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Stationary phase gene expression of *Mycobacterium tuberculosis* following a progressive nutrient depletion: a model for persistent organisms?☆, ☆☆

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Summary

The majority of individuals infected with TB develop a latent infection, in which organisms survive within the body while evading the host immune system. Such persistent bacilli are capable of surviving several months of combinatorial antibiotic treatment. Evidence suggests that stationary phase bacteria adapt to increase their tolerance to environmental stresses. We have developed a unique in vitro model of dormancy based on the characterization of a single, large volume fermenter culture of *M. tuberculosis*, as it adapts to stationary phase. Cells are maintained in controlled and defined aerobic conditions (50% dissolved oxygen tension), using probes that measure dissolved oxygen tension, temperature, and pH. Microarray analysis has been used in conjunction with viability and nutrient depletion assays to dissect differential gene expression. Following exponential phase growth the gradual depletion of glucose/glycerol resulted in a small population of survivors that were characterized for periods in excess of 100 days. Bacilli adapting to nutrient depletion displayed characteristics associated with persistence in vivo, including entry into a non-replicative state and the up-regulation of genes involved in β-oxidation of fatty acids and virulence. A reduced population of non-replicating bacilli went on to adapt sufficiently to re-initiate cellular division.

Keywords

Tuberculosis; Persistence; Metabolism; Microarray; Stationary phase

Introduction

The majority of infective cases involving *Mycobacterium tuberculosis* result in a protective immune response that limits active infection but fails to eradicate a sub-population of bacilli.[1] It is such persistent bacteria that are thought to be responsible for the prolonged antibiotic regimens required for effective treatment of tuberculosis.[2] In the Cornell mouse

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model, mice treated with antibiotics until they are estimated by culture to have reached a sterile state, still contain *M. tuberculosis* DNA equivalent to 10^5 organisms per organ[3] and the presence of mRNA is still detectable.[4] This suggests that, following chemotherapy, a small percentage of the original bacterial population may still be present.

The metabolic state of these persisters has become an area of intense research and evidence suggests that persistent bacilli are metabolically active,[4] perhaps more akin to the observed activity of stationary phase cultures of *Escherichia coli*.[5] On progression into a carbon limited stationary phase, cultures of *M. smegmatis* gain increasing resistance to a variety of environmental perturbations, such as oxidative, acid, or osmotic stress. Bacilli also demonstrate the ability to sense limiting concentrations of glycerol and initiate a biological shutdown capable of allowing survival for at least 650 days.[6] A carbon starvation model using *M. tuberculosis* was recently used to assess the mRNA profile of a population of cells over a 96 h period, following a sudden withdrawal of nutrients.[7] Changes in expression of regulatory genes detected by microarray were concurrent with a reduction in mRNA species involved with energy metabolism and cell division. Their study also demonstrated that cells starved in this way for six weeks were more resistant to anti-tuberculous drugs.

In contrast to previous models of in vitro persistence,[7,8] the model introduced in this study allowed for the maintenance of a long-term stationary phase culture via the flexibility of a fermenter vessel, in which parameters such as pH, temperature, and dissolved oxygen tension, could be accurately controlled. A 750 ml culture was maintained and sampled for over 100 days. The culture was held at a dissolved oxygen tension (DOT) of 50% throughout this period to assess survival tactics of bacilli in a nutrient exhausted state when oxygen availability was not a limiting factor. Whole genome microarrays were employed to profile gene expression throughout this time period, with the advantage that all samples were generated from the same population. Statistical analysis of these profiles suggest that surviving bacilli utilized fatty acids as their predominant energy source via pathways of β-oxidation, and that many genes postulated as virulence determinants were up-regulated.

Materials and methods

Inoculation and control of carbon starvation culture conditions

A 1 l chemostat vessel fitted with a titanium top plate (as detailed previously[9]) was adapted for use as a closed, single culture fermenter vessel and sterilized containing 750 ml volume of Middlebrook 7H9 broth with ADC supplement, 0.2% glycerol, and 0.2% Tween 80. Following inoculation with *M. tuberculosis* (strain H37Rv) the culture was continuously monitored by a Anglicon Microlab Fermentation System (Brighton Systems, Newhaven, UK) linked to sensor probes inserted into the culture able to measure pH, temperature, and dissolved oxygen tension (DOT). This electronic controller allowed temperature to be maintained at a defined level of 37°C, and DOT to be stabilized at 50% (air saturation at 37°C) via the addition of either nitrogen gas or air to the culture. Increased cell dispersion was accomplished with the aid of a magnetic stirrer, which also improved the uptake of gas into the culture. Cultures were maintained in this stable aerophilic environment throughout exponential phase growth, and the subsequent nutrient depleted stationary phase.

Viable counts

For each assessment, ten-fold dilutions of culture in sterile deionized water were plated in triplicate onto Middlebrook 7H10 + OADC agar. Plates were incubated at 37°C±2°C for 3 weeks before *M. tuberculosis* colonies were counted.

