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Identification of *Mycobacterium avium* genes associated with the resistance to host antimicrobial peptides.

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Abstract

Antimicrobial peptides are an important component of the innate immune defense. *Mycobacterium avium* subsp *hominissuis* (*M. avium*) is an organism that establishes contact with the respiratory and gastrointestinal mucosa as a necessary step for infection. *M. avium* is resistant to high concentrations of polymyxin B, a surrogate for antimicrobial peptides. To determine gene-encoding proteins that are associated with this resistance, we screened a transposon library of *M. avium* strain 104 for susceptibility to polymyxin B. Ten susceptible mutants were identified and the inactivated genes sequenced. The greatest majority of the genes were related to cell wall synthesis and permeability. The mutants were then examined for their ability to enter macrophages and to survive macrophage killing. Three clones among the mutants had impaired uptake by macrophages compared to the wild-type strain, and all ten clones were attenuated in macrophages. The mutants were shown also to be susceptible to cathelicidin (LL-37), in contrast to the wild-type bacterium. All but one of the mutants were significantly attenuated in mice. In conclusion, this study indicated that the *M. avium* envelope is the primary defense against host antimicrobial peptides.

Introduction

Mycobacterium avium subsp *hominisuis* (hereafter *M. avium*) is a pathogen that infects humans and other mammals by crossing mucosal barriers. In humans *M. avium* causes disease in both immunocompromised and immune competent individuals (Ashitani *et al.*, 2001; Marras & Daley, 2002). Indication of infection in most of the patients is connected to signs and symptoms associated with the respiratory tract or systemic disease (Ashitani *et al.*, 2001; Marras & Daley, 2002).

In order to cause infection by crossing the respiratory and intestinal mucosas, *M. avium* has to resist to the action of antimicrobial peptides present on mucosal surfaces. Human intestinal mucosa secretes beta-defensins, cathelicidin and Reg IIIβ (Bevins & Salzman, 2011; Ouellette, 1999), while chiefly cathelicidin and defensins are found in the respiratory tract mucosa (Bals, 2000). Antimicrobial peptides play an important role in the host innate response against a number of bacteria (Sorensen *et al.*, 2008) and perhaps against Mycobacteria.

High concentrations of antimicrobial peptides are encountered in the mucus layer, preventing bacteria to move closer to the mucosal surface. In the intestinal lumen, *M. avium* has to cross two layers of mucus, one of them with large concentrations of antimicrobial peptides (Johansson *et al.*, 2011). In fact, work by Hansson's group has shown that while the external mucus layer contains many bacteria and low concentration of antimicrobial peptides, the most inner layer of mucus is rich in antimicrobial peptides and generally deficient in microorganisms (Johansson *et al.*, 2008). *M. avium* does not have flagellum or any other mechanisms to move across the mucus layers toward the mucosa. Therefore, in order for the bacterium to establish contact with mucosal surface, it should possess a mechanism that

would confer resistance to the harmful environment of the mucus, perhaps for an extended period of time. We now have evidence that *M. avium* does not bind to mucin, which may facilitate the bacterial migration in the mucus (manuscript in preparation). However, the pathogen should also be able to resist the action of antimicrobial peptides.

Prior studies have suggested the *Mycobacterium tuberculosis* may be susceptible to rabbit and human defensins and to other antimicrobial peptides (Miyakawa *et al.*, 1996). In addition, other studies have also shown that neutrophil proteins, HNP-1, 2 and 3, have bactericidal activity against organisms of the *M. avium* complex (Ogata *et al.*, 1992). More recently, it has been shown that *Mycobacterium avium* subsp *paratuberculosis*, a close species to *M. avium* subsp *hominssuis,* resist to the action of several antimicrobial peptides (Alonso-Hearn *et al.*, 2010).

Maloney and colleagues observed that *M. tuberculosis* cell surface phospholipids are in their majority lysinylated (Maloney *et al.*, 2009). The authors also demonstrated that in the absence of lysX gene, involved in the lysilynation of surface phospholipids, the bacterium becomes susceptible to antimicrobial peptides in vitro and is attenuated, compared with the wild-type bacterium, in vivo (Maloney *et al.*, 2009). The findings indicate that the susceptibility of pathogenic mycobacteria to antimicrobial peptides depend upon the components on the bacterial surface, which the expression can vary depending on the environment. PhoP, a major regulator of cell wall in many bacteria species, including *M.tuberculosis*, is associated with the resistance to antimicrobial peptides as well *(Ryndak et al., 2008).*

Antimicrobial peptides have been shown to possess antibacterial, antifungal and antiviral activities in model systems in vitro (Carroll *et al.*, 2010). Antimicrobial peptides are also produced by activated phagocytes and previous work has demonstrated their role in the

phagocyte’s mechanisms of killing (Brogden, 2005).

Macrophages and neutrophils contain antimicrobial peptides that participate in the killing of intracellular bacteria. The bactericidal molecules are delivered from the lysosome into the pathogen’s vacuole, where they can be inserted in the bacterial cell envelope (Duplantier & van Hoek, 2013). Because virulent mycobacteria inhibit the fusion of the phagosome with lysosome, much of the bactericidal peptides are probably not delivered to the bacterial environment (Sturgill-Koszycki *et al.*, 1994).

