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A complex regulatory network controlling intrinsic multidrug resistance in *Mycobacterium smegmatis*

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SUMMARY

Mycobacteria are intrinsically resistant to a variety of stresses including many antibiotics. Although a number of pathways have been described to account for the observed resistances, the mechanisms that control the expression of genes required in these processes remain poorly defined. Here we report the role of a predicted anti-sigma factor, MSMEG_6129 and a predicted eukaryotic like serine/threonine protein kinase, MSMEG_5437, in the intrinsic resistance of *Mycobacterium smegmatis* to a variety of stresses including the genotoxic agent mitomycin C, hydrogen peroxide and at least four different antibiotics - isoniazid, chloramphenicol, erythromycin and tetracycline. We show that MSMEG_5437 influences the phosphorylation state of MSMEG_6129. Further, MSMEG_6129 controls the expression of a plethora of genes including efflux pumps, ABC transporters, catalases and transcription factors, either directly or *via* regulators like WhiB7, which account for the observed multi-drug resistance phenotypes. MSMEG_6129 in turn phosphorylates a contiguously located putative anti-anti-sigma factor, MSMEG_6127. We therefore propose that MSMEG_5437, MSMEG_6129 and MSMEG_6127 are components of a master regulatory network, upstream of *whiB7*, that controls the activity of one or more of the 28 sigma factors in *M. smegmatis*. Together, this network controls the expression of a regulon required for resistance to several unrelated antibiotics.

Keywords

Anti-sigma factor; intrinsic drug resistance; whiB7

INTRODUCTION

The genus *Mycobacterium* includes both pathogenic and saprophytic species that are able to survive exposure to environmental stresses, including oxidative and genotoxic stress, hypoxia, nutrient starvation, and exposure to multiple antibiotics (Hingley-Wilson et al., 2003, Zahrt and Deretic, 2002). Mycobacterial responses to stress are of particular interest for understanding the pathogenesis of *Mycobacterium tuberculosis* and its sensitivity and reaction to antibiotics (McKinney, 2000). A variety of such pathways and regulators have been described (Zahrt and Deretic, 2002, Rustad et al., 2009, McKinney et al., 2000,

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Rodrigue et al., 2006, Missiakas and Raina, 1998). The ability of *M. tuberculosis* to persist for many years in its human host, and the requirement for lengthy antibiotic regimens to eliminate drug sensitive strains reflects the effectiveness of the responses to stressful environments. Many of the processes are likely to be adapted from stress responses that are common to mycobacteria and related strains, including saprophytes such as *Mycobacterium smegmatis*. The detailed pathways, signals, regulatory responses and molecular interactions are not yet well understood; however, it is clear that mycobacteria have very complex and overlapping regulatory pathways reflected in the *M. tuberculosis* proteome of about 190 transcription regulators including sigma factors, two component systems, protein kinases as well as over a hundred transcription activators and repressors (Bishai, 1998).

Transcriptional reprogramming in bacteria is mediated by a complex array of regulatory factors including a repertoire of sigma factors that reversibly associate with RNA polymerase to enable transcription initiation at specific subsets of promoters (Gruber and Gross, 2003). Alternate sigma factors are often regulated post-translationally by anti-sigma factors that function by binding to and sequestering their cognate sigma factors thus preventing promoter recognition by RNA polymerase (Helmann, 1999). Anti-anti-sigma factors relieve this repression by binding to the anti-sigma factor (Hughes and Mathee, 1998). This complex set of three-partner-switching interactions ultimately determines the concentrations of active sigma factors and consequently the gene expression profile, and has been best studied in *Bacillus* spp (Kroos et al., 1999).

Transcription regulation in mycobacteria appears more intricate and presents significant departures from the less complex systems of *Bacillus* and *Escherichia coli*. *Mycobacterium tuberculosis* encodes 11 alternate sigma factors and *M. smegmatis* encodes at least 26 (Rodrigue et al., 2006), each of which is presumably regulated by its cognate set of regulators. To date regulators of only four mycobacterial sigma factors - σ^H , σ^F , σ^E , σ^L have been experimentally demonstrated and highlight the intricacies of regulation (Barik et al., 2010, Beaucher et al., 2002, Greenstein et al., 2007, Malik et al., 2009, Park et al., 2008, Raman et al., 2001, Song et al., 2003, Dainese et al., 2006). The oxidative stress responsive alternate sigma factor, σ^H , is controlled by the anti-sigma factor RshA (Park et al., 2008, Raman et al., 2001, Song et al., 2003). RshA is a redox sensor and sequesters σ^H in a reducing environment; under oxidizing conditions disulfide bond formation between cysteines in RshA releases σ^H repression. The RshA- σ^H interaction has also been shown to be under further control of phosphorylation by the eukaryotic like STPK, PknB, such that phosphorylation of RshA leads to dissociation of the complex (Raman et al., 2001, Park et al., 2008, Song et al., 2003). The stress responsive σ^F is also under a complex set of regulatory mechanism involving the anti-sigma factor UsfX which is in turn controlled by two anti-anti-sigma factors RsfA and RsfB (Beaucher et al., 2002). RsfA is redox sensor and regulates UsfX under reducing conditions while RsfB is controlled by phosphorylation. Furthermore, unlike other studied anti-sigma factors, UsfX does not phosphorylate RsfB and the kinase that phosphorylates RsfB is hitherto unknown (Beaucher et al., 2002, Malik et al., 2009, Greenstein et al., 2007). σ^E is regulated by RseA in a redox dependent manner; pknB dependent phosphorylation of RseA is further required for cleavage of RseA thereby activating the σ^E regulon (Barik et al., 2010).

Mycobacteria are intrinsically resistant to several commonly used antibiotics (da Silva et al., 2011). This has been attributed to the highly impermeable mycolic acid containing cell wall as well as to the constitutive and inducible expression of several active drug efflux mechanisms. Three transcriptional regulators, LfrR, MarR and WhiB7, are known to affect drug susceptibility (Morris et al., 2005, Buroni et al., 2006, McDermott et al., 1998) of which *M. tuberculosis whiB7* is best studied and has been shown to influence sensitivity to five unrelated antibiotics (Morris et al., 2005). Further, it is known that levels of drug resistance are influenced by the degrees of expression of the drug targets and their regulators – including InhA for isoniazid (Larsen et al., 2002), EthA that catalyzes the activation of ethionamide (Engohang-Ndong et al., 2004) and LfrA the fluoroquinolone efflux pump (Buroni et al., 2006) and are thus likely to be influenced by the complex regulatory transcription pathways. A recent demonstration of the involvement of SigI in isoniazid resistance in *M. tuberculosis* (Lee et al., 2012b) as well as SigA in multi-drug resistance in *M. smegmatis* (Burian et al., 2013) further underscores the significance of global transcription modulation in intrinsic resistance of mycobacteria to antibiotics.

Here we present data that suggests the existence of a master regulatory network upstream of *whiB7*, comprised of an anti-sigma factor (MSMEG_6129) and an anti-anti sigma factor (MSMEG_6127), that influences resistance in *M. smegmatis* to mitomycin C, hydrogen peroxide (H₂O₂) and several unrelated antibiotics by controlling a large regulon including ABC transporters and drug efflux pumps, catalases, WhiB7 and other transcription factors. Conceivably this circuit is itself responsive to regulatory proteins and signals that fine-tune its activity, one of which we demonstrate is MSMEG_5437, a eukaryotic like serine/threonine protein kinase.

