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The Intrinsic Antibiotic Resistance to β-Lactams, Macrolides, and Fluoroquinolones of Mycobacteria Is Mediated by the *whiB7* and *tap* Genes

K. V. Shur[a, *], D. A. Maslov[a], N. E. Mikheecheva[a, b], N. I. Akimova[a], O. B. Bekker[a, **], and V. N. Danilenko[a]

[a] *Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, 119991 Russia*
[b] *Moscow Institute of Physics and Technology (State University), Dolgoprudny, Moscow oblast, 141700 Russia*
**e-mail: shurkirill@gmail.com*
***e-mail: obbekker@mail.ru*

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Abstract—The *M. tuberculosis* resistome includes a number of genes involved in intrinsic drug resistance. One of the major members of this system is the *whiB7* gene encoding a transcription factor, which regulates expression of a number of genes—its regulon. In this study, we analyze the impact on intrinsic drug resistance levels of the *whiB7* and *tap* genes and their prevalent mutants. The mutations found within *whiB7* and *tap* genes in the genomes of strains belonging to Beijing and EAI-Manila lineages allowed us to investigate the new role of these genes in the intrinsic drug resistance of mycobacteria to β-lactams, fluoroquinolones, and macrolides and to assume their significance in the development of *M. tuberculosis* genotypes. Here we also established that the mutation in the *tap* gene – insC_{581}, previously described, as a marker of the Beijing lineage, cannot be the marker due to its absence in a number of Beijing strains.

Keywords: Mycobacterium tuberculosis, model object *Mycobacterium smegmatis*, intrinsic drug resistance, *whiB7* gene, WhiB7 regulon, *tap* gene

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Mycobacterium tuberculosis is the causative agent of tuberculosis – one of the most dangerous diseases and the major cause of deaths due to bacterial infections. One of the main problems arising in the treatment of tuberculosis is the pathogen's drug resistance to existing antibiotics, which can be classified as intrinsic or acquired [1, 2]. Intrinsic drug resistance is mediated by a large number of genes and genetic systems, which compose the resistome [3]. The *M. tuberculosis* resistome includes genes encoding proteins of different classes: cellular transporters (IniBAC, EfpA, etc. [4]), target (Erm(37) [5], etc.) and drug's chemical structure (Eis [6], etc.) modifiers, stress response transcription factors (SoxRS, MarRAB, or Rob [7, 8]), and many others [9]. *whiB7* is one of the most important genes of the resistome. It encodes a transcriptional activator of the genes involved in the processes of drug resistance, virulence, and survival within the host macrophages [10, 11].

WhiB7 controls the expression of genes involved in virulence and survival within macrophages [12–14]. Studies using microarray analysis (DNA microarray) showed that the WhiB7 transcription factor upregulates the expression of 12 genes constituting the WhiB7 regulon and a system of intrinsic drug resistance to a wide range of antibiotics [15]. This system is an important element of the resistome in various *Actinobacteria* (*M. tuberculosis*, *M. bovis*, *M. africanum*, *S. lividans*, *M. smegmatis*, etc.) [15]. Now it is proven that only four out of 12 genes in the WhiB7 regulon take part in the implementation of drug resistance to antibiotics of different classes (macrolides, tetracyclines, lincosamide, pleuromutilin, phenicols, and aminoglycosides): *erm* [16, 17], *tap* [18, 19], *eis* [20], and *rv1473* [21, 22]. Activation of the transcription of these genes is realized through the binding of the A/T-hook at the C-terminus of the WhiB7 protein to the A/T-rich promoter region of the regulon's gene [15, 23]. When binding the promoter region of the regulon's gene, WhiB7 forms a complex with σ-factor A (SigA), which in turn becomes the initiator of the gene's transcription [22]. It was found that the regulatory region of the *whiB7* gene includes a similar A/T-rich site, indicating this gene's expression autoregulation [11].

