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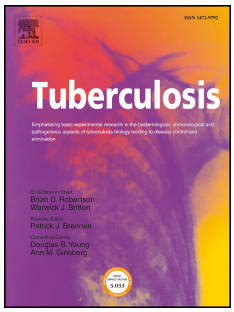

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A *kat*G S315T or an *ahpC* promoter mutation mediate *Mycobacterium tuberculosis* resistance to 2-Thiophen Carboxylic Acid Hydrazide, an inhibitor resembling the anti-tubercular drugs Isoniazid and Ethionamide

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Running title: Resistance to TCH in *M. tuberculosis*.

Abstract: Clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium bovis* are differentially susceptible to 2-Thiophen Hydrazide (TCH); however its mechanism of action or the reasons for that difference are unknown. We report herein that under our experimental conditions, TCH inhibits *M. tuberculosis* in solid but not in liquid medium, and that in spite of resembling Isoniazid and Ethionamide, it does not affect mycolic acid synthesis. To understand the mechanisms of action of TCH we isolated *M. tuberculosis* TCH resistant mutants which fell into two groups; one resistant to TCH and Isoniazid but not to Ethionamide or Triclosan, and the other resistant only to TCH with no, or marginal, cross resistance to Isoniazid. A S315T *kat*G mutation conferred resistance to TCH while *kat*G expression from a plasmid reduced *M. tuberculosis* MIC to this drug, suggesting a possible involvement of KatG in TCH activation. Whole genome sequencing of mutants from this second group revealed a single mutation in the alkylhydroperoxide reductase *ahp*C promoter locus in half of the mutants, while the remaining contained mutations in dispensable genes. This is the first report of the genetics underlying the action of TCH and of the involvement of *ahpC* as the sole basis for resistance to an anti-tubercular compound.

Keywords: 2-Thiophen Carboxylic Acid Hydrazide, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, Non Tuberculous Mycobacteria**,** Alkylhydroperoxidase, TCH resistant mutants.

Introduction.

The differentiation between slow growing mycobacteria has been an issue for the microbiology diagnostic and research laboratories for many years. Prior to the advent of nucleic acid amplification techniques, the differentiation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* from the Non Tuberculous Mycobacteria (NTM) was based on the utilization of selective inhibitors, such as *p*-nitrobenzoic acid (PNB); this latter compound has been used in multiple formats ranging from simple test tube assays to radiometric assays (Collins and Levett 1989) and more recently a microscopic-observation-drug-susceptibility (MODS)-based technique (Agarwal, Dhole *et al.* 2014). Along with the PNB assay, 2-Thiophen Hydrazide (TCH) was useful and reliable in differentiating *M. tuberculosis* from *M. bovis*; while *M. bovis* strains (including the vaccine BCG strain) grew up to TCH concentrations of 1 µg/ml, *M. tuberculosis* strains were able to grow with up to 5 µg/ml (Kaeppler 1964, Vestal and Kubica 1967). The test was widely used due to its simplicity, only requiring culturing of the isolate on Lowenstein-Jensen medium containing TCH at the designated concentrations. However, a classification of *M. tuberculosis* clinical strains discriminated between "Asian " (or "Indian") strains and "European" or "classical" strains on the basis of TCH susceptibility (Grange, Aber *et al.* 1977, Grange, Aber *et al.* 1978); puzzlingly while Asian strains displayed an increased sensitivity to TCH (between 1 and 5 µg/ml), "classical" strains were resistant to 5 µg/ml of TCH (Grange, Yates *et al.* 1985). The reason for the difference between Asian and classical strains was never deciphered. Importantly the claim that the TCH test could not be used on *M. tuberculosis* strains that were resistant to the cornerstone TB drug Isoniazid (4-Pyridinecarboxylic acid hydrazide, INH) led Yates and collaborators to study the relationship between resistance to both INH and TCH in *M.tuberculosis* strains by isolating spontaneous mutants and addressing the existence of cross-resistance (Yates, Grange *et al.* 1984). Their study concluded that the TCH assay was a valid method for subdividing *M. tuberculosis* strains for epidemiological purposes regardless of the INH resistance state since clinical strains very

rarely showed such cross-resistance *in vivo* (Yates, Grange *et al.* 1984). In summary, although TCH was used for more than fifty years in mycobacteriology laboratories as part of a simple culture test not only to differentiate *M. tuberculosis* from *M. bovis* but also to subdivide *M. tuberculosis* strains, there is a lack of knowledge on the rationale for its use and its mechanism(s) of action. Moreover, susceptibility to TCH in *M. bovis* and *M. tuberculosis* strains is in itself an intriguing difference between two species that are so closely related and that show highly comparable susceptibility to other anti-tubercular drugs with the exception of pyrazinamide (Scorpio and Zhang 1996). The differential susceptibility to TCH may reflect metabolic or even target differences between members of the genus *Mycobacterium*.

In order to gain insight in the mechanism(s) of action of TCH we herein describe the isolation and characterization of *M. tuberculosis* mutants resistant to TCH; we also report on the identification of *katG* and *ahpC* as genes involved in the TCH resistance phenotype, two genes that play important roles in resistance to INH. Although the mechanism(s) of action of the drug remain to be elucidated, we herein define a framework to decipher the mechanism of action of TCH in *M. tuberculosis* and *M. bovis*.

Materials and Methods.

Bacterial strains, growth media and growth conditions. *M. tuberculosis* H37Rv, *M. bovis* var BCG Pasteur and *Mycobacterium smegmatis* mc^2155 were from laboratory stocks. An INH resistant (INH^R) *M. tuberculosis* clinical isolate (INM27833, bearing a Ser315Thr mutation in *katG* as confirmed by DNA sequencing) was kindly provided by Dr. N. Simboli (Mycobacteriology service, National Institute for Microbiology "Carlos G. Malbrán", Buenos Aires, Argentina); *Mycobacterium avium*, *Mycobacterium marinum* and *Mycobaterium kansasii* clinical isolates identified to species level at a national reference center were used as NTM species and were the

generous gift of Dr. N. Morcillo (Mycobacteriology Service, Hospital Cetrángolo, Buenos Aires, Argentina). *M. tuberculosis* H37Rv, its derivative strains obtained through this work, *M. bovis* var BCG strain Pasteur and NTM were propagated in Middlebrook 7H9 broth medium supplemented with 0.5% glycerol, 10% ADS (Albumin- Dextrose NaCl supplement) and 0.05% (w/v) Tween 80 (7H9-ADS-Gly-Tween for short unless otherwise stated). Middlebrook 7H9-ADS-Gly with the addition of agar 1.5% (w/v) was routinely used as solid media. *Escherichia coli* strain DH5α was used for cloning experiments and was grown in Luria–Bertani (LB) broth or agar medium. Culture media were supplemented with kanamycin (20 μg/ ml) when required.

All chemicals and solvents were from Sigma- Aldrich (Mo) unless stated differently.

