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Identification of *Mycobacterium avium* genes expressed during *in vivo* infection and the role of the Oligopeptide transporter OppA in virulence

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ABSTRACT

M. avium causes disseminated disease in patients with AIDS and other immunosuppressive conditions and pulmonary infections in individuals with chronic lung diseases. Much still need to be learn about the mechanisms of *M. avium* pathogenesis. Using a mouse model of disseminated *M. avium* disease, we applied an *in vivo* expression technology system and identified *M. avium* genes up-regulated in different organs of mice during early stage of infection. The *M. avium oppA* gene, involved in an active transport of oligopeptides across the cell membrane, was found highly expressed in lung, liver and spleen of mice. Mutation in the transport domain of the *oppA* gene resulted in bacterial attenuation in both macrophages and in mice. Using protein-protein interaction assay, it was determined that two hypothetical small proteins, MAV_2941 (73aa) and MAV_4320 (45aa), interact with OppA. MAV_2941 was shown to be secreted by the bacterium into the macrophage cytoplasm. Mutations in MAV_2941 was associated with significant impairment of growth in macrophages. Understanding the mechanisms involved in the functions of MAV_2941 and MAV_4320 is warranted.

INTRODUCTION

Mycobacterium avium subsp *hominissuis* (hereafter *M. avium*) is a common bacterial pathogen responsible for opportunistic infections in HIV-positive individuals, immunocompetent patients with underlying chronic lung diseases but in apparently healthy population [1,2]. *M. avium* infects preferentially blood monocytes and tissue macrophages, and successfully survives within them. Pathogenic mycobacteria had adapted to unfavorable environments in the host by interfering with many of the defense mechanisms. When ingested by macrophages, *M. avium* prevents phagosome acidification [3] and fusion of phagosomes with lysosomes [4]. Bacterial components that play important roles in the early phase of infection are usually significant contributors to the adaptation to specific host niches. Previously, we have described genes regulated within macrophages by using *M. avium* promoter trap-GFP library [5]. In addition, the screening of an *M. avium* transposon library for clones deficient in uptake by human mononuclear cells identified both a PPE gene and a Pathogenicity Island (PI) [6]. The latter was associated with the ability of the bacterium to infect both amoeba and mammalian macrophages. The work also characterized the binding of *M. avium* protein encoded by the PI's ORF7 to actin, which results in cytoskeleton rearrangement and phosphorylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The finding indicated that the ability to infect amoeba has equipped *M. avium* with novel virulence factors, many times not shared with other mycobacteria, including *M. tuberculosis*. Recent evidence that GAPDH is involved in the vesicle transport of eukaryotic secreted proteins from the endoplasmic reticulum to the Golgi apparatus [7] suggests that the binding may be part of the pathogen's strategy to modify early events in vacuole

trafficking.

Despite the knowledge obtained on *M. avium* genes associated with pathogenesis, the knowledge is still limited about mycobacterial virulence determinants. The *In Vivo* Expression Technology (IVET) has been developed to identify genes specifically expressed at different stages of infection *in vivo*. Different from the transposon approach, the IVET system does not require generating a chromosomal gene disruption. The IVET approach, based on *lacZ* and *purA*, was initially adapted for *Salmonella typhimurium*, and genes encoding for various virulence factors were identified in a mouse model of septicemia as well as in a rat model of chronic lung infection [8]. *In vivo* selection, based on auxotrophic markers, has also been reported for *Pseudomonas aeruginosa* [9] and *Klebsiella pneumoniae* [10]. Comparable CAT-based IVET strategies have been used to unveil novel virulence genes from *Yersinia enterocolitica* in a mouse model [11]. In addition, *Mycobacterium tuberculosis* genes, preferentially expressed during macrophage infection and *in vivo*, were identified through *inhA*-based IVET system [12,13]. Although the IVET system has the ability to discover potential virulence determinants, it also has limitations, since many of the bacterial genes are associated with metabolism or other basic metabolic functions.

To select for *M. avium* virulence determinants involved in mechanisms of survival in different organs in mice, we developed an IVET system based on a quinolone-resistance gene as a selection marker. DNA gyrase, an enzyme essential for DNA super coiling and required for DNA replication and gene transcription, has been shown in mycobacteria to be a target for the quinolone family of drugs [14]. We then created a *M. avium*-promoter library upstream of the promoter-less resistant *gyrA* gene. The screening for *M. avium*

tissue-specific virulence determinants expressed during the early phase of mouse infection, successfully identified *M. avium* genes regulated *in vivo*. Here we describe that one of those genes, expressed in the lung, spleen and liver is associated with the transport of *M. avium* proteins to the macrophage cytoplasm.

MATERIALS AND METHODS

Construction of IVET vector. Quinolone-resistant *M. avium* au2 and CDC1 clinical isolates were obtained from Dr. Clark Inderlied, Children's Hospital Los Angeles. The *gyrA* and *gyrB* genes were amplified using the sense (F) and antisense (R) primers provided in Table 1, and sequenced to determine the point mutations. The *E. coli*-*Mycobacterium* IVET test vector was constructed from pMV261 His-tag containing plasmid by removing the *hps60* promoter and inserting the strong sigma-70 (G13) promoter of *M. marinum* in *NheI* site. The *gyrA*-resistant gene with a point mutation at amino acid 87 was cloned in front of G13 promoter. In order to ensure that *gyrA* expression from the *M. avium* promoter-trap library was the direct result of activation of bacterial promoters, the *trpA* transcriptional terminator was placed, in advance, upstream of the G13 promoter. The IVET test vector named pLDG13GyrA and the control vector pLDGyrA, without the G13 promoter, were transformed into *M. avium* 104 and *M. smegmatis* mc² 155. The *M. avium* wild-type and construct clones were then assayed for minimal inhibitory concentration (MIC) using the broth macrodilution method, as previously described [15].

***M. avium* promoter-trap IVET library construction and *in vivo* screening.** *M. avium* library was created by the following method: *M. avium* 104 genomic DNA was partially digested with *Sau3AI* restriction enzyme. After electrophoresis on a 1% agarose gel, fragments with 300-1500 bp size were extracted from the gel and cloned into the dephosphorylated *BclI* site of pLDGyrA plasmid upstream of the resistant *gyrA* gene (Fig. 1A). The resulting library was expanded in *E. coli* DH10BTM competent cells (Life Technologies). Screening of *E. coli* transformants for restriction analysis showed that

90% of the library contained DNA inserts. Approximately 30,000 clones of *E. coli* containing IVET constructs were used as the source plasmid for further construction of the library in *M. avium*. Mycobacterial competent cells were prepared by washing bacterial pellet four-times with cold washing buffer (10% glycerol, 0.1% Tween 80). The competent cells in amount of 100 to 200 μ l were combined with 3 to 7 μ l of library and transferred to a prechilled 0.2-cm cuvette. Electroporation was carried out under the following conditions: capacitance, 25 μ F; resistance, 1,000 Ω ; voltage, 2.5 kV. Clones were recovered on Middlebrook 7H10 agar plates containing 400 μ g/ml kanamycin. Over 12,000 *M. avium* clones representing approximately three-times coverage of the genome were stored in pools of one thousand.

