Transcriptional Response of *Mycobacterium tuberculosis* to Cigarette Smoke Condensate

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Smoking is known to be an added risk factor for tuberculosis (TB), with nearly a quarter of the TB cases attributed to cigarette smokers in the 22 countries with the highest TB burden. Many studies have indicated a link between risk of active TB and cigarette smoke. Smoking is also known to significantly decrease TB cure and treatment completion rate and increase mortality rates. Cigarette smoke contains thousands of volatile compounds including carcinogens, toxins, reactive solids, and oxidants in both particulate and gaseous phase. Yet, to date, limited studies have analyzed the impact of cigarette smoke components on *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB. Here we report the impact of cigarette smoke condensate (CSC) on *Mtb* in vitro. We show that exposure of virulent *Mtb* to cigarette smoke increases the mutation frequency of the pathogen and strongly induces the expression of the regulon controlled by SigH—a global transcriptional regulator of oxidative stress. SigH has previously been shown to be required for *Mtb* to respond to oxidative stress, survival, and granuloma formation in vivo. A high-SigH expression phenotype is known to be associated with greater virulence of *Mtb*. In patients with pulmonary TB who smoke, these changes may therefore play an important, yet unexplored, role in the treatment efficacy by potentially enhancing the virulence of tubercle bacilli.

**Keywords:** smoking, cigarette smoke, tuberculosis, sigH, mycobacterium

**INTRODUCTION**

Cigarette smoking is practiced by an estimated 20.7% of the world's population, 80% of which belong to low- and middle-income countries. Smoking is considered an added risk factor for tuberculosis (TB), with nearly a quarter of the TB cases attributed to cigarette smokers in the 22 countries with the highest TB burden (WHO, 2019). Many studies have indicated a link between risk of active TB and cigarette smoke, with some predicting as much as a fivefold increase in the risk of active TB in smokers compared to non-smokers (Yu et al., 1988; Jha et al., 2008; Lin et al., 2009; Rao et al., 2012, 2017; Ozturk et al., 2014; Smith et al., 2015; Bronner Murrison et al., 2016). Smoking also significantly decreases TB cure and treatment completion and increases mortality rates (Chiang et al., 2012; Bonacci et al., 2013).

Whole cigarette smoke contains thousands of volatile compounds including carcinogens, toxins, reactive solids, and oxidants in both gaseous and particulate phase. Generation of whole
cigarette smoke under laboratory conditions (containing both the soluble and insoluble fractions of cigarettes smoke that would be inhaled into the lungs of a smoker) is challenging. Two forms of cigarette smoke, cigarette smoke extract (CSE) and cigarette smoke condensate (CSC), are therefore routinely used for investigating the effect of cigarette smoke components in vitro and in situ. CSE is obtained by dissolving the water-soluble gas and particulate phase, generated from puffing a lit cigarette, in a phosphate-buffered saline solution. CSC consists of the water-insoluble fraction and is generated by passing the cigarette smoke through a filter pad and eluting the particles collected on the filter in a solvent. The compositions of CSE and CSC differ, with nicotine for instance making up 55.8% of CSC but only 8.98% of CSE (Mutepe et al., 2013). This is important to note when using either for investigating the effect of cigarette smoke. CSC has been used in many research studies to study the effect of cigarette smoke (Baboni et al., 2010; Mutepe et al., 2013; Cockeran et al., 2014; Semlali et al., 2014; Cholo et al., 2020).

Some of the compounds of cigarette smoke are pro- and others anti-inflammatory (Borgerding and Klus, 2005; Stampfli and Anderson, 2009; Kim et al., 2018). It increases the permeability of the epithelium, impairs mucociliary clearance, alters metabolism of cells, and induces oxidative and nitrosative damage to cell membranes and DNA—thereby ultimately causing apoptosis. Although smoking increases the production of pro-inflammatory cells, it compromises their ability to phagocytose and kill bacteria, thereby increasing bacterial burden (Stampfli and Anderson, 2009; Feng et al., 2011; Shaler et al., 2013; Agarwal et al., 2014; O’Leary et al., 2014; Aryanpur et al., 2016a,b; Bai et al., 2017). Smoking also causes genomic DNA mutations that are associated with cancers (Alexandrov et al., 2020).

The respiratory tract microbiota differs significantly between smokers and non-smokers (Charlson et al., 2010; Yu et al., 2017). Although many studies have investigated the effect of cigarette smoke on the host cells and responses, limited studies have been done to investigate the effect of cigarette smoke on human pathogens that commonly infect the respiratory tract. Cigarette smoke increases oxidative stress and biofilm formation in Pseudomonas aeruginosa (Chien et al., 2020), Staphylococcus aureus (Kulkarni et al., 2012; Hwang J. H. et al., 2016), Streptococcus mutans (Baboni et al., 2010), and the sino-nasal microbiota of smokers (Goldstein-Daruech et al., 2011). Increased cell adhesion, favoring persistent colonization, is observed in S. aureus and methicillin-resistant S. aureus (MRSA) (Kulkarni et al., 2012; Crotty Alexander et al., 2015; McEachern et al., 2015; Hwang J. H. et al., 2016). An upregulation of virulence genes and concomitant increased virulence is observed in P. aeruginosa and S. aureus (McEachern et al., 2015; Hwang J. H. et al., 2016; Kulkarni et al., 2016; Chien et al., 2020). Expression of virulence and adhesion factors are, however, not upregulated in Streptococcus pneumoniae (Manna et al., 2018), while biofilm formation is (Mutepe et al., 2013). Mycobacterium tuberculosis (Mtbb) biofilm formation is also increased upon exposure to CSC (Cholo et al., 2020). Detoxifying enzymes, efflux pumps, and osmoregulator transporters, which can provide a direct defense against the cigarette smoke, are upregulated, while fatty and lipoteichoic acid synthesis is downregulated in S. pneumoniae (Manna et al., 2018). These different responses could suggest a bacterial specific cigarette smoke response.

It was suggested that the aforementioned changes may be heritable and therefore likely due to genetic modifications brought about by the cigarette smoke exposure. Resistance to antimicrobial agents is for instance observed as a result of cigarette smoke exposure (Miyahara et al., 2011; Crotty Alexander et al., 2015, 2018; Hwang J. H. et al., 2016; Hwang J. Y. et al., 2016; Chien et al., 2020). Although this resistance is linked to changes in the cell wall in MRSA (McEachern et al., 2015), cigarette smoke exposure was directly linked to clinically relevant mutations in drug [rifampicin (RIF) and ciprofloxacin] resistance genes of P. aeruginosa (Miyahara et al., 2011). In Salmonella typhimurium, the induction of mutant variants is also observed upon exposure to cigarette smoke components (Claxton et al., 1989; Auffeherde and Grossmann, 2007, 2008). This mutability is smoke concentration, composition, time of exposure, and strain dependent (Mizusaki et al., 1977; Morin et al., 1987; Camoirano et al., 1994; Yim and Hee, 2001; Yim et al., 2002; Auffeherde and Grossmann, 2007, 2008; Roemer et al., 2016; Thorne et al., 2016). It was previously suggested that cigarette smoke should also be considered as a mutagenic substance to Mtbb, the causative agent of TB (McGrath et al., 2014). To our knowledge, the effect of CSC on the transcriptional response of Mtbb has, however, not been investigated. In this study, the effects of CSC on the survival of Mtbb, mutation frequency, and gene expression profile in vitro are explored.

MATERIALS AND METHODS

Bacterial Culture Conditions

Mtbb CDC1551 (BEI: NR-13649) was cultured in 7H9 containing 0.05% Tween-80 and supplemented with 0.2% glycerol and 10% oleic acid, albumin, dextrose, catalase (OADC) (Middlebrook) (subsequently referred to as 7H9 OADC). For exposure to CSC, 7H9 supplemented with 0.2% glycerol and dextrose (20 g/l) containing 0.05% Tween-80 (referred to as 7H9 dextrose) was used. CSC (Murty Pharmaceuticals, Lexington, KY, United States) was diluted fivefold in dimethyl sulfoxide (DMSO) to prepare working stock of 8 mg/ml which was added to 7H9 dextrose where indicated. Bacteria were also cultured on 7H11 agar (Difco) supplemented with 0.5% glycerol and 10% OADC (prepared in the laboratory: 0.06% oleic acid, 5% albumin, 2% dextrose, 0.004% catalase, 0.85% sodium chloride, and filter sterilized) (subsequently referred to as 7H11 agar) for colony-forming unit (CFU) enumeration. A RIF stock solution of 10 mg/ml was prepared in 10% DMSO 90% water solution and used where necessary at 2 μg/ml final concentration in 7H11 agar.