Determination of TCH Minimum Inhibitory Concentration. Cultures of each mycobacterial species were started from fresh Middlebrook 7H9-ADS-Gly plates; to this end a loop full of growth was taken from each plate, resuspended in 7H9-ADS-Gly 0.05% Tween 80 (with the exception of *M. smegmatis* for which 0.5% Tween was used) and incubated at 37°C (except for *M. marinum* which was incubated at 30°C) for 5 days (*M. smegmatis, M. marinum*), 7 days (*M. kansasii*), or 30 days (*M. tuberculosis*, *M. bovis* var BCG and *M. avium*). The cultures were kept 1-2 h at room temperature with no agitation to allow clumps to settle; afterwards aliquots were withdrawn and diluted (1/50 for *M. smegmatis*, 1/10 for the remaining species) in fresh 7H9-ADS-Gly medium. Cultures were incubated at 37°C (except for *M. marinum* which was incubated at 30°C) with shaking until saturation. Colony Forming Units (CFU) were determined by plating ten-fold dilutions of each strain on 7H9-ADS-Gly solid medium, dilutions calculated to contain 10^3- 10^4 CFU were plated on the same solid medium containing TCH (prepared in distilled water at 100 mg/ml) at increasing concentrations ranging from 0.5 to 100 μg/ml. Plates were incubated at appropriate conditions as described above before visual inspection and colony counting. The drug

concentration at which CFUs in the presence of the drug were 0.1% of the CFUs present in medium without drug was taken as MIC $_{99.9}$.

Isolation of spontaneous TCH resistant mutants. Spontaneous TCH-resistant (TCH^R) mutants were isolated from five independent cultures of *M. tuberculosis* H37Rv started from ~ 10^6 CFU/ml and grown in 7H9-ADS-Gly supplemented with 0.05% Tween 80. The culture was incubated with shaking at 37°C until saturation. One hundred µl aliquots of tenfold serial dilutions (10^0- 10^{-3}) of each culture were plated on 7H9-ADS-Gly supplemented with 1.5% (w/v) agar in the presence of TCH at 25, 50 and 100 µg/ml. Plates with no TCH were used to determine total CFUs by plating 100 µl aliquots of the 10^{-5}-10^{-8} dilutions followed by incubation at 37°C for 30 days. Ten colonies arising on plates from each culture at different TCH concentrations were streaked on fresh solid media devoid of drug and tested to confirm their MIC to TCH.

Characterization of TCH^R mutants. The isolated *M. tuberculosis* mutants were analyzed for growth features (colony morphology and size) and growth rate in 7H9-ADS-Gly-Tween liquid broth. Resistance to other anti-tubercular drugs inhibitors of mycolic acid biosynthesis was analyzed by plating dilutions containing ≈ 10^3 CFU of each mutant on 7H9-ADS-Gly agar plates containing INH (0.02; 0.05; 0.1; 0.25; 0.5 and 1 µg/ml); Ethionamide (ETH) or Triclosan (TRC), at 0.5; 1.0; 2.5; 5 and 10 µg/ml. CFUs were determined after incubation for 30 days at 37°C.

Analysis of the *'de novo'* synthesis of lipids.

In vivo labeling, extraction and analysis of lipids from liquid cultures of *M. tuberculosis* were performed as described by Vilchéze *et al.* (Vilcheze, Morbidoni *et al.* 2000). Briefly cultures were

grown in 7H9-ADS-Gly-Tween broth at 37°C with agitation up to mid-log phase and treated for 18 h with the chosen concentrations of TCH (50 or 100 µg/ml, that is 10x and 20x MIC values) or INH (0,5 µg/ml corresponding to 10x MIC value); at this point 1 µCi/ ml of [1-^{14}C] acetate was added to each culture followed by further incubation for 18 h. When the influence of the time of contact with the drug was evaluated, cultures were incubated with TCH at the chosen concentrations for 24 or 48 h. In order to perform "*in vivo*" labeling on solid medium we used a protocol described by Nandakumar *et al.* (Nandakumar, Nathan *et al.* 2014). Briefly, 1 ml of *M. tuberculosis* H37Rv culture ($O.D._{600}$ ~ 1.0) was centrifuged and the bacterial pellet transferred onto a nitrocellulose disk placed on top of plates containing solid 7H9-ADS-Gly medium; after 3 days of incubation at 37°C the disk was transferred to a fresh plate of the same solid medium containing 50 µg/ml TCH. After further 48 h of incubation, a 5 µl aliquot of [1-^{14}C] acetate diluted in 45 µl of 7H9 broth was carefully added to the surface of the disk. After 24 h of incubation the disk was removed and placed into a centrifuge tube containing 1 ml of ice-chilled 7H9 broth, followed by gentle shaking by vortex and removal of the disk. The resulting ^{14}C-labelled cells were harvested by centrifugation at 5,000 rpm, washed twice with distilled water and kept frozen until use. The extraction and analysis of fatty acids and mycolic acids was done as follows: ^{14}C-labelled control (no drug added) and treated cells were subjected to alkaline hydrolysis in 15% (w/v) tetrabutylammonium hydroxide (TBAH, Fluka) at 105°C for 8 h, followed by the addition of 2 ml of CH_2Cl_2 and 100 µl of CH_3I. The entire reaction mixture was then mixed by rotation at room temperature for 1 h and centrifuged, and the lower organic phase was carefully removed, washed with water, and dried at 55°C under a nitrogen stream. The resulting pellet was extracted with ethyl ether and dried again before adding a small volume of CH_2Cl_2. Aliquots (10 µl, 10% of the total extract and representing ~ 40,000 cpm) containing the obtained mixtures of fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were subjected to analytical one-dimensional thin layer chromatography (TLC), on silica gel plates (5735 silica gel 60 F254; Merck) using hexane: ethyl acetate 95:5 v/v for three

developments. Similarly, one-dimensional argentation TLC was carried on using silica gel plates dipped in $AgNO_3$ to separate saturated from unsaturated fatty acids, in this case petroleum ether/diethyl ether (85:15 v/v) was used as eluent for three runs. For two-dimensional silver ion argentation TLC (2D-TLC), an aliquot of each sample containing the mixture of FAMEs and MAMEs (≈ 80,000cpm each) was applied to silica gel plates previously impregnated with $AgNO_3$ (80% of the length of the plate). The plates were developed in the first direction (without argentic impregnation) twice with hexane/ethyl acetate (95:5 v/v) and, after rotating the plate, in the second direction (containing silver ions) three times with petroleum ether/diethyl ether (85:15 v/v). In all cases detection of radiolabeled species was done by autoradiography. The autoradiograms were obtained after exposure at -80°C for 2-3 days on X-ray film.

Genome DNA preparation and whole genome sequencing of TCH^R mutants.

