An efficient method for random mutagenesis in *Mycobacterium avium* subsp. *hominissuis* and for screening of mutants affected in virulence

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1 Introduction

Historically, human civilisation has been badly affected by deadly bacterial diseases and epidemics, where large scale deaths from tuberculosis, typhus, cholera, plague, diphtheria, dysentery, typhoid and pneumonia have caused havoc with humanity. Similarly the non-bacterial diseases like smallpox and malaria have also killed many humans. Today, better therapeutic methods, immunisation and antibiotic treatments have reduced morbidity and mortality rates from bacterial diseases. However, identification of new bacterial pathogens in human environment in the past decades has intimated us to put more research and study to control these new emerging infectious diseases.

1.1 Mycobacteria

1.1.1 The Mycobacterium genus

The *Mycobacterium* (*M.*) genus is best recognised for its pathogens *M. tuberculosis* (*M. tb*) and *M. leprae*, the infectious agents of tuberculosis and leprosy respectively. Mycobacteria are known for their acid-fast properties, cell wall with mycolic acids, and high percentage (61% to 71%) of guanine plus cytosine contents in the genome [1]. Mycobacteria are obligatory aerobic, straight or slightly curved rod shaped bacteria, with a length of 1 to10 microns and a width of 0.2 to 0.6 microns. The *Mycobacterium* genus belongs to the family *Mycobacteriaceae*, by now this genus is comprised of 167 species and subspecies [2]. The genus is divided into three groups; *Mycobacterium tuberculosis* complex, *M. leprae* and all other non tuberculosis causing mycobacteria, referred to as non tuberculous mycobacteria.

1.1.2 The Mycobacterium tuberculosis complex (MTBC)

MTBC species that cause tuberculosis in human include *M. tuberculosis*, *M. bovis, M. africanum*, *M. microti, M. canetti, M. caprae* and *M. pinnipedii* [3]. MTBC mode of transmission is not through vectors but is airborne, from infected animals and ingestion of contaminated food. An infection dose of 10 bacilli by inhalation is enough to cause the disease [4], with a capability of long latent period. Mainly antibiotic therapy or in some cases surgery is undertaken to treat the disease as attenuated

live vaccine (bacillus Calmette-Guérin: BCG) gives limited protection. Still tuberculosis causes deaths of more than one million people each year and World Health Organisation estimated that one-third of the global population is at present infected with *M. tb* [5].

1.1.3 The non tuberculous mycobacteria (NTM)

Since the discovery of “tuberkelbazillus” in 1882 by Robert Koch, *M. tb* was considered the most significant species in the genus *Mycobacterium*. Following the discovery, for many years (70 to 80 years), it was regarded the most valued organism, whereas NTM from human, animal or environmental sources were normally rejected for their little importance [6, 7]. Since 1950s numerous isolation of NTM, under certain conditions prompted some researchers to realise that, these organisms may be clinically important. Ultimately with the discovery of acquired immuno-deficiency syndrome (AIDS) in 1981 and the years to come, NTM got greater recognition and concern from scientific world. It was the most common opportunistic bacterial infection assigned to AIDS patients, in time when the incidence of tuberculosis started to decline [8]. NTM infections have now been recognised in most of the world, as rates of infection and disease appear to be rising in many developed countries [9, 10, 11]. Reasons for this observation include better diagnostic methods, rise in AIDS patients and other immuno-compromised individuals, rise in life expectancy for patients with increased susceptibility, and recognition of these microbes as potential pathogens.

Many terms have been coined for these mycobacteria as “atypical,” “anonymous,” “opportunist,” “tuberculoid,” “mycobacteria other than tuberculosis” and/or non tuberculosis mycobacteria”. Hereafter the last name will be used for the sake of uniformity.

The NTM disease is not a single unit disease, but is an overall designation for broncho-pulmonary diseases, lymphadenitis, skin infections, soft tissue infections, joint infections and disseminated diseases mostly in immuno-compromised persons [11]. Unlike tuberculosis, NTM disease transmission from human to human has not been reported.

NTM mainly occur ubiquitously in the environment, and are very often isolated from natural water sources, tap water, biofilms, aerosols, soil, dust, domestic and wild animals, and foods [12-14], They have been isolated from showerhead biofilms [15],

household plumbing [16], potting soil [17] and thus surround human environment. Interestingly, it had been also isolated from the water aboard the Russian space station Mir [18].