MIC Determination

A serial twofold dilution series of CSC working solution (8 mg/ml in DMSO), DMSO, and 7H9 dextrose was prepared in 7H9 dextrose in a 96-well plate. Mtbb cultures at day 4 of growth were diluted to optical density (OD) of 0.002 and equal volumes added to these serial dilutions (total 100 μl). After 3 weeks of

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growth at 37°C, resazurin (30 µl of a 0.02% stock) was added to each well and color development was allowed overnight. Pink wells indicated growth and blue, no growth. DMSO wells served as control for the effect of the CSC diluent on the growth of Mtb while the 7H9 dextrose wells served as positive Mtb growth controls. Three independent replicate experiments were done.

**Cigarette Smoke Condensate Exposure Survival Assays**

Starter cultures were cultured in 7H9 OADC for 6 days, subcultured to OD 0.05, and allowed to grow for 4 days. Bacteria were collected, washed twice in 7H9 dextrose to remove catalase, resuspended in 7H9 dextrose, and sonicated to remove clumps. After filtering through a 0.2-micron cell strainer (Falcon) to remove clumps, the cultures were diluted to an OD of 0.2. For survival assays, these cultures were split into 10-ml cultures and exposed to 50 µg/ml CSC or equal volume of DMSO only (unexposed) for 3, 6, 24, and 48 h. Dilution series were prepared at each time point in phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Tween-80 and proper dilutions plated on 7H11 agar. CFUs were enumerated after 3–4 weeks. The percentage survival was calculated by dividing the CFU/mL of CSC exposed cultures with that of the DMSO-exposed (unexposed) cultures, which was set as 100% survival.

**Mycobacterium tuberculosis Mutation Frequency Determination**

Rifampicin resistance is generally acquired by mutations in the rpoB gene of Mtb (Hirano et al., 1999). The acquisition of these mutations and subsequent ability of the bacteria to grow in the presence of RIF have been used in many studies to study the mutation frequency and rate in Mtb (McGrath et al., 2014). These assays were adapted for mutation frequency determination after exposure to CSC. Exposure to the diluent of CSC, DMSO, was taken as the mutation rate without exposure, to exclude any influence of the diluent. Preparation of cultures was done as for the CSC survival assays. Cultures were split into 30-ml cultures and exposed to 50 µg/ml CSC or equal volume of DMSO (unexposed) for 3 and 24 h. Dilution series of two separate cultures were prepared in PBS containing 0.5% Tween-80 and proper dilutions plated on 7H11 agar. Undiluted cultures (10 ml, 1 ml, and 100 µl) were also plated on 7H11 agar containing 2 µg/ml RIF. CFU enumeration was done after 4 weeks to exclude the presence of satellite colonies. The mutation frequency in the presence of CSC was calculated by using the average CFU in the two cultures exposed to CSC plated on 7H11 agar RIF (representing the RIF resistant mutants) and dividing it by the average CFU in the two cultures exposed to CSC plated on 7H11 agar (total bacterial count). The mutation frequency was calculated in the same way for the DMSO (unexposed) cultures. Three independent exposure experiments were done.

**RNA- Sequencing**

The Zymo-Seq RiboFree Total RNA Library Kit was used for library preparation according to the manufacturer’s instructions. RNA (500 ng) was used and ribosomal depletion done for 30 min, followed by 14 cycles of PCR. RNA-seq was done using the Illumina NextSeq 500 V2.5 kit according to the manufacturer’s instructions on the NextSeq 500 machine by the UTHSCSA Genome Sequencing Facility. Sequencing reads were subjected to secondary data analysis using the Partek Flow (Partek Inc., Chesterfield, MO, United States) pipeline. In short, reads were filtered based on quality of Phred 30 with minimum read lengths of 25 nucleotides and aligned to the Mtb CDC1551 reference genome sequence (European Nucleotide Archive ASM858v1) using the RNA-seq aligner STAR v2.5.3a. Post-alignment quality assessment, transcript abundance estimation according to ASM858v1 transcriptome annotation by expectation-maximization algorithm, and sample read count normalization using Transcripts Per Kilobase Million (TPM) were done.

**Differential Gene Expression Analysis**

Read count data (not normalized) were uploaded into the iDEP91 online RNA-seq analysis software (Ge et al., 2018). The minimal counts per million (CPM) were kept at default of 0.5 in at least one library. Count data were transformed for clustering and principal component analysis using EdgeR: log2 (CPM + c) and pseudocount (c) of 4. Gene IDs were not converted to Ensembl. Where multiple transcripts were present, full stops were replaced with underscores in the read count files to enable recognition of the transcripts as distinct. Missing-value imputation was done.
and the Mycobrowser website of CSC was determined by literature search on Google Scholar. The function of genes differentially expressed in the presence of CSC was considered the main factor, reference DMSO used to CSC was considered as a factor, Exposure Option on the IDEP91 software, with an FDR of 0.1 and minimum fold change of 2.0. Time was considered as a factor. Exposure of gene median. Principal component analysis was done. Differentially expressed genes were identified using the DESeq2 R package via access from GEO. Raw and analyzed data can be accessed from GEO via the accession number GSE172041.

Confirmation of Gene Expression by RT-qPCR

Some genes, which were differentially regulated by exposure to CSC according to RNA-seq data, were selected for confirmation of gene expression levels by RT-qPCR. RNA (500 ng) was reverse transcribed using random primers and the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions in a Veriti 96-well thermal cycler (Thermo Fisher Scientific). No reverse transcriptase controls were done to confirm the absence of DNA in the RNA samples. PCR was done using 0.5-μM primers (Table 1) and the Applied Biosystems PowerUp SYBR Green Master Mix kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was diluted 100-fold before use in the PCR reactions. PCR conditions used were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 18 s, and 72°C for 30 s. Melt curve analysis was done (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s) to confirm the presence of only one PCR product and the absence of primer dimers. The ΔΔCt method was used and normalization done with sigA as a housekeeping gene.

Statistical Calculations

All graphs were plotted using GraphPad Prism 8.02 or IDEP91 software available online, as indicated above (Ge et al., 2018). Multiple t-tests were done, without assuming equal deviation or correction for multiple comparisons, and p ≤ 0.05 was considered statistically significant.

### RESULTS

#### Effect of Cigarette Smoke Condensate on Mycobacterium tuberculosis Growth and Survival

The effect of nicotine, one of the cigarette smoke components, on non-tuberculous mycobacteria and CSC on *Mtb* was previously investigated and shown to have no effect on the growth in vitro (Greenstein et al., 2011; Cholo et al., 2020). Since the effect of CSC was previously investigated in H37Rv strain and in 7H9 OADC media (containing catalase which mops up reactive oxygen species), we determined the effect of CSC on the growth and survival of the CDC1551 strain in the absence of catalase to determine the concentrations of CSC to be used in this study. Firstly, we investigated the concentration at which CSC inhibits *Mtb* growth by doing MIC assays. The growth of *Mtb* was present in all wells containing only 7H9 dextrose. No growth was observed at a CSC concentration of 125 μg/ml, while growth was still present at 62.5 μg/ml, making the MIC between 62.5 and 125 μg/ml. This was as a result of CSC and not the diluent, DMSO, since growth was observed up to 3.125% DMSO and only 0.78% DMSO was present in the 62.5-μg/ml CSC well. Based on the MIC, *Mtb* was exposed to 25, 50, and 75 μg/ml CSC or DMSO (0 μg/ml) for 3, 6, 24, and 48 h in 7H9 dextrose media. Survival was assessed by CFU enumeration and relative to DMSO exposure, which was set as 100% survival. No significant difference in bacterial survival upon exposure to CSC was observed at the different CSC concentrations or time points tested (Figure 1A).

#### Effect of Cigarette Smoke Condensate on Mutation Frequency of Mycobacterium tuberculosis

Since CSC contains a whole array of toxic compounds (Borgerding and Klus, 2005; Kim et al., 2018) and was shown to be mutagenic in other bacteria (Claxton et al., 1989; Auferheide and Grossmann, 2007, 2008; Miyahara et al., 2011), the effect of CSC on mutation frequency was investigated. Growth on RIF was considered as genotypic development of RIF resistance. The frequency of RIF mutations in the presence of CSC (50 μg/ml) was compared to the frequency of RIF mutations in the diluent of CSC, DMSO. The mutation frequency in the presence of CSC was significantly (2.32-fold) higher than in DMSO (Figure 1B).

