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Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance

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***Objectives*: The high prevalence of isoniazid-resistant *Mycobacterium tuberculosis* is often explained by a high mutation rate for this trait, although detailed information to support this theory is absent. We studied the development of isoniazid resistance *in vitro*, making use of a laboratory strain of *M. tuberculosis*.**

***Methods*: Spontaneous isoniazid-resistant mutants were characterized by molecular methods allowing identification of the most commonly encountered resistance-conferring mutations. Additionally, we determined the *in vitro* mutation rates for isoniazid and rifampicin resistance, and characterized the genome of a triple-resistant strain.**

***Results*: Results confirm that the *in vitro* mutation rate for isoniazid resistance (3.2×10^{-7} mutations/cell division) is much higher than the rate for rifampicin resistance (9.8×10^{-9} mutations/cell division). However, in the majority of the *in vitro* mutants *katG* was partially or completely deleted and neither of the two most common *in vivo* mutations, *katG*-S315T or *inhA*-C(-)15T, were found in 120 isogenic mutants. This implies that clinically prevalent resistance mutations were present in <0.8% of isoniazid-resistant strains selected *in vitro* (95% CI 0%–2.5%). The triple-resistant strain had acquired isoniazid resistance via a 49 kbp deletion, which included *katG*. Apart from previously identified resistance-conferring mutations, three additional point mutations were acquired during sequential selection steps.**

***Conclusions*: These outcomes demonstrate that the *in vivo* mechanism of isoniazid resistance is not reflected by *in vitro* experiments. We therefore conclude that the high *in vitro* mutation rate for isoniazid resistance is not a satisfactory explanation for the fact that isoniazid monoresistance is significantly more widespread than monoresistance to rifampicin.**

Keywords: tuberculosis, drug resistance, mutation rate

Introduction

Although the global incidence of tuberculosis (TB) is declining, drug resistance is rapidly emerging and spreading.[1] Insufficient control measures have led to a drastic increase in the prevalence of drug-resistant strains and, more importantly, the degree of drug resistance; in certain high-burden countries the nature of multidrug-resistant TB (MDR-TB) has rapidly evolved from incidental to epidemic.[1]

The production of new and more effective antibiotics partially addresses this problem, but without a more detailed comprehension of the molecular mechanisms that lead to successful (M)DR strains, these new compounds may also be quickly rendered ineffective by the generation of extensively drug-resistant TB (XDR-TB).

Isoniazid and rifampicin are the two most commonly used anti-TB drugs and the backbone of any successful TB treatment programme. MDR-TB is consequently defined as TB that is resistant to at least isoniazid and rifampicin. Monoresistance to isoniazid is significantly more prevalent than monoresistance to rifampicin and MDR-TB; based on the most recent data it is estimated that 13.3% of all TB cases globally involve isoniazid

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monoresistance, versus 0.6% rifampicin monoresistance and 5.3% MDR-TB cases.[1]

Shortly after the introduction of isoniazid in the 1950s, the first resistant *Mycobacterium tuberculosis* mutants were isolated and it was observed that many of these strains had reduced catalase activity.[2] It was first assumed that deletion of *katG*, coding for the only identified catalase–peroxidase in *M. tuberculosis*, was the main mechanism responsible for resistance to isoniazid.[3] However, subsequent studies demonstrated that, although conferring high levels of resistance, this is not the primary mechanism.[4–7] Although any mutation reducing KatG activity results in resistance, relatively few mutations in *katG* account for the majority of isoniazid resistance in clinical isolates; resistance to isoniazid is primarily conferred by the mutations *katG*-S315T (45%–75%) and *inhA*-C(-)15T (15%–25%).[8–13]

Biochemical and *in vitro* studies have shown that the most prevalent mutant, *katG*-S315T, still retains a significant part of its catalase activity,[7,14–17] which appears to be sufficient for the preservation of virulence.[18,19] Strains carrying this mutation typically have an intermediate (1–5 mg/L) level of resistance to isoniazid,[11,15,20,21] since KatG has reduced abilities to activate isoniazid, but has not completely lost this function. Mutations in the regulatory region of *inhA* usually lead to a low-level resistance (0.2–1.0 mg/L).[15,21]

Mutations that lead to complete loss of function of KatG, such as deletions, insertions or other frameshifts, are found in a small proportion of the isoniazid-resistant strains.[22] On average, partial or complete *katG* deletion mutants are reported in only ~5% of resistant isolates,[13,15,20,23,24] but higher proportions have been reported on occasion.[5,6] Additionally, strains with isoniazid resistance due to deletion of the *katG* gene are less likely to be clustered than strains with the *katG*-S315T mutation, a mutation significantly associated with successful dissemination.[12,13,25,26]

Isolates resistant due to inactivation of KatG often have additional mutations in the regulatory region or coding sequence of *ahpC*, coding for an alkylhydroperoxidase.[27,28] Originally, researchers assumed these mutations were alternative mechanisms of isoniazid resistance,[29,30] but subsequent studies showed that the majority of the mutations in *ahpC* or the intergenic region *oxyR-ahpC* were compensating for the loss of *katG* function.[31,32]

It is frequently assumed that the mutation rate for isoniazid resistance is ~100 times higher than for rifampicin and, consequently, that isoniazid resistance precedes resistance to rifampicin in the development of MDR-TB. However, there are no empirical grounds for these assumptions. We therefore studied the emergence of isoniazid resistance *in vitro*, making use of well-characterized *M. tuberculosis* laboratory strains. We determined the *in vitro* mutation rate to isoniazid and characterized spontaneous isoniazid-resistant mutants by sequencing, multiplex ligation-dependent probe amplification (MLPA)[33] and comparative genome hybridization (CGH).

