The pleiotropic transcriptional response of *Mycobacterium tuberculosis* to vitamin C is robust and overlaps with the bacterial response to multiple intracellular stresses

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*Mycobacterium tuberculosis* (Mtb) owes its success as a pathogen in large measure to its ability to exist in a persistent state of ‘dormancy’ resulting in a lifelong latent tuberculosis (TB) infection. An understanding of bacterial adaptation during dormancy will help in devising approaches to counter latent TB infection. *In vitro* models have provided valuable insights into bacterial adaptation; however, they have limitations because they do not disclose the bacterial response to the intracellular environment wherein the bacteria are simultaneously exposed to multiple stresses. We describe the pleiotropic response of Mtb in the vitamin C (vit C) model of dormancy developed in our laboratory. Vit C mediates a rapid regulation of genes representing ~14 % of the genome in Mtb cultures. The upregulated genes were better represented in lipid, intermediary metabolism and regulatory protein categories. The downregulated genes mainly related to virulence, detoxification, information pathways and cell wall processes. A comparison of this response to that in other models indicates that vit C generates a multiple-stress environment for axenic Mtb cultures that resembles a macrophage-like environment. The bacterial response to vit C resembles responses to gaseous stresses such as hypoxia and nitric oxide, oxidative and nitrosative stresses, nutrient starvation and, notably, the activated macrophage environment itself. These responses demonstrate that the influence of vit C on Mtb gene expression extends well beyond the DevR dormancy regulon. A detailed characterization of the response to vit C is expected to disclose useful strategies to counter the adaptive mechanisms essential to Mtb dormancy.

**INTRODUCTION**

*Mycobacterium tuberculosis* (Mtb) is the aetiological agent of tuberculosis (TB) and its success as a pathogen is attributed to its ability to mount an adaptive response to the hostile intracellular environment and enter into prolonged periods of ‘dormancy’ resulting in a lifelong latent TB infection. The eradication of latent TB infection from the community is undoubtedly a key component of TB control strategies. In this context, a thorough understanding of the physiological and metabolic state of dormant bacilli holds the key for devising approaches to counter latent TB infection.

*In vitro* models of dormancy have provided us with valuable insights into the unique properties and metabolic activity of dormant Mtb (Sikri & Tyagi, 2013). Hypoxia was established as a physiologically relevant stress signal (Wayne & Diaz, 1967; Wayne, 1976) and it is the best understood trigger for dormancy. An *in vitro* model of bacterial dormancy under hypoxia was described first by Wayne’s laboratory (Wayne & Hayes, 1996). Subsequent variations of the hypoxia model were introduced, including the standing of cultures in tube and plate formats (Hu et al., 1998; Saini et al., 2004), the rapid generation of defined
hypoxia (Sherman et al., 2001) and bacterial culturing in vented cap flasks under low oxygen tension (Florczyk et al., 2003). The survival mechanisms adopted by intracellular Mtb under conditions of nutrient limitation were delineated in an in vitro nutrient starvation model (Loebel et al., 1993; Betts et al., 2002). An aerobic nutrient depletion adaptation model was described where the dissolved oxygen tension was maintained at 50 %, which allowed the maintenance of long-term stationary phase culture (Hampshire et al., 2004). The model was based on the hypothesis that a bacterial subpopulation of Mtb that survives in vitro during the stationary phase may represent the population of the bacilli that persist during infection. A multiple-stress model was developed more recently, which combined the stresses of hypoxia, high CO₂, nutrient limitation and acidic stress and therefore more closely approximated the environment faced by intracellular dormant bacteria (Deb et al., 2009). While these models have provided valuable insights into bacterial adaptation to various environmental cues, they are generally limited to assessing the bacterial response to one stress signal at a time. Hence they do not provide the complex intracellular environment encountered by bacteria during infection wherein bacteria are exposed simultaneously to stresses that include reactive oxygen and reactive nitrogen species, acidic pH, nutrient limitation, antimicrobial peptides, cell membrane-perturbing agents, etc. (Schnappinger et al., 2003; Russell, 2011).

Vitamin C (vit C) is an essential dietary nutrient in humans that is involved in a wide range of biological processes including collagen biosynthesis, as a cofactor of several enzymes, facilitating iron transport and as a physiological antioxidant (Mandl et al., 2009). Studies have described the beneficial effects of vit C in treating human and experimental TB (McConkey & Smith, 1933; Hemila et al., 1999). The action of vit C in infectious diseases is attributed to host protection from oxidative damage by reactive oxygen and nitrogen intermediates (ROIs and RNIs) (Jariwalla & Harakeh, 1996). A serendipitous discovery was made in our laboratory that vit C treatment of Mtb resulted in a rapid induction of the DevR (DosR) dormancy regulon and led to the development of bacterial ‘dormancy’. Vit C also elicited acidic and oxidative stress responses and induced an isoniazid (INH) tolerant phenotype in the treated bacteria both in axenic cultures and in the THP-1 cell infection model (Taneja et al., 2010). These responses suggested that vit C potentiates an early induction of the ‘dormancy phenotype’ under aerobic conditions, and bacteria may exhibit physiological adaptations similar to those observed under conditions of gradual O₂ depletion (hypoxia) and NO exposure. In order to obtain an insight into the global response of Mtb, we analysed the gene expression changes in axenic cultures upon exposure to vit C. A comparative transcriptome analysis revealed a considerable overlap in the bacterial response to vit C and various intracellular stresses, indicating that the response to vit C is pleiotropic and mimics bacterial responses to multiple intracellular stresses.