DNA was extracted from cultures of selected mutants according to standard lab protocols; in brief 10% (w/v) glycine was added to fresh late log phase mycobacterial cultures and the incubation continued for 12 h. One ml from fresh cultures of each TCH^R mutant and the parental strain were transferred to a 2 ml microcentrifuge tube, cells were inactivated by placing the suspensions in a heating block at 80°C for 1 h; after cooling down, 70 µl 10% SDS solution and 50 µl of Proteinase K stock (10 mg/ml) were added to each cell suspension. The tubes containing the samples were gently inverted a few times until viscosity was evident. Afterwards, the tubes were incubated at 60°C for 1 h. After this time, 100 µl 5M NaCl and 100 µl 10% CTAB (both solutions pre-warmed at 60°C) were added to the Proteinase K-SDS treated cell suspensions and the incubation continued for 30 min. When the treatments were completed, the cell suspensions were briefly frozen (15 min) at -80°C followed by 15 min incubation at 60°C and frozen again at -80°C for 30 min. The frozen samples were warmed to room temperature and 700 µl of chloroform/isoamyl alcohol (24:1 v/v) was added to each tube; samples were inverted gently for 30 sec or until phase homogeneity. The

tubes were then centrifuged at 13,000 rpm at room temperature and the resulting aqueous layer was gently withdrawn with a large bore tip to prevent DNA shearing and transferred to a new microcentrifuge tube. The DNA was precipitated by adding 0.1 vol 3M sodium acetate (pH 5.2) and 1 vol isopropanol to the aqueous fractions. The tubes contents were slowly mixed by inversion and placed at 48°C for 1h. Upon centrifugation at 12,000 rpm for 30 min at room temperature the supernatant was removed, and the pellet DNA was gently washed twice with cold 70% ethanol. After a new centrifugation step the ethanol was removed and the pellet allowed to dry. The genomic DNA was dissolved overnight in TE buffer and kept at -20°C until use.

Genome sequence analysis. The Illumina Genome Analyzer IIx system was used for whole genome sequencing (WGS). DNA was fragmented by sonication, end-repaired and indexed adapters ligated. Libraries were size selected on 2.5% TAE agarose gels. Library material was isolated from gel slices using the QiaQuick MinElute Gel Extraction kit (Qiagen). Purified libraries were quantified using a Qubit™ fluorometer (Invitrogen) and a Quant-iT™ double-stranded DNA High-Sensitivity Assay Kit (Invitrogen). Clustering and sequencing of the material was carried out as per the manufacturer's instructions, v2 Single Read Cluster Kits and v3 SBS kits (Illumina) were utilized for all sequencing.

Whole genome sequencing data was aligned using the published *M. tuberculosis* H37Rv reference genome (NC_000962, from NCBI) with bowtie2 (Langmead and Salzberg 2012). Samtools (Li, Handsaker *et al*. 2009) and bcftools (https://github.com/samtools/bcftools) were used to predict single nucleotide variants (SNV). Each SNV had to be supported by at least 4 uniquely mapped reads at the position, with a SNV quality greater than 90, which corresponds to a false positive rate (FPR) lower than $10e^{-9}$.

Cloning of *M. tuberculosis katG*. PCR amplification of *M. tuberculosis katG* was performed using the following primers: 5´-**GAATTC**GTGCCCGAGCAACACCCACC-3´ (*katG* Forward) and 5´-**AAGCTT**CCGAATCAGCGCACGTCGAAC- 3´ (*katG* Reverse) where the underlined bold sequence corresponds to sites for restriction enzymes *Eco*RI and *Hind*III respectively, using *M. tuberculosis* chromosomal DNA as template. The amplification product was purified from agarose gels and cloned using the pGEM-T Easy cloning vector (Invitrogen) followed by electroporation into *E. coli*. Clones containing inserts of the expected size (as determined by restriction enzyme digestion) were sequenced at a commercial facility and inserts that showed no mutations were cloned into the integrative shuttle *E. coli*-mycobacteria vector pMV361. One such construct was propagated in *E. coli* and upon plasmid preparation, introduced into *M. smegmatis* mc^2155 and *M. tuberculosis* H37Rv by electroporation following standard protocols (Snapper, Melton *et al.* 1990).

Analysis of TCH stability.

Sample preparation. TCH was added to 7H9-ADS-Gly (final concentration 5 µg/ml) and incubated at 37 °C for 72 h. Then 1 ml of the mixture was extracted with ethyl acetate (3 x 150 µl) and the combined organic extracts were evaporated under N_2 stream, diluted with $CHCl_3$ up to 1 mg/ml final concentration and submitted for GC-MS analysis. A solution of TCH in distilled water was also prepared (5 mg/ml) and incubated, extracted and diluted following the same procedure described above. A sample of pure TCH dissolved in $CHCl_3$ was also used as a control.

GC-MS analysis. The analysis was performed using a Shimadzu GC-MS QP 2010 Plus equipped with a SPB-1 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The carrier gas was helium, at a flow rate of 1 ml/min. Column temperature was initially 50 °C for 3 min, then gradually increased to 300 °C at 10 °C/ min, and kept at that temperature for 5 min. For GC-MS detection an electron ionization system was used with ionization energy of 70 eV, with full scan

between 60 to 600 m/z. Injector and detector temperatures were set at 250 and 230 °C, respectively. The injection volume was 1 µl in split mode (1:5).

^{1}H NMR analysis. Spectra were acquired on a Bruker Avance II 300 MHz (75.13 MHz) using D_2O as solvent of a solution of pure TCH at a concentration of 5mg/ml (final volume 500 µl). Chemical shifts (δ) were reported in ppm downfield from tetramethylsilane (TMS) at 0 ppm.

Chemical oxidation of TCH and INH by Mn(III) pyrophosphate. The chemical oxidation of TCH was studied using manganese (III) pyrophosphate as oxidant following the protocols described by Nguyen *et al.* (Nguyen, Claparols *et al.* 2001, Nguyen, Quemard *et al.* 2002). Briefly, the reaction was performed in 100 mM phosphate buffer (pH 7.5) containing 4mM manganese (III) pyrophosphate, with 2mM NAD^+ as acceptor. Both INH (as a positive control) and TCH were used at 2mM. The mixtures were stirred at room temperature for 20 min and the reaction products run on TLC silica plates with ethanol in the case of TCH, or ethyl acetate/ethanol (90/10) in the case of INH, as mobile phase. Afterwards, the developed plates were inspected under UV light (254 nm) or using a *p*-anisaldehyde solution (3.7 ml of *p*-anisaldehyde in a mix of 135 ml of absolute ethanol, 5 ml of concentrated sulfuric acid and 1.5 ml of glacial acetic acid); in this case the TLC plates were dipped in the solution followed by heating at 120 °C for 3 minutes.

Results.

TCH is active only on members of the *M. tuberculosis* complex. Growth in the presence of TCH differentiates between *M. tuberculosis* and *M. bovis* clinical isolates (MIC of 5 µg/ml and 1 µg/ml, respectively); however little is known on the molecular basis for this phenotype, nor as to how TCH works in these species. In order to address these questions we first tested TCH on different mycobacterial species on 7H9-ADS-Gly agar, which is routinely used as solid chemically defined media as opposed to the standard Lowenstein-Jensen (L-J) medium generally used in most clinical mycobacteriology laboratories. Our results showed that TCH displayed the same level of activity on Middlebrook solid defined media as on standard L-J media, with wild type *M. bovis* and *M. bovis* var BCG being inhibited by the presence of 1 µg/ml and *M. tuberculosis* H37Rv being inhibited by 5-10 µg/ml. Longer incubation times (60 days) did not reveal mycobacterial growth. NTM showed no inhibition of growth even in the presence of very high TCH concentrations (100 µg/ml) as has widely been observed in clinical mycobacteriology settings. However, using the fast growing NTM *M. smegmatis* we observed a transient inhibition of growth in solid medium at TCH concentrations higher than 5 µg/ml and up to 100 µg/ml; while growth on plates containing high concentrations of TCH was marginal after 3 days of incubation at 37°C, colonies started to appear afterwards reaching comparable numbers than the control plates by day 12 (Fig. 1). This behavior was not seen when testing other NTM, which grew at all times unabated.