M. avium comes in contact with both the mucosal surface and phagocytic cells, and therefore we initiated the investigation on the resistance of the pathogen to antimicrobial peptides by screening a transposon library. Our screen was able to identify several genes associated with resistance to antimicrobial peptides, and beginning to understand how the bacterium can defend itself against the action of powerful bactericidal molecules.

Material and Methods

Bacterial strains and growth conditions:

Mycobacterium avium subsp *hominissuis* 104 is a virulent strain isolated from the blood of an AIDS patient. The bacterium was cultured on Middlebrook 7H11 agar supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC; Difco Laboratories, Detroit MI) or grown in Middlebrook 7H9 broth enriched with 0.2% glycerol and OADC. Bacteria were used in log phase as well as stationary phase of growth.

Escherichia coli DH5α (Stratagene, San Diego, CA) was grown on Luria-Bertani broth or agar plate (Difco Laboratories). Antimicrobial concentrations were added to the culture medium as indicated: Polymyxin B (32 µg/ml), Kanamycin (200 µg/ml).

MIC Determination

The inoculum was prepared by picking up 5 to 10 colonies (only transparent colonies) from a 7H10 agar plate and transferring the colonies onto 7H9 broth and allow to grow for 24 h. The minimal inhibitory concentrations (MICs) were determined by seeding 10^5 CFU of MAC 104 mutants or the wild-type bacterium into 96-well round-bottom plates in presence of Middlebrook 7H9 broth, 10% OADC and serial dilutions of polymyxin B. Controls included inoculum undiluted without drug and inoculum diluted 1:10 without drug. After 5 and 10 days of incubation at 37^{o}C, turbidities were compared to no antibiotic controls. Samples were plated onto 7H10 agar plates to confirm the results. Significant activity was defined as a reduction of 2 or more orders of magnitude over the period of the test.

M. avium transposon library and screening:

An *M. avium* 104 transposon library was constructed as previously described (Li *et al.*, 2010). It was duplicated into 96-well-flat-bottomed tissue culture plates to test for susceptibility to

polymyxin B (Sigma, St. Louis, MO), a cationic antimicrobial peptide surrogate. *M. avium* minimum inhibitory concentration (MIC) to polymyxin B was found to be > 500 µg/ml. Bacterial clones were then exposed to 32 µg/ml of polymyxin B and incubated at 37^{o}C. Growth was measured after 7 days of incubation by comparing turbidity of wells with or without polymyxin B. Clones showing susceptibility (no growth) in presence of polymyxin B were re-tested by plating onto 7H10 agar plates to confirm the phenotype.

Sequencing of clones and analysis

Genes interrupted in identificated clones were sequenced using the method as previously described (Danelishvili *et al.*, 2007). DNA sequences were obtained at the Center for Gene Research and Biocomputing, Oregon State University. Database search and analysis were performed using BLAST. The *M. avium* sequence DNA from the NCBI database was used to confirm the obtained sequences.

Susceptibility to cathelicidin

Purified LL-37 peptide was purchased from Ana Spec (Fremont, CA) and used at concentrations of 5 and 10 µg/ml in PBS. Bacteria were exposed to both concentrations of LL-37 at 37^{o}C for 3 h and an aliquot of the suspension was removed and plated onto 7H10 plates for quantification of the number of colonies.

Macrophage assay

To determine the susceptibility to macrophage killing, wild-type bacterium and clones were incubated with THP-1 macrophage monolayers containing 1 x 10^5 cells (previously stimulated with phorbol-esther, 50 µg/ml) at a multiplicity of infection (MOI) of 5. Phagocytosis was allowed to happen at 37^{o}C for 2 h in presence of RPMI-1643 media containing heat-inactivated fetal calf serum. Then the monolayers were washed with Hank's buffered salt

solution (HBSS). Some wells were then lysed with sterile water to release viable intracellular bacteria. The lysate suspension was serially diluted and then plated onto 7H11 agar plates to quantify the number of viable bacteria. After establishing the number of bacteria taken by macrophage monolayers at 2 hours, the remaining monolayers were followed for 4 days either without stimulation or with stimulation with 100 U of 1,25 dihydroxyvitamin D3 (1,25 vit D3). Monolayers were then lysed using the same procedure described above. The number of CFU at day 4 were compared to the number of CFU at 2 h after infection to determine the increase or reduction in the number of bacteria.

In vivo virulence assessment

C57/BL6 bg+/bg- mice were infected via the caudal vein with approximately 3×10^7 bacteria (MAC 104 and mutants). Some mice were sacrificed after 1 week post-infection to establish the baseline level of bacteria in spleen and liver. The remaining mice were sacrificed 4 weeks after infection. After, splenic and hepatic tissues were respectively removed and homogenized in 3 ml and 5 ml (respectively) of 7H9 broth containing 20% glycerol. Spleen and liver homogenates were serially diluted and plated onto &h11 agar plates. Plates were then incubated at 37^oC. After 10 days, bacterial infection load was determined by counting CFU. A total of 10 mice/experimental group and 7 mice for the 1 day inoculum determination, were used.

Statistical Analysis

A Student's T test was employed to compare experimental groups and control in all the experiments. P value smaller than 0.05 was considered statistically significant.