**METHODS**

**Strains, culture conditions and RNA isolation.** Mtb strains (Table 1) expanded from −70 °C frozen stocks were subcultured twice in Dubos medium containing 0.5% BSA, 0.75% glucose and 0.085% NaCl plus 0.1% Tween-80 (DTA medium) with shaking at 220 r.p.m. at 37 °C. For microarray analysis and validation by reverse transcriptase quantitative PCR (RT-qPCR), 100 ml bacterial cultures were grown in 250 ml Erlenmeyer flasks to OD₅₉₅ ~0.1–0.2 (path length 0.56 cm, Micro plate reader 680; Bio-Rad) and treated with 10 mM vit C (Sigma Aldrich) for 8 h prior to RNA isolation.

For devS and dosT studies, single complements of devS and dosT sensor kinases were constructed in an Mtb AdevSAdosT mutant strain (Table 1). Full-length devS (1737 bp) and dosT (1722 bp) were amplified from Mtb H37Rv genomic DNA using primer pairs: devSNdeIF and devSXbalR for devS amplification, and dosTNdeIF and dosTXbalR for dosT amplification (Table S1, available in the online Supplementary Material). The amplified products were cloned in pJFR19, an Mtb integrative plasmid (Chauhan et al., 2006), downstream of the constitutive acetamidase promoter. The constructs were electroporated individually into the Mtb AdevSAdosT mutant strain to generate single-sensor kinase complements expressing DosT and DevS. The complemented gene sequences were verified by DNA sequence analysis and sensor kinase expression was confirmed by Western blot analysis (not shown). For RNA isolation from devS- and dosT-complemented strains, 10 ml of cultures were grown in 50 ml tubes to OD₅₉₅ ~0.1–0.2 and treated with 10 mM vit C for 1 h. Thereafter, RNA was isolated from these cultures as described in Chauhan & Tyagi (2008) and analysed by RT-qPCR as described below.

**Microarray data extraction and analysis.** Total RNA (1 µg) from three replicates of vit C-treated (10 mM for 8 h as described above) and untreated cultures was processed for microarray analysis at Genotypic India as described (De Majumdar et al., 2012). The expression data were filtered on the basis of fold change values in vit C-treated versus untreated cultures. Genes that were at least 1.8-fold induced or repressed, with a P-value ≤ 0.05, were considered to be significantly differentially regulated genes (DRGs). Benjamini–Hochberg false discovery rate correction was then applied to the P-values for these DRGs (Table S2). Student’s t-test was used for statistical analysis.

The raw data are deposited with NCBI under GEO accession no. GSE60376 (http://www.ncbi.nlm.nih.gov/geo/).

**Intracellular bacterial RNA isolation.** THP-1 cells were grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 10 % FBS, 1.46 g l-glutamine 1⁻¹, 2 g sodium bicarbonate l⁻¹ and 2.3 g HEPES l⁻¹. Forty million THP-1 cells in 175 cm² tissue culture flasks were infected with Mtb at an m.o.i. of 10:1 (bacteria:cell) as described previously (Taneja et al., 2010). At 8 h post-infection, THP-1 cells were lysed and bacteria isolated as described previously (Monahan et al., 2001), with minor variations. The resulting bacterial pellet was resuspended in 1 ml TRI reagent and RNA was isolated as described above.

**RT-qPCR.** Total bacterial RNA (500 ng) isolated from various strains and from the THP-1 infection set-up was reverse transcribed to cDNA and analysed by qPCR using gene-specific primers (Table S1) and iQ SyBr Green Supermix (CFX96 Real-Time PCR-detection system; Bio-Rad). Reaction conditions were 94 °C (10 min) followed by 40 cycles of 94 °C (20 s), 55–65 °C (20 s) and 72 °C (20 s). Transcript levels in various RNA samples were normalized using 16S rRNA, and the fold change in expression in vit C-treated axenic cultures or intracellular Mtb was calculated with respect to untreated aerobic axenic cultures as described previously (Taneja et al., 2010).
Comparison of dormancy models. Mtb H37Rv genes differentially regulated in response to vit C treatment were compared to the data from other dormancy models. These datasets were obtained from NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) and included responses observed under hypoxia [Wayne hypoxia model wherein hypoxia develops gradually by the consumption of oxygen in sealed culture tubes (Voskuil et al., 2004) and the enduring hypoxia model wherein severe hypoxia (0.2% oxygen with N₂ balance) is developed rapidly (Rustad et al., 2008)], low pH (Fisher et al., 2002, Rohde et al., 2007), nutrient starvation (Betts et al., 2002; Hampshire et al., 2004), oxidative stress (Schnappinger et al., 2003; Voskuil et al., 2011), nitrosative stress (Voskuil et al., 2011) and in response to both vit C (Vilchéze et al., 2013) and macrophage infection (Schnappinger et al., 2003). The datasets were filtered for up-regulated and downregulated genes and then compared to vit C response using PERL programs for the commonly up- and downregulated genes.