#### Transcriptional Response to Cigarette Smoke Condensate

RNA was extracted from *Mtb* cultures exposed to 50 μg/ml CSC and DMSO for 3 and 24 h. To investigate the transcriptional response of *Mtb* to CSC, RNA-sequencing was done and reads mapped to the CDC1551 reference genome. Comparable total reads per million counts were observed for all the samples (Figure 2A). PCA showed that time was the biggest factor in differential gene expression (Figure 2B). This is likely due to multiple factors including the composition and concentration of CSC that likely changed as a result of the volatile nature of CSC. Bacterial growth was also observed previously between

### Table 1: Gene-specific primers used for RT-qPCR analysis.

| Gene primer | Sequence (5′−3′) |
|-------------|-----------------|
| SigAFor     | TGCAGTCGGTGCTGGACAC |
| SigARrev    | CTCGTAACGGGCTCCTTGTGA |
| MT3320For   | GTCCAACGGCGAGATTTCTCT |
| MT3320Rrev  | CTTGCCGTCGCTTCCTTGTGA |
| MT1608For   | ACGGAAGATACGCGACTGAG |
| MT1608Rrev  | GCCAAACGGCGAGATTTCTCT |
| MT1259For   | GTGCGCTGATGCTGCACACCA |
| MT1259Rrev  | GCCAAACGGCGAGATTTCTCT |
| MT0997For   | ACCTCTGCGTGAAAGGTGCTC |
| MT0997Rrev  | TGCACCCCCGGTCACTGGG |

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M. tuberculosis Response to Cigarette Smoke

**FIGURE 1** | Survival and mutation frequency in Mtb exposed to CSC. (A) Survival of Mtb upon exposure to 25, 50, and 75 μg/ml CSC for 3, 6, 24, and 48 h relative to survival upon exposure to the diluent, DMSO, which was set as 100%. Multiple t-tests were done (n = 3), without assuming standard deviation and without correction for multiple comparisons. No significant differences were observed. (B) Frequency of RIF-resistant mutants, determined by CFU on 7H11 agar RIF (2 μg/ml), compared to bacterial count in the culture, determined by plating dilution series on 7H11 agar. Three independent experiments (n = 3) were done and are indicated in different colors. The fold difference in mutation frequency between CSC and DMSO is indicated in the corresponding color with black indicating the mean fold change. The error bars indicate the mean and standard deviation. A paired, two-tailed t-test indicated significant increase in mutation frequency upon exposure to CSC (p = 0.034). *p < 0.05.

**FIGURE 2** | RNA-seq analysis. (A) Total read count per million for all samples. (B) PCA separated by CSC exposure followed by time for all the samples (C) PCA for 24-h samples indicating divergence of the batch B samples (batch A = replicate 1, batch B = replicate 2, and batch C = replicate 3). DMSO samples are indicated in blue and CSC in red. Graphs were generated using the iDEP91 software.

the 3- and 24-h time points, contributing to the different bacterial state and transcription at the two time points. Larger variations may also mask some smaller differences in the effect of CSC during the analysis. A separate analysis of the 3- and 24-h data was therefore done. Of the 4,007 genes for which expression was assessed at 3 h of exposure, 3,968 genes passed
Cigarette smoke condensate inhibited Mtb’s growth at a concentration between 62.5 and 125 μg/ml in vitro cultures. Mtb’s survival upon exposure to CSC was not significantly affected, although slightly lower CFUs were observed upon CSC exposure compared to DMSO (Figure 1A). CSC previously did not affect the growth of Mtb H37Rv in planktonic 7H9 OADC cultures in 96-well plates (Cholo et al., 2020). One of the main components of cigarette smoke, nicotine, did not affect growth of non-tuberculous mycobacteria in culture (Greenstein et al., 2011) but did promote the growth of Mtb in epithelial cells (Miramontes et al., 2021). An initial bacteriostatic effect of CSC, within the first 24 h, was observed during survival assays, although doubling of bacteria occurred after 48 h. This is likely due to the dissipation, degradation, and/or chemical changes of the volatile compounds within CSC within the 24-h period. Additionally, interaction of CSE with plastic containers and degradation and/or evaporation of CSE was previously shown to influence the bioavailability of the cigarette smoke components in cell culture experiments (Bourgeois et al., 2016). The volatile nature of CSC is therefore a limiting factor when attempting to explore the effects of more long-term CSC exposure on Mtb. It would be interesting to investigate the effects of cigarette smoking on Mtb load and virulence factors in animal models such as a macaque model, which closely mimics the immune response in humans.

A 2.32-fold higher mutation frequency was observed after 24 h of exposure to CSC compared to exposure to DMSO (Figure 1B). Strain background, CSC concentration, composition, and time of exposure likely influenced the mutability of Mtb upon exposure to cigarette smoke (Mizusaki et al., 1977; Morin et al., 1987; Camoirano et al., 1994; Yim and Hee, 2001; Yim et al., 2002; Aufderheide and Gressmann, 2007, 2008; Bourgeois et al., 2016; Roemer et al., 2016; Thorne et al., 2016). It was also shown that the effect of CSE on bacteria was highly dependent on the bacterial-load-to-CSE ratio (Bourgeois et al., 2016). The effect of the ratio of Mtb to CSC concentration, bacterial growth phase, and strain background on the mutability of CSC should therefore be investigated in future studies.

Initially, the intention was to investigate the mutation rate and not just frequency. Preliminary experiments, however, indicated a negative mutation rate (data not shown), likely as a result of bacterial growth and death rate, since these factors had a significant effect on mutation rate calculations in previous studies (Frenoy and Bonhoeffer, 2018). A slight drop in the Mtb CFU was observed upon exposure to CSC, although this death did not cause significant percentage survival differences compared to DMSO controls (Figure 1A). Traditionally Luria–Delbrück assays, which use extremely low bacterial counts (thereby allowing exclusion of preexisting mutants from the cultures) and culturing for a prolonged time, are done to assess the mutation frequency and rate. This technique was, however, not usable due to the extremely volatile nature of CSC and inability to culture a low bacterial burden culture for an extended time. Another study has since also used cultures at mid-log growth for mutation rate analysis (Naz et al., 2021). Due to the volatile nature of CSC, the compounds to which Mtb were exposed likely also changed during the exposure time, thereby making the exposure conditions variable and mutation rate assessment challenging, if not impossible. Due to its slow growth, the mutation rate of Mtb CDC1551 is extremely low.

Mutation rate is also dependent on the drug used for mutation assessment (Ford et al., 2013). The use of phenotypic CPM filtering. Clustering of CSC-exposed and DMSO-exposed replicates/batches was observed. For the 24-h-exposed samples, PCA indicated clustering for replicates 1 and 3 (batches A and C) while replicate 2 (batch B) did not cluster as expected (Figure 2C). Replicate 2 samples were therefore excluded from all subsequent analyses for the 24-h samples. Of the 4,007 genes identified in the remaining four 24-h samples, 3,939 genes passed the CPM filter.

Following exposure to CSC for 3 h, 59 genes were upregulated more than twofold while only one was downregulated (Table 2). Genes upregulated upon exposure to CSC at 3 h included those encoding sigma factors [MT3320 (sigH), MT1259 (sigE), MT2783 (sigB), and MT3319], ESAT-6-associated proline–proline–glutamate (PPE) proteins [MT1068 (PPE15) and MT1850 (PPE29)], and other virulence and reactivation-associated genes [MT1608 (mmpL6) and (MT0148)]. Multiple genes which are regulated by SigH were upregulated, including genes encoding oxidoreductases (MT3569 and MT0083) and thioredoxin reductases [MT0838 (thiX), MT1517 (trxB), MT4032 (trxB2), and MT4033 (trxC)]. Genes encoding proteins involved in stress response such as MT0365 (dnaK), MT0366 (grpE), MT0397 (clpB), and MT3438) and genes previously shown to be induced by DNA damage [MT2881 and MT3142 (dinP)] were upregulated.

Since heavy metals are a known component of cigarette smoke (Borgerding and Klus, 2005), it was not surprising that the copper-responsive regulon and other genes encoding copper responsive/export systems [MT0995 (csoR), MT0996, MT0997 (ctpV), MT0998, MT0196 (mymT), MT0869, MT0873, and MT3039] as well as molybdenum [MT3301 (moeZ)] and cadmium [MT2719 (cadI)] responsive genes were upregulated by CSC. At 24 h of exposure to CSC, only seven genes were upregulated and three downregulated. Only five genes, which were upregulated at 3 h of CSC exposure, remained upregulated at 24 h including a methyltransferase (MT0586), MT1608 (mmpL6), a probable dehydrogenase MT3140, possible dioxygenase (MT3250), and TetR/MarR regulator (MT3249) encoding genes. Two genes encoding MarR family transcriptional regulators and bacterioferritin [MT0706_1 and MT3949 (bfbB)] were upregulated at 24 h, but not 3 h of CSC exposure. RNA-seq results were confirmed by RT-qPCR for selected genes (Figure 3).