NTM differs in virulence, colony morphology and antibiotic resistance. It also differs in growth rate; includes slow growing and fast growing NTM, where many species from both have the ability to cause diseases in humans [19, 20] and animals. Presently they are classified on the basis of 16S ribosomal deoxyribonucleic acid (rDNA) sequence.

1.1.4 *Mycobacterium avium* complex (MAC)

Along with many other species, NTM also includes MAC, which is comprised of *M. intracellulare*, *M. scrofulaceum* and *M. avium* species. *M. avium* can be further divided into 4 subspecies: *M. avium* subsp. *paratuberculosis* (MAP) causes the Johne's disease in ruminants; *M. avium* subsp. *avium* (MAA) causes avian Tb; *M. avium* subsp. *silvaticum* infect notably wood pigeons and cranes; and finally *M. avium* subsp. *hominissuis* which causes disease in humans [21]. Restriction fragment length polymorphism (RFLP) typing, using insertion sequence IS*1245* as a probe, is a standardised tool for the molecular typing of *M. avium* [22, 23]. This deoxyribonucleic acid (DNA) typing method has been used in many epidemiological studies [13, 23-27] and is useful in differentiation between *M. avium* subspecies *avium* and *hominissuis* [28]. The insertion element IS*1245* is present in all *M. avium* except *M. avium* subsp. *paratuberculosis* [29], its distribution and copy number is highly variable in the genome from specie to specie. *M. avium* IS*1245* RFLP results from human and porcine sources possess highly multiple IS*1245* patterns, whereas bird type shows typically three bands for the same [30].

MAC species has been mainly isolated from water and soil all around the world. *M. avium* infections could be linked to long exposure to soil [31] or household water supply [32]. They can grow and survive an environment with low pH [12, 33], hot or cold water [34], antibiotics and disinfectants [35-37]. *M. avium* undergo dormant state in response to starved environment [38] and in addition they can also show resistance to heavy metals [39]. MAC to some extent can tolerate chlorine or chloramine concentration used for disinfection by municipal water systems [37]. This proves the wide range distribution of MAC and shows how it can challenge the

human immune system and serve as an environmental source for colonisation and infection of immuno-compromised individuals.

Recently, MAC clinical isolation has been increased with the increase in immuno-compromised individuals or longer life expectancy for AIDS patients [40]. Lung infections as well as lymphadenitis are most often caused by *M. avium* [11, 41], which is considered to be among the clinically most important NTM [42, 43]. In addition to having virulence, MAC are also more stress tolerant than most of the other NTM species [37, 39].

1.1.5 *Mycobacterium avium* subsp. *hominissuis* (MAH)

MAH is ubiquitous, opportunistic pathogen commonly isolated from soil and water [44, 45]. It customarily causes disseminated infections in humans with HIV, but otherwise can also infect healthy individuals with underlying diseases [8] and is also attributed for children lymphadenitis [46]. MAH has been isolated from pig, cattle, dogs, birds, deer, horses and other animals [47-51]. All these animals could be MAH reservoir, but it does not necessarily mean that MAH is zoonotic in nature.

MAH unique features include presence of numerous replicates of IS*1245*, varying 16S-23S rDNA internal transcribed spacer sequence and the capacity to survive and grow in temperatures between 24°C to 45°C [21]. Furthermore MAH is negative for IS*901*, whereas MAA is positive for the same insertion element [52].

MAH exhibit different distinguishable colony morphotypes on Congo-red plates (smooth opaque, smooth transparent and rough) which display different degrees of virulence. Smooth transparent and rough colonies are considered to be more virulent than smooth opaque colonies [53, 54]. The transparent morphotype is mainly isolated from patients and shows increased growth in macrophages and mouse models [55], and greater resistance to antibiotics. The colony morphotype is associated with the glycopeptidolipid (GPL) composition [56]. By inducing the release of various pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) or tumor necrosis factor-alpha (TNF-α), GPL modulates the immune response against mycobacteria [57].

1.2 Mycobacterial cell wall

The cell wall structure is an important factor determining virulence of *M. avium* [56]. The mycobacterial plasma membrane is covered by a thick layer of cell wall (Figure 1). The inner part of the cell wall is insoluble and mainly composed of a large cell wall core or complex [58]. It is formed of covalently linked structures peptidoglycan (PG), arabinogalactan (AG) and mycolic acids (MA) [59]. They are covalently bonded together and extend towards outside from plasma membrane, with the inner end as PG and on outer end MAs. Most of the drugs synthesised target this MA-AG-PG complex.

