The Genetic Requirements for Fast and Slow Growth in Mycobacteria

Dany J. V. Beste, Mateus Espasa, Bhushan Bonde, Andrzej M. Kierzek, Graham R. Stewart, Johnjoe McFadden*

FHMS, University of Surrey, Guildford, United Kingdom

Abstract

*Mycobacterium tuberculosis* infects a third of the world’s population. *Primary tuberculosis* involving active fast bacterial replication is often followed by asymptomatic *latent tuberculosis*, which is characterised by slow or non-replicating bacteria. Reactivation of the latent infection involving a switch back to active bacterial replication can lead to post-primary transmissible tuberculosis. Mycobacterial mechanisms involved in slow growth or switching growth rate provide rational targets for the development of new drugs against persistent mycobacterial infection. Using chemostat culture to control growth rate, we screened a transposon mutant library by Transposon site hybridization (TraSH) selection to define the genetic requirements for slow and fast growth of *Mycobacterium bovis* (BCG) and for the requirements of switching growth rate. We identified 84 genes that are exclusively required for slow growth (69 hours doubling time) and 256 genes required for switching from slow to fast growth. To validate these findings we performed experiments using individual *M. tuberculosis* and *M. bovis* BCG knock out mutants. We have demonstrated that growth rate control is a carefully orchestrated process which requires a distinct set of genes encoding several virulence determinants, gene regulators, and metabolic enzymes. The *mce1* locus appears to be a component of the switch to slow growth rate, which is consistent with the proposed role in virulence of *M. tuberculosis*. These results suggest novel perspectives for unravelling the mechanisms involved in the switch between acute and persistent TB infections and provide a means to study aspects of this important phenomenon *in vitro*.

Introduction

With the average daily death toll from tuberculosis at 4,500 the worldwide burden of this disease is overwhelming [1]. Control measures are being severely thwarted by the worldwide spread of antibiotic resistant strains of *Mycobacterium tuberculosis*. In 2008 WHO recorded the highest rates of multi-drug (MDR) resistant strains of TB and the spread of a virtually untreatable form of the disease caused by extensively resistant strains (X-DR) [1]. Novel anti-TB drugs are urgently required which shorten the lengthy drug regimen required to treat TB and also have activity against drug resistant strains. The identification of genes which provide essential but unique functions in *M. tuberculosis* greatly facilitates drug discovery programs and also provides further information about the complex biology of this highly successful pathogen.

Slow growth rate is amongst one of the remarkable features of *M. tuberculosis*. This pathogen can only achieve a maximum growth rate equivalent to a doubling time of about 16 hours in optimal laboratory conditions whilst in the human host growth rates vary upon the site and stage of infection. Tuberculosis is characterized by two distinct phases an acute phase where the bacteria are actively growing and a persistent phase where the bacteria are in a slow growing or non-growing state [2]. This ability to persist for decades in a state refractory to immune clearance but primed for reactivation is key to the success of *M. tuberculosis* and represents an important barrier to the control of tuberculosis as presently used chemotherapies are largely inactive against non-dividing cells. The identification of pathways and genes essential to establish and maintain persistence would facilitate the development of drugs which target this phase of infection.

Although very little is known about the state of the tubercle bacillus during persistence, it is generally agreed that the organism replicates slowly or not at all during this stage of disease. To study this component of the biology of *M. tuberculosis* we utilized chemostat culture as this is the only method available to control growth rate. In addition, the use of continuous culture in a chemostat offers many advantages over batch culture enabling the experimenter to vary the growth rate whilst maintaining cells in a constant physical and chemical environment. Our previous studies into the physiology of *Mycobacterium bovis* BCG (the vaccine strain of the tubercle bacillus) demonstrated that growth rate modulated the biomass composition [3]. In addition, the transcriptomic fingerprint for slow growing BCG showed a high correlation to the profile of *M. tuberculosis* growing in a macrophage [4] and also TB patient’s sputum [5].

Whilst transcriptomics has proved to be an excellent tool for studying the response of microbes to the environment it has been shown to have limited value in predicting the contribution of individual genes to the fitness of *M. tuberculosis* [6,7]. The current study aims to further probe the physiological and gene regulatory
state of slow-growing M. tuberculosis by mutational analysis. Transposon site hybridisation (TraSH) is a microarray based transposon tracking strategy to monitor the fitness of mutants in mixed populations under different conditions. This powerful functional genomic tool was developed using M. tuberculosis and has now been applied successfully to a variety of pathogens [6,9]. Sassetti and co-workers have used TraSH to identify essential genes for the growth of M. tuberculosis in minimal glucose medium [8] as well as infection of mice [10] and survival in a macrophage [7]. We build on this work to identify genes required for slow and fast growth on minimal glycerol media in the controlled environment of a chemostat. This data provides an invaluable resource for mycobacterial researchers and will facilitate the characterisation of genes with unknown roles and provide further functional clues for some important virulence genes.

