Published in final edited form as:

Tuberculosis (Edinb). 2010 January ; 90(1): 50–56. doi:10.1016/j.tube.2009.10.004.

***Mce2* operon mutant strain of *Mycobacterium tuberculosis* is attenuated in C57BL/6 mice**

Olivera Marjanovic¹, Toshiko Miyata¹, Amador Goodridge¹, Lon V. Kendall², and Lee W. Riley¹

¹School of Public Health, University of California, Berkeley, CA 94720, USA

²Laboratory Animal Resources, Colorado State University, Fort Collins, CO 80523, USA

Summary

Mycobacterium tuberculosis genome contains four related sets of an operon called *mce* (*mce1-4*). The disruption of one of these operons, *mce1*, causes *M. tuberculosis* to become hypervirulent, whereas the *mce3* and *mce4* operon mutants are attenuated in mice. This study examined the phenotype of the *mce2* operon mutant. The deletion of *mce2* operon in *M. tuberculosis* H37Rv had no effect on bacterial growth in 7H9 liquid broth or survival within macrophages. However, RAW macrophage-like cells infected with the mutant strain were reduced in their ability to produce TNF- α , IL-6 and MCP-1. In C57BL/6 mouse lungs, the *mce2* operon mutant and wild type H37Rv replicated similarly up to 20 weeks of infection. However, by 56 weeks of infection, all mice infected with the wild type H37Rv had died, while 80% of those infected with the mutant remained alive ($P < 0.0001$). The proportion of affected lung parenchyma in mice infected with the mutant was substantially less than that of mice infected with the wild type for the same time periods of infection. These observations suggest that the *mce2* operon mutant is attenuated, and that this attenuation is related not to the bacterial burden but to the mutant's decreased ability to elicit a type of immune response and lung pathology detrimental to the survival of the animal.

Keywords

Mycobacterium tuberculosis; *mce* operons; *mce2* operon mutant

1. Introduction

It is estimated that 1/3 of the world's population is latently infected with *M. tuberculosis*, the etiologic agent of tuberculosis [1]. Of these latently infected individuals, only about 10% develop active disease. Thus the majority of infected people carry this organism for lifetime. The mechanism underlying this latent infection and reactivation from this latent state in an animal host is still poorly understood.

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Corresponding Author: Lee W. Riley, (lriley@berkeley.edu), Phone: 510-642-9200, Fax: 510-642-8250.

Competing interests: None declared.

Ethical Approval: Not required.

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M. tuberculosis genome contains four homologous copies of an operon called *mce* (*mce1-4*) (Supp. Fig. 1). All four *mce* operons carry six *mce* genes (*mceA-F*) and two *yrbE* genes (*yrbEA-B*). The *mce* genes encode proteins that localize to the cell wall [2-4], whereas *yrbE* genes encode integral membrane proteins [4]. In 1993 Arruda et al. reported that *mce1A* conferred upon a nonpathogenic *E. coli* an ability to enter and survive within mammalian cells [5]. The *mce* operon genes exhibit differential expression profiles under different growth conditions *in vitro* as well as throughout various phases of *M. tuberculosis* infection *in vivo* [6-8]. Casali et al. has shown that *mce1* operon genes are negatively regulated intracellularly by a transcriptional regulator (*mce1R*) which belongs to the GntR family of transcriptional regulators [9]. Uchida et al. has shown that *M. tuberculosis* strain disrupted in *mce1R* gene exhibits enhanced virulence in mice, suggesting that the level of expression of *mce1* operon gene products affects infection outcome [10]. On the other hand, *mce3* operon genes are regulated by a gene Rv1963 which belongs to the *tetR* family of transcriptional regulators [11, 12]. Most recently, Santangelo and colleagues demonstrated that *mce2* operon's expression is regulated by Rv0586 gene which encodes a putative GntR-like regulator [11].

Previously, it was shown that *M. tuberculosis* strain disrupted in *mce1* operon fails to establish latent infection in the mouse model of tuberculosis [3]. Mice infected with the *mce1* operon mutant strain died much earlier than those infected with the wild type strain. In addition, histological examination of lung sections revealed an aberrant pro-inflammatory response in mice infected with the *mce1* operon mutant strain. On the other hand, *M. tuberculosis* strains disrupted in *mce3* and *mce4* operons displayed an attenuated phenotype in the mouse model of infection [13]. Thus, these studies suggest that despite the high level of homology among different *mce* operons, they appear to have distinct functions during the course of *M. tuberculosis* infection in mice. Therefore, in this study, we set out to examine the phenotype of *mce2* operon mutant using an approach similar to that done with *mce1*, 3 and 4 operon mutants.