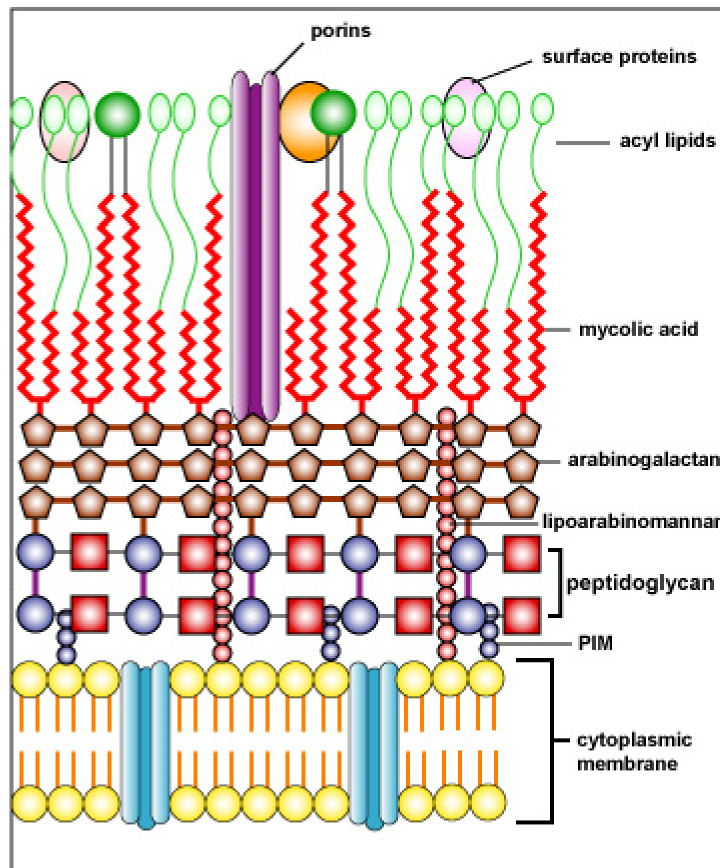


Figure 1. Cell wall of mycobacteria

(Source:http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit4/innate/u1fig11.html)

The outer part of the cell wall is lipid-rich, it is the most important determinant of NTM ecology and epidemiology [60-63]. The components of the outer cell wall are regarded as the signalling and effector molecules of mycobacteria [58] due to their corresponding role with the immune response. The outer part of the cell wall is

soluble and contains various freely associated lipids with long and short-chain fatty acids interacting with the short and long-chains fatty acids of the inner part of cell wall. The lipid-linked carbohydrates couple with the outer part of the cell wall and are consist of lipoarabinomannan (LAM), lipomannan, phthiocerol-containing lipids and the phosphatidylinositol mannosides, that are intercalated with the mycolic acids. Most of these lipids are specific for mycobacteria.

In slow-growing mycobacteria like *M. tuberculosis*, *M. leprae and M. avium*, the LAMs are capped with mannose residues and are known as ManLAMs [64-66], while *M. smegmatis* and *M. fortuitum*, the fast-growing mycobacteria, have phosphoinositol-capped LAMs referred as PILAMs [67]. But LAMs are uncapped in *M. chelonae* and are known as AraLAMs [64, 66].

The outer most layer, generally called the capsule, mainly consists of polysaccharides (glucan and arabinomannan). NTM resistance to disinfectants and antibiotics is related to the cell wall-associated permeability barrier [35, 68, 69] and is one of the distinguishing acid-fast properties, tested by the Ziehl-Neelsen stain [70]. This staining is most commonly used as a diagnostic tool to identify mycobacteria. It is based on acid resistance of mycobacteria due to presence of mycolic acids and waxy material in their envelope. The red colour due to carbol fuchsin treatment of mycobacterial cells can not be removed even after treatment with acid. Finally mycobacteria appear as red rods under microscope. Other bacteria can not retain the red stain, when counter-stained with methylene blue and will appear as blue under microscope.

The long-chain mycolic acids in the outer membrane confer hydrophobicity, impermeability and slow growth to mycobacteria [61]. Bendinger *et al.* [71] also proposed that these mycolic acids could be involved in selective attachment to surfaces. Evidence for partial permeability to hydrophilic molecules in *M. chelonae* has been reported by Jarlier and Nikaido [72].

