$iniBAC$ induction Is Vitamin B12- and MutAB-dependent in \textit{Mycobacterium marinum}*

Maikel Boot, Marion Sparrius, Kin Ki Jim, Susanna Commandeur, Alexander Speer, Robert van de Weerd, and Wilbert Bitter

From the Department of Medical Microbiology and Infection Control, VU University Medical Center, De Boelelaan 1108, 1081 HZ, Amsterdam, The Netherlands

Tuberculosis can be treated with a 6-month regimen of antibiotics. Although the targets of most of the first-line antibiotics have been identified, less research has focused on the intracellular stress responses that follow upon treatment with antibiotics. Studying the roles of these stress genes may lead to the identification of crucial stress-coping mechanisms that can provide additional drug targets to increase treatment efficacy. A three-gene operon with unknown function that is strongly up-regulated upon treatment with isoniazid and ethambutol is the $iniBAC$ operon. We have reproduced these findings and show that $iniBAC$ genes are also induced in infected host cells, with higher variability. Next, we set out to elucidate the genetic network that results in $iniBAC$ induction in \textit{Mycobacterium marinum}. By transposon mutagenesis, we identified that the operon is highly induced by mutations in genes encoding enzymes of the vitamin B12 biosynthesis pathway and the vitamin B12-dependent methylmalonyl-CoA-mutase MutAB. Lipid analysis showed that amutA::tn mutant has decreased phthiocerol dimycocerosates levels, suggesting a link between $iniBAC$ induction and the production of methyl-branched lipids. Moreover, a similar screen in \textit{Mycobacterium bovis} BCG identified that phthiocerol dimycocerosate biosynthesis mutants cause the up-regulation of $iniBAC$ genes. Based on these data, we propose that $iniBAC$ is induced in response to mutations that cause defects in the biosynthesis of methyl-branched lipids. The resulting metabolic stress caused by these mutations or caused by ethambutol or isoniazid treatment may be relieved by $iniBAC$ to increase the chance of bacterial survival.

\textit{Mycobacterium tuberculosis}, the causative agent of tuberculosis, is responsible for 1.5 million deaths annually, making it the most deadly bacterial pathogen known to mankind (1). Although tuberculosis can be treated effectively with a long term multidrug regimen, failure to complete treatment has led to increasing occurrence of drug resistance, which in turn complicates treatment (1, 2). Therefore, the need to develop new anti-mycobacterial compounds is evident. An attractive target for drug therapy is the mycobacterial cell wall, which is composed of non-canonical elements providing the opportunity to specifically target mycobacteria (3). In the past several antibiotics that target the mycobacterial cell wall have been identified and have proven to be effective in treatment of tuberculosis. Well-known examples of specific anti-cell wall antibiotics for mycobacteria are ethambutol (EMB)\(^2\) and isoniazid (INH). Ethambutol targets the polymerization of arabinan, an essential component of the mycobacterial cell wall involved in covalently linking the peptidoglycan layer to the outer membrane (4). The prodrug isoniazid is activated by the peroxidase KatG and subsequently forms adducts with NAD+ to inhibit InhA, a NADH-dependent enoyl-ACP (acyl carrier protein) reductase (5). As a consequence, biosynthesis of mycolic acids, the most abundant lipid of the mycobacterial outer membrane, is inhibited, causing an aberrant and lethal deformation of the cell wall (6). Recent research efforts have emphasized that also other essential steps in the biogenesis of the mycobacterial cell wall can serve as new targets for anti-tuberculosis interventions (7, 8).

Although our knowledge on cell wall biosynthesis in mycobacteria has greatly increased over the last decades, the bacterial stress responses that follow after treatment with antibiotics targeting the mycobacterial cell wall have yet to be elucidated. By mapping the intra-bacterial responses upon treatment with antibiotics, key pathways that are necessary to cope with antibiotics or with antibiotic-induced stress can be identified. Moreover, mapping bacterial stress levels may help to explain the observed heterogeneity, displayed by bacterial subpopulations, to antibiotic treatment within patients (9). Together, these findings might pave the way for novel drug targets that sensitize or enhance currently used antibiotics.

Despite the availability of gene expression data after exposure of mycobacteria to anti-tuberculosis drugs, there is no comprehensive map of the stress response pathways that follow upon treatment. Moreover, antibiotic stress regulation pathways were not addressed in general. Insights into the pathways that mycobacteria deploy when confronted with antibiotic treatment will lead to a better understanding of the bacterial adaptive strategies and may consequently pinpoint drug-induced blind spots that can be targeted by novel compounds. Some individual genes or gene clusters that are regulated in

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1 To whom correspondence should be addressed. Tel.: 31-20-4448319; E-mail: w.bitter@vumc.nl.

2 The abbreviations used are: EMB, ethambutol; INH, isoniazid; CLC, carp leukocyte cell; PDIM, phthiocerol dimycocerosate; MIC, minimal inhibitory concentration; hpi, hours post infection; DLP, dynamin-like protein.
response to antibiotic treatment have been well characterized. For instance, the acyl carrier protein kasA has been found to be up-regulated in response to isoniazid (10). Another set of genes that was found to be highly induced by sublethal concentrations of ethambutol and isoniazid in *M. tuberculosis* and *Mycobacterium bovis* BCG is the iniBAC operon (11, 12). This operon was found to be induced specifically by these two cell wall targeting antibiotics but not by general cell wall stress caused by disruption of the membrane integrity through the activity of granulysin or lysozyme (12). Further studies by the same group showed that overexpression of iniA seemed to confer tolerance to INH treatment in *M. bovis* BCG but not in *M. tuberculosis* (13). This suggests a function for iniA in coping with the stress that is induced by INH or EMB treatment in *M. bovis* BCG. Despite these early studies, not much is known about the pathways that lead to induction of these three genes with unknown function.

In *Escherichia coli*, disruption of cell wall biosynthesis can be sensed by extracytoplasmic function sigma factor E (14). Similarly, mycobacterial sigE has been shown to respond to heat-shock and oxidative stresses (15). Although sigE is not a specific sensor for cell wall stress, other sigma factors may play a role in the stress network that is activated upon iniBAC induction. A few publications have addressed the regulation of the iniBAC operon. Colangeli et al. (16) have shown that lsr2, among regulating a large set of other genes, negatively regulates iniBAC expression, whereas recent studies have also implicated operon regulation by mtrA and the alleged INH binding regulation element inbr (17, 18).