RESULTS

MSMEG_5437 is required for *M. smegmatis* tolerance to mitomycin C and antibiotics

In a Transposon Site Hybridization (TraSH)-based search (Murry et al., 2008, Sassetti et al., 2001) for genes responsive to mitomycin C (MMC) in *M. smegmatis*, we identified 28 novel genes corresponding to a variety of different pathways or processes that are at least five-fold underrepresented under MMC exposure (Table S1). Three putative regulatory proteins, MSMEG_5437, MSMEG_6921 and MSMEG_2538 – encoding a predicted serine-threonine kinase, a conserved hypothetical regulator, and a MarA-like transcription factor respectively – are of particular interest as potential components of a genotoxic stress-responsive pathway (Table S1). Isogenic deletion strains of the three genes constructed by recombineering (van Kessel and Hatfull, 2008) are hypersensitive to 30 ng/ml MMC by at least one order of magnitude compared to wild-type *M. smegmatis* (Fig. 1a). Of these, *mc*²*155: 5437* shows the greatest sensitivity towards MMC. However the MSMEG_5437-dependent response is unlikely to be within the DNA repair pathway *per se*, because the mutant is unaltered in its response to either ultraviolet light or hydrogen peroxide (H₂O₂) (Fig. S1). Surprisingly, the mutant has a pronounced increase in sensitivity to the unrelated antibiotics isoniazid, tetracycline and chloramphenicol, suggesting a central involvement of MSMEG_5437 in response to environmental stresses (Fig. 1b). The antibiotic susceptibility profile is, however,

quite specific and no change in resistance was observed with rifampicin, ethionamide, ethambutol, or ciprofloxacin (Fig. S1).

BLASTP searches of MSMEG_5437 against the NCBI protein database revealed similarity to the eukaryotic-like serine/threonine protein kinases (STPKs) found in bacteria, and MSMEG_5437 is one of 18 putative STPKs (*pknA* through *pknL*) identified in the genome sequence of *M. smegmatis*. MSMEG_5437 shows sequence conservation to the catalytic kinase domain that is well conserved in all mycobacterial kinases and it is difficult to discern which class of kinases it is functionally related to. However, MSMEG_5437 contains almost all of the conserved signature motifs of the catalytic domains of eukaryotic STPKs that is also present in mycobacterial kinases (Av-Gay and Everett, 2000) including the critical catalytic lysine residue at position 126 (Fig. 1c). To determine the *in vivo* targets of MSMEG_5437 we performed 2-DIGE analysis of total protein from wild type and *mc*²155: 5437 bacteria followed by phosphoprotein staining. A prominent decrease in the *in vivo* phosphoprotein levels in the mutant is consistent with it either acting directly as a kinase, or being required for the activity of other *M. smegmatis* kinases (Fig. 1d). We could identify a total of 52 protein species that were differentially phosphorylated in the mutant; of these 18 were completely undetectable in *mc*²155: 5437 and their identity was determined using mass-spectrometry. These proteins represent a broad array of diverse predicted functions (Table 1).

MSMEG_6129 is responsive to MSMEG_5437 and is required for sensitivity to antibiotics and Mitomycin C (MMC)

It is plausible that at least a subset of the proteins whose phosphorylation is reduced in the *mc*²155: 5437 mutant is involved in pathways contributing to increased antibiotic susceptibility. We initially chose to construct isogenic deletion mutations in MSMEG_0059, MSMEG_6091, MSMEG_0456, MSMEG_6427 and MSMEG_6129 as representatives of different functional categories; of these only the phenotype of *mc*²155: 6129 was striking. When compared to wild type *mc*²155, *mc*²155: 6129 showed increased resistance to MMC, INH, tetracycline and chloramphenicol, rather than increased sensitivity (Fig. 2a). The two mutant strains were different from each other and the wild type in their sensitivities to antibiotics when grown in agar as well as in their minimum inhibitory concentrations in liquid media (Fig. 2b and Table 2). This is in contrast to *mc*²155: 5437, which showed increased susceptibility to the same drugs. Interestingly, *mc*²155: 6129 also showed strongly increased sensitivity to hydrogen peroxide (Fig. 2c), a phenotype not observed with *mc*²155: 5437. Genetic complementation of *mc*²155: 6129 with an exogenous copy of MSMEG_6129 restores the phenotype to that of wild type bacteria (Fig. 2d) confirming that the MSMEG_6129 is responsible for the phenotype.

Proposed model for the function of MSMEG_6129 in antibiotic resistance

Bioinformatic analyses using BLAST and HHPRED suggest that MSMEG_6129 is an anti-sigma factor. Furthermore, the adjacent MSMEG_6127 is a predicted anti-anti sigma factor. This was immediately striking as the phenotypes of *mc*²155: 5437 and *mc*²155: 6129 can be accounted for by assuming that the unphosphorylated form of MSMEG_6129 binds to a cognate sigma factor, and prevents it from associating with RNA Polymerase to transcribe a

regulon influencing drug sensitivities. The proposed model is shown in Figure 3. Although MSMEG_6129 was identified as a phosphorylation substrate of MSMEG_5437 in the 2-DIGE analysis, we have been unable to demonstrate phosphorylation of MSMEG_6129 by MSMEG_5437 *in vitro* using purified proteins. While it is likely that MSMEG_5437 has an indirect influence on the phosphorylation status of MSMEG_6129, it is possible that phosphorylation may require activation of MSMEG_5437 or additional cofactors and it is too early to dismiss a direct interaction without further biochemical analyses. In the absence of MSMEG_5437, as in the *mc*²155: 5437 strain, MSMEG_6129 is constitutively unphosphorylated resulting in the sequestration of its cognate sigma factor and down regulation of the antibiotic resistance regulon and consequent antibiotic sensitivity. Deletion of MSMEG_6129 would therefore be expected to increase expression of the regulon with resulting antibiotic resistance. The model also predicts that an overexpression of MSMEG_6129 would shift the balance towards increased sequestration of the sigma factor and mimic the phenotype of a *mc*²155: 5437 mutant. Indeed we find that overexpression of MSMEG_6129 from an acetamide inducible promoter renders the bacteria sensitive to isoniazid, chloramphenicol and tetracycline to a level exhibited by *mc*²155: 5437 (Fig. 4).

MSMEG_6127, a predicted anti-anti-sigma, is a phosphorylation substrate of MSMEG_6129

The adjacent organization of MSMEG_6129 and the anti-anti sigma factor (MSMEG_6127) raises the possibility that the two interact with each other and that MSMEG_6127 is the cognate negative regulator of MSMEG_6129. In most systems studied to date the tripartite interactions between sigma, anti-sigma and anti-anti-sigma factors is regulated by phosphorelay signals in which the anti-sigma factor typically phosphorylates the anti-anti-sigma factor. Figure 5a shows robust *in vitro* phosphorylation of MSMEG_6127 by MSMEG_6129 using overexpressed proteins. Analysis of the sites phosphorylated in MSMEG_6127 by MSMEG_6129 *in vitro* using LC-MS/MS shows eight ser/thr residues that are phosphorylated in MSMEG_6127 and includes Ser63 which coincides with the consensus phosphorylation site in the anti-anti sigma factor family (Fig. 5b) consistent with the idea that MSMEG_6129 and MSMEG_6127 constitute a cognate anti-sigma: anti-anti-sigma pair thereby lending support to the model proposed in Figure 3.

Although a homologue of MSMEG_6129 could not be identified in *M. tuberculosis*, MSMEG_6127 shows 62% similarity (37% identity) to the *M. tuberculosis* RsfB protein, which is the cognate anti-anti-sigma factor of UsfX, the negative regulator of σ^F in *M. tuberculosis*. Because MSMEG_6129 can phosphorylate MSMEG_6127 *in vitro*, we therefore tested the ability of MSMEG_6129 to phosphorylate the *M. tuberculosis* homologue of MSMEG_6127, RsfB. We find that indeed RsfB can function as a substrate of MSMEG_6129 *in vitro* (Fig. 5c).