Four genes of the regulon responsible for drug resistance can be divided into two functionally different classes: genes encoding cell transporters (*tap* [19]

Table 1. Bacterial strains and plasmids used in the work

Strains, plasmid vectors	Description	Reference
Strains		
M. smegmatis mc^2 155	Mutant strain with increased frequency of transformation	[29]
E. coli DH5α	Strain used for production of plasmids F- f 80 ΔlacZΔM15, Δ (lacZYA-argF), U169 (Promega)	[30]
Plasmid vectors		
pMIND	Shuttle expression vector with the origins of replication in the cells of *Mycobacterium* and *E. coli;* Hyg^r, Km^r	[31]
pMINDKM–	Modified pMIND with deletion of the kanamycin resistance gene; Km^r	[32]
pMWB7	pMINDKM-containing *whiB7* gene (*Bam*HI + *Spe*I)	
pMWB7M	pMINDKM-containing *whiB7*ΔG_{191} gene (*Bam*HI + *Spe*I)	
pMTAP	pMINDKM-containing *tap* gene (*Bam*HI + *Spe*I) from *M. tuberculosis*	
pMTAPM	pMINDKM-containing *tap* (insC_{581}) gene (*Bam*HI + *Spe*I) from *M. tuberculosis*	

and *rv1473* [21]) and genes encoding modifiers of the drug's chemical structure (*eis* encoding aminoglycoside acetyltransferase specific to kanamycin [24] and *erm* 23S rRNA (adenine-(2058)-N(6))-methyltransferase providing resistance to macrolides, lincosamides, and streptogramins) [15].

M. smegmatis mc^2 155 has a similar system of intrinsic drug resistance, but it has a larger number of resistance genes compared to *M. tuberculosis*. In addition, *M. smegmatis* is characterized by rapid growth (two to three days), nonpathogenicity, and effective transformation. These characteristics allow using *M. smegmatis* mc^2 155 as the model of *M. tuberculosis* to study the resistome genes [10].

In the present study, we focused on the *whiB7* and *tap* genes [25], whose products are involved not only in the implementation of the drug resistance but also in the processes of the immune response control and survival of mycobacteria within the host organism [26]. Search for and analysis of the emergence of mutations in the *tap* and *whiB7* genes of *M. tuberculosis* can be an effective way to study the foundations of the origin of resistance and virulence of the pathogen [27]. In addition, our work examines the relationship between the phenotype (drug resistance), genotype, and mutations in the *tap* and *whiB7* genes.

To achieve these goals, we used sequence data from clinical isolates of *M. tuberculosis*, as well as the sequence of the genomes of *M. tuberculosis* from the NCBI Genome database.

MATERIAL AND METHODS

Bacterial Strains, Plasmid Vectors, and Cultivation Conditions

Escherichia coli DH5α were cultivated in LB medium (Amresco, United States), with stirring at 200–250 rpm at 37°C. Strains of *M. smegmatis* were cultured on tryptone soya agar M290 (HiMedia, India) and in Lemco-TW (5 g/L bacto-peptone (Oxoid, United Kingdom), 5 g/L Lemco Powder (Oxoid, United Kingdom), 5 g/L NaCl and 0.05% Tween 80 (ICN Biomedicals, United States)) at 37°C. If necessary, antibiotics were added: ampicillin 150 μg/mL or hygromycin B (250 μg/mL for *E. coli* DH5α; 50 μg/mL for *M. smegmatis*). The strains and genetic constructs used are shown in Table 1.

Site-Directed Mutagenesis

We used the Nelson point mutations method [28]. The mutant *whiB7* gene with a deletion (delG_{191}) was made using primers carrying the mutation (whiB7Mt_dir and WhiB7_mix) and the terminal WhiB7Mt_mix_dir and WhiB7Mt_mix_rev (Table 2). The basis for the creation of the mutant gene were an artificially synthesized *whiB7* gene with codons that were adapted for optimal expression in *E. coli* cells and the sequence encoding the N-terminal A/T-hook, amplified from the genomic DNA of *M. tuberculosis* H37Rv.