GFP reporter assay. Mtb expressing GFP reporter gene from the *Rv3134c* promoter (p-3134c-1 plasmid, pPFV27 containing a DevR-dependent *Rv3134c* promoter; Chauhan & Tyagi, 2008) was grown in DTA medium as described above to OD₅₉₅ 0.1–0.2. Culture aliquots of 200 μl were dispensed into a 96-well black clear-bottom plate. The bacterial cells were exposed to vit C and the plates were incubated under standing conditions at 37 °C. *Rv3134c* promoter activity was assessed by measuring GFP fluorescence (excitation at 483 nm/ emission at 515 nm) as described (Chauhan & Tyagi, 2008). A promoterless GFP vector control was used as control and its fluorescence values were subtracted from the test values. The data are plotted as relative fluorescence units (RFU/OD) from triplicate values.

Mycobacterial growth indicator tube (MGIT) assay. Mtb H37Rv culture was diluted to OD₅₉₅ ~0.1–0.2 in mycobacterial growth indicator tubes (MGIT; Becton Dickinson). Vit C was added to tubes containing Mtb cultures or medium only (final concentration 10 mM). Control tubes contained untreated culture or medium only. The tubes were incubated by standing up to 4 h at 37 °C and the fluorescence was captured under a long-wavelength UV lamp. Fluorescence intensities are expressed as fold change in intensity with respect to untreated medium control.

RESULTS

Rapid and extensive changes in Mtb gene expression occur in response to vit C

Approximately 14% of the entire genome, comprising 280 genes, was identified as being overexpressed and another 14% (283 genes) as being repressed. These DRGs were classified into various categories based on TubercuList functional classes (http://genolist.pasteur.fr/TubercuList; Fig. 1, Table S2). Genes were considered differentially regulated if they were up- or downregulated by ≥1.8-fold with a 95% data confidence interval (P-value ≤ 0.05). DRGs mainly belonged to the ‘virulence, detoxification, adaptation’, ‘lipid metabolism’, ‘cell wall and cell processes’ and ‘intermediary metabolism and respiration’ functional categories. Thirty-four (16%) of the 210 genes in the ‘virulence, detoxification and adaptation’ category were significantly downregulated (by up to ~4.5-fold), while 21 genes (10%) were significantly upregulated (by up to ~61-fold). ‘Lipid metabolism’ was a major category, with 34 genes (~14%) being upregulated by up to ~41-fold and 19 (~8%) downregulated by up to ~3-fold. Upon exposure to vit C, 55 (~7%) out of 736 genes belonging to the ‘cell wall and cell processes’ functions were significantly downregulated (by up to ~4-fold), as opposed to 30 genes (~4%) being induced (by up to ~47-fold). Out of the 889 genes on the microarray chip belonging to the category ‘intermediary metabolism and respiration’, 81 (~9%) were upregulated and 56 (~6%) were downregulated, by up to ~26-fold and ~4-fold, respectively. Some of the major functional groups of DRGs are discussed below.

*mce1* operon

The repressed set includes members of the *mce* operons implicated in facilitating bacterial entry into host cells (Arruda et al., 1993; Flesselles et al., 1999). The 13-gene *mce1* operon, *Rv0166* to *Rv0178*, was downregulated. This operon is downregulated under multiple stresses, including hypoxia, nutrient starvation and detergent exposure (Manganelli et al., 2001; Sherman et al., 2001; Betts et al., 2002), and also in infected bone marrow-derived murine macrophages (Schnappinger et al., 2003) and RAW murine macrophages (Casali et al., 2006), suggesting that vit C may in some way mimic the intracellular environment.

Chaperones and heat-shock proteins

Several genes encoding chaperones, including *hsp*, *hspX*, *dnaK*, *dnaJ1*, *grpE*, *clpB*, *htpX* and *hsp*, were upregulated. The gene *hspR* encodes a regulator of the *dnaK* (hsp70)
The expression of HspR-dependent genes occurs on the release of repression under heat shock (Stewart et al., 2001). ClpB reportedly interacts with DnaK (Raman et al., 2001), and HspX, a member of the DevR dormancy regulon, is required for growth within macrophages (Yuan et al., 1998). The gene hsp (also known as acr2), which bears ~40% similarity to hspX, is highly upregulated along with the HspR regulon during heat shock (Stewart et al., 2002). These genes are reported to be upregulated under other stresses, including hypoxic, oxidative and pH stresses, as well as within macrophages (Schnappinger et al., 2003; Rohde et al., 2007; Rustad et al., 2008; Voskuil et al., 2004, 2011).

**Detoxification**

The adaptive responses of Mtb to reactive oxygen and nitrogen species such as H₂O₂ and diethylenetriamine (DETA)/NO have been described previously (Schnappinger et al., 2003; Voskuil et al., 2011) and show a considerable similarity to the vit C-induced stress response (Fig. 2). katG and ahpC, the two best-studied H₂O₂ responsive genes, were upregulated; katG encodes catalase and is induced by ascorbic acid and H₂O₂ (Mulder et al., 1999; Sala et al., 2003). The ahpC, ahpD, lpd and dltT genes, encoding the subunits of the NADH-dependent peroxidase and peroxynitrite reductase (Bryk et al., 2002), were also moderately upregulated (by up to ~2.3-fold) in response to vit C addition.