In this study we aimed to elucidate the genetic network that revolves around the induction of the iniBAC operon. We found that the operon can be induced by mutations in vitamin B12 biosynthesis or the vitamin B12-dependent methylmalonyl-CoA mutase MutAB in *Mycobacterium marinum*. Mutants in *mutAB* show decreased phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL) levels but no altered antibiotic susceptibility.

**Results**

The iniBAC Operon Is Induced upon EMB and INH Treatment in *M. marinum*—To investigate the response of the iniBAC operon to stress, we generated a reporter gene construct by placing the gene encoding green fluorescent protein mEos3.1 under control of the *M. marinum* iniB promoter region, optimized as previously reported for *M. marinum* (12). The reporter construct was transformed into *M. marinum* cells, and activity was assessed using the inducing antibiotics INH or EMB. The initial induction experiments were carried out by the addition of the antibiotics EMB or INH at their minimal inhibitory concentration (MIC) followed by flow cytometry analysis. Starting from day 1, we observed a strong induction of the iniBAC reporter construct after the addition of the antibiotic, as compared with the non-treated *M. marinum* cells; fluorescence was 114-fold (EMB) and 121-fold (INH) higher. The induction increased over time and reached a 357-fold difference by day 5 for EMB and 643-fold for INH (Fig. 1A).

Next, we tested reporter activity in cell infection experiments. Carp leukocyte cells (CLC) and THP-1 macrophages were infected with *M. marinum* harboring the iniBAC reporter construct, and analysis of induction kinetics was tracked by flow cytometry. To differentiate between infected and non-infected macrophages and to assess which proportion of the infected cells expressed the iniBAC reporter construct, the red fluorescent protein crimson E2 was constitutively expressed on an episomal construct. We combined this with an integrative version of the stress marker containing the iniB reporter and mEos3.1. As such we could determine which proportion of the total pool of infected, crimson-positive cells was also iniBAC-induced and, thus, mEos3.1 positive. After infection, the phagocytized bacteria were challenged with $1 \times {\text{MIC}}$ of EMB or INH, and flow cytometry was used to measure induction of the iniBAC reporter on days 0, 1, 2, and 3. Fig. 1B shows a representative measurement of an experiment performed in triplicate. In THP-1 cells the population of macrophages that contained bacteria with induced iniBAC increased to 41.6% (S.D. 0.4%) on day 3 for ethambutol. Isoniazid showed a faster induction pattern in THP-1 cells with 13.4% (S.D. 0.4%) becoming mEos3.1-positive on day 1, increasing to 38.6% (S.D. 0.7%) on day 3 (Fig. 1, B and C). In CLC infection assays, similar patterns were observed (Fig. 1, B and C).

In summary, the induction of the iniBAC reporter during intracellular infection seems to increase over time but does not seem to affect all bacteria, which was observed in culture. THP-1 cells were also analyzed by confocal imaging on days 1 and 2 (Fig. 2). Confocal imaging confirmed the observations of flow cytometry assays. Moreover, the EMB- and INH-treated macrophages showed a clear pattern of heterogeneity from day 2 onward, coinciding with the significant increase in induction seen by flow cytometry for both antibiotics.

In conclusion, we have confirmed that *M. marinum* expresses iniBAC upon treatment with EMB and INH. Furthermore, this induction is observed in culture and in two different cell types using a cell infection assay. However, induction is more variable during cell infection as compared with culture.

**Mutations in Vitamin B12 Biosynthesis and the Vitamin B12-dependent mutAB Cause Up-regulation of iniBAC in *M. marinum*—**Next we set out to probe which genes and regulation networks play a role in iniBAC induction. For this, a wild type *M. marinum* M^LUS^A containing an exosomal variant of the iniBAC stress marker was used as a parent strain to create a transposon library. The resulting mutants were plated out and selected for high fluorescence. Analysis of the transposon insertion site was performed for a total of 50 positive mutants. Strikingly, 32 of the 50 transposons insertions were identified in genes that are responsible for cobalamin synthesis, the so-called cob family genes (Fig. 3A). These genes are part of the set of 25 genes that are required for vitamin B12 biosynthesis in mycobacteria (19). Furthermore, 12 mutants were identified in methylmalonyl-CoA-mutase encoding genes MutAB or in the gene coding for its alleged foldase, meaB (20). The MutAB enzyme plays a role in propionate metabolism, converting (S)-methylmalonyl-CoA, a precursor for methyl-branched lipids, to (R)-methylmalonyl-CoA, which is further processed to succinyl-CoA to enter the tricarboxylic acid or glyoxylate cycle (Fig. 3B). The methyl-branched lipids synthesized from (S)-methylmalonyl-CoA include PDIM, diacyl trehalose/pentaacyl trehalose, and SL-1 (21). MutAB is vitamin B12-depen-
dent, suggesting that the mutants affected in vitamin B12 biosynthesis were most likely identified because of their downstream effect on MutAB. Besides identification of most known cobalamin biosynthesis genes, this screen also confirms that MMAR_4563, is probably coding for the vitamin B12 biosynthesis enzyme CobF (data not shown), and MaeB is indeed functionally linked to MutAB. Other mutants that showed induction, albeit to a lesser extent, include mutations in \( \text{pks16}, \text{mshD}, \text{or mas} \), genes that are all involved in cell wall lipid biosynthesis (Table 1).

**FIGURE 1.** The in\( \text{iBAC} \) operon is induced upon treatment with EMB and INH both in culture and in \( \text{vitro} \). A, flow cytometry plots of untreated cultures (black line) compared with cultures treated with ethambutol (red line) and isoniazid (blue line) with the MIC concentration. For each condition 30,000 events are plotted, mean fluorescence intensity is shown on the \( x \) axis per the amount of cells (\( y \) axis). Induction folds are indicated per day and calculated as treated sample mean fluorescence intensity divided by untreated sample mean fluorescence intensity. B, plots of induction patterns in THP-1 and CLC cells on day 3 after infection. EMB and INH were added 3 hpi. C, induction over time in THP-1 cells and CLC cells, plotted as the percentage of cells that fall into the “mEos3.1 positive” gate. The graph shows the average percentage of induction of three independent experiments. The S.D. values were too small to be displayed. \( \text{RFI} \), relative fluorescence intensity.