Materials and methods

Growth of bacteria

The *M. tuberculosis* parent strains that all experiments were conducted with, MTB72 (ATCC 35801) and H37Rv (ATCC 27294), were initially susceptible to all antimicrobials used in this study. Bacteria were cultured in Middlebrook medium 7H9 (Difco, BD, Sparks, MD, USA), supplemented with oleic acid/albumin/dextrose/catalase (OADC Enrichment, BD, Sparks, MD, USA), in a shaking incubator at 37°C. A liquid starting culture was made by inoculating pure colonies from Löwenstein–Jensen or Coletsos slopes into 10 mL of culture medium. When these cultures reached the logarithmic growth phase, 0.5 mL was transferred to 9.5 mL of fresh, non-selective medium and bacteria were cultured until mid-logarithmic growth. Oxidative stress was applied by addition of H_2O_2 (31% solution Ultrapur, Merck KGaA, Darmstadt, Germany) at a subinhibitory concentration of 50 mM, 24 h before the bacteria were harvested. All procedures with live mycobacteria were performed in a Biosafety Level III laboratory.

Growth in macrophages

THP-1 cells. Human macrophage-like THP-1 cells (ATCC TIB-202) were grown in 8 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum, 1% HEPES and 1% L-glutamine (all from Gibco, Invitrogen, Breda, The Netherlands). The cells were grown in 75 cm^2 flasks at 37°C in a humidified chamber with a 5% CO_2 atmosphere. When a density of $\sim 1 \times 10^6$ cells/mL was reached, cells were treated with 16 nM phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands). After 1 h, *M. tuberculosis* bacteria were added and after 5 h the PMA and bacteria that were not phagocytosed were washed away with medium twice.

M. tuberculosis. Log-phase cultures of *M. tuberculosis* strain MTB72 were allowed to stand for 5 min and then the upper 500 μL was filtered with a 5 μm filter (Millipore). After filtration, the concentration of the dispersed bacterial suspensions was determined by measuring the absorbance at 420 nm, an A_{420} of 0.15 corresponding to 1×10^8 bacteria/mL. Bacteria were added to the macrophages in such a way that a multiplicity of infection of ~1/10 was maintained. An acid-fast stain was made and analysed by light microscopy (magnification: ×100) in order to determine if bacteria were properly associated with the macrophages.

Selection of isoniazid-resistant mutants. In three experiments, intracellular isoniazid-resistant bacteria were selected by addition of 1 mg/L isoniazid to the flasks after 3 days growth of macrophages + bacteria. After 4 h of incubation with isoniazid, the supernatant was discarded and cells were washed with fresh medium. Macrophages were scraped off the bottom of the flask with a cell scraper and taken up in 2 mL sterile water in a new tube. The macrophages were disrupted by application of osmotic stress and sonication in a water bath, and bacteria were subsequently plated on solid Middlebrook medium (MB7H11) containing 1 mg/L isoniazid.

In one experiment, the pre-selection of resistant mutants in the cells was omitted and after disruption of the macrophages an aliquot of the bacteria was used for determination of cfu. From this experiment, the mutation frequency in macrophages was calculated.

Proportion of mutants

To determine the mutation frequency (i.e. the proportion of resistant mutants), four 0.5 mL aliquots of log-phase cultures were plated onto solid MB7H11 medium (Difco, BD, Sparks, MD, USA) containing 0.4, 1.0 or 20 mg/L isoniazid (Sigma–Aldrich Chemie). Serial dilutions of the cultures were plated on non-selective medium to determine the cfu present. The mutation frequency was calculated by dividing the number of resistant colonies by the cfu plated. From each experiment, a number of resistant mutants were picked from

the antibiotic-containing plates and DNA was isolated for further molecular characterization.