Biochemical analysis of glucose and glycerol depletion

Depletion of available carbon sources from the medium was assessed using commercially available kits. Initially, samples were double filtered through 0.2 μm filters (Sartorius) to remove cellular material. Glucose and glycerol levels were then determined using either a neat of five-fold dilution of filtered sample according to the manufacturers instructions (Glucose: Sigma Diagnostics, product #115-A; Glycerol: R-Biopharm, Glasgow, UK).

RNA extraction and purification

Samples (20 ml) were removed from the culture during mid-exponential phase growth (day 5) and various time points during stationary phase. These samples were added directly into 4 volumes of 5 M GTC solution (5 M guanidinium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol; as detailed by Butcher et al.[10]). This procedure immediately blocks further transcriptional changes and stabilizes the mRNA so as to reflect the true mRNA representation in the bacterial population under study. Following an incubation step of 1 h at room temperature, cellular material was pelleted by centrifugation at 900*g* for 30 min, re-suspended in Trizol reagent (Life Technologies), and shaken in a reciprocal shaker (Hybaid) at maximum setting 6.5 for 45 seconds in the presence of 0.5 ml of 0.1 mm glass beads. RNA was then double chloroform extracted and precipitated into iso-propanol (containing 0.3 M sodium acetate). Pelleted material was washed in 70% ethanol and dissolved in 800 μl H_2O. DNA contamination was removed using deoxyribonuclease 1 (DNase I amplification grade, Life Technologies), following which samples were again precipitated in ethanol, washed, and dissolved in H_2O. Material for microarray was further purified using the RNeasy® clean up kit (Qiagen).

Microarray hybridization and data capture

Whole genome microarrays were prepared and hybridized as described previously.[11] The array consisted of a total of 3924 PCR-generated gene specific elements, designed to minimize cross hybridization using available genome information,[12] which were spotted onto polylysine coated glass slides (See http://bugs.sghms.ac.uk/). Each array was the result of a hybridization between 2 μg of extracted RNA (DNase treated) and a control. The control channel was prepared using 2 μg of H37Rv genomic DNA, extracted following a previously published method,[13] as the template for random primers. Resulting products were then Cy-labelled in a 90 min incubation at 37°C, following the addition of 5 μl 10X REact 2 Buffer, 1 μl dNTPs (5 mM dA/G/TTP, 2 mM dCTP), 5U Klenow, and 1.5 μl Cy3/Cy5 (all Life Technologies, except Klenow: Amersham Pharmacia Biotech). Microarray scanning was performed with an Affymetrix 428 scanner, following which fluorescent spot intensities were quantified using ImaGene 4.1 software (BioDiscovery Inc, USA).

Data handling and normalization

Data from ImaGene files were processed using the statistical computing package R (http://www.cran.r-project.org), which allowed the flexibility to pre-process/filter data prior to normalization. Data were also analysed in GeneSpring (version 6, Silicon Genetics, www.sigenetics.com). RNA extractions were taken at each of seven time points: One during exponential phase growth (day 5), and the others following entry into stationary phase at days 18, 49, 62, 75, 102, and 104. Each RNA extraction was used to generate technical replicates for a specific time point. Data from each time point was then normalized to allow comparison of gene expression. This was accomplished by following a method of array surface normalization published previously,[14] which accounts for signal/background variation across the entire array. We used $\log_2$(RNA signal/DNA signal) values, with all signal values below 1.0 being set to 1.0. A two-dimensional loess surface to log ratio was

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then calculated to allow correction of spot values. Following this, the lower 20th percentile of data was removed to reduce unwanted variability. Gene values from technical replicates were then averaged and all resulting values had their day 5 (mid-exponential growth) counterpart subtracted. Thus, a comparison between gene expression at day 5 and expression at all other time points was created. The calculation of *p*-values by *t*-test highlighted genes showing significantly different expression to that observed during exponential phase growth. Finally, all *p*-values were corrected using the Benjamini-Hochberg method to take into account multiple experiment testing.

Taqman verification of microarray output

Oligonucleotide primers and probes to *groES, mas, mpt83, nuoA, Rv0188, Rv2745c, sigB* and *sigA* were designed using the software Primer Express (PerkinElmer, California, USA). All probes were labelled with the fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5′ end and *N; N; N'; N'*-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end (both MWG Biotech, Milton Keynes, UK), with the exception of *sigA*, which had VIC® (PerkinElmer) attached to the 5′ end. All primers and probes are listed in Table S1 (on the web) with their respective modifications and concentrations in reactions. All comparisons of mRNA levels were carried out as multiplex reactions using *sigA* as a reference in combination with one other gene. TaqMan Universal One step Master mix (PerkinElmer) was used in amplification reactions, and each sample was processed in duplicate along with a multiscribe negative to control for possible genomic DNA contamination. Each was carried out in a final volume of 25 μl and cycled under the following conditions: 30 min at 48°C, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. In order to normalize the individual reactions 6-carboxy-x-rhodamin (ROX) was always included as passive reference dye. The difference between cycle threshold value (Ct-value) was used to indicate template quantity, based on the baseline-corrected normalized fluorescence (ΔRn), with the difference in *sigA* Ct-value being subtracted as a constant control.