Results

Susceptibility in vitro

A transposon library was screened in vitro against polymyxin B, a surrogate for antimicrobial peptides, for clones susceptible to 32 µg/ml. Approximately 2,400 clones were evaluated for susceptibility to polymyxin B. The wild-type *M. avium* showed significant resistance to antimicrobial peptides and polymyxin B, with a Minimum Inhibitory Concentration (MIC) greater than 500 µg/ml. Ten mutants exhibited three or more fold reduction in growth in presence of 32 µg/ml of polymyxin B. All ten mutants failed to show increased susceptibility to sub-inhibitory concentrations of INH and clarithromycin but were more susceptible to ethambutol (data not shown).

Identification of mutations in *M. avium* clones

Transposon insertion locations were elucidated by amplifying flanking regions of Tn5367, as previously report (Danelishvili *et al.*, 2007; Li *et al.*, 2010). Table 1 lists all the mutants identified. All of the genes interrupted either play a role in generic cellular function, or encode for hypothetical proteins that are upstream to genes involved on cell wall permeability or still, are directly involved in cell wall permeability or fatty acid biosynthesis.

Inactivation of Kas B (MAV_2191 mutant #9) results in the synthesis of mycotic acids that are 2-4 carbons shorter than the mycolic acid in wild-type bacterium (Gao *et al.*, 2003). Kas B inhibition strikingly increases cell wall permeability to lipophilic compounds but has shown little effect on resistance to hydrophilic compounds (Gao *et al.*, 2003).

MAV_0119, a gene interrupted in mutant #3, encodes for a hypothetical protein that shows similarities to phosphatidylethanolanine N-methyltransferase, a principle component of cell membranes.

Mutants 1, 5, 6 and 7 are deficient in genes involved in cell wall synthesis. Tn5367 was located in the MAV_0216, a gene encoding for a hypothetical protein. This protein has similarity with the cutinase superfamily and analysis of the surrounding genes suggests that the transposon may have interrupted an operon, thereby suppressing the upstream genes which are associated with cell wall permeability (acyl-GA synthase, polyketide synthase, acetyl/propionyl CoA carboxylase beta unit).

The transposon also interrupted a polyketide synthase (pks), analogue to the *Mycobacterium avium* subsp *paratuberculosis* pks 12 and *M. tuberculosis* pks 12. The pks 12 is involved in the synthesis of dimycocerosate phthiocerol, a major cell wall lipid. Dimycocerosate phthiocerol is an integral element of the cell wall of pathogenic mycobacteria and has been hypothesized to provide cell wall impermeability (Camacho *et al.,* 2001).

Susceptibility to LL-37 (cathelicidin)

Humans, in contrast to many other mammals, have only one cathelicidin gene, and its expression leads to bactericidal activity in many tested systems. To evaluate if cathelicidin had comparable activity to polymyxin B, we exposed the wild-type bacterium and the transposon mutants obtained to different concentrations of LL-37 and determined the number of viable bacteria after 3 h. Almost all the mutants showed susceptibility to 5 µg/ml, while the wild-type bacterium apparently resisted the bactericidal effect of LL-37. All of the mutants were susceptible to 10 µg/ml of cathelicidin.

Macrophage uptake and killing

To determine whether alterations in the bacterial cell envelope has any impact on uptake and survival in macrophages we examined the interaction of the clones with macrophages. As shown in Table 2, three among the clones tested had significant decrease in the ability to

infect macrophages, although not all the mutations were associated with impact on phagocytosis. Three among all the mutants had their uptake by macrophages impaired at 30 min while for two of the clones the phenotype was still observed at later time point when compared with the uptake of the wild-type strain.

In Table 3, the results demonstrate that all of the clones were attenuated in non-stimulated macrophages. While some of the clones were still able to replicate within macrophages, four of the mutants had significant decrease in the number of intracellular bacteria compared with the wild-type bacteria and with the number of intracellular bacteria at the time after infection. It is also of note that when macrophages were stimulated with 1,25 vit D_3, the killing of intracellular *M. avium* strains increased substantially. 1,25 vit D_3 induces the synthesis of cathelicidin by macrophages (Liu *et al.*, 2006). Past work has demonstrated that the killing of *M. avium* in macrophages following stimulation 1,25 with vit D_3 is due to the secretion of TNF-α, GM-CSF and antimicrobial peptides (Bermudez *et al.*, 1990).

In vivo studies

To examine whether the mutations in *M. avium* led to attenuation in vivo, C57/BL-6 mice were infected with the bactericidal strains I.V. and at week 4 after infection, spleen and liver of the mice were harvested and the number of bacteria/organ determined. As displayed in Table 5, only the mutant 5 (inactivation of MAV_3616) did not show attenuation in vivo. All other tested mutants were attenuated. Mutants 2, 3, 9 and 10 had severe impairment of virulence as demonstrated by significant decrease in colony counts in both spleen and liver of mice.

Discussion

The innate immunity plays an important role in detecting and eradicating pathogens, although the details of the complex interactions between players remain incompletely known (Brogden *et al.*, 2003). Studies have stressed the importance of epithelial-derived as well as phagocyte-expressed antimicrobial peptides and observations in mice deficient in genes encoding for cathelicidin confirmed the increased susceptibility to infections (Brogden *et al.*, 2003; van der Does *et al.*, 2012).