Results and Discussion

Genes required for growth on Roisin’s glycerol minimal media

In the experiments described by Sassetti et al (2003) transposon libraries of M. tuberculosis and M. bovis BCG were constructed and recovered on 7H10 agar and TraSH analysis was used to identify genes which were essential for the survival of both species on this media. The experiments described here used just the BCG transposon library generated by Sassetti et al (2003) for TraSH analysis to investigate the genetic basis of growth control in a chemostat model. Initially we determined the genes that were ‘additionally essential’ for growth in the defined minimal media used for chemostat cultivations: Roisin’s minimal broth. The additionally essential genes discussed here are defined as genes which were identified in this study as essential for growth of M. bovis BCG on Roisin’s minimal glycerol medium but had not been previously identified by Sassetti et al (2003) as essential for the growth of M. bovis BCG and M. tuberculosis on 7H10 medium.

A late log phase culture of the BCG transposon library [8] was inoculated into a 2 L bioreactor operated in batch mode. Cells were harvested after the OD600 reached approximately 1.0. The composition of the output mutant pools was compared by TraSH as described using randomly labeled genomic DNA as a control probe [10]. Microarray signals were analysed using a rank based statistic, Rank products (RP) [11]. RP is a non-parametric statistical method which has demonstrated robustness in microarray analysis and has been shown to have a higher sensitivity and selectivity in comparison with t-test based statistical methods [12]. This method detects genes that consistently have the largest positive or negative log-ratio.

Roisin’s media is a chemically defined glycerol-limited media that contains only one source of carbon plus Tween 80 as a dispersal agent, ammonia as a nitrogen source and lacks citrate, biotin or pyridoxine [3]. In contrast 7H10 is composed of several potential carbon sources including glutamic acid, glycerol, glucose, oleic acid and bovine serum albumin and also contains the cofactors biotin and pyridoxine hydrochloride and the buffer sodium citrate facilitating citrate mediated iron transport. In the absence of citrate mycobacteria grown on Roisin’s would be reliant on the siderophore mycobactin for iron transport. We therefore expected to find that the set of ‘additionally essential’ genes required to grow on Roisin’s media to be principally those involved in glycerol uptake and metabolism, ammonia uptake and genes required for de novo biosynthesis of biotin, pyridoxine and mycobactin.

A total of 241 genes (Dataset S1) that were not essential for survival in 7H10 media [8] were identified here as uniquely required for growth in Roisin’s minimal medium. Several of these, including MmpL3, ideR, embA, have previously been shown to be essential for the growth of M. tuberculosis on media containing both glucose and glycerol [13,14] so it is unclear why they were identified as non-essential in the original screen performed by Sassetti and colleagues (2003).

Under laboratory conditions glycerol is the favored carbon source of M. tuberculosis and the vaccine strain M. bovis BCG. M. bovis, however is unable to use glycerol as a sole carbon source as a result of a single nucleotide polymorphism in the gene glykD [15]. During the creation of M. bovis BCG the serial passaging of M. bovis on glycerinated medium selected for the correction of this mutation [15]. The route for glycerol utilization is generally assumed to proceed via glycerol kinase followed by dehydrogenation [15]. However, many bacteria utilize an alternative pathway whereby glycerol is first oxidized by glycerol dehydrogenase before being phosphorylated [16]. Glycerol dehydrogenase activity has been detected in M. tuberculosis [17], but no gene encoding this activity has been annotated in the genome and several genes encoding putative alcohol dehydrogenases are also present. The results presented here show that glykK was essential for growth on Roisin’s minimal glycerol media (p = 1.48×10^-5). To clarify the situation, and also to independently validate the TraSH data, an individual gene knock-out of glykK was constructed in both M. bovis BCG and M. tuberculosis. The resulting mutants displayed a dysgonic growth phenotype when grown on 7H11 media and failed to grow on Roisin’s agar (Figure 1) confirming the TraSH result and indicating that the glycerol kinase pathway is indeed essential for glycerol metabolism.

The experimental data indicated that, amongst biotin biosynthetic genes, only bioD was essential for growth in the biotin free Roisin’s media. However, bind with a pfp value of 0.114 was just below the cut-off value for essentiality. Surprisingly none of the genes involved mycobactin synthesis were essential for growth in Roisin’s minimal medium. M. tuberculosis is peculiar in its ability to produce both cell-associated (mycobactin) and secreted (carboxymycobactin) siderophores to capture iron. The cross feeding of the mbt mutants by carboxymycobactin released from neighboring cells may enable mbt mutants to overcome their siderophore deficiency. However mbkB, which, encodes the enzyme required for the initial steps in mycobactin T synthesis, had a pfp value of 0.21 and therefore may have a role in the optimal growth of mycobacteria in this media. Deletion of the mbkB gene has been shown to disrupt the biosynthesis of both mycobactin and carboxymycobactin [18]. Cross feeding could also enable pyridoxine synthesis mutants to overcome the inability to synthesise this cofactor.