Results and discussion

A single microbiological culture of *M. tuberculosis* was continuously monitored and maintained at 50% DOT for in excess of 100 days, while allowing extraction of culture material for the biochemical measurement of glycerol and glucose depletion, and assessment of the bacterial population by CFU counts. The culture pH showed an initial drop from 6.71 to 6.43 within the first 10 days, following which little variation was observed over the remainder of experiment, resulting in a final pH of 6.42 (day 104, data not shown). Glucose and glycerol levels in the 7H9 broth were both initially at 2 g l^{-1}. The rate at which they were utilized is shown in Fig. 1. Glucose dropped rapidly down to ≈200 mg l^{-1} within a period of 5 days post-inoculation but thereafter only demonstrated a gradual depletion over the remainder of the culture period, with a final measurement of 78 mg l^{-1} at day 109. In contrast, glycerol utilization within the culture was less rapid but reduced to undetectable levels between days 12 and 18. According to colony forming unit counts, the population of bacilli entered stationary phase by day 15, the cell density having risen to greater than 1×10^9 cells ml^{-1} in the preceding logarithmic stage. However, the exhaustion of nutrients then led to a drop in viability, leaving about 0.01% of the original viable population (Fig. 2). The observed progressive drop in viability occurred following entry into stationary phase for a period of about 60 days, resulting in a measurement of 1.6×10^5 CFU/ml by day 78. After this period, the drop in viability ceased, and the remaining three measurements showed increases consistent with the emergence of a small population of bacilli adapted sufficiently to nutrient depletion to re-establish cell division. The final measurement taken at day 111 was 2.1×10^6 CFU/ml, indicating an approximate four-fold increase in viable bacilli following the measurement taken at day 78.

Statistical interpretation of array data

RNA was extracted from culture material during mid-exponential phase growth (5 days post inoculation) and through out stationary phase (see arrows, Fig. 2). A lower amount of RNA was obtained from stationary phase extractions but quantities were still sufficient to produce replicates in all time points apart from day 102. An R^2 value was calculated for each set of replicates in the time course, and plots were generated to demonstrate how the removal of low intensity fractions of data affected R^2 (Fig. 3). The R^2 values calculated were consistently high enough (0.621–0.839) to suggest that the majority of variation in the time-course data was due to changes in gene expression between time points, rather than variation between technical replicates. Further to this, removal of low intensity values ($\log_2$RNA/DNA) had a beneficial effect, increasing the value of R^2 as successive fractions were removed (Fig. 3). This demonstrated that most unwanted variation in the time course data resulted from spots with low hybridization levels, and all further analysis was carried out on a data set with the bottom 20th percentile removed.

Differences in gene expression were initially viewed using a normalization strategy that compared the expression of surviving bacilli at days 18, 35, 49, 62, 75, 102, and 104 to that seen in exponentially growing cells at day 5. In particular, this highlighted a relatively consistent pattern of gene expression, following entry into stationary phase, up to day 75 (Fig. 4). A correlation tree generated by comparing data from individual arrays also demonstrates that mRNA profiles produced from exponentially growing cells were quite distinct from those from the same population in stationary phase up until day 75 (Fig. 5). All genes that were significantly up or down-regulated ($p \leq 0.05$) at all time-points including days 18, 35, 49, 62, and 75 are displayed in Tables S2 and S3, see the web. Arrays from days 102 and 104 seem to cluster more closely to those from exponentially growing cells (Fig. 5). Viable count data may be suggestive that the population began dividing again by these latter time points, and this could explain the apparent shift back to expression profiles similar to those observed from exponentially growing cells.

Taqman verification

A set of 7 genes were selected for quantitative analyses of mRNA levels in sampled material from day 5 and day 18, to verify the results obtained using microarray. The results of assessed genes are displayed in Fig. 6. Of the genes tested, both methods gave satisfactorily similar results in 6 out of the 7 genes tested, when comparing the regulation between the two time points. Only the regulation predicted for groES highlighted differences between the predictive capacity of the two methods. This latter result may be explained as an array artefact resulting from the up-regulation of whiB3 on the opposite strand. This may have caused transcriptional read through into groES, highlighting a problem in the interpretation of data from PCR generated microarrays,[14] which should be taken into consideration when analysing array data.

Biological analysis of microarray data

We developed a defined model of latency based on the hypothesis that the bacilli able to survive entry into stationary phase in vitro may be similar to the bacilli that cause persistence in vivo. A comparison of expression profiles generated from the bacterial population in logarithmic phase growth (day 5) and those generated from stationary phase cells highlighted a subset of genes with significantly differential regulation for the period of time including days 18, 49, 62, and 75 (Fig. 4). These genes have been grouped according to functional classification in Table S4 (on the web). Functional classes that contain more significantly up-regulated than down-regulated genes include those involved with fatty acid degradation, the glyoxylate shunt, anaerobic respiration, amino acid/amine degradation, RNA synthesis, RNA modification and DNA transcription. Conversely, functional classes

that contain more significantly down-regulated, as opposed to up-regulated genes, include carbon degradation, *de novo* generation of ATP, and purine/pyrimidine synthesis.