**iniBAC Induction in Vitamin B12 Biosynthesis Mutants Can Be Rescued by Exogenous Vitamin B12**—To confirm that the effects seen on the induction of the in\( \text{iBAC} \) stress marker were due to abrogated vitamin B12 biosynthesis, we compared the induction of three mutants, \( \text{mutA::tn, mshD::tn, and cobN::tn.} \) Without the addition of vitamin B12, both \( \text{mutA::tn} \) and \( \text{cobN::tn} \) showed clear expression of mCherry (Fig. 4A). As expected, the \( \text{cobN} \) transposon mutant clearly reverts from high in\( \text{iBAC} \) induction to a near background expression level by the addition of 10 \( \mu \text{g/ml} \) vitamin B12 (Fig. 4B). We also tested
which concentration of vitamin B12 was required to phenotypically complement the cob mutants. Lowering the concentration to $\leq 100$ ng/ml vitamin B12 abrogated complementation, which was also confirmed by flow cytometry (data not shown). Because usually only trace amounts of vitamin B12 are required, this means that *M. marinum* does not efficiently transport vitamin B12 under these conditions. The other two mutants, *mutA*::tn and *mshD*::tn, were unaffected by the addition of vitamin B12, as confirmed by fluorescence microscopy (data not shown).

Besides complementation with vitamin B12, a genetic complementation was also performed for three mutants, i.e. *cobB*::tn, *mshD*::tn, and *mutA*::tn. The resulting complementing strains were analyzed by flow cytometry for fluorescence induction of the stress marker as compared with their mutant parent strain. This confirmed that complementation of *cobB*::tn and *mshD*::tn was achieved by introducing an intact copy of the respective gene (Fig. 4C). The *mutA*::tn mutant did not complement when only *mutA* was reintroduced in the mutant strain, suggesting that the transposon mutant we obtained in *mutA* likely also disturbs expression of the downstream genes *mutB* and *MMAR_2304* (*meaB* orthologue). Complementation was achieved when we introduced *mutA*, *mutB*, and *MMAR_2304*, as fluorescence induction was restored to wild type levels.

Addition of Acetic Acid or Propionic Acid to Transposon Mutants in *mutA* or *cobB* Does Not Affect Viability or iniBAC Induction—To assess whether we could find the metabolic cue that induces iniBAC, we performed an experiment on HdB minimal medium plates containing only acetic acid, propionic

![Diagram of METABOLIC PATHWAY](image)

**FIGURE 2.** Induction of iniBAC in THP-1 macrophages reveals heterogeneity. A, THP-1 cells 1 day after infection without antibiotics (−), treated with 1 μg/ml EMB or with 10 μg/ml INH. In each panel a representation of three images is shown, where the THP-1 nucleus is stained with DAPI (blue), and *M. marinum* constitutively expresses CrimsonE2 (red) and conditionally expresses mEos3.1 as a measure for iniBAC induction (green). B, same layout as in A but for day 2 after infection of THP-1 cells.

**FIGURE 3.** Mutants that show a strong up-regulation of iniBAC are in the vitamin B12 biosynthesis pathway or mutAB-associated. A, a pie chart depicts the distribution of mutants that were found in the transposon mutagenesis screen. In total, 50 mutants were analyzed genetically. B, schematic overview of the propionate degradation pathway adjusted from (20). PCC, propionyl-CoA carboxylase; MMCE, methylmalonyl-CoA epimerase; SCS, succinate synthase; MCS, methyl citrate synthase; MCD, methyl citrate dehydratase; AOX, aconitase; MCL, 2-methylisocitrate lyase; PDHC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid.
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Table 1
List of M. marinum mutants identified in transposon mutagenesis screen

This table contains an overview of the unique M. marinum mutants that were identified in this study. The H37Rv orthologue, if present, the gene length and the number of unique mutants per gene are indicated.

| Gene ID     | Gene product      | H37Rv orthologue | Gene length | Transposon position(s) within gene (bp from 5' of gene) |
|-------------|-------------------|------------------|-------------|-------------------------------------------------------|
| MMAR_0989  | PE_PGRS           |                 | 3153        | 2149                                                  |
| MMAR_1350  | tetR repressor    | Rv3208           | 684         | 421                                                   |
| MMAR_1768  | mas               | Rv2940c          | 5619        | 1173                                                  |
| MMAR_1885  | cobB              | Rv29848          | 1377        | 148, 194, 416, 429, 597, 610, 905, 1271, 1290, 1293, 1324 |
| MMAR_2302  | mutA              | Rv1492           | 1878        | 14, 225, 427, 1836                                    |
| MMAR_2303  | mutB              | Rv1493           | 2262        | 6, 41, 521, 1095, 1724, 2179                           |
| MMAR_2304  | meaB              | Rv1496           | 1011        | 204, 379                                              |
| MMAR_3037  | cobN              | Rv2062c          | 1357        | 76, 129, 311, 1486, 3374, 3394                         |
| MMAR_3045  | cobH              | Rv2065           | 627         | 123                                                   |
| MMAR_3046  | cobI              | Rv2066           | 1470        | 256                                                   |
| MMAR_3052  | cobk              | Rv2070c          | 729         | 11                                                    |
| MMAR_3053  | cobM              | Rv2071c          | 740         | 715                                                   |
| MMAR_3054  | cobL              | Rv2072c          | 1173        | 1093                                                  |
| MMAR_3251  | cobU              | Rv2054c          | 561         | 75                                                    |
| MMAR_3252  | cobT              | Rv2207           | 1077        | 525                                                   |
| MMAR_3307  | cobC              | Rv2231c          | 1053        | 273                                                   |
| MMAR_3311  | cobD              | Rv2236c          | 942         | 792                                                   |
| MMAR_4476  | pks16             | Rv1013           | 1635        | 652                                                   |
| MMAR_4563  | cobF              | none             | 744         | 30, 134, 155, 232, 610                                |
| MMAR_4863  | mshD              | Rv0819           | 963         | 469, 526                                               |

Figure 4. M. marinum deficient in vitamin B12 production can be complemented by the addition of exogenous vitamin B12 in vitro. A, mutants mutA::tn, mshD::tn, and cobN::tn compared with a WT M. marinum containing the pSMT3-IniB-mCherry and WT M. marinum without the addition of vitamin B12 on the plate. B, the same mutations when exposed to 10 μg/ml vitamin B12 (cyanocobalamin). Genetic complementation (C) was also performed by introduction of an integrative vector containing an intact copy of the mutated genes. C, the resulting strains were analyzed in triplicate by flow cytometry and are compared in mean fluorescence induction (MFI) to their mutant counterparts.