Comparison of glycerol essentiality results with flux balance analysis (FBA) model predictions of a genome-scale metabolic model

Genome-scale metabolic models provide a valuable framework to interpret essentiality screens as they allow predictions of gene essentiality using current knowledge of the metabolic network whilst also providing an insight into incomplete or incorrect metabolic knowledge. The glycerol TraSH results were compared with gene essentiality predictions using a genome-scale metabolic network of M. tuberculosis (GSMN-TB) [19]. The gene essentiality scan was performed using the minimal biomass e as described previously [19]. In addition the carbon source was changed to glycerol and the biotin and citrate gates were closed. Overall there was a good correlation between the GSMN-TB model and the observed gene essentialities as 76.66% of the predictions were identical to the experimental results (additionally essential genes only). This analysis identified some interesting inconsistencies (false
positives and false negatives) which reveal important information about the metabolism of tuberculosis and this information can also be used to make informed alterations to the GSMN. The set of false negatives (genes predicted as dispensable by the model but shown to be essential experimentally) included genes which had potential homologues with annotations of only moderate confidence and maybe unable to replace the activity of their deleted isoenzyme. For example, fabD which is a major component of M. tuberculosis fatty acid synthase II has a possible homologue fabD2 which is present in the GSMN. The demonstration that fabD is essential suggests that fabD2 is unable to functionally complement the activities of fabD. Several genes were also predicted as functionally redundant due to the presence in the model of alternative pathways that can substitute for genes predicted to be essential. For instance, the model predicted that the gene glpK is non-essential due to the presence of the alternative glycerol pathway described above. This alternative glycerol utilization pathway has now been deleted from the GSMN-TB model.

Amongst the false negative model predictions was the malate dehydrogenase gene mdh (Rv1240), which was identified previously as non-essential in the original TraSH screen [8] but was identified as essential in the TraSH analysis presented here. The non-essentiality in the model was due to the predicted activity of “malic enzyme” (malate dehydrogenase decarboxylating, me2) that catalyses the oxidative decarboxylation of L-malate to pyruvate and which, together with pyruvate carboxylase, can potentially convert malate to oxaloacetate and thereby complement in-silico mdh mutants. To confirm this result we attempted to construct mdh KO mutants in both M. tuberculosis and M. bovis BCG. Despite numerous attempts we were unable to delete the mdh gene even when the transformants were recovered on 7H11 media which contains glucose in addition to glycerol. When a second copy of the wild type mdh gene was integrated into the attB site in the chromosome, we could easily isolate mutant strains with deletions in the original mdh gene demonstrating that this gene was essential for growth on both glucose and glycerol containing medium. The result indicates that either malic enzyme and/or pyruvate carboxylase are not active in Roisin’s medium (at least in the direction pyruvate to acetate). To date, activity of these enzymes has not been demonstrated in M. tuberculosis.

Rv1099c, a fructose 1, 6 bisphosphatase (glpX) was predicted as essential for gluconeogenesis by the GSMN but this was not confirmed experimentally by the TraSH analysis. Rv1099c has been shown to have GlpX activity and can complement an E. coli mutant lacking fructose 1, 6 bisphosphatase [20]. In addition, Rv1099c was identified as essential for in vivo growth in a mouse model of TB by TraSH analysis [10], which is consistent with the requirement for gluconeogenesis in vivo. It is therefore puzzling why it is not required for growth on glycerol in vitro. The Rv2131c gene has been shown to possess fructose-1, 6-bisphosphatase activity in addition to inositol monophosphatase activity and could therefore potentially substitute for glpX [21].

Genetic requirements for growth in a carbon-limited chemostat

In order to identify genes that are important for both the control and establishment of fast and slow growth rates competitive chemostat cultivations were performed. The experiments were carried out at two different growth rates (D = 0.03 h^-1 and D = 0.01 h^-1) equivalent to doubling times (td) of 69 h and 23 h respectively. The physiological and transcriptomic profile of BCG cells at these growth rates have been described previously [3,4]. Subsequently, continuous cultures at a dilution rate of 0.03 h^-1 were switched to a dilution rate of 0.01 h^-1 and vice versa (Figure 2). This strategy was used to identify genes important for the change from one growth rate to another. The cultures were sampled in the batch phase just prior to the start of continuous culture and after ~11 generations of chemostat culture. DNA was extracted from the surviving bacterial cells and mutant gene identity and abundance assessed by TraSH. This analysis identified mutants which were negatively selected for during either slow (F) (td = 69 h) (Dataset S2, Figure 3) or fast (S) (td = 23 h) (Dataset S3, Figure 3) rates and also mutants attenuated in the switch from fast to slow (F-S) (Dataset S4) or slow to fast (S-F) (Dataset S5).

