The Role of MSMEG_0004/Rv0004 During DNA Damage in Mycobacteria

XXX, Leslie Weiss, and Christina Stallings, Ph.D.

Department of Molecular Microbiology, Washington University in St. Louis School of Medicine, Saint Louis, MO

Scanning electron micrograph of *M. tuberculosis*. Image: CDC/Dr. Ray Butler, credit: Janice Haney Carr

Why Mycobacteria and MSMEG_0004?

According to World Health Organization estimates, there were 9.4 million new cases of Tuberculosis (TB) and 1.7 million TB-related deaths in 2009.[1] TB is an infectious bacterial disease caused by *Mycobacterium tuberculosis (Mtb)*, an incredibly resilient pathogen that can remain dormant while withstanding a continuous bombardment of oxidative, nitrosative and genotoxic stresses.[2,3] Furthermore, all drug-resistant strains of *Mtb* to date acquire their drug resistance without gaining new DNA; *Mtb* instead develops its drug-resistance through mutations in DNA replication and repair.[6] Together, these unique characteristics provide a strong basis for studying proteins that may be involved in mycobacterial DNA damage and repair.

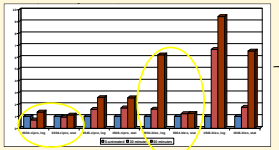
MSMEG_0004 (*rv0004* in *Mtb*) was identified in a microarray analysis as upregulated during log phase DNA damage.[4,5] As the fourth gene from the origin, it is well-conserved in both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, a widely-used, nonpathogenic model for *M. tuberculosis*. *MSMEG_0004/rv0004* also shares the same operon as gene *recF*, which codes for the DNA repair and replication enzyme RecF. MSMEG_0004 and RecF may be similarly involved in mycobacterial DNA replication or repair. The goal of this project then, is to reveal the role and function of MSMEG_0004 in mycobacteria.

Experiments

- Make deletion mutant (no *MSMEG_0004*)
  - Phage recombination[4]
  - Two-step allelic exchange
- Measure *MSMEG_0004* transcription levels after inducing stress response
  - Oxidative stress, ciprofloxacin, DNA breaks
  - Quantitative real-time PCR analysis
- Search for other proteins that interact with MSMEG_0004
  - Co-immunoprecipitation of MSMEG_0004 with other proteins in cell lysate
    - → Create overexpression complements: (additional copy of gene for gathering more protein)
      1) MSMEG_0004 with N-terminus tag
      2) MSMEG_0004 not tagged

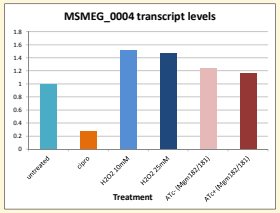
Results

Quantitative Real-time PCR



Preliminary qRT-PCR of genes found upregulated in microarray assay

→ *MSMEG_0004* data circled in yellow
→ Treatments: ciprofloxacin and bleomycin
→ To be repeated in the future



(Above): Ratios of treated vs. untreated transcript levels, both normalized.

Treatment	Average-fold change
Ciprofloxacin: • inhibits DNA gyrase • Interferes with DNA replication	0.2693
10mM of H_2O_2: • oxidative stress • low level of exposure	1.524
25mM of H_2O_2: • oxidative stress • higher level of exposure	1.474
ATc- (Mgm182/181): • no ATc added • low-level of stress	1.243
ATc+ (Mgm182/181): • treated w ATc for 45 min. • high-level of stress	1.161

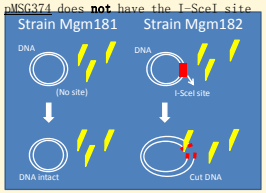
Mgm181 vs. Mgm182

(Wild-type *M. Smegmatis*) (plasmid

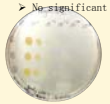
Mgm181 = mc^2155 + pMSG374
Mgm182 = mc^2155 + pMSG375

pMSG375 contains I-SceI target site (I-SceI = stress-induced homing endonuclease)

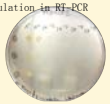
pMSG374 does **not** have the I-SceI site



- I-SceI activity results in double-stranded DNA breaks
  - Anhydrotetracycline was used to induce high-level activity of I-SceI
- Ratio of Mgm182/Mgm181 measures the transcriptional response to genotoxic stress
- MSMEG_0004 transcription was found to be upregulated 2-fold in initial microarray
  - No significant upregulation in RT-PCR



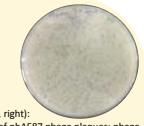
Untreated



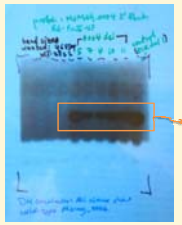
Treated w/ ATc for 45 min.
→ Visible killing of cells

Deleting *MSMEG_0004*

Method 1: Phage Recombination



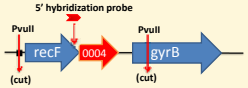
(Above, right): Plate of phAE87 phage plaques; phage were used to replace *MSMEG_0004* with an antibiotic resistance cassette



(Above): Southern blot showing all wild-type restriction patterns .