KatG is involved in resistance to TCH in *M. tuberculosis*. As mentioned above, early reports indicated that *M. tuberculosis* INH^R strains could also be TCH^R (Yates, Grange *et al.* 1984), an observation also made by Parsons and colleagues for *M. bovis* (Parsons, Brosch *et al.* 2002). INH and TCH are also structurally similar, both bearing a hydrazide group (Fig. 2A). These observations, in the context of current understanding on the basis of the mechanism of action of

INH (summarized in Fig 2A and 2B), led us to hypothesize that the catalase/peroxidase KatG could be involved in the resistance to TCH. In first instance we tested the susceptibility of *M. tuberculosis* INM 27833, a clinical *M. tuberculosis* INH^R strain having a S315T mutation affecting the *katG* catalase/peroxidase. Our results indicated that this strain was not affected by TCH up to 50 µg/ml (Fig. 2C and Table 1). Conversely a *M. tuberculosis* H37Rv strain containing an extra copy of *M. tuberculosis katG* cloned into the integrative vector pMV361 was more susceptible to TCH showing a 2-fold decrease in MIC value (Fig. 2D and Table 1). Based on those results, we next tested whether a non- enzymatic oxidizing method already proven for INH -incubation with Mn(III) pyrophosphate as described by Nguyen et al, (Nguyen, Claparols *et al.* 2001, Nguyen, Quemard *et al.* 2002)- would cause a comparable effect on TCH. That was indeed the case, with a rapid *in vitro* conversion of both INH and TCH under our experimental conditions (Fig S1). Taken as a whole, these results suggest that -like INH- TCH may be activated by KatG, supporting earlier phenotypic observations (Yates, Grange *et al.* 1984, Parsons, Brosch *et al.* 2002). However, direct evidence of the KatG-mediated needs to be confirmed.

Mycolic acid biosynthesis is not affected by TCH in *M. tuberculosis* and *M. bovis* BCG. Since TCH is structurally related to INH, we next set out to study if they share the same molecular target. It is well known that INH affects mycolic acid biosynthesis through inhibition of the enoyl-ACP-reductase InhA, one of the components of the mycobacterial FASII system. To test whether TCH targeted mycolic acid biosynthesis, we performed *M. tuberculosis* and *M. bovis* BCG Pasteur "*in vivo*" labeling using the radioactive fatty acid precursor [1-^{14}C] acetate. To this end, mid-log cultures ($OD_{600\ nm} \approx 0.7$) were left untreated or treated for 24 h with 2.5 and 5-fold the MIC of TCH (25 µg/ml and 50 µg/ml); after this, the radioactive precursor was added and the culture was incubated for another 24 h before cell collection and fatty acid and mycolic acid extraction. The analysis of mycolic acids and fatty acids by mono-dimensional TLC showed no change in the

intensity or composition of the labeled mycolic or fatty acids at either drug concentration even when longer exposure times to the drug (two doubling times) were used (Fig. 3); in an identical manner monodimensional argentation TLC showed no changes in mycolic acids, saturated or unsaturated fatty acids (data not shown). Careful scrutiny of radiolabeled fatty acids and mycolic acids extracted from the TCH-treated *M. tuberculosis* cultures by 2D-TLC showed no change of the mycolic acid pattern, nor any novel spots that could be related to an effect on their biosynthesis (data not shown). In all cases treatment with INH (0.5 µg/ml) used as a control gave the expected results causing the well-known loss of mycolic acids and the hallmark accumulation of fatty acids. Taken together, these results suggest a mode of action for TCH that is distinct from that of INH and ETH.

TCH lacks activity in liquid culture under standard growth conditions. While assaying the effects of different exposure times to the drug in 7H9-ADS-Gly-Tween medium, we surprisingly found no noticeable changes on growth rate (judged by spectrophotometric measurements) of TCH treated cultures even when very high concentrations of drug (up to 100 µg/ml) and longer times of exposure (up to 72 h) were used (Fig. 4). The presence of agar and Tween are the only differences between liquid and solid Middlebrook 7H9-ADS-Gly, thus we ruled out a contribution of the tensioactive agent Tween 80 by following growth at OD_{600nm} in medium devoid of it. Our results again showed that TCH was inactive regardless of the presence or absence of the tensioactive, hence ruling it out as a contributing factor to the lack on TCH activity in liquid medium (data not shown). We next hypothesized that differences in oxygen availability between liquid and solid media may play a role in the activity of TCH or through metabolic changes of the mycobacterial cells. To test this, cultures were grown under low agitation (30 rpm) conditions on 7H9-ADS-Gly-Tween in the presence of TCH (100µg/ml). However, we again failed to detect any difference in growth rate under the mentioned conditions as judged by turbidity measurements (data not shown).

The same experiment using static cultures gave comparable results, that is, no activity of TCH in liquid medium was detected. Although resuspension of cultures grown in static conditions did not yield accurate measurements due to mycobacterial aggregation, it was clear that the turbidity of TCH treated cultures was comparable to control cultures while INH treated cultures showed a decline in turbidity (data not shown). Preliminary results showed that an intermediate concentration of TCH (25 µg/ml) did not alter growth of cultures containing a reduced number of bacilli ($2x10^5$ or $2x10^6$ bacilli/ml) (data not shown). Based on those puzzling results and in order to circumvent this problem, we next tested the activity of TCH on the synthesis of mycolic acids in *M. tuberculosis* growing on solid medium by following a protocol recently described in which the addition of the radioactive precursor was added on top of small quantities of cells (roughly $2x10^7$ CFU) growing on solid medium (Nandakumar, Nathan *et al.* 2014). In spite of this strategy, we could not detect any difference in the pattern of fatty acids and mycolic acids regardless of the presence or absence of TCH; however, INH used as a control totally inhibited mycolic acid synthesis in *M. tuberculosis* as expected (Fig. 5). In summary, the inhibitory activity of TCH was restricted to solid media under our assay conditions and did not affect mycolic acid synthesis.

