WHOLE-GENOME SEQUENCING AND COMPARATIVE GENOMIC ANALYSIS OF *MYCOBACTERIUM SMEGMATIS* MUTANTS RESISTANT TO IMIDAZO[1,2-*b*][1,2,4,5]TETRAZINES, ANTITUBERCULOSIS DRUG CANDIDATES

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The spread of multidrug and extensively drug-resistant *Mycobacterium tuberculosis* urges the development of novel antituberculosis drugs. Previously, we studied the compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5] tetrazines capable of inhibiting serine/threonine protein kinases (STPK) in the original *M. smegmatis aphVIII+* test-system. To unveil the mechanism of action of drug candidates, it is necessary to search for mutations in the mycobacterial genome that confer resistance to these compounds. The aim of our work was to find and describe such mutations in *M. smegmatis* strains. We carried out the whole-genome sequencing of 9 mutants resistant to 3 imidazo[1,2-*b*][1,2,4,5]tetrazines. Seven of 9 mutant strains were found to have the Y52H mutation in the highly conserved mycobacterial gene *MSMEG_1601* encoding a protein with an unknown function. Additionally, three of those 7 strains were shown to have two mutations in the *MSMEG_1380* encoding a transcriptional regulator. The remaining 2 mutant strains had mutations in *MSMEG_0641* and *MSMEG_2087* genes encoding transporter-proteins. No mutations were found in STPK genes, meaning that they might be not the primary targets of the studied compounds. Further investigation of MSMEG_1601 function may be of interest as this protein might be the biological target or a part of a new mechanism underlying resistance to antituberculosis drug candidates.

Keywords: *Mycobacterium smegmatis*, drug resistance, resistance mutations, whole-genome sequencing, substituted imidazotetrazines, tuberculosis

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ПОЛНОГЕНОМНОЕ СЕКВЕНИРОВАНИЕ И СРАВНИТЕЛЬНЫЙ ГЕНОМНЫЙ АНАЛИЗ МУТАНТОВ *MYCOBACTERIUM SMEGMATIS*, УСТОЙЧИВЫХ К СОЕДИНЕНИЯМ КЛАССА ЗАМЕЩЕННЫХ ИМИДАЗО[1,2-*b*][1,2,4,5] ТЕТРАЗИНОВ – КАНДИДАТОВ В ПРОТИВОТУБЕРКУЛЕЗНЫЕ ПРЕПАРАТЫ

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Распространение штаммов *Mycobacterium tuberculosis* с множественной и широкой лекарственной устойчивостью требует разработки новых противотуберкулезных препаратов. Ранее нами были исследованы соединения класса замещенных имидазо[1,2-*b*][1,2,4,5]тетразинов, показавшие способность ингибировать серин-треониновые протеинкиназы в оригинальной тест-системе *M. smegmatis aphVIII+*. Для определения механизма действия кандидатов в лекарственные препараты необходимо исследование мутаций в геноме микобактерий, приводящих к устойчивости к этим препаратам. Целью работы было найти и охарактеризовать мутации, определяющие устойчивость штаммов *M. smegmatis*. Проводили полногеномное секвенирование девяти мутантов, устойчивых к трем соединениям класса замещенных имидазо[1,2-*b*] [1,2,4,5]тетразинов. В семи из девяти мутантных штаммов обнаружена мутация (Y52H) в гене *MSMEG_1601*, кодирующем белок с неизвестной функцией и являющемся консервативным для микобактерий, причем в трех штаммах дополнительно обнаружены две мутации в гене *MSMEG_1380*, кодирующем транскрипционный регулятор. В двух оставшихся мутантных штаммах обнаружены мутации в генах *MSMEG_0641* и *MSMEG_2087*, кодирующих белки-транспортеры. Мутаций в генах, кодирующих СТПК, обнаружено не было. Вероятно, они не являются основными мишенями исследуемых соединений. Дальнейшее изучение функции белка MSMEG_1601 представляет интерес в случае, если этот белок является новой биомишенью, либо частью нового механизма реализации устойчивости к потенциальным противотуберкулезным препаратам.