There was very little overlap between the mutants attenuated at fast growth rate and slow growth rate (Figure 3) but five genes
Figure 2. TraSH screen for mutants with reduced fitness at different growth rates in a carbon limited chemostat. A transposon mutant library of *Mycobacterium bovis* BCG was inoculated into the chemostat and after an initial phase in batch culture was grown at a doubling time of 23 h (D = 0.03 h⁻¹) and samples removed for analysis by transposon site hybridisation (TraSH). The transposon mutant library was similarly inoculated into the chemostat at a growth rate corresponding to a doubling time of 69 h (D = 0.01 h⁻¹) and samples again removed for TraSH analysis. Finally, cultures established at the fast growth rate were switched to the slow growth rate, and visa versa, to identify mutants unable to switch between the different growth rates.

doi:10.1371/journal.pone.0005349.g002

Figure 3. Mutants with reduced fitness at (A) fast (td = 23 h) and (B) slow (td = 69 h) growth rate in a carbon limited chemostat. Venn diagram shows the overlapping genes.

doi:10.1371/journal.pone.0005349.g003
were required for optimal growth in the chemostat irrespective of the growth rate (Figure 3). These included regX3, mmpL4 and mma which have all been shown previously to have an important role in the virulence of M. tuberculosis in a mouse model but without any observed alteration in their in vitro growth rate. The finding that they are also required for optimal growth in the chemostat suggests that they play a role in maintaining growth in carbon-limiting conditions. A very subtle growth defect during the stationary phase of growth was reported for the regX3 deletion mutant of M. tuberculosis [22] when grown in Dubos medium in which carbon would also be limiting growth during stationary phase. RegX3 appears to play a role in the regulation of which carbon would also have been limiting growth during the batch phase (results not shown) but had a significantly reduced fitness during slow growth rate, independently confirming the TraSH data (Figure 4). However, when the experiment was repeated using a wild type M. tuberculosis H37Rv and a M. tuberculosis hspR KO mutant [27] the mutation conferred a growth defect relative to the wild type strain during the batch phase of growth whereas there was no additional reduction of fitness observed during chemostat culture at slow growth rate (Figure 4). The result appears to indicate a difference in growth control between M. tuberculosis and M. bovis BCG.

Genes required for slow growth rate

TraSH analysis of slowly growing BCG identified 89 genes (Dataset S2; Figure 3) important for the establishment and maintenance of this growth rate. As has been reported previously there was a very poor correlation between the transcriptome of slowly growing BCG and the TraSH essentiality data [6,7]. Only 9 genes (cobQ1, pumT, fprB, ctpV, Rv1227c, Rv2014, tspG, Rv2943, Rv3691) were required for slow growth rate that had also been identified as transcriptionally upregulated in this condition [19].

Essential genes were divided into functional categories using the gene ontology developed as part of the genome sequencing project (http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/) and a chi square test used to calculate functional groups with a significant fraction of genes identified as being required for slow growth. Nine functional categories were highlighted as being significantly involved in slow growth: electron transport; ATP-proton motive force; cobalamin biosynthesis; modification of fatty and mycolic acids; degradation of RNA; conserved membrane proteins; detoxification; virulence and other IS elements.

| Input Pool | Output pool | Gene Name and Function |
|------------|-------------|------------------------|
| Batch      | Fast growth rate (tₜ=23 h) | Rv0596c (antitoxin), pknH (serine-threonine protein kinase), Rv1962 (antitoxin) |
| Batch      | Slow growth rate (tₜ=69 h) | hspR (heat shock regulator), Rv2103c (toxin), Rv2494 (toxin), ppsA and ppsB (DIM biosynthesis and important in virulence), nucC and NuoN (aerobic/anaerobic respiration), cmaA2 (cyclopropane-mycolic acid synthase) |
| Batch to Fast growth rate | Slow growth rate | Rv0485 (transcriptional regulator), MprA (mycobacterial persistence regulator), NuoE (aerobic/anaerobic respiration), cmaA2 (cyclopropane-mycolic acid synthase, mutant hypervirulent in mice) |
| Batch to Slow growth rate | Fast growth rate | Rv0172 and lprK (part of mce1 operon), dosT (histidine kinase sensor for Dos regulon) and sigF, sigJ (alternative sigma factors involved in virulence and persistence), whiB1 (transcriptional regulatory protein) |

Several genes that appeared to be required for slow growth are of particular note (Table 1). Genes encoding the pin domain proteins (Rv2103c and Rv2494) were identified with mutants that had reduced fitness at slow growth rate. These proteins are putative toxin genes and have been proposed to play a role in promoting slow growth during stressful environments [24]. Interestingly, mutants in two anti-toxin genes (Rv0596c and Rv1962) were identified as having reduced fitness at the fast growth rate. Several of the genes apparently involved in slow growth have previously been associated with virulence, such as (ppsA and ppsB) which are essential for the synthesis of phospho-cholesterol dimyocerosates (DIM). DIM mutants have high cell wall permeability and are severely attenuated in mouse models of TB [25,26]. Several known regulators were also identified as being involved in slow growth including the heat shock protein repressor gene hspR. Strains of M. tuberculosis lacking hspR over-express heat shock proteins and have defects specifically during the chronic or persistent phase of TB infection, a feature which was proposed to be due to enhanced immune recognition [27]. To verify the involvement of this regulator in maintenance of slow growth, competitive chemostat experiments were performed using M. bovis BCG strains (wild type and ΔhspR). The strains grew identically during the batch phase (results not shown) but had a significantly reduced fitness during slow growth rate, independently confirming the TraSH data (Figure 4). When the experiment was repeated using a wild type M. tuberculosis H37Rv and a M. tuberculosis hspR KO mutant [27] the mutation conferred a growth defect relative to the wild type strain during the batch phase of growth whereas there was no additional reduction of fitness observed during chemostat culture at slow growth rate (Figure 4). The result appears to indicate a difference in growth control between M. tuberculosis and M. bovis BCG.