1.3 Pathogenesis of *Mycobacterium avium*

M. avium is a facultative intracellular pathogen, and can be acquired directly from the environment, either through intestinal or respiratory route. It has the ability to invade epithelial cells and move across the mucosal layer of the human body. These bacilli can resist the immune system and infect macrophages, but activated macrophages

can reduce or kill intracellular mycobacteria. Phagocytosis of *M. avium* is supposed to be mediated via binding of the bacteria to a variety of receptors including complement receptor (CR) 1, CR2, CR3, CR4, the mannosyl-fucosyl-receptor, the fibronectin receptor, the integrin receptor $\alpha(v)\beta_3$, and the transferrin receptor [73-76]. Uptake by epithelial cells involves actin polymerisation mediated via activation of the small G-protein Cdc42 [77]. *M. avium* inhibits the acidification of the phagosome and the fusion of the phagosome with lysosomes [78, 79]. The inhibition of the phagolysosome requires a close contact between the mycobacterial surface and the phagosome membrane, which in turn depends on the presence of sufficient cholesterol in the plasma membrane [80]. Blocking the phagosome maturation also limits the intersection of mycobacteria-containing phagosomes with the intracellular trafficking pathways of antigen-presenting molecules which results in a decrease of Major histocompatibility complex (MHC) class II molecules [81].

1.4 Mutagenesis in MAC and identification of virulence genes

Mutagenesis is the induction of genetic changes by point mutations (by single nucleotide change), deletion or insertion (of several nucleotides or whole genes), or duplication (repeated sequence) in the DNA sequence. Single or many genes are often replaced (knocked out) by allelic exchange substrates, which flank a selectable marker and possess homology to the gene of interest or the sequences flanking this gene. Homologous recombination is useful in generating mutant strains, by replacing the gene of interest with a selectable marker.
In *E. coli* and other fast growing bacteria, homologous replacement is a well established model and is widely used. Since it is known that not all *M .avium* strains could be transformed [82] and furthermore in slow growing mycobacteria, inefficient DNA uptake, and relatively high rates of illegitimate recombination [83] hinders inactivation of genes by allelic replacement. On the other hand illegitimate recombination can be useful in generating random mutants.
Only few virulence genes from *M. avium* have been defined due to difficulties in generating site-directed mutants. Members of the MAC are characterised by the presence of highly antigenic glycolipids and GPLs on their cell wall. Belisle *et al.* [84] for the first time cloned and expressed genes encoding oligosccharide of serovar 2-specific GPL of cell wall from pathogenic *M. avium*, in a non-pathogenic

M. smegmatis. Colony morphology in *M. avium* is associated with virulence and it has been observed that rough colony spontaneous mutants were unable to synthesise GPL due to deletions in parts of genes responsible for their synthesis [85]. In *M. intracellulare* the cell wall is free from these GPL surface antigens [86]. A mutation in *mtf*D gene encoding a 3-O-methyltransferase, which is involved in the methylation of GPLs, leads to an elevated pro-inflammatory response of infected macrophages and an attenuated virulence in mice [87].

The first virulence-associated gene described for *M. avium* was the *mig* gene (*macrophage induced* gene), which is induced in macrophages and upon exposure to acidic pH. Transfer of *mig* into *M. smegmatis* moderately enhanced their intracellular survival [88]. Mig, a medium-chain acyl-CoA synthase, plays a role in fatty acid metabolism and may be involved in degradation and synthesis of fatty acids or in detoxification of free fatty acids [89].

The first drug resistance-linked genes identified by mutation were Maa1979 and Maa2520 in MAC. Knock out of the earlier mentioned gene led to changes in colony morphology via binding to Congo-red and showed susceptibility to drugs, while the later gene exhibited morphological changes along with reduced intracellular survival in THP-1 macrophages [90]. The PPE proteins, characterised by a proline-proline-glutamic acid motif, also have been associated with virulence of *M. avium*. An attenuated PPE mutant described by Li *et al.* [91] was not able to prevent the acidification of the phagosome and the phagosome/lysosome fusion.

Cloning of six genes related to invasion of mucosal epithelial cells in *M. avium* along with a strong L5 mycobacterial phage promoter into a plasmid and then transformation in *M. smegmatis* led to essential expression of these genes altogether. This suggested that invasion of intestinal mucosa was an orchestrated event, rather than an advent of single factor or gene [92].