The *in vivo* experiments were approved by the IACUC committee of the Oregon State University. Female C57 BL/6 black mice, 6 weeks old, were purchased from Jackson Laboratories, and housed at the Laboratory of Animal Research Center at Oregon State University. Thirty-two mice were divided into four groups of eight control and twenty-four experimental mice (eight in three *M. avium* library pool infection groups) (Fig. 1B). While the control group was infected intravenously through the tail vein with 100 μ l aliquots containing 10^7 colony forming units (CFU)/ml of wild-type *M. avium* 104, the experimental group was infected in a similar manner with IVET library pools of *M. avium*. Two mice from each group were sacrificed at day 2 to establish the baseline infection. The rest of the animals were treated with moxifloxacin 150 mg/kg daily. After 2 weeks of treatment, mice were sacrificed from both control and experimental groups and spleens, livers and lungs were harvested. Surviving bacteria were isolated from all mice. At least one hundred colonies were screened from each mouse. The bacteria were

quantified on 7H10 Middlebrook plates with or without kanamycin 400 µg/ml. No bacteria grew in control group of infected mice treated with 400 µg/ml of kanamycin. The *gyrA* overexpressing plasmids were isolated from the mycobacterium and sequenced at the Central Service Laboratory (CSL), Center for Genome Research and Biocomputing (CGRB), Oregon State University, Corvallis. Database search and sequence comparisons were performed at the National Center for Biotechnology Information (www.ncbi.nih.gov) using BLAST network service.

Real-Time PCR quantification. To confirm that the changes in *M. avium* gene expression level *in vivo* were similar to those seen from *M. avium* promoter IVET library, twelve genes were selected for Real-Time analysis. Bacterial RNAs from control (broth grown) and experimental (mice organs) samples, obtained at the same time points as the IVET experiment, were extracted and processed for Real-Time PCR, as previously described [5]. The intracellular bacteria were extracted from organ tissues as follow: tissues were lysed with 0.5% SDS. Lysed cells from suspension were removed by differential centrifugation at 300 rpm for 5 min and the supernatant was removed and centrifuged at 3,500 rpm for 10 min to recover intact bacterial cells. The resulting bacterial pellets were re-suspended in Trisol (Life Technologies). RNA extraction was performed as previously described [5]. Samples were cleaned up with RNA clean kit (QIAGEN), treated with DNase, and concentration and quality of RNA was verified with $A_{260/280}$ ratio. Reverse transcription was performed using the SuperScript® III Reverse Transcriptase kit (Life technologies) according to the manufacturer's protocol. Quantitation of the expression of *M. avium* genes was carried out in the iCycler iQ (Bio-Rad) with SYBR Green I assay using gene-specific primers (Table 1). The calculated

threshold cycle (Ct) for each gene amplification was normalized to the Ct of the 16S rRNA gene amplified from the corresponding sample and the fold change in gene expression was calculated as previously described [5].

Mutant constructs. To study the role of *oppA*, MAV_2941 and MAV_4320 in the pathogenesis of *M. avium*, the PCR-based mutations were generated in these genes. Mutations were generated by simply including the desired nucleotide change in the primer sequence. More specifically, in the transport domain of the OppA aspartic acid (Asp) was replaced with glutamic acid (Glu) acid at 523aa position and asparagine (Asn) with lysine (Lys) at 527aa position. Two mutations at 2aa replacing glycine (Gly) with Glu and at 73aa replacing arginine (Arg) with Lys were generated in MAV_2941; while in the MAV_4320 gene Glu was replaced with Gly at 4aa of N-terminus and Arg was changed with Gly at 42aa of C-terminus. The mutations in the gene were confirmed by sequencing before cloning. Mutated genes were then cloned into *EcoRI* site of the IVET test vector (pLDG13GyrA with His-tag) containing the resistant *gyrA* gene. The construct plasmids were transformed into the wild-type *M. avium*, as described above, and plated on 7H10 agar plates containing kanamycin (km) 400 µg/ml (marker for pLDG13). To confirm if km resistant clones of *M. avium* truly contained transformed constructs, PCR was performed for km and mutated *M. avium* genes using primers designed based on the plasmid sequence. In addition, *M. avium* mutated clones were grown in 7H9 broth and the western blot analysis was performed for mutated OppA, MAV_2941 and MAV_4320 proteins using His-antibody. To verify the differences in macrophage survival during infection with wild-type and mutated-overexpressed clones, THP-1 human monocyte cells (ATCC) were differentiated into macrophages by adding

50ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich). Cells then were seeded at 80% confluency in 24-well plates, infected with either the *M. avium* wild-type, the wild-type containing the pLDG13GyrA vector or mutated-overexpressed clones for 1 hour. After bacterial infection, the wells were washed three times with HBSS and incubated with 200 µg/ml of amikacin for 1 h to kill and prevent the extracellular growth. To establish the baseline infection, monolayers from each groups were lysed at 2 h infection with 0.25 % SDS for 10 min, serially diluted and plated onto Middlebrook 7H10 agar or containing appropriate antibiotics for colony forming unit (CFU) counts. Rest of monolayers were incubated for 3 and 5 days, in presence of RPMI-1640 supplemented with 10% FBS and 0.6 µg/ml of ciprofloxacin to predominantly overexpress the mutated genes. After each time point, cells were lysed and dilutions were plated onto agar plates to determine the number of CFU. The CFUs were recorded from antibiotic-treated as well as untreated macrophages.

***In vivo* survival study.** Thirty-six C57 beige mice were divided into two groups of eighteen and each group was infected intravenously with approximately 1×10^8 either *M. avium* LDG13GyrA or OppA clone expressing mutated gene. Both groups received intraperitoneal injection of 150 mg/kg moxifloxacin every 2 days. The dose of moxifloxacin was based on the studies that show that treatment with 150 mg/kg is bactericidal for *M. avium* [16]. After two days of initial infection, six mice were harvested from each group and bacterial loads were determined in spleens by plating homogenized tissues onto 7H10 Middlebrook plates. Twenty-four mice (twelve in each group) were followed for additional three weeks. Then, the experiment was terminated and the bacterial loads were determined in the mouse spleens.

Protein-protein interaction screening by BacterioMatch II Two-Hybrid system. The Bacteriomatch II two-hybrid system (Stratagene, La Jolla CA) was used to construct and co-transform the bait pBT:*oppA* and the prey pTRG:MAC-library [17] into the reporter strain of *E. coli*. Transformants were plated onto LB agar plates containing 400 µg/ml carbenicillin, 12.5 µg/ml tetracycline, 34.5 µg/ml chloramphenicol, and 50 µg/ml kanamycin and incubated at 30°C overnight. Next day, all grown *E. coli* colonies were transferred onto LB plates containing the same antibiotics, except carbenicillin, and containing the following additional reagents: 80 µg/ml X-Gal and 200 µM phenylethyl β-D-thio galactoside in dimethylformamide. The clones that turned blue in presence of X-Gal at 30°C incubation were selected and sequenced. The OppA positive interactions with MAV_2941 and MAV_4320 proteins were repeated and confirmed with the following co-transfections of pBT:MAV_2941 or pBT:MAV_4320 with pTRG:*oppA* vector, as described above.