MSMEG_6129 controls a diverse array of genes

While most anti-sigma factors are located in the vicinity of their cognate σ factors, the identity of the σ factor(s) regulated by MSMEG_6129 is not immediately obvious from the genome organization. However, the genes under its control can be identified by comparing the transcriptome of *mc*²155: 6129 with wild-type *M. smegmatis* using high throughput cDNA sequencing (RNAseq). A total of 11342404 reads aligning to ORFs were obtained

for wild type mc^{2155} and 11927188 reads for $mc^{2155}: 6129$ of which 74% and 81% respectively correspond to mRNA. The number of transcripts of each gene in $mc^{2155}: 6129$ was normalized to a million reads for both the strains and the number of reads per ORF were compared in both strains for each ORF. In order to facilitate our analysis we chose to examine those transcripts whose levels changed at least 2.5 fold in the $mc^{2155}: 6129$ strain as compared to the wild-type. While the expression of most genes remain unchanged, we found 181 genes with induced expression and 40 genes with reduced expression in $mc^{2155}: 6129$ (Figure 6a and Table S2). The disproportionately higher number of induced genes is consistent with the suggested anti-sigma factor function of MSMEG_6129, a deletion of which would result in dysregulated availability of its cognate sigma factors and subsequently increased transcription of downstream genes.

Among the 181 upregulated genes, 84 are hypothetical proteins of unknown function. Of the remaining, two classes of proteins efflux pumps and transcription regulators are most relevant to the observed phenotype of $mc^{2155}: 6129$ and are discussed below (Table 3). The downregulation of catalase genes is intriguing and their significance is also discussed. The results of RNAseq were also verified using qPCR for the pertinent genes from the above three categories (Fig. 6b).

Upregulation of known efflux pumps in $mc^{2155}: 6129$ may account for resistance to Tetracycline and Chloramphenicol

Nine efflux pumps and transporters are seen to be upregulated in $mc^{2155}: 6129$ (Table 3). This is of immediate attention as intrinsic resistance of *M. tuberculosis* to several antibiotics has been partially linked to the activities of efflux pumps in the cell envelope (da Silva et al., 2011). Transcript levels of MSMEG_5187 which encodes the TetV shows a 9 fold increase in $mc^{2155}: 6129$ as compared to wildtype; TetV is a well-characterized efflux pump that is known to confer resistance to tetracycline in mycobacteria and could account for the observed tolerance of the mutant to tetracycline. MSMEG_5047 and MSMEG_6245 encode a drug resistance transporter of the Bcr/CflA subfamily and a chloramphenicol resistance protein respectively both of which belong to the major facilitator superfamily (MFS) of transporters that extrude a variety of chemically unrelated compounds including chloramphenicol. The increased transcription of these could account for the observed tolerance of the mutant to chloramphenicol. In addition to the above efflux proteins, four ABC family of transporters are also up-regulated in $mc^{2155}: 6129$ which could contribute to the efflux of either these or other drugs not yet tested.

Downregulation of catalase genes may account for INH resistance and H_2O_2 sensitivity in $mc^{2155}: 6129$

While the resistance to tetracycline and chloramphenicol can be accounted for by the overexpression of efflux genes from the RNAseq data, it is not immediately obvious why $mc^{2155}: 6129$ is resistant to isoniazid or sensitive to H_2O_2 . It is noteworthy that a deletion in MSMEG_6129 results in a 2–3 fold decrease in expression of two catalase encoding genes, MSMEG_6213 and MSMEG_6232 (Table 3). Catalase (encoded by *katG* in *M. tuberculosis*) is known to activate the prodrug isoniazid which then inhibits InhA, involved in mycolic acid biosynthesis (Chouchane et al., 2000, Baulard et al., 2000). The

M. smegmatis genome encodes eight catalases, although the specific role of each of these in the activation of INH or detoxification of H₂O₂ is unknown. The present transcriptomics data however suggests the involvement of MSMEG_6213 and MSMEG_6232 in the above functions. Moreover, although mc²155: 6129 is resistant to isoniazid, its sensitivity to ethionamide, a structural analogue of INH not activated by catalase (Baulard et al., 2000), remains unchanged which further supports the idea that the observed resistance to isoniazid occurs at the stage of drug activation and not at a downstream stage common to both isoniazid and ethionamide. This decrease in catalase expression could also offer an explanation for the observed increase in sensitivity of the mutant to H₂O₂.

Upregulation of *M. smegmatis whiB7* and other transcription regulators in mc²155: 6129

The expression of fourteen transcription regulators are seen to be upregulated in mc²155: 6129 (Table 3). Of these, three are WhiB-family of transcription factors-MSMEG_0051 (*whiB6*), MSMEG_1953 (*whiB7*), and MSMEG_6199 (*whiB2*). Interestingly, *M. tuberculosis whiB7* has previously been shown to be involved in the intrinsic resistance of *M. tuberculosis* to multiple antibiotics by regulating the expression of known drug resistance genes including *tap* (Rv1258c), Rv1473 and Rv1988 (Morris et al., 2005, Ainsa et al., 1998). A knock out of *whiB7* renders *M. tuberculosis* more sensitive to several unrelated antibiotics including chloramphenicol, tetracycline and erythromycin whereas overexpression of *whiB7* results in increased resistance to these antibiotics (Morris et al., 2005). A gene replacement mutant of MSMEG_1953 was constructed using recombineering and tested for its sensitivity to isoniazid, chloramphenicol, tetracycline and erythromycin. As with an *M. tuberculosis whiB7* mutant, MSMEG_1953 was sensitive to chloramphenicol, tetracycline and erythromycin suggesting overlaps in the regulatory mechanisms of antibiotic resistance in *M. smegmatis* and *M. tuberculosis* (Table 4). Similar results were also observed by Burian et al who showed that *M. smegmatis whiB7* can be induced with erythromycin (Burian et al., 2012).

In addition to the WhiB family of transcription regulators, we also observe the upregulation of three transcription regulators, MSMEG_1025, MSMEG_2305 and MSMEG_6441, that belong to the TetR family, members of which are known to encode proteins involved in multidrug resistance and pathogenicity of gram-positive and gram-negative bacteria (Engohang-Ndong et al., 2004, Ramos et al., 2005). Upregulation of MSMEG_1025, MSMEG_2305 and MSMEG_6441 in mc²155: 6129 could contribute directly or indirectly to the observed multi-drug resistance phenotype of mc²155: 6129.