The sulfur assimilation genes cysD and cysN are induced by iron limitation and repressed by the presence of cysteine, a major end-product of sulfur assimilation. These genes are also induced upon exposure to oxidative stress, suggesting regulation of sulfur assimilation by Mtb in response to toxic oxidants (Pinto et al., 2004). These genes were overexpressed on exposure to vit C, implying that Mtb mounts a robust counter-response to oxidative stress. The thioredoxin-encoding gene, trxB1, known to be induced in response to H₂O₂ stress (Voskuil et al., 2011), was also found to be induced, while other thioredoxin genes (trxA, trxB2 and trxC) were modestly overexpressed and did not satisfy the applied cut-off for classifying a gene as differentially expressed.

Oxidative stress is an important component of the host defence against bacteria. Our findings imply that Mtb perceives oxidative stress from vit C and mounts a rapid and robust counter-response to this stress. It is implied that the response to vit C that occurs through enhanced chaperone function and detoxification mechanisms is protective and is likely to restore the function of cellular molecules that might be damaged by ROIs.

**Lipid and mycolic acid metabolism**

The gene ino1 was upregulated. This gene encodes myo-inositol-1-phosphate synthase, which is involved at the first step of inositol biosynthesis (Movahedzadeh et al., 2004).
and is a component of cell wall constituents and of the redox buffer mycothiol (Fahey, 2001). Other upregulated genes include accA2 and accD2, which are believed to be involved in mycolic acid biosynthesis (Barry et al., 2007), as well as fadE5, fadE13 and fadD19, having fatty acid degradation function (Schnappinger et al., 2003; Muñoz-Elias & McKinney, 2006), and putative acyl-CoA dehydrogenases fadE1, fadE6, fadE26, fadE28 and fadE32, indicating that the bacterium might alter its metabolism to utilize fatty acids as a carbon source upon vit C exposure. Genes scoA and scoB of the citE–scoA operon, whose gene products are responsible for the utilization of ketones (Goulding et al., 2007), were also upregulated. The gene scoB was earlier shown to be upregulated under anaerobic conditions (Starck et al., 2004). pks1-papA1, involved in sulfolipid synthesis and which are known to be induced in infected bone marrow-derived macrophages (Rohde et al., 2012), were also upregulated in response to vit C. Triglycerides have been shown to accumulate in Mtb in response to multiple stresses, and of particular interest is the induction of tgs1, a signature gene for triglyceride accumulation in dormant bacteria (Deb et al., 2009). The upregulation of tgs1 in response to vit C is consistent with the induction of dormancy-like conditions in Mtb.

The downregulated genes of this category included those whose products are mainly involved in mycolic acid modifications. One of these, nmaA3, encodes a methyltransferase that generates methoxymycolates (Behr et al., 2000). Other repressed genes include umaA, which may encode a methyltransferase that modifies cell wall mycolic acids (zu Bentrup & Russell, 2001), and desA3, known to encode an aerobic desaturase involved in the synthesis of oleic acid, a precursor of mycobacterial membrane lipids (Okuyama et al., 1967; Walker et al., 1970). A number of genes involved in fatty acid degradation were also downregulated (fadD5, fadD10, fadD22, fadD29 and fadD30). The apparent upregulation of genes implicated in the same process (see above) likely reflects the selective usage of redundant genes of fatty acid metabolism in Mtb to enable bacterial adaptation to various environmental cues.

**Replication, transcription and translation functions**

A large number of genes of this category were downregulated, mainly those encoding proteins involved in replication, transcription and translation functions. For example, dnaA and dnaB, encoding proteins that interact with the origin of replication (Yamamoto et al., 2002) and unwind DNA during replication (Biswa & Tsodikov, 2008), respectively, were downregulated. The genes encoding GreA transcription elongation factor as well as 30S and 50S ribosomal proteins (rpsL, rpsJ, rplC, rpsQ, rplN, rplX, rpmE and rpmB2) were also downregulated, indicating a scaled-down requirement for mRNA and protein synthesis during bacterial adaptation to a ‘dormant’ state. In contrast, nrdB and nrdZ, encoding class I and II ribonucleotide reductases, respectively, were upregulated, suggesting the need for ribonucleotide reductase-catalysed production of dNTPs under conditions of limited chromosomal replication (Muñoz-Elias et al., 2005). The gene nrdB is reportedly involved in adaptation to nitrosative stress (Högberg et al., 2004), while nrdZ belongs to the DevR dormancy regulon.