Lack of activity of TCH in liquid medium is not due to inactivation in liquid medium. An extensive literature search did not reveal any report on TCH stability. In order to assess whether liquid media may favor chemical alterations in TCH leading to lack of inhibitory activity, we examined the stability of the drug under the assay conditions by dissolving TCH in 7H9-ADS-Gly media at 5mg/ml; additionally, a sample of the drug dissolved in water was prepared at the same concentration for comparative purposes. Both samples were incubated at 37 °C for 72h, and afterwards, extracted with ethyl acetate, evaporated, resuspended in $CHCl_3$ and analyzed by GC-MS. The chromatogram of the TCH in water showed only one main peak at 16.43 min without any other peaks that would be evidence of decomposition (Fig. S2A). TCH dissolved in 7H9-ADS-Gly

showed a main peak at the same retention time, with only traces of compounds belonging to the media, demonstrating the stability of TCH under the assay conditions (Fig. S2A). The identity of the main peak was corroborated by comparing the mass spectra of the standard drug (Fig. S2B) that matched the NIST database spectrum (Fig. S2C). The GC-MS analysis provided evidence of the stability of TCH in aqueous media, but the ionization method may have prevented the detection of a polar or thermally sensitive product; therefore we complemented the analysis by ^{1}H NMR. A sample of TCH (5mg/ml) in D_2O was incubated a 37 °C for 72h and the spectra were acquired every 24 h. Our results clearly showed that the spectra did not acquire any new signal that could indicate the presence of a decomposition product (Fig. S3). Thus, the chemical stability of TCH does not seem to change in aqueous solution and rules it out as a factor underlying the lack of TCH activity in liquid medium.

Whole genome sequencing identifies *ahpC* mutations conferring resistance to TCH. Because of the specific in vitro conditions in which TCH showed activity, and to reveal the target(s) for TCH, we isolated spontaneous TCH^R *M. tuberculosis* mutants on 7H9-ADS-Gly agar plates containing TCH at 25, 50 and 100 µg/ml. Mutants were obtained at a frequency of 10^{-7}- 10^{-8}. TCH resistant mutants fell into two groups depending on the cross resistance to INH displayed. The first group showed cross resistance to INH (MIC 0.5µg/ml) and had a high resistance to TCH (>100µg/ml) with the exception of mutant TCH25.1 which showed a medium level of resistance to TCH (25µg/ml). The second group had no cross resistance to INH and displayed a wide range of resistance to TCH (25 to >100µg/ml) (Table 2). Colony morphology and growth rates of the TCH^R mutants were similar to the ones of the parental strain (data not shown).

To gain understanding on the molecular mechanism(s) of action of TCH we performed whole WGS on 7 mutants from our set of TCH^R, INH^S mutants (obtained from independent cultures) and mutant

TCHR25.1 which was of interest due to its resistance phenotype. Our results identified a mutation (c-81t) in the promoter region of *Rv2428* (*ahp*C), encoding an alkyl hydroperoxidase in half (4/8) of the mutants sequenced. Interestingly, this mutation has previously been shown to occur in INH^R resistant strains but only in association with mutations causing loss or reduction of KatG function (Vilcheze and Jacobs 2014). In addition, it has previously been shown that this mutation caused a 2-fold increase in *ahpC* expression (Zhang, Dhandayuthapani *et al.* 1996, Heym, Stavropoulos *et al.* 1997). Thus, increased expression of AhpC seems to confer increased resistance to TCH with no cross resistance to INH in *M. tuberculosis*. The remaining mutations in the TCH^R, INH^S mutants occurred in the following genes: *glc*B (*Rv1837c*), *Rv2731, ppe*52 (*Rv3144c*), and the intergenic region between genes *Rv3716c* and *Rv3717* (Table 3). Interestingly, mutant TCH R25.1 displayed two mutations, one affecting gene *Rv3220c*, encoding a sensor of a two-component histidine kinase system, and a mutation upstream of *Rv0312* that encodes a proline-threonine-rich protein of unknown function. Some of the above mentioned genes have been shown to be non-essential by transposon mutagenesis (Sassetti, Boyd *et al.* 2001) or by gene knock-out (Parish, Smith *et al.* 2003); thus their role in resistance to TCH is not obvious.

Discussion.

Conditions affecting TCH activity on *M. tuberculosis*. The last two decades revealed the mechanism of action of several anti-tubercular drugs including two important pro-drugs, INH and ETH (2-ethylpyridine-4-carbothioamide). Through a combination of genetics and biochemistry it was shown that INH is activated by the non-essential mycobacterial catalase/peroxidase encoded by the gene *katG* (Heym, Alzari *et al.* 1995, Heym, Saint-Joanis *et al.* 1999). Mutations in *katG* account for more than 90% of the INH resistant phenotypes of clinical *M. tuberculosis* strains (Jagielski, Bakula *et al.* 2015; Torres, Paul *et al.* 2015). A combined interdisciplinary approach

spanning biochemistry, genetics, chemistry and physics revealed the mechanism of action of INH (Vilcheze and Jacobs 2007). Once activated, INH forms a variety of unstable radicals one of which reacts non enzymatically with NAD^+ and NADP targets InhA, a vital acyl-ACP enoyl reductase involved in mycolic acid biosynthesis (Dessen, Quemard *et al.* 1995, Quemard, Sacchettini *et al.* 1995). These extremely long α-alkyl β-hydroxy fatty acids are essential to maintain the mycobacterial cell wall structure (Vilcheze, Morbidoni *et al.* 2000; Barkan, Liu *et al.* 2009). Although activated by a different enzyme, ETH is also a pro-drug and an inhibitor of *inhA* through the same mechanism of action (Banerjee, Dubnau *et al.* 1994).

As is widely known in clinical mycobacteriology laboratories, TCH, an hydrazide structurally related to INH and ETH, was not active on NTM (MIC≥100); however, we observed that *M. smegmatis* was able to grow at 100 µg/ml TCH albeit with a significant growth delay; moreover the fact that colony size and numbers equalized upon long incubation times suggested a metabolic adaptation of *M. smegmatis* to the toxicity of TCH (Fig. 1). Given that TCH displays a clear activity on *M. tuberculosis* and *M. bovis*, we focus on *M. tuberculosis* as model organism to gain insight on this drug mechanism(s) of action. Due to structural similarities between TCH, INH and ETH, we hypothesized that TCH would be a pro-drug and also an inhibitor of the synthesis of mycolic acids in *M. tuberculosis* complex species. In our hands, expression of *M. tuberculosis katG* from a plasmid decreased the susceptibility to TCH in wild-type *M. tuberculosis* H37Rv (Fig. 2D). Moreover, a clinical *M. tuberculosis* strain containing a S315T mutation in *katG*, the most widely described mutation causing resistance to INH, showed increased resistance to TCH when compared to the wild-type strain (Fig. 2C). Also, preliminary experiments showed that both INH (used as a control) and TCH were modified by Mn(III) pyrophosphate in the presence of NAD^+ as was previously described for INH (Nguyen, Claparols *et al.* 2001, Nguyen, Quemard *et al.* 2002) (Fig. S1). Taken together, those results suggest that TCH is most likely a pro-drug and that KatG is its

possible activator. In this regard, more work to confirm the role of KatG and the identity of the radicals generated is warranted.