**DISCUSSION**

The significant link between cigarette smoking and TB disease has been investigated on other respiratory bacteria, no studies have, to our knowledge, investigated the effect of CSC on the transcriptional profile or mutagenesis of Mtb.

Cigarette smoke condensate inhibited Mtb’s growth at a concentration between 62.5 and 125 μg/ml in vitro cultures. Mtb’s survival upon exposure to CSC was not significantly affected, although slightly lower CFUs were observed upon CSC exposure compared to DMSO (Figure 1A). CSC previously did not affect the growth of Mtb H37Rv in planktonic 7H9 OADC cultures in 96-well plates (Cholo et al., 2020). One of the main components of cigarette smoke, nicotine, did not affect growth of non-tuberculous mycobacteria in culture (Greenstein et al., 2011) but did promote the growth of Mtb in epithelial cells (Miramontes et al., 2021). An initial bacteriostatic effect of CSC, within the first 24 h, was observed during survival assays, although doubling of bacteria occurred after 48 h. This is likely due to the dissipation, degradation, and/or chemical changes of the volatile compounds within CSC within the 24-h period. Additionally, interaction of CSE with plastic containers and degradation and/or evaporation of CSE was previously shown to influence the bioavailability of the cigarette smoke components in cell culture experiments (Bourgeois et al., 2016). The volatile nature of CSC is therefore a limiting factor when attempting to explore the effects of more long-term CSC exposure on Mtb. It would be interesting to investigate the effects of cigarette smoking on Mtb load and virulence factors in animal models such as a macaque model, which closely mimics the immune response in humans.

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## TABLE 2 | Genes differentially expressed in Mtb after 3 and 24 h of CSC exposure.

| Gene ID | Gene ID | Gene name | Function | References | log2Fold Change at 3 h | p-value at 3 h | log2Fold Change at 24 h | p-value at 24 h |
|---------|---------|-----------|----------|------------|------------------------|----------------|------------------------|----------------|
| **Upregulated genes:** | | | | | | | | |
| MT0083  | Rv0077c | ethA2     | Putative oxidoreductase protein | Wohlenkugel et al., 2017 | 4.23 | 9.31E-45 | 3.53 | 1.96E-29 |
| MT0148  | Rv0140  |          | Mtb reactivation-associated proteins | Wayne and Hayes, 1996 | 2.43 | 1.12E-02 | 4.49E-30 | |
| MT0196  | Rv0196  | MymT     | Copper-binding metallothionein | Gold et al., 2008 | 2.38 | 4.49E-30 | 2.47E-05 | |
| MT0366* | Rv0350  | dnaK     | Important for stress-responsive regulator heat shock protein R (HspR) function | Bandyopadhyay et al., 2012 | 1.13 | 5.80E-06 | 2.47E-05 | |
| MT0366* | Rv0351  | grpE     | Heat shock stress-responsive element | Bandyopadhyay et al., 2012 | 1.12 | 1.08E-05 | 1.33 | 1.90E-07 |
| MT0397* | Rv0384c | clpB     | Essential stress regulator of Mtb and endows survival advantage to dormant bacilli | Tripathi et al., 2020 | 1.13 | 4.49E-30 | 1.33 | 1.90E-07 |
| MT0586  | Rv0560c |          | Methyltransferase | Chen et al., 2019 | 4.52 | 2.77E-202 | 3.53 | 1.96E-29 |
| MT0706.1| Rv0678  | Mar family of transcriptional regulator | Radhakrishnan et al., 2014 | – | – | 1.33 | 2.01E-03 |
| MT0838  | Rv0816c | thiX     | Putative thioredoxin | Rahiwi et al., 2020 | 1.54 | 1.54E-05 | 1.84 | 4.40E-47 |
| MT0869  | Rv0846c | groupB   | Putative multicopper oxidase | Rowland and Niederweis, 2012 | 1.84 | 4.40E-47 | 1.33 | 2.01E-03 |
| MT0870  | Rv0847  | lipS     | Probable lipoproteins | Sutcliffe and Harrington, 2004 | 1.49 | 5.55E-29 | 2.47E-05 | |
| MT0873  | Rv0850c |          | Putative transposase, copper-responsive | Festa et al., 2011 | 1.05 | 2.04E-05 | 1.33 | 2.01E-03 |
| MT0996  | Rv0968c | csoR     | Copper-dependent regulation of the copper responsive operon | Liu et al., 2007 | 2.39 | 2.63E-03 | 1.49 | 5.55E-05 |
| MT0996  | Rv0968c |          | Hypothetical protein, part of the copper responsive operon | Festa et al., 2011 | 2.32 | 1.38E-03 | 2.32 | 1.38E-03 |
| MT0997  | Rv0969c | ctpV     | Putative copper exporter, required for full virulence in Mtb | Ward et al., 2010 | 2.31 | 9.52E-04 | 2.31 | 9.52E-04 |
| MT0998  | Rv0970  |          | Part of cos operon, response to copper | Liu et al., 2007 | 2.44 | 5.30E-04 | 2.44 | 5.30E-04 |
| MT1020* | Rv0991c |          | Redox-regulated molecular chaperone | Becker et al., 2020 | 1.65 | 2.40E-08 | 2.65 | 2.40E-08 |
| MT1068  | Rv1039c | PPE15    | Duplicated from ESAT-6 (esx) gene cluster region 5 | Gey van Pittius et al., 2006 | 1.71 | 1.21E-03 | 3.16 | 7.54E-33 |
| MT1259  | Rv1221  | SigE     | ECF subfamily sigma subunit E | Manganelli et al., 2001 | 1.81 | 3.99E-03 | 3.16 | 7.54E-33 |
| MT1376.1*| Rv1335  | CysO     | Part of the cysteine biosynthesis pathway | Agren et al., 2008 | 1.13 | 1.17E-04 | 1.13 | 1.17E-04 |
| MT1517* | Rv1471  | trxB     | Thioredoxin reductase, disulfide reductase | Akif et al., 2008 | 2.12 | 1.09E-03 | 2.12 | 1.09E-03 |
| MT1579* | Rv1528c |          | Conserved hypothetical | | 2.17 | 3.64E-09 | 2.17 | 3.64E-09 |
| MT1579* | Rv1528c |          | Conserved hypothetical | | 2.17 | 3.64E-09 | 2.17 | 3.64E-09 |
| MT1608  | Rv1557  | mmpL6    | Conserved large membrane protein involved in oxidative stress response | Arumugam et al., 2019 | 2.41 | 2.04E-35 | 3.16 | 7.54E-33 |
| MT1711  | Rv1673c |          | Conserved hypothetical | | 1.01 | 6.73E-03 | 1.33 | 2.17E-06 |
| MT1850  | Rv1801c | PPE29    | PPE protein-associated with ESAT-6 (esx) gene cluster region 5 | Gey van Pittius et al., 2006 | 1.52 | 6.54E-06 | 1.52 | 6.54E-06 |
| MT1924  | Rv1875  |          | Possible pyridoxine 5-phosphate oxidase | Mashadi et al., 2011 | 1.23 | 1.27E-06 | 1.23 | 1.27E-06 |
| MT2066  | Rv2011c |          | Putative MarR family transcriptional regulator, unknown function | Lee et al., 2018 | 2.63 | 3.92E-10 | 2.63 | 3.92E-10 |
| MT2067  | Rv2012  |          | Conserved hypothetical protein | | 2.38 | 1.25E-05 | 2.38 | 1.25E-05 |
| MT2468* | Rv2397c | cysA     | ABC import systems functioning as a sulfate importer | Soni et al., 2020 | 1.01 | 4.03E-05 | 2.38 | 1.25E-05 |
| MT2469* | Rv2398c | cysW     | Sulfur metabolism | Zeng et al., 2013 | 1.03 | 2.05E-05 | 2.38 | 1.25E-05 |