![Heat map depicting the expression of genes involved in the core response of Mtb to oxidative and nitrosative stresses generated by treatment with vit C (this study), H2O2 for 40 min (Schnappinger et al., 2003; Voskuil et al., 2011) and DETA/NO for 40 min (Voskuil et al., 2011). * Data from Schnappinger et al. (2003). Blank cells indicate that data were not available for the genes.](http://mic.sgmjournals.org/)

**Fig. 2.** Response of Mtb to oxidative and nitrosative stresses. The heat map depicts the expression of genes involved in the core response of Mtb to oxidative and nitrosative stresses generated by treatment with vit C (this study), H2O2 for 40 min (Schnappinger et al., 2003; Voskuil et al., 2011) and DETA/NO for 40 min (Voskuil et al., 2011). * Data from Schnappinger et al. (2003). Blank cells indicate that data were not available for the genes.
and is induced by hypoxia and low doses of NO (Voskuil et al., 2003; Roberts et al., 2004).

This shift in gene expression is indicative of the fact that vit C-exposed bacteria adapt to a dormant form characterized by cessation of replication and reduction in gene transcription and translation, probably to reduce energy consumption and to divert resources to functions more essential for adaptation and survival under stress conditions.

**Transport**

The phosphate-specific transporter operon pstB-pstC1-pstA2 was downregulated, as were several lipoprotein-coding genes, including lprK, lppA, lppW, lppX and lpaF. Of these, lprK (of the mce1 operon) is believed to be involved in cell invasion and is repressed by RNI (Arruda et al., 1993; Ohno et al., 2003). The lppX gene may be involved in the transport of phthiocerol dimycocerosates to the bacterial outer membrane and its disruption leads to attenuation of virulence of Mtb (Sulzenbacher et al., 2006). The lppA gene product has been implicated in host–pathogen interactions as it is found only in pathogenic mycobacteria (Graña et al., 2010). These changes in gene expression of the ‘cell wall and cell processes’ category indicate that vit C triggers major changes in the bacterial cell wall and possibly in bacterial virulence and pathogenesis. Metal-ion transporter-coding genes, like ctpV and ctpG, were also upregulated. The gene ctpV, encoding an exporter, is induced during infection of macrophages (Graham & Clark-Curtiss, 1999) and in the Wayne hypoxia model (Muttucumaru et al., 2004) and is required for copper efflux as well as for detoxification and full virulence of Mtb during copper stress (Ward et al., 2010). Studies have suggested that copper homeostasis mechanisms may play a role in the outcome of bacterial infections (Ward et al., 2010). Like ctpV, ctpG is also copper inducible (Rowland & Niederweis, 2012), but it is predicted to function in the efflux of zinc (Argüello et al., 2007).

**Metabolism**

The overexpressed genes in this category included those that are considered to be important for maintenance of infection and that shift the metabolism to a state of energy conservation. Genes such as pckA, coding for phosphoenolpyruvate carboxykinase of the gluconeogenesis pathway, and icl, encoding isocitrate lyase of the glyoxylate pathway, were upregulated. These genes are upregulated in lung infection in mice (McKinney et al., 2000), and it has been established recently that gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical to establish and maintain Mtb infection (Marrero et al., 2010).

**Respiration**

The expression of ndh, which encodes type II (non-proton-pumping type) NADH dehydrogenase, was upregulated, while the genes encoding subunits of the aerobic NADH dehydrogenase (nuoA–M) were repressed. The downregulation of genes required for aerobic respiration observed in this study is consistent with previous observations that type I NADH dehydrogenase is downregulated during the NRP-1 stage of hypoxia. The increased transcription of cydA and cydB of the cydABCD cluster might facilitate efficient utilization of oxygen by high-oxygen affinity cytochrome bd-type menaquinol oxidase, as also seen in the NRP-1 stage of dormancy (Bosshoff et al., 2004).

Mtb may also utilize other substrates, such as nitrate, which was shown to accumulate in chronically infected tissues (Malm et al., 2009). Indeed, the NirBD assimilatory nitrate reductase-encoding genes were upregulated in vit C-treated cultures. Likewise, narG and narJ genes of the narGJHI cluster, encoding nitrate reductase, were modestly upregulated while the narX-nark2 nitrite efflux system, which supports the activity of nitrate reductase (Sohaskey & Wayne, 2003), was highly upregulated. These observations suggest that vit C mediates changes in gene expression that may help bacteria to maintain an energy-efficient state, as observed in the case of hypoxic stress.

**Regulators**

The massive transcriptional response of Mtb to vit C treatment pointed towards the involvement of specific regulators. Indeed, 25 genes, constituting ~15 % of all Mtb regulators, were significantly upregulated in response to vit C. These included the iron-dependent regulator genes furA and ideR, whose products are negative regulators of the iron-scavenging response and are highly induced within macrophages (Schnappinger et al., 2003; Voskuil et al., 2011) and also during hypoxia (Voskuil et al., 2004). The upregulation of these genes is suggestive of an iron-deficient environment in the presence of vit C or may aid the repair/replacement of iron-containing proteins which may be damaged, as noted in the bacterial response to stresses caused by ROIs and RNIs (Schnappinger et al., 2003).