Surprisingly, we failed to observe a concentration dependent killing in liquid media in spite of the good anti-tubercular activity (comparable to that of ETH) that TCH exerted in solid media. In our hands, TCH lacked activity on liquid medium even at concentrations 10-fold higher than MIC, regardless of growth conditions. The utilization of 7H9-ADS-Gly-Tween as liquid (in which TCH is inactive) and solid (where TCH is active) media ruled out medium composition as a factor on the drug activity. We also discarded oxygen as a critical factor for activity since TCH did not produce any killing effect whether liquid cultures were incubating with or without agitation under conditions usually met in clinical laboratories. Discrepancies in MIC values for Pyrazinamide in liquid media have been traced back to an inoculum effect, most likely due to metabolic activity of the growing bacilli that may change the medium pH and thus reduce the efficacy of this drug, active in acidic media (Zhang and Mitchison 2003). Importantly, it has recently been reported that the activity of bedaquiline in liquid medium is affected by the inoculum size used for the assay (Lounis, Vranckx *et al.* 2016); although of importance for clinical practice, the reasons for that discrepancy remain unknown. Our preliminary results showed that TCH (25 µg/ml) did not exert any visible activity in liquid medium (7H9-ADS-gly-Tw) even when a reduced inoculum size was used ($\approx 2x10^5$ CFU/ml) (Franceschelli, J.J., personal communication), the reasons for that failure remain to be determined.

There are no experimental data on TCH stability and physical properties, with only some theoretical studies of its spectroscopic properties (Balachandran, Janaki *et al.* 2014). However, our studies discarded instability of the compound in liquid medium as a possible reason for the lack of TCH activity (Fig. S2A-C). To our knowledge this is the first report on a compound exerting anti-tubercular activity only on solid media; and thus, a thrilling challenge to solve and a warning note for the screening of novel anti-tubercular drugs that are usually performed in liquid media.

FASII is not a target for TCH. Due to structural similarities between TCH and the well-characterized mycolic acids inhibitors INH and ETH, and our results showing a comparable *in vitro* oxidation of both INH and TCH by Mn(III) pyrophosphate (Fig. S1) we hypothesized that TCH could be a pro-drug acting as an inhibitor of the FASII cycle by inactivation of the enoyl-ACP reductase InhA. In order to test that, we performed "*in vivo*" radiolabeling on cells growing in solid medium, thus overcoming the lack of activity of TCH in liquid media under our experimental conditions. Yet, in spite of being able to see the inhibition of the synthesis of mycolic acids by INH, TCH gave a fatty acid profile indistinguishable from the one obtained from cells grown in the absence of any inhibitor (Figs. 3 and 5). The results remained unchanged after extended exposure to the drug; thus the mechanism of action of TCH is not related to inhibition of the synthesis of mycolic acids.

Mutations in the *ahpC* promoter confer resistance to TCH. Our screen for spontaneous TCH^R mutants yielded ≈ 50% that displayed cross-resistance to INH (but not to ETH or TRC); a second group, with a TCH^R INH^S ETH^S TRC^S phenotype (thus suggesting the presence of a mutation conferring resistance to TCH that was not accompanied by resistance to those well characterized InhA inhibitors) was of most interest to us. We therefore performed WGS on 7 randomly chosen TCH^R mutants and one mutant displaying a medium level TCH^R high level INH^R phenotype. Surprisingly, four of these mutants showed a previously described SNV in the promoter region of *ahp*C (Rv2428.), a gene encoding an alkylhydroperoxydase. The mutation, c-81t, was reported by several groups as present in *M. tuberculosis* INH^R mutant strains and shown as causing an increase in *ahpC* expression (Sherman, Mdluli *et al.* 1996, Zhang, Dhandayuthapani *et al.* 1996, Heym, Stavropoulos *et al.* 1997). The mutant strain TCHR25.1 showed a mutation in a two-component

system for which no role on INH resistance has been previously been described; however the impact of the mutation on the function of the protein was not evident. The remaining SNVs fell either in intergenic regions or non-essential genes. The potential contribution of these latter mutations to TCH resistance will need further studies. Importantly, the mutants containing the c-81t change were isolated from four of the five independent cultures used for TCH^R mutant screening and represented half of the TCH^R $INH^S ETH^S$ TRC^S mutants randomly selected for WGS analysis (4/8), thus strongly implicating a role for *ahpC* expression in resistance to TCH.

Different approaches indicates that *ahp*C is not an essential gene in *M. tuberculosis* and *M. bovis* (Wilson, de Lisle *et al.* 1998, Springer, Master *et al.* 2001, DeJesus, Gerrick *et al.* 2017), thus suggesting that *ahpC* does not fulfill the requirements to be considered a target for TCH but plays an important role in resistance to this compound.

The link between resistance to INH and the presence of mutations in the *M. tuberculosis katG* and *ahpC* genes have been studied for almost 20 years. While the role of KatG in the INH^R phenotype is clear as the necessary activator of the INH pro-drug, the precise role of *ahpC* has proven more difficult to elucidate. Mutations in the promoter region and in the *ahpC* coding sequence in INH^R strains have been described in detail (Vilcheze and Jacobs 2014). A survey of the literature shows that mutations in the *ahpC* promoter region leading to overexpression of the enzyme are described in 29% of INH^R strains (Kelley, Rouse *et al.* 1997, Pagan-Ramos, Song *et al.* 1998, Rinder, Thomschke *et al.* 1998, Dalla Costa, Ribeiro *et al.* 2009, Vilcheze and Jacobs 2014). Importantly, one study reported that 20% of INH^R clinical isolates and 8% of the INH^S isolates contained the same mutations in the *oxyR-ahpC* intergenic region (Baker, Brown *et al.* 2005). Moreover, over-expression of *ahpC* failed to increase *M. tuberculosis* MIC to INH suggesting that this enzyme is not directly related to INH resistance (Heym, Stavropoulos *et al.* 1997). Thus, our results describing low level resistance to INH within the isolated TCH^R mutants are in agreement with previous publications. Although a matter of debate, it is generally accepted that *ahpC* mutations arise as a

compensatory mechanism in strains that have lost *katG* (Sherman, Mdluli *et al.* 1996), from this perspective we are herein reporting that *ahp*C mutants may arise without *kat*G mutations being present.

It is important to mention that it has previously been described that *M. bovis* BCG Pasteur shows up-regulation of *ahp*C (Springer, Master *et al.* 2001). Moreover, recently a very comprehensive study by Abdalah *et al.* analyzed the global transcriptional profile and gene expression differences, as well as quantitative protein analysis, between BCG strains. Their results showed a larger amount of AhpC when BCG strains Pasteur (3.4-fold), Phipps (4-fold), Danish (1.9-fold), Tokyo (1.8-fold) and Birkhaug (2.9-fold) were compared to *M. bovis* 2122/97 (Abdallah, Hill-Cawthorne *et al.* 2015). Similarly, an increased amount of AhpD (2-3-fold for all the BCG strains mentioned) was detected, however no SNVs were present in the promoter region of *ahp*C (Abdallah, Hill-Cawthorne *et al.* 2015). As BCG Pasteur shows equal levels of inhibition by TCH as *M. bovis*, it is therefore not the case that simple up-regulation of *ahp*C expression is sufficient for increased resistance to TCH, at least in *M. bovis* lineage strains. Indeed, BCG strains have several SNVs and large insertion/deletions which may explain the lack of concordance between increased *ahp*C expression and TCH susceptibility. Despite these observations, the fact that the only SNV found in our TCH resistant strains was a well described mutation causing overexpression of *ahp*C strongly supports the idea of this protein as an important factor contributing to TCH resistance in these *M. tuberculosis* mutants.

