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Polymorphisms in Isoniazid and Prothionamide Resistance Genes of the *Mycobacterium tuberculosis* Complex▽

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Sequence analyses of 74 strains that encompassed major phylogenetic lineages of the *Mycobacterium tuberculosis* complex revealed 10 polymorphisms in *mshA* (*Rv0486*) and four polymorphisms in *inhA* (*Rv1484*) that were not responsible for isoniazid or prothionamide resistance. Instead, some of these mutations were phylogenetically informative. This genetic diversity must be taken into consideration for drug development and for the design of molecular tests for drug resistance.

The *Mycobacterium tuberculosis* complex (MTBC), the causative agents of tuberculosis (TB), claimed the lives of 1.8 million people in 2008 (38, 39). In many settings, the efforts to control TB are threatened by the emergence and transmission of drug-resistant TB-causing strains (7). Yet the traditional phenotypic techniques for the detection of these resistant strains are slow and, therefore, add to the myriad of barriers to successful treatment of TB (3, 9, 20, 26). To overcome this challenge, genotypic drug susceptibility testing has been introduced to obtain more rapid results (26). Most recently, the World Health Organization has endorsed the Cepheid Xpert MTB/RIF assay which can detect rifampin resistance directly from sputum within 2 h (4, 30).

A clear understanding of the relationship between genotype and phenotype is a prerequisite for molecular resistance tests. In the past, however, several studies were confounded by false associations between polymorphisms and drug resistance. As a result, phylogenetically informative single-nucleotide polymorphisms (SNPs) were incorrectly regarded as resistance-conferring mutations (10, 15, 21–23). This also occurred in a recent study by Brossier et al., who undertook a comprehensive study of ethionamide (ETH) resistance genes in clinical MTBC strains (5). Some of the mutations detected in this study are in fact neutral polymorphisms rather than resistance-conferring mutations. Indeed, the −102G/A mutation in the promoter region of *inhA* (*Rv1484*), the shared target of ETH and isoniazid (INH), is a known marker for *Mycobacterium africanum* West African 1 (16). Similarly, the N111S mutation in *mshA* (*Rv0486*), a gene required for mycothiol biosynthesis that plays an unknown role in activating ETH, occurs in the Erdman laboratory strain that is sensitive to both INH and ETH and belongs to the Haarlem genotype (1, 32). Moreover, we previously found the *mshA* A187V change in two closely related modern Beijing strains (25). Both were sensitive to prothionamide (PRO; regularly used and tested in Germany) and, consequently, probably also to ETH, given that the two drugs display a high degree of cross-resistance (28).

To investigate in greater detail the role of the above-mentioned *mshA* polymorphisms and any polymorphisms that might exist in the coding sequence of *inhA*, we analyzed a reference collection of 74 strains that encompassed major phylogenetic lineages of MTBC (35, 36).

Genomic DNA was isolated as described previously (31). We amplified and sequenced *mshA* and *inhA* using the primer pairs Rv0486_5′ (5′-CCG CTA CCG CCA TCA CCG ACT T-3′) and Rv0486_3′ (5′-GGC CGC ACG CAG CAC AAT-3′) and Rv1484_5′ (5′-AAA CGT GAC CGC GAA TGT GG-3′) and Rv1484_3′ (5′-CAG CTC CGC GAT CAG TGT GC-3′). PCR was performed in 25 μl reaction mixtures using Qiagen HotStarTaq DNA polymerase with 500 nM each primer. Cycling conditions for *Rv0486* were 95°C for 10 min and 35 cycles of 95°C for 30 s, 58°C for 35 s, and 72°C for 90 s, followed by a final elongation of 72°C for 10 min, and cycling conditions for *Rv1484* were 95°C for 10 min and 35 cycles of 95°C for 30 s, 66°C for 30 s, and 72°C for 90 s, followed by 72°C for 10 min. The PCR products thus obtained were sequenced using an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems, CA) and an ABI Prism BigDye terminator kit, version 3.1, according to the manufacturer's instructions. The sequence data were analyzed using SeqScape Software, version 2.6 (Applied Biosystems), with the *M. tuberculosis* H37Rv (GenBank accession number AL123456.2) sequence as reference (*Rv0486* sequence data, bp −180 to bp +801, and *Rv1484* sequence data, bp −90 to bp +810). Drug susceptibility testing was performed using the Bactec MGIT 960 system (Becton Dickinson, MD). Testing of INH was carried out according to the manufacturer's instructions, and testing for PRO was performed as described previously (27).