2. Materials and Methods

2.1 Disruption of the *mce2* operon

An *mce2* operon mutant was generated by disruption of a DNA segment between *yrbE2A-mce2F* genes in wild type H37Rv bacterial strain (Supp. Fig. 2). The mutation was carried out with a strategy described by Parish and Stoker [14]. Briefly, we disrupted the genes by amplifying 2kb DNA regions upstream and downstream of the targeted segment. Shown below are primer sets used to generate upstream and downstream 2kb DNA regions flanking the *mce2* operon genes.

Upstream flanking region primer set:

Forward primer: 5' TTATAAGTACTCAGCACCGCGCCGTTGTCTGA 3'

Reverse Primer: 5' TTATAAAGCTTTGCCGACTCGCCTGGCTAAC 3'

Downstream flanking region primer set:

Forward primer: 5' TTATAAAGCTTGCCGGCGCGATCAGGTACCG 3'

Reverse primer: 5'TTATAGCGGCCGCTTGACTATCCGGGATCCAGCG 3'

Following digestion with appropriate restriction enzymes these PCR products were subcloned into the p2NIL vector [14]. This vector carries the kanamycin resistance gene for selection in both *E. coli* and *M. tuberculosis*, an origin of replication E (*oriE*) for replication in *E. coli*, a small multiple cloning site (MCS), the *lacZ* gene for screening on X-Gal, and the sucrose sensitivity gene *sacB*. An unmarked mutation was created by a two-step counter-

selection procedure. In the first step, single cross-over constructs were obtained as a result of plasmid DNA insertion into *M. tuberculosis* genome. At this point the transformed bacteria were blue in color, kanamycin resistant, and sucrose sensitive. A single cross-over colony was then grown in 7H9 +ADC (10%) media without antibiotic for one week to allow the second cross-over event to occur. The bacteria were then plated on 2% sucrose and X-gal plates to select for the loss of the integrated plasmid. Colonies that appeared white were picked and PCR screened for the expected mutation. Finally, Southern blot was performed to confirm presence of the mutation.

2.2 In-vitro growth kinetic analysis of mutant strain

M. tuberculosis strains were first grown to mid-log phase. An aliquot of the culture was passed through a 5 µm pore filter to prepare single cell suspensions. Optical density of these cultures was recorded at 600 nm and bacteria were inoculated into fresh 7H9+ADC media. Subsequently, these cultures were propagated in 5% humidified CO₂ incubator for 21 days. Every other day, 1 ml of the cultures was taken and an optical density at 600 nm recorded to generate a growth curve for each bacterial strain.

2.3 Determination of survival and cytokine and chemokine expression in RAW macrophages infected with the *mce2* operon mutant strain

The RAW 264.7 murine macrophage-like cell line (ATCC, MD) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, NY) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, CA) at 37°C in a 5% CO₂ humidified incubator. Cell concentration was determined by a hemocytometer. Cells were plated at a density of 1×10^5 cells/well in 24 well tissue culture plates and incubated overnight to achieve approximately 70% confluency. The macrophages were infected at a multiplicity of infection (MOI) of 1:1. After 6 hours of infection, macrophages were washed 3 times with DMEM to remove extracellular bacteria.

To examine the intracellular invasion, growth, and survival of bacteria, we lysed the macrophages with 1ml of PBS - 0.5% Triton-X-100 and plated cell lysates at serial dilutions onto 7H11 agar at 6, 24, 48, and 72 hours post infection. Colony forming units (CFU's) were enumerated 21 days post plating. Supernatants of the above cell cultures were used to measure TNF- α , IL-6, IL-12, IL-10, IL-4 and MCP-1 produced by RAW macrophages in response to infection with the wild type H37Rv or the *mce2* operon mutant strain. Lipopolysaccharide (1mg/ml) (Sigma) was used as a positive control and uninfected macrophages served as a negative control for each experiment. We also used *mce1* operon mutant strain as an additional control. The above cytokines or chemokines were measured by ELISA with the reagents purchased from eBioscience.

