Final Report: Waikato Medical Research Foundation Project #258

The Role of the FitAB Toxin-Antitoxin System in the Maintenance of the Carrier Population of *Neisseria gonorrhoeae*

29-08-2018

Summary

The aim of this research project is to characterise the role of a toxin-antitoxin system (the FitAB system) in intracellular replication and cellular trafficking of the gonococcus (GC) *Neisseria gonorrhoeae*. Trafficking and intracellular replication are incredibly important for establishment of infection, in addition intracellular replication is hypothesised to play a role in the maintenance of an asymptomatic carrier population. We have characterised the biochemical activity of the FitB protein, and have determined it cuts RNA in a sequence specific manner. The length of RNA FitB cuts is different to previously characterised VapC enzymes, leading to development of a new method to determine sequence specificity. We optimised intracellular replication experiments using A549 lung epithelial cells and FitAB gene deletion strains. We see a marked difference between wild-type and deletion strains, which confirms the FitAB TA system plays a role in intracellular replication. We have taken RNA samples from various time points during this experiment and are awaiting the transcriptomes for these from the Beijing Genomics Institute (BGI).

Background

A toxin-antitoxin (TA) system was identified in a mutagenesis screen for mutants with the ability to traffic across epithelial cells faster compared to the wild type (1). GC that lack the *fitAB* (fast intracellular trafficking) operon grow normally extracellularly, but have an increased rate of replication within epithelial cells and as such increases the rate at which the mutant can traverse the epithelium (1).

The FitAB toxin-antitoxin system belongs to the VapBC TA family. Members of the VapBC family are hypothesized to play role in the persistence of *Mycobacterium tuberculosis* and other bacterial pathogens (2). The VapBC system is characterized by an overlapping, bicistronic operon, where the antitoxin (VapB/FitA) is upstream of the toxin (VapC/FitB). The antitoxin consists of a DNA binding domain that binds to its own promoter to regulate expression and also binds to VapC inhibiting its activity. VapC belongs to the PIN domain family of proteins, which cause growth arrest by virtue of their ribonuclease activity (3).

Proposed Aims of the Project

The overall aim of this project is to determine the cellular target of the of the FitAB toxin antitoxin system within *Neisseria gonorrhoeae* to better understand the mechanism of intracellular replication and persistence of this organism.

Research Design & Objectives Achieved

The FitAB, VapBC toxin-antitoxin system has been shown to slow intracellular trafficking of the bacterium across epithelial cells. However the mechanism by which it does this is unknown. Using a combination of *in vitro* and *in vivo* experiments we are elucidating this mechanism.

(1) *Express and purify the FitAB system using E. coli as a host*

Mattison et al (2006) expressed the FitAB system in *Escherichia coli* using the pET28b vector encoding the intact *fitAB* operon for expression with a C-terminal His-tag. I plan to use the same expression and purification strategy as Mattison et al (2006). Genes encoding the *fitAB* operon will be synthesised by GeneArt and cloned into pET28b for expression in *E. coli.*

We have cloned the FitAB locus into the pET28B plasmid and completed expression trials to optimise expression of FitAB in our lab.

(2) *Isolate VapC for use in ribonuclease activity assays*

Trypsin will be trialled for removal of VapB, as per the method developed for isolation of mycobacterial VapC proteins (4, 5). If this is not successful a variety of other methods will be trialled to disrupt the VapBC interaction and purify VapC i.e. pH, salt and low concentrations of detergent.

We took a new approach and can express FitB within E. coli, negating the need for trypsin treatment of the FitAB complex and subsequent purification.

(3) *Determine the ribonuclease sequence specificity of VapC*

Based on the structural similarity of FitB to VapC proteins from *Pyrobaculum aerophilum* that we have characterised (6, 7) and the conservation of four acidic residues in the active site of FitB we will test the protein for ribonuclease activity. The pentaprobe ribonuclease activity assay screen developed in our lab for characterisation of VapC proteins (4) will be used to determine the sequence specificity of FitB. The pentaprobe system is a set of plasmids from which RNA can be transcribed that covers every combination of five bases. RNA oligonucleotides covering pentaprobe RNAs cut by VapC/FitB are then made and analysed for specific cut site recognition by MALDI-TOF mass spectrometry.