Bacteria were continuously aerated at 50% DOT, and consistent with this we did not observe a general up-regulation of genes associated with the previously published hypoxia induced dormancy regulon.[15] However, 2 genes from this regulon (*Rv2629* and *Rv2630*) were significantly up regulated. In addition, 4 genes involved in anaerobic respiration were up regulated (*narG, cysH, nirA* and *fdhF*) and 5 genes involved in aerobic respiration (*nuoA/D/J/L* and *glpD2*) were down regulated, even in the presence of high levels of oxygen. This may highlight a subset of genes within regulons normally associated with hypoxia that can be independently regulated as a consequence of the altered metabolic state associated with stationary phase.

Metabolic adaptation

It has been suggested previously that the rise in the levels of isocitrate lyase in response to macrophage ingestion allows bacilli to survive via the β-oxidation of fatty acids, which are found within human tissue.[16] Isocitrate lyase is the product of *aceA*, one of only two genes in our model that were significantly up-regulated in the glyoxylate shunt (the other being *aceAa*), increasing in expression steadily from a 2.6-fold up at day 18, to 9.4-fold by day 75. This is in contrast to the findings of Betts et al who found no up–regulation of this enzyme in their nutrient limitation model, in which bacteria were instantaneously starved and assessed for up to 4 days afterwards.[7] The gradual rise of *aceA* seen throughout our time course may not have been apparent at a time point earlier than day 18.

Within the TCA cycle the up-regulation of *gltA1* was also apparent (approximately 2 fold at day 18, rising thereafter as high as 8.3-fold). In *E. coli* GltA catalyses the condensation of oxaloacetate and acetyl coenzyme-A to produce citrate plus coenzyme-A;[17] in *M. tuberculosis* it may also act to feed proprionyl CoA through the glyoxylate and carboxylic acid cycles.[18] This enzyme is therefore likely to be up-regulated to process the increasing levels of CoA carriers resulting from β-oxidation. The only other heavily up-regulated gene associated with the TCA cycle is *pckA*, the rate limiting step in gluconeogenesis (2-fold at day 18, rising thereafter as high as 2.8-fold), suggesting that a portion of the cycling carbon units required rerouting into gluconeogenesis. These data infer that the metabolic regulation of cells adapting to stationary phase was very similar to that observed in bacilli adapting to the phagosomal environment of a macrophage.[18]

The likelihood that β-oxidation of fatty acids was occurring during this period is also verified by the observation that a number of genes involved in fatty acid, butanoate, and propanoate metabolism were also up-regulated. The replenishment of acetyl-coA via such metabolic pathways requires the four steps of dehydrogenation, hydration, oxidation, and thiolysis. Examples of a number of genes capable of carrying out these four steps are displayed in Table S5 (on the web). Only the hydratases listed are not deemed significantly up-regulated according to our criteria ($p \leq 0.05$), although *echA11* and *echA12* were both up-regulated throughout, had relatively small error bars in replicates, and were not thought to be falsely assigned due to read through of opposing strand genes. The up-regulation of *fadA4* and *scoA* ($p \leq 0.05$) was also accompanied by *scoB*, and these enzymes in particular, would be vital to the balanced utilization of ketones resulting from β-oxidation. In summation, the depletion of glycerol and glucose created a surviving population relying on the balanced β-oxidation of fatty acids to drive the supply of central metabolites via the glyoxylate shunt and gluconeogenesis.

Transcription, translation and protein regulation

The sigma factors expressed by *sigB*, *sigE*, and *sigH* are known to alter the expression of a subsets of genes necessary to adapt to stresses in vivo.[19] Published work to date demonstrates that, although different types of stress tend to be associated with the up-regulation of different sigma factors, a hierarchy of control exists, in which *sigH* is at the top.[19] The up-regulation of all three of these sigma factors was seen throughout the period of stationary phase adaptation between day 18 and 75 ($p \leq 0.05$), suggesting that nutrient depleted bacteria were increasing the expression of genes thought to be involved in virulence and persistence processes.[20–22]

The down-regulation of ATP synthase genes in bacilli following entry into stationary phase has been characterized in this and a previous study.[7] A total of 202 genes in the *M. tuberculosis* genome encode proteins that contain the 'ATP/GTP binding site motif A'. Reduced levels of cellular ATP should make such proteins 'expensive' to the cell. However, 21 of these genes were significantly up-regulated during stationary adaptation and are displayed in Table S6 (on the web). The majority of the genes in (Table S6, on the web) impose a tighter control on the levels of transcription, translation and protein levels within surviving bacteria, which are discussed below. Increasing expression of *rpoA* suggests that functioning RNA polymerases were vital to the survival of the stationary population, supporting the previous findings that persistent bacilli are transcriptionally active.[4] The up-regulation of transcripts from *rpoB*, *rpoC* and *Rv1390* was also recorded, the protein derivatives of which do not have ATP/GTP binding motifs, but are required for RpoA function. The inclusion of *rpsD* (producing the 30 s protein S4) also suggests the importance of functioning ribosomes, as the S4 component is known to regulate their formation in *Bacillus stearothermophilus*, being one of the first proteins to interact with the rRNA.[23] The up-regulation of *ftsH* in stationary bacilli indicates their increased requirement for ribosomal transfer of polypeptide chains to translocation machinery, since homologues of FtsH have previously been demonstrated to act in this way.[24] (Table S6, on the web) also contains *infB*, *miaA*, and *rhlE*, all of which have been demonstrated to affect transcriptional regulation in other bacterial species,[25–27] in which they have been hypothesized to promote alterations in the levels of specific mRNA species. The data also indicates that the levels of protein appear to be more tightly regulated, by the inclusion of the protease genes *clpC* and *clpX* in Table S6, on the web (*clpP* and *clpP2* were also significantly up-regulated). Proteolysis functions as a key regulatory mechanism and impacts not only on the stability of key metabolic enzymes but also on the effective removal of terminally damaged polypeptides.[28] The ClpXP protease in *E. coli* is involved with DNA damage repair and stationary-phase gene expression.[29]