Western blot analysis of *M. avium* secreted proteins. Acetamidase promoter expression vector pJAM2, kindly provided by J. A. Triccas and B. Gicquel (Pasteur Institute, Paris, France), was used to construct the pJAM2:MAV_2941 and pJAM2:MAV_4320 plasmids. The primers indicated in Table 1 were used to amplify MAV_2941 and MAV_4320 genes from the *M. avium* chromosomal DNA, and PCR fragments were then cloned in frame with the His-tag at the *Bam*HI restriction site. The constructs were introduced into *M. avium* 104 strains. The positive *M. avium* clones were grown to log phase in M63 medium supplemented with 1 mM MgSO₄, 0.5% Tween-80 and 2% succinate for non-induced cultures or 2% succinate and 2% acetamide for induced cultures, as previously described [18]. The THP-1 macrophages were infected

with *M. avium* pJAM2:MAV_2941, or pJAM2:MAV_4320, induced and non-induced clones for 48 h. After which, cells were lysed with CellLytic™ M reagent (Sigma) and centrifuged at $10,000 \times g$ for 15 min to remove bacterial pellet and cell debris from the suspension. Pre-cleared cell lysates were incubated with His-agarose conjugate primary antibody overnight at 4° C. Samples were separated on 16.5% precast Tricine gels, transferred into nitrocellulose membranes and blocked overnight in a 1:1 dilution of TBS in Odyssey blocking buffer (Li-Cor, Lincoln NE). Next day, membranes were incubated with anti-rabbit IgG linked to AlexaFluor680 secondary antibody (Li-Cor, Lincoln NE) at a dilution of 1:5000 for 1 h and scanned using Odyssey Imager (Li-Cor).

β -lactamase assay for *M. avium* protein secretion. In order to confirm the western blot results for *M. avium* protein secretion and demonstrate the cellular translocation, MAV_2941 and MAV_4320 genes generated with *HindIII* and *EcoRI* restriction sites were cloned into pLDG13:*bla*(-) plasmid containing TEM-1 β -lactamase gene (*bla*) lacking the first 69 nucleotides important for β -lactamase enzyme secretion. The constructs were transformed and expressed in *M. avium* 104 strain. *M. avium* clones containing either a vector with *bla*(-) reporter gene lacking a signal sequence (negative control), a vector expressing the full-size *bla*(+) reporter gene (positive control) or a vector expressing the *bla*(-)_MAV_2941 and *bla*(-)_MAV_4320 fusion (experimental clones) were used for macrophage infection assay. Human macrophages (10^5) were seeded into 96-well plates or in 2-chamber slides and loaded with the fluorescent substrate CCF2-AM at the room temperature using manufacturer's protocol (Life Technologies). After, THP-1 cells were infected with *M. avium* clones expressing *bla*(-), *bla*(+), MAV_2941:*bla*(-) and MAV_4320:*bla*(-) fusion proteins at a multiplicity of

infection of 10:1 and incubated at 37°C in 5% CO₂. After 2 h infection cells were washed three times with Hank's balanced salt solution (Life Technologies) to remove extracellular bacteria. Readings were recorded at 4h, 8h, 12h, 16h, 20h and 24h post-infection with two filter sets: excitation 405 ± 20 nm/emission 460 ± 40 nm and excitation 405 ± 20 nm/emission 530 ± 30 nm using Tecan Infinity 200 cytofluorometer. Fluorescence micrographs were captured with CCF2 filter set (Leica).

Statistical Analysis. The data represents the mean of three experiments ± the standard deviation. Significance of the differences between experimental groups and control groups was analyzed by the Student's *t*-test. A *p* value of < 0.05 was considered significant.

RESULTS

Feasibility study of IVET vector *in vitro*. As previously reported, substitutions of amino acid residues within the DNA gyrase subunits A and B are involved in quinolone resistance, mostly at codons corresponding to amino acids 83 and 87 in the *gyrA* and amino acids 426, 447, and 464 in the *gyrB* [19]. In addition, the corresponding enzymes are at least 10-fold less sensitive to the inhibitory effects of quinolones, when compared to the corresponding wild-type DNA gyrases. Sequencing of *gyrA* and *gyrB* genes of the quinolone resistant clinical isolates of *M. avium* identified the *gyrA* gene with a point mutation at amino acid 87, which was used for construction of the IVET test vector. *In vitro* susceptibility of *M. smegmatis* and *M. avium* wild-type and resistant *gyrA* expressing clones to ciprofloxacin and moxifloxacin is presented on Table 2. Bacteria were tested against quinolones in range of 0.15 to 65 µg/ml concentrations by the broth microdilution method [15]. Ciprofloxacin MIC for *M. smegmatis* and *M. avium* wild-type and clones, containing the control plasmid, were 0.3 µg/ml and 0.625 µg/ml, respectively to the strain. Mycobacteria containing the IVET test vector had their growth inhibited at significant higher concentrations of ciprofloxacin and moxifloxacin compared to the wild-type and the plasmid containing controls. After functional confirmation of *gyrA* resistance, we removed G13 promoter resulting pLDGyrA plasmid and used the promoterless vector for library creation.

Infection with the *M. avium* promoter-trap IVET library and treatment *in vivo*.

Mice infected with *M. avium* wild-type and IVET library pools were treated for 2 weeks and effect of moxifloxacin treatment was determined by bacterial loads in different organs. As shown on Table 3, daily treatment of *M. avium*-infected mice with high dose

of moxifloxacin resulted in significant decrease in the number of intracellular bacteria compared with the control group at two weeks. Treatment of wild-type infected mice significantly reduced the number of intracellular *M. avium* in contrast to treatment of IVET library-infected mice at the same time points. In addition, bacterial loads from two weeks infected tissues of sensitive *M. avium* 104 were plated on 7H10/kanamycin 400 µg/ml agar medium. The wild-type bacterium did not grow on kanamycin plates in contrast to resistant *M. avium* containing expressed IVET vector.

Identification of *M. avium* genes expressed *in vivo*. Partial screening of the IVET library (Fig. 1B) identified 15 *M. avium* clones displaying resistance to moxifloxacin treatment *in vivo*. The reason moxifloxacin was used instead of ciprofloxacin was the low MIC and greater tissue level compared to ciprofloxacin. Among sequences are gene promoters associated with bacterial metabolic pathways, virulence and detoxification, while the majority of genes have unknown function (Table 4). A number of these promoters are activated specifically in different organs of mice after two weeks of *M. avium* infection.

***M. avium* gene expression analysis by Real-Time quantitative PCR.** To confirm that the changes in *M. avium* gene expression *in vivo* match to the ones observed by the screening of the *M. avium* promoter IVET library, twelve genes were selected for Real-Time analysis. The chosen genes were MAV_0464 (*oppA*) (a component of an hypothetical ABC-transporter), which showed expression in all organs of mice, as well as tissue-specific genes MAV_0469, MAV_0470, MAV_5017 (spleen), MAV_0769, MAV_2480, MAV_3104 and MAV_4357 (liver), MAV_0638, MAV_3164, MAV_4033 and MAV_4237 (lung). The expression levels of target genes from intracellular bacteria

in livers, spleens and lungs, and from broth-grown bacteria, were normalized to the expression level of the endogenous reference 16S rRNA in each sample. There was significant expression of all selected genes by bacteria from tissue, confirming the IVET results (Fig. 2). The *M. avium* oligopeptide transporter *oppA* showed highest up-regulation (38-fold) in spleen compared to the expression of the rest of genes.

Mutations in OppA transport domain result in *M. avium* impairment in

macrophages and in mice. The OppA protein, a component of the ABC-transporter family, has been shown to be involved in the transport of bacterial proteins or peptides important for survival *in vivo* [20]. To identify if *M. avium* oligopeptide transporter had impact on bacterial survival, we mutated the *oppA* gene in the domain associated with the transport of bacterial proteins. *M. avium* clone containing the mutated *oppA* construct was grown in 7H9 Middlebrook broth in presence of ciprofloxacin to dominantly express mutated gene. The expression of the mutated OppA protein in *M. avium* was analyzed by the western blotting using His antibody (Fig.4C). We then studied the survival of the mutant clone in THP-1 cells. As shown in Figure 3A the mutated clone was attenuated in macrophages compared with the wild-type bacterium.