Genes required for fast growth rate

Only 29 genes were attenuated for survival at the fast growth rate (D=0.03 h⁻¹). As the maximum growth rate (μmax) of M. bovis BCG in Roisin’s medium in continuous culture is 0.033 [Beste, 2005 1564/id] the fast growth rate is very close to μmax and therefore fewer additionally essential genes would be expected. With this relatively small number of genes it was not possible to find any significant functional associations.

Genetic requirements for the shift from slow to fast growth rate

A total of 256 genes were identified as important in the switch from slow growth rate to fast. Significant functional categories
and the switch from fast to slow but not the switch from slow to fast growth) almost all the top-ranked genes belong to the mce1 operon. This was not due to simple overgrowth of a single mutant since independent mutants representing inactivation of at least 11 of the 13 genes in the mce1 operon were over-represented in these conditions. In addition mutations in the putative ATPase, Rs0655 which has been functionally linked to the mce1 operon [38] and also Rs0199 and Rs0290 which are closely related to genes present in the mce1 operon [39] also had a positive fitness affect. It appears therefore that inactivation of any of the genes in the mce1 operon and their associated genes give rise to mutants with the same competitive growth advantage.

This result seemed initially to be contradictory. Why would mce1 operon mutants be advantageous at slow, during the switch from slow to fast growth rate and at fast growth rate but not for the switch from slow to fast growth rate? The common factor in these three conditions is a switch from a fast to a slower growth rate. This is still compatible with the finding that the mutants also had a growth advantage at fast growth rate in the chemostat since this experiment involved a shift from growing at maximal growth rate in batch to the fast growth rate in the chemostat (which is nevertheless slower than the rate in batch). To validate this result a M. tuberculosis mce1 operon mutant was constructed (see methods) and competitive chemostat experiments performed at the slow growth rate after an initial period in batch. The results of two independent experiments showed that although there was no difference in fitness between the wild type and mutant during batch culture (results not shown) the mutant had dramatic competitive advantage at slow growth rate in the chemostat as evidenced by the outgrowth of the mce1 mutant from a starting mutant:WT ratio of 1 to a ratio of 100 after 35 days of continuous culture confirming the TraSH data (Figure 5). After 35 days a “quasi steady state” was obtained between the wildtype and mutant, a phenomenon which has been described previously in chemostat cultures [40]. The reason for the maintenance of competing strains in the chemostat remains controversial and is subject to continuing mathematical investigation [41,42]. When the dilution rate was then switched to a fast growth rate the selective advantage was lost, the wild type recovered and both strains returned to approximately equivalent amounts (Figure 5).

The ability of the wild type to recover at fast growth rate in this competition was intriguing and unexpected. However, the system becomes dynamic after the shift in the dilution rate and so the growth rate of both strains will depend on factors not apparent in steady state cultures. These observations may provide a clue to the function of the mce1 operon but require further focused investigations.

The mce1 operon has been the centre of much research and debate. Interest in this operon dates back to 1993 when Arruda et al demonstrated that mce1A was able to transfer to Escherichia coli the ability to invade eukaryotic cells [43]. The genome sequencing project revealed that the mce1A gene is part of an operon of 13 genes and that this operon is duplicated four times (mce2, mce3, mce4) in the genome of M. tuberculosis [44]. The role of the genes in M. tuberculosis remains highly controversial although there is an increasing consensus that each operon (which resembles a multi subunit ABC transport system) encodes a transporter system. The mce1 locus has recently been shown to be involved in the transport of cholesterol [38,45]. However, the role of mce1 remains mysterious. Riley and colleagues have reported a hypervirulent phenotype for mce1 gene knock-outs in a murine model of TB [46,47]; whereas a transposon mce1 mutant used by Sassetti and Rubin (2003) and also Joshi et al (2006) [38] had a growth defect in early infection in competition experiments. An in vitro phenotype

Figure 4. Competitive chemostat experiments: Wild type Vs ΔhspR. Average CFU ratios of (i) wild type H37Rv-ΔhspR (closed circles) and wild type BCG-ΔhspR (closed triangles) during continuous culture at a dilution rate of 0.01 h\(^{-1}\) (t\(=\)69 h) were plotted as a function of time. Data were normalized so that the ratio at the start of chemostat culture equaled 1. Only the BCG ΔhspR strain was attenuated for slow growth rate in the chemostat.

doi:10.1371/journal.pone.0005349.g004

included energy metabolism, miscellaneous oxidoreductases and oxygenases, PPE family and also the unknown function class indicating that a large number of uncharacterized gene products are required for the shift from slow to fast growth rate. Amongst these genes are several regulators including the stress response sigma factors sigF and sigJ and the transcriptional regulators whiB1 and whiB6. Although previous studies have failed to find an in vitro growth effect for either sigF [28,28] or sigJ [29] mutants our results indicate that the genes are involved in the switch from slow to fast growth rate. SigF mutants have reduced lethality in both mouse [28] and guinea pig models [30] of TB and also have altered cell membrane properties [31,32] whereas sigJ is dispensable for in vivo growth but appears to be involved in protection against hydrogen peroxide [29]. The resuscitation promoting factor gene nfpE (Rv2450c) was also identified as being essential for the switch from slow to fast growth rate.