We found one mutation upstream of the *mshA* coding region and a total of six synonymous and seven nonsynonymous mutations within the coding regions of *mshA* and *inhA* (Table 1). Based on the susceptibility results for PRO and INH, all of these mutations were polymorphisms rather than markers for

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TABLE 1. Sequencing and drug susceptibility results of 74 MTBC strains[a]

Sample	Genotype[b]	SNP(s) (uppercase in codons) and resulting mutation(s) in:		Drug susceptibility test result[c]	
		mshA (*Rv0486*)	*inhA* (*Rv1484*)	PRO	INH
12594/02	Beijing	gCa/gTa A187V			
1500/03	Beijing	gCa/gTa A187V		ND	
1934/03	Beijing	gCa/gTa A187V		ND	
3256/02	Beijing	gCa/gTa A187V		R	R
3329/02	Beijing	gCa/gTa A187V			R
4445/02	Beijing	gCa/gTa A187V			R
1417/02	Cameroon				
1428/02	Cameroon				
5390/02	Cameroon			ND	
5400/02	Cameroon			ND	
1797/03	EAI				
4850/03	EAI	gGg/gCg G106A, gAc/gCc D218A			
947/01	EAI				
10459/03	Euro-American				
12591/02	Euro-American				
8870/03	Euro-American				R
10469/01	Ghana			R	R
10493/01	Ghana				R
2570/02	Ghana				
9679/00	H37Rv ATCC 27294				
2336/02	Haarlem	aAc/aGc N111S			
9532/03	Haarlem	aAc/aGc N111S			
4130/02	Haarlem	aAc/aGc N111S			
1449/02	*M. africanum* 1a				
1473/02	*M. africanum* 1a				
5434/02	*M. africanum* 1a			ND	
10473/01	*M. africanum* 1b			ND	
10494/01	*M. africanum* 1b				
1443/02	*M. africanum* 1b				
5432/02	*M. africanum* 1b			ND	R
10476/01	*M. africanum* 2	Cta/Tta L261L	gTg/gCg V78A		R
10514/01	*M. africanum* 2		gTg/gCg V78A		R
10517/01	*M. africanum* 2		gTg/gCg V78A		
5468/02	*M. africanum* 2		gTg/gCg V78A	ND	
9550/00	*M. africanum* 2 ATCC 25420		gTg/gCg V78A		
4258/00	*M. bovis*			ND	
751/01	*M. bovis*				
7540/01	*M. bovis*			ND	
9564/00	*M. bovis* ATCC 19210	Ggg/Agg G106R			
3040/99	*M. canettii*	ggG/ggC G106G, gaC/gaT D208D	ggT/ggC G205G		
3041/99	*M. canettii*	−132C/A,[d] ggG/ggC G106G	ggT/ggC G205G		
3151/08	*M. canettii*	−132C/A,[d] ggG/ggC G106G	ggT/ggC G205G	ND	
1694/00	*M. caprae*				
8986/99	*M. caprae*			ND	
9577/99	*M. caprae*				
416/01	*M. microti* llama			ND	
1479/00	*M. microti* vole			ND	
8753/00	*M. microti* llama				
7011/02	*M. pinnipedii* seal		Ccg/Tcg P107S		
7739/01	*M. pinnipedii* seal		Ccg/Tcg P107S		
10264/03	Tur	Ctg/Ttg L244L			
10529/03	Tur	Ctg/Ttg L244L			
11313/03	Tur	Ctg/Ttg L244L			
2169/99	Uganda I			ND	
2201/99	Uganda I			ND	R
2333/99	Uganda I		ctC/ctT L88L	ND	
2176/99	Uganda II			ND	
2191/99	Uganda II		ctC/ctT L88L	ND	
2253/99	Uganda II			ND	
4412/04	X-type				
8431/05	X-type			ND	
9953/04	X-type				

[a] Strains with no data listed were susceptible, and the wild-type sequence was detected; strains of the following genotypes which were sensitive to both PRO and INH and did not harbor any mutations are not listed: Delhi/CAS (samples 2637/02, 7936/01, and 7507/01), LAM (samples 7968/03, 8885/03, and 946/03), S-type (samples 2151/03, 2318/06, and 6411/05), and Ural (samples 1657/03, 8431/03, and 2679/03).