Other overexpressed regulators included the secreted tyrosine phosphatase ptpA, encoding a key protein in Mtb survival and pathogenicity, which is induced intracellularly and inhibits phagosome acidification (Wong et al., 2011). The gene pknK, encoding PknK serine–threonine protein kinase, was induced; the involvement of PknK in translation control by downregulating the expression of tRNA genes as a function of growth has been reported (Malhotra et al., 2012). The genes encoding PknB and PknA protein kinases, predominantly expressed in the exponential phase of growth (Kang et al., 2005), were repressed in vit C-treated cultures. Such alterations in the expression of regulatory proteins suggest that Mtb utilizes multiple regulatory networks to overcome environmental stresses for its survival. Among the sigma factors, the genes encoding sigma factors B and E were upregulated, while sigK was downregulated. Of these, sigB and sigE were upregulated during oxidative stress (Voskuil et al., 2011).
Vitamin C mimics intracellular stresses for Mtb

**Vit C triggers rapid induction of the DevR dormancy regulon**

A robust induction of the DevR dormancy regulon was observed in the transcriptional response of Mtb H37Rv to vit C (Fig. 3), which closely matched the bacterial response to gaseous stresses, including hypoxia (Voskuil et al., 2004; Rustad et al., 2008), nitric oxide (NO) (Voskuil et al., 2011) and carbon monoxide (CO) (Kumar et al., 2008). The 8 h vit C response closely resembled the induction response observed between 6 and 14 days in the Wayne model (Voskuil et al., 2004), implying the vit C model to be a rapid hypoxia model. It also resembled, albeit to a lesser extent in terms of the magnitude of induction, the enduring hypoxic response between 4 and 12 h (Rustad et al., 2008). Likewise, a 40 min exposure to 0.05–1 mM DETA/NO (Voskuil et al., 2011) and a 3 h exposure to CO (Kumar et al., 2008) seem to closely resemble the response to vit C.

The MGIT assay is based on the change in fluorescence intensity of a ruthenium dichloride derivative according to the oxygen level in the tube. The depletion of oxygen results in an increase in fluorescence of this compound and is routinely used to monitor metabolic activity and growth of bacteria (Gentle & Yeh, 1999). This assay established that vit C treatment led to a depletion of oxygen; a rapid increase in fluorescence, indicative of hypoxia generation, was observed in MGIT culture/medium tubes spiked with vit C and not in control culture/medium (Fig. 4a, b). The induction of the DevR regulon was confirmed by the

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**Fig. 3.** DevR regulon induction in response to various gaseous signals. The heat map depicts the expression of the DevR regulon in our vit C model, the Wayne hypoxia model (Voskuil et al., 2004), enduring hypoxic response (Rustad et al., 2008), and NO stress (Voskuil et al., 2011) and CO stress models (Kumar et al., 2008). Blank cells indicate that data were not available for those genes.
Rv3134c GFP reporter assay, where vit C exposure led to a robust and rapid development of fluorescence in a dose-dependent manner (Fig. 4c). The extent of hypoxia generation by vit C corresponds to 30% dissolved oxygen concentration (Taneja et al., 2010), which is adequate for GFP to mature to its fluorescent form in the plate assay (Chauhan & Tyagi, 2008). The generation of hypoxia by vit C was further confirmed by the physiological response of treated Mtb cultures; a rapid and preferential induction of the DevR regulon was noted after vit C treatment in Mtb Comp dosT and not Comp devS cultures in RT-qPCR study (~83-fold versus 13-fold induction of hspX and ~13-fold versus ~4-fold induction of tgs1 in Comp dosT versus Comp devS strains). An unpaired two-tailed t-test analysis of the two Comp strains versus Mtb H37Rv was performed; the difference in fold expression of the regulon genes between Mtb Comp dosT and H37Rv strains was not significant (P=0.22) whereas that between Mtb Comp devS and H37Rv was significant (P=0.04). These results imply that near-complete restoration of regulon expression occurred in DosT-complemented bacteria and not in DevS-complemented bacteria after 1 h vit C exposure (Fig. 4d).

**Vit C-induced transcriptional response is largely independent of DevR**

The differential expression of representative genes from various functional groups identified by microarray analysis was analysed by RT-qPCR in axenic cultures of Mtb H37Rv and ΔdevR strains to assess whether the vit C-mediated transcriptional response was DevR-dependent. A high correlation (Pearson correlation r=0.915 and P<0.0001) between microarray and quantitative expression data was observed, which provided an overall confirmation of the microarray data (Fig. S1).
With the exception of the regulon genes, which were expressed in a DevR-dependent manner (Fig. 5, bottom panel), all genes from different Tuberculist functional categories that were analysed by RT-qPCR were expressed similarly in both the Mtb H37Rv and ΔdevR strains (Fig. 5). In the ‘intermediary metabolism’ group, the aerobic NADH dehydrogenases (operons nuoA–G and nuoH–N) were downregulated in both strains. Expression analysis of the first and the last genes of both these operons indicated their downregulation, which is consistent with the microarray data. Similarly, the genes encoding enzymes participating in gluconeogenesis, glyoxylate shunt and cholesterol
metabolism were upregulated in both strains. These included the
phosphoenolpyruvate carboxykinase-encoding gene pckA,
isoctate lyase-encoding gene icl, and cyp125, which forms a
part of the cholesterol degradation gene cluster. Other genes
upregulated in this category include Rv3083 and trxB1,
with roles in mycolic acid biosynthesis and antioxidant
defence, respectively. Lipid metabolism genes fadD13 and
fadb were also upregulated, as discussed previously. Several
genes from the ‘virulence, detoxification and adaptation’
category were validated, including the overexpressed genes
clpB, htpX and hsp, the oxidative stress response genes ahpC
and katG, and the enhanced intracellular survival-coding
genes eis. Rv1771, yrbE1A and Rv0178 were downregulated.
Rv1771 is believed to encode the terminal enzyme of the vit
C biosynthetic pathway, and yrbE1A and Rv0178 form the
first and last genes of the mce1 operon. Gene furA, the
independent regulator, also believed to be involved in the
oxidative stress response, was overexpressed in the ‘regulat-
ory proteins’ category in both strains. In the ‘cell wall and
cell process’ category, rpfD, encoding a resuscitation
promotion factor, and espA, a member of the Esx-1 secretion
system, were validated. The repressed set of genes from the
same category included the pstB1 and pstC phosphate
transporter genes and the cell division protein genes gid and
rodA. Lastly, in the ‘information pathways’ category, lrS2,
involved in cell division, was repressed. Also, sigma factors
sigB and sigE were upregulated, while genes involved in
replication and protein synthesis, dnaB, rpsJ and rplN, were
downregulated.