Fast to slow growth rate

Only 19 genes had reduced fitness during the shift from fast to slow growth rate. Of note amongst this list was mprA, a two component regulator which has been shown to have a role in the entry and maintenance of a persistent infection in mice [36]. Absence of mprA also increases growth in resting macrophages [36] and increases the expression of stress related genes [37].

Positive fitness effects

Some mutations led to enhanced growth and are therefore positively selected in the competitive growth experiment. These mutants were identified using rank products, ranking on the largest positive log ratio. This generated lists of genes whose loss led to a significant selective advantage over the wild type strain in each condition (Dataset S6, S7, S8, S9). The most striking feature of this data was that for three of the four conditions examined (slow, fast
for mce1 mutants has not previously been reported but our results show that mce1 mutants have a competitive advantage over wild type strains during the switch to slow growth rate in a carbon limited chemostat.

Our finding that mce1 mutants are overrepresented in slow growth rate cultures is both intriguing and puzzling. The finding has some similarities to ‘evolutionary cheating’ in E. coli whereby mutants that fail to enter, or exit early from, the non-dividing stationary phase state are overrepresented in stationary phase cultures [48]. We posit a similar scenario for M. tuberculosis whereby (for unknown reasons) mce1 mutants are unable to enter, or exit early from, the slow growth state and are thereby overrepresented in slow growth rate cultures. This hypothesis is consistent with data showing that mce1 expression is turned off initially during murine infection but is then switched back on in a later stage of infection [49]. If this hypothesis is correct then the underlying mechanism may be involved in the observed hypervirulent phenotype of the mce1 knock out strain in vivo [46] and could have implications for understanding the switch from acute to persistent infections in the host.

Conclusion

The data presented here demonstrate that maintaining growth at slow and fast growth rate and switching between these states is a carefully controlled process in mycobacteria involving a unique set of genes and not simply acceleration or deceleration of the same cellular processes. Several of these genes are transcriptional regulators (such as hopR) and many have previously been implicated as involved in virulence including persistence. The results indicate that growth control in M. tuberculosis is a complex process and that the phenomenon of growth control, as measured in the chemostat, may have relevance to virulence (including persistence) in the host. Many of the genes that we identified as involved in growth control in the chemostat have not previously been associated with any phenotype in vitro and therefore the results potentially also provide a fruitful experimental route towards unraveling the role of M. tuberculosis genes whose function is currently unknown. Perhaps the most surprising finding of this study was the demonstration that the mce1 operon is involved in growth control of M. tuberculosis. This result is very interesting but begs the question: how does mce1 influence growth? Whilst our experiments do not identify what this role is they do provide a means to study this phenomenon in vitro.

Materials and Methods

Chemostat cultivations

A library of transposon mutants constructed in M. bovis BCG Pasteur was kindly provided by Eric J. Rubin [8]. An aliquot of the transposon insertion library was cultured in Rosin’s minimal medium, without the addition of biotin [3] until an OD$_{600}$ of 1.0. This pre-culture was transferred into a 2 L bioreactor as previously described [3]. After inoculation the culture was grown as a closed system until the OD$_{600}$ reached approximately 1.0. Continuous culturing was then started at a known dilution rate of 0.03 h$^{-1}$ (equivalent to a doubling time $t_d$ of 24 h) or 0.01 h$^{-1}$ ($t_d$ = 69 h). Six to eight volume changes were allowed for selection of mutants with growth defects or enhanced survival at each dilution rate. Culture samples were harvested from the chemostat in the batch phase and during continuous culture and used to isolate genomic DNA. Cultures established at the fast growth rate were switched to the slow growth rate, and vice versa in order to identify mutants unable to switch between different growth rates (Figure 1). Genomic DNA was extracted from independent triplicate or quadruplicate chemostat cultures using standard methods. TRASH probes were generated using the protocol described by Sasetti et al. (2001).

For the competitive chemostat cultivations of individual mutant versus wild type equivalent amounts of antibiotic tagged mutant and wild type strains were inoculated into bioreactors and grown at a dilution rate of 0.01 h$^{-1}$ or 0.03 h$^{-1}$. Samples were removed from the chemostat once every generation during continuous culture. The numbers of wild type and mutant bacteria were determined by plating serially diluted samples onto 7H11 agar (for total bacterial counts) and 7H11 agar containing hygromycin at 50 μg ml$^{-1}$ (to enumerate the cfu of the mutant).

