Author's response to reviews

Title: Identification of Gene Targets against Dormant Phase Mycobacterium tuberculosis Infections

Authors:

Dennis J Murphy (Dennis.6.Murphy@gsk.com)
James R Brown (James.R.Brown@gsk.com)

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Author's response to reviews: see over
Dr Annabel Phillips  
Senior Assistant Editor  
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Re: MS: 1024182486134272 - Identification of Gene Targets against Dormant Phase Mycobacterium tuberculosis Infections  

Dear Dr. Phillips:

We have uploaded a revised version of our manuscript which incorporates many of the changes suggested by the three reviewers. We very much appreciate their constructive comments as well as their overall positive support for acceptance. We describe below our responses to the specific comments of each reviewer.

Reviewer: Valerie Mizrahi  
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Major Compulsory Revisions
1. Pg. 11, line 16-18. The authors state that “the foremost assumption is that genes that are highly expressed in a number of experimental situations are important for persistence of non-replicating M. tuberculosis”. A consequence of this assumption is that the list of potential dormancy phase targets is heavily biased towards genes/pathways that are highly induced under hypoxia or nutrient starvation in vitro, in macrophages, in mouse lung and in hollow fibre implants in mice. However, one of the most important findings from the study by Rengarajan et al. (PNAS 2005; 102: 8327-8332) is the remarkable lack of concordance between genes that are regulated and those that are essential for survival in macrophages with the majority of genes required for survival in macrophages being constitutively expressed. The authors must reconcile their conclusions regarding target prioritization with these findings.

Response: In the macrophage model used by Rengarajan et al., the M. tuberculosis bacterium is in growth phase mode and genes essential for growth are known to be highly expressed for most
species of bacteria. However, in its nonreplicative dormancy phase, *M. tuberculosis* undergones major metabolic changes and reduces its growth potential. Thus differential gene expression patterns are to be expected when contrasting bacteria cultured under simulated granuloma environments characterized by low nutrients and hypoxia to the more nutrient rich, growth inducing environment of the macrophage. The overall goal of our study was to identify gene targets specific to the eradication of dormancy phase, *M. tuberculosis*, which are likely to be non-replication related targets. We attended to clarify this position and reconcile our study with Rengarajan et al. by adding to the Discussion section:

p. 12-13 “As an example, Rengarajan et al. found that genes expressed under hypoxic or low nutrient conditions simulating the granuloma environment, are not necessarily essential for intracellular growth in macrophages. [12] In bacteria, growth essential genes are often constitutively expressed while virulence and environmental response genes tend be highly regulated yet essential during those periods of high induction. Thus one would expect that different groups of genes play distinctive roles, and have transient importance for viability, in the non-replicative versus replicative phases of the bacterium’s life cycle.”

2. Pg. 12, lines 2-4 and Table 2. It is not at all clear how – if at all – the growth/ gene attenuation score obtained by meta-analysis of the three TraSH datasets were used to determine which genes were selected for inclusion in the “prioritized” targets and target classes given in Table 2. This point must be clarified in order to explain why, for example, Rv2005c, which has a growth attenuation score of zero was included in Table 2, but Rv2004c, which has the highest growth inhibition score (as reported on pg. 9) and an upregulation score of 11.9995 was not. This question is particularly relevant in light of the statement that “Targets that show both up-regulation in the dormant state and inhibit growth are certainly more interesting, but it may well be the case that genes [that] are required for survival in the non-replicating state have no effect on growth”. Similarly, the authors must explain if and how the downregulation score was used as a criterion for target/ pathway selection. For instance, why is sigJ, which only shows a low downregulation score, included in Table 2?

Response:

As mentioned earlier, the objective of this study was to integrate a meta-analysis of gene expression and essential data with biochemical and pharmacological rationale in order to propose potential targets against dormancy phase TB infections. While computational methods can yield a list of hit scoring genes, it is necessary to reconcile proposed targets with other components of the involved pathway. Thus we decided that it would be useful for the reader to see the values and descriptions of all genes associated with proposals for targeting specific pathways. We have tried to clarify this approach by adding to the Discussion section:

p. 13 “Here we discuss the relative tractability of proposed dormancy phase modulated genes and pathways as drug targets for the eradication of dormancy phase M. tuberculosis. The individual genes and scores discussed below are listed in Table 2. Since not every gene in a particular pathway scored highly, we included additional low scoring pathway proteins in Table 2 in order to provide context for targeting particular pathways.”
Also the Table 2 header now reads:

“Table 2. Pathways and associated genes which are potential TB dormancy phase targets. Multiple genes in the same pathway are included for score comparisons.”

To reply to the specific question about Rv2004c, this gene is currently annotated only as a conserved hypothetical protein, so its biochemical function is unknown. Thus despite the attractiveness of the growth inhibition result, there is no practical way at present to target this gene.