Fluctuation assay

The mutation rates (i.e. the chance of a mutation occurring per generation) for isoniazid and rifampicin were determined by performing a fluctuation assay, making use of the p_0-method.[34]

A 10 mL starting culture was prepared by inoculating strain MTB72 from a slope, to minimize the presence of spontaneous mutants. When bacterial growth reached mid-logarithmic phase, the culture was vortexed vigorously to homogenize the bacterial suspension. Clumped cells were allowed to settle for 3 min and 55 new 1 mL cultures were made by transferring 1 μL of this cell suspension (∼1000 bacteria) to a 2 mL screwcap tube containing 1 mL of MB7H9 medium + OADC. Two to three sterile glass beads were added to each culture, to ensure a continuous homogeneous suspension. The 1 mL cultures were incubated in a shaking incubator at 37°C for ∼10 generations (∼8 days), ensuring a ≥1000-fold increase in cell number. The cultures were then centrifuged at 5000 **g** for 8 min and 850 μL of supernatant was discarded. For each culture the remaining 150 μL was plated in one well of a square 25-well replica plate (Greiner, Germany) containing 3 mL of MB7H11 supplemented with either 8 mg/L rifampicin (Sigma–Aldrich Chemie) or 1.0 mg/L isoniazid. The number of cfu plated in each well was estimated by making serial dilutions of the five remaining 1 mL cultures and plating onto non-selective MB7H11. The two 25-well replica plates were allowed to dry in a biosafety laminar flow cabinet, until all liquid was absorbed into the solid medium. The plates were then sealed and incubated at 37°C. After 3–4 weeks incubation, the proportion of squares without growth was counted and the mutation rate was calculated.[34]

DNA isolation

Separate mutant colonies, selected on isoniazid-containing solid medium, were picked and suspended in 150 μL of Tris/EDTA buffer containing 1% Triton X-100 (BDH Laboratory Supplies, Poole, England) and then heated at 95°C for 30 min. After lysis, cells were centrifuged at 5000 **g** for 3 min and 130 μL of the supernatant was collected as a DNA sample.

For the experiment using pooled isolates, four separate colonies were suspended in a single tube and DNA was extracted simultaneously.

PCR and sequencing

A 233 bp fragment, surrounding codon 315 of *katG*, was amplified from selected isolates by PCR using forward primer katG-315_FW 5′-CATGAACGACGTCGAAACAG-3′ and reverse primer katG-315_RV 5′-CGAGGAAACTGTTGTCCCAT-3′. In addition, a 300 bp fragment of *katG* surrounding codon 463 was amplified using forward primer katG-463_FW 5′-TCCCGTTGCGAG ATACCTT-3′ and reverse primer katG-463_RV 5′-AGGGTGCG AATGACCTTG-3′. Isolates were scored as Δ*katG* if one or both of the PCR products were absent. From a proportion of the strains that were positive for the katG-315 PCR, the PCR fragment was sequenced in both directions according to protocols previously published.[35]

From another subselection of our isoniazid-resistant isolates, a 251 bp fragment spanning the intergenic region of *oxyR-ahpC* was amplified and sequenced, using forward primer oxyR-ahpC_FW 5′-TGCTGAACCACTGCTTTGC-3′ and reverse primer oxyR-ahpC_RV 5′-GGTCACCGCCGATGAGAGC-3′.

Results of all PCRs were analysed by agarose gel electrophoresis using a 2% agarose gel (Multi-Purpose agarose, Roche Diagnostics GmbH, Mannheim, Germany).

MLPA

M. tuberculosis-specific MLPA was performed as previously published,[33] except in the present study only four probes were included (Table 1), allowing results to be easily interpreted on an agarose gel. The 16S rRNA probe (202 bp) targets a region specific for members of the MTB complex and was used as internal control. Probes katG-315 (160 bp) and inhA-15 (178 bp) identify the two most prevalent isoniazid resistance-conferring mutations. Probe katG-463 targets a point mutation in codon 463 of *katG* specific for principal genotypic groups (PGGs) 2 and 3 that was present in all strains used in this study. The absence of the *katG*-463 or *katG*-315 products identified a deletion of these regions.

CGH

Strain RB14, a rifampicin- and rifabutin-resistant isolate derived from MTB72,[35,36] was plated on solid medium containing 20 mg/L isoniazid. Upon growth of isoniazid-resistant mutants, 13 colonies were picked, purified and analysed by PCR and MLPA, as described in this study. Seven out of 13 (54%) were negative for both the *katG*-315 and the *katG*-463 PCR, indicating a substantial deletion of the *katG* region. We selected one of these deletion mutants of RB14, RB14H5, for further analysis. In order to determine the exact size of the deletion, the genomes of strain RB14H5 and MTB72, its wild-type parent, were compared by CGH (NimbleGen CGH Whole-Genome Tiling Arrays, Roche NimbleGen, Madison, WI, USA). DNA was extracted from liquid cultures and processed, purified and shipped according to the company's requirements. Data obtained from the CGH experiment was interpreted and analysed with SignalMap v1.9 and Microsoft Excel. Primers were designed to amplify likely regions of

Table 1. MLPA probes used in this study to screen isoniazid-resistant mutants

MLPA probe	Target	Length of ligated probes	Phenotype
16S rRNA	16S rRNA gene	202 bp	member of MTB complex
katG-315	*katG* codon 315 ACC	160 bp	isoniazid-resistant via *katG*-S315T
inhA-15	*inhA* (-)15 'T' allele	178 bp	isoniazid-resistant via *inhA*-C(-)15T
katG-463	*katG* codon 463 CGG	319 bp	PGG 2 or 3

Indicated are the name of the probe, the region or point mutation it targets, the length of the ligated probes and the phenotype that is associated with the point mutation.