In conclusion, we have confirmed a role for KatG in the resistance to TCH, reinforcing previous phenotypic observations of cross resistance between TCH and INH. In addition, we isolated TCH resistant mutants with a drug susceptibility profile consistent with its lack of activity on InhA, and finally and importantly, we unveiled *ahpC* expression as a player in resistance to TCH, a compound with anti-tubercular activity. These data provide new clues that we hope will finally lead to the identification of the molecular target for TCH.

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655 **Figure legends**

656 **Figure 1**: Growth of *M. smegmatis* mc^2155 in solid 7H9-ADS-Gly medium containing increasing
657 concentrations of TCH. Aliquots of 100 µl containing aprox 10^2-10^3 cfu were spread on each plate.
658 Plates were incubated for 15 days at 37°C and growth monitored daily.

659 **Figure 2**: Role of KatG in the activity of TCH in *M. tuberculosis*. (A) Chemical structures of INH,
660 ETH and TCH. (B) KatG mediated activation of INH and adduct formation of the activated INH
661 radical; proposed activation of TCH. Panels C and D: Susceptibility to TCH or INH of *M.*
662 *tuberculosis* NM27388 (containing a Ser315Thr mutation in KatG) (C) or *M. tuberculosis* H37Rv
663 pMV361::*katG* (D). Aliquots of 100 µl of each culture containing approximately 10^2–10^3 cfu were
664 spread on each half plate of 7H9-ADS-Gly solid media plates containing INH or TCH at the
665 indicated concentration. Plates were incubated for 30 days at 37°C before visual inspection.

666 **Figure 3**: Analysis of the effect of TCH on fatty acid and mycolic acid biosynthesis in
667 *M. tuberculosis* H37Rv. TLC of FAMEs and MAMEs extracted from *M. tuberculosis* H37Rv
668 cultures growing in the presence of TCH (exposure time 24 or 48 h, drug concentration 25 or 50
669 µg/ml) or INH (0.5 µg/ml). Comparable counts (≈40 000 cpm) were loaded onto silica gel TLC
670 plates, which were developed three times in hexane/ethyl actetate (95:5, v/v). Plates were exposed
671 to X-ray film and for 48-72 h at -80°C before developing. FAME, fatty acid methyl ester; MAMEs
672 mycolic acid methyl esters (α, α-mycolic acids; M, metoxi-mycolic acids; K, keto-mycolic acids).

673 **Figure 4**: TCH does not affect *M. tuberculosis* growth in liquid medium. *M. tuberculosis* H37Rv
674 was grown at 37°C in the presence of TCH (25 or 50 µg/ml) or INH (0.5 µg/ml). Growth was
675 monitored by samples absorbance (OD_{600nm}). Three independent replicates were carried on with
676 comparable results.

677

Figure 5: Analysis of the effect of TCH on mycolic acid biosynthesis in *M. tuberculosis* H37Rv grown in solid medium. Aliquots of mid-log cultures of *M. tuberculosis* H37Rv were exposed to solid medium containing either TCH (50 µg/ml), INH (0.5 µg/ml) or left untreated and labeled lipids were extracted as described in Materials and Methods. 2D-TLC analysis of ^{14}C-acetate labeled cultures was performed by loading comparable counts (≈80,000 cpm) on silica plates impregnated with $AgNO_3$. The plates were developed twice in hexane:ethyl acetate (95:5 v/v) in the first direction and three times in petroleum ether:diethyl ether (85:15 v/v) in the second direction. OAME, oleic acid methyl ester; SFAMEs, saturated fatty acids methyl esters; MAMEs mycolic acid methyl esters (α, α-mycolic acids; M, metoxi-mycolic acids; K, keto-mycolic acids).

Table 1. MICs of *M. tuberculosis* strains against TCH and INH.

Strain	MIC (µg/mL)	
	TCH	INH
H37Rv	10	0,1
INM27833 (KatG S315T)	>50	>0,5
H37Rv pMV361::*kat*G	≤5	≤0,05

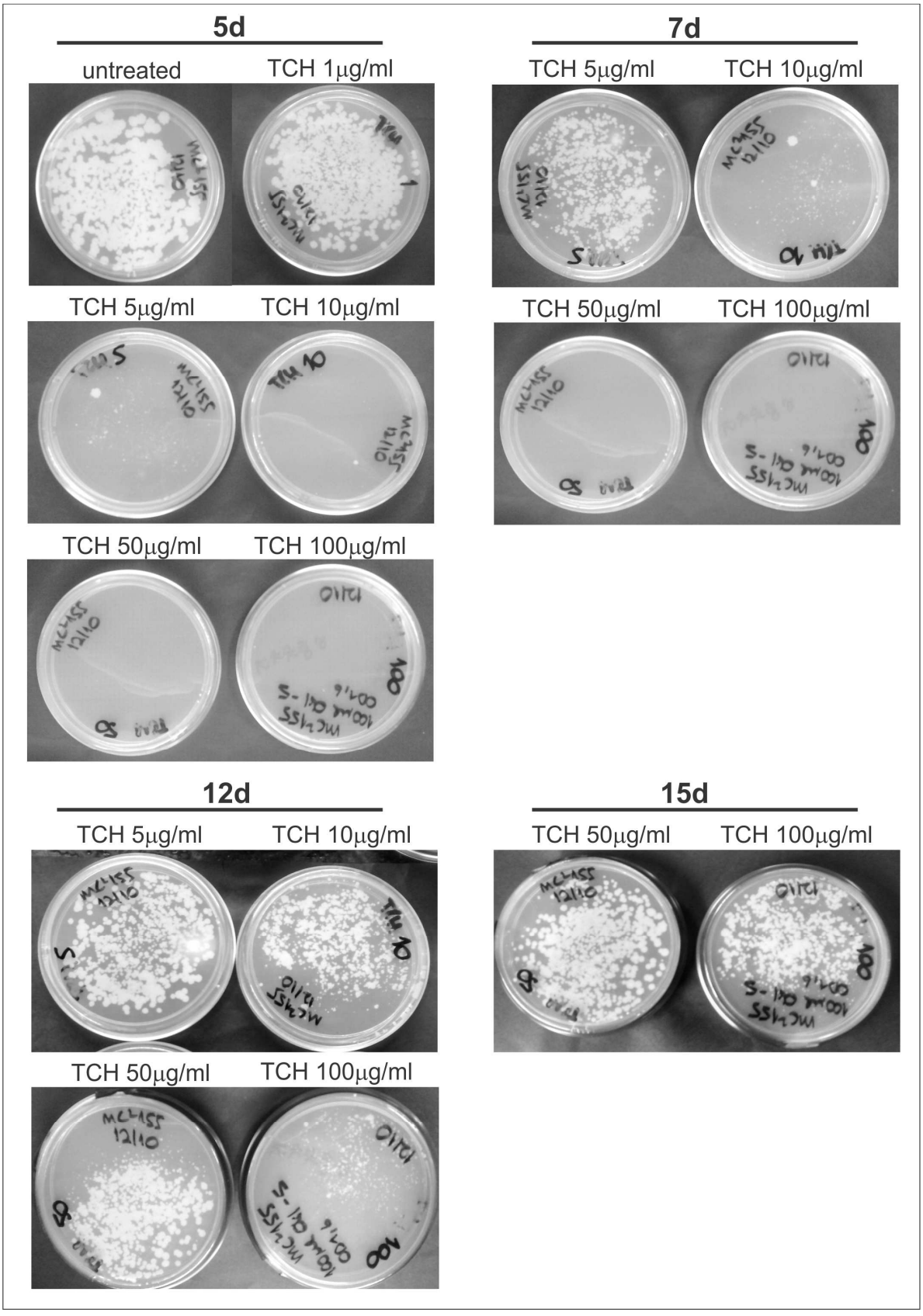
Table 2. MICs of TCH^{R} *M. tuberculosis* mutants against TCH, INH, ETH and TRC