2.4 Mouse Infection

To maintain virulence, *M. tuberculosis* strains were passaged once in mice prior to use in this experiment. Eight week old female C57BL/6 mice (Jackson Laboratories) were infected with the wild type H37Rv *M. tuberculosis*, *mce2* or *mce1* operon mutant strains via the aerosol route using the Inhalation Exposure System (Glas-co, Terre Haute, Ind.). We confirmed the low dose inoculum conditions by enumerating CFU counts recovered from the lungs of three mice per infection strain 24 hours post infection. Lungs were homogenized and plated onto 7H11 + OADC (10%) + cyclohexamide (100 µg/ml) agar plates and these plates were read for CFU's 21 days later – doses of infection were 76, 57 and 153 CFU's per right lung in mice infected with H37Rv, *mce2* and *mce1* operon mutants respectively. Additionally, at different time points post-infection (3, 6, 12 and 20 weeks), the right lung from three mice per strain was collected, homogenized in PBS-Tween (0.05%), appropriately diluted, and plated onto 7H11 agar plates containing OADC and

cyclohexamide (100 ug/ml). Twenty one days later, these plates were examined to assess bacterial load in the mouse lung.

2.5 Mouse survival study

Three groups of 10 mice each, infected with either Wt H37Rv, *mce2* or *mce1* operon mutant strains were followed until they exhibited moribund features (loss of weight, failure to groom, ruffled fur, and lethargy) that occur just prior to death. At this point, mice were anesthetized and then euthanized by cervical dislocation. Health status of the mice was monitored daily by the veterinary staff of the North Animal Facility of UC Berkeley.

2.6 Mouse lung gross pathology and histology

The mouse left lungs fixed in 10% neutral (PBS) buffer formalin were examined and photographed with Digital Nikon 4300 camera. These fixed lungs were embedded in paraffin, sectioned, and stained for histology with hematoxylin and eosin (H&E). Sectioning and staining were performed by Histology Consultation Services, Everson, Washington. For comparative purposes, sections were obtained from the same region of all lungs: 3 sections were obtained from each lung from 3 mice (superior, middle and posterior sections of the lung). Histopathology of 3, 6, 12 and 20 weeks post infection were analyzed. Pathological examination was done by a veterinary pathologist, who was blinded to the sources of the lung sections.

2.7 Statistics

Data are presented from representative experiments as the mean $\pm$ SE of triplicate infections. All experiments were repeated at least 3 times. Differences between the means of each treatment group were analyzed by Student's *t* test and were considered significant at $P < 0.05$.

Mouse survival was compared by Kaplan-Meier curve.

3. Results

3.1 Disruption of the *mce2* operon

Probes (2kb downstream and upstream flanking regions) to differentiate *mce2* operon mutant vs. parental bacterial strain were generated. Upon digestion with *Hind*III and *Xmn*I and probing with the downstream flanking region (Probe# 2), the expected DNA bands of 4312 bp for the *mce2* mutant and 4708 bp for the parental strain, respectively, were found. However, when the upstream flanking region was used as a probe (Probe #1), the expected DNA bands were 4323 bp for the *mce2* mutant and 8332 bp for the parental strain. As shown in the Supp. Fig. 3, the desired *mce2* operon mutation was successfully generated on the wild type H37Rv bacterial strain background.

3.2 In-vitro growth kinetic analysis of *mce2* operon mutant strain

We wished to test whether *mce2* operon deletion had any effect on the ability of the mutant bacterium to grow *in vitro* in 7H9 liquid broth. Bacteria were inoculated into fresh 7H9+ADC media at an optical density recorded at 600 nm of 0.005. Their growth was then monitored for 21 days. Fig. 1A shows that *mce2* operon deletion had no effect on the mutant's ability to grow in liquid broth.

3.3 Determination of survival and cytokine and chemokines expression in RAW macrophages infected with the *mce2* operon mutant strain

The concentrations of TNF- α , IL-6, IL-12, IL-4, IL-10 and MCP-1 in supernatants of RAW macrophages were measured. We also infected cells with *mce1* operon mutant as control. At an MOI of 1:1, we detected production of TNF- α , IL-6, MCP-1, and IL-10 in response to wild type and *mce2* operon mutant infection. Production of IL-4 and IL-12 was below the limit of detection in the supernatant of cells infected by these strains.