difference identified and any mutations present were confirmed by cycle sequencing [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Results

Deletions in katG are the most common mechanism of resistance to isoniazid in vitro

Two initial independent experiments with MTB72 were performed, using 20 mg/L isoniazid to select resistant mutants. Mutation frequencies of 4.40×10^{-6} and 7.56×10^{-6} were measured, and PCR assays targeting the regions flanking codons 315 and 463 of *katG* identified deletions in 4/20 and 18/20 mutants, respectively (Table 2). None of the mutants with an intact *katG* gene carried a mutation, as determined by sequencing, except for one strain that had acquired a TGG(W)→CGG(F) mutation in codon 321. Deletions in *katG* were confirmed by MLPA, as well as the absence of typical mutations *katG*-S315T or *inhA*-C(-)15T in all the characterized mutants.

We hypothesized that the high mutation frequencies and domination of *katG* deletions over isoniazid resistance-conferring mutations were either due to the absence of significant oxidative stress *in vitro* or the concentration of isoniazid used. To test the first hypothesis, the experiment was repeated after exposure to 50 mM H_2O_2, in an attempt to select against the occurrence of *katG* deletions in favour of clinically relevant mutations. The mutation frequency was comparable to that obtained in previous results (3.6×10^{-6}, see Table 2) and 17/20 (85%) selected mutants had a deletion in *katG*. None of the analysed strains had acquired resistance mutations in *katG*-315 or *inhA*-(-)15.

We then lowered the concentration of isoniazid to 1 mg/L. Again, two independent experiments were performed, both resulting in average to high proportions of *katG* deletion mutants (13/37 and 15/20) and none of the targeted clinically prevalent mutations in 57 colonies analysed. The mutation frequencies were 1.78×10^{-6} and 4.17×10^{-6}, respectively. Addition of H_2O_2 to the medium slightly increased the mutation frequency to 1.60×10^{-5}, but the majority (14/20) of the selected mutants had still acquired a deletion in *katG* (Table 2).

Five independent experiments were also conducted to determine the mutation frequency for rifampicin, resulting in an average mutation frequency of 6.86×10^{-8} using 8 mg/L rifampicin (Table 2). This was 43.4 times lower than the average mutation frequency observed with 1 mg/L isoniazid. In addition, fluctuation assays were performed to determine the mutation rates for isoniazid and rifampicin. The estimated mutation rate for 1 mg/L isoniazid was 3.2×10^{-7} mutations/cell division, 32.3 times higher than the estimated rate for 8 mg/L rifampicin (9.81×10^{-9} mutations/cell division, Table 2).

Less than 1% of in vitro selected isoniazid-resistant mutants carry a clinically prevalent mutation

Being unsuccessful in our attempt to select for isoniazid-resistant strains with either of the two most clinically prevalent mutations, we decided to screen a larger pool of isoniazid-resistant mutants derived from MTB72, in order to estimate the prevalence of the most common clinical mutations *in vitro*. We used the methods described above to select isoniazid-resistant mutants with 0.4 mg/L isoniazid.

The mutation frequency was 8.02×10^{-6} (Table 3) and an initial characterization of 29 mutants showed that 28% (8/29, Table 3) had acquired a deletion in *katG*. To increase the chance

Table 2. Characterization of the spontaneous isoniazid-resistant mutants derived from strain MTB72

Antibiotic concentration	Selection condition	Mutation frequency	Mutation rate	No. of Δ*katG* (%)	No. of targeted mutations
20 mg/L INH	—	7.56×10^{-6}	—	18/20 (90%)	0/20
	—	4.40×10^{-6}	—	4/20 (20%)	0/20
	$+H_2O_2$	3.60×10^{-6}	—	17/20 (85%)	0/20
1 mg/L INH	—	4.17×10^{-6}	—	15/20 (75%)	0/20
	—	1.78×10^{-6}	—	13/37 (35%)	0/37
	$+H_2O_2$	1.60×10^{-5}	—	14/20 (70%)	0/20
	+THP1	1.84×10^{-6}	—	5/24 (21%)	0/24
	+THP1 + INH	—	—	1/10 (10%)	0/10
	+THP1 + INH	—	—	0/5 (0%)	0/5
	+THP1 + INH	—	—	6/20 (30%)	0/20
1 mg/L INH	—	2.98×10^{-6}[a]	3.20×10^{-7}	—	—
8 mg/L RIF	—	6.86×10^{-8}[b]	9.81×10^{-9}	—	—

INH, isoniazid; RIF, rifampicin; THP1, macrophage; MTB, *M. tuberculosis*.
The concentration of isoniazid was the concentration in the solid medium on which the bacteria were selected. The selection conditions are additional criteria to select against Δ*katG* mutants, in an attempt to favour the selection of clinically relevant mutants. Mutation frequency is the proportion of mutants. Mutation rate is the estimated number of mutants per generation. No. of Δ*katG* is the number of *katG* deletion mutants (of the characterized mutants). No. of targeted mutations is the number of *katG*-S315T or *inhA*-C(-)15T found in the characterized mutants.
[a]Average of data depicted in this table.
[b]Average of five independent experiments.