3. In addition to the criticisms above, the list of genes shown in Table 2 would be far more convincing and useful if expanded to include other parameters pertaining to their quality and tractability as targets such as druggability, availability of a structure, presence/absence of a human homologue, uniqueness of the reaction catalysed, and so on.

Response:

The tractability of each gene is discussed in detail in the text. However, we have added to Table 2 under the column “Brief Annotations”, human homolog (with BLAST E-values; E-value greater than 1e-10 used as cutoff) and relevant crystal structures, if available.

4. Why were the DeaDMan gene essentiality data of Lamichhane et al. (Infect. Immun. 2005; 73:2533-2540) not taken into consideration when calculating the growth attenuation scores?

Response:

We are aware of the Lamichhane et al. paper but did not use their gene essentiality data mainly because it is not a complete genome screen (530 out of 3990 ORFs) unlike the included published TrasSH analyses. Weighting a partial dataset would seem to add too much bias to our analysis. In addition, they used a different strain of M. tuberculosis, CDC 1551 rather than H37Rv. A cursory review of the genes listed showed substantial overlap with other published essential studies.

5. Table 1. The differences in maximum scores given to the various data sets make some sense for the expression data on the grounds of relevance as a dormancy model. However, how does this scoring system apply to the three TraSH data sets, which were all arbitrarily given a maximum score of 5? Are they all equally relevant as dormancy models and, if not, why are they scored equally? (One might argue that none of these is relevant to dormancy and that the only relevant data sets would be those that identify genes that are conditionally essential under the conditions deemed to be relevant to dormancy, such as hypoxia, starvation, etc. This question relates to the issue of the value/weighting placed on the growth attenuation score as an indicator for target prioritization, as outlined above.

Response:
As the reviewer seems to logically discuss, there is the issue that no TraSH experiment has been performed, or could be performed, on M. tuberculosis in dormancy phase since gene essentiality is determined by affect on growth. Rather than trying to speculate which of the growth phase TraSH studies most closely approximated dormancy phase conditions, in light of no clear best candidate, we decided to simply apply equal weight to all 3 datasets (Table1).

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Minor Essential Revisions
6. Figure 2 is confusing and requires clarification. What is meant by “murine”? The authors should also make it clear that these data pertain only to the expression analysis data and not to the gene essentiality data.

Response:

Caption now reads:

“Fig. 2. Overlap of the top 400 highest-scoring genes (~10% of the genome) from each of the three types of gene expression models of dormancy. Murine refers to M. tuberculosis cells isolated from mouse macrophages, subcutaneous hollow fiber, and lung.”

7. There are many typographical and other errors in the manuscript which must be corrected.
Response:

Typos corrected and terms clarified. New page numbers given in brackets:

a. Pg. 4, line 12: ethambutol. Done [p.4]
b. Pg. 6, line 9: delete “that” Done [p. 6]
c. Pg. 6, bottom line: delete “.” after “step 2” Done [p. 7]
d. Pg. 7, 4 lines from bottom: replace “in vitro” with “hypoxia” Done [p. 8]
e. Pg. 9, line 5: remove underline from “dosR” Done [p. 9]
f. Pg. 9, lines 16-18. What is meant by the statement that “DevS, a regulator of dosR (see below) inhibits growth in vitro”? Are the authors referring to the positive growth/ gene attenuation score for this gene? Done. [p. 10] Essential according to TraSH study. Citation included for Sassetti CM, et al. Mol Microbiol 2003, 48: 77-84.
g. Pg. 11, line 14: delete “and” Done [p. 12]
h. Pg. 11, line 18: change to “criterion” Done [p. 12]
i. Pg. 12, line 3, insert “that” after “genes” Done [p. 13] Sentence reworded:
“Thus one would expect that different groups of genes play distinctive roles, and have transient importance for viability in the non-replicative versus replicative phases of the bacterium’s life cycle.”
j. Pg. 13 sub-heading and elsewhere in the text: stick to one nomenclature for the Dos/Dev system, i.e. either use DosR/S/T throughout, or DevR/S/T, not a mixture of the two. Done. We decided to use Dev since this gene annotation is the standard across bacteria in GenBank.
k. Pg. 13, line 21: Consolidate reference numbers into a single set. Done [p. 13]
l. Pg. 14, line 5: Replace “Saini’s” with “Tyagi’s”. Done [p. 15] Sentence reworded: Paving the way for testing this in the near future are recent papers which describe a validated high-throughput screening assay, [50] and x-ray crystal structures of the C-terminus of devR, with and without DNA bound. [51].
m. Pg. 14, lines 6-7. This sentence requires elaboration/clarification. What is meant by “component 2”? Is it the regulator of a two-component regulator system? Done [p. 14] Sentence reworded: “These systems consist of a sensor kinase (component 1) which autophosphorylates histidine, then transphosphorylates a conserved aspartate of its cognate transcriptional regulator (component 2).”