To examine whether the mutated *oppA* clone was also attenuated *in vivo*, we infected C57BL/6 mice with *M. avium* carrying the plasmid pLDG13GryA (control) or *M. avium oppA* mutated clone and treated mice with 150 mg/kg of moxifloxacin to stimulate the expression of the mutated *oppA* gene. As observed in Figure 3B, at week 3 following infection, there was a significant attenuation of the *oppA* mutant. The results showed that while the control bacterium grew over time in the spleen, the mutated clone decreased in viability ($p < 0.05$).

***M. avium* small proteins interacting with OppA.** To test the hypothesis that OppA might transport small proteins to the surface of the bacterium and/or secrete them into the cytoplasm of host cells, we used BacterioMatch II Two-Hybrid system to identify OppA-interacting protein(s). With the co-transformation of the bait pBT:*oppA* and pTRG:MAC-library into the reporter strain of *Escherichia coli*, we determined that two hypothetical proteins, MAV_2941 (73aa) and MAV_4320 (45aa), directly interact with OppA (Fig. 4A).

Role of MAV_2941 and MAV_4320 in *M. avium* survival. To define the role of MAV_2941 and MAV_4320 proteins in the pathogenesis of *M. avium*, we generated two-point mutations in the N- and C-terminus of MAV_2941, and in the N- and C-terminus of MAV_4320 as described in Material and Methods. The expression of His-tagged mutated MAV_2941 and MAV_4320 proteins in *M. avium* 104 strain is shown in Figure 4C. The molecular weight of the mutated proteins was expected. The reason we opted to create a dominant negative strain was that gene knockout is very difficult to be accomplished in *M. avium*, and is even more difficult if the target gene is small, like MAV_2941 and MAV_4320. To rule out a possibility of growth defect, mutated clones were grown in 7H9 broth and growth rates were compared with wild-type bacterium. Both clones grew similarly to *M. avium* 104. To evaluate uptake by macrophage and intracellular survival, THP-1 mononuclear phagocytes were infected for 1 h and growth monitored for 3 and 5 days in presence or absence of minimal inhibitory concentration of ciprofloxacin (to induce the expression of the mutated genes, which were cloned fused with the *gyrA*). The intracellular growth of *M. avium* clones was then compared to the wild-type bacterium (Fig. 4B). All clones had similar ability to infect macrophages, however, after 3 and 5

days of infection, the MAV_2941 mutated clone showed impairment of growth in THP-1 cells ($p=0.07$ for day 3 and $p<0.05$ for day 5, Fig. 4B).

***M. avium* MAV_2941 is secreted into the host cell.** To determine whether MAV_2941 and MAV_4320 were secreted into the cytoplasm of host cells, we used the β -lactamase assay. THP-1 phagocytes were infected with *M. avium* clones expressing *bla*(-) lacking the signal peptide (Fig. 5A), *bla*(+) containing the signal peptide (Fig. 5B),

MAV_2941:*bla*(-) (Fig. 5C) or MAV_4320:*bla*(-) (Fig. 5D). Using a cell-permeable fluorescent substrate CCF2-AM for β -lactamase, detectable hydrolysis was observed during *bla*(+), positive control, and MAV_2941:*bla*(-) infection (Fig. 5B and C) at 20h post-infection. An endogenous β -lactamase hydrolysis of the substrate was quantified by cytofluorometry (Fig. 5E), confirming MAV_2941 protein secretion in human

macrophages. We also analyzed the intracellular number of bacteria, which was found to be similar in all groups (Fig. 5F). In addition, we fused both proteins to 6-His-tag of pJAM2 vector and over-expressed them in *M. avium*, as previously described [18]. The THP-1 cells were infected with *M. avium* clones for 24 h, and then macrophages were lysed, separated from the intracellular bacteria and processed for western blot analysis using His-tag antibody. As shown in Figure 5G, both proteins were expressed in *M. avium*, but only MAV_2941 was detected in the cytoplasmic fraction of macrophages.

Identification of the MAV_2941 motif and structural homologies. MAV_2941 possibly belongs to the operon composed of MAV_2940-MAV_2938 genes. A gene and protein homology search between *M. avium* and other non-mycobacterial species databases using BLAST network at NCBI, identified MAV_2941 as a unique mycobacterial gene with 68% GC content. When MAV_2941 was compared against

other mycobacterial databases, it was determined that this gene is 98% homologous to *M. avium* subsp. *paratuberculosis* and 79% homologous to *M. intracellulare* gene. However, the coding protein sequence is interrupted by a frame shifts in both sequenced strains of *M. avium* subsp. *paratuberculosis* and *M. intracellulare*. MAV_2941 is absent in *M. tuberculosis*. Domain search at CBS Prediction (<http://www.cbs.dtu.dk/services>), or MAST (Motif alignment and search tool, <http://meme.nbcr.net>) services did not show any conserved domains or common motifs in the MAV_2941 protein. The comparison was also performed against non-annotated contigs of water- and soil-borne amoeba species, common environmental hosts for *M. avium*, in the Sanger Institute (<http://www.sanger.ac.uk>) database. This search, using the *Dictyostelium discoideum* OmniBlast Server, identified high-scoring segment match of MAV_2941 protein to contig JC3V2, annotated as phosphatidylinositol kinase-like kinase (PIKK; Gene ID: 8623319 tra1). The further structural homology search, using ExPASy Proteomics (<http://ca.expasy.org>) ClustalW alignment, identified *Homo sapiens* phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3), a homolog to *D. discoideum* PIKK and structurally similar to *M. avium* MAV_2941 protein. Our search for MAV_4320 protein could not identify the conserved domain, common motif or similarity to any bacterial species besides *M. avium* subsp. *paratuberculosis* with 92% identity.

DISCUSSION

Screening of a *M. avium* IVET library in mice identified a bacterial oligopeptide permease A (*oppA*) highly expressed in the liver, spleen and lung of mice. The OppA belongs to the ATP-binding cassette (ABC) transporter family, and past studies reported OppA as one of the solute-binding proteins playing an important role in the transport of bacterial nutrient across the cell envelope. The process is accomplished by using the energy produced by the hydrolysis of ATP [21]. The ABC transporter group is involved in the survival of different bacteria [22,23] and in *Streptococcus pneumoniae* appears to modulate gene expression in response to environment cues [24]. In eukaryotic cells, the oligopeptide permease has also been reported to play a role in specific physiological processes, including drug efflux from cancer cells [25], as well as, association with host protection against *Y. pestis* in a mouse model [26]. The *M. tuberculosis* oligopeptide permease (Opp) has been shown to be linked to the expression of virulence-associated lipids and PE-family proteins, and its loss resulted in the delay of the death of chronically-infected animals [27]. In another study OppA has been linked with cytokine secretion and apoptosis of *M.tuberculosis*-infected macrophages [28].