Cloning

Cloning of the target genes was carried out using chemically competent cells of *E. coli* DH5α and electrocompetent *M. smegmatis* mc^2 155. Amplification

Table 2. Primers used in the work

Primers and their application	5' → 3' primer sequence	Restriction site
Primers for *whiB7* site-directed mutagenesis		
whiB7Mt_dir	TTTTGAATTCATGAGCGTGCTGACC	*Eco*RI
whiB7Mt_mix_dir	AACGCGCCGAGCCGTGGGCGTTTGGGGTGGTGA	
whiB7_mix-	TCACCACCCCAAACGCCCACGGCTCGGCGCGTT	
whiB7Mt_mix_rev	TTTTAAGCTTGACTCACGATCGAGCGCTCGATCGTGAGTCAAGCTTAAAA	*Hind*III
Primers for Sanger sequencing		
WB7sLF	TGGCAGAGATCATCCGTC	
WB7sLR	GCAGGTCAGAAAATCGGT	
Rv1258-1F	GAAGGCACGGCGCTTGACGAC	
Rv1258-1R	CGCGATCATCAAGCCACCGAT	
Rv1258-2F	ATGACGGCTCGTGACTCGATGCT	
Rv1258-2R	CGACCAGCCCGGTGCACACGATG	
Rv1258-3F	TCGGCCTGGTGTACGGACCCATC	
Rv1258-3R	AAGGGTCAAAGGCCCGGTCTTGC	
Primers for cloning		
pM-wb7-f	GACAGGATCCGGAGGAAATGTTATGAGCGTGCTGACCGTTCC	*Bam*HI
pM-wb7-r	ATGCACTAGTTTACGCCACGGCATCTTTACGCG	*Spe*I
pM-wb7M-r	ATGCACTAGTTTAGACTCACGATCGAGCCTTGGT	*Spe*I
PM1258_F	GACAGGATCCGGAGGAAATGTTATGAGAAACAGCAACCGCGG	*Bam*HI
PM1258_R	ATGCACTAGTTTATCACTGAGCCGATCCTACGGG	*Spe*I

(PCR) was conducted with the High Fidelity PCR Enzyme Mix (Thermo Fisher Scientific, United States) according to the manufacturer's manual. For restriction, Thermo Fisher Scientific restriction enzymes were used according to the manufacturer's manual. Ligation of restricted DNA fragments into the vectors was performed using T4 DNA ligase (Thermo Fisher Scientific) for one hour at 22°C. Sanger sequencing of the obtained genetic constructions was carried out in SRI PCM FMBA of Russia (http://rcpcm.org/). If necessary, additional primers were used for sequencing. All the oligonucleotides used are presented in Table 2.

In Silico Analysis

An in-house algorithm based on BLAST was used [33] (Perl programming language (https://www.perl.org/)) for the analysis of mutations; the genomes of *M. tuberculosis* deposited in GenBank were used [34]. Only those DNA sequences for which it was possible to determine the phylogenetic strain affiliation (1741 strains) were selected for the research. The identification of different lineages was based on the identification of specific SNP in the toxin–antitoxin systems' genes in *M. tuberculosis* [11, 28]. The genome of *M. tuberculosis* strain H37Rv was used as the reference genome for the analysis of mutations *in silico*.

M. tuberculosis Clinical Isolates Analysis

The present study analyzed DNA material previously described by Maslov et al. [35]. This collection includes 65 samples of DNA extracted from clinical isolates of *M. tuberculosis* divided into two groups: group A—41 samples characterized by the resistance to at least one antibiotic; group B—24 control samples sensitive to antibiotics. All the isolates were typed with spoligotyping and by analysis of mutations in the toxin–antitoxin systems' genes [35, 36]. The collection includes 43 isolates belonging to the Beijing-modern lineage (16 of which belong to Beijing B0/W-148), one to X, two to Ural, and 19 to LAM9.