Vit C-mediated stresses mimic the macrophage
environment

Mtb is exposed to multiple intracellular stresses, including
surfactant, acidic pH, hypoxia, nutrient limitation, oxidative
and nitrosative stresses, iron limitation and exposure to
gaseous stresses such as NO and CO. In vitro dormancy
models are generally designed to study bacterial responses to
a single stress confronted in vivo (Sikri & Tyagi, 2013).
However, it is imperative to have a comprehensive under-
standing of bacterial adaptation to the combined effect of
these stresses rather than to only one or two isolated stress
signals.

To gain a comprehensive insight into the nature of stress(es)
produced by vit C, our differential gene expression data (Table
S2) were compared to those data obtained from various
dormancy models (Fig. 6, Table S3). Notably, a con-
siderable overlap in gene activation responses was observed
with the enduring hypoxic response (Rustad et al., 2008), the
RNI/ROI response (Schnappinger et al., 2003; Voskuil et al.,
2011) and the activated macrophage model (Schnappinger
et al., 2003). Of special note is the overlap of 162 genes from
the activated macrophage dataset (Schnappinger et al., 2003)
with the upregulated genes of the vit C dataset (Table S4). The
DevR regulon constituted a notable proportion of this
common response. A significant extent of similarity was also
noted with the enduring hypoxic response (Rustad et al.,
2008): ~82% of genes overlapped in the upregulated set and
~70% in the downregulated set. A similar trend was noted for
the Wayne hypoxia model between days 4 and 20 (non-
replicating persistence stages) (Voskuil et al., 2004). Vit C
causes an immediate drop in the pH of the growth medium to
~5.5 (Taneja et al., 2010), and lowers it further to pH ~4.5
over a period of 4 days (data not shown). The occurrence of
pH stress is indicated by an overlap of the vit C response with
the acid stress response (Fisher et al., 2002; Rohde et al.,
2007). In addition, nutrient stress (Betts et al., 2002) also seems to
have been created by the presence of vit C (Fig. 6).

Only five genes were upregulated uniquely in the vit C model
versus other models included in this comparative analysis.
These included two genes belonging to the PE/PPE family
(PE_PGRS44 and PE27A) and three encoding conserved
hypothetical proteins (Rv2393, Rv2160A and Rv2325c).
Likewise, 16 genes were uniquely downregulated in the vit
C dataset. Of these, seven genes encoded either conserved
hypothetical proteins or unknown proteins (Rv0100, Rv0964c,
Rv1116A, Rv2295, Rv2481c, Rv2530A and Rv3435c), one
belonged to the ‘phage-related proteins’ category (Rv1055),
two were membrane protein-coding genes (Rv0666 and
Rv2723), one a possible regulator (Rv2779c) and one mce-
related gene (Rv0590A), while the rest were metabolism-
related genes (Rv0097, TB7.3, Rv0947c and glpQ2).

Since the in vitro vit C-based dormancy model seems to
incorporate the major adaptive responses of Mtb, as seen in
the context of the host cell, we validated these predictions
by RT-qPCR analysis of RNA isolated from intracellular
Mtb in the THP-1 cell infection model. The genes selected
for validation were representative of the common responses
observed in the vit C model, activated macrophage, hypoxia
and ROI/RNI stress models. An overall consistent pattern of
gene expression was observed in the Mtb THP-1 infection
model and axenic Mtb cultures treated with vit C (Figs 7 and
S2; Pearson correlation coefficient r = 0.58 with P < 0.001),
suggesting a similarity in bacterial responses to vit C and
intracellular stresses.