In summary, we show that IniBAC induction is declining in vivo, with virtually no induction after 120 h. The decrease in induction was seen for all three mutants, indicating that the induction has not changed in this period of time (Fig. 6), which is not unexpected because the bacteria were already highly expressing mCherry and the protein is relatively stable. However, after 120 h post infection (hpi), a clear reduction of mCherry was observed in all three mutants, whereas the green fluorescent signal, i.e. mEos3.1, was still present or even increased.

In summary, we show that iniBAC induction is declining in vivo, with virtually no induction after 120 h. The decrease in induction was seen for all tested mutants and, therefore, seems to be independent of vitamin B12 availability.

Mutants in mutA and cobB have decreased levels of methyl-branched lipids PDIM and PGL—Because MutAB mediates the isomerization of (R)-methyl-malonyl-CoA to succinyl-CoA, we hypothesized that a mutA transposon mutant could have an excess amount of (R)-methylmalonyl-CoA. Consequently, this could influence the amount of methyl-branched lipids that are synthesized, such as PGLs/PDIMs (Fig. 3B). To...
FIGURE 5. The addition of acetic acid, propionic acid, and valeric acid to M. marinum WT and cobB::tn and mutA::tn mutants shows no obvious effect on bacterial viability. A, the left row indicates the bacterial fold dilution, and below each panel the corresponding carbon source and concentration are denoted. B, flow cytometry was performed to quantify the induction on glucose, acetic acid, and propionic acid.

FIGURE 6. iniBAC induction of transposon mutants in mutA, cobN, and mshD is transient and decreases over time. Confocal images of zebrafish embryos infected with WT M. marinum MUSA, mutA::tn, cobN::tn, or mshD::tn containing a plasmid pmv-hsp60-mEos3.1 and piniB-mCherry. Zebrafish were injected in the hindbrain ventricle and fixed in PFA. The top panel shows an fluorescence induction overlay of mCherry (stress marker) and mEos3.1 (total infection) for 24 hpi, whereas the lower panel shows this for time point 120 hpi.
assess whether this was the case, we performed TLC analysis to detect both PDIM and PGL variants for WT MUSA and transposon mutants in genes mas, mutA, and cobB. Strikingly, when comparing the amount of PDIMs of the mutA and cobB transposon mutants, a significant reduction in PDIM types A, B, and C can be distinguished (Fig. 7A). This reduction is phenotypically similar to a mas transposon mutant, which is known to be strongly affected in PDIM production (22). Moreover, PGL production was also severely decreased in mutA::tn and cobB::tn compared with WT, whereas the mas mutant, as expected, showed a null phenotype for this lipid (Fig. 7B). In conclusion, abrogation of vitamin B12 biosynthesis or mutation of mutAB caused a decrease in methyl-branched lipids PGL and PDIM in M. marinum.

The Antibiotic Susceptibility of mutAB and cobB Mutants to First-line Antibiotics Is Unchanged—To see whether the mutations that induce iniBAC are also changed in antibiotic susceptibility, we determined the MIC values of mutA::tn, mutB::tn, mshD::tn, and cobB::tn upon exposure to five first-line antibiotics. There were no differences observed for the four mutants tested, indicating that, although differences in PDIM and PGL can be seen for mutA::tn and cobB::tn, this does not seem to affect antibiotic susceptibility (Table 2). This finding seems to contradict the previously published results by Colangeli et al. (13), where they stated that up-regulation of iniA in M. tuberculosis caused a moderate but significantly increased resistance phenotype against ethambutol.

The Induction of iniBAC Is Not Caused by General Cell Wall Stress—The array of mutants that show an increased induction of the iniBAC operon seem to revolve around methyl-branched lipid biosynthesis. To see whether a defect in lipid biosynthesis and cell envelope integrity in general would also cause induction of the operon, we transformed a kasB::tn mutant, a mutant known to produce truncated mycolic acids, with the iniBAC stress marker (23). This mutant shows reduced growth and is attenuated in zebrafish embryos. Comparison of induction on plate to a mutA::tn and mshD::tn mutant revealed that the kasB::tn mutant does not seem to be highly induced on plate (data not shown).

Subsequent analysis by flow cytometry revealed that the iniBAC operon is indeed only 1.4-fold induced in this mutant, comparable with a mshD::tn mutant, whereas a mutA transposon mutant showed a 14.2-fold induction compared with a WT expressing the stress marker (Fig. 8). Therefore, not all genes that cause cell wall alterations contribute equally to the induction of the iniBAC operon.

Mutants in PDIM Biosynthesis Operon Show Induction of iniBAC in M. bovis BCG—Although most genes coding for vitamin B12 biosynthesis enzymes are present in species belonging to the M. tuberculosis complex, research by Warner et al. (24) has shown that M. tuberculosis does not seem produce vitamin B12 in vitro. This bacterium, therefore, seems to depend on the import of exogenous vitamin B12 (24, 25).

Because of this difference, we wondered what genes would affect iniBAC induction in these species. To explore this, we created a transposon mutant library in an M. bovis BCG Copenhagen strain containing the iniBAC stress reporter. In total, we identified 29 mutants that showed a high induction of the stress reporter. Genetic analysis of these mutants showed that we did not obtain any transposon insertion in genes involved in the vitamin B12 biosynthesis pathway, as expected. Most of these mutants were affected in the PDIM biosynthesis operon, including tesA, ppsB-E, papA5, and mas, similar to what had been found for M. marinum. A list of the unique mutants identified in this screen can be found in Table 3.