Metabolism of sulphur

A total of 7 genes involved in the metabolism of sulphur were significantly up-regulated, including *cysG, cysD, cysQ, cysH, cysA, cysN* and *cysT* (the latter 4 also appear in Table S6, on the web). Sulphur is an essential component in maintaining translation initiation and redox maintenance, the metabolism of which is controlled by the *cys* genes. In *Salmonella typhimurium cysG* is involved with the production of precursors to siroheme, a cofactor required for SO_3^{2-} reduction, that is also vital for the reduction of nitrite.[30] Sulphation also requires the presence of the activated sulphate form phosphoadenosine 5′-phosphosulphate, an important intermediate of the sulphate assimilation pathway, and this would likely be accomplished by the sulphurylase encoded by *cysD* and *cysN*. Furthermore, the inclusion of *cysT* and *cysA* (producing a permease and sulphate transport ATP-binding protein) is demonstrative that the surviving population is relying more on scavenging for cysteine containing proteins from the extra-cellular environment.[31]

Stationary phase operons

Many of the genes that are significantly up-regulated (Tables S2) during stationary phase adaptation are located in 'operon like clusters'. One such cluster of genes seen in our study (*rsbW, Rv3288c, Rv3289c*, and *lat*) has previously been identified as up-regulated in the stationary phase survival study by Betts et al., although we saw no up-regulation of the proposed transcriptional regulator *Rv3291c*.[7] Other gene clusters present include *Rv0982-Rv0984* (*moaB2*), *Rv1460-Rv1463, Rv3138-Rv3141* (*pflA, fadE24, fadE23*, and *fadB4*, respectively), and *Rv3458c-Rv3460c* (*rpsD, rpsK*, and *rpsM*, respectively). The largest cluster of genes includes *Rv3864, Rv3867-Rv3871, Rv3876*, and *Rv3878*, the majority of which fall within the RD-1 region deleted from strain BCG Pasteur.[32] In addition, the genes *cfp10/esat6* (*Rv3874/Rv3875*) were both up-regulated between day 18–61 (approximately 5-fold or higher), but by day 75 *cfp10* dropped to the equivalent of 4-fold below the level of expression observed during exponential phase. The product of *esat-6* is an example of a potent immunogen in diseased patients, that only seems to be present within the *M. tuberculosis* complex.[33] The inclusion of *Rv0287/cfp-7* in the genes displayed in (Table S6, on the web) also suggests the importance of other WXG100 members in stationary phase, that have previously been proposed as part of a novel secretory network.[34] The observed regulation of this region in our stationary phase model would therefore imply that stationary bacilli were increasing their expression of virulence associated genes.

Cell wall lipids

The ability of *M. tuberculosis* to synthesize and transport the complex lipid phthiocerol dimycocerosate (PDIM) to the exterior of the cell has previously been suggested as a crucial factor in virulence.[35] The production and transport of PDIM is a complex cellular process involving a number of genes. The best characterized are the *ppsA-E* gene cluster and *mas*, described to be involved with the production of the components necessary to form PDIM. All genes associated with the mas gene cluster were up-regulated throughout the period of stationary phase adaptation (*mas, fadD26, fadD28, mmpL7*, and *ppsA-E*). In addition, at day 75 a sharp rise in expression of *fadD26, fadD28* and *mas* was apparent, to levels of 11-, 23-, and 30-fold up, respectively. Further more, the up-regulation of *pks2, pks3/4, pks10* and *pks13* was also apparent during this period. A *pks10* knockout mutant has been shown to be incapable of synthesizing DIM,[36] and mutants in *pks3/4* have been shown to be defective in the production of mycolipenic acids, thought only to be present in virulent strains.[37] Additionally, the *mas* like gene *pks2* is responsible for encoding a hepta/octa-methyl branched fatty acid synthase, suggested to be important in the generation of sulpholipids that also have a postulated role in virulence.[38] Taken together these data indicate that bacilli adapting to stationary phase up-regulate the production of selected cell wall lipids that have been associated with virulence in models of *M. tuberculosis* infection.[35]

Rv0539 is present in (Table S6, on the web), representing a further example of an 'expensively' up-regulated gene containing the 'ATP/GTP binding site motif A'. In the recently re-annotated version of the *M. tuberculosis* genome, *Rv0539* was suggested to show homology to a dolichyl-phosphate-mannose synthase precursor from *Trypanosoma brucei*.[39] Many moieties in the mycobacterial cell wall may be capped with mannan by such enzymes, and certain lipids modified in this way are thought to enhance virulence.[40] The requirement for mannan indicates the importance of gluconeogenesis to stationary phase bacteria, demonstrated by the up-regulation of *pckA* in this study.