[b] CAS, Central Asian; EAI, East African Indian; LAM, Latin American Mediterranean.

[c] PRO, prothionamide; INH, isoniazid; R, resistant; ND, not determined.

[d] Leads to synonymous ctC/ctA L411L change in *Rv0485*.

antibiotic resistance, as they occurred independently from phenotypic resistance. Instead, some were phylogenetically informative. Among these was the previously discussed N111S mutation in *mshA*, which appeared to be a marker for the Haarlem genotype as a whole rather than being specific to just the Erdman strain (1, 32, 34). All six modern Beijing strains displayed the A187V change in *mshA*, thereby showing that this mutation was not confined to the two modern Beijing strains alluded to earlier (25).

To analyze the distribution of *mshA* A187V further, we turned to a number of previously published data sets in which this mutation was detected but not discussed. Seven modern Beijing strains (T67 and T85; HN878 and X122; CHIN+, SA+, and V+) that were sequenced as part of the *M. tuberculosis* Phylogeographic Diversity Sequencing Project (MtbPDSP) or by Ioerger et al. and Schürch et al. harbored this mutation, whereas five ancestral Beijing strains from said studies (98 1833; R1207; CHIN−, SA−, and V−) had a wild-type sequence (6, 12, 14, 18, 29; S. Gagneux, personal communication). A more in-depth follow-up of this particular position in 16 additional Beijing strains revealed that *mshA* A187V was present not only in five modern Beijing strains (NLA009600299, NLA000701561, NLA000701003, NLA000700911, and NLA000100595) but also in four strains (NLA000801248, NLA000602040, NLA009601196, and NLA009501317) that were phylogenetically intermediate between ancestral and modern Beijing strains (29). Strain 00 1695 from the MtbPDSP which has the *mshA* A187V mutation also falls into this category (S. Gagneux, personal communication). Consequently, this mutation should be regarded as specific to the Beijing genotype rather than as a marker for the genotype as a whole or just modern strains. Importantly, HN878 was fully drug sensitive, confirming our prior suggestion that this mutation did not confer resistance to either ETH or PRO (18).

With the exception of the aforementioned upstream mutation, no polymorphisms in *inhA* had been described in the literature. Consequently, we were the first to identify the P107S mutation in *inhA*, which appeared to be a marker for the *Mycobacterium pinnipedii* seal genotype rather than for INH or PRO resistance. In contrast, V78A had been described in two independent studies and was one of only a few mutations in the *inhA* coding region, rather than its promoter, that were associated with INH resistance (2, 19, 24). Instead, our results showed that this mutation was a marker for *M. africanum* West African 2 (WA2) strains as opposed to drug resistance. This agrees with the MtbPDSP, which detected this change only in the WA2 strains 4141/04 and GM 0981 (6, 12, 14).

In summary, our results demonstrate that natural genetic diversity confounded a number of prior studies investigating resistance mechanisms in clinical MTBC isolates (2, 5, 24). Moreover, the fact that *inhA* is not fully conserved has further practical consequences beyond the genotype-phenotype relationship of INH/PRO cross-resistance. The Global Alliance for TB Drug Development is currently working with GlaxoSmith-Kline to develop a new inhibitor against *inhA* which has already entered the lead optimization stage (13). Given that the highest prevalence of WA2 strains is observed in countries with some of the highest prevalence of TB worldwide (e.g., in 2009, approximately 1,229 per 100,000 inhabitants of Sierra Leone suffered from active disease, 18% of which is estimated to be caused by WA2) (8, 17, 37), it is paramount that these lead compounds are tested against the mutants discovered in this study to exclude the possibility of intrinsic antibiotic resistance. The same applies to the recently discovered *inhA* inhibitors, which display bactericidal activities under both aerobic and anaerobic conditions but have only been tested against strains from the Euro-American lineage (11, 33).

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