To determine whether these mutations had an effect on bacterial viability, MIC values for five first-line antibiotics were
TABLE 2

MIC determination on M. marinum mutants shows no change in antibiotic susceptibility

Measurements were performed in triplicate. The concentration at which no visible growth was seen is denoted as the MIC value in μg/ml. For the triplicate measurements, three identical values were found. As such, the S.D. is zero and, therefore, not written behind the values in the table.

| Strain       | Rifampicin | Ethambutol | Isoniazid | Streptomycin | Ciprofloxacin |
|--------------|------------|------------|-----------|--------------|---------------|
| WT           | 0.32       | 1.6        | 32        | 4            | 4             |
| mutA::tn     | 0.32       | 0.8        | 32        | 4            | 4             |
| mutB::tn     | 0.32       | 0.8        | 32        | 4            | 4             |
| cobB::tn     | 0.32       | 0.8        | 32        | 4            | 4             |
| mshD::tn     | 0.32       | 0.8        | 32        | 4            | 4             |

FIGURE 8. A flow cytometry plot analyzing the iniBAC expression of the transposon mutants in kasB (in blue), mshD (in green), and mutA (in red) versus a WT M. marinum (in black) harboring the stress marker construct with mCherry.

determined for the M. bovis BCG transposon mutants papA5::tn and mas::tn. Both mutants were compared with their WT parent strain harboring the stress marker. No differences of >2-fold were observed when comparing mutant to parent, again indicating that there is no correlation between iniBAC up-regulation and antibiotic susceptibility (Table 4), as was also observed in M. marinum. Together, these data show that mutants in two different mycobacterial species mutations or changes in PDIM biosynthesis induce the iniBAC operon, further supporting the link between iniBAC induction and the biosynthesis of methyl-branched lipids.

Discussion

In this work we show that the induction of the iniBAC operon as a result of EMB or INH treatment takes place both in cultured M. marinum and in intracellular bacteria, confirming previous observations of iniBAC induction in Mycobacterium smegmatis and M. bovis BCG by Alland et al. (12). We also show that the induction seems to be heterogeneous within THP-1 macrophages. Heterogeneity upon antibiotic treatment is a phenomenon that is well known for mycobacteria, and this phenotype plays an important role in patient treatment outcomes (9). Therefore, the observed variation in iniBAC induction underlines the importance of studying antibiotic response networks.

Previously, induction of the iniBAC operon was defined as a general cell wall stress operon that was induced upon the addition of cell wall biogenesis antibiotics EMB and INH (12, 13). In this study we show that the induction of iniBAC seems to be more specific and linked to the biosynthesis of methyl-branched lipids. M. marinum transposon mutants in mutAB or cob genes responsible for vitamin B12 biosynthesis caused strong induction of iniBAC. Moreover, these strains showed a strong decrease in the amount of PDIM and PGL lipids but no altered antibiotic susceptibility. Interestingly, in M. bovis BCG, mutations in the PDIM biosynthesis operon cause induction of the iniBAC operon and, therefore, point to a similar induction signal as the PDIM-deprived M. marinum transposon mutants.

One theory that could explain why the iniBAC operon is highly induced in both species is that one of the lipid precursors (S)-methylmalonyl-CoA, (R)-methylmalonyl-CoA, or propionyl-CoA accumulates. Mycobacteria are highly sensitive to increases in the pool of intermediates of fatty acids but also propionyl-CoA and propionyl-CoA itself. High levels of propionyl-CoA have been found to be toxic unless detoxified by the methyl citrate cycle (26). Because the methyl citrate cycle is intact in the iniBAC-inducing mutants, the accumulation of (S)-methylmalonyl-CoA or (R)-methylmalonyl seem to be the most likely accumulating candidates. In M. marinum, prevention of conversion of (R)-methylmalonyl-CoA to succinyl-CoA causes induction of the iniBAC operon, whereas M. bovis BCG probably does not have MutAB activity in culture due to an incomplete vitamin B12 biosynthesis pathway. Subsequently, M. marinum shows lowered PDIM levels upon mutations in the methyl-malonyl pathway, and M. bovis BCG shows induction of the operon when PDIM biosynthesis is disturbed, possibly leading to accumulation of a toxic intermediate. When we assessed growth of WT M. marinum and its isogenic cobB::tn and mutA::tn mutants on minimal medium, there was no clear difference in growth or iniBAC induction for any of the carbon sources used. This suggests that metabolites of the different pathways indicated in Fig. 3 do not seem to play a direct role in iniBAC stress.

Another hypothesis is that a misbalance occurs between the methyl-malonyl pathway because of disturbance of outflow due to mutations in PDIM biosynthesis or blocking of MutAB and the methyl citrate cycle. This is supported by work from Lee et al. (27), where it was found that the relative abundance of acetyl-CoA determined the toxicity of propionyl-CoA or its three-carbon intermediates. Because in M. marinum both pathways are constitutively active in vitro, a blockade can lead to an overflow of the methyl-citrate cycle and may cause iniBAC induction. The iniBAC operon might be involved in rescuing the bacterium from these otherwise toxic intermediates. It seems that the outflow of possibly accumulated (S)-methylmalonyl-CoA and (R)-methylmalonyl-CoA is not simply managed by conversion to methyl-branched lipids. As we did not assess the relative balance of these two intermediates, it might be worthwhile to investigate the relative abundance of acetyl-CoA and propionyl-CoA by metabolomics. Surprisingly, the
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**TABLE 3**