Adaptation to nutrient depletion

A rapid reduction in the ability of bacteria to synthesize protein leads to the accumulation of the stringent response signalling molecule (p)ppGpp which in turn reduces transcription of stable RNA genes.[41] The enzyme Rel_{mtb} (the only ppGpp synthase homologue in *M.*

tuberculosis) is required for survival of mycobacteria under long term in vitro starvation conditions.[42] Microarray studies characterising the response of *M. tuberculosis* to both a fall in available nutrients and hypoxia, have demonstrated a rise in rel_{mtb}.[7,43] However, both studies characterized a fairly short-term response to such conditions. Our time course data suggests that the levels of rel_{mtb} transcript increased at day 18 (≈3-fold increase) but then showed gradually decreasing levels at all other time points. These data would support previously published data that rel_{mtb} is involved with an initial adaptation to depleting nutrients that facilitates long-term survival.[42] The type of regulatory pattern seen for rel_{mtb} was also observed for *aceAb*, which is heavily up-regulated at day 18 but then appears to drop substantially following this. Such examples may be representative of a larger subset of genes required for the initial transition in growth. It has previously been suggested that increasing levels of (p)ppGpp in *E. coli* may be the sole inducer of *qor*, a gene shown to be up-regulated in response to conditions of stress, which seems to function by reducing the quinone pool.[44] However, in our model following the drop in expression of rel_{mtb} at day 18, *qor* stayed up-regulated through out the time course and even showed a substantial increase at day 75, suggesting that other levels of regulation are present.

Re-activation of growth

The data between days 18 and 75 have been used to highlight the survival tactics of a bacterial population entering stationary phase following the depletion of glucose and glycerol from a media source that was being continually aerated at a DOT of 50%. However, there are still definite differences between profiles at each of these time points. The correlation tree presented (Fig. 5) demonstrates great similarity between arrays processed from days 48 and 61, but arrays from day 18 and day 75 grouped on two subtly different branches. The transient up-regulation of a subset of stringent response genes discussed may be the reason for differences in expression at day 18. It is also possible that differences in expression at day 75 may be attributable to a population now progressing towards a controlled division of cells. The viable count measurements taken at days 78 and 96 suggest that cell numbers approximately doubled over this period (see Fig. 2), and counts recorded at day 111 were about four-fold higher than those from day 78. Changes in gene expression patterns were consistent with a chromosomal preparation for division at day 75. The up-regulation of *dinG*, *dnaE1*, *dnaZX*, *polA*, *mesJ*, *rodA*, *Rv0605/Rv2792c/Rv2886c* (resolvases), *Rv1708/Rv3213c* (possible role in chromosome partitioning), *Rv2309c* (integrase), and the cell division protein *Rv0435c* was observed at this time point. The function of many of these genes would seem to be involved with repair of the chromosome prior to the resetting of stalled replication forks.[45] The possibility that DNA repair was occurring at day 75 is also supported by the observation that, with the exception of *recX* and *recR*, the remaining *rec* genes became up-regulated at this time point. Both *recX*, producing a RecA regulator, and *recR*, involved with the RecBC-independent process of DNA repair, were up-regulated on transition into stationary phase but became gradually down regulated from this point onwards. Regulation of *priA* is also consistent with these observations, being heavily down-regulated following entry into stationary phase, but rising much closer to the level of expression seen in exponential phase growth at day 75. In *E. coli*, the helicase PriA has been implicated in loading replisomes back onto failed replication forks and is thought vital for their repair.[46–48] The up regulation of priA therefore suggests that cells were beginning to reinitiate chromosomal division. Furthermore, the up-regulation of *rodA* observed at day 75 also support the hypothesis that a reactivation of cellular division was imminent. In *E. coli* side wall murein synthesis during elongation of the cell wall is thought to be carried out by pbp proteins that are not fully functional without the presence of RodA.[49] The increase of RodA within the population of surviving bacilli would therefore indicate the commencement of cell wall elongation.

Conclusion

The aerobic nutrient depletion–adaptation model presented in this paper was based on the hypothesis that the sub-population of bacteria in vitro that survive entry into stationary phase may represent the persistent bacilli that develop during *M. tuberculosis* infection. A continued adaptive response to the depletion of glucose and glycerol from such a culture was observed up to 75 days post inoculation. Bacilli up-regulated a number of the genes thought to be important during the infective process in vivo, including many associated with virulence. Other genes that remained up-regulated during this period suggest that surviving bacteria were metabolising fatty acids, either present in the culture supernatant or part of an endogenous intracellular supply, while more tightly controlling a number of other metabolic functions, including cellular division. The proposal that a re-activation event occurred following day 75 suggests that the surviving sub-population had adapted sufficiently to the depletion of glucose/glycerol to begin dividing again, perhaps akin to a reactivation event in vivo.