Statistical Analysis

The Pearson correlation coefficient (r) was used to assess the connection between detected mutations and phenotype of drug resistance/belonging to a specific lineage.

Determination of Drug Resistance

The level of *M. smegmatis* drug susceptibility was assessed by the paper disk method [37], as well as by searching for minimum inhibitory concentrations (MIC) of antibiotics in liquid medium.

The culture of *M. smegmatis* cells was grown in liquid Lemco-TW medium with the addition of selective antibiotic hygromycin B (Sigma-Aldrich, United States) at a concentration of 50 μg/mL and tetracycline as an inducer at a concentration of 20 ng/mL (Sigma-Aldrich, United States). The cell culture was grown to optical density $OD_{600} = 1.2$, and 5 mL of 20-fold dilution (1 part of cell suspension, 9 parts of sterile water, and 10 parts of M290 medium) was used as a top solid layer for dishes with M290 medium. Sterile paper disks ($d = 7$ mm) containing different antibiotics were placed on the surface of the upper layer of the dish with the analyzed culture of *M. smegmatis*.

To determine minimum inhibitory concentrations of *M. smegmatis* growth, the method of serial twofold dilutions of antibiotics in liquid Lemco-TW medium (0.1% Tween 80) was used. The growth or its absence was evaluated visually and confirmed by spectrophotometry after 24 h of incubation at 37°C.

All experiments for determination of drug susceptibility were performed in three independent repeats. Due to the fact that the method of determining the MIC in a liquid medium has a low resolution and does not assess a low level of resistance, it was used to test the control antibiotics (chloramphenicol for WhiB7 and streptomycin for Tap) and to assess the level of drug susceptibility of *M. smegmatis* transformants obtained by the method of paper disks.

RESULTS

Search for Whib7 Orthologs in M. smegmatis mc² 155

To confirm the adequacy of the selected model organism and to study the role of allelic variants of the *whiB7* and *tap* genes, orthologous genes of *tap* and *whiB7* from *M. smegmatis* mc² 155 were sought.

From published data, it is known that the *whiB7* ortholog in *M. smegmatis* mc² 155 is *MSMEG_1953* [38]. Analysis of the structural features of this ortholog related it to [Fe–Su]-dependent transcription factors with A/T-hook to bind to the promoter region of the regulated gene (Fig. 1). Thus, the genome of *M. smegmatis* contains the *MSMEG_1953* gene, being orthologous to *whiB7* of *M. tuberculosis*. The genome of *M. smegmatis* mc² 155 contains other orthologs of the WhiB7 regulon genes of *M. tuberculosis*: *MSMEG_1646* (*erm38*), *MSMEG_3140* (*rv1473*), *MSMEG_3513* (*eis*) [10, 39]; however, there is no functional ortholog of the *tap* gene. The gene *MSMEG_5033* is annotated as pseudogene and contains a shift of the reading frame in the 95th codon (from 419).

Search for Genetic Polymorphism of the WhiB7 Regulon among the Sequenced Genomes of M. tuberculosis

The analysis of 1741 sequences of various *M. tuberculosis* isolates' genomic DNA revealed the presence of polymorphism in *whiB7* and *tap* genes. The number of strains with mutations belonging to different lin-

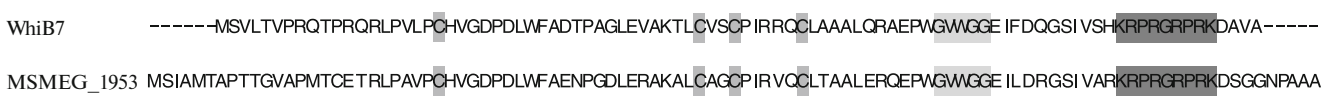

Fig. 1. Alignment of orthologous proteins WhiB7 from *M. tuberculosis* and MSMEG_1953 from *M. smegmatis* mc^2 155. Four conservative cysteine residues are highlighted in light gray, the tryptophan-containing/glycine-rich motif is highlighted in gray, and DNA-binding A/T-hook is highlighted in dark gray.

eages is represented in Table 3. Specific mutations and their positions are shown in Fig. 2.