Mycobacteria are a group of pathogens that infects many host cells but preferentially macrophages. Mycobacteria, therefore, must have significant number of strategies to be able to cause disease in mammals. One of the mechanisms used by the host to eliminate pathogens is the production of antimicrobial peptides molecules that are both released on the mucosal surfaces and intracellularly in phagocytic cells (Becknell *et al.*, 2013; Hansdottir *et al.*, 2008; van der Does *et al.*, 2012). Studies in the past have demonstrated that human defensins have bactericidal and/or bacteriostatic activity in vitro against *Mycobacterium avium* subsp *hominissuis* (Ogata *et al.*, 1992; Shin & Jo, 2011) and *Mycobacterium tuberculosis* (Miyakawa *et al.*, 1996; Rivas-Santiago *et al.*, 2006; Shin & Jo, 2011). In addition, more recent observations have supported the activity of cathelicidin (LL-37) against *M. tuberculosis* (Rivas-Santiago *et al.*, 2006; Rivas-Santiago *et al.*, 2008; Sonawane *et al.*, 2011; van der Does *et al.*, 2012). Cathelicidin expression in humans can be stimulated by the presence of 1,25-dihydroxyvitamin D_3 (1,25 vit D3) and a number of studies have shown evidence that *M. tuberculosis* and *M. avium* infections can be attenuated by controlling bacterial replication in macrophages following stimulation by 1,25 vit D3 (Bermudez *et al.*, 1990; Rivas-Santiago *et al.*, 2008; Yuk *et al.*, 2009). In contrast, other groups have been less

successful in establishing the correlation between *M. tuberculosis* survival and antimicrobial peptide production (Rivas-Santiago *et al.*, 2006; Sow *et al.*, 2011). In fact, work by Maloney and colleagues (Maloney *et al.*, 2009) described that mutation in the LysX protein of *M. tuberculosis*, a lysyl-transferase synthetase, makes the bacterium susceptible to the action of antimicrobial peptides (Maloney *et al.*, 2009), suggesting that in conditions which the protein is expressed and lysilynation occurs, the bacterium is potentially resistant to antimicrobial peptide molecules.

M. avium subsp *hominissuis* is even more resistant to antibiotics than *M. tuberculosis* and because the ability to survive in harsh environments as well as within environmental hosts (Inderlied *et al.*, 1993) containing a diverse array of killing mechanisms, it is assumed to have a harder cell wall to penetrate. To improve the understanding about susceptibility to antimicrobial peptides, we decided to screen a transposon bank of mutants to the action of polymyxin B, a surrogate for bactericidal peptides, and test the identified mutants with increased susceptibility to the antimicrobial, in a number of model systems in vitro and in vivo. The results of this study indicate that inactivation of cell wall synthesis/maintenance related genes leads to susceptibility to antimicrobial peptides and in the majority of the mutant strains, decrease of the ability to attenuation in macrophages and in mice. Interestingly, three of the mutations were associated with decreased of uptake by macrophages at 30 min and 2 h. This observation has several implications. First, because the phagocytosis assay was carried out in absence of opsonizing components of the serum, the results indicate that alterations in bacterial cell wall may impair uptake and make the bacteria more difficult to be ingested by phagocytes. The fact that fewer viable bacteria were isolated from macrophages at 1 h, may indicate that they were killed upon uptake. The other implication is that mutant bacteria may

enter macrophages by a pathway which is not the usual “pathogen-related” pathway, therefore increasing the likelihood that they will be subject to phagocyte bactericidal arsenal. However, we could not demonstrate that by inhibiting rapid mechanisms of killing (superoxide and nitric oxide-dependent) had any effect on the number of intracellular bacteria (data not shown).

Antimicrobial peptides are small molecules produced and secreted by epithelial cells and phagocytes (van der Does *et al.*, 2012). Many studies have demonstrated that mycobacterium infection results in increased production of the bactericidal molecules, including cathelicidin (Rivas-Santiago *et al.*, 2008; Shin & Jo, 2011). More recently, it has been shown that LL-37 regulates the transcription of autophagy-related genes, such as beclin-1 and atg 5, and still, other macrophage functions, suggesting that it does not only have direct anti-bacterial activity but also participates actively in the activation and regulation of other innate immune functions. The macrophages, 1,25 Vit D_3, and cathelicidin are involved in the killing of pathogens (Yuk *et al.*, 2009). In addition, *M. tuberculosis* but no *M. avium hominissuis* killing in macrophages has been linked to autophagy (Gutierrez *et al.*, 2004).

All the mutants identified in our work when exposed to sub-inhibitory concentrations of clarithromycin and INH did not show increased susceptibility to the antibiotics. However, they all were more susceptible to ethambutol (data not shown). This observation may have correlation with the particular action of ethambutol on the cell wall of *M. avium* subsp *hominissuis (*Mikusova et al., 1995) or that the bacterial cell wall works a partial barrier to the compound.