TNF- α concentration in supernatants increased over time reaching approximately 4,200 pg/ml after 72 hours post infection with the wild type H37Rv strain. Infection with the *mce2* operon mutant led to significantly lower amounts of TNF- α production, reaching about 3,300 pg/ml at 72 hours post-infection ($P=0.0124$). The most significant difference was observed in the cells infected with *mce1* operon mutant, confirming the results previously shown by Shimono et al. [3]. After 72 hours, only about 2,100 pg/ml of TNF- α ($P=0.0002$) was detected in the supernatants of cells infected with the *mce1* operon mutant strain (Fig. 2A).

Concentration of IL-6 in RAW cell supernatants increased over 72 hours for all strains tested. There was about half as much of this cytokine produced in response to *mce2* operon mutant compared to when the cells were infected with the wild type strain (50pg/ml vs. 100 pg/ml $P=0.047$). Infection of cells with the *mce1* operon mutant resulted in very low levels of IL-6, nearly indistinguishable from negative control (uninfected cells) (Fig. 2B).

MCP-1 production by RAW cells infected with different mycobacterial strains was similar at all time points other than 24 hours post infection. At 24 hours post infection, there was a significant decrease in MCP-1 produced in response to infection with *mce2* operon mutant compared to wild type (2000 pg/ml vs. 4000 pg/ml $P=0.003$). In cells infected with the *mce1* operon mutant, levels of MCP-1 produced at this time point were lower than those of *mce2* operon infected cells (1,800 pg/ml $P=0.0002$) (Fig. 2C).

We noted no significant differences in IL-10 concentrations in supernatants of RAW macrophages infected with different bacterial strains (data not shown). Our results show that all *M. tuberculosis* *mce* operon mutant strains elicited different levels of cytokine and chemokine production by infected RAW macrophages. To determine whether intracellular bacterial growth rates were related to these differences in cytokine and chemokine production, we monitored intracellular growth rates of the mycobacterial strains in RAW macrophages. The numbers of CFU obtained from RAW cell lysates was similar at day 0 for all *M. tuberculosis* strains tested and remained as such over the next 72 hours (Fig. 1B).

3.4 Bacterial burden in mouse lungs infected with *mce2* operon mutant strain

We observed similar bacterial burdens in the lungs of C57BL/6 mice infected with Wt H37Rv, *mce2* and *mce1* operon mutant strains up to 20 weeks post infection (Fig. 3). In addition, at time of death, all mice infected with different bacterial strains contained similar bacterial burdens in their lungs $\sim 10^6$ CFU (data not shown).

3.5 Determination of mouse morbidity

We observed that mice infected with *mce1* operon mutant started to die much earlier than those infected with the wild type H37Rv and *mce2* operon mutant strains (Fig. 4). More specifically, mice infected with *mce1* operon mutant strain began to die at 25 weeks post infection while those infected with the wild type H37Rv and *mce2* operon mutant strain began dying at 44.5 and 47.4 weeks post infection, respectively. All 10 of the mice infected with the *mce1* operon mutant were dead by 54 weeks post infection. All mice infected with

the wild type strain died by week 56 post infection while 80% of the mice infected with the *mce2* operon mutant remained alive at this time point.

3.6 Gross pathology and histology

By 12 and 20 weeks of infection, gross mouse lung pathology examination revealed fewer and more diffuse granulomas in mice infected with the *mce2* operon mutant compared to those infected with the wild type strain (Fig. 5). Temporal histopathological assessment of mouse lungs showed slower progression of pulmonary lesions in mice infected with the *mce2* operon mutant strain relative to wild type (Fig. 6). We also confirmed the earlier finding by Shimono et al. [3] of severe lung pathology in mice infected with the *mce1* operon mutant.

The pattern of inflammatory cell infiltrate in all groups of infected mice was similar, with a predominant mixture of lymphocytes and foamy and epithelial macrophages intermingled with minimal numbers of neutrophils. At 3 weeks and 20 weeks of infection, the proportion of lung parenchymal lesion was greater in mice infected with wild type and the *mce1* operon mutant than in mice infected with the *mce2* mutant (Fig. 6). At 20 weeks, less than 25% of the lung parenchyma had granulomatous lesions in mice infected with the *mce2* operon mutant, while the others had 50-75% involvement (Fig. 6).

