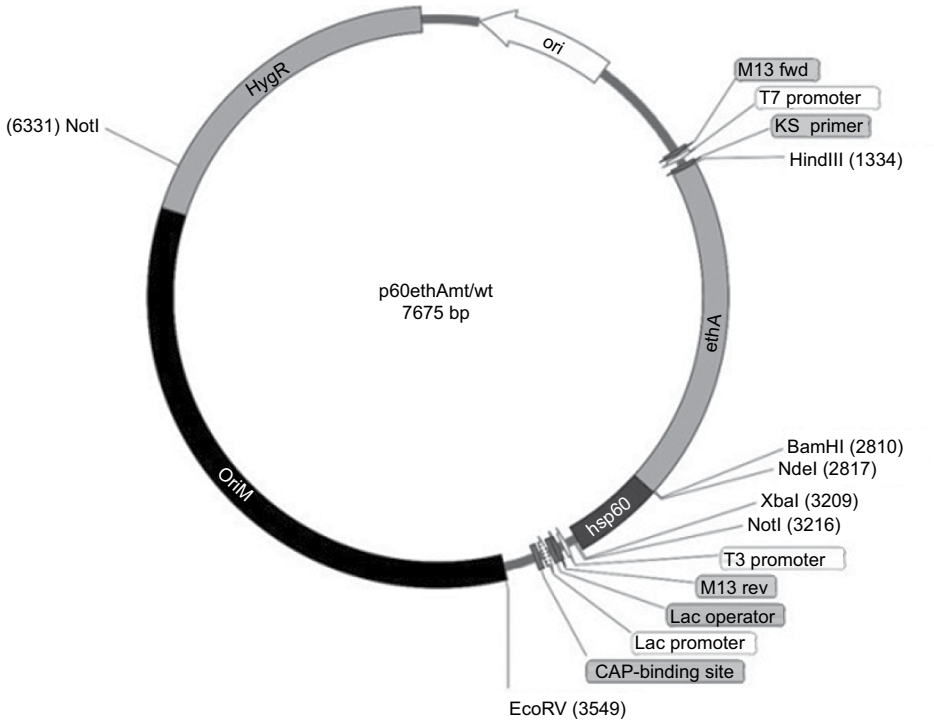

Figure 2 *E. coli*–mycobacteria shuttle plasmids p60ethA$^{Mt/Wt}$.
Notes: *OriE*, origin of replication region in *E. coli*; *OriM*, origin of replication in mycobacteria; *hyg*, hygromycin-resistant gene; *ethA*, *ethA*^{wt} or *ethA*^{W21R}.
Abbreviation: *E. coli*, *Escherichia coli*.

Table 1 PCR and sequencing primers used to delineate target-based spontaneous genotypic resistance mechanisms of *M. bovis* BCG Tice

Resistance to	Primer pairs	Nucleotide sequences (5′–3′)	Upstream extension (base)	Downstream extension (base)	Product length (bp)
PRO	EthRf5/EthRr5	TTTTCCAGGATGGCGTAGC/CCGACCGGATCGTCAACA	185	263	1099
	EthAf/EthAr	CCTGGCAGCTTACTACGTGTC/CGGCATCATCGTCGTCTG	75	54	1599
	inhAf/inhAr	TCACGGCGGTAGAAGAGCA/CCACGCAGATGTCGCAAAGA	548	326	1684
	KatGf/KatGr	TGCGAAAGATCCAACCCTC/AGACCAACCGTGTAGGCAAAT	276	317	2816
	Ndhf/Ndhr	ACTTGGCTCCGCACGGCTAT/ATCCGGCGACGGCATTCA	217	109	1718
	ahpCf/ahpCr	CGACTGGCTCATATCGAGAAT/AATACCTGCGGATTTCGTGT	216	180	984
EthAf2	GGAATTC*CATATG*ACCGAGCACCTCGACGTT				
EthAr2	CCC*AAGCTT*CTAAACCCCCACCGGGGCA				
hyg-f	GTGACACAAGAATCCCTG				
hyg-r	TCAGGCGCCGGGGGCGGTG				

Note: Primers for each gene amplification were extended with ~150 bp upstream and downstream of start and stop codons.
Abbreviations: *M. bovis*, *Mycobacterium bovis*; PRO, prothionamide.

Table 2 MICs of PRO and ETA for wild-type and recombinant strains

Serial number	Strain	Mutations	MICs (μg/mL)	
			PRO	ETA
1	*M. tuberculosis* H37Rv:p60*ethA*$_{Mt}$	W21R	32	32
2	*M. tuberculosis* H37Rv:p60*ethA*$_{Wt}$	–	0.25	0.25
3	*M. tuberculosis* H37Rv Wt	–	0.5	0.5
4	*M. bovis* BCG Tice BCG:p60*ethA*$_{Mt}$	W21R	32	32
5	*M. bovis* BCG Tice BCG:p60*ethA*$_{Wt}$	–	0.5	0.5
6	*M. bovis* BCG Tice Wt	–	0.25	0.25

Abbreviations: ETA, ethionamide; *M. bovis*, *Mycobacterium bovis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; PRO, prothionamide.

China: 1) State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China, and 2) State Key Laboratory of Respiratory Disease, Department of Clinical Laboratory, Guangzhou Chest Hospital, Guangzhou, China. The facilities are compliant with biosafety level 2+ and 3 requirements for handling infectious materials.

Disclosure

The authors report no conflicts of interest in this work.

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