n. Pg. 14, line 8: insert “of” after “combination”. Done [p. 15]
o. Pg. 14, line 10: insert a full stop after “[47]”. Done [p. 15]
p. Pg. 17, line 9: “bacillus”. Done [p. 18]
q. Pg. 18, line 2: What is meant by “of this enzyme”? Which enzyme? There are several enzymes involved in pantothenate biosynthesis. p. 19) Done. “Supporting this notion are inhibitors of pantothenate kinase inhibit the growth of the low GC Gram-positive bacterium, Staphylococcus aureus, in a typical MIC assay. [80]”

r. Pg. 18, line 4. What do the authors mean by the statement that the pantothenate biosynthesis knockouts of M. tuberculosis “do not behave as expected”? The purpose of assessing auxotrophs of M. tuberculosis in vivo is to assess the accessibility of the organism to exogenous sources of the co-factor/ vitamin/ amino acid etc. whose de novo biosynthesis has been disrupted. The published data suggest that M. tuberculosis can access enough exogenous pantothenate to survive. [p. 19] Done. Now reads: “However pantothenate biosynthesis knockouts in M. tuberculosis in mouse infection experiments suggest the operation of some salvage mechanism.”

s. Pg. 24, line 9: Insert full stop after “[104]” Done [p. 26 – now reference #105]
t. General: The combined TraSH data score is referred to “gene attenuation score” in Table S1, but as ‘growth attenuation score” in Table 2. Done. Table S1 now reads growth attenuation score.
u. References list: All references must be standardized to conform to a single format (e.g. ref. 33, 96 have the titles in capitals; some references abbreviate the journal title, whereas others do not) Done – An issue with Reference Manager which was used to generate the references and citations.
v. Table 2: Change “mprA” to “mprAB” Done [Table 2]
w. Figure 1, Step 2: insert “on” after “based”. Done [Figure 1]
x. Figure 3: complete the legend “intermediary metabolism and re…”; also “conserved in M. bovis” Done [Figure 3]

Discretionary Revisions (which the author can choose to ignore)
8. The Discussion section of the manuscript is very long. In some cases, the biological/biochemical context of each target/pathway is described in excessive detail and could be
considerably shortened, particularly if this paper is targeted at TB researchers who would be quite familiar with the published literature.

Response:

The changes we have made should help to clarify concepts and improve readability. Since this paper is in BMC Microbiology rather than a TB specialist journal, we feel that this paper should be more broadly accessible to non-TB researchers as well.

Reviewer: Martin Voskuil

No references in text to any of the supplemental figures?
Response: Yes, they are there:
Table S1, p 7.
Table S2, p. 9
Table S3, p. 11

Confusion would be avoided if the authors chose to use either DosR/S/T or DevR/S/T throughout the paper and not switch between them. I would suggest Dos as it stands for “dormancy survival” not “differential expression virulence” (Dev), which is clearly not the role of the DosR regulon. – Done throughout. As mentioned above, we decided to use Dev since this gene annotation is the standard across bacteria in GenBank.

Page 2, line 4: incidents should be incidence.
Done [p. 2]

Page 2, lines 5-7: It is not proven that drug treatment is complicated by dormant Mtb. Done sentence reworded:
“Eradication of TB is affected by the ability of the bacterium to survive up to decades in a dormant state primarily in hypoxic granulomas in the lung and to cause recurrent infections.”

Page 4, line 16: add non-replicating to “dormant phase” as there is no evidence that in the murine or macrophage models bacilli are “dormant”.
Done [p. 4]

Page 4, line 17: say four main types and add macrophage since that is mentioned in following sentences.
Done [p. 4]

Page 5, line 15: switch “methods” with studies, as there are methods to inactivate genes, but few studies have been conducted. -
Done [p. 5]
Page 7, and Figure 1: What criteria are used to determine “relevance to dormant state”, this seems very subjective.

Response: By way of clarification, the first paragraph of the Results now has:

[p. 7]“A zero to five scoring system was developed based upon two criteria. The first criterion was the overall relevance of the experimental conditions to persistence in the granuloma. The mouse macrophage and whole animals studies model the immediate response of M. tuberculosis to immune attack and long term survival in the host. The granuloma itself is characterized by avascularization and necrosis which have been modeled by the hypoxic and starvation conditions. The maximum score for a particular experimental dataset was adjusted based on potential relevance to the clinical occurrence of dormancy phase M. tuberculosis infections. For studies with multiple time series sampling, increasing weight was given to later time-points samples. The second criteria involved the rank order of gene expression in a particular study which allowed for cross study comparisons. (See Table 1 and Methods for details on the scoring scheme.)”