Ключевые слова: *Mycobacterium smegmatis*, лекарственная устойчивость, мутации устойчивости, полногеномное секвенирование, замещенные имидазотетразины, туберкулез

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According to the World Health Organization, over 2 billion people (1/3 of the world population) are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), one of the deadliest infectious diseases that kills 10.8 million people every year [1]. The key challenge in the fight against TB is the emergence and spread of mycobacterial strains resistant to both rifampicin and isoniazid (multidrug-resistant TB, MDR-TB) and those additionally resistant to fluoroquinolones and one of the second-line injectable drugs (extensively drug-resistant TB, XDR-TB) [2, 3]. Therefore, the development of antituberculosis drugs with a novel mechanism of action is a key objective in fighting TB.

Previously, we studied the antimycobacterial activity of compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines [4] that showed inhibiting activity on mycobacterial serine/threonine protein kinases (STPK) in the original validated test-system *M. smegmatis aphVIII+* [5]. However, to confirm the mechanism of action of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines, as well as the mechanism underlying resistance to these compounds, it was necessary to identify resistance-conferring mutations using *M. smegmatis* as a model organism [6].

The aim of this study was to sequence *M. smegmatis* mutants resistant to 3 compounds (TSV-395, TSV-402 and NIK-1283) representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines and to carry out their comparative genomic analysis.

METHODS

Mycobacterial strains and culturing

For this study we selected the following mycobacterial strains: 1) *M. smegmatis mc2* 155 (wild type); 2) *M. smegmatis* at^R*8, at*^{R}*9, at*^{R}*10* resistant to TSV-395; 3) *M. smegmatis* at^R*1, at*^{R}*2, at*^{R}*11* resistant to TSV-402; 4) *M. smegmatis* at^R*14, at*^{R}*17, at*^{R}*19* resistant to NIK-1283. The selected mutant strains exhibited cross-resistance to all three tested compounds.

Mycobacteria were grown in the liquid Middlebrook 7H9 broth (Himedia, India) supplemented with OADC (Himedia, India), 0.1% Tween-80 and 0.1% glycerol at 37 °C and 250 r/min.

DNA isolation and whole-genome sequencing

Mycobacterial DNA was isolated from 15 ml of the liquid culture according to the protocol described in [7]. After preliminary isolation, DNA was treated with RNase A (Thermo Fischer Scientific, USA) and extracted in the phenol-chloroform-isoamyl alcohol solution (25 : 24 : 1).

DNA libraries were prepared using Nextera kits (Illumina, USA); sequencing was carried out on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 2 x 315 bp (Illumina, USA). Sequencing of the wild-type strain genomic DNA was conducted with the MiSeq Reagent Kit v2 2x150 bp (Illumina, USA). The obtained data were submitted to the NCBI Sequence Read Archive (SRA) (entry ID SRP145443).

Processing of whole-genome sequencing data and comparative genomic analysis

The obtained reads were aligned to the reference genome (NC_008596.1, PRJNA57701) using the BWA-MEM algorithm [8]. The pileup was generated by mpileup (-B -f) in SAMtools [9]. Single nucleotide variants were called by running mpileup2snp (--min-avg-qual 30 --min-var-freq 0.80 --p-value 0.01 --output-vcf 1) in VarScan 2.3.9 [10]. Annotation was created using vcf_annotate.pl (courtesy of Natalya Mikheecheva of the Laboratory of Bacterial Genetics, Vavilov Institute of General Genetics). The non-synonymous single nucleotide variants found within open reading frames and absent in the wild-type strain were selected for further analysis. The similarity search was conducted in BLAST (https://blast.ncbi.nlm.nih.gov).