(Continued)
| Gene ID  | Gene ID CDC1551 | Gene ID H37Rv | Gene name | Function | References | log2Fold Change at 3 h | p-value | log2Fold Change at 24 h | p-value |
|---------|----------------|---------------|-----------|----------|------------|------------------------|---------|--------------------------|---------|
| MT2719  | Rv2641         | cadI          | Cdmi-Inducible protein | Hotter et al., 2001 | 2.44 | 9.77E-02 |
| MT2780  | Rv2707         | Rv2710        | SigB       | ECF subfamily sigma subunit B | Lee et al., 2008 | 1.01 | 2.85E-05 | 3.89 | 4.24E-02 |
| MT2783* | Rv2713c        | MT2780        | Cell-binding protein, important in cell invasion | Chapeton-Montes et al., 2008 | 1.08 | 2.41E-08 |
| MT2881  | Rv2167c        | MT2783        | MT2881     | Probable transposase, DNA damage induced | Boshoff et al., 2003 | 3.89 | 4.24E-02 |
| MT2981  | Rv2913c        | Rv2962c       | MT2981     | Probable D-amino acid hydrolase | Av-Gay and Everett, 2000 | 1.20 | 3.64E-09 |
| MT3037  | MT3037         | MT3037        | MT3037     | Hypothetical protein | 1.60 | 7.51E-11 |
| MT3038  | Rv2962c        | MT3037        | MT3038     | Probable glycosyltransferase | Perez et al., 2004 | 1.82 | 5.28E-03 |
| MT3039  | Rv2963         | MT3038        | MT3039     | Probable integral membrane | Ward et al., 2010 | 2.43 | 8.89E-05 |
| MT3041  | Rv2964         | MT3041        | MT3041     | PentU      | Phong et al., 2015 | 2.11 | 8.24E-03 |
| MT3041.1| MT3041.1       | MT3041.1      | MT3041.1  | Hypothetical protein | 1.86 | 1.10E-04 |
| MT3139.1| MT3139.1       | MT3139.1      | MT3139.1  | Hypothetical protein | 1.04 | 2.51E-16 |
| MT3140* | Rv3054c        | MT3140        | MT3140    | Probable NADPH dehydrogenases | Murphy and Brown, 2007 | 4.68 | 2.15E-06 | 1.61 | 6.20E-05 |
| MT3141  | Rv3055         | MT3141        | MT3141    | Possible TetR-family transcriptional regulatory protein | Tyagi et al., 2015 | 1.89 | 3.46E-15 |
| MT3142  | Rv3056         | MT3142        | MT3142    | DinP      | Kana et al., 2010 | 1.65 | 1.32E-15 |
| MT3150  | Rv3065         | MT3150        | MT3150    | EmrE      | Poole and Fruci, 2016 | 1.55 | 1.58E-13 |
| MT3151  | Rv3066         | MT3151        | MT3151    | TetR-like DNA-binding regulator | Bollia et al., 2012 | 1.33 | 2.07E-07 |
| MT3248  | MT3248         | MT3248        | MT3248    | Ppe protein | Kurboul et al., 2008 | 1.38 | 6.60E-15 |
| MT3249  | Rv3160c        | MT3249        | MT3249    | Possible TetR/AcrR transcriptional regulator | 2.10 | 9.87E-30 | 1.42 | 6.91E-04 |
| MT3250  | Rv3161c        | MT3250        | MT3250    | Predicted to encode a dehydrogenase, involved in drug resistance | Melief et al., 2019 | 2.51 | 1.65E-33 | 1.69 | 1.21E-06 |
| MT3263  | Rv3174         | MT3263        | MT3263    | Putative dehydrogenase/reductase | Bacon et al., 2004 | 1.80 | 4.70E-03 |
| MT3264  | Rv3175         | MT3264        | MT3264    | Possible amida (aminohydrolase) | 1.44 | 5.28E-03 |
| MT3266  | Rv3177         | MT3266        | MT3266    | Putative peroxidase | Reyes et al., 2012 | 2.78 | 4.78E-08 |
| MT3301* | Rv3206c        | MT3301        | MT3301*   | Molybdenum cofactor biosynthesis and cysteine biosynthesis | Voss et al., 2011 | 1.16 | 2.66E-06 |
| MT3318  | MT3318         | MT3318        | MT3318    | Conserved hypothetical | 1.25 | 2.00E-10 |
| MT3319* | Rv3222c        | MT3319        | MT3319*   | Hypothetical protein sigma factor associated | Thakur et al., 2007 | 1.03 | 2.36E-09 |
| MT3320* | Rv3223c        | MT3320        | MT3320*   | SigH      | ECF subfamily sigma subunit H | Mehra and Kaushal, 2009 | 1.04 | 3.72E-09 |
| MT3438  | Rv3334         | MT3438        | MT3438    | MerR transcriptional regulator, linked to early and enduring hypoxic response | Sun et al., 2018 | 1.47 | 8.61E-05 |
| MT3569* | Rv3463         | MT3569        | MT3569*   | Predicted oxidoreductases | Coulson et al., 2017 | 2.58 | 3.71E-04 |
| MT3949  | Rv3841         | MT3949        | MT3949    | BfrB     | Fruin | – | – | 1.14 | 0.001109 |
| MT4032* | Rv3913         | MT4032        | MT4032*   | TpxB2     | Thioredoxin reductase | Olison et al., 2013 | 1.24 | 3.78E-08 |
| MT4033* | Rv3914         | MT4033        | MT4033*   | TpxC     | Thioredoxin reductase, disulfide reductase | Akif et al., 2008 | 1.21 | 1.68E-06 |

**Downregulated genes:**

- MT0356: Rv0341 induB Glycine-rich cell wall protein which promotes Mtb growth in hostile environments (in vitro)
- MT3830: Rv3727 Similar to phytoene dehydrogenase precursor
- MT3831: Rv3728 Possible sugar transporter, major facilitator superfamily

*Indicate genes directly or indirectly regulated by SigH.
Mtb detected. Mutations driven by CSC which could lead to drug resistance. This is in agreement with what was previously observed for other bacteria (Claxton et al., 1989; Goldstein-Daruech et al., 2011; Kulkarni et al., 2012; Hwang J. H. et al., 2016), allowing evasion of the host immune system, it would also be interesting to explore the effect of CSC on the complete virulence (bacterial burden as well as pathology) in the murine model (Kaushal et al., 2002), and for Mtb mice with ablation in the vaccine efficacy of BCG (Sadagopal et al., 2009). Thus, it has been established that in vivo infection in lungs of mice with Mtb leads to accumulation of frameshift mutations that result in a high SigH expression phenotype, and this correlates with greater virulence (Safi et al., 2019). Similarly, it has been suggested that multiplication of genome copies of SigH and the resulting increase in antioxidant activity are responsible for the ablation in the vaccine efficacy of BCG (Sadagopal et al., 2009).

RIF resistance as a sole indicator of mutations in Mtb and accepting genotypic drug resistance without confirming rpoB mutations in the Mtb colonies (which grew on the RIF plates) are therefore major limitations of this study. The increased mutation frequency determined here does, however, suggest that cigarette smoke could have mutagenic effects on Mtb, which could lead to drug resistance. This is in agreement with what was previously observed for other bacteria (Claxton et al., 1989; Außerheide and Gressmann, 2007, 2008; Miyahara et al., 2011). It is therefore necessary to assess mutation frequency by whole-genome sequencing, where mutations in multiple genes are detected. Mutations driven by CSC which could lead to Mtb drug resistance may not be related to known mutations in drug resistance genes, but instead to changes in the cell wall or other virulence factors of Mtb. Using whole-genome sequencing will provide the most complete understanding of the mutagenic properties of CSC on Mtb. Since the cell wall of Mtb is unique (Alderwick et al., 2007) and cigarette smoke has been shown to change the charge on other bacterial surfaces (Crotty Alexander et al., 2015; Hwang J. H. et al., 2016), allowing evasion of the host immune system, it would also be interesting to explore the effect of CSC on the Mtb cell wall.

It is also important to remember that CSC contains only the insoluble fractions of cigarette smoke and although it contains many of the chemicals that make up cigarette smoke, limited water-soluble chemicals would be present due to the preparation method (Borgerding and Klus, 2005; Stampfl and Anderson, 2009; Kim et al., 2018). CSC was chosen since it is commercially available and we made use of a single batch during experiments to ensure consistency in its composition. Other methods would require smoking of cigarettes and collection of the water-soluble fractions (CSE), but this would limit the consistency of the composition of the preparation between laboratories and batches and was therefore avoided.

The transcriptional response of Mtb to CSC was explored. During the 3-h exposure, the expression of numerous genes was upregulated, while only one was downregulated (Table 2). This likely represents the immediate response to the toxic compounds and oxidative and nitrosative stress generated by CSC. By 24 h, the expression of only five of the 59 genes upregulated at 3 h remained upregulated. The transcriptional response to CSC therefore seems to be a more immediate and short-lived response.