We have finished pentaprobe assay screens with purified FitB protein. FitB diplays sequence specific ribonuclease activity. FitB does not cut small RNA oligonucleotides, we observed no activity of FitB on RNA sequences <35 bases. Our method for determination of FitB sequence specificity relies on small RNA oligonucleotides for mass spectrometry analysis. We have therefore developed a new method for determining sequence specificity of FitB using a RNA circularisation and a variety of molecular biology enzymes. We are optimising this method at present.

(4) *Carry out RNAseq to compare the transcriptome of the deletion strain against the wild-type under intracellular growth conditions (within epithelial cells)*

Epithelial cells (A431) will be cultured in the lab, infected with 16-18 hour *N. gonorrhoeae* WT or Δ*fitAB* cultures as per intracellular growth assays carried out in Hopper el al (2000). The intracellular CFU 6-10 hours post infection will be measured and RNA extracted at these time points for RNAseq analysis. Comparison of differentially expressed genes between the WT and *fitAB* deletion strains will help elucidate the biological target.

The intracellular replication experiments took a lot of optimisation to achieve the same results as in Hopper et a (2000). We had difficulties in obtaining enough RNA from the experiment for RNA-Seq as the amount of bacterial RNA (which we want to analyse) is very low compared to the amount of RNA from the A549 cells. We have refined the RNA extraction protocol further and have sent samples to BGI for transcriptome analyses. We expect the data in the next 4 weeks.

(5) *Synthesise the biological target for in vitro ribonuclease activity assays*
Genes that are differentially expressed in the RNAseq experiment will be analysed for the presence of the ribonuclease cut site identified in (3). RNA transcripts of these genes will be synthesised *in vitro* and tested with purified FitB for activity.

This objective relies on achieving (4) which was not achieved by the end of this grant.

I have taken maternity leave over the time I have had the funding from the WMRF and returned to work at 0.5 FTE. Combined with unanticipated protocol optimisation and equipment failure (MALDI-TOF mass spectrometer) it has taken me longer than anticipated to achieve the aims of the project.

Results and Discussion

Intracellular replication assays in lung epithelial cells (A549) show that deletion of the *fitAB* operon results in a marked increase in intracellular replication up until 10 hours post gentamicin treatment, whereupon the number of bacteria within epithelial cells plummets drastically (Figure 1). The wild-type however steadily increases, suggesting that FitAB is in some way regulating intracellular replication (Figure 1). This is consistent with characterisation of the *vapBC* operon from *Mycobacterium smegmatis*, which regulates glycerol uptake and metabolism, and as such 'fine-tunes' cellular metabolism. However, the phenotype seen here for the *fitAB N. gonorrhoeae* deletion strain is much more pronounced.

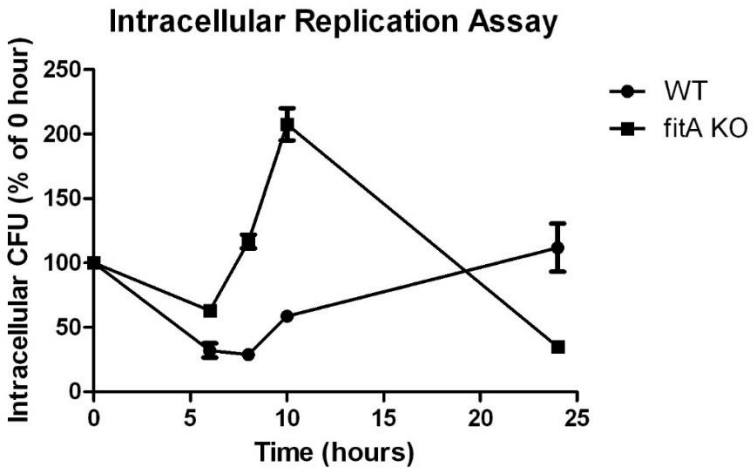

Figure One. Intracellular Replication of Neisseria gonorrhoeae wild-type and *fitAB* deletion (fitA KO) strains. A549 lung epithelial cells were infected with GC and incubated for 16 hours followed by gentamicin treatment for one hour to remove extracellular bacteria. Colony forming units (CFUs) from intracellular bacteria were monitored 6, 8, 10 and 24 hours post gentamicin treatment.