The gene expression data were also compared to the recently
published microarray data for vit C-treated Mtb cultures
(Vilchéze et al., 2013). The overlap between the two datasets
was minimal (Table S5), which may be attributed to differ-
ences in the experimental set-up in the two studies, including
culture media, the concentration and duration of vit C
treatment (10 mM for 8 h versus 4 mM for 48 h) and culture
conditions (flasks versus roller bottles). Worthy of special
mention is the iron content (as ferric ammonium citrate) of
the culture media; the iron content of Dubos medium (50 mg
l⁻¹; our study) is actually 20% higher than that of
Middlebrook 7H9 medium (40 mg l⁻¹; Vilchéze et al.,
2013). Therefore, the discrepancy in observation cannot be
attributed to a lower concentration of iron in Dubos versus
Middlebrook media as suggested (Vilchéze et al., 2013).
The duration of exposure to vit C may have contributed to the
noted differences in bacterial gene expression in the two
studies.
**Fig. 6.** Genetic response of Mtb to various stresses. The expression of DRGs [genes that were upregulated (n=280) or downregulated (n=283) 1.8-fold (P ≤ 0.05)] in the vit C model was compared to various dormancy models. Red, genes upregulated ≥ 1.8-fold; green, genes downregulated ≥ 1.8-fold; and yellow, genes showing up- and downregulation across various time points/conditions. Blank spaces represent genes that were neither induced nor repressed 1.8-fold in the models used for the comparison. (EHR, enduring hypoxic response; IC, intracellular; mφ, macrophage; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates).
On the basis of the substantial overlaps observed between bacterial responses in the infection model and various *in vitro* models (Fig. 6), we propose that (1) vit C per se generates a multiple-stress environment for the bacterial cell, and more importantly, (2) the vit C model mimics an activated macrophage-like condition in axenic bacterial cultures (Fig. 6).

**DISCUSSION**

We have deciphered the most significant genetic responses of Mtb to a short-term exposure to vit C. A comparison of these responses to various stress responses reported in the literature reveals the pleiotropic properties of vit C. A striking observation was the rapid and robust induction of the DevR dormancy regulon, which closely overlaps with the response observed in all hypoxia models. We confirmed that vit C mediates the generation of hypoxia, which triggers a rapid induction of the DevR regulon in a primarily DosT-dependent manner. These observations are consistent with (1) earlier reports of hypoxia generation by vit C (Scarpa *et al.*, 1983; Taneja *et al.*, 2010), (2) the known function of DosT as an oxygen sensor (Kumar *et al.*, 2007; Sousa *et al.*, 2007) and (3) the role of the DosT oxygen sensor in rapid induction of the DevR regulon (Honaker *et al.*, 2010; Taneja *et al.*, 2010). However, our findings are not in agreement with those of Honaker *et al.*

**Fig. 7.** Validation of intracellular stress responses by RT-qPCR. Fold change in gene expression of Mtb isolated from THP-1 macrophages versus *in vitro*-cultured Mtb treated with 10 mM vit C was determined by RT-qPCR analysis of genes commonly expressed in various stress models. Fold change values for both sets were calculated with respect to axenic aerobic Mtb cultures and expressed as mean ± SD of three to six independent replicates and represented as a heat map.
(2010), which implicated DevS, and not DosT, in vit C-mediated induction of the DevR regulon. The difference may be attributed to the absence of hypoxia in their experimental set-up.

A second significant finding of this study is that vit C per se generates multiple stresses that resemble the macrophage environment, and in turn Mtb elicits a powerful and comprehensive counter-response that overlaps substantially with responses observed in other models. It was reported recently that exposure to vit C leads to the killing of Mtb cultures in vitro (Vilchéez et al., 2013). We and others have shown that Mtb viability is unaffected by vit C at concentrations up to 20 mM (Honaker et al., 2010, Taneja et al., 2010; Ghodbane et al., 2014). Furthermore, we have shown previously that vit C-treated axenic cultures and intracellular Mtb bacteria are viable and a significant proportion of the bacteria acquire an isoniazid-tolerant ‘dormant’ phenotype (Taneja et al., 2010). An analysis of the transcriptional response to vit C observed in the present study suggests that protective functions are rapidly activated that could facilitate the adaptation of Mtb to vit C. In summary, transcriptome analysis of Mtb indicates that vit C closely mimics the macrophase-like environment and forms the basis of a simple and rapid model to decipher the pleiotropic adaptation response of Mtb to intracellular stresses.

ACKNOWLEDGEMENTS

We are thankful to Dr David Sherman, Seattle Biomedical Research Institute, USA for providing Mtb ΔdevR and Mtb ΔdevSΔdosT strains, and to Dr Neil Stoker, St George’s, University of London, UK for Mtb H37Rv. The contribution of Dr Kohinoor Kaur in the construction of devS and dosT-complemented strains of Mtb is duly acknowledged. We are grateful to Dr Sudha Rao and Mohd Aiyaz of Genotypic India, Bengaluru, India for helpful discussions of the data. J. S. T. is thankful to the Department of Biotechnology, Government of India for a Tata Innovation Fellowship and to the Department of Science and Technology for the J. C. Bose National fellowship. K. S. thanks CSIR for a Senior Research fellowship and S. D. B. thanks the Indian Council of Medical Research for a Senior Research Fellowship. M. N. and P. K. are grateful to the Department of Biotechnology, Government of India for financial assistance. The facilities of Biotechnology Information System (BTIS) and Sushma Rani of the Department of Biotechnology at AIIMS are also highly acknowledged for assisting in microarray data analysis.

REFERENCES


Edited by: B. Minam

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