Based on the results of our study, interference with mycolic acid synthesis, synthesis of dimycocerosyl phthiocerol, a major cell wall lipid, which has been associated with cell wall

299 permeability and other genes linked to cell wall synthesis, enhanced susceptibility to
300 antimicrobial peptides. *M. avium* probably faces the challenge of antimicrobial peptides in the
301 mucosal surface. It is plausible to speculate that *M. avium* when in the intestinal tract
302 environment contains a cell envelope that is resistant to antimicrobial peptides. In
303 macrophages, however, because *M. avium* is able to inhibit phagosome-lysosome fusion
304 (Sturgill-Koszycki *et al.*, 1994), the contact with antimicrobial peptides may not occur in
305 principle , or at least not for all the intracellular bacteria. To explain the increased
306 susceptibility observed both in macrophages and in vivo, one must consider the fact that other
307 pathways, such as autophagy may contribute to the attenuation observed. Alternatively, some
308 of the attenuation observed with mutants when in macrophages and in mice may be explained
309 by a combination of factors in addition to the inability to inhibit phagosome-lysosome fusion.
310 Therefore, mechanism of susceptibility of the mutants in macrophages is probably multi-fold.
311 A mutant deficient in polyketide synthase has been previously described, but an association
312 with superoxide anion, or nitric oxide production by macrophages has not been established
313 (Li *et al.*, 2010). In fact, the mechanisms associated macrophage killing of organisms
314 belonging to the *M. avium* complex is poorly understood. The mutant #8, with inactivation of
315 an olygosyltrehalose synthase, may illuminate a possible mechanism, since several
316 microorganisms respond to environmental stresses by accumulating high levels of trehalose
317 (Zaragoza *et al.*, 2003). Trehalose is the only detectable free sugar in mycobacteria. Inability
318 to respond properly to environmental stresses and challenges may explain in part, the
319 susceptibility of this particular mutant in both macrophages and mice.
320 In summary, by screening a transposon library for increased susceptibility to polymyxin B, we
321 identified a number of *M. avium* mutants that are susceptible to the action of cathelicidin and

322 are attenuated in both, macrophages and mice. These findings are important because they
323 unveil potential targets for therapy or prevention of the infection as well as they offer new
324 insights on the pathogenicity of *M. avium*.
325

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References

Alonso-Hearn, M., Eckstein, T. M., Sommer, S. & Bermudez, L. E. (2010). A Mycobacterium avium subsp. paratuberculosis LuxR regulates cell envelope and virulence. *Innate immunity* **16**, 235-247.

Ashitani, J., Mukae, H., Hiratsuka, T., Nakazato, M., Kumamoto, K. & Matsukura, S. (2001). Plasma and BAL fluid concentrations of antimicrobial peptides in patients with Mycobacterium avium-intracellulare infection. *Chest* **119**, 1131-1137.

Bals, R. (2000). Epithelial antimicrobial peptides in host defense against infection. *Respir Res* **1**, 141-150.

Becknell, B., Spencer, J. D., Carpenter, A. R., Chen, X., Singh, A., Ploeger, S., Kline, J., Ellsworth, P., Li, B. & other authors (2013). Expression and antimicrobial function of Beta-defensin 1 in the lower urinary tract. *PLoS One* **8**, e77714.

Bermudez, L. E., Young, L. S. & Gupta, S. (1990). 1,25 Dihydroxyvitamin D3-dependent inhibition of growth or killing of Mycobacterium avium complex in human macrophages is mediated by TNF and GM-CSF. *Cell Immunol* **127**, 432-441.

Bevins, C. L. & Salzman, N. H. (2011). Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol* **9**, 356-368.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**, 238-250.

Brogden, K. A., Ackermann, M., McCray, P. B., Jr. & Tack, B. F. (2003). Antimicrobial peptides in animals and their role in host defences. *Int J Antimicrob Agents* **22**, 465-478.

Camacho, L. R., Constant, P., Raynaud, C., Laneelle, M. A., Triccas, J. A., Gicquel, B., Daffe, M. & Guilhot, C. (2001). Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. *J Biol Chem* **276**, 19845-19854.

Carroll, J., Field, D., O'Connor, P. M., Cotter, P. D., Coffey, A., Hill, C., Ross, R. P. & O'Mahony, J. (2010). Gene encoded antimicrobial peptides, a template for the design of novel anti-mycobacterial drugs. *Bioeng Bugs* **1**, 408-412.

Danelishvili, L., Wu, M., Stang, B., Harriff, M., Cirillo, S. L., Cirillo, J. D., Bildfell, R., Arbogast, B. & Bermudez, L. E. (2007). Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. *Proc Natl Acad Sci U S A* **104**, 11038-11043.

Duplantier, A. J. & van Hoek, M. L. (2013). The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds. *Front Immunol* **4**, 143.

Gao, L. Y., Laval, F., Lawson, E. H., Groger, R. K., Woodruff, A., Morisaki, J. H., Cox, J. S., Daffe, M. & Brown, E. J. (2003). Requirement for kasB in Mycobacterium mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. *Mol Microbiol* **49**, 1547-1563.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I. & Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766.

Hansdottir, S., Monick, M. M., Hinde, S. L., Lovan, N., Look, D. C. & Hunninghake, G. W. (2008). Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol* **181**, 7090-7099.

Inderlied, C. B., Kemper, C. A. & Bermudez, L. E. (1993). The Mycobacterium avium complex. *Clin Microbiol Rev* **6**, 266-310.