We had hoped to see at least one strain with a non-wild type band size, but deletion of *MSMEG_0004* was unsuccessful.

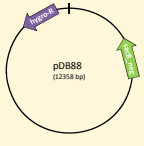
(All wild-type bands, 2922 base pairs long)



Method 2: Two-Step Allelic Exchange

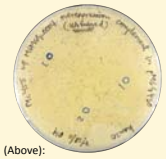
(Above): **1% agarose gel with PCR products**
→MSMEG_0004 5' and 3' flanking sequences
→to be cloned into pDB88

Clone into pDB88

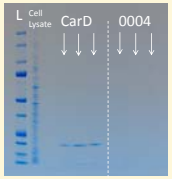


(Above): **Vector pDB88**[7]
→ Negative selection with sucrose
→ Positive selection with Hygromycin

Co-Immunoprecipitation



(Above): Final plate of *M. Smegmatis* that contains the HA tagged MSMEG_0004 overexpression complements used in Co-IP (Non-HA tagged not shown)



(Above): Western blot of the MSMEG_0004 co-immunoprecipitation experiment.

(CarD samples were included as positive controls.)

Possible reasons why Co-IP showed no bands in MSMEG_0004 lanes:

1) **HA tagged MSMEG_0004 is unstable or not expressed at sufficiently high levels**
2) **N-terminus HA tag is lethal for cells**

Conclusions

Transcript levels of *MSMEG_0004* were monitored with quantitative real-time PCR. Treatment with Ciprofloxacin, which inhibits DNA replication, down-regulated *MSMEG_0004* 0.27 fold; 10mM and 25mM of H_2O_2 (an oxidative stressor) both increased transcription by 1.5 fold. These numbers suggest that *MSMEG_0004* transcription increases under oxidative stress and decreases when DNA replication is inhibited.

MSMEG_0004, like many genes close to the origin of replication, is very difficult to delete or mutate. After an unsuccessful attempt to delete *MSMEG_0004* via phage recombination, we are currently in the process of deleting *MSMEG_0004* using the conventional two-step allelic exchange system. Once we have created deletion mutants, we shall further analyze the deletion mutant's survival rates, perform DNA repair plasmid assays and search for any proteins or nucleic acid binding partners. Any additional data regarding MSMEG_0004 will take us closer to elucidating the function and role of MSMEG_0004 in *M. smegmatis* and its pathogenic counterpart, *M. tuberculosis.*

Future Directions

- Finish making the deletion mutants via allelic exchange
- qRT-PCR analysis with different stresses
  - Bleomycin, nitric oxide, acid stress
  - Starvation – oxygen (hypoxia), phosphate deprivation
- Continue searching for interacting partners
  1) Gel shift experiment: protein-DNA interactions
  2) Co-IP repeated using a different tag or antibody for identification
- DNA repair plasmid assays to confirm/rule out the role of MSMEG_0004 as a DNA repair enzyme

References

1. World Health Organization. *Tuberculosis*. November 2010. Fact sheet No. 104. (http://www.who.int/mediacentre/factsheets/fs104/en/)
2. Hassett, D.J. and M.S. Cohen, *Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells.* Faseb J, 1989. 3(14): p. 2574-82.
3. MacMicking, J., Q.W. Xie, and C. Nathan, *Nitric oxide and macrophage function.* Annu Rev Immunol, 1997. 15: p. 323-50.
4. Stallings CL, Stephanou NC, Chu L, Hochschild A, Nickels BE, Glickman MS. *CarD is an essential regulator of rRNA transcription required for Mycobacterium tuberculosis persistence.* Cell. 2009 Jul 10;138(1):146-59.
5. Stallings CL, Chu L, Li, LX, Glickman MS. *Catalytic and non-catalytic roles for the mono-ADP ribosyltransferase Arr in the mycobacterial DNA damage response*. PLoS One. 2011; 6(7): e21807.
6. Ramaswamy S, Musser JM. *Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update*. Tuber Lung Dis. 1998;79(1):3-29.
7. Barkan D, Stallings CL, Glickman MS. *An improved counterselectable marker system for mycobacterial recombination using galK and 2-deoxy-galactose*. Gene. 2011 Jan 1;470(1-2):31-6. Epub 2010 Sep 17.

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