We created point mutations in the transport domain of *oppA* gene and demonstrated that the mutated *M. avium* clone had significant impairment of growth in human phagocytes and in mice. We hypothesized that, besides the oligopeptides, OppA might export small proteins to the surface of the bacterium and deliver them to the cytoplasm of macrophages. Using two-hybrid approach, it was observed that both MAV_2941 and MAV_4320 proteins recognized OppA. Further investigation demonstrated that just MAV_2941, but not MAV_4320, was secreted into the cytoplasm of infected THP-1

mononuclear phagocytes. It certainly would be reassuring if we had determined that the OppA mutant does not secrete MAV_2941; however, technical limitations of working with *M. avium* prevent it. Nonetheless, our data clearly demonstrates that both OppA and MAV_2941 are involved in virulence.

M. avium MAV_2941 is a small protein with structural identity to a protein motif in its environmental host soil-borne amoebae *Dictyostelium*. The protein, phosphatidylinositol kinase-like kinase, was similar to the accessory domain of *Homo sapiens* PIK3C3. Since *M. avium* evolved survival strategies in environmental protozoan, as evidenced before to occur between bacteria and *Entamoeba* [29], it is plausible to speculate that the bacterium acquired MAV_2941 from another amoeba-adapted bacterium. *M. avium* replicates within *Acanthamoeba castellanii* and inhibits the lysosome fusion with vacuoles [30]. It is most likely that some *M. avium* strategies, to infect efficiently as well as survive in macrophages, originated under survival pressure within environmental amoebae.

Dictyostelium is a genetically tractable host, and the vesicular maturation and transport mechanisms involved in the endocytic pathway are well characterized [31]. In amoeba, phosphatidylinositol 3-kinase (PI3K) signaling has been shown to contribute to an array of cellular functions, including actin polymerization, bacterial phagocytosis and vesicular trafficking [29,32]. *Homo sapiens* PIK3C3, also named hVPS34 (vacuolar protein sorting 34), is the sole member of the class III PI3K. The role of PI3K in the regulation of vesicular trafficking of the endosomal system and more specifically in the phagosome maturation, has been previously described [33]. In *Salmonella*-containing vacuoles the activity of the effector SopB is linked to the inhibition of vacuole-lysosome fusion through dissociation of host endocytic trafficking proteins [34,35]. During *M.*

tuberculosis infection, hVPS34 recruitment has a critical role in the phagosome maturation [36]. *M. tuberculosis* lipoarabinomannan and a PI3P phosphatase, SapM, have been shown to inhibit PI3P generation, effecting the normal phagosome recruitment [37]. *M. tuberculosis* infection promotes targeting of immunity-related GTPases to the phagosome membranes via lipid-mediated interaction with PI3K and, thus, altering the phagolysosome biogenesis [38]. Future studies will address the possibility of MAV_2941 has a role in altering phagosome maturation.

Author's contribution:

LD: Designed studies, performed experiments, wrote the paper

BS: Performed experiments

LEB: Designed studies, wrote the paper, senior author

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Table 1. Sense (F) and Antisense (R) primers.

Experiment	Target	Primers	PCR product (bp)
	<i>gyrA</i>	5'-ATGACTGACACCACGCT-3'F 5'-CTAGCCGTCCGACCCC-3'R	2,520
	<i>gyrB</i>	5'-GTGGCTGCCAGAAAG-3'F 5'-TTAAACGTCTAGGAAG-3'R	2,034
Real-time PCR:			
	MAV_0464/oppA	5'-ATCCTGGATCGACTC-3'F 5'-TTGGTGCTCAGCGCC-3'R	200
	MAV_0469	5'-GTGACCGTCTTCGAC-3'F 5'-TGCGCGTAGCTGGAC-3'R	200
	MAV_0470	5'-ATGCTGGCCGAGCCC-3'F 5'-GTCAGCACCGTGACG-3'R	200
	MAV_5017	5'-GTGGGCTATCTGCGC-3'F 5'-GCTGGGTGACGGCGC-3'R	200
	MAV_0769	5'-TTCCAGCGACATCCG-3'F 5'-CGCGGGGGAACGACA-3R	200
	MAV_2480	5'-AAGTTCTTGGACCGC-3'F 5'-GTCACCACGAGCGAC-3'R	200
	MAV_3104	5'-GTGGTCTTCGAGTCG-3'F 5'-ACGCTGTGGTCCGGG-3'R	200
	MAV_4357	5'-GTGCGCGACCACCTC-3'F 5'-CACACCACGAGTCCG-3'R	200
	MAV_0638	5'-TTGACGGATCACCT-3'F 5'-ATCTCGCCAGCGGT-3'R	200
	MAV_3164	5'-ATGAATGTTCGTCGAT-3'F 5'-CGCCATCGGCGTGGC-3'R	200
	MAV_4033	5'-GTGACGGTCCCGAAA-3'F 5'-ATCTTCGACTTGTTG-3'R	200
	MAV_4237	5'-GACGTTGTGCTGGAA-3'F 5'-GTAGGGGTGGGCATC-3'F	200
Mutation:			
	MAV_0464/oppA	5'-TTTGAATTCGTGGCCGTGCTGGCC-3'F 5'- TTTGAATTCAGCCTTGACGATGTTCTCGT ACTCGGGCAGCCCCTTCCA-3'R	1,605 219
	MAV_2941	5'-TTTGAATTCTTGGAGCATGAACCG-3'F 5'-TTTGAATTCCTTGCCCGAACGGCC-3'R	
	MAV_4320	5'-TTTGAATTCGTGACCACAGGGACC-3F 5'-TTTGAATTCTTGGCCCGCCCGGAA-3'R	135

Transformant screening:	km	5'-ATGAGCCATATTCAA-3'F 5'-TTAGAAAAACTCATC-3'R	816
	pLDG13GyrA	5'-TTTGGGTGGGGCTGC-3'F 5'-CCTCGAGCAAGACGT-3'R	depending on gene
Overexpression in pJAM2:	MAV_2941	5'-TTTGGATCCTTGGGGCATGAACCG-3'F 5'-TTTGGATCCCCTGCCCCGAACGGCC-3'R	219
	MAV_4320	5'-TTTGGATCCGTGACCACAGAAACC-3'F 5'-TTTGGATCCTTGGCCGGCCCCGGAA-3'R	135
	MAV_2941	5'-TTTAAGCTTTTGGGGCATGAACCG-3'F 5'-TTTGAATTCCCTGCCCCGAACGGCC-3'R	219
β-lactamase:	MAV_4320	5'-TTTAAGCTTGTGACCACAGAAACC-3'F 5'-TTTGAATTCTTGGCCGGCCCCGGAA-3'R	135

Table 2. MICs of ciprofloxacin and moxifloxacin for mycobacterium clones

Bacteria	Ciprofloxacin MIC (µg/ml)	Moxifloxacin MIC (µg/ml)
<i>M. avium</i> au2	> 20	> 10
<i>M. avium</i> 104	0.6	0.3
<i>M. avium</i> 104 pLDGyrA	0.6	0.3
<i>M. avium</i> 104 pLDG13GyrA	> 10	> 5
<i>M. smegmatis</i> mc ² 155	0.3	0.15
<i>M. smegmatis</i> mc ² 155 pLDG13	0.3	0.15
<i>M. smegmatis</i> mc ² 155 pLDG13GyrA	1.25	1.25