We have also characterised the growth of WT and *fitAB* deletion strains in culture. Most noticeable is the difference in the extracellular pH (of the growth media) between the two strains (Figure 2). Even though there is no difference in growth in culture between the strains, the acidic pH of the culture media of the deletion strain suggests an 'uncoupling' of cellular metabolism compared to the wild-type.

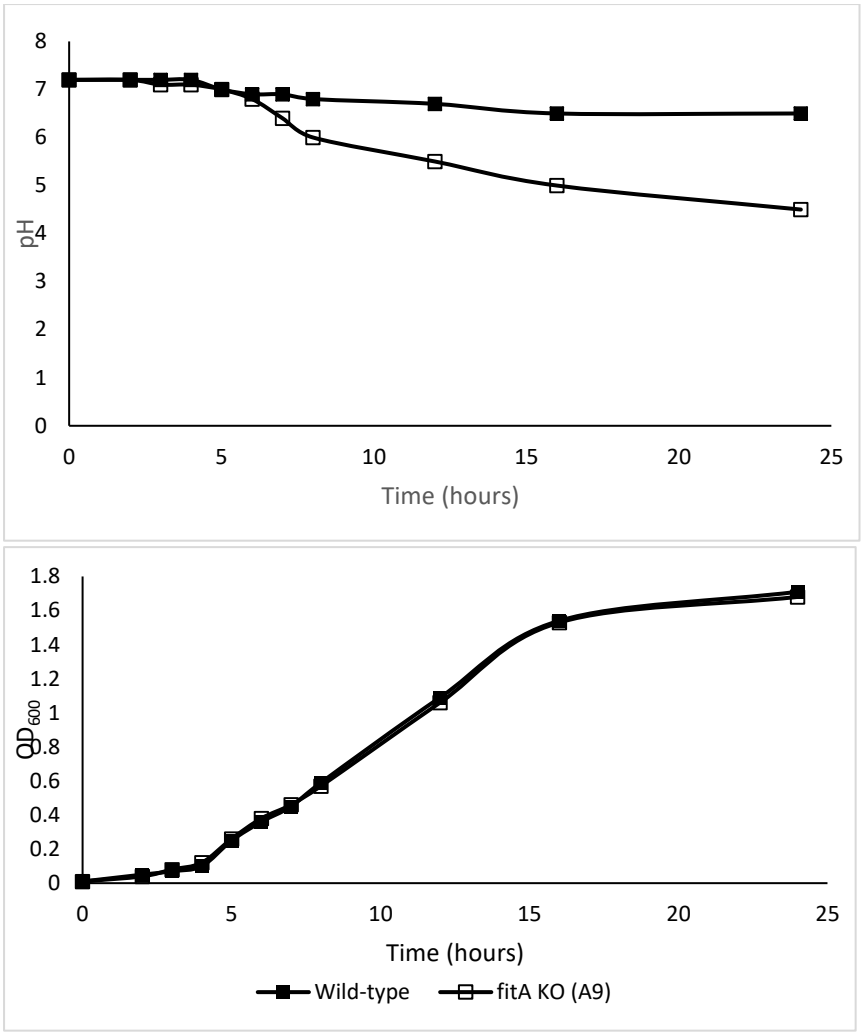

Figure Two. Characterisation of wild-type and the *fitAB* deletion strain in culture. Monitoring pH of the growth media shows that the deletion strain has a marked decrease in the pH of the media (upper graph) this is also demonstrated by the colour change in the culture media, that includes phenol red as a pH indicator (right panel). The growth rates of WT and fitAB deletion strains are the same in culture (lower graph).