Microarrays and hybridizations

Fluorescently labeled cDNA were produced by reverse transcription of the TRASH probes (1 μg) with Superscript II (Invitrogen) in the presence of Cy3-dCTP (Amersham Pharmacia) using random hexamer oligonucleotides to prime cDNA synthesis. The reference control sample of Cy-3 labeled genomic DNA used for the glycerol minimal media experiments was prepared by mixing Cy3-labeled dCTP and BCG genomic DNA with Klenow DNA polymerase in the presence of Cy3-dCTP (Amersham Pharmacia) for the glycerol minimal media experiments was prepared by mixing Cy3-labeled dCTP and BCG genomic DNA with Klenow DNA polymerase in the presence of random primers. The reaction was incubated for a minimum of 90 minutes at 37°C.

The DNA microarrays provided by the Bacterial Microarray Group at St Georges (http://bugs.sgul.ac.uk/index.php) were constructed from PCR-amplified ORF-specific DNA, representing all of the predicted open reading frames from the M. tuberculosis H37Rv genome spotted in duplicate. The array designs are available in BuGI@Sbase (Accession No. A-BUGS-1 and A-BUGS-2; http://bugs.sgul.ac.uk/A-BUGS-1) and also ArrayExpress (Accession No. A-BUGS-1 and A-BUGS-2). Pre-hybridisation, hybridisation and washing were performed as described by Stewart et al. [50].
Data processing and statistical analysis

Microarrays were scanned using a 428 Array Scanner (Affymetrix). Fluorescence intensity data from each array were automatically quantified using BlueFuse for microarrays (version 3.3) software (Bluegene). The spot confidence was also evaluated by BlueFuse and low quality spots (flag E) were rejected. Duplicate spots were averaged and filtered so that only genes for which there was a value for at least 4 replicates were statistically analysed.

Microarray data were analysed by the Rank Product analysis [11] using the Bioconductor package RankProd [51] in R [52]. This generates a list of genes ranked according to log ratio and also calculates a conservative estimate of the percentage of false positives (pfp) or false discovery rate (FDR). The pfp considers the problem of multiple testing and does not require an additional correction method. The genes with FPF values smaller than 0.1 (10%) were regarded as differentially represented. Fully annotated microarray data have been deposited in BpG@Sbase (accession number E-BUGS-83; http://bugs.sgl.ac.uk/E-BUGS-83) and also ArrayExpress (accession number E-BUGS-83).

Genetic manipulations

For the construction of mch, glpK and mcl mutants, E. coli strain DH5α was grown in solid or liquid Luria-Bertani (LB) medium as described by Sambrook et al. [53]. M. bovis BCG cells were propagated in Middlebrook 7H9 broth or 7H11 agar containing 5% (v/v) OADC enrichment media supplement (Becton Dickenson), 0.5% glycerol plus 0.03% Tween 80 for liquid cultures. For mycobacteria, when selection was required, kanamycin at 20 μg ml⁻¹, X-gal at 50 μg ml⁻¹, hygromycin at 50 g ml⁻¹ and sucrose at 2% (w/v) were added to the culture media.

Mutants of M. bovis BCG and M. tuberculosis were constructed using the strategy described by Stewart et al. 2001. Approximately 1 kb regions flanking mch and glpK were amplified by PCR using the Roche Expand High Fidelity PCR system. Fragments were cloned either side of the hygromycin cassette into the suicide vector pG5, which is a pSMT100 [27] plasmid carrying a kanamycin resistance gene in addition to the sacB counterselectable marker. The resulting plasmid was electroporated into M. tuberculosis H37Rv and M. bovis BCG as described previously [54]. Double crossovers were selected for on 7H11 agar supplemented with kanamycin, hygromycin and X-gal. To restore the wild-type phenotype a cloned glpK gene was reintroduced into ΔglpK M. bovis BCG and ΔglpK M. tuberculosis on the pKinta plasmid [50]. To facilitate the inactivation of the mch gene, a diploid strain was constructed by integrating a second copy of the gene at the attB site [50]. Deletion of the chromosomal copy of mch was performed as described above. PCR and Southern analysis were performed to verify the expected genotypes.

The mcl operon was disrupted in M. tuberculosis strain H37Rv using a suicide vector kindly provided by Lee Riley and the two step counter selection cloning strategy described by Parish and Stoker [54]. This vector targets the gene Rxv0168. It was previously demonstrated that Rxv0168 mutants do not express any of the genes in the mcl operon [46]. Potential mutants were verified by PCR and Southern blot hybridization.

References

1. World Health Organisation (2008) Tuberculosis Fact Sheet.
2. Stewart GR, Robertson BD, Young DB (2003) Tuberculosis: a problem with persistence. Nat Rev Micro 1: 97–105.
3. Beste DJV, Peters J, Hooper T, Avignone-Rossa C, Bushell ME, et al. (2005) Compiling a Molecular Inventory for Mycobacterium bovis BCG at Two Growth Rates: Evidence for Growth Rate-Mediated Regulation of Ribosome Biosynthesis and Lipid Metabolism. J Bacteriol 187: 1677–1684.
4. Beste DJV, Laing E, Boone B, Avignone-Rossa C, Bushell ME, et al. (2007) Transcriptomic Analysis Identifies Growth Rate Modulation as a Component of the Adaptation of Mycobacteria to Survival inside the Macrophage. J Bacteriol 189: 3969–3976.
5. Garton NJ, Waddell SJ, Sherratt AL, Lee SM, Smith RJ, et al. (2008) Cytological and Transcript Analyses Reveal Fat and Lazy Persister-Like Bacilli in Tuberculous Sputum. PLoS Medicine 5: e75.
6. Kendall SL, Rison SCG, Movahedzadeh F, Fritz R, Steker NG (2004) What do microarrays really tell us about M. tuberculosis? Trends in Microbiology 12: 537–544.