4. Discussion

We found that *M. tuberculosis* H37Rv disrupted in the *mce2* operon is attenuated in the mouse model of TB. We first showed that the *mce2* operon is not essential for bacterial viability *in vitro* (Fig. 1). This finding is in contrast to that of Sassetti and colleagues, who used transposon site hybridization methodology (TRaSH) to show that *yrbE2B* gene (a gene in the *mce2* operon) is essential for bacterial growth *in vitro* [15]. The difference is not surprising due to the possible polar effects when transposon mutagenesis is used to disrupt a gene in an operon, as opposed to the in-frame mutation we created for this study. The *mce2* operon mutant created by Gioffre et al. also grew normally *in vitro* [16].

Furthermore, the ability of *mce2* operon mutant bacteria to survive within naïve RAW macrophages was similar to that of wild type parental strain. Therefore, we conclude that the genes encoded by the *mce2* operon play no role in bacterial replication *in vitro* or its survival within RAW macrophages.

However, we observed that the levels of TNF- α , IL-6 and MCP-1 were significantly reduced in response to infection of RAW cells with the *mce2* operon mutant compared to infection with the wild type H37Rv strain. While cytokine expression patterns observed in RAW cells may not necessarily reflect the *in vivo* effect of the mutant, we did examine cytokine expression patterns in the mouse lungs at 3, 6, and 12 weeks of infection. However, the results of such measurements involving mixed cells were uninterpretable (data not shown), as we had observed with lung cells from mice infected with the *mce1* operon mutant we reported previously [10]. Nevertheless, we were able to reproduce an earlier work, which showed that the *mce1* operon mutant was severely deficient in its ability to induce macrophage pro-inflammatory response [3]. We note that, as depicted in Fig. 6, histopathology studies of the lungs are more illustrative of the differences in pro-inflammatory response than cytokine measurements in these types of infection models. We also note that although both *mce1* and *mce2* operon mutants induced significantly lower levels of pro-inflammatory cytokines than did the wild type H37Rv strain in RAW cells, the levels induced by the *mce1* operon mutant was significantly lower compared to those induced by the *mce2* operon mutant.

Senaratne et al showed that *mce3* and *4* operon mutants, which are attenuated in mice, and wild type *M. tuberculosis* induced similar levels of the same cytokines in RAW macrophages infected ex-vivo [13]. Thus, the *mce2* operon mutant has a phenotype distinct from the other 3 *mce* operon mutants; compared to the wild type strain, it is attenuated in mice (like the *mce3* and *4* mutants), and induces less amount of cytokines in RAW cells (like the *mce1* operon mutant). These observations suggest that the *in vitro* analyses of cytokines produced by macrophages—which may reflect what happens acutely in an *in vivo* infection—are not predictive of chronic *in vivo* infection outcomes.

The mouse lung histopathologic findings are consistent with the attenuation we observed in mice infected with the *mce2* operon mutant. At 56 weeks post infection, all of the mice infected with the wild type strain had died, whereas 80% of mice infected with the *mce2* operon mutant still remained alive. It is interesting to note that mice infected with the *mce2* mutant contained a similar number of bacteria in their lungs at all time points post infection when compared to wild type infected mice. Therefore, the attenuation observed with the *mce2* operon mutant cannot be attributed to a lower bacterial burden.

Gioffre et al. has also demonstrated that disruption of the *mce2* operon led to the attenuation of this strain's virulence in BALB/c mice when intratracheal route of infection was used [16]. However, in their study, the *mce2* operon mutant's attenuation in mice was associated with decreased bacterial burden and delayed granuloma formation. The *mce2* operon mutant examined by Gioffre et al. was constructed by allelic interruption of the *mce2A* gene, which is not dissimilar to the way our *mce2A* mutant was constructed. However, the route of infection and infectious inoculum sizes were different--intratracheal and intraperitoneal (as opposed to the aerosol route of infection we used)--at 2 logs higher number of bacteria. Finally, their mouse infection was carried out up to 20 weeks, in contrast to ours which lasted 56 weeks. In their study, all mice infected with the wild type were dead by 9 weeks, which is most likely due to the high inoculum size (2.5×10^4) and the more susceptible mouse strain (BALB/c) used [16].