In the Methods:

[p. 31] “The data sets and scoring weights compiled for this analysis are listed in Table 1. A zero to five scoring system was developed that utilized both the relevance of the experimental conditions to the dormant state and the rank order of expression.

The maximum score for a particular experimental dataset was adjusted based on our judgment of relevance to the clinical occurrence of dormancy phase M. tuberculosis infections. For studies with multiple time series sampling, increasing weight was given to later time-points samples.”

Page 7, line 13: change “expressed genes” to induced genes as only induction from log phase is being measured in the referenced papers. This is actually a significant weakness in the analysis since just because a gene is not induced it doesn’t mean it isn’t on and important.

Done [p. 8]

Page 8 Section of multi-gene trends and figure 3 doesn’t appear to be very informative are important, could be removed.

Response:
We feel that this section is brief and provides an overview of genome-wide analysis results which are not covered in the other sections. Therefore, we would prefer to see it remain.

Figure 4, needs a y-axis legend, not clear what the figure is saying.
Response:
We added a legend, “No. of genes”. Fig. 4A and Fig. 4B are referenced in the text.

Page 9: is the dosR regulon information found in a supplemental figure?
Yes, this information is part of Table S2.
Page 9, lines 16-18: The statement starting with “DosS, a regulator” is incorrect and not demonstrated in Voskuil JEM 2003. There is no defect in a DosR mutant in a macrophage model and not in growth in vitro, but there is in the Wayne model.

Response:

Voskuil JEM 2003 was an incorrectly placed citation since the references were to growth inhibition in vitro and in mouse macrophages as inferred from TraSH results. The statement now reads:

[p. 10] “Only six of the 53 genes have an effect on growth in the gene disruption experiments (TraSH), supporting the idea that their main role is in shifting to and maintaining the non-replicative state. DevS, a regulator of devR (see below) inhibits growth in vitro [13] and significantly in the mouse macrophage model, [14] consistent with the response to nitric oxide. [27]”

Page 9, line 18: What is the reference for the USP having a growth defect? This should be referenced?

Response:

[p. 10] Again, this was inferred from the TraSH experiments. The proper reference has been added.

Page 13, line 8: latent should be chronic, since murine infections do not accurately mimic latent infections in humans.

Respond:

This sentence has been changed to:

[p. 14] “Mice lacking iNOS are rapidly killed by M. tuberculosis, whereas mice with functional iNOS yield chronic infections.”

Page 13, lines 10-11: These references do not say anything about the DosR regulon not being on and thus Mtb continues to grow. Currently there is little evidence to support this hypothesis. In fact in an iNOS KO mouse the regulon is still on, although at a lower level. See Shi et al PNAS 2003.

Response:

We substituted “activated” with “upregulated” which implies that the regulon is modulated not silenced. This sentence now reads:

[p. 14] “Without the nitric oxide signal, the regulon is not upregulated and cell division continues until the host is killed. [41] [42]”
Page 16, line 1: What would be the benefit over Rif treatment of targeting DosR?

Response:
In this section, the discussion concerns the targeting of other transcriptional targets rather than DosR. To clarify, the sentence has been modified:

[p. 17] “However, any novel anti-TB strategy based on the targeting of transcriptional components such as sigma-factors, would need to demonstrate some advantage over current rifampin therapy which also disrupts transcription through the inhibition of RNA polymerase activity.”

Page 17, last sentence: What do unusual carbohydrates have to do with Ac-CoA?

Response:
Ac-CoA is a critical intermediate in the biosynthesis of carbohydrates which, in turn, are critical cell membrane components. To clarify, we have re-worded this section as:

[p. 19] “Fatty acids, via breakdown to Ac-CoA and use of the glyoxylate shunt, provide the carbon for carbohydrate synthesis and acetyl-coenzyme A appears to be the gate through which much of the utilized carbon pool must pass. The M. tuberculosis membrane, being rich in unusual carbohydrates [78] [79], is likely critically dependent upon this carbon flux through Ac-CoA for the maintenance of its integrity.”

Page 19, second to last sentence: What is the “pH rate behavior”?
Done [p. 21]: The term “kinetic behavior” has been substituted.

Page 27, line 13: Reference should be Sherman not Stewart.
Done [p. 29]: The reference is actually Stewart and has been changed in the text.

The third reviewer, Dr. Helen Billman-Jacobe, had no suggestions for either major or minor revisions.

We greatly appreciate the comments of all the reviewers which we feel have strengthened the manuscript. Hopefully, this manuscript is now acceptable for publication.

Please feel free to contact us if you have any further questions or comments. Thank you for your editorial efforts as well.

Sincerely yours,

Dennis J. Murphy
James R. Brown