Table 3. Characterization of MTB72-derived isoniazid-resistant mutants initially selected with 0.4 mg/L isoniazid

Acquired MIC	Mutation frequency	No. of Δ*katG* (%)	No. of targeted mutations (%)
Undefined	8.02×10^{-6}	8/29 (28%)	0/29 (<3.5%)
0.4 < MIC < 2.0	—	3/30 (10%)	0/30 (<3.3%)
MIC > 2.0[a]	—	—	0/120 (<0.8%)

A subdivision of two groups of mutants was made on the basis of their acquired isoniazid MIC, indicated in the column 'acquired MIC'. MIC is expressed in terms of mg/L isoniazid. Mutation frequency is the proportion of mutants. No. of Δ*katG* is the number of *katG* deletion mutants. No. of targeted mutations is the number of *katG*-S315T or *inhA*-C(-)15T found in the characterized mutants.
[a]DNA samples were derived from mutants pooled in sets of four.

of finding clinically prevalent mutations, additional mutants were picked from the original plates (0.4 mg/L isoniazid) and plated in duplicate on plates containing 0.4 mg/L isoniazid and 2.0 mg/L isoniazid. Thirty colonies were picked that grew on 0.4 mg/L isoniazid but showed no growth on 2.0 mg/L isoniazid and, thus, had an MIC between 0.4 and 2.0 mg/L isoniazid. Of these 30 selected mutants, three (10%) had acquired deletions in *katG* (Table 3). No mutations were found in *katG*-315 or *inhA*-(-)15 by MLPA, but sequencing of *katG* from 10 of these low-MIC mutants with intact *katG* revealed an ACT(T)→ATT(I) mutation in codon 271 of *katG* in one of the strains.

From the panel of mutants selected with 0.4 mg/L isoniazid, an additional subpopulation with an MIC > 2.0 mg/L was analysed by MLPA, targeting the *inhA*-15 and *katG*-315 mutant alleles. In order to facilitate analysis, mutant colonies were pooled in sets of four before DNA was extracted and a total of 120 mutants were screened in this fashion. The suitability of the method for detecting a single mutant in a mixed sample using MLPA was confirmed by analysis of artificial mixtures made with wild-type *M. tuberculosis* DNA and DNA from clinical strains carrying either the *katG*-S315T or *inhA*-C(-)15T mutation (Figure 1). We were unable to measure the number and percentage of Δ*katG* mutants with this method, since samples were mixtures of four colonies and only samples in which all four colonies had a deletion in *katG* would be detected. No mutations were found in these 120 isoniazid-resistant *in vitro* mutants, indicating that the prevalence of clinically relevant mutations [*katG*-S315T or *inhA*-C(-)15T] *in vitro* is <0.8% (95% CI 0%–2.5%), under the conditions described (Table 3).

Representative experiments were also performed with model strain H37Rv. Selection of spontaneous resistant mutants with 0.4 mg/L isoniazid resulted in a mutation frequency of 2.3×10^{-6}. From the resistant mutants, a subselection was made of strains with an MIC between 0.4 and 2.0 mg/L isoniazid. MLPA analysis of 30 of these selected strains showed that 3.3% (1/30) had acquired a deletion in *katG*, but no mutations were detected in *katG*-315 or *inhA*-(-)15. This indicates that also for strain H37Rv <3.3% of the isoniazid-resistant H37Rv mutants carried a clinical mutation.

No adaptive mutations were found in the oxyR-ahpC intergenic region of a random selection of ΔkatG mutants

In order to exclude the possibility that the high prevalence of Δ*katG* mutants in our experiments was due to the acquisition of mutations in the *oxyR-ahpC* intergenic region, we sequenced a 251 bp fragment spanning the *oxyR-ahpC* intergenic region of a selection of our MTB72-derived isoniazid-resistant mutants. This panel of strains represented the different isoniazid concentrations and additional selection conditions used in this study, and all strains had acquired deletions in various regions of *katG*. In total, we screened 21 Δ*katG* mutants for adaptive mutations in *oxyR-ahpC*. The sequence of all analysed mutants was identical to that of the wild-type parent MTB72, indicating that none of the mutants had acquired mutations in this region.