Thus, in our study, the longer survival time of mice infected with the *mce2* operon mutant could be attributed to a lower bacterial burden and the mouse strain (C57BL/6) we used. In contrast to the *mce1* operon mutant, which also induced low levels of cytokine production by RAW cells [3], the *mce2* operon mutant showed a lung histology more similar to that produced by wild type H37Rv. That is, the granulomas were tightly organized, with the distribution and number of lymphocytes and macrophages similar to that seen in lungs of mice infected with wild type H37Rv. This could be because the *mce2* mutant induced higher levels of pro-inflammatory cytokines than did the *mce1* mutant. The main difference was in the proportion of the lung parenchyma affected (Fig. 6). Death of mice seen with the *mce1* operon mutant and wild type H37Rv infections appears to be associated with the large proportion (>70%) of the lung parenchymas affected compared to that in mice infected with the *mce2* operon mutant at the same time points of infection (Fig. 6).

Thus, this and the previous studies of the *mce* operon mutants show that each mutant exhibits a distinct phenotype *in vivo* and *in vitro*. We and others have recently shown that the *M. tuberculosis mce* operon families may serve as lipid transporters [17, 18]. The disruption of lipid-modulating functions could alter the lipid-rich *M. tuberculosis* cell wall composition, which in turn could affect how a host mounts an immune response. Thus, the differences we saw in mouse immune response and clinical outcome may be mediated not necessarily by the differences in bacterial protein products expressed or not expressed by the respective operons but by the structural changes in cell wall that result from the disruption of these operons. These observations suggest the importance of a system-biology approach to

characterize a host-pathogen relationship that determines the ultimate fate of an infection with *M. tuberculosis*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Nicola Casali and Ryan Senaratne for helpful discussions.

Funding: This project was supported in part by the Center for AIDS Research Grant and NIAID Grant RO1AI035266.

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Fig. 1.

Mycobacterium tuberculosis growth in vitro in liquid broth and its survival within RAW macrophages. (A) *M. tuberculosis* H37Rv wild type strain and *mce2* operon mutant were both grown in 7H9+ADC. The experiment was done in triplicate and these data are based on one of three independent experiments with similar results. (B) Bacterial survival within RAW macrophages over 72 hour time period. Raw cells were infected with wild type H37Rv, *mce1* or *mce2* operon mutant strains at an MOI of 1:1 and bacterial survival monitored over 72 hours. The experiment was done in triplicate and these data are based on one of three independent experiments with similar results.

Fig. 2.

Kinetic analysis of TNF- α (A), IL-6 (B), and MCP-1 (C) production by RAW macrophages infected with H37Rv wild type or *mce* mutant strains at multiplicity of infection (MOI) of 1:1. Cells were infected and cytokine production assessed over 72 hour time period post infection. Controls included uninfected RAW cells, *mce1* operon mutant or LPS (not shown). The experiment was done in triplicate and these data are from one of three independent experiments with similar results. Comparison for statistical significance was made between *mce* operon mutants vs wild type. (*) Indicates significance where $P < 0.05$.

Fig. 3.

Bacterial replication in mouse lungs at different time points post infection. Recovery of bacteria is determined by cfu enumeration per mouse lung at indicated weeks; the number of cfu is expressed as the mean $\pm$ standard deviation (n=3).

Fig. 4. Survival kinetics of C57BL/6 mice infected with *M. tuberculosis* H37Rv wild type, *mce2* operon and *mce1* operon mutant strains. Ten mice per strain were initially infected via aerosol infection route and followed for the indicated number of weeks. By week 56 all mice infected with the H37Rv wild type and *mce1* operon mutant strain were dead while only 20% of mice infected with the *mce2* operon mutant were affected.

Fig. 5.

Gross pathology of mouse lungs infected with wild type H37Rv, *mce2* operon mutant or *mce1* operon mutant strains. Lungs were harvested at different time points post infection. Uninfected mouse lung is shown to represent negative control. Three mice per infection point per mouse strain were sacrificed and the lung was removed for analysis. The lung shown is a representative of such lungs. Ruler marks represent 1mm increments.

Fig. 6. Histology of pulmonary lesions in infected mice. Sections of C57BL/6 mouse lungs magnified x20 (A and B) and x400 (C) and stained with H&E. Mouse lungs were harvested at 3 weeks post infection (A) and 20 weeks post infection (B and C). Also shown is the picture of uninfected mouse lung section (D) magnified x20 and x400. Three mice per infection point were sacrificed. Micrographs shown are representative of multiple mouse lung sections taken from each lung.