Johansson, M. E., Larsson, J. M. & Hansson, G. C. (2011). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A* **108 Suppl 1**, 4659-4665.

Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L. & Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* **105**, 15064-15069.

Li, Y. J., Danelishvili, L., Wagner, D., Petrofsky, M. & Bermudez, L. E. (2010). Identification of virulence determinants of Mycobacterium avium that impact on the ability to resist host killing mechanisms. *J Med Microbiol* **59**, 8-16.

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., Schauber, J., Wu, K. & other authors (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **311**, 1770-1773.

Maloney, E., Stankowska, D., Zhang, J., Fol, M., Cheng, Q. J., Lun, S., Bishai, W. R., Rajagopalan, M., Chatterjee, D. & other authors (2009). The two-domain LysX protein of Mycobacterium tuberculosis is required for production of lysinylated phosphatidylglycerol and resistance to cationic antimicrobial peptides. *PLoS Pathog* **5**, e1000534.

Marras, T. K. & Daley, C. L. (2002). Epidemiology of human pulmonary infection with nontuberculous mycobacteria. *Clin Chest Med* **23**, 553-567.

Mikusova, K., Slayden, R. A., Besra, G. S. & Brennan, P. J. (1995). Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob Agents Chemother* **39**, 2484-2489.

Miyakawa, Y., Ratnakar, P., Rao, A. G., Costello, M. L., Mathieu-Costello, O., Lehrer, R. I. & Catanzaro, A. (1996). In vitro activity of the antimicrobial peptides human and rabbit defensins and porcine leukocyte protegrin against Mycobacterium tuberculosis. *Infect Immun* **64**, 926-932.

Ogata, K., Linzer, B. A., Zuberi, R. I., Ganz, T., Lehrer, R. I. & Catanzaro, A. (1992). Activity of defensins from human neutrophilic granulocytes against Mycobacterium avium-Mycobacterium intracellulare. *Infect Immun* **60**, 4720-4725.

Ouellette, A. J. (1999). IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *Am J Physiol* **277**, G257-261.

Rivas-Santiago, B., Sada, E., Tsutsumi, V., Aguilar-Leon, D., Contreras, J. L. & Hernandez-Pando, R. (2006). beta-Defensin gene expression during the course of experimental tuberculosis infection. *J Infect Dis* **194**, 697-701.

Rivas-Santiago, B., Hernandez-Pando, R., Carranza, C., Juarez, E., Contreras, J. L., Aguilar-Leon, D., Torres, M. & Sada, E. (2008). Expression of cathelicidin LL-37 during Mycobacterium tuberculosis infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. *Infect Immun* **76**, 935-941.

Ryndak , M., Wang, S., Smith, I. (2008). PhoP, a key player in Mycobacterium tuberculosis virulence. Trends Microbiology **16**, 528-534

Shin, D. M. & Jo, E. K. (2011). Antimicrobial Peptides in Innate Immunity against Mycobacteria. *Immune Netw* **11**, 245-252.

Sonawane, A., Santos, J. C., Mishra, B. B., Jena, P., Progida, C., Sorensen, O. E., Gallo, R., Appelberg, R. & Griffiths, G. (2011). Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages. *Cell Microbiol* **13**, 1601-1617.

Sorensen, O. E., Borregaard, N. & Cole, A. M. (2008). Antimicrobial peptides in innate immune responses. *Contrib Microbiol* **15**, 61-77.

Sow, F. B., Nandakumar, S., Velu, V., Kellar, K. L., Schlesinger, L. S., Amara, R. R., Lafuse, W. P., Shinnick, T. M. & Sable, S. B. (2011). Mycobacterium tuberculosis components stimulate production of the antimicrobial peptide hepcidin. *Tuberculosis (Edinb)* **91**, 314-321.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. & other authors (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**, 678-681.

van der Does, A. M., Bergman, P., Agerberth, B. & Lindbom, L. (2012). Induction of the human cathelicidin LL-37 as a novel treatment against bacterial infections. *J Leukoc Biol* **92**, 735-742.

Yuk, J. M., Shin, D. M., Lee, H. M., Yang, C. S., Jin, H. S., Kim, K. K., Lee, Z. W., Lee, S. H., Kim, J. M. & other authors (2009). Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* **6**, 231-243.

Zaragoza, O., Gonzalez-Parraga, P., Pedreno, Y., Alvarez-Peral, F. J. & Arguelles, J. C. (2003). Trehalose accumulation induced during the oxidative stress response is independent of TPS1 mRNA levels in Candida albicans. *Int Microbiol* **6**, 121-125.

Table 1. Genes identified in the *M. avium* mutants associated with susceptibility to polymyxin B

Mutant#	Gene	Accession	Function
1	MAV_4265	ABK69290	Aldehyde dehydrogenase (NAD) family protein
2	MAV_3253	YP_882435	Hypothetical protein from the daunorubicin resistance gene cluster
3	MAV_0119	YP_879415	Thiopurine S-methyltransferase (tpmt)
4	MAV_0216	ABK65610	Cutinase superfamily protein
5	MAV_3616	ABK66306	Long-chain specific acyl-CoA dehydrogenase
6	MAV_4687	ABK68276	Dihydrolipoamide dehydrogenase
7	MAV_3373	YP_882794	Methyltransferase, UbiE/COQ5 family protein
8	MAV_3210	YP-882392	Glycogen debranching enxyme GlgX
9	MAV_2191	ABK67230	Beta-ketoacyl-acyl carrier protein (ACP) synthase (KAS), type II
10	MAV_2450	YP_881643	Erythronolide synthase (polyketide synthase), modules 3 and 4

Table 2. Macrophage infection assay. Infection at 30 min and 2 h comparing the wild-type *M. avium* 104 and mutant clones.