The *mtr*AB genes are a two-component regulatory system, determining the composition and permeability of the cell wall. Cangelosi *et al.* [93] demonstrated a mutation inactivating MtrB, a histidine protein kinase, impairs survival of *M. avium* in macrophages. Similarly when *pstAB* gene, required for synthesis of the core GPL was inactivated by mutation, resulted in impaired attachments to polyvinyl chloride surfaces, yet have no effect on attachment to plastic and glass surfaces [94]. The gene *fad*D2 homologous to Rv0270 in *M. tuberculosis* encoding an acyl-CoA synthase involved in degradation of fatty acids, plays a role in invasion of epithelial

cells [95] and in cytoskeletal rearrangement via activation of Cdc42 [77]. Lack of induction of cytoskeletal rearrangement results in a 50% reduction of invasion efficiency [95].

A *M. avium* strain with mutation in gene *MAV_2928*, homologous to *M. tuberculosis* PPE gene *rv1787*, was impaired in its ability to replicate in macrophages and prevented phagosome lysosome fusion. Furthermore, the environment in phagosomes containing the mutant varied with respect to the concentration of zinc, manganese, calcium and potassium. *MAV_2928* may thus be involved in the establishment of the intracellular environment of *M. avium*-infected cells [76]. A mutation in gene *MAV_2450* (polyketide synthase, homologous to *pks* gene of *M. tuberculosis*), *MAV_4292*, *MAV_0385* and *MAV_4264* showed survival in low pH in macrophage vacuoles in mice [43]. Furthermore, *MAV_4012* was related to oxidative substances sensitivity.

1.5 Mycobacterial intracellular survival

In mycobacterial infection, the early phase is very critical, when cytokines play an important role in controlling the infection. *M. avium* is a facultative intracellular pathogen and can survive in macrophages. The intracellular survival is often attributed to its thick cell wall and certain surface linked components that combine to form a strong mycobacterial defense mechanism [96, 97].

Macrophages reduces the internal pH of phagosomes below 5, this is an ideal condition for lysosomal enzyme activity and survival of microorganisms is hampered [79]. For survival, intracellular pathogens adopt certain strategies, e.g. *Listeria monocytogens* and *Shigella* species has been reported to move from acidic phagosomes into cytoplasm [98]. *Legionella pneumophila* [99] and *Toxoplasma gondii* [100] were also involved in survival at reduced phagosomal acidity.

Cytokines are small, low molecular weight, non-structural and soluble proteins. They are produced by one cell to influence the function of another cell, either at normal or pathological condition and thus play a major role in intercellular communication of the immune system. They exert influence on leukocytes (lymphocytes, granulocytes, monocytes and macrophages) instantly after contact with a foreign antigen. The Cytokine term is a collective name for interleukins: produced by leukocytes, Lymphokines: produced by lymphocytes, Monokines: produced by monocytes. On

the one hand cytokines are classified into five general categories: interleukins, interferons, colony-stimulating factors, tumor necrosis factors and growth factors. On the other hand on the basis of their role in infection, they are divided into two groups. Pro-inflammatory which promote inflammation (e.g. TNF-α, IL-1β, IFN-γ) and anti-inflammatory which equilibrate the effect of inflammatory reactions (e.g. IL-10).

Along with interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) proves to be the major cytokine, when it comes to the defence against mycobacteria. It helps in phagocytosis, intracellular eradication, MHC molecule expression and granuloma formation [101-103].

The pro-inflammatory cytokine interleukin-1beta (IL-1β) is produced by monocytes and monocyte-derived macrophages. It is potentially highly active in response to infection and injury [104]. It activates T-cells to release cytokines, most prominently INF-γ, resulting in activation of macrophages for elimination of germs [105, 106].

Interleukin-10 (IL-10), an anti-inflammatory cytokine, is capable to inhibit cytokine production (e.g. TNF-α and IL-8) by activated human monocytes [107] and interferon gamma secretion by THP-1 cells [108]. It has a positive effect on *M. avium* intracellular survival by inhibiting cytokine production from activated murine macrophages [109] and therefore has a significant role in pathogenesis of MAC infection.

Recognition of *M. avium* by mouse macrophages involves binding of a 20 to 25 kDa lipoprotein from the cell envelope of *M. avium* to TLR2. This interaction leads to bacteriostasis of *M. avium* in a MyD88-dependent way [110]. Even though the expression of TNF-α is also induced via TLR2-signalling, its role in growth restriction of *M. avium* is unclear [110]. IFN-γ is considered to be a key cytokine for killing of *M. avium* and its expression is promoted by IL-18 secreted by *M. avium*-infected human macrophages [111].