List of M. bovis BCG mutants identified in transposon mutagenesis screen

| Gene ID | Gene product | H37Rv orthologue | Gene length | Transposon position(s) within gene |
|---------|--------------|------------------|-------------|----------------------------------|
| Mb0016c | phbA         | Rv0016c          | 1476        | 252, 652, 995                    |
| Mb0673  | Carotenoid oxygenase | Rv0654         | 1506        | 178                              |
| Mb1041  | pks16        | Rv1013           | 1635        | 538                              |
| Mb1069c | PE protein   | Rv1040c          | 828         | 260                              |
| Mb1145c | NFS transporter | Rv1140c         | 1557        | 661                              |
| Mb1951c | PPE34        | Rv1917           | 4380        | 942                              |
| Mb2370  | lppQ         | Rv2341           | 420         | 8                                |
| Mb2720c | suhB         | Rv2701c          | 873         | 340                              |
| Mb2953  | tesA         | Rv2938           | 786         | 96                               |
| Mb2956  | ppsB         | Rv2932           | 4617        | 330, 1385, 3528                  |
| Mb2958  | ppsC         | Rv2933           | 6567        | 2764, 3227                      |
| Mb2959  | ppsD         | Rv2934           | 5484        | 244, 903, 1370, 5342             |
| Mb2960  | ppsE         | Rv2935           | 4467        | 1370, 4172                      |
| Mb2964  | papA5        | Rv2939           | 1269        | 99, 361                          |
| Mb2965c | mas          | Rv2940c          | 6336        | 778, 1400, 3259                  |
| Mb3024  | lppY         | Rv2999           | 966         | 877                              |

**TABLE 4**

MIC values for the M. bovis BCG mutants

| Strain   | Rifampicin | Ethambutol | Isoniazid | Streptomycin | Ciprofloxacin |
|----------|------------|------------|-----------|--------------|---------------|
| WT-B     | 0.0008     | 2          | 0.5       | 0.45         | 0.25          |
| papA5::tn| 0.0016     | 2          | 0.5       | 0.27         | 0.25          |
| WT-B4    | 0.01       | 2          | 0.5       | 0.45         | 0.25          |
| mas::tn  | 0.005      | 2          | 0.5       | 0.45         | 0.25          |

**M. marinum** mutAB mutants have lower PGL and PDIM levels, confirming research by Jain et al. (28) who showed that a mutAB overexpression strain has increased PDIM levels. Therefore, a regulation pathway may be in place that reduces the methymalonyl pathway when toxic metabolites in this cycle reach a certain threshold. Alternatively, another unidentified outflow of these metabolites is present, as some of these enzymes in the pathway are duplicated in M. marinum.

The function of the inibAC operon has been previously addressed by Colangeli et al. (13). They predicted that IniA might serve as an efflux pump that leads to tolerance to EMB and INH treatment (13). We also tested this hypothesis using our overexpression mutants. In our system inibAC overexpression took place under more natural conditions and, therefore, possibly included overexpression of accessory genes that have not been identified yet. Based on the antibiotic susceptibility assays with high expressing inibAC mutants in M. marinum and M. bovis BCG, we notice that induction of the operon did not alter antibiotic susceptibility. Our results, therefore, do not support the hypothesis that this operon forms an efflux pump that influences bacterial resistance to EMB and INH.

In line with this, sequence analysis shows that both iniA and iniC contain a GTPase domain that shows a high similarity to dynamin family proteins. This suggests that these proteins might have a function that allows them to vesiculate possible toxic intermediates. Bacterial dynamin-like proteins (DLPs) have first been discovered in a cyanobacterium, *Nostoc punctiforme* (29). It was shown that an N. punctiforme DLP could form tubulations in lipid bilayers and facilitate both fission and fusion of vesicles in a GTP-dependent manner (30). More recently, *E. coli* has been shown to contain a triplet of DLPs, *leoABC*, that mediate toxin release via membrane vesicles. It is worth noting that bacterial DLPs are often found in tandem, like *E. coli* leoA and leoC, and mycobacterial iniA and iniC (31). It is tempting to hypothesize that the inibAC genes are dynamin-like proteins that allow vesiculation of toxic molecules in mycobacteria, and this is definitely something that future research can address. Recently, it has been suggested that DLP of *Bacillus subtilis* is involved in membrane stress (32). Perhaps overproduction of some mycobacterial lipids, such as PDIM, could also trigger membrane stress.

In this work we established that *M. marinum* produces vitamin B12 in vitro and that MMAR_4563 is indeed a functional gene in this biosynthesis route, as previously suggested by Weerdenburg et al. (33). Therefore, we propose to call this gene cobF. The vitamin B12 mutants that we identified seem to have reduced PDIM levels. Although we did not confirm reversion of PDIM levels upon vitamin B12 supplementation by TLC, we assume that this is the case. Because PDIMs are important for virulence in mycobacteria (34), this may suggest a direct link between vitamin B12 biosynthesis, cell homeostasis, and virulence. Evidence for the effect of vitamin B12 homeostasis and virulence was presented by Weerdenburg et al. (33), where it was shown that mutations in the vitamin B12 biosynthesis genes caused an increased fitness for *M. marinum* in *Acanthamoeba* infection experiments. Although there are two enzymes in *M. marinum* that need vitamin B12 as a cofactor, methionine synthase MetH and methymalonyl-CoA mutase MutAB (24, 25), based on our results the absence of MutAB could explain the observed virulence effects because the mycobacterial lipid profile is altered. In this paper and a previous study by Gopinath et al. (25), it was shown that mycobacteria
are able to take up vitamin B12 in vitro. Because vitamin B12 is present in the host (35), there is a reservoir for vitamin B12 that can be scavenged during infection, thereby altering lipid expression.

Although our in vivo experiments show that iniBAC induction is absent after 120 hpi independent of vitamin B12 availability, we cannot rule out that vitamin B12 plays a role in vivo. In light of this we find it interesting that both mutA*:tn and cobN*:tn mutants showed a significantly milder infection phenotype than mshD*:tn and WT M. marinum, indicating that abrogation of the MutAB pathway or vitamin B12 biosynthesis may influence the outcome of infection.

Future experiments should be performed to elucidate the role of the iniBAC genes in EMB and INH-induced stress. Most importantly, the possibility that iniBAC plays a role in coping with toxic intermediates may very well lead to the identification of novel therapeutic targets that allow potentiation of current mycobacterial drug therapy.

Experimental Procedures

Bacterial Strains and Culturing Conditions—E. coli strain DH5α was used to for cloning and propagation of plasmid DNA. Cultures were grown at 37 °C in LB broth with the addition of antibiotics hygromycin B (50 μg/ml), kanamycin (25 μg/ml), or streptomycin (30 μg/ml) where necessary.