Table 3. Moxifloxacin treatment of *M. avium* infected mice

Bacterium	Drug dose (mg/kg)	Number of bacteria (mean \pm SE)		
		Liver	Spleen	Lung
<i>M. avium</i> 104	150	2.1 \pm 0.9x10 ^{4a}	1.5 \pm 1.3x10 ^{5a}	1.0 \pm 0.3x10 ^{4a}
<i>M. avium</i> IVET-library pool #1	150	4.4 \pm 0.6x10 ^{6a,b}	2.3 \pm 0.9x10 ^{7a,b}	1.5 \pm 0.3x10 ^{6a,b}
<i>M. avium</i> IVET-library pool #2	150	3.7 \pm 0.9x10 ^{6a,b}	5.1 \pm 0.3x10 ^{7a,b}	0.9 \pm 0.5x10 ^{6a,b}
<i>M. avium</i> IVET-library pool #3	150	4.6 \pm 0.8x10 ^{6a,b}	4.2 \pm 0.3x10 ^{7a,b}	1.7 \pm 1.2x10 ^{6a,b}
Untreated controls:	At day 2	2.4 \pm 1.6x10 ⁶	1.7 \pm 1.2x10 ⁷	0.7 \pm 0.5x10 ⁶
	At 2 weeks	1.4 \pm 1.1x10 ⁷	3.2 \pm 0.9x10 ⁸	1.0 \pm 0.3x10 ⁷

Values are CFU per gram of tissue;

^a, $P < 0.05$ compared with control at 2 weeks;

^b, $P < 0.05$ compared to treatment of *M. avium* 104 infection.

Table 4. *M. avium* genes identified with IVET system in the early phase of infection

Organ	Gene	Product description
Spleen	MAV_0464/ <i>oppA</i>	Extracellular solute-binding protein, family protein 5. ABC-type oligopeptide transport system, periplasmic component.
	MAV_0469	HAD-superfamily protein subfamily protein IB hydrolase.
	MAV_0470	Hypothetical protein. Helicase/secretion neighborhood CpaE-like protein.
	MAV_3674/ <i>ald</i>	Alanine dehydrogenase/PNT, N-terminal domain.
	MAV_4466/ <i>rplV</i>	50S ribosomal protein L22. Binds specifically to 23S rRNA during the early stage of 50S assembly.
	MAV_5017	Hypothetical protein. Belongs to ABC-type transport system involved in resistance to organic solvents, permease component.
Liver	MAV_0024	Forkhead associated domain (FHA). FHA domains may bind to phosphothreonine, phosphoserine and phosphotyrosine.
	MAV_0464/ <i>oppA</i>	Extracellular solute-binding protein, family protein 5. ABC-type oligopeptide transport system, periplasmic component.
	MAV_0769	LytR/CpsA/Psr family protein. Cell envelop-related transcriptional attenuator.
	MAV_2480	Phospholipase, patatin family protein. Predicted esterase of the alpha-beta hydrolase superfamily.
	MAV_3104	Macrolide ABC transporter ATP-binding protein.
	MAV_4357	Hypothetical protein. Function unknown.
Lung	MAV_0464/ <i>oppA</i>	Extracellular solute-binding protein, family protein 5. ABC-type oligopeptide transport system, periplasmic component.
	MAV_0638	Conserved hypothetical protein. Predicted nucleic-acid-binding protein containing a Zn-ribbon.
	MAV_3164	Cytochrome D ubiquinol oxidase subunit 1.
	MAV_4033/ <i>nuoA</i>	NADH dehydrogenase subunit A.
	MAV_4237/ <i>yfdE</i>	Hypothetical protein. Predicted acyl-CoA transferases/carnitine dehydratase.

FIGURE LEGEND

Fig. 1. *M. avium* IVET system.

(A) Plasmid map of pLDGyrA promoter library.

(B) Schematic representation of the IVET library screening *in vivo*.

Fig. 2. Real-Time PCR quantification of *M. avium*-selected genes in spleens, livers and lungs.

Organs were harvested and intracellular bacterial RNAs were used to determine the number of copies of cDNAs for target and reference genes. Quantitation of the expression of *M. avium* genes was carried out with SYBR Green I assay by Real-Time PCR detection system using gene-specific primers. The data represent the average of three independent experiments \pm SD.

Fig. 3. Functional studies of *M. avium* OppA.

(A) Survival assay for *M. avium* clone expressing mutated OppA protein *in vitro*. THP-1 cells were infected with *M. avium* wild-type and mutated OppA clone for 3 and 5 days; CFUs were recorded from 0.6 μ g/ml ciprofloxacin-treated, as well as untreated macrophages. The number of bacteria taken up by the macrophages was: *M. avium* 104: $5.8 \pm 0.4 \times 10^5$; *M. avium* with pLDG13GyrA $5.6 \pm 0.3 \times 10^5$; and *M. avium* pLDG13GyrA + OppA/mutated $5.7 \pm 0.2 \times 10^5$. Results represent means \pm standard error of the mean of three independent experiments. **, $p < 0.01$, the significance of differences between the wild-type and *M. avium* mutated OppA clone.

(B) Survival assay for *M. avium* clone expressing mutated OppA protein *in vivo*. Mice were infected with approximately 1×10^8 bacteria. *, $p < 0.05$, the significance of differences between *M. avium* mutated OppA clone and the bacteria carrying the plasmid pLDG13GyrA at 2 days or 3 weeks post-infection.

Fig. 4. *M. avium* protein-protein interaction studies.

(A) A bacterial two-hybrid system was performed to identify and then reconfirm interaction between OppA and *M. avium* proteins. The known interaction (1) pBT:LGF2 + pTRG:GAL11^P serves as a positive control in protein-protein interaction studies. The pair of the following bait and target vectors (2) pBT:*oppA* + pTRG:vector (negative control); (3) pBT:*oppA* + pTRG:MAV_2941; (4) pBT:*oppA* + pTRG:MAV_4320; (5) pBT:vector + pTRG:*oppA* (negative control); (6) pBT:MAV_2941+ pTRG: *oppA*; (7) pBT:MAV_4320+ pTRG: *oppA* was cotransformed into BacterioMatch II reporter *E. coli*-competent cells and positive clones were selected on LB agar plates containing tetracycline, chloramphenicol, kanamycin, X-Gal and phenylethyl β-D-thio galactoside.

(B) Survival assay for *M. avium* clones expressing mutated MAV_2941 and MAV_4320 proteins. THP-1 cells, infected with *M. avium* wild-type and clones expressing mutated MAV_2941 and MAV_4320 protein, were treated with minimal inhibitory concentration of ciprofloxacin. Bacterial CFUs were recorded from treated and untreated macrophages at 3 and 5 days post-infection. Data represent the mean ± standard error. The significance of differences between *M. avium* wild-type and mutated MAV_2941 and MAV_4320 clones was evaluated as **, $p < 0.01$ and *, $p < 0.05$ at the same time point of infection.

(C) Western blotting against His-tagged mutated OppA (57kDa), MAV_2941 (8kDa) and MAV_4320 (5kDa) proteins. *M. avium* clones, containing mutated genes in pLDG13GyrA vector, were grown in 7H9 broth with 0.6 μg/ml of ciprofloxacin (mutations described in Material and Method section). Cells were collected at exponential growth phase and lysed with rapid mechanical disruption in bead-beater. The pre-cleared cell lysates were separated on 16.5% Tricine gels and processed for western blot analysis

using His primary antibody.

Fig. 5. MAV_2941 protein secretion into the cytoplasm of THP-1 cells visualized by β -lactamase-catalyzed hydrolysis of CCF2-AM.