MSMEG_6129 functions upstream of *M. smegmatis whiB7* controlling both *whiB7* dependent and independent pathways

Gene expression profiling of *M. smegmatis* mc²155: 6129 shows striking overlap with the *M. tuberculosis* strain overexpressing WhiB7 (Morris et al., 2005). We observe that MSMEG_5187 and MSMEG_5102, homologues of Rv1258c (the tetracycline efflux pump) and Rv1473 (a putative macrolide transporter) respectively, as well as homologues of acetyl transferases, cutinases and ureases are upregulated in mc²155: 6129 (Table S2) similar to that in the *M. tuberculosis* strain overexpressing WhiB7 (Burian et al., 2012). Furthermore, *M. smegmatis* MSMEG_1953 (*whiB7*) is upregulated >7 fold in mc²155: 6129. Taken

together, the data suggest that MSMEG_6129 is an upstream regulator controlling the expression of MSMEG_1953 (*whiB7*), which in turn activates a regulon including antibiotic efflux pumps (MSMEG_5187 and MSMEG_5102). The model therefore predicts mc²155: *6129* to display resistance to the other antibiotics that an mc²155: *1953* strain is sensitive to and is found to be indeed more tolerant to erythromycin as compared to wild type bacteria (Fig. 7).

mc²155: *1953* however does not display sensitivity to isoniazid suggesting that the observed resistance of mc²155: *6129* to isoniazid occurs via a MSMEG_1953 independent pathway (Table 4). This is consistent with previous observations of Burian et al. who demonstrated that transcription of MSMEG_1953 is induced by several antibiotics but not by isoniazid (Burian et al., 2012). The entire spectrum of phenotypes exhibited by mc²155: *6129* is therefore not entirely mediated by MSMEG_1953. We envisage a scenario in which MSMEG_6129 controls the expression of several genes, directly or indirectly through regulators like MSMEG_1953 (*whiB7*), which finally determines its behavior in the presence of antibiotics and oxidative stresses.

DISCUSSION

The extreme resistance of *Mycobacterium spp.* to a variety of antibiotics has traditionally been attributed to their waxy and highly impermeable cell envelope that forms a physical barrier against external threats. However, the contribution of metabolic plasticity that minimizes the effect of antibiotics and other chemicals that do permeate this barrier has been less appreciated thus far but is beginning to gain prominence from recent studies that highlight the role of transcriptional regulators like *whiB7* and *sigI* (Lee et al., 2012a, Morris et al., 2005). This opens up broader questions as to how a complex and intertwined transcription regulatory circuit, central to which is the large pool of sigma factors and their upstream regulators, facilitates intrinsic drug resistance in mycobacteria. The results presented here identify new factors and provide novel insights into a mechanism of global transcriptional regulation that directly influences the sensitivity of mycobacteria to antibiotics.

We envisage a scenario in which the anti-sigma factor, MSMEG_6129, controls the expression of a regulon, either directly or indirectly through transcriptional regulators like *WhiB7*, by negatively regulating an unidentified sigma factor that controls several cellular responses including the response to multiple unrelated antibiotics and oxidative stresses. Moreover, the interaction between MSMEG_6129 and MSMEG_6127 suggest that MSMEG_6129 (the anti-sigma factor) is itself under the control of the MSMEG_6127 (its cognate anti-anti sigma factor) via a phosphorylation dependent mechanism such that phosphorylation of MSMEG_6127 by MSMEG_6129 could release the anti-sigma factor for negative regulation of sigma factor and its downstream genes. This circuit is presumably under further control of an upstream signaling pathway involving MSMEG_5437 that affects the phosphorylation status of MSMEG_6129. The inverse phenotypes of MSMEG_5437 and MSMEG_6129 suggests that the phosphorylated form of the anti-sigma factor is functionally inactive and makes the sigma factor available to transcribe downstream genes involved in antibiotic resistance. It is interesting that MSMEG_5437 shows homology to

receptor eukaryotic-like (Hanks-type) ser/thr kinases; 9 of 11 *M. tuberculosis* Hanks-type kinases are transmembrane kinases and presumably transduce extracellular signals. This makes MSMEG_5437 an attractive target to sense the external milieu and relay the signal to the downstream sigma factors. Although MSMEG_5437 shows homology primarily to the catalytic domains of STPKs along its entire length of 287 amino acids, we note the presence of a significant transmembrane domain (residues 201–227) identified using TMPred (Hofmann, 1993). Further experiments will be required to demonstrate the role of MSMEG_5437 as a membrane sensor. Although at present we do not have compelling evidence for MSMEG_5437 to be a membrane associated kinase, it is of interest that another cytoplasmic mycobacterial STPK, *pknG*, has been previously implicated in intrinsic drug resistance in *M. smegmatis* such that a deletion in *pknG* rendered the bacteria sensitive to erythromycin, vancomycin, rifampin and ethambutol (Wolff et al., 2009).

The identity of the sigma factor regulated by MSMEG_6129 however remains elusive. We explored MSMEG_1804 and MSMEG_1970 as highly likely candidates—MSMEG_1804 is a SigF homologue that has been shown to be under RsfB regulation and MSMEG_1970 is a putative sigma factor with induced expression in *mc²155: 6129*. However, isogenic deletions of MSMEG_1804 and MSMEG_1970 were indistinguishable from wild type in their resistance to isoniazid, chloramphenicol and tetracycline consistent with previous reports from Gebhard et al (Gebhard et al., 2008) who showed that the isoniazid sensitivity of *sigF* remains unchanged. Interestingly, a recent study identified a direct physical interaction between WhiB7 and σ^A (Burian et al., 2013), implying that expression of WhiB7 and its regulon are dependent on σ^A activity. This raises the possibility that MSMEG_6129 exerts its effect via interaction with σ^A and offers a mechanism in which the abundance and promoter occupancy of σ^A could be regulated by MSMEG_6129 over a dynamic range to facilitate mycobacterial adaptation to its changing environment.

While MSMEG_6129 plays a pivotal role in regulating intrinsic resistance to multiple antibiotics in *M. smegmatis*, a homologue in *M. tuberculosis* is conspicuously absent even though the two species show similar drug tolerance behaviors. At least two lines of evidence presented here suggest that transcription pathways that regulate antibiotic resistance, while not identical, show substantial overlap between the two species. First, *M. smegmatis whiB7* (MSMEG_1953) is highly sensitive to tetracycline, chloramphenicol and erythromycin similar to that of an *M. tuberculosis whiB7* strain (Fig. 7 and Table 4). In addition, the genes regulated by WhiB7 in both species show significant overlap (Table S2 and (Morris et al., 2005)). Therefore, it is highly likely that a functional homologue of MSMEG_6129 is present in *M. tuberculosis* that regulates intrinsic drug resistance pathways in the pathogen. This relationship between sigma factors and intrinsic drug tolerance through a phosphorelay mechanism offers a means to render mycobacteria more sensitive to the currently available antibiotic regime.

However it can't be ruled out that *M. smegmatis* has diverged from *M. tuberculosis*, with the involvement of additional factors. This is supported by the presence of 26 alternate sigma factors in *M. smegmatis*, as against 11 in *M. tuberculosis*. The requirement for an additional regulatory network in *M. smegmatis* to control intrinsic drug resistance may not be entirely

unexpected considering the competitive habitat of this saprophyte that is shared with other soil bacteria capable of synthesizing a plethora of antibiotics.

EXPERIMENTAL PROCEDURES

Media and Strains

Mycobacterium smegmatis was grown at 37°C in Middlebrook 7H9 (DIFCO) supplemented with 10% ADC and 0.05% Tween 20. Antibiotics were added as required to indicated amounts. *M. smegmatis* overexpressing MSMEG_6129 and MSMEG_6127 were grown in Middlebrook 7H9 containing 0.2% succinate to an OD of 0.4 and induced with 0.02% acetamide for an additional 18h wherever indicated. Gene replacement mutants were constructed using recombineering as described previously (van Kessel and Hatfull, 2007) using recombineering functions encoded on pJV53. The recombineering construct for each gene was generated by cloning in the multiple cloning sites flanking the hygromycin cassette of pYUB854. The left arm and right arms were generated using the following primer pairs respectively: MSMEG_5437 – ctcgttgagcatctagagcggccacca/gttgcgcgcgaggcgtgtggcgagg and cagcgggtgagctagcctcgcga/gaggagcagcccacagatctgtgtg, MSMEG_6129- cgagcgtgaaatgcgtctagagccat/ggttgagccaaaagcctaaggggtg and gttgatgcacgcctagccgacct/cgcgcgatcgacatcctcagaggtg, MSMEG_1953-ttctcggcgaatctagagatccgggt/ccgccacgattcttaaggatcgtgcc and ctcgaccgcggaagccttgcgcgc/ggaagtgcgatcgaagatctcag, MSMEG_1804-ccggcagcggaaacttaaggagtggcgcg/gcggaaaggcacctctagacgatctctcgcgc and gagcgggtcggtagctcgcagatgc/cttgccccctcagaccgtggaccac, MSMEG_1970-gcccgaggcctcgtccatcgc/cgttgccaggagtctagagcagcgtcgaagc and ctggaggcaatggagtagcgcgatcgtgc/ggtgaactcgtcagggcacgggcgtcag. Upon confirmation of the mutant by PCR, the recombineering plasmid was cured by repeated passage through antibiotic free medium.