7. Rengarajan J, Bloom BR, Rubin EJ (2005) Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. PNAS 102: 8327–8332.

8. Sassetti CM, Boyd DH, Rubin EJ (2003) Genes required for mycobacterial growth defined by high density mutagenesis. Molecular Microbiology 40: 77–84.

9. Chan K, King JC, Falkow S (2003) Microarray-Based Detection of Salmonella enterica Serovar Typhimurium Transposon Mutants That Cannot Survive in Macrophages and Mice. Infect Immun 73: 5438–5449.

10. Sassetti CM, Rubin EJ (2005) Genetic requirements for mycobacterial survival during infection. Proceedings of the National Academy of Sciences of the United States of America 100: 12989–12994.

11. Breiling R, Armengaud P, Amstmann A, Herzyk P (2006) Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. Fems Letters 573: 83–92.

12. Hong F, Breiling R (2008) A comparison of meta-analysis methods for detecting differentially expressed genes in microarray experiments. Bioinformatics 24: 374–382.

13. Lewin EM, Voskuil MI, Gold B, Schoehn EM, Smith J (2002) IdeR, an essential gene in Mycobacterium tuberculosis: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. Infect Immun 70: 3571–3581.

14. Domenec P, Reed MB, Barry CE III (2005) Contribution of the Mycobacterium tuberculosis MmpL protein family to virulence and Drug Resistance. Infect Immun 73: 3492–3501.

15. Keating LA, Wheeler PR, Sansour H, Irwold JK, Dale J, et al. (2005) The pyrazinamide requirement of some members of the Mycobacterium tuberculosis complex is due to an inactive pyruvate kinase: implications for in vivo growth. Microbiology 56: 163–174.

16. Lin ECC (1976) Glycerol Dissimulation and its Regulation in Bacteria. Annual Review of Microbiology 30: 533–578.

17. Goldman DS (1963) ENZYME SYSTEMS IN THE MYCOBACTERIA XV.: The pyruvate requirement of some members of the Mycobacterium tuberculosis complex is due to an inactive pyruvate kinase: implications for in vivo growth. Microbiology 56: 163–174.

18. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, et al. (2000) The salicylate-derived mycobactin siderophores of Mycobacterium tuberculosis are essential for growth in macrophages. Proceedings of the National Academy of Sciences of the United States of America 97: 1252–1257.

19. Beste D, Hooper T, Stewart G, Bonde B, Avignone-Rossa, et al. (2007) GSMN-Complex Components Regulatory System SenX3-RegX3 Regulates Phosphate-Dependent SenX3-RegX3 Transcriptional Activity. Science 319: 446–450.

20. Glover RT, Kriakov J, Garforth SJ, Baughn AD, Jacobs WR Jr (2007) The Two-Component System sensor is required for virulence of Mycobacterium tuberculosis. FEMS Microbiology 237: 415–423.

21. Stewart GR, Saldanha JW, Hunt DM, Hoar DN, Colston MJ, et al. (2004) A partner for the Mycobacterium tuberculosis Lrp sensor, MprA. Mol Microbiology 56: 163–174.

22. Rickman L, Saldanha JW, Hunt DM, Hoar DN, Colston MJ, et al. (2004) A partner for the Mycobacterium tuberculosis Lrp sensor, MprA. Mol Microbiology 56: 163–174.

23. Pandey DP, Gerdes K (2005) Toxin-antitoxin loci are highly abundant in free-living mycobacteria. FEBS Letters 533: 578–582.

24. El Hajji M, Rapaport A (2009) Practical coexistence of two species in the chemostat: A slow-fast characterization. Mathematical Biosciences 218: 33–39.

25. Lobry G, Harmand J (2006) A new hypothesis to explain the coexistence of n species in the presence of a single resource. Comptes Rendus Biologies 329: 40–46.

26. Rengarajan J, Bloom BR, Rubin EJ (2005) Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. PNAS 102: 8327–8332.

27. Stewart GR, Snevin VA, Wald G, Russell T, Tormay P, et al. (2001) Overexpression of heat-shock proteins reduces survival of Mycobacterium tuberculosis in the chronic phase of infection. Nat Med 7: 732–737.

28. Chen K, King JC, Falkow S (2003) Microarray-Based Detection of Salmonella enterica Serovar Typhimurium Transposon Mutants That Cannot Survive in Macrophages and Mice. Infect Immun 73: 5438–5449.

29. Chan K, King JC, Falkow S (2003) Microarray-Based Detection of Salmonella enterica Serovar Typhimurium Transposon Mutants That Cannot Survive in Macrophages and Mice. Infect Immun 73: 5438–5449.