Due to the fact that *whiB7* is an ancient and conservative gene providing natural drug resistance, in the studied sample, mutations in the gene *whiB7* were discovered only in 25 genomes. For analysis, we used the NCBI Genome database (http://www.ncbi.nlm.nih.gov/genome/genomes/166). For each genome, phylogenetic affiliation was established by analyzing the toxin–antitoxin systems' genes [36]. Isolates (558) from the Beijing line, 500 LAM, 199 EAI, and 565 isolates from other lines were genotyped (see Table 3). Analysis of SNPs allowed us to identify only one mutation potentially influencing the functional activity of WhiB7: a deletion at the position G_{191}. The G_{191} deletion causes the shift of the reading frame immediately before the A/T-hook. Thus, the mutant protein loses the ability to initiate transcription. This mutation is characteristic of the representatives of the EAI-Manila lineage with a correlation coefficient $r = 0.92$ (95% confidence interval 0.91–0.93, p-value < 0.001). Polymorphic variants in which mutational changes do not affect important structural elements of the WhiB7 protein were not included in further analysis.

Similarly, the search for mutations in the *tap* gene was conducted. The greatest percentage of mutations was observed in the genomes of *M. tuberculosis* belonging to the Beijing line. Detailed analysis

Table 3. SNPs in the *whiB7* and *tap* genes of *M. tuberculosis*

Line	Sublinage	Number of strains	Number of strains with SNPs* in *tap* gene	Percentage of SNPs in sublinage	Number of strains with SNPs* in *whiB7* gene
Beijing	Beijing-ancestral	175	34	19.4	
	Beijing-modern	297	296	100.0	4
	Beijing-modern-B0/W148	86	85	100.0	
Cameroon		6		0.0	
Haarlem		49	5 (4** + 1)	10.2	
X		80	2	2.5	
LAM	LAM	311	2	1.3	
	F15/LAM4/KZN	150		0.0	2
	LAM1	30	7	22.6	
	LAM2	9		0.0	
SMI		5		0.0	
Ural		83		0.0	
Ghana		37		0.0	
S		109		0.0	
EAI	EAI	180	25 (17 + 8)	13.9	3
	EAI-Manila	19	19**	100.0	19
Deli-CAS		103	2	1.9	
H37Rv-like		12		0.0	
	TOTAL	1741	477	30.2	28

* Nonsynonymous SNPs.
** Synonymous SNPs.

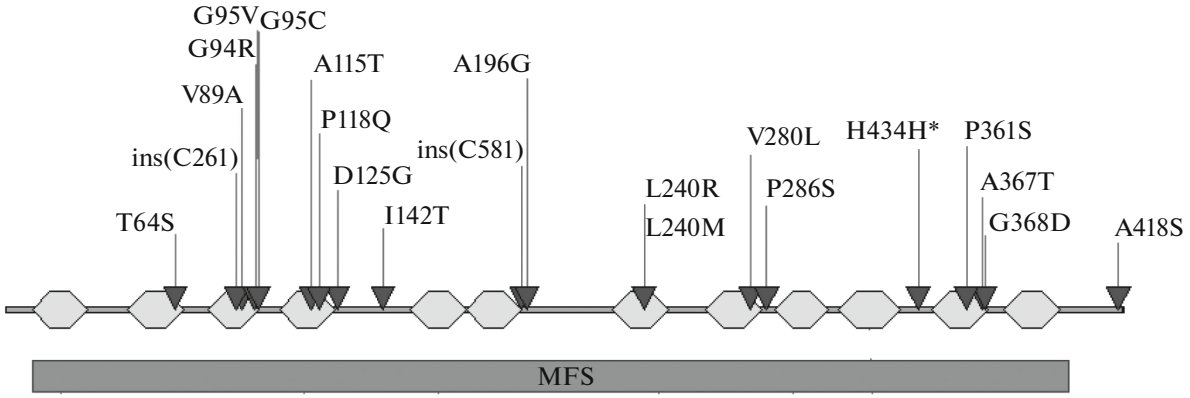

Fig. 2. Map of mutations in the *tap* gene. Structural elements of the Tap protein (419aa): hexagons—transmembrane domains; gray color indicates the sequence of the MFS (major facilitator superfamily) domain; arrows indicate replacements of amino acids.