Protein overexpression and purification

MSMEG_6127 and MSMEG_6129 were amplified from *M. smegmatis* genomic DNA and using the primer pairs caaacaccgtcggaggtgcatatgac/ctaccgggtgcgacactcagtgtagccaccaggtccatagacagac/gtcgacaggtggtgaagcttccgagtt, cloned in pET21a (Novagen) and transformed into BL21(DE3) Star, grown to an A₆₀₀ of 0.4 and induced with 1mM IPTG. The cells were lysed in a buffer containing 50mM Tris-HCl (pH 8.0), 300mM NaCl and 5% glycerol and the clarified lysate was loaded on a Ni-NTA column (Qiagen). Non-specifically bound proteins were removed by washing with lysis buffer containing 20mM imidazole and the histidine tagged protein was eluted with 100mM imidazole.

Identification of mitomycin C responsive genes in *M. smegmatis*

For construction of transposon libraries, *M. smegmatis* cultures were grown to OD₆₀₀ of 0.6 in Middlebrook 7H9 + ADC, washed several times with with MP buffer (50 mM Tris, pH 7.5/150 mM NaCl/10 mM MgSO₄/2 mM CaCl₂) and resuspended in 1/10 of the original culture volume of MP buffer. Cells were infected with 10¹⁰ phage MycoMarT7/ml of original culture for 3 h at 37°C. Transduced cells were plated on 7H10+ADC + kanamycin (40µg/ml) at 37°C. Libraries were prepared by scraping approximately 50,000 colonies off

plates and freezing at -80°C . The mutant library was grown in liquid media to an OD of 0.3 and exposed to 30ng/ml of mitomycin C for 12 hours, a concentration that was previously determined to be sublethal to wild type bacteria. An equal volume of culture was set aside without mitomycin C exposure and served as the control. Genomic DNA was isolated from each sample and conditional lethal genes identified using hybridization on microarrays obtained from The Institute of Genomic research (TIGR) as described previously (Murry et al., 2008).

Antibiotic and H_2O_2 Sensitivity Assays

Wild type *mc²155* and mutant strains were grown to an A_{600} of 0.6–0.7 tested for their susceptibility to antibiotics by spotting a 10 fold serial dilution initially on Middlebrook 7H10 (DIFCO) plates containing a range of each drug: mitomycin C (20–40ng/ml), isoniazid (1–16 $\mu\text{g/ml}$), rifampicin (1–16 $\mu\text{g/ml}$), ethionamide (8–128 $\mu\text{g/ml}$), tetracycline (0.05–1 $\mu\text{g/ml}$), chloramphenicol (8–128 $\mu\text{g/ml}$) and erythromycin (20–60 $\mu\text{g/ml}$). The concentration of antibiotic showing the most prominent difference in each case was then used in subsequent experiments. Antibiotic susceptibility on liquid media was assayed by inoculating the desired strain in a two fold dilution series of each antibiotic at an initial A_{600} of 0.0004. The cultures were incubated at 37°C and the A_{600} was measured after 36hs. Wild type *mc²155* and mutant strains were grown to an A_{600} of 0.3 and exposed to varying concentrations of H_2O_2 (0, 4, 6, 16, 32, 64 and 128mM) at 37°C for 30minutes. Following exposure each sample was rapidly diluted in Middlebrook 7H9 and 10 fold serial dilutions were spotted on Middlebrook 7H10 agar plates.

In vitro kinase assays and determination of phosphorylation sites

5 μM of purified anti-anti sigma factor (MSMEG_6127 or RsfB) was mixed with increasing amounts of MSMEG_6129 to molar ratios of 1:1, 1:2, 1:4, 1:8 in a buffer containing 50mM TrisHCl, (pH 8.0), 50mM NaCl, 10mM MgCl_2 and 10mM MnCl_2 . Reactions were started by the addition of 1 μCi of [$\gamma\text{-P}^{32}$] ATP and 15 μM ATP, incubated at 25°C for 30 mins and terminated by the addition of 20mM EDTA. The proteins were separated on a 15% SDS PAGE gel and visualized by autoradiography.

5 μM of MSMEG_6127 purified from *E. coli* was mixed with 10 μM of MSMEG_6129 in a buffer containing 50mM TrisHCl, (pH 8.0), 50mM NaCl, 10mM MgCl_2 and 10mM MnCl_2 and 15 μM ATP at 25°C for 30 mins. The samples were separated on a 15% SDS/PAGE, stained with coomassie blue followed by excision of MSMEG_6127 from the gel. The phosphorylated residues were identified using MALDI tandem MS/MS (Applied Biomics, Inc.).

2D gel electrophoresis and phosphoprotein staining

Phosphoprotein analysis was performed by Applied Biomics, Inc. CA. In brief, the wild type and *mc²155: 5437* strains were grown to exponential phase and the protein extracts are differentially labeled with Cy-3 and Cy-5 and separated by 2-dimensional gel electrophoresis. Phosphorylated proteins are then stained with a fluorescent phospho-protein staining solution followed by destaining. The changes in abundance of phosphorylated proteins between the two samples was then identified using the ImageQuant and DeCyder

softwares. 18 protein spots were completely absent in *mc²155: 5437* and were excised from the gel and identified by MALDI TOF/TOF (tandem mass spectrometry MS/MS).

RNA preparation and RNA-seq analysis and qPCR

Total RNA was prepared from wild type and *mc²155: 6129* strains grown to exponential phase in Middlebrook 7H9-ADC using the Qiagen RNA preparation kit followed by two DNase I treatments. Approximately 5µg total RNA samples were treated with the Ribo-Zero™ rRNA removal procedure (Epicentre) to enrich for mRNA. Approximately 500ng of each sample was then used to synthesize cDNA by reverse transcription using the Ovation® RNA-Seq System (Nugen) followed by library preparation using the Encore NGS Multiplex System I (Nugen) and high throughput sequencing on the Illumina platform.

The sequence data were demultiplexed using FASTQ Groomer and Barcode Splitter on the Galaxy platform. Each read was trimmed by 6bp at the left end and 2bp at the right end, then aligned to the annotated *M. smegmatis* genome using Bowtie with standard settings (Galaxy). The number of reads corresponding to each ORF was calculated, and the data were normalized to a million reads as well as to the size of each ORF, and expressed as Reads per Kilobase per million mapped reads (RPKM) and are available as Excel files.