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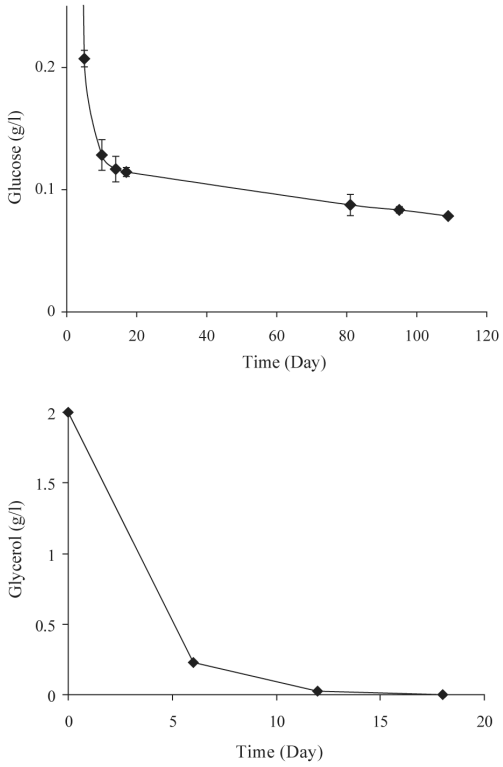

Figure 1.
Nutrient depletion levels observed in the 50% DOT culture of *M. tuberculosis*. Error bars represent two standard deviations from the mean. Cultures started with $2gl^{-1}$ of both glycerol and glucose. Identical data were also produced from a separate culture that was set up and run for 50 days (data not shown).

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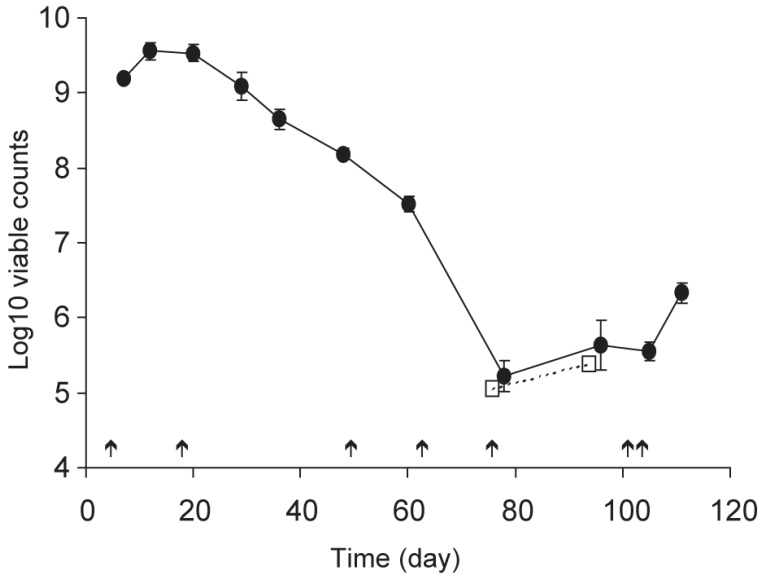

Figure 2.

Viable counts for 50% DOT culture. Values displayed represent the average of two dilution series plated out in triplicate. Error bars represent two standard deviations from the mean. Almost identical viability levels were recorded in another culture that was set up and run for 50 days (data not shown). Arrows displayed across the *x*-axis demonstrate the time points at which RNA was extracted from the culture for microarray analysis. Boxes connected by dotted line represent the difference in viable counts expected if the number of viable bacilli recorded at day 78 had doubled by the next reading.

Time (day)	Replicate R^2
Day 5	0.621
Day 18	0.755
Day 49	0.839
Day 62	0.822
Day 75	0.711
Day 102	n/a
Day 104	0.814

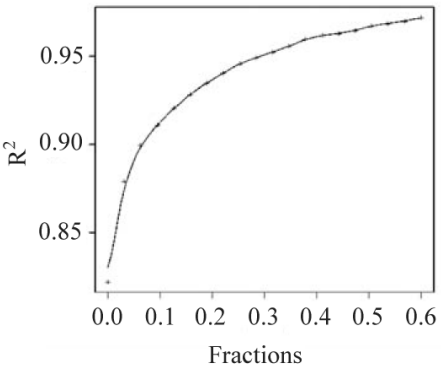

Figure 3.
Tabulated R^2 values for array replicates (n = 5, 3, 3, 3, 2, 1, and 2 for days 5, 18, 49, 62, 75, 102, and 104, respectively) at each individual time point in the 50% DOT culture. Changes in R^2 for an individual time point (Day 62, 50% DOT) as increasing fractions of low intensity data are removed is also displayed.