		Phagocytosis	
	Strains/genes	**30 min**	**2 h**
	MAC 104 (WT)	$4.9 \pm 0.4 \times 10^5$	$8.2 \pm 0.3 \times 10^5$
1	(4C8)/MAV_4265	$3.4 \pm 0.4 \times 10^5$	$7.1 \pm 0.5 \times 10^5$
2	(6E10)/MAV_3253	$5.3 \pm 0.5 \times 10^5$	$8.8 \pm 0.6 \times 10^5$
3	(7D10)/MAV_0119	$5.4 \pm 0.6 \times 10^5$	$8.4 \pm 0.6 \times 10^5$
4	(2G6)/MAV_0216	$8.8 \pm 0.3 \times 10^{4(*)}$	$6.9 \pm 0.4 \times 10^5$
5	(36H10)/MAV_3616	$5.6 \pm 0.4 \times 10^{4(*)}$	$2.3 \pm 0.4 \times 10^{4(*)}$
6	(23B4)/MAV_4687	$5.0 \pm 0.6 \times 10^5$	$8.4 \pm 0.4 \times 10^5$
7	(2H2)/MAV_3373	$5.8 \pm 0.6 \times 10^5$	$8.0 \pm 0.5 \times 10^5$
8	(25E10)/MAV_3210	$4.4 \pm 0.5 \times 10^5$	$7.5 \pm 0.7 \times 10^5$
9	(2C6)/MAV_2191	$4.9 \pm 0.6 \times 10^5$	$8.2 \pm 0.6 \times 10^5$
10	(1C2)/MAV_2450	$5.3 \pm 0.6 \times 10^{4(*)}$	$3.1 \pm 0.4 \times 10^{5(*)}$

$^{(*)}$ $p<0.05$ compared with *M. avium* 104 wild-type

Table 3. Macrophage survival assay comparing the ability of the wild-type *M. avium* 104 with mutant clones.

		CFU/10^5 macrophage lysate			
	Strains/genes	**1 h**	**4 days (with 1.25 vit $D_{3)}$**	**4 days (without 1.25 vit $D_{3)}$**	**Outcome**
	MAC 104 (WT)	$6.1 \pm 0.3 \times 10^5$	$5.7 \pm 0.4 \times 10^4$	$3.0 \pm 0.4 \times 10^6$	
1	(4C8)/MAV_4265	$3.4 \pm 0.4 \times 10^5$	$1.6 \pm 0.3 \times 10^4$	$6.2 \pm 0.3 \times 10^{5(*)}$	impaired
2	(6E10)/MAV_3253	$3.3 \pm 0.5 \times 10^5$	$8.9 \pm 0.5 \times 10^3$	$5.4 \pm 0.4 \times 10^{5(*)}$	impaired
3	(7D10)/MAV_0119	$5.9 \pm 0.5 \times 10^5$	$2.1 \pm 0.5 \times 10^5$	$7.9 \pm 0.4 \times 10^{5(*)}$	impaired
4	(2G6)/MAV_0216	$4.6 \pm 0.7 \times 10^4$	$7.3 \pm 0.4 \times 10^4$	$2.3 \pm 0.5 \times 10^{5(*)}$	decreased
5	(36H10)/MAV_3616	$2.3 \pm 0.4 \times 10^5$	$3.3 \pm 0.6 \times 10^4$	$4.0 \pm 0.3 \times 10^{5(*)}$	impaired
6	(23B4)/MAV_4687	$3.7 \pm 0.3 \times 10^5$	$1.1 \pm 0.4 \times 10^5$	$7.3 \pm 0.5 \times 10^{5(*)}$	impaired
7	(2H2)/MAV_3373	$3.8 \pm 0.5 \times 10^5$	$5.0 \pm 0.3 \times 10^4$	$2.9 \pm 0.3 \times 10^{5(*)}$	decreased
8	(25E10)/MAV_3210	$2.1 \pm 0.5 \times 10^5$	$6.9 \pm 0.5 \times 10^4$	$5.1 \pm 0.7 \times 10^{5(*)}$	impaired
9	(2C6)/MAV_2191	$1.6 \pm 0.4 \times 10^5$	$2.2 \pm 0.4 \times 10^4$	$8.4 \pm 0.3 \times 10^{4(*)}$	decreased
10	(1C2)/MAV_2450	$2.0 \pm 0.6 \times 10^4$	$1.7 \pm 0.3 \times 10^3$	$6.0 \pm 0.3 \times 10^{3(*)}$	decreased

$^{(*)}$ $p<0.05$ comparing the growth of the mutant strain with the wild-type growth

Table 4. Activity of LL-37 against *M. avium* 104 and mutant clones.