Primers for qRT-PCR were generated using PrimerExpress software (Applied Biosystems). cDNA was generated using random hexamers and Maxima reverse transcriptase (Fermentas), and qRT-PCR performed using the Maxima SYBR Green qPCR Master Mix (Fermentas). Applied Biosystems 7300 Real-Time PCR System was used with cycling conditions of: 50°C for 2', 95°C for 10', and 40 cycles of 95°C for 15", 60°C for 1'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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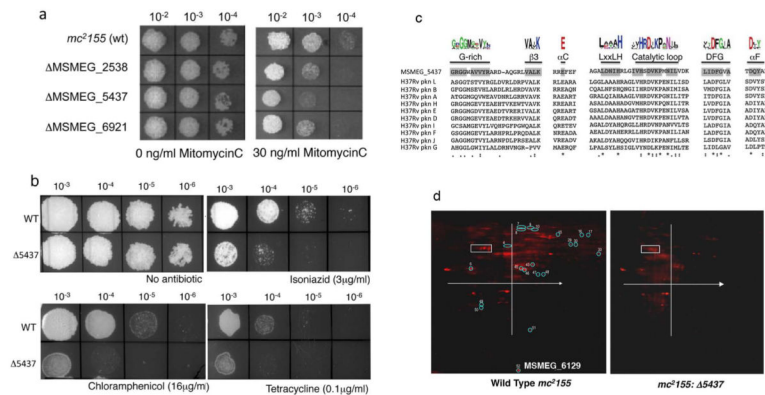


Figure 1. MSMEG_5437, a predicted STPK, is required for *M. smegmatis* tolerance to MitomycinC and antibiotics

(a) Ten fold dilutions of gene replacement mutants in MSMEG_2538, MSMEG_5437 and MSMEG_6921 were spotted on Middlebrook 7H10 containing 30ng/ml of MMC with MSMEG_5437 being the most sensitive of the mutants. (b) Ten fold serial dilutions of wild type and *mc*²155: 5437 were spotted on Middlebrook 7H10 containing indicated concentrations of isoniazid, chloramphenicol and tetracycline as well as a control lacking antibiotic. The mutant is hypersensitive to all three antibiotics tested. (c) Multiple sequence alignment of MSMEG_5437 with the eukaryotic like STPKs in *M. tuberculosis* showing the conserved signature motifs of bacterial ser/thr kinases and the conserved kinase domain in MSMEG_5437 shaded in grey. Deviations in the sequence of MSMEG_5437 are observed in the glycine rich motif and the catalytic loop and are marked. (d) Total cell lysate was prepared from wild type *mc*²155 and *mc*²155: 5437 and separated by 2-D gel electrophoresis. The gels were fixed and stained with a fluorescent phosphoprotein that stains all phosphorylated proteins. Differentially phosphorylated proteins were located using a combination of ImageQuant and DeCyder softwares. The white box and x and y axis on each slide are for orientation purposes. Fifty one differentially phosphorylated proteins were identified and are circled and numbered. Twenty one protein spots were completely absent in *mc*²155: 5437 and were excised from the gel and identified by MALDI TOF/TOF (tandem mass spectrometry MS/MS). Identity of these spots is presented in Table 1.

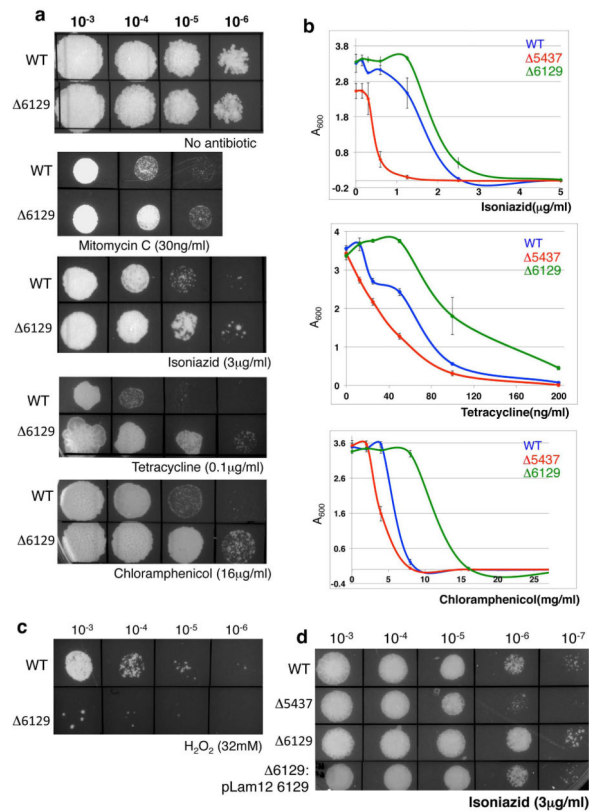


Figure 2. *M. smegmatis mc²155: 6129* is resistant to multiple antibiotics but hypersensitive to H₂O₂

(a) Ten fold serial dilutions of wild type and *mc²155: 6129* were spotted on Middlebrook 7H10 containing indicated concentrations of antibiotics. (b) Survival curves of wild type, *mc²155: 5437* and *mc²155: 6129* grown for 36 hours in liquid media containing the indicated antibiotics is shown in panels on the right. *mc²155: 6129* is more resistant to all the drugs tested as compared to the wildtype parent strain. Error bars represent standard deviation of means of three independent experiments. (c) *mc²155: 6129* shows almost 100% killing when exposed to 32mM H₂O₂ for 30 mins, a concentration where the viability of wildtype is unchanged. (d) Overexpression of MSMEG_6129 in wildtype cells makes the bacteria hypersensitive to isoniazid similar to *mc²155: 5437* whereas exogenous expression of MSMEG_6129 in *mc²155: 6129* restores isoniazid sensitivity to wild type levels.

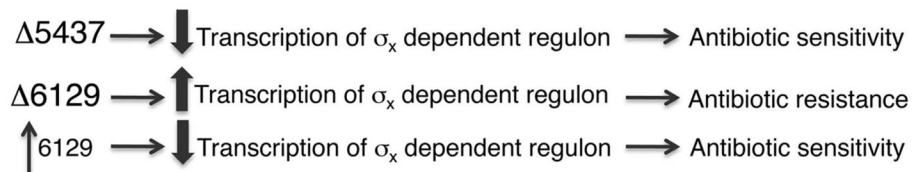
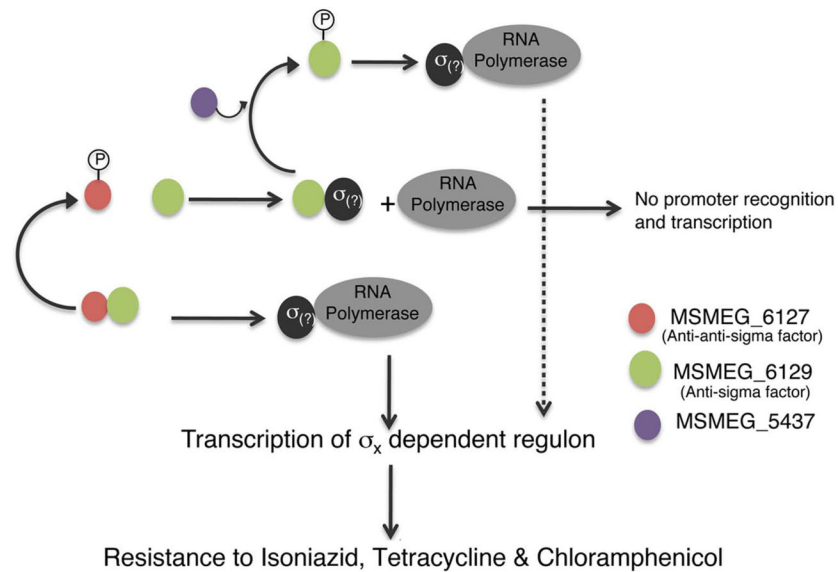


Figure 3. Proposed model showing the interaction of transcription regulatory factors in antibiotic resistance in *M. smegmatis*

We propose that MSMEG_6129 (green) regulates a hitherto unknown sigma factor required for transcription of antibiotic resistance genes. MSMEG_6129 is presumably under negative regulation by MSMEG_6127 (red) and MSMEG_5437 (purple).