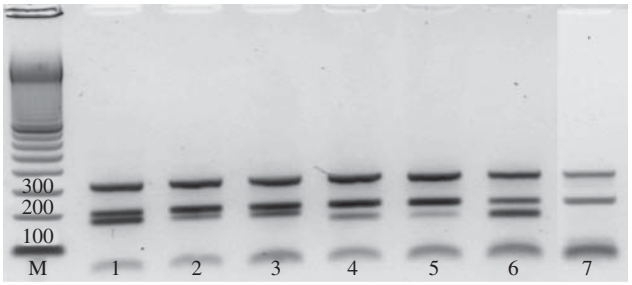

Figure 1. Agarose gel showing separation of MLPA products. Samples shown are artificial mixtures made with DNA from isoniazid-resistant clinical isolates and DNA from wild-type strain MTB72. From top to bottom, bands represent amplified probes targeting *katG*-L463R (319 bp), 16S rRNA (202 bp) and *inhA*-C(-)15T (178 bp, lanes 1–3) or *katG*-S315T (160 bp, lanes 4–6). Lane 1, 100% *inhA*-C(-)15T mutant; lane 2, 10% *inhA*-C(-)15T mutant + 90% wild-type; lane 3, 20% *inhA*-C(-)15T mutant + 80% wild-type; lane 4, 20% *katG*-S315T mutant + 80% wild-type; lane 5, 10% *katG*-S315T mutant + 90% wild-type; lane 6, 100% *katG*-S315T mutant; lane 7, 100% wild-type. M denotes a 100 bp marker, numbers on the first three bands correspond to the respective sizes (in bp).

In vitro-selected ΔkatG mutant has a 49 kbp deletion, but is viable in vitro

The genomes of MTB72 and RB14H5 were screened by CGH, in order to obtain information about the mutations that RB14H5 acquired during the sequential selection steps. Purified DNA from both strains was compared with the sequence of strain CDC1551 (GenBank AE000516). The CGH data indicated a large deletion in the RB14H5 genome, compared with that of MTB72 [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. A PCR with primers targeting the flanking regions confirmed that a 49 kbp fragment

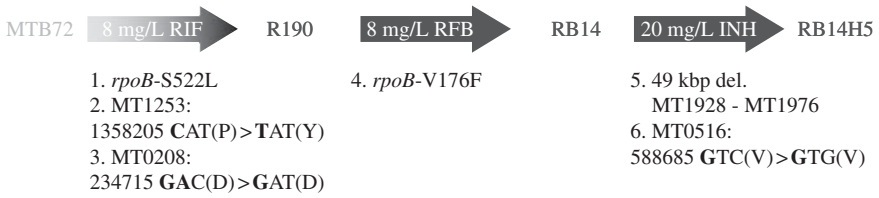

Figure 2. Schematic representation of the three rounds of antibiotic selection that strain MTB72 was subjected to. The selection steps finally resulted in the triple-resistant strain RB14H5. The position of the drug resistance-conferring mutations (1, 4 and 5) and additional mutations (2, 3 and 6) indicates in which selection step they were acquired. Gene ID and coordinates of mutations 2, 3, 5 and 6 are based on the annotated genome sequence of strain CDC1551 (GenBank AE000516). R190 and RB14 are the names of the intermediate strains. RIF, rifampicin; RFB, rifabutin; INH, isoniazid; kbp, kilobase pairs; del., deletion.

spanning 47 genes (MT1928–MT1976), including the whole of *katG*, was deleted from the RB14H5 genome [Table S2, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/), and Figure S1]. The exact location of the deletion, 2127129–2176164, was determined by sequencing of this PCR product.

Additional SNPs were accumulated during in vitro selection

With the data obtained by CGH, not only the extent of the *katG* deletion could be determined, but also other mutations throughout the genome of strain RB14H5 could be identified. The two previously identified mutations in *rpoB* conferring resistance to rifampicin and rifabutin, S522L and V176F, respectively,[35,36] were confirmed by CGH. In addition, three single nucleotide polymorphisms (SNPs) were identified in genes MT1253, MT0208 and MT0516, corresponding to genes Rv1215c, Rv198c and Rv0498 in H37Rv (GenBank AL123456), respectively. PCR and sequencing of these regions in RB14H5 confirmed a C→T mutation in MT1253, a G→A mutation in MT0208 and a C→G mutation in MT0516, of which only the mutation in MT1253 was non-synonymous (Figure 2).

The regions where mutations were identified in RB14H5 were screened in the intermediate strains isolated after each antibiotic selection step, to determine when these additional mutations occurred. After selection with rifampicin, two SNPs, in MT1253 and MT0208, were identified in addition to the mutation in *rpoB*. Selection with rifabutin resulted only in a resistance-conferring mutation, *rpoB*-V176F, but the 49 kbp deletion was accompanied by the mutation in MT0516 after selection with isoniazid (Figure 2).

Discussion

Despite the increasing prevalence of resistant *M. tuberculosis*, isoniazid remains an essential component of current TB treatment programmes. In an attempt to shed more light on the molecular mechanisms leading to the development of MDR-TB, we initially screened *in vitro*-selected isoniazid-resistant mutants of *M. tuberculosis* strain MTB72, using different concentrations of isoniazid. None of the characterized strains carried the mutations that are predominantly responsible for isoniazid resistance in clinical isolates [*katG*-S315T or *inhA*-C(-)15T]. Moreover, a large proportion of the resistant clones acquired resistance to isoniazid by deletion of *katG*, a genotype that is rarely observed in clinical isolates. In agreement with a previous report,[37] we measured a higher mutation rate for isoniazid resistance than for rifampicin resistance *in vitro*. However, the dramatic difference between the *in vivo* and *in vitro* spectrum of isoniazid resistance mutations suggests that an *in vivo* mutation rate for isoniazid resistance equal to or potentially lower than the rifampicin resistance rate ($\sim 10^{-8}$ mutations/cell division) is much more feasible. These findings indicate the importance of genetic characterization of mutants before results obtained in the laboratory are extrapolated to the clinical setting.