			LL-37 concentration	
	Strain/gene	**Inoculum**	**5 µg/ml**	**10 µg/ml**
	MAC 104 (WT)	$2.4 \pm 0.3 \times 10^4$	$2.6 \pm 0.3 \times 10^4$	$2.3 \pm 0.4 \times 10^4$
1	(4C8)/MAV_4265	$3.1 \pm 0.4 \times 10^4$	$2.1 \pm 0.4 \times 10^4$	$1.0 \pm 0.3 \times 10^{4(*)}$
2	(6E10)/MAV_3253	$2.6 \pm 0.2 \times 10^4$	$2.6 \pm 0.4 \times 10^4$	$9.3 \pm 0.2 \times 10^{3(*)}$
3	(7D10)/MAV_0119	$2.5 \pm 0.3 \times 10^4$	$2.0 \pm 0.4 \times 10^4$	$8.9 \pm 0.5 \times 10^{3(*)}$
4	(2G6)/MAV_0216	$2.8 \pm 0.3 \times 10^4$	$9.7 \pm 0.5 \times 10^{3(*)}$	$6.1 \pm 0.3 \times 10^{3(*)}$
5	(36H10)/MAV_3616	$2.7 \pm 0.2 \times 10^4$	$1.2 \pm 0.2 \times 10^{4(*)}$	$7.8 \pm 0.2 \times 10^{3(*)}$
6	(23B4)/MAV_4687	$3.0 \pm 0.4 \times 10^4$	$1.4 \pm 0.3 \times 10^{4(*)}$	$6.7 \pm 0.4 \times 10^{3(*)}$
7	(2H2)/MAV_3373	$3.1 \pm 0.4 \times 10^4$	$1.1 \pm 0.3 \times 10^{4(*)}$	$7.5 \pm 0.4 \times 10^{3(*)}$
8	(25E10)/MAV_3210	$2.6 \pm 0.2 \times 10^4$	$8.1 \pm 0.5 \times 10^{5(*)}$	$5.3 \pm 0.6 \times 10^{3(*)}$
9	(2C6)/MAV_2191	$2.9 \pm 0.3 \times 10^4$	$9.6 \pm 0.5 \times 10^{4(*)}$	$6.4 \pm 0.3 \times 10^{3(*)}$
10	(1C2)/MAV_2450	$2.7 \pm 0.4 \times 10^4$	$9.8 \pm 0.3 \times 10^{4(*)}$	$5.9 \pm 0.5 \times 10^{3(*)}$

$^{(*)}$ $p<0.05$ compared to WT *M. avium* 104 control.

Bacteria were exposed to 5 µg/ml or 10 µg/ml of recombinant LL-37 for 3 h and then plated onto 7H10 agar.

Table 5. Evaluation of virulence of the mutations in comparison to the wild-type *M. avium* 104 in C57 BL/6 mice

	Mutant gene	CFU/organ [§] 1 day		4 weeks	
		Liver	Spleen	Liver	Spleen
	MAC 104 (WT)	$2.6 \pm 0.4 \times 10^5$	$3.8 \pm 0.5 \times 10^5$	$4.2 \pm 0.6 \times 10^6$	$7.4 \pm 0.5 \times 10^7$
1	(4C8)/MAV_4265	_	_	$3.2 \pm 0.3 \times 10^{4(*)}$	$1.0 \pm 0.6 \times 10^{(*)(\pm)}$
2	(6E10)/MAV_3253	_	_	$< 2.0 \times 10^{2(\pm)}$	$< 2.0 \times 10^{2(\pm)}$
3	(7D10)/MAV_0119	_	_	$1.4 \pm 0.3 \times 10^{4(*)}$	$5.6 \pm 0.5 \times 10^{3(*)(\pm)}$
4	(2G6)/MAV_0216	_	_	$2.9 \pm 0.4 \times 10^{5(*)}$	$3.8 \pm 0.5 \times 10^{5(*)}$
5	(36H10)/MAV_3616	_	_	$3.1 \pm 0.5 \times 10^6$	$8.2 \pm 0.3 \times 10^7$
6	(23B4)/MAV_4687	_	_	$1.9 \pm 0.6 \times 10^{6(*)}$	$6.0 \pm 0.4 \times 10^{6(*)}$
7	(2H2)/MAV_3373	_	_	$8.8 \pm 0.4 \times 10^{5(*)}$	$4.1 \pm 0.6 \times 10^{6(*)}$
8	(25E10)/MAV_3210	_	_	$1.7 \pm 0.5 \times 10^{5(*)}$	$1.2 \pm 0.5 \times 10^{5(*)}$
9	(2C6)/MAV_2191	_	_	$3.7 \pm 0.3 \times 10^{4(*)(\pm)}$	$2.1 \pm 0.6 \times 10^{2(*)(\pm)}$
10	(1C2)/MAV_2450	_	_	$4.1 \pm 0.3 \times 10^{4(*)(\pm)}$	$2.7 \pm 0.3 \times 10^{2(*)(\pm)}$

[(*)] $p < 0.05$ compared with *M. avium* wild-type control at 4 weeks
[(±)] $p < 0.05$ compared with wild-type control at 1 day, inoculum
[§] The results represent the mean ± SEM