Strain/mutant	MIC (µg/mL)			
	TCH	INH	ETH	TRC
H37Rv	5-10	0.05	5	10
TCH R25.1	25	0.5	5	10
TCH R25.12, TCH R25.13, TCH R25.10	25-50	0.05	5	10
TCH R25.11	50-100	0.05	5	10
TCH R50.6, TCH R50.7, TCH R50.8, TCH R50.9	≥100	0.1	5	10
TCH R50.1, TCH R50.2, TCH R50.3, TCH R50.4, TCH R25.2 TCH R25.3	≥100	0.5	5	10

Table 3. SNPs identified in *M. tuberculosis* TCH-resistant mutants.

TCH^{R} mutant	Coordinate	Gene	Rv number	Mutation	Aminoacid substitution	Comment
25.1	380435	Intergenic		c → g	N/A	-121bp upstream of *Rv0312*, conserved hypothetical proline and threonine rich protein
	3596244	*Rv3220c*	Rv3220c	a → g	S431P	Two component sensor kinase
25.11	3510642	*PPE52*	Rv3144c	c → t	G226S	SNPs also identified in Ioerger *et al* [#]
25.12	4160982	Intergenic		g →a	N/A	-19bp upstream of *Rv3716c* and -116bp upstream of *Rv3717c*
25.13	3043105	*Rv2731*	Rv2731	g → a	G27R	Conserved alanine and arginine rich protein
50.6	2086466	*glc*B	Rv1837c	g → a	A172A	
	2726112	Intergenic		c → t	N/A	-81bp Upstream of *ahpC* (Rv2428)
50.7	2726112	Intergenic		c → t	N/A	-81bp Upstream of *ahpC* (Rv2428)
50.8	2726112	Intergenic		c → t	N/A	-81bp Upstream of *ahpC* (Rv2428)
50.9	2726112	Intergenic		c → t	N/A	-81bp Upstream of *ahpC* (Rv2428)

[#]J Bacteriol. 2010 Jul;192(14):3645-53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untreated
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7d
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TCH 50μg/ml
TCH 100μg/ml
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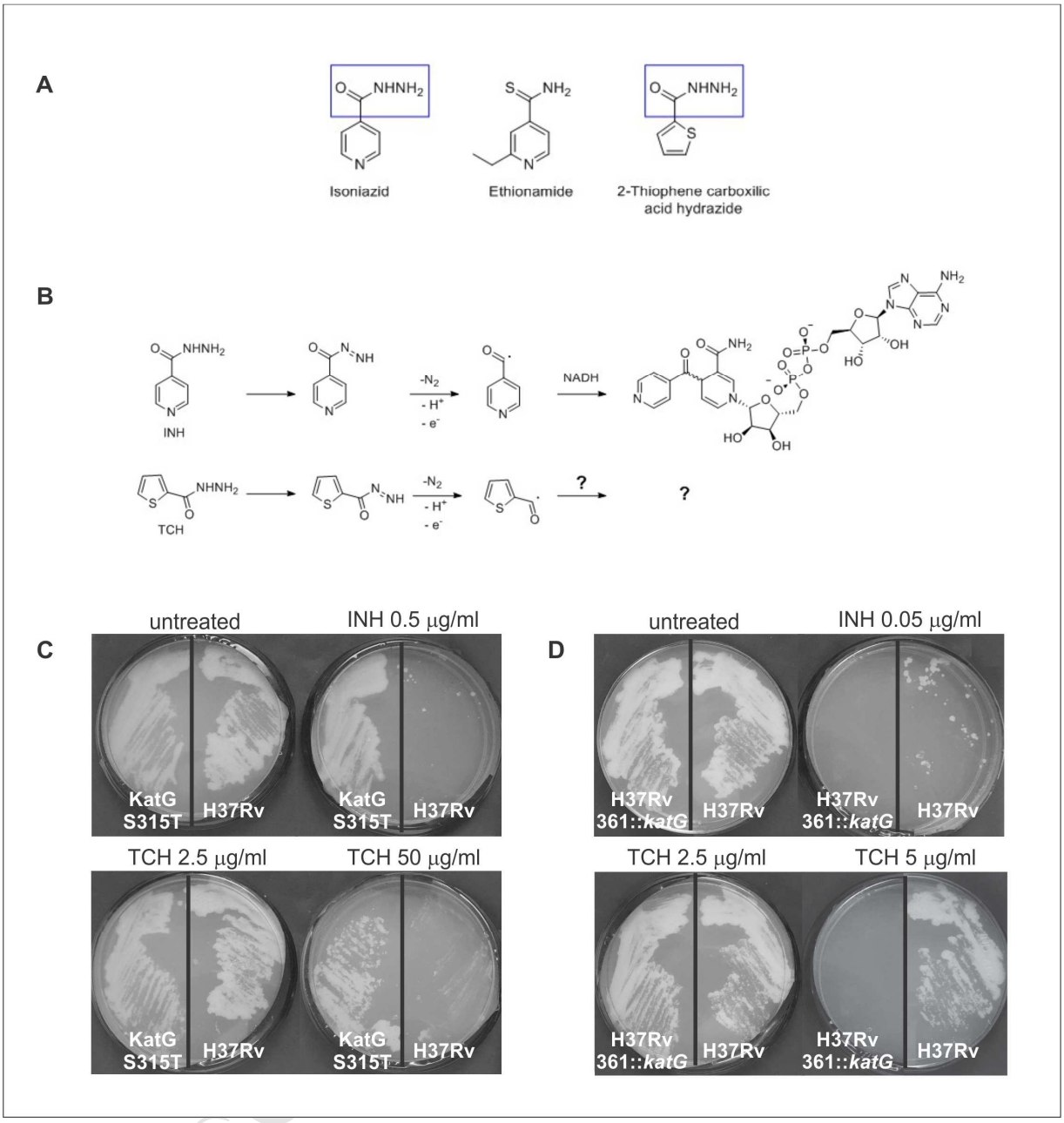
TCH 100μg/ml


A
Isoniazid
Ethionamide
2-Thiophene carboxilic acid hydrazide
B
INH
TCH
-N2
- H+
- e-
NADH
?
C
untreated
INH 0.5 μg/ml
KatG S315T
H37Rv
TCH 2.5 μg/ml
TCH 50 μg/ml
D
untreated
INH 0.05 μg/ml
H37Rv 361::katG
H37Rv
TCH 2.5 μg/ml
TCH 5 μg/ml