KatG protects bacteria from the oxidative burst encountered when *M. tuberculosis* cells reside in macrophages or granuloma.[18,38] *In vitro* conditions are significantly less challenging, not in the least because of the extracellular catalases in the medium, protecting the bacteria from harmful reactive oxygen species. We speculated that these protective culture conditions allowed for the observed high *in vitro* prevalence of Δ*katG* mutants, which are highly resistant to isoniazid, but susceptible to oxidative insult.[28,31,38] However, addition of 50 mM H_2O_2 did not alter the spectrum of mutations (Table 2). Attempts to select isoniazid-resistant mutants from mycobacteria grown in medium without added catalase have been unsuccessful, as bacterial growth of the wild-type strain MTB72 was inadequate in medium without catalase.

Conditions that seemed to have an effect on the proportion of *katG* deletion mutants, but not on the presence of *katG*-S315T or *inhA*-C(-)15T mutants, were selection of mutants with low levels of isoniazid (0.4–2.0 mg/L, Tables 2 and 3) and growth/selection in human macrophages. The average proportion of Δ*katG* mutants selected with 20 mg/L was 65% (39/60), with 1 mg/L was 55% (42/77) and with 0.4 mg/L was 28% (8/29, Tables 2 and 3), suggesting that Δ*katG* mutants are more likely to be highly resistant.

Moreover, from the mutants selected with 0.4 mg/L isoniazid that had an MIC between 0.4 and 2.0 mg/L, only 10% (3/30, Table 3) had a deletion in the *katG* region. Growth in THP1 cells reduced the proportion of deletion mutants, selected with 1 mg/L isoniazid, to 20% (12/59, Table 2). These results show that it is possible to reduce the proportion of deletion mutants under specific conditions, but even in strains selected under these conditions neither of the two most prevalent clinical mutations were identified.

The mutants selected with 0.4 mg/L isoniazid (Table 3) that were stratified on the basis of their MICs were all originally selected on the same plate. In total, 179 isolates selected with 0.4 mg/L isoniazid (29+30+120, Table 3) were analysed by MLPA from this plate. None of these 179 isolates acquired either of the targeted mutations; thus, the prevalence of these

mutations *in vitro* was <0.6% (<1/179, 95% CI 0%–1.6%) under the conditions described. Similar results were also obtained with strain H37Rv (<3.3%), suggesting that this is a general phenomenon, rather than a strain-specific effect. This observation is also supported by the absence of reports of spontaneous *in vitro* isoniazid-resistant mutants that carry clinical mutations. In fact, we are aware of only one study that reports the selection of an isoniazid-resistant *katG*-S315T mutant,[21] but this mutant was acquired after a mouse was infected and treated with isoniazid. It is possible that none of the selection conditions we used were suitable for the selection of the targeted mutations, perhaps because further adaptation is required to obtain an MIC > 0.4 mg/L. However, this would be true for all previous reports of *in vitro* studies.

The general assumption is that isoniazid resistance *in vivo* is much more widespread due to a higher mutation rate, particularly in *katG*.[39] Indeed, the *M. tuberculosis katG* gene is located within a genetically unstable region,[40] suggesting an increased propensity for mutation. Based on this instability, one would expect to observe a whole spectrum of *katG* mutations in resistant clinical isolates, but in practice the majority of clinically resistant strains contain the same mutations. However, the genetic instability of the *katG* region is a likely explanation for the high prevalence of Δ*katG* mutants in our and other laboratory studies.

The empirical evidence for the high mutation rate hypothesis is extracted from a few studies in which fluctuation assays with isoniazid were performed.[37] These studies report a significantly higher mutation rate for isoniazid compared with that for rifampicin, although mutants were not genetically characterized. We obtained similar results for the *in vitro* mutation rates when selecting with isoniazid and rifampicin (Table 2). However, we suspect that this does not explain the high prevalence of isoniazid resistance *in vivo*, since none of the isoniazid-resistant mutants we screened carried the mutations that are most frequently observed in clinical isolates. In fact, merely two or three mutations account for isoniazid resistance in the majority of resistant clinical isolates.[8–13] For rifampicin resistance there are three *rpoB* mutations that are seen in 80%–95% of clinical isolates.[9,41–43] The *in vivo* mutation rate for isoniazid resistance is therefore more likely to be similar to or lower than the rate for rifampicin resistance, as was also suggested by others.[44] This would still lead to a higher prevalence of isoniazid-monoresistant isolates if one takes into account that isoniazid has been used to treat TB much longer than rifampicin. In addition, isoniazid has been regularly administered as monotherapy, whereas rifampicin is almost exclusively part of a combination therapy, thereby reducing the chances of drug resistance.