*M. marinum* wild type M*USA* (36) was used as a basis for mycobacterial experiments, and mutants were derived from this parent strain. *M. marinum* M*USA* and *M. bovis* BCG Copenhagen were used for transposon mutagenesis experiments. Cultures were grown in Middlebrook 7H9 liquid medium supplemented with Middlebrook ADC and 0.05% Tween 80 with the addition of the aforementioned antibiotic concentrations when required. Additional antibiotics isoniazid (Sigma) and ethambutol (Sigma) were routinely added to log-phase cultures at the indicated final concentrations. Cyanocobalamin (Sigma) was added where indicated at a concentration of 10 μg/ml. Middlebrook 7H10 solid agar supplemented with Middlebrook OADC was used for bacterial plating. Both cultures and plates were grown at 30 °C. Sauton plates were prepared according to a protocol published by D. W. Allen (37). Human monocytic cell line THP-1 and carp leukocyte cell line CLC were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) and Glutamax (Gibco). THP-1 cells were grown in 37 °C with 5% CO₂, whereas CLCs were grown at 30 °C with 5% CO₂.

Construction of Plasmids—All plasmids used in this study are listed in Table 5, whereas all primers used in this study can be found in Table 6. Anchored primers containing either an Xbal site or BamHI site were used to amplify a 588-bp fragment that included the iniB promoter from *M. marinum* M*USA* genomic DNA. The resulting product and a PAL5000 derivative containing pSTM3-hsp60-mCherry (38) were both digested with Xbal and BamHI, mixed, and ligated, resulting in pSTM3-pheniB- mCherry. A 3' promoter truncation was created as described by Alland et al. (12) by modeling the *M. bovis* BCG promoter sequence onto the *M. marinum* M*USA* promoter sequence. Anchored primers containing an Xbal site for the 5' primer and BamHI site for the 3' primer were used to amplify a 211-bp fragment from *M. marinum* M*USA* genomic DNA. The insert and pAL5000 derivative were digested with Xbal and BamHI, mixed, and ligated, creating pSTM3-pheniB4-mCherry. Subsequently, GFP variant mEos3.1 (39) was amplified with anchored primers (BamHI for the 5' primer and BsrGI for the 3' primer) and subsequently digested together with pSTM3-pheniB4- mCherry with BamHI and BsrGI creating pSTM3-pheniB4- mEos3.1. Integrative plasmids were derived from pMV361, and all integrative constructs were created with Infusion (Clontech) cloning (40). The insert piniB4-mEos3.1 was amplified from pSTM3-pheniB4-mEos3.1 cloned into Infusion into a promoterless version of pMV361 that was digested with PmlI and NsiI creating pMV-piniB4-mEos3.1. A previously constructed variant pMV-hsp60-mEos3.1 (41) was digested with NheI and ClaI to serve as a backbone for complementation constructs pMV-hsp60-cobB and pMV-hsp60-mshD. Both cobB and mshD were amplified from *M. marinum* M*USA* genomic DNA and cloned into the digested vector with Infusion to create pMV-hsp60-cobB and pMV-hsp60- mshD, respectively. The aforementioned promoterless version of pMV361 was digested with HindIII to introduce mutA and mutAB+MMAR_2304 to create pMV-mutA and pMV-mutAB-MMAR_2304.

Transposon Mutagenesis—Transposon mutagenesis was performed in *M. marinum* M*USA* and *M. bovis* BCG Copenhagen, both containing pSTM3-pheniB4-mCherry. Both strains were infected with the mycobacterial phage phiMycMarT7 that contains the Himar 1 transposon (42). Transposon mutants were assessed for increased mCherry expression, indicating induction of *iniBAC* by fluorescence microscopy. Colonies that showed a vast increase in fluorescence were isolated.

### Table 5

| Name                              | Features                                      | Source       |
|-----------------------------------|-----------------------------------------------|--------------|
| pSTM3-hsp60-mCherry               | hsp60 promoter, mCherry, hygR                 | (38)         |
| pSTM3-piniB-mCherry               | iniB promoter, mCherry, hygR                  | This study   |
| pSTM3-piniB4-mEos3.1              | Truncated iniB promoter, mEos3.1, hygR        | This study   |
| pMV361                            | hsp60 promoter, L5 attP and aph, oriE, strR   | (40)         |
| pMV-piniB4-mEos3.1                | Truncated iniB promoter, mEos3.1, L5 attP and aph, oriE, strR | This study |
| pMV-hsp60-cobB                    | hsp60 promoter, cobB, L5 attP and aph, oriE, strR | This study |
| pMV361-nohsp60                    | L5 attP and aph, oriE, strR                    | This study   |
| pMV-mutA                          | mutA, L5 attP and aph, oriE, strR             | This study   |
| pMV-mutAB                          | mutA, mutB, MMAR_2304, L5 attP and aph, oriE, strR | This study |
| pTEC19-MSP-crimsonE2              | msp promoter, crimsonE2, hygR                  | Addgene      |
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**TABLE 6**
List of primers used in this study

| Name                | Sequence (5′ → 3′)                                                                 |
|---------------------|-----------------------------------------------------------------------------------|
| iniB-FW             | TTCTAGAAGCTCCTGGGCTGGCGGATTT                                                      |
| iniB-RV             | TTGGATCCCTGGGCTGGCGGATTT                                                          |
| iniB4-FW            | TTGGATCCCTGGGCTGGCGGATTT                                                          |
| iniB-REV            | TTTGGATGGCCGAGTGCAGTGA                                                            |
| iniB4mEos-FW        | TTTGGATGGCCGAGTGCAGTGA                                                            |
| iniB4mEos-RV        | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-hsp60mEos-FW    | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-hsp60mEos-RV    | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-B4-mEos-FW      | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-B4-mEos-RV      | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-cobB-FW         | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-cobB-RV         | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-mutA-FW         | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-mutA-RV         | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-msd-FW          | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-msd-RV          | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-mutAB2304-FW    | TTTGGATGGCCGAGTGCAGTGA                                                            |
| pmv-mutAB2304-RV    | TTTGGATGGCCGAGTGCAGTGA                                                            |

and the transposon insertion site was determined by ligation-mediated PCR as described previously (36).

