Final report: Waikato Medical Research Foundation Project #239

The role of PhoH2 in the pathogenesis of *Mycobacterium tuberculosis*

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Summary/Scientific statement/Aim:

The overall aim of this research project was to advance the understanding of the biological role of toxin-antitoxin systems in *M. tuberculosis* by determining the biological role of our recently characterised variation of a toxin-antitoxin system, PhoAT-PhoH2 in mycobacteria. By studying our deletion strain of *phoH2* in *Mycobacterium smegmatis*, using phenotypic microarrays, in house growth experiments, and RNA sequencing technologies we have deduced a new hypothesis surrounding the biological role for PhoH2 in mycobacteria along with a series of potential biological targets.

Background:

Most work thus far has focussed on understanding the role of VapC proteins in the context of VapBC toxin-antitoxin systems, which reside in high numbers in the causative agent of Tuberculosis, *Mycobacterium tuberculosis*. Here, VapC, a toxin protein, forms a complex with its cognate antitoxin (VapB). All VapC toxins are RNAses [1] and elicit their toxin activity by virtue of their sequence-specific mRNAse activity. Specifically in *M. smegmatis*, VapC when free of its antitoxin, acts as a regulator of metabolic flux specifically targeting carbon metabolism [2]. This presents an interesting question - what is the role of the vast number of VapC toxins in *M. tuberulcosis* given the single VapC toxin in *M. smegmatis* is a regulator of carbon metabolism? Is the role of these proteins to elicit tight-knit control over the cells metabolic processes?

VapC proteins in addition to being found in the genome alongside their cognate antitoxins are also found fused with RNA binding domains and AAA+ ATPase domains [3]. We have recently investigated PhoH2 proteins from mycobacteria [4]. These proteins are composed of a VapC RNAse protein fused with a PhoH protein. Little to no work had previously been done on these proteins, thus our primarily biochemical investigation was the first to reveal that PhoH2 is posing as a variation of a toxin-antitoxin, now together with an RNA helicase (RNA unwinding) protein, and was the first to identify the sequence-specific activity of PhoH2 proteins, as well as confirm that PhoH proteins are RNA helicases capable of unwinding RNA. Based on these observations we predicted that PhoH2 would contribute to the regulation of further metabolic processes in *M. tuberculosis* and *M. smegmatis*.

This funding received from the Waikato Medical Research Foundation has enabled us to explore this avenue using phenotypic microarrays, in house growth experiments, and RNA sequencing technologies to tease out the biological role of PhoH2 from *M. smegmatis*.

Results and Discussion:

Initial experimentation using phenotypic microarrays was carried using the Biolog Omnilog systems to compare the growth phenotypes of *M. smegmatis* mc^{2}155 wildtype and mc^{2}155 *ΔphoH2* deletion strain on different metabolites of nitrogen, carbon and phosphorus. The results of these experiments showed that with a handful of conditions, the *phoH2* deletion strain differed in its growth characteristics from the wildtype strain. Each of these conditions was followed up with in house growth experiments to try to validate the difference in growth. None of the phenotypes observed with the Biolog Omnilog system were able to be reproduced with in house growth experiments that replaced the carbon or nitrogen source with that identified in the Biolog Omnilog screen (Figure 1).

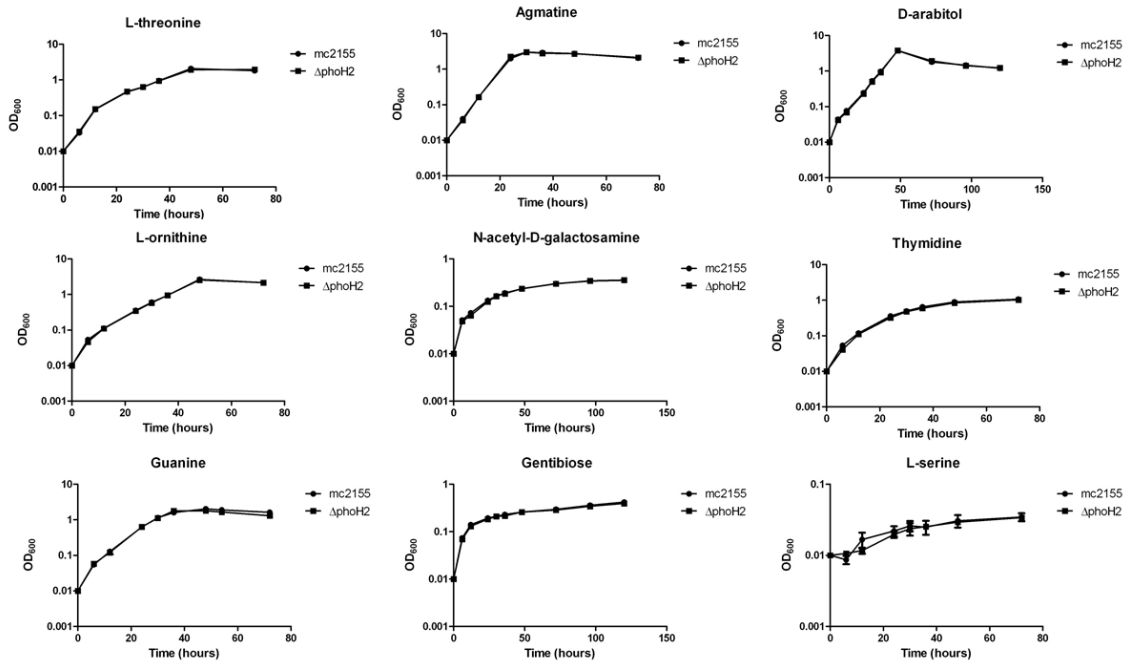

Figure 1. Growth curves of mc^{2}155 and mc^{2}155 *ΔphoH2* with various carbon or nitrogen sources identified from the Biolog Omnilog phenotypic Microarray. No difference in growth is observed between mc^{2}155 and mc^{2}155 *ΔphoH2*.