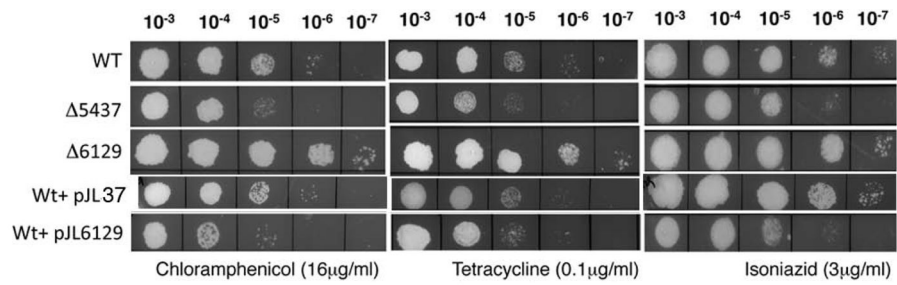


Figure 4. Overexpression of MSMEG_6129 in wild type mc^2155 results in antibiotic sensitivity Ten fold serial dilutions of wild type, $mc^2155: 5437$, $mc^2155: 6129$ and mc^2155+ pJL37MSMEG_6129 (a strain overexpressing MSMEG_6129) were spotted on Middlebrook 7H10 containing indicated concentrations of antibiotics. Overexpression of MSMEG_6129 results in antibiotic sensitivity to levels observed for $mc^2155: 5437$.

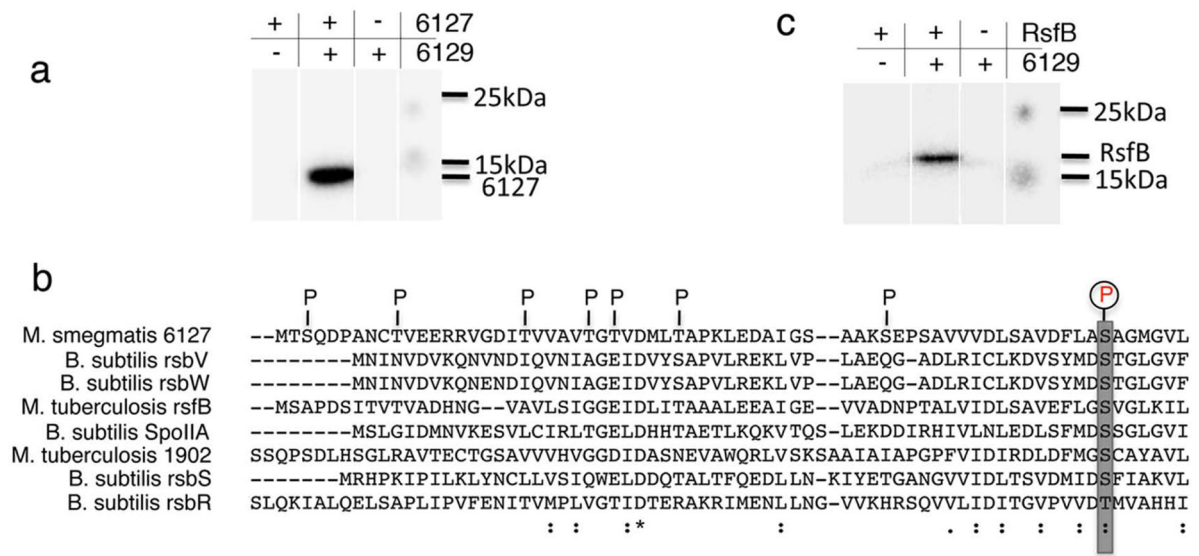


Figure 5. MSMEG_6129 phosphorylates MSMEG_6127 *in vitro*

(a) MSMEG_6127 and (b) *M. tuberculosis* RsfB purified from *E. coli* were incubated with purified MSMEG_6129 in kinase buffer containing 1 μ Ci of γ -P³² ATP at 25°C for 30 mins and separated on a 15% SDS-PAGE followed by autoradiography. The positions of MSMEG_6127 and RsfB are marked as well as the 15 kDa and 25 kDa standards. (c) The position of residues phosphorylated in MSMEG_6127 by MSMEG_6129 identified by MALDI TOF/TOF are indicated and include the consensus anti-sigma factor phosphorylation site at position S63.

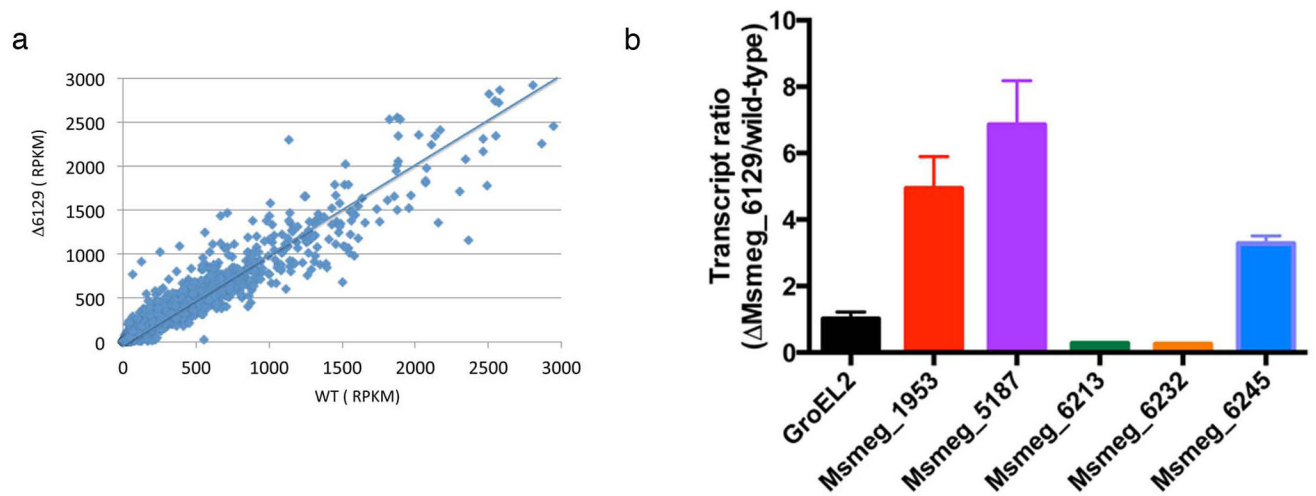


Figure 6. Deletion of MSMEG_6129 results in induction of antibiotic resistance genes (a) Scatter plot of mRNA levels of 6716 ORFs in *mc*²¹⁵⁵ and an isogenic mutant of MSMEG_6129. (b) The amount of transcript of MSMEG_1953, MSMEG_5187, MSMEG_6213, MSMEG_6232 and MSMEG_6245 in the wild type as well as *mc*²¹⁵⁵: 6129 was quantified using qPCR and expressed as a fold overexpression in *mc*²¹⁵⁵: 6129 over wild type. GroEL1 was used as an endogenous control and is shown to remain unchanged in the 2 strains. Error bars represent standard deviation of means of three independent experiments