demonstrated that the most common mutation in *tap* was C_{581} insertion ($r = 0.81$, 95% confidence interval 0.79–0.82, p-value <0.001). This mutation may be a phylogenetic marker of the separation of the strain into sublineages inside the Beijing cluster, as well as a marker of the microevolution of intrinsic drug resistance: 19.4% of isolates have a mutation in C_{581} in the Beijing-ancestral subline and 100% in younger sublineages Beijing-modern and -B0/W148. In addition to the insertion associated with a Beijing lineage, a number of other SNPs were discovered, some of which may be associated with certain lineages/sublineages. In eight genomes, mutation T(504)A ($r = 0.22$, 95% confidence interval 0.18–0.27, p-value < 0.001) was detected, and in 17 genomes G(838)C ($r = 0.29$, 95% confidence interval 0.25–0.33, p-value < 0.001) in the EAI line; T(1029)C, in the EAI-Manila ($r = 0.97$, 95% confidence interval 0.97–0.98, p-value < 0.001); and T(528)C, in four isolates of the Haarlem line ($r = 0.28$, 95% confidence interval 0.24–0.32, p-value < 0.001). Detection frequencies of the other mutations were not statistically significantly connected with the *M. tuberculosis* lineages (Table 3).

Search for whiB7 and tap Gene Polymorphism in the Collection of M. tuberculosis Clinical Isolates

Analysis of the collection of 64 clinical isolates of *M. tuberculosis* with different levels of drug resistance, genotypes, and other factors [35] did not reveal mutations in the *whiB7* gene, which also confirms that this gene sequence is highly conservative. Analysis of *tap* gene sequences showed the presence of the C_{581} insertion in strains from the Beijing lineage ($r = 0.96$), which corresponds to data obtained for 1741 strains of *M. tuberculosis*.

Study of the Effect of Mutations in the whiB7 and tap Genes on M. smegmatis Drug Susceptibility

In silico analysis of the mutant *whiB7*ΔG_{191} showed that the shift of the reading frame led to the loss of the A/T-hook and, as a consequence, to the loss of the ability to form a protein–DNA complex.

M. smegmatis transformants containing the plasmid vectors pMWB7 and pMWB7M (including *whiB7* and *whiB7*ΔG_{191} genes) with overexpression of *whiB7* gene were analyzed using the paper disks method for changes in the level of the resistance to antibiotics of different chemical classes (β-lactams, tetracyclines, macrolides, lincosamides, fluoroquinolones, aminoglycosides, etc.). The transformants containing the *whiB7* gene of *M. tuberculosis* showed an increase in the level of drug resistance to aminoglycosides, macrolides, tetracyclines, chloramphenicol, and β-laktam antibiotic imipenem. The drug susceptibility of the cells carrying the plasmid vector with mutant variant of the *whiB7* gene remained at the level of the negative control (plasmid without the gene *whiB7*) (Fig. 3a). At the moment, the gene of WhiB7 regulon responsible for the drug resistance to β-lactam antibiotics is not identified.

M. smegmatis mc^2 155 has a nonfunctional sequence of the *tap* gene in its genome. Thus, the presence of the nonfunctional ortholog in the *M. smegmatis* genome allows evaluating the spectrum of drug resistance defined by this cellular transporter in the absence of *tap* overexpression.

According to the study of the drug resistance, it was discovered that Tap defined a slightly different range of resistance than was previously described. In addition to the classic targets—streptomycin (aminoglycoside) and tetracycline [19, 22, 40]—there was an increase in drug resistance to macrolides and fluoroquinolones in *M. smegmatis* transformants containing the gene of functionally active Tap protein from *M. tuberculosis* (Fig. 3b).