Interestingly, sigH, a global regulator (Manganelli et al., 2002), and its downstream regulon were induced by CSC (Table 2). SigH is a global stress response regulator which is induced upon exposure to oxidative and nitrosative stress (Mehra and Kaushal, 2009). Additionally, mmpL6, encoding for conserved large membrane proteins, which is also involved in oxidative stress response (Arumugam et al., 2019), was upregulated at both 3 h and remained upregulated at 24 h of CSC exposure (Table 2). Increased oxidative stress was also previously suggested to occur due to exposure to the same CSC (the same preparation used in our study) in Mtb H37Rv, since catalase, known to remove oxidative stress, caused significant attenuation of the CSC-mediated changes in biofilm formation in Mtb (Cholo et al., 2020). The upregulation of oxidative stress response genes is also consistent with the increased oxidative stress observed in other bacteria upon cigarette smoke exposure (Baboni et al., 2010; Goldstein-Daruech et al., 2011; Kulkarni et al., 2012; Hwang J. H. et al., 2016; Chien et al., 2020).

Upregulated genes also included genes known to be directly transcribed under the influence of SigH, e.g., trxB2/trxC operon which encodes for thioredoxin/thioredoxin reductases that play a role in mitigating oxidative stress and downstream sigma factors sigE and sigB which further amplify the antioxidative function of sigH. Also induced was the expression of heat shock/chaperonin proteins dnaK and grpE, and clpB, which are critical in resolving protein aggregates that may result during oxidative stress. These genes are coregulated with SigH and SigE (Mehra and Kaushal, 2009). The SigH and SigE regulons are two of the most important regulons allowing response to oxidative stress in Mtb (Voskuil et al., 2011). Sixteen genes that are known to be controlled by SigH were upregulated by exposure of Mtb to CSC indicating true upregulation of a regulon.

Interestingly, RNA-seq results did not show changes in expression of genes involved in the hypoxia response, e.g., dosR, dosS, or dosT (Mehra et al., 2015). Since SigH is required for pathology in the murine model (Kaushal et al., 2002), and for the complete virulence (bacterial burden as well as pathology) in the non-human primate model (Mehra et al., 2012), clearly the antioxidative function promoted by SigH provides Mtb with a clear survival advantage during the innate phase of the infection (Kernodle, 2012). Several other studies point to the importance of SigH and its antioxidant function in the virulence of Mtb. Thus, it has been established that in vivo infection in lungs of mice with Mtb leads to accumulation of frameshift mutations that result in a high SigH expression phenotype, and this correlates with greater virulence (Safi et al., 2019). Similarly, it has been suggested that multiplication of genome copies of SigH and the resulting increase in antioxidant activity are responsible for the ablation in the vaccine efficacy of BCG (Sadagopal et al., 2009).
Not only is SigH critical for complete TB virulence and lung granuloma formation, but also lack of genes downstream of this key regulator results in disease ablation. Thus, a ΔsigE mutant is also attenuated in animal models (Manganelli et al., 2001), while lack of thioredoxins or their cognate reductases results in control of infection and lack of disease (Lin et al., 2016). Similarly, the ΔsigH mutant is efficient as a vaccine in protecting both rhesus (Kaushal et al., 2015) and cynomolgus (Singh et al., in process) macaques against lethal challenge with Mtb.

Our results therefore indicate that CSC does not cause a hypoxic influence on Mtb but rather generates an oxidative stress response. The high-SigH expression phenotype is clearly associated with greater virulence in multiple animal models and has been postulated to correlate with drug tolerance, treatment failure, and relapse of human TB (Safi et al., 2019). While our results were not performed in the context of Mtb contained within host phagocytes, they have significant implications. Induction of SigH-mediated response by CSC in Mtb suggests that chronic exposure to cigarette smoke may provide a pathogenic advantage to Mtb in individuals with pulmonary TB (which may be, in part, driven by increased sigH expression in vivo). An investigation of the effect of cigarette smoke on Mtb gene expression should therefore be explored in animal models of cigarette smoke exposure. This would allow controlled exposure and assessment of the effect on both host and bacteria for short-term cigarette smoke exposure. It would also be interesting to investigate the oxidative stress response of Mtb collected from TB patients who are long-term smokers and compare to Mtb from shorter-term smokers or non-smokers.

Cigarette smoke condensate contains numerous heavy metals including cadmium, lead, copper, zinc, and selenium. The average concentrations of these metals are also higher in the blood of smokers than non-smokers (Massadeh et al., 2010). The initial induction of the copper exporter MT0997 (ctpV) at 3 h of CSC exposure may have caused loss of numerous metals due to non-specific metal export (Agranoff and Krishna, 2004). Since CSC contains iron and iron drives Fenton chemistry and causes oxidative stress, brfB upregulation at 24 h would prevent iron-mediated oxidation, thereby protecting lipids, DNA, and proteins from the potentially toxic effects of iron (Sharma et al., 2016). The expression of copper response regulator CsoR, Rv0970, another cos response member gene, and cadII, which responds to cadmium, was also induced in Mtb treated with CSC.

This study indicates that the effect of cigarette smoke on the host immune response is not the only factor which is driving the link observed between smoking and TB. The effect of cigarette smoke on the bacteria themselves may very well be a driving force in the increased risk to develop TB, lack of treatment efficacy, and increased mortality observed in smokers. This may in part occur due to cigarette smoke driving genetic mutations as well as inducing virulence factors in the bacteria, thereby allowing more effective bacterial survival.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ncbi.nlm.nih.gov/genbank/, GSE172041.

AUTHOR CONTRIBUTIONS

DK contributed funding and overall concept and design, and edited and wrote the manuscript. DW performed all research, conceptualized the study, analyzed the data, and wrote the manuscript. CM validated some data. SM provided resources, funding, and concept and edited and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abdalla, A. E., Yan, S., Zeng, J., Deng, W., Xie, L., and Xie, J. (2020). Mycobacterium tuberculosis Rv0341 promotes Mycobacterium survival in in vitro hostile environments and within macrophages and induces cytokine expression. Pathogens 9:454. doi: 10.3390/pathogens9060454

Agarwal, A. R., Yin, F., and Cadenas, E. (2014). Short-term cigarette smoke exposure leads to metabolic alterations in lung alveolar cells. Am. J. Respir. Cell Mol. Biol. 51, 284–293. doi: 10.1165/rcmb.2013-0523OC

Agranoff, D., and Krishna, S. (2004). Metal ion transport and regulation in Mycobacterium tuberculosis. Front. Biosci. 9:2996–3006. doi: 10.2741/1454