In light of these results, a growth experiment was carried out under standard conditions (LB media with 0.05% tyloxapol) and samples were taken across the growth curve for RNA extractions and subsequent RNA sequencing analysis (Figure 2).

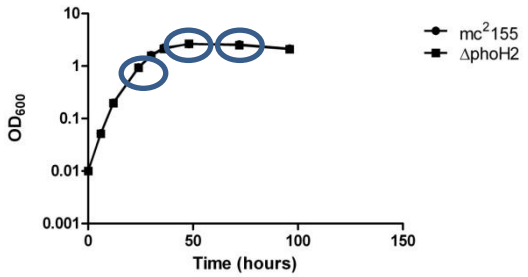

Figure 2. Growth curve of mc²155 and mc²155 *ΔphoH2* in $LB_{tyloxapol}$ media. Time points selected for RNA extractions for RNA sequencing are indicated by the blue circles (24, 48 and 72 hours).

RNA was extracted from the culture samples and sent to the Beijing Genomics Institute (BGI) for transcriptomic analyses that included Bioinformatics.

The transcriptomes received from BGI were further analysed and results revealed an upregulation of genes belonging to the SigF regulon. SigF is a sigma factor that directs the activity RNA polymerase and regulates expression of genes and proteins involved with the adaptation of *M. smegmatis* to stationary phase, oxidative stress and heat/cold shock [5].

Experimentation to confirm this phenotype is in progress, these current experiments aim to test the susceptibility of mc²155 wildtype, mc²155 *ΔphoH2* and strains of *M. smegmatis* that overexpress PhoH2, to H_2O_2 oxidative or heat stress using the approach described in [5]. If indeed susceptibility is greater with strains that overexpress PhoH2, suggesting regulation of this regulon by PhoH2, RNA will be extracted for qRT-PCR analysis of SigF regulon genes. In order to test if PhoH2 acts directly on SigF mRNA a series of PCR products that span the SigF operon have been amplified from *M. smegmatis* DNA (Figure 3). These PCR products have been transcribed into RNA and are to be used as substrates in unwinding and degradation assays with PhoH2 protein, to determine whether SigF mRNA is a direct target of PhoH2.

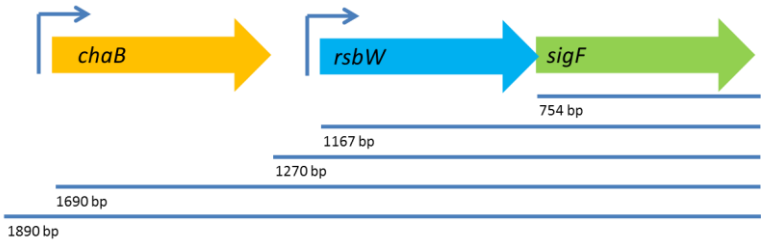

Figure 3. Schematic of the SigF operon and surrounding genes. PCR products generated that span the SigF operon (blue lines) that have been transcribed into RNA to be used as substrates for PhoH2 in RNA unwinding and degradation assays.

The results of these experiments will confirm whether PhoH2 is acting as a new regulator of SigF and therefore posing as a new central regulator of the stress response in *M. smegmatis*. This will enable us to explore similar targets in *M. tuberculosis* to gain a better understanding of the role of PhoH2 in this pathogenic organism. This work will also enable us to look into whether this regulation can be interfered with thus determining if these proteins pose as suitable candidates for drug design.

Conclusion:

Using RNA sequencing technologies we have teased out the first potential biological role of PhoH2 in mycobacteria. Our results suggest that PhoH2 is posing as a new central regulator of the stress response in *M. smegmatis* by regulating the expression of sigma factor SigF. This new hypothesis is currently being validated and complements existing hypotheses surrounding the role of VapBC toxin-antitoxin systems that are proposed to play a role in the tight-knit control over the cells metabolic processes. Now along with PhoH2, a variation of a toxin-antitoxin system, the role of these proteins is pointing towards intricate regulation of cellular metabolism.

References:

1. Ahidjo, B.A., et al., VapC Toxins from Mycobacterium tuberculosis Are Ribonucleases that Differentially Inhibit Growth and Are Neutralized by Cognate VapB Antitoxins. PLoS One, 2011. 6(6): p. e21738.
2. McKenzie, J.L., et al., A VapBC Toxin-Antitoxin Module is a Post-Transcriptional Regulator of Metabolic Flux in Mycobacteria. Journal of Bacteriology, 2012. 194: p. 2189-2204.
3. Arcus, V.L., et al., The PIN-domain ribonucleases and the prokaryotic VapBC toxin-antitoxin array. Protein Engineering, Design & Selection, 2011: p. 1-8.
4. Andrews, E.S.V. and V.L. Arcus, The mycobacterial PhoH2 proteins are type II toxin antitoxins coupled to RNA helicase domains. Tuberculosis, 2015. 95: p. 385-394.
5. Humpel, A., et al., The SigF Regulon in Mycobacterium smegmatis Reveals Roles in Adaptation to Stationary Phase, Heat, and Oxidative Stress. Journal of Bacteriology, 2010. 192(10): p. 2491-2502.

Dissemination of results:

Poster presentation at the Maurice Wilkins Centre Early Career Researcher meeting December 2016.

Statement of how the funds were used:

Consumables and services including: molecular biology reagents, protein expression and purification, other general consumables, and DNA sequencing: $13,590.79

RNA sequencing (Beijing Genomics Institute): $5771.80

The remaining balance of $357.41 is to be used for RNA biology and RNA helicase text books.