The FRET-based fluorescent substrate CCF2-AM is rapidly cleaved by the endogenous β -lactamase enzyme, which converts CCF2-AM into its negatively charged form, producing a blue fluorescent signal at excitation 409 nm and emission 450 nm. In the absence of β -lactamase activity, the charged form of the CCF2-AM fluoresces green (at excitation 409 nm/ emission 520 nm). β -lactamase hydrolysis in: **(A)** Macrophages infected with *M. avium bla(-)* clone; **(B)** Cells infected with *M. avium* clone expressing full-length *bla(+)* protein; **(C)** Cells infected with *M. avium* clone expressing MAV_2941:*bla(-)* fusion lacking β -lactamase signal sequence; and **(D)** *M. avium* clone expressing MAV_4320:*bla(-)* fusion protein. Bar, 10 μ m.

(E) Hydrolysis of CCF2-AM by β -lactamase. Human macrophages were loaded with the cell permeable fluorescent substrate CCF2-AM for 3h and then infected with *M. avium* clones expressing *bla(-)*, *bla(+)*, MAV_2941:*bla(-)* and MAV_4320:*bla(-)* fusion proteins in 96-well plates. The increase in β -lactamase enzymatic activity (blue fluorescence readings at excitation 405 nm with bandwidth 20 and emission 460 nm with bandwidth 40 nm) was measured with the rate of CCF2 hydrolysis. Readings were recorded using Infinity 200 cytofluorometer (Tecan). The hydrolysis of CCF2-AM by β -lactamase was captured at 20h post-infection. The data represent the average of three independent experiments \pm SD. **, $p < 0.01$ the difference between *M. avium bla(-)* and MAV_2941:*bla(-)* infection groups.

(F) The percentage of *M. avium*-infected macrophages is presented as a function of the

number of ingested *M. avium* *bla*(-), *bla*(+), MAV_2941:*bla*(-) and MAV_4320:*bla*(-) clones after 2 h of incubation. The numbers represent the mean \pm SD of three independent experiments performed in duplicate.

(G) THP-1 cells were infected with *M. avium* clones containing over-expressed His-tagged proteins. At 24 h post-infection, cells were lysed with 0.25% SDS, centrifuged and supernatants were processed for western blot analysis. Lines: 1. *M. avium* expressing His:MAV_4320 protein (control); 2. His:MAV_4320 protein in the THP-1 cytoplasmic fraction; 3. *M. avium* expressing His:MAV_2941 protein (control); 4. His:MAV_2941 protein from cytoplasmic fraction of macrophages.