Agren, D., Schnell, R., Oehlmann, W., Singh, M., and Schneider, G. (2008). Cysteine synthase (CysM) of Mycobacterium tuberculosis is an O-phosphoserine sulfhydrylase: evidence for an alternative cysteine
biosynthesis pathway in mycobacteria. J. Biol. Chem. 283, 31567–31574. doi: 10.1074/jbc.M804877200
Akif, M., Khare, G., Tyagi, A. K., Mande, S. C., and Sardeasi, A. A. (2008). Functional studies of multiple thioredoxins from Mycobacterium tuberculosis. J. Bacteriol. 190, 7087–7095. doi: 10.1128/JB.00139-08
Alderwick, L. J., Birch, H. L., Mishra, A. K., Eggeling, L., and Besra, G. S. (2007). Structure, function and biosynthesis of the Mycobacterium tuberculosis cell wall: arabinogalactan and lipoproteinribomannan assembly with a view to discovering new drug targets. Biochem. Soc. Trans. 35(PT 5), 1325–1338. doi: 10.1042/BST0351325
Alexandrov, L. B., Kim, J., Haradhvala, N. I., Huang, M. N., Tian Ng, A. W., Wu, Y., et al. (2020). The repertoire of mutational signatures in human cancer. Nature 578, 94–101. doi: 10.1038/s41586-020-1943-3
Arumugam, P., Shankaran, D., Bothra, A., Gandotra, S., and Rao, V. (2019). The MmpS6-MmpL6 operon is an oxidative stress response system providing selective advantage to Mycobacterium tuberculosis in Stress. J. Infect. Dis. 219, 459–469. doi: 10.1093/infdis/jiy526
Aryanpur, M., Masjadi, M. R., Mortaz, E., Hosseini, M., Jamaati, H., Tabarsi, P., Aufderheide, M., and Gressmann, H. (2008). Mutagenicity of native cigarette mainstream smoke and its gas/vapour phase by use of different tester strains. Mutat. Res. 656, 82–87. doi: 10.1016/j.mrrev.2008.07.008
Av-Gay, Y., and Everett, M. (2000). The eukaryotic-like Ser/Thr protein kinases of Aryanpur, M., Masjedi, M. R., Mortaz, E., Hosseini, M., Jamaati, H., Tabarsi, P., Aufderheide, M., and Gressmann, H. (2008). Characterizing the Mycobacterium tuberculosis Rv2707 protein and determining its sequences which specifically bind to two human cell lines. Protein Sci. 17, 342–351. doi: 10.1110/ps.073083308
Charlson, E. S., Chen, J., Casters-Allen, R., Bittinger, K., Li, H., Sinha, R., et al. (2010). Disordered microbial communities in the upper respiratory tract of cigarette smokers. PLoS One 5:e15216. doi: 10.1371/journal.pone.0015216
Chen, C., Han, X., Yan, Q., Wang, C., Jia, L., Taj, A., et al. (2019). The inhibitory effect of GlimU acetyltransferase inhibitor TPSA on Mycobacterium tuberculosis may be affected due to its methylation by methyltransferase Rv0560c. Front. Cell. Infect. Microbiol. 9:251. doi: 10.3389/fcimb.2019.00251
Chiang, Y. C., Lin, Y. M., Lee, J. A., Lee, C. N., and Chen, H. Y. (2012). Tobacco consumption is a reversible risk factor associated with reduced successful treatment outcomes of anti-tuberculosis therapy. Int J Infect Dis. 16, e130–e135. doi: 10.1016/j.ijid.2011.07.007
Chen, J., Huang, J. H., Nilaad, S., Masso-Silva, J. A., Jeong, Ahn S, McCaehn, E. K., et al. (2020). Cigarette smoke exposure promotes virulence of Pseudomonas aeruginosa and induces resistance to neutrophil killing. Infect. Immun. 88:e00527-20. doi: 10.1128/IAI.00527-20
Cholo, M. C., Raselio, S. S. M., Venter, E., Venter, C., and Anderson, R. (2020). Effects of cigarette smoke condensate on growth and biofilm formation by Mycobacterium tuberculosis. Biomed. Res. Int. 2020:8237402. doi: 10.1155/2020/8237402
Claxton, L. D., Morin, R. S., Hughes, T. J., and Lewtas, J. (1989). A genotoxic assessment of environmental tobacco smoke using bacterial bioassays. Mutat. Res. 222, 81–99. doi: 10.1016/0165-1110(89)90022-0
Cockeran, R., Herbert, J. A., Mitchell, T. J., Dix- Peel, T., Dickens, C., Anderson, R., et al. (2014). Exposure of a 23F serotype strain of Streptococcus pneumoniae to cigarette smoke condensate is associated with selective upregulation of genes encoding the two-component regulatory system 11 (TCS11). Biomed. Res. Int. 2014:976347. doi: 10.1154/2014/976347
Coulosn, G. B., Johnson, B. K., Zheng, H., Colvin, C. J., Fillinge, R. J., Haiderer, E. R., et al. (2017). Targeting Mycobacterium tuberculosis sensitivity to thiostress at acidic pH kills the bacterium and potentiates antibiotics. Cell Chem. Biol. 24, 993-1004.e4. doi: 10.1016/j.chembiol.2017.06.018
Crotty Alexander, L. E., Drummond, C. A., Hepokoski, M., Mathew, D., Moshensky, A., Willeford, A., et al. (2018). Chronic inhalation of e-cigarette vapor containing nicotine disrupts airway barrier function and induces systemic inflammation and multiorgan fibrosis in mice. Am. J. Physiol. Integr. Comp. Physiol. 314, R834–R847. doi: 10.1152/ajpregu.00270.2017
Crotty Alexander, L. E., Vyas, A., Schraufnagel, D. E., and Malhotra, A. (2015). Electronic cigarettes: the new face of nicotine delivery and addiction. J. Thorac. Dis. 7, E248–E251. doi: 10.3978/j.issn.2072-1439.2015.07.37
De Rossi, E., Arrigo, P., Bellinzoni, M., Silva, P. A., Martin, C., Ainsa, J. A., et al. (2002). The multidrug transporters belonging to major facilitator superfamily of Mycobacterium tuberculosis. Mol. Med. 8, 714–724.
Feng, Y., Kong, Y., Barnes, P. F., Huang, F. F., Klucar, P., Wang, X., et al. (2011). Exposure to cigarette smoke inhibits the pulmonary T-cell response to influenza virus and Mycobacterium tuberculosis. Infect. Immun. 79, 229–237. doi: 10.1128/IAI.00709-10
Festa, R. A., Jones, M. B., Butler-Wu, S., Sinsimer, D., Gerads, R., Bishai, W. R., et al. (2013). Targeting Mycobacterium tuberculosis sensitivity to thiostress at acidic pH kills the bacterium and potentiates antibiotics. Cell Chem. Biol. 20, 1119-1131. doi: 10.1016/j.chembiol.2013.07.023
Fosk, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., et al. (2013). Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat. Genet. 45, 784–790. doi: 10.1038/ng.2656
Gold, B., Deng, H., Bryk, R., Vargas, D., Eliezer, D., Roberts, J., et al. (2008). Localization and characterization of the ESAT-6 (esx) gene cluster regions. *BMC Evol. Biol.* 8:290. doi: 10.1186/1471-2148-8-290

Gey van Pittius, N. C., Sampson, S. L., Lee, H., Kim, Y., van Helden, P. D., Frenoy, A., and Bonhoeffer, S. (2018). Death and population dynamics affect *M. tuberculosis* replication. *Cell. Mol. Biol.* 55, 586–601. doi: 10.1128/CMbol.01273-07

Lee, S. J., Lee, I. G., Lee, K. Y., Kim, D. G., Eun, H. J., Yoon, H. J., et al. (2016). Two distinct mechanisms of transcriptional regulation by the redox sensor YodB. *Proc. Natl. Acad. Sci. U. S. A.* 113, E5202–E5211. doi: 10.1073/pnas.1604427113

Lin, H. H., Ezzati, M., Chang, H. Y., and Murray, M. (2009). Association between tobacco smoking and active tuberculosis in Taiwan: prospective cohort study. *Am. J. Respir. Crit. Care Med.* 180, 475–480. doi: 10.1164/rccm.200904-0549OC

Kulkarni, R., Antala, S., Wang, A., Aamaral, F. E., Rampersaud, R., Larussa, S. J., et al. (2012). Cigarette smoke increases Staphylococcus aureus biofilm formation via oxidative stress. *Infect. Immun.* 80, 3804–3811. doi: 10.1128/IAI.00689-12

Kulkarni, R., Caskey, J., Singh, S. K., Paudel, S., Baral, P., Schexnayder, M., et al. (2016). Cigarette smoke extract-exposed methicillin-resistant *Staphylococcus aureus* regulates leukocyte function for pulmonary persistence. *Am. J. Respir. Crit. Care Med.* 195, 699–707. doi: 10.1164/rccm.2015-0397OC
Miyahara, E., Nishie, M., Takuji, S., Miyanoara, H., Nishi, J., Yoshike, K., et al. (2011). Environmental mutagens may be implicated in the emergence of drug-resistant microorganisms. *FEBS Microbiol. Lett.* 317, 109–116. doi: 10.1111/j.1574-6968.2011.02215.x

Mizusaki, S., Okamoto, H., Akiyama, A., and Fukuhara, Y. (1977). Relation between chemical constituents of tobacco and mutagenic activity of cigarette smoke condensate. *Mutat. Res.* 48, 319–325. doi: 10.1016/0027-5107(77)90175-0

Morin, R. S., Tulis, J. J., and Claxton, L. D. (1987). The effect of solvent and extraction methods on the bacterial mutagenicity of sidestream cigarette smoke. *Toxicol. Lett.* 38, 279–290. doi: 10.1016/0378-4274(87)90010-5

Murphy, D. J., and Brown, J. R. (2007). Identification of gene targets against dormant phase *Mycobacterium tuberculosis* infections. *BMC Infect. Dis.* 7:84. doi: 10.1186/1471-2334-7-84

Mutepe, N. D., Cockeran, R., Steel, H. C., Theron, A. J., Mitchell, T. J., Feldman, C., et al. (2013). Structure of cigarette smoke condensate on pneumococal biolfim formation and pneumolysin. *Eur. Respir. J.* 41, 392–395. doi: 10.1183/09031936.00213211

Naz, S., Dabral, S., Nagarajan, S. N., Arora, D., Singh, L. V., Kumar, P., et al. (2021). Compromised base excision repair pathway in *Mycobacterium tuberculosis* impairs superior adaptability in the host. *PLoS Pathog.* 17:e1009452. doi: 10.1371/journal.ppat.1009452