Previously, researchers have shown that a whole spectrum of different Δ*katG* mutants can be found both *in vitro* and *in vivo*.[13,15,40,45] In our study, partial deletions of the *katG* gene close to the 5′ terminus would not have been detected with the methods used, possibly resulting in an underestimation of the deletion mutants present. The few *in vitro* studies on isoniazid resistance that have been performed have indeed determined that the majority of the mutants were catalase-negative.[45] Furthermore, one group of researchers have implicitly stated that almost all *in vitro* isoniazid mutants identified in their laboratory were Δ*katG* strains, regardless of the concentrations of isoniazid used.[44]

Throughout the course of this work, only two isoniazid-resistant mutants were identified that contained point mutations in the *katG* gene: one mutant acquired a TGG(W)→CGG(F) mutation in codon 321; and the other an ACT(T)→ATT(I) mutation in codon 271. Neither of these mutations has been observed in clinical isolates, although other mutations in codon 321 have been reported and associated with very low quantities of KatG, and, therefore, significantly reduced catalase/peroxidase activity.[7,16,22]

None of our selected isoniazid-resistant strains carried a mutation in the *oxyR-ahpC* intergenic region, even after exposure to H_2O_2. Sherman *et al.*[28] suggested that either a second selection event or passaging was probably necessary for *in vitro*-generated mutants to acquire the additional mutation in *oxyR-ahpC*. This may, in part, explain why this mutation was not identified in any of our strains, although there is at least one report of an isoniazid-resistant mutant that had acquired a mutation in *oxyR-ahpC in vitro*.[46]

The failure to detect any of the clinically prevalent mutations in *katG*, *inhA* or *ahpC* in our study, in combination with results of others, suggests that the current *in vitro* model is not representative of the *in vivo* situation. These observations also argue against the view that the primary route to MDR-TB is invariably via isoniazid monoresistance. In fact, there is evidence that isoniazid resistance does not always precede rifampicin resistance in the development of MDR in clinical settings.

In a recent report, a rifampicin-monoresistant *M. tuberculosis* strain was disseminated and subsequently acquired isoniazid and other drug resistance via the common resistance mechanisms, including *katG*-S315T.[47] Moreover, a recent outbreak in London started with an isoniazid-resistant strain, but upon dissemination the strain acquired rifampicin resistance via unusual mutations in *rpoB*.[48] This is interesting as the genetic background of bacteria probably plays a role in the evolutionary routes they can and will follow. The presence of pre-existing drug resistance mutations can be of influence on the acquisition of additional drug resistance[36] and several studies have shown that different genotypes of *M. tuberculosis* appear to have different preferred resistance mechanisms.[12,13,49–51] Similar observations were made in our laboratory and by others.[35,52] The occurrence of atypical drug resistance mutations may therefore be an indication of suboptimal genetic routes or unusual conditions.

We used CGH to compare the genomes of triple-resistant strain RB14H5 and that of its wild-type parent strain, MTB72. Earlier, we had determined that RB14H5 has a non-functioning catalase–peroxidase in addition to a double mutation in *rpoB*, which moderately increases the stress levels.[36] Remarkably, strain RB14H5 had acquired a 49 kbp deletion, spanning 47 genes including the complete *katG* gene, but nonetheless it remained viable *in vitro*. We are aware of only one other report of a strain with an equally large deletion in the *katG* region.[40]

Strain RB14H5 acquired three other SNPs in addition to the major deletion and the drug resistance mutations (Figure 2). It is unknown if these SNPs are adaptive mutations or more opportunistic 'hitchhike' mutations and whether results obtained are representative of the *in vivo* situation.

The genetic analysis of bacteria is generally focused on previously identified targets, such as genes involved with antibiotic resistance or repetitive sequences for fingerprinting purposes. One consequence of this bias is that additional mutations elsewhere in the genome are missed. Isogenic strains generated in the laboratory may not be as isogenic as assumed and new phenotypes may consequently be attributed to the wrong

mutation.[53] CGH allows the whole genome to be screened for additional mutations that may have occurred in a strain of interest compared with the wild-type template. It would be interesting to compare clinical strains in this fashion, for instance sequential isolates obtained from a single patient.

Additionally, CGH can be valuable in detecting drug resistance-conferring mutations;[54] in some geographical locations up to 25% of clinical strains have acquired isoniazid resistance via unidentified mechanisms.[10,13,39,43]

The present study shows that characterization of *in vitro* mutants is essential in order to draw accurate conclusions and that results obtained in the laboratory are not automatically applicable to the *in vivo* situation. Combined MLPA and CGH analysis of representative clinical isolates should be used to elucidate preferred routes to MDR. Based on our results it can be concluded that the high *in vitro* mutation rate for isoniazid resistance is not a satisfactory explanation for the widespread isoniazid resistance found in clinical isolates, as there is no substantial evidence that the *in vivo* mutation rate for isoniazid resistance is significantly higher than that for rifampicin resistance. Moreover, the outcomes of our study in combination with reports of others lead us to question the current view that development of a successful MDR strain predominantly begins with isoniazid monoresistance. A more detailed study of the genetic mechanisms by which *M. tuberculosis* strains adapt to their environment is warranted, hopefully leading to identification of genotypes or drug resistance profiles that are most likely to result in virulent strains.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Table S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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