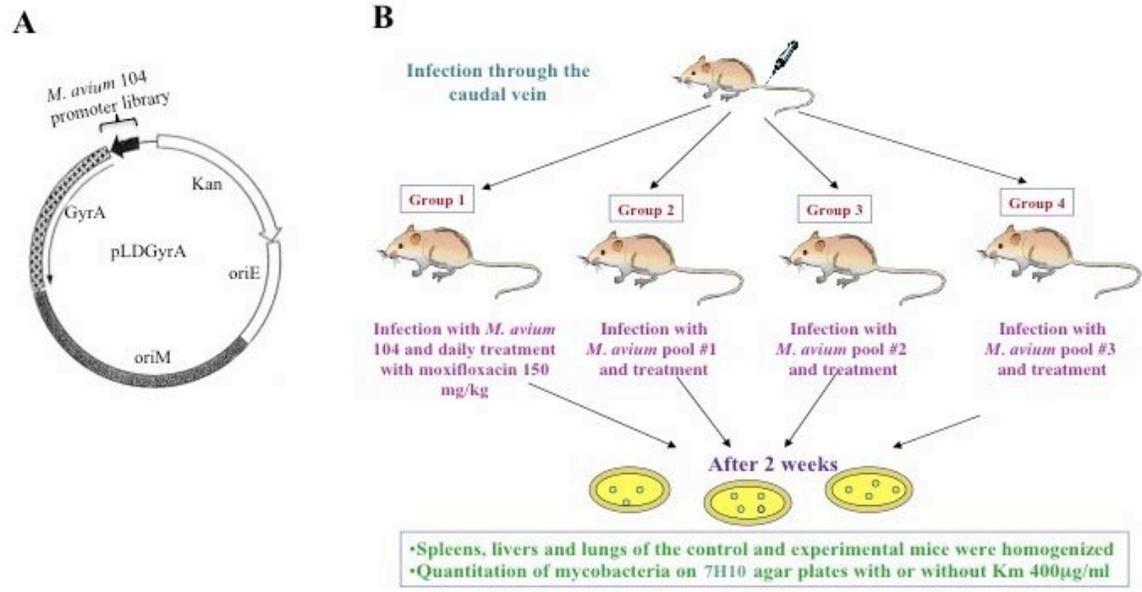


Figure 1

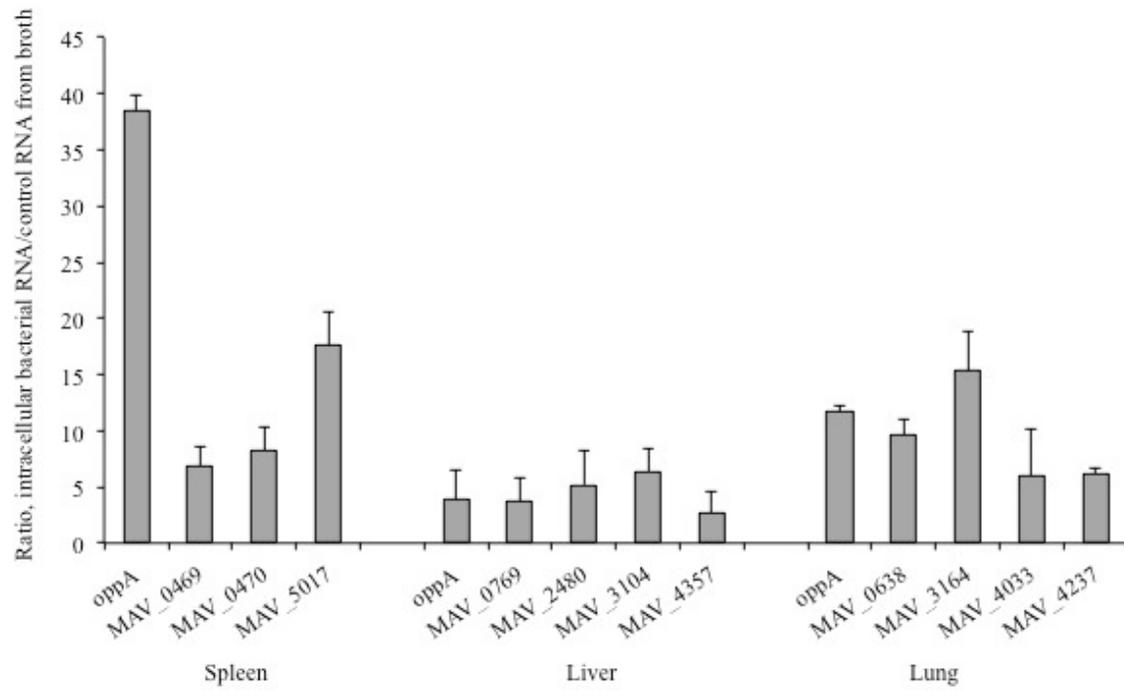


Figure 2

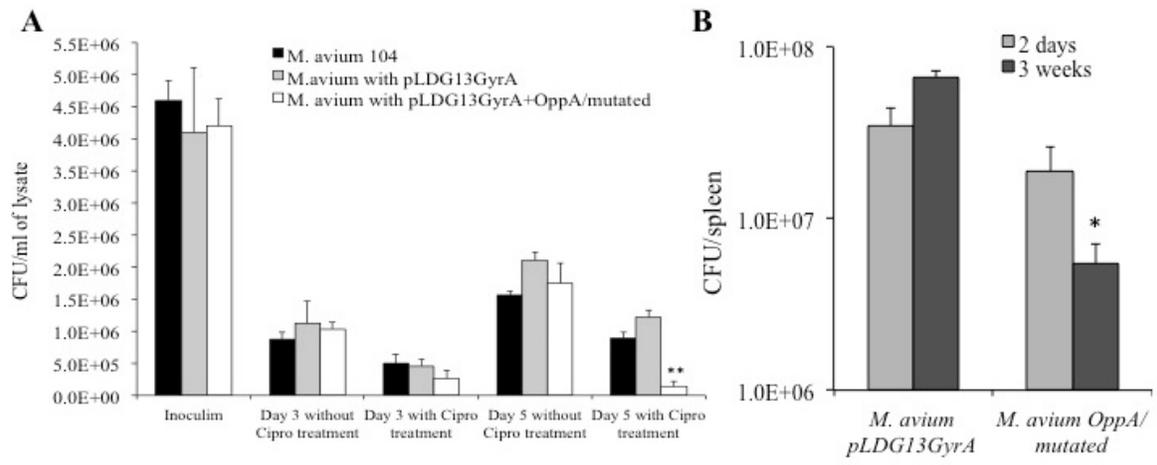


Figure 3

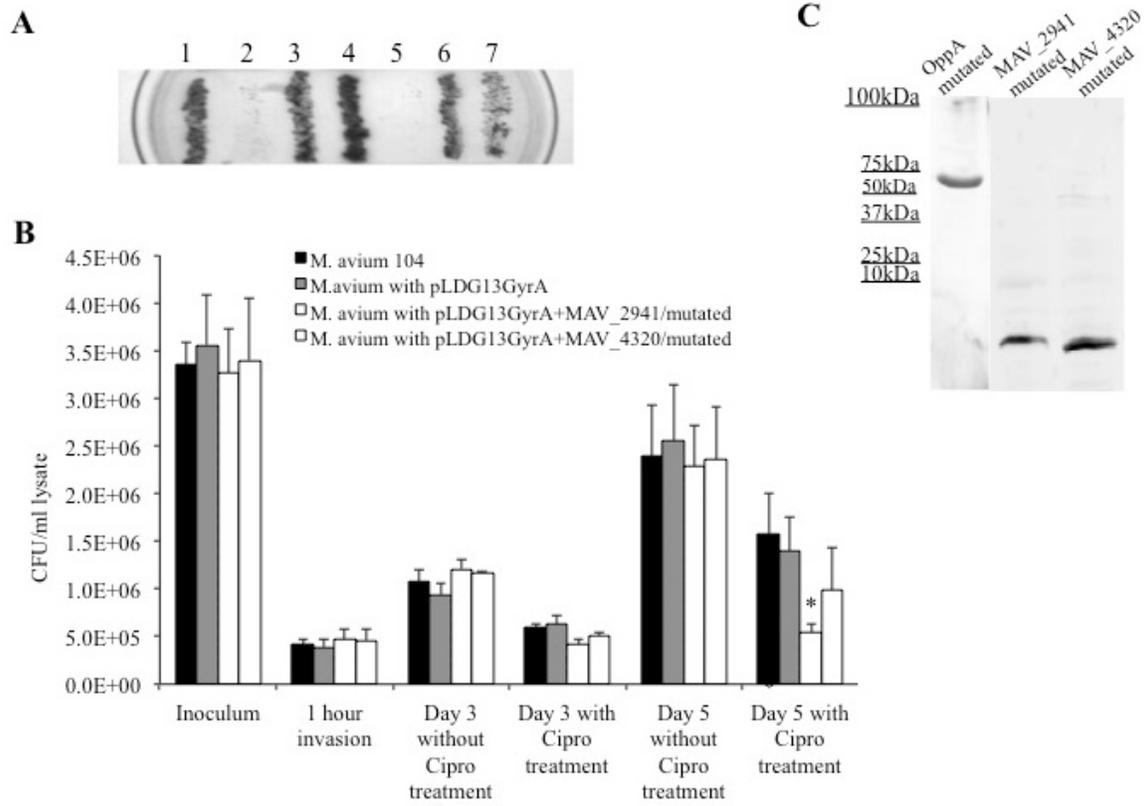


Figure 4

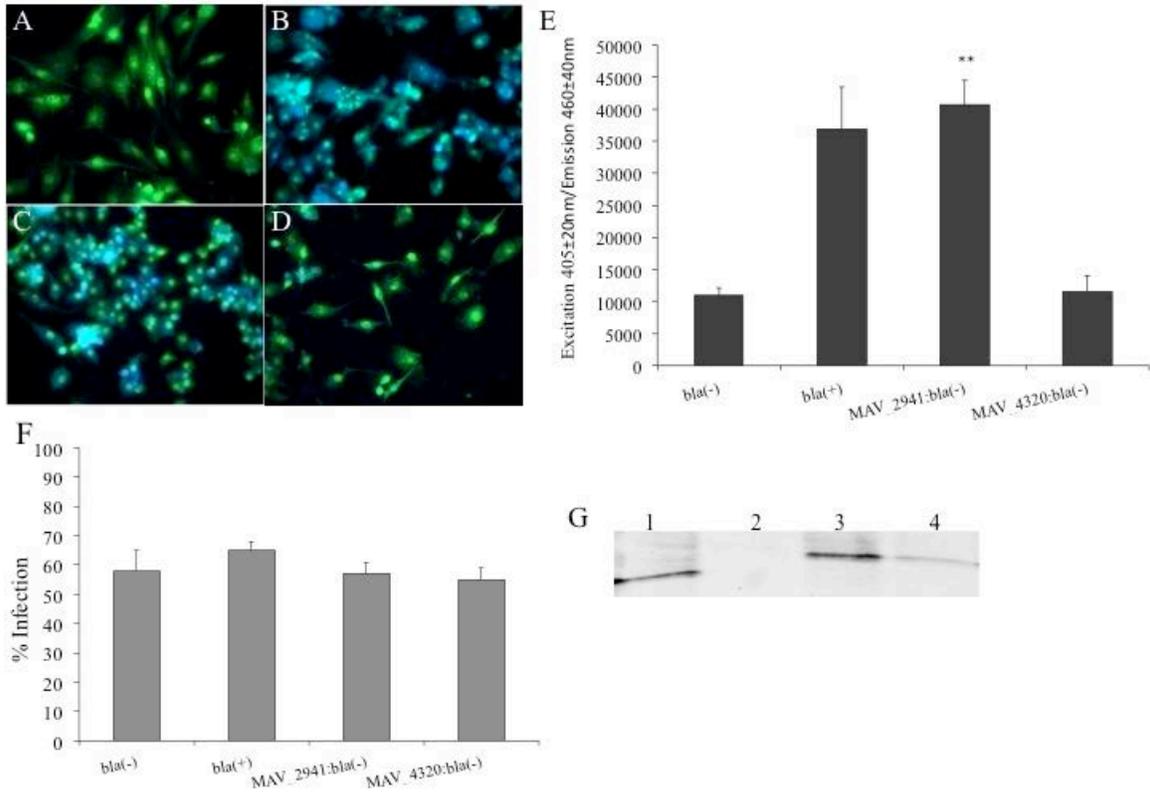


Figure 5