O’Leary, S. M., Coleman, M. M., Chew, W. M., Morrow, C., McLaughlin, A. M., Gleeson, L. E., et al. (2014). Cigarette smoking impairs human pulmonary immunity to *Mycobacterium tuberculosis*. *Am. J. Respir. Crit. Care Med.* 190, 1430–1436. doi: 10.1164/rcrm.2014-0385OC

Olson, A. L., Neumann, T. S., Cai, S., and Sem, D. S. (2013). Solution structures of *Mycobacterium tuberculosis* thioredoxin C and models of intact thioredoxin system suggest new approaches to inhibitor and drug design. *Proteins* 81, 675–689. doi: 10.1002/prot.24228

Ozturk, A. B., Kilicaslan, Z., and Issever, H. (2014). Effect of smoking and indoor air pollution on the risk of tuberculosis: smoking, indoor air pollution and tuberculosis. *Tuberk. Toraks.* 62, 1–6. doi: 10.5578/tt.7013

Perez, E., Constant, P., Laval, F., Lemassu, A., Laneelle, M. A., Daffe, M., et al. (2004). Molecular dissection of the role of two methyltransferases in the biosynthesis of phenolglycolipids and phthiocerol dimycoserosate in the *Mycobacterium tuberculosis* complex. *J. Biol. Chem.* 279, 42584–42592. doi: 10.1074/jbc.M406134200

Phong, T. Q., Ha do, T. T., Volker, U., and Hammer, E. (2015). Using a label free quantitative proteomics approach to identify changes in protein abundance in multidrug-resistant *Mycobacterium tuberculosis*. *Indian J. Microbiol.* 55, 219–230. doi: 10.1007/s12088-015-0511-2

Poole, K., and Fruci, M. (2016). “Antimicrobial drug efflux systems as components of bacterial stress responses,” in *Efflux-Mediated Antimicrobial Resistance in Bacteria*, eds X. Z. Li, C. Elkins, and H. Gzsurgkza (Cham: Atdos). doi: 10.1007/978-3-319-39658-3_26

Radhakrishnan, A., Kumar, N., Wright, C. C., Chou, T. H., Tringides, M. L., Bolla, M., et al. (2013). Crystal structure of the transcriptional regulator Rv0678 of *Mycobacterium tuberculosis* in multidrug-resistant *M. tuberculosis* strain suggest new approaches to inhibitor and drug design. *Proteins* 81, 519–529. doi: 10.1002/prot.24228

Rahalwes, K. C., Osman, S. H., and Morita, Y. S. (2020). Role of LmeA, a *Mycobacterium tuberculosis* glpK produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U. S. A.* 116, 19665–19674. doi: 10.1073/pnas.1907651116

Semlali, A., Killer, K., Alazani, H., Chmielewski, W., and Rouabhia, M. (2014). Cigarette smoke condensate increases C. albicans adhesion, growth, biofilm formation, and EAP1, HWP1 and SAP2 gene expression. *BMC Microbiol.* 14:61. doi: 10.1186/1471-2180-14-61

Shaler, C. R., Horvath, C. N., McCormick, S., Jeyanathan, M., Khera, A., Zganiacz, A., et al. (2013). Continuous and discontinuous cigarette smoke exposure differentially affects protective Th1 immunity against pulmonary tuberculosis. *PLoS One* 8:e59185. doi: 10.1371/journal.pone.0059185

Sharma, D., Lata, M., Faheem, M., Khan, A. U., Joshi, B., Venkatesan, K., et al. (2016). *M. tuberculosis* ferritin (Rv3841): potential involvement in Aminakic (AK) & Kanamycin (KM) resistance. *Biochem. Biophys. Res. Commun.* 478, 908–912. doi: 10.1016/j.bbrc.2016.08.049

Smith, G. S., Van Den Eeden, S. K., Baxter, R., Shan, J., Van Rie, A., Herrig, A. H., et al. (2015). Cigarette smoking and pulmonary tuberculosis in northern California. *J. Epidemiol. Commun. Health* 69, 568–573. doi: 10.1136/jech-2014-204292

Sonu, X., Zhang, L., Jiang, J., Ng, M., Cui, Z., Mai, J., et al. (2018). Transcription factors Rv0081 and Rv3334 connect the early and the enduring hypoxic response of *Mycobacterium tuberculosis*. *Virulence* 9, 1468–1482. doi: 10.1080/22127051.2020.1714458

Stampfl, M. R., and Anderson, G. P. (2009). How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat. Rev. Immunol.* 9, 377–384. doi: 10.1038/nri2530

Sun, X., Zhang, L., Jiang, J., Ng, M., Cui, Z., Mai, J., et al. (2018). Smoking and indoor air pollution on the risk of tuberculosis: smoking, indoor air pollution and tuberculosis. *Tuberk. Toraks.* 62, 1–6. doi: 10.5578/tt.7013

Thorne, D., Crooks, I., Hollings, M., Seymour, A., Meredith, C., and Gaca, M. (2016). The mutagenic assessment of an electronic-cigarette and reference cigarette smoke using the Ames assay in strains TA98 and TA100. *Mutat. Res.* 812, 29–38. doi: 10.1016/j.mrmt.2016.10.005

Tripathi, P., Singh, L. K., Kumari, S., Hakiem, O. R., and Batra, J. K. (2020). ClpB is an essential stress regulator of *Mycobacterium tuberculosis* and endows survival advantage to dormant bacilli. *Int. J. Med. Microbiol.* 310:151402. doi: 10.1016/j.micm.2020.151402

Tyagi, P., Dharmanara, A. T., Bhaskar, A., Chakrapani, H., and Singh, A. (2015). *Mycobacterium tuberculosis* has diminished capacity to counteract redox stress induced by elevated levels of endogenous superoxide. *Free Radic. Biol. Med.* 84, 344–354. doi: 10.1016/j.freeradbiomed.2015.03.008

Voskuil, M. I., Bartek, I. L., Visconti, K., and Schoolnik, G. K. (2011). The response of *Mycobacterium tuberculosis* to reactive oxygen and nitrogen species. *Mutat. Res.* 733, 155–163. doi: 10.1016/j.mrmt.2010.11.005

Voss, M., Nimtz, M., and Leimkühler, S. (2011). Elucidation of the dual role of mycobacterial MoeZR in molybdenum cofactor biosynthesis and cysteine biosynthesis. *PLoS One* 6:e28170. doi: 10.1371/journal.pone.0028170
Ward, S. K., Abomoelak, B., Hoye, E. A., Steinberg, H., and Talaat, A. M. (2010). CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 77, 1096–1110. doi: 10.1111/j.1365-2958.2010.07273.x

Wayne, L. G., and Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect. Immun.* 64, 2062–2069.

WHO (2019). New Data on Tuberculosis Trends in 202 Countries. Available online at: https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-report-2019

Wohlkonig, A., Remaut, H., Moune, M., Tanina, A., Meyer, F., Desroses, M., et al. (2017). Structural analysis of the interaction between spiroisoxazoline SMART-420 and the *Mycobacterium tuberculosis* repressor EthR2. *Biochem. Biophys. Res. Commun.* 487, 403–408. doi: 10.1016/j.bbrc.2017.04.074

Yim, J. J., Yoo, C. G., Lee, C. T., Kim, Y. W., Han, S. K., and Shim, Y. S. (2002). Lack of association between glutathione S-transferase P1 polymorphism and COPD in Koreans. *Lung* 180, 119–125. doi: 10.1007/s004080000086

Yim, S. H., and Hee, S. S. (2001). Bacterial mutagenicity of some tobacco aromatic nitrogen bases and their mixtures. *Mutat. Res.* 492, 13–27. doi: 10.1016/s1383-5718(01)00152-8

Yu, G., Phillips, S., Gail, M. H., Goedert, J. J., Humphrys, M. S., Ravel, J., et al. (2017). The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome* 5:3. doi: 10.1186/s40168-016-0226-6

Yu, G. P., Hsieh, C. C., and Peng, J. (1988). Risk factors associated with the prevalence of pulmonary tuberculosis among sanitary workers in Shanghai. *Tubercle* 69, 105–112. doi: 10.1016/0041-3879(88)90072-4

Zeng, L., Shi, T., Zhao, Q., and Xie, J. (2013). *Mycobacterium* sulfur metabolism and implications for novel drug targets. *Cell Biochem. Biophys.* 65, 77–83. doi: 10.1007/s12013-012-9410-x

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