Using *Escherichia coli* as a host we can express both the FitAB complex and FitB alone. Using IMAC and gel filtration chromatography we have successfully purified these proteins, enabling subsequence functional characterisation. Using previously established protocols from our lab we show that FitB shows sequence specific ribonuclease activity. This is shown in Figure 3, across the pentaprobe RNAs (922, 923, 924, 925, 926, 927 and 932) every combination of 5 bases is covered. Different banding patterns are seen with different pentaprobe RNAs, indicating sequence specificity. In addition we show that this is Magnesium dependent as inclusion of EDTA abolishes ribonuclease activity. We are in the process of determining the exact nucleotide sequence that FitB targets using a combination of RNA oligonucleotides based on pentaprobe 924 and mass spectrometry.

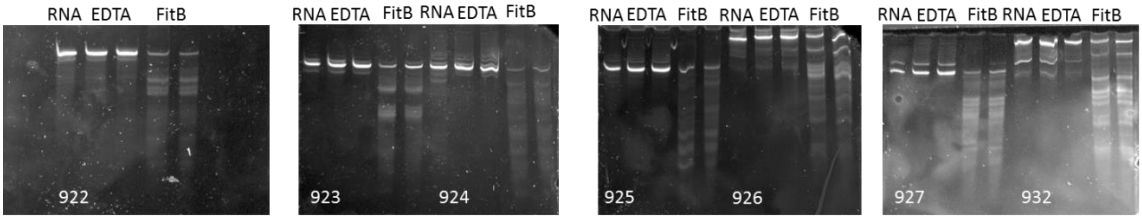

Figure Three. Pentaprobe ribonuclease activity assays. FitB shows sequence specific ribonuclease activity (lanes labelled FitB), inclusion of EDTA abolishes activity by chelating magnesium (lanes labelled EDTA) controls include EDTA and RNA only (no FitB) to check integrity of RNA and for ribonuclease contamination. EDTA and FitB reactions performed in duplicate for each pentaprobe RNA (922, 923 etc.)

We have determined that the FitAB toxin-antitoxin system plays an important role in intracellular replication. We have optimised our RNA extraction protocol from these and are awaiting our transcriptome data for 8 and 10 hour time points. We also show that FitB has sequence specific ribonuclease activity that is magnesium dependent, and have developed a new method to identify its sequence specificity. We are in the process of determining the exact sequence it targets. Reconciliation of transcriptomic data with the sequence specificity of FitB will determine the role of FitAB in intracellular replication, and in doing so provide hints as to the mechanism of persistence of *N. gonorrhoeae*.

References:

1. Hopper S, Wilbur JS, Vasquez BL, Larson J, Clary S, Mehr IJ, et al. Isolation of Neisseria gonorrhoeaeMutants That Show Enhanced Trafficking across Polarized T84 Epithelial Monolayers. Infection and Immunity. 2000;68(2):896-905.
2. Sala A, Bordes P, Genevaux P. Multiple toxin-antitoxin systems in *Mycobacterium tuberculosis*. Toxins. 2014;6(3):1002-20.
3. Arcus VL, McKenzie JL, Robson J, Cook GM. The PIN-domain ribonucleases and the prokaryotic VapBC toxin–antitoxin array. Protein Engineering Design and Selection. 2011;24(1-2):33-40.
4. McKenzie JL, Duyvestyn JM, Smith T, Bendak K, MacKay J, Cursons R, et al. Determination of ribonuclease sequence-specificity using Pentaprobes and mass spectrometry. RNA. 2012;18(6):1267-78.
5. McKenzie JL, Robson J, Berney M, Smith TC, Ruthe A, Gardner PP, et al. A VapBC Toxin-Antitoxin Module Is a Posttranscriptional Regulator of Metabolic Flux in Mycobacteria. Journal of Bacteriology. 2012;194(9):2189-204.
6. Arcus VL, Bäckbro K, Roos A, Daniel EL, Baker EN. Distant Structural Homology Leads to the Functional Characterization of an Archaeal PIN Domain as an Exonuclease. Journal of Biological Chemistry. 2004;279(16):16471-8.
7. Bunker RD, McKenzie JL, Baker EN, Arcus VL. Crystal structure of PAE0151 from *Pyrobaculum aerophilum*, a PIN-domain (VapC) protein from a toxin-antitoxin operon. Proteins: Structure, Function, and Bioinformatics. 2008;72(1):510-8.