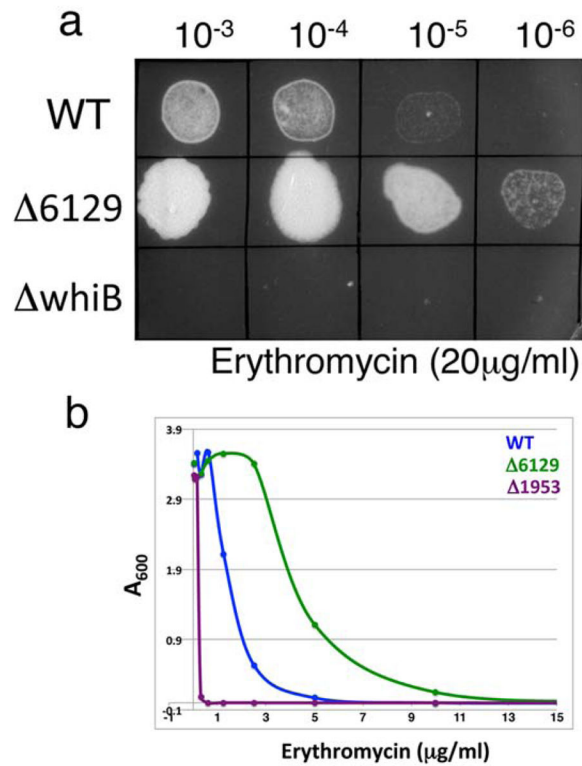


Figure 7. Drug sensitivity of *mc*²155: 6129 can be predicted from the known phenotype of *mc*²155: 1953

(a) Ten fold serial dilutions of wild type, *mc*²155: 6129 and *mc*²155: *whiB7* were spotted on Middlebrook 7H10 containing 20 μg/ml erythromycin. (b) Survival curves of wild type, *mc*²155: 5437 and *mc*²155: 6129 in liquid media containing the indicated concentration of erythromycin. *mc*²155: 6129 shows increased resistance to erythromycin when compared to the wildtype parent strain as predicted.

Table 1
Identification of phosphoproteins absent in *mc*²155: 5437 using MALDI-TOF

Total cell lysate was prepared from wild type *mc*²155 and *mc*²155: 5437 were separated by 2-D gel electrophoresis and the phosphorylated proteins identified by fluorescent staining. Differentially phosphorylated proteins were located using a combination of ImageQuant and DeCyder softwares. Of the 51 differentially phosphorylated proteins 21 protein spots were completely absent in *mc*²155: 5437 and were excised from the gel and identified by MALDI-TOF/TOF (tandem mass spectrometry MS/MS).

Spot #	<i>M. smegmatis</i> ID	<i>M. tuberculosis</i> ID	Protein ID
4	MSMEG_0059	Rv3868	ATPase, AAA family protein
9	MSMEG_1881	Rv3240c	preprotein translocase subunit SecA
10	MSMEG_6091	Rv3596c	negative regulator of genetic competence ClpC/mecB
16, 17	MSMEG_0456	Rv0006	DNA gyrase subunit A
15	MSMEG_0005	Rv0005	DNA gyrase subunit B
30	MSMEG_1670	Rv3318	succinate dehydrogenase flavoprotein subunit
33	MSMEG_6761	Rv3302c	glycerol-3-phosphate dehydrogenase 2
8	MSMEG_3642	Rv1832	glycine dehydrogenase
45, 46, 47	MSMEG_2080	Rv3140	putative acyl-CoA dehydrogenase
29	MSMEG_1807	Rv3285	acetyl-/propionyl-coenzyme A carboxylase alpha chain
7	MSMEG_2412	Rv2967c	pyruvate carboxylase
48	MSMEG_3046	Rv1383	carbamoyl phosphate synthase small subunit
49	MSMEG_0702	Rv0791c	monooxygenase
50	MSMEG_2351	Rv3029c	electron transfer flavoprotein, beta subunit
51	MSMEG_6427	Rv3846	[Mn] superoxide dismutase
52	MSMEG_6129	-	Putative anti-sigma factor
43	MSMEG_1401	Rv0685	elongation factor Tu
6	MSMEG_3102	Rv1448c	transaldolase

Table 2
Survival of wild type *mc*²155: 5437 and *mc*²155: 6129 in antibiotic containing liquid media

The Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) of Isoniazid, Tetracycline and Chloramphenicol are shown for wild type, *mc*²155: 5437 and *mc*²155: 6129.

	WT	MSMEG_5437	MSMEG_6129
Isoniazid	5	2.5	10
Tetracycline	100	75	200
Chloramphenicol	16	8	32

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Table 3
Deletion of MSMEG_6129 results in induction of antibiotic resistance genes

Total RNA was prepared from wild type and mc²155: 6129 strains followed by high throughput cDNA sequencing. The data is normalized to a million reads as well as for gene length for both strains (Reads per million bases per kilobase of ORF= RPKM) and expressed as a ratio of RPKM values of the wild type strain and the mutant. The 3 categories of proteins where the ratio of RPKM values of 6129 to the wild type strain is >2.5 and are immediately relevant to the observed drug resistance phenotype of mc²155: 6129 are shown. The results of RNA seq were verified using qPCR for the pertinent genes from the above three categories highlighted in grey.

Category	Gene ID	Fold change	Gene description
		in 6129	
Drug Resistance & ABC Transporters	MSMEG_5187	9	tetracycline-resistance determinant TetV
	MSMEG_5102	7.5	ABC transporter ATP-binding protein
	MSMEG_3203	4.3	transporter, LysE family
	MSMEG_5047	3.7	drug resistance transporter (Bcr/CfIA)
	MSMEG_0422	3.5	Transferase. Toxin production & resistance
	MSMEG_5659	3.1	ABC transporter, ATP-binding protein
	MSMEG_5660	3	ABC transporter ATP-binding protein
	MSMEG_6245	2.9	chloramphenicol resistance protein
	MSMEG_6595	2.6	ABC transporter complex
Transcription Regulators	MSMEG_1953	6.5	transcription factor WhiB7
	MSMEG_0051	6	transcription factor WhiB6 family
	MSMEG_1025	37	TetR-family transcriptional regulator
	MSMEG_1420	3.7	probable transcriptional regulatory protein
	MSMEG_5610	3.6	putative transcriptional regulator family
	MSMEG_2305	3.4	TetR-family transcriptional regulator
	MSMEG_6441	2.9	transcriptional regulator, TetR family
	MSMEG_0473	2.8	transcriptional regulator, LuxR family
	MSMEG_1117	2.8	transcriptional regulator
	MSMEG_1970	2.7	sigma factor
	MSMEG_5872	2.7	DNA-binding response regulator PhoP
	MSMEG_0545	2.6	transcriptional regulator, LuxR family
	MSMEG_6199	2.6	Transcription factor WhiB2
	MSMEG_2905	2.5	DNA binding transcription factor
Catalases	MSMEG_6213	-2.5	Manganese containing catalase
	MSMEG_6232	-2.6	catalase KatA

Table 4
Survival of wild type *mc*²¹⁵⁵ and *mc*^{2155: 1953} in antibiotic containing liquid media

The Minimum Inhibitory Concentration ($\mu\text{g/ml}$) of Isoniazid, Tetracycline and Chloramphenicol is shown for wild type, *mc*^{2155: 1953} and compared to known literature values for *M. tuberculosis*.

	<i>M. smegmatis</i>		<i>M. tuberculosis</i>	
	WT	MSMEG_1953	WT	whiB7
Isoniazid	5.0	5.8	n/a	n/a
Tetracycline	100	25	20	20
Chloramphenicol	16	5	8	4
Erythromycin	5	0.6	80	40
Spectinomycin	100	25	128	16

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