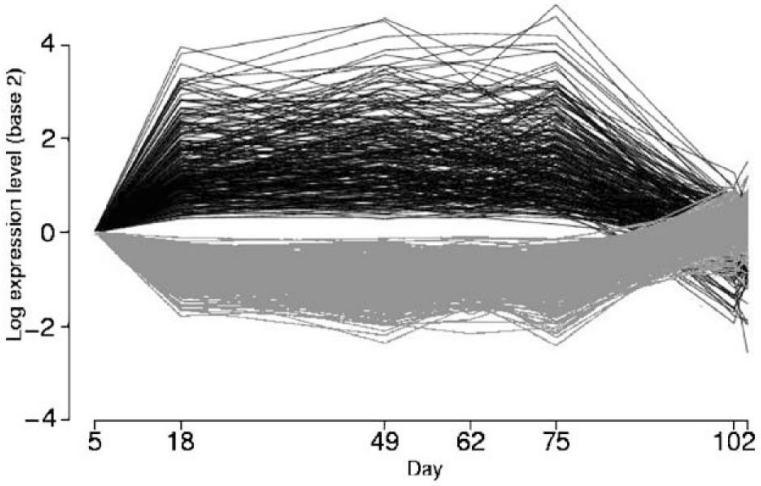

Figure 4.

The regulation of two sets of genes are displayed through out the entire time course (day 5 to day 104). The two sets of genes displayed consist of those deemed either significantly up-regulated (black lines) or down-regulated (grey lines) through out a period of stationary phase adaptation between days 18 and 75. Every time point has been normalized so that the gene expression levels recorded are compared to exponential phase growth (day 5). Following day 75, the consistently similar pattern of expression observed in the genes displayed broke down, coinciding with the suggestion from viable count data that bacillary division had recommenced.

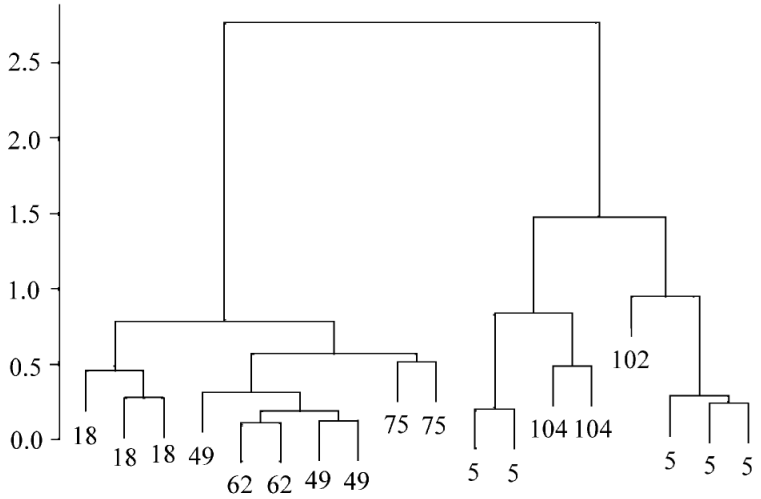

Figure 5.
Correlation tree calculated using each array within the 50% DOT stationary phase time course. The bottom 20th percentile of data was removed before a Spearman rank correlation was used to cluster individual arrays. Clustering is displayed as day 5 (5 arrays); day 18 (3 arrays); day 49 (3 arrays); day 62 (2 arrays); day 75 (2 arrays); day 102 (1 array); and day 104 (2 arrays). Array profiles resulting from days 18–75 were quite separate and distinct from those generated from exponential phase growth.

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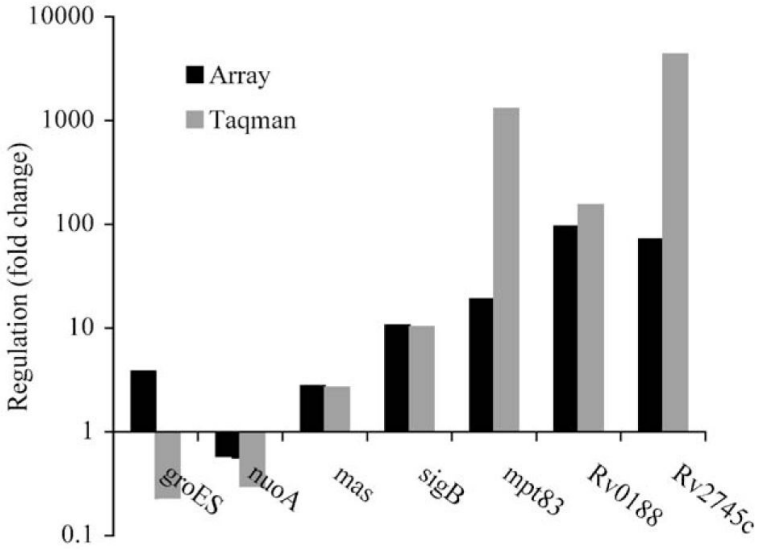

Figure 6.
Comparison of the results attained when assessing mRNA changes between day 5 and day 18 of the time course using either loess normalized microarray data or quantitative RT PCR (Taqman). Similarity in the two separate experimental techniques was observed in all but one of the genes assessed. Transcriptional read through may have affected the microarray data for *groES*.[14]