RESULTS

Comparative genomic analysis

After genome assembly, we conducted a comparative genomic analysis of mutant and wild-type strains. The following unique single nucleotide polymorphisms were identified:

1) CGT to AGT substitution in codon 233 (R>S) of *MSMEG_0641* (binding-protein-dependent transporters inner membrane component) in the mutant at^{R}10;

2) ACG to GTG substitution in codon 52 (T>V) of *MSMEG_1380* (transcriptional regulator) in the mutant at^{R}19;

3) insertions of VG amino acids at position 51 of *MSMEG_1380* (transcriptional regulator) in the mutants at^{R}11 and at^{R}17;

4) TAC to CAC substitution in codon 52 (Y>H) of *MSMEG_1601* (hypothetical protein) in the mutants at^{R}1, at^{R}2, at^{R}8, at^{R}11, at^{R}14, at^{R}17, and at^{R}19;

5) TAC to TGC substitution in codon 188 (Y>C) of *MSMEG_2087* (transporter small conductance mechanosensitive ion channel (MscS) family protein) in the mutant at^{R}9.

Genes containing the above-mentioned mutations are not pseudogenes but the functions of the proteins they encode have not been confirmed experimentally.

Identification of homologous genes in the genome of *M. tuberculosis*

The similarity search carried out in BLAST returned the homologs of *M. tuberculosis* proteins with the above-mentioned mutations (Table).

DISCUSSION

The crucial phase in the development of any novel antibacterial drug is the study of its mechanism of action. Obtaining mutants resistant to the studied compound and the identification of mutations underlying this resistance is a classical approach to the detection of possible targets for an antibiotic. We have conducted the comparative genomic analysis of 9 mutants

Table. Characteristics of the closest homologs of *M. tuberculosis* proteins with the mutations that presumably confer resistance to antituberculosis drugs

Protein	Family	Function	The closest homolog in *M. tuberculosis* (gene locus)	Identity of the amino acid sequence (%)	Amino acid sequence coverage (%)
MSMEG_0641	DppC ABC transporters	Transport of amino acids and inorganic compounds	*dppB (rv3665c)*	35	98
MSMEG_1380	AcrR/TetR_N	Transcriptional regulators	*rv0067c*	33	71
MSMEG_1601	Unknown	Unknown	*rv3412c*	87	100
MSMEG_2087	MscS	Mechanosensitive ion channels	*rv3104c*	69	89

cross-resistant to all three studied compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines. Having analyzed the mutants' genomes, we selected the most plausible drivers of drug resistance: 5 mutations in 4 genes.

Two mutations were identified in genes encoding a transmembrane transporter (MSMEG_0641) and a mechanosensitive channel (MSMEG_2087); these mutations can affect transport of the studied compounds into and out of the cell. Two mutations were found in the *MSMEG_1380* gene encoding a TetR family transcriptional regulator. TetR proteins can participate in the regulation of drug resistance by controlling expression of different membrane transporters. For example, the TetR protein of *M. abscessus* activates expression of cell transporters MmpS5/MmpL5 implicated in the resistance to thioacetazone derivatives [11].

Of all the identified mutations, the most promising for further research might be the mutation in the *MSMEG_1601* gene, as it is present in 7 out of 9 mutants. This is a highly conserved mycobacterial gene: it is found in all representatives of the *Mycobacterium* genus, including *M. leprae* with its very reduced genome, and in some other actinobacteria, and belongs to the so called "mycobacterial core hypotheticals" (highly conserved proteins with unknown functions) [12], though it is not vital for the growth of mycobacteria *in vitro* [13]. The proteomic analysis of different *M. tuberculosis* lineages demonstrated that the Rv3412 protein homologous to MSMEG_1601 is found in greater abundance in virulent strains, including a LAM strain, in comparison with attenuated strains of *M. bovis* BCG. This allowed the authors to suppose a possible implication of the Rv3412 protein in the infection process [14].

CONCLUSIONS

We have discovered 5 mutations in 4 genes that possibly confer resistance to substituted imidazo[1,2-*b*][1,2,4,5]tetrazines. The contribution of each mutations is yet to be confirmed by reverse genetics. However, it is already clear that one of them located within the *MSMEG_1601* gene represents a certain interest: unlike other mutant genes, *MSMEG_1601* is not linked to transmembrane transport and might be a direct biological target for substituted imidazo[1,2-*b*][1,2,4,5]tetrazines.

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