**Flow Cytometry Analysis**—The induction of the iniBAC was assessed by flow cytometry. Bacteria were grown to an $A_{600}$ of 0.3, and antibiotics were added at the indicated concentrations for a specified amount of time. For each time point, 1 ml was culture was collected by centrifugation and washed with PBS containing 0.05% Tween 80. The bacterial pellet was collected by centrifugation, and bacterial cells were resuspended in PBS containing 0.05% Tween 80 and subsequently analyzed on a BD Accuri C6 flow cytometer (BD Biosciences) equipped with a 488-nm laser and 585/40-nm filter for mEos3.1 and a 640-nm laser and 675/25 filter to detect CrimsonE2. In total, 30,000 gated events were collected per sample per time point, and data were analyzed using BD CFlow software.

*M. marinum* MUSA Infection of THP-1 Cells and CLCs—For infection of THP-1 and CLCs, $1 \times 10^7$ cells per well were seeded in 100-mm plates (Corning) and grown for 24 h. THP-1 monocytes were differentiated for 48 h by the addition of 25 ng/ml phorbol 12-myristate 13-acetate (Sigma). *M. marinum* containing plasmid MSP-Crimson alone and a strain with both MSP-crimson and integrative construct pMV-piniB4-mEos3.1 were grown to an $A_{600}$ of 0.8 and washed in RPMI 1640 previous to infection. Bacterial cells were subsequently added at a multiplicity of infection of 5. THP-1 cells were incubated at 33 °C and CLCs at 30 °C. After three h, extracellular mycobacteria were removed by washing 3 times with RPMI 1640. Amikacin was added to a concentration of 200 µg/ml for 3 h to kill any remaining extracellular mycobacterial cells. This point was considered time point 0 h, and cells were subsequently harvested at 0, 24, 48, and 72 hpi. THP-1 and CLC cells were both collected by the addition of 1× Accutase® (Sigma). Infected cells were spun down at 1000 rpm and washed with RPMI 1640 and resuspended in PBS for flow cytometry analysis. For confocal analysis, cells were fixedated in 4% PFA in PBS, washed in PBS, and spun down on a coverslip using a cytospin. Vectashield (Vector Laboratories) with DAPI was added on the coverslip, and cells were subsequently analyzed with a Leica TCS SP8 confocal microscope.

Sole Carbon Source Experiments on HdB Plates—HdB plates were prepared as previously described by Hartman and De Bont (44). Acetic acid, propionic acid, and valeric acid (Sigma) were dissolved by sonication in HdB medium containing 0.02% tyloxapol to an end concentration of 0.1%. Glucose was added to a final concentration of 1%, and a plate with no carbon source was taken along as growth control. Bacterial cells were washed 3× in PBS containing 0.02% tyloxapol and subsequently 3 µl of 10-fold dilutions of WT *M. marinum*, and transposon mutants in cobB and mutA were added to the plates. Plates were incubated for 14 days and imaged to visualize bacterial growth. Flow cytometry was performed by resuspending the resulting bacterial colonies in PBS plus 0.05% Tween.

**Infection of Zebrafish Embryos**—To visualize *M. marinum* in zebrafish embryos, pMV-hsp60-mEos3.1 was introduced in *M. marinum* MUSA strains that already contained the stress marker construct pSMT3-piniB4-mCherry. Zebrafish embryos were injected with 2000 cfus by microinjection in the hindbrain ventricle as previously described (45, 46). Infection was monitored at specified times by means of fluorescence microscopy. For confocal analysis, zebrafish embryos were fixated in 4% paraformaldehyde dissolved in PBS for 24 h and subsequently stored in 0.5% parafomaldehyde. For confocal microscopy, zebrafish were immobilized in 1% low melting point agarose in PBS and imaged with a Leica TCS SP8 confocal microscope.

**Mycobacterial Lipids Extraction and TLC Analysis**—*M. marinum* transposon mutants mas::tn, cobB::tn, and mutA::tn were grown to an $A_{600}$ of 1.0. Then, 50 absorbance units were collected by centrifugation and washed three times with PBS. The extraction of the cell envelope lipids was performed in three steps to harvest the apolar, polar, and mycolic acid lipid fractions as previously described (47, 48). Both apolar and polar lipid fractions were analyzed with two-dimensional TLC for PDIM and the LOS lipid contents system A and system E, respectively, as earlier described by Besra (43). Briefly, equal amounts of lipid fractions were spotted on silica-60 TLC plates (Merck) and separated by a two-dimensional TLC system A (one-dimensional, petroleum ether:ethyl acetate 98/2 (v/v)); two-dimensional, petroleum ether:acetone 98/2 (v/v)) and system E (one-dimensional, chloroform:methanol:H$_2$O 60/30/6 (v/v/v) and two-dimensional, chloroform:acetic acid:methanol: H$_2$O 40/25/3/6 (v/v/v/v)). Subsequently, the lipids were visualized by addition of 5% molybdisphosphoric acid in methanol.
(for PDIM analysis) or 0.2 g of orcinol in 100 ml of 20% H2SO4 coloring agents (for LOS analysis). Lipid contents were visualized by TLC-plate charring at 150 °C for 10 min.

Antibiotic Susceptibility Assay—Both M. marinum and M. bovis BCG transposon mutants were grown in 7H9 supplemented with ADC to an A600 of 0.6–1. Subsequently 10⁴ cells were distributed per well in a 96-well plate, and serial 2-fold dilutions of ciprofloxacin, ethambutol, isoniazid, streptomycin, and rifampicin were added (Sigma). The MIC was determined as the concentration of antibiotics that inhibited visible growth. Wells without the addition of antibiotics were used as a growth control, and experiments were performed at least in triplicate.

Author Contributions—W. B. and M. B. conceived and designed the experiments. M. S., R. v d W., K. K. J., A. S. S. C., and M. B. performed the experiments. W. B. and M. B. analyzed the data. W. B. and M. B. wrote the paper.

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