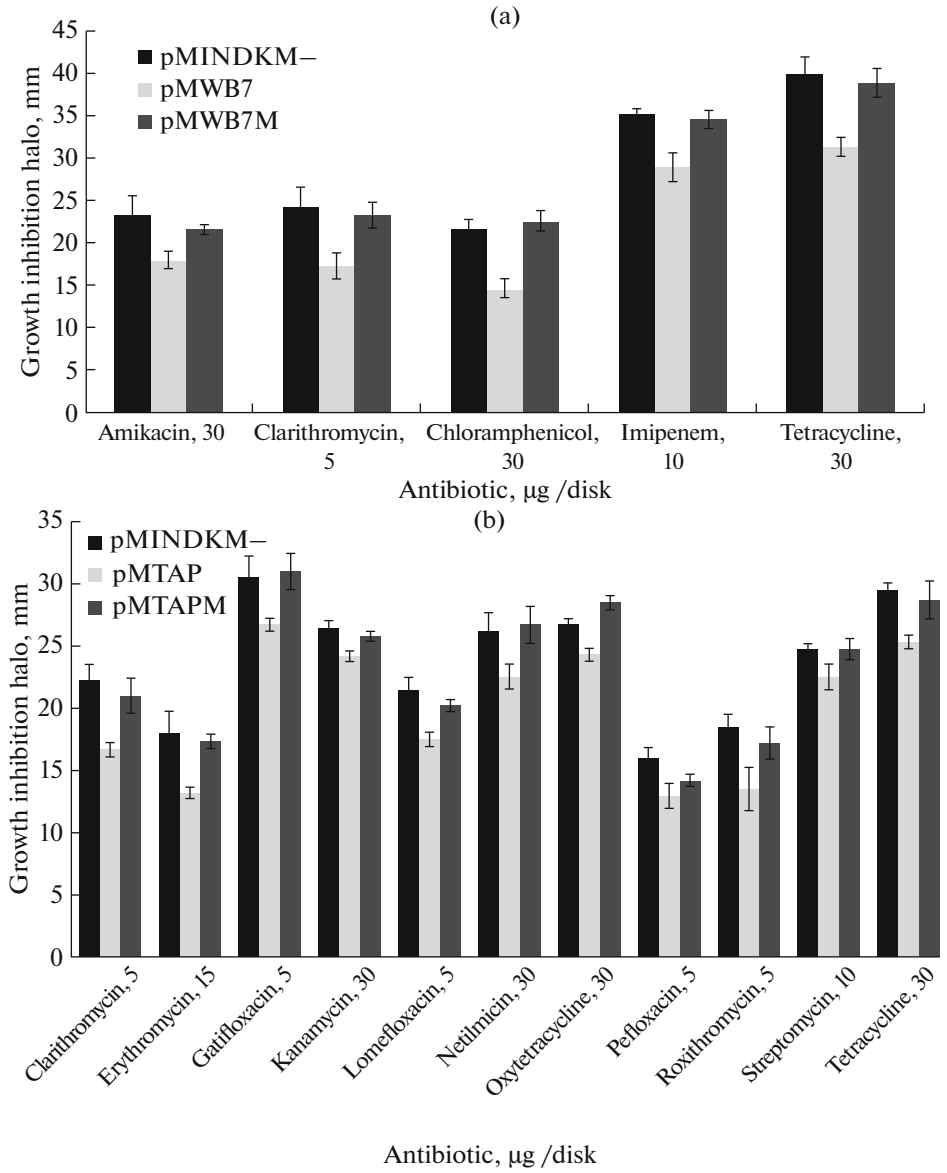

Fig. 3. Diameter of the growth inhibition halo of *M. smegmatis* transformants. Error bar—standard deviation. (a) Allelic variants of the *whib7* gene; (b) allelic variants of the *tap* gene.

MICs for cell cultures of *M. smegmatis* transformants containing constructs with *whiB7* or *tap* genes from *M. tuberculosis* were defined to assess the level of drug resistance defined by WhiB7 and Tap (Table 4). Any changes in drug sensitivity to fluoroquinolones, kanamycin and β-lactam antibiotics were not detected. This difference can be explained by the fact that the method of determining the MIC in a liquid medium makes it possible to detect a twofold increase/attenuation of drug sensitivity to the antibiotic, while the method of paper disks makes it possible to quantify the level of drug sensitivity to capture even weak changes.

DISCUSSION

Today it is known that different isolates of *M. tuberculosis* belong to various genotype lineages with unique phylogenetic markers and phenotype characteristics. The currently created model of dividing *M. tuberculosis* into genotypes requires additions and corrections in a connection with the development of closed region-associated lineages as well as for studying mechanisms and ways of evolution of successful genotypes (Beijing, LAM, etc.). In recent works, Köser et al. and Villellas et al. [41, 42] showed new phylogenetic markers (insertion in the *tap* gene) for identification of isolates from Beijing-modern sub-

Table 4. Determination of MICs (μg/mL) of antibiotics for *M. smegmatis* strains

Transformant	Cm	Clr	Stm	Ofx	Tet
pMINDKM–	32	0.25	0.125	0.5	0.0625
pMWB7	64	0.5	–	–	0.125
pMWB7M	32	0.25	–	–	0.0625
pMTAP	–	0.5	0.25	0.5	–
pMTAPM	–	0.25	0.125	0.5	–

Cm—chloramphenicol; Clr—clarithromycin; Stm—streptomycin; Ofx—ofloxacin; Tet—tetracycline.

line. In the present work, we showed that, although such insertion was typical of the Beijing line ($r = 0.81$), its presence could not be informative for intragroup phylogeny of the *M. tuberculosis* isolates of the Beijing line. Apparently, on the basis of different representation of this mutation among Beijing sublines (~18% in Beijing-ancestral and >99% in Beijing-modern and Beijing-B0/W148), we can assume that this mutation is a marker of the evolution of the Beijing genotype from a single ancient clone of the Beijing-ancestral to the contemporary Beijing-modern and Beijing-B0/W148 sublines that emerged in the era of antibiotics. The search for genetic markers that can split existing lines into sublinages using functional markers is promising. Such an example can be a mutation in the *whiB7* gene with a high coefficient of correlation with the line EAI-Manila ($r = 0.92$). This functional marker may indicate the development of an isolated Philippine population of drug sensitive strains and their origin from a single clone with the mutation.

Interestingly, young Beijing sublineages with the insertion in the *tap* gene (Beijing-modern and Beijing-B0/W148) develop rapidly and spread quickly. This allows concluding that the intrinsic drug resistance is controlled by other genes at a higher level. Perhaps, functionally useful *tap* gene was necessary for *M. tuberculosis* ancestors for protection from antibiotics produced by soil bacteria (e.g., *Streptomyces* spp.) and now can be considered as an atavism. The study of genes of the *M. tuberculosis* resistome could be an alternative, as well as the investigation of the relationship between mutations in these genes and the occurrence of induced drug resistance, virulence, transmissibility, etc.

The obtained data on changes in drug susceptibility in the transformants containing the *whiB7* and *tap* genes allowed extending the knowledge on the spectrum of intrinsic drug resistance defined by the WhiB7 regulon. Data on resistance to β-lactam antibiotics, macrolides, and fluoroquinolones should be further explored at the transcriptional level [40]. It is also necessary to establish the role of the WhiB7 regulon in the process of the implementation of drug resistance, namely, as the factor of cross-resistance in the presence of antibiotics of other stresses. Our study showed the necessity of an antistress system in the survival of bacteria, but the evolutionary-phylogenetic analysis showed that the evolution of the Beijing line is "crowding out" the genes of the resistome while developing virulence and transmissibility systems [42].

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