The ability to survive and even replicate inside the phagosomes of macrophages is an important virulence factor of mycobacteria. Intracellular *M. avium* survives antibacterial activities such as nitric oxide and reactive oxygen species and the mechanisms leading to killing of *M. avium* are still unknown [43].

Infection experiments with macrophages give information on the early host response to mycobacterial infections [112]. Different types of macrophages or monocytic cells have been employed to assess mycobacterial virulence and among these the human macrophage-like cell line THP-1 has proven a suitable system for virulence testing

[112, 113]. It was shown that THP-1 cells are similar to primary human monocyte-derived macrophages with respect to their ability to take up mycobacteria and hinder their growth [114].

1.6 NTM survival in free living amoeba

Free living amoebas (FLA) are unicellular, cell wall-free eukaryotic organisms and occur either as Trophozoite: a motile phagocyte surviving under favourable conditions, or as Cyst: a non-motile phase under desiccation or extreme conditions. MAC has been shown to infect and survive in *Acanthamoeba* (*A.*) spp. [115-117] and *Dictyostellium* spp. [118, 119] and even to remain viable in amoebal cysts [116, 120]. Water is a common habitat for both, often FLA phagocytes mycobacteria, where the later has the ability to prevent lysosomal elimination and grow in FLA. Mycobacterial survival in amoeba cyst gives an advantage to survive the harmful environmental condition and protect it against antimicrobials, changes in pH and temperature. *M. avium* subspecies *avium* and *paratuberculosis* endure in FLA [115, 121, 122], which in turn enhances virulence [123], boosts survival under adverse conditions and resistance against antibiotics [124]. Tenant and Bermudez [117] proved 5 out of 20 genes by real-time PCR, which are up-regulated upon *M. avium* infection of *A. castellanii*.

Since *M. avium* is phagocytosed by FLA, in this study the possibility of an interaction with *A. castellanii* was examined. This may provide information on the pathogenesis and virulence of the MAC infection.

1.7 Objectives

MAH is an opportunistic pathogen causing disease in immuno-compromised individuals such as elderly people and it is also often associated with lymphadenitis in children less than 5 years. Samples from AIDS patients have often revealed MAH infections, but horizontal transmission between humans is not yet reported. *M. avium* has been shown to be taken up and multiply both within human macrophages [125] and FLA [117]. Recently, over the last decade, an increasing incidence of NTM infections has been reported in the developed countries [9, 126, 127].

M. avium exists freely in the environment and has been isolated from water, soil, pigs, pets and other animals. Environmental substrates including water, biofilm, soil, dust are regarded as source of infection, but still this needs to be confirmed.

The complex cell wall, slow growth rate and difficulties in genetic manipulations, due to high percentage of Guanine and Cytosine in the genome, are possible reasons, which limit the identification of virulence markers from MAH. Only few virulence genes from *M. avium* have been defined due to difficulties in generating site-directed *M. avium* mutants.

The aim of the project consists of two parts: first to establish a system of random mutagenesis in MAH in order to identify virulence markers and secondly to identify a set of certain phenotypic methods, which can be easily carried out in order to recognise virulence associated mutants.

2 Materials and Methods

2.1 Bacteria strains and growth conditions

Mycobacterial strains (Table 1 and 5) were grown in Middlebrook 7H9 broth (BD Biosciences, Heidelberg, Germany), supplemented with either 10% ADC (2 g glucose, 5 g BSA, 0.85 g NaCl in 100 ml dH_2O) or 10% OADC (BD Biosciences, Heidelberg, Germany) and 0.05% Tween 80 (Roth, Karlsruhe, Germany) without shaking, and on Middlebrook 7H11 agar (BD Biosciences, Heidelberg, Germany), supplemented with 10% ADC or OADC and 0.5% Glycerol (Roth, Karlsruhe, Germany) at 37°C. For selection of recombinant mycobacteria, media was supplemented when required with 50 µg ml^{-1} kanamycin (Roth, Karlsruhe, Germany) or/and 50 µg ml^{-1} hygromycin B (Roth, Karlsruhe, Germany). Additionally, 100 µg ml^{-1} Congo-red (Roth, Karlsruhe, Germany) was added to Middlebrook agar for Congo-red plating. *Escherichia* (*E.*) *coli* DH5α (Table 1) was cultured in/on Luria-Bertani (LB) broth and agar at 37°C. For selection of recombinant *E. coli*, media was supplemented when required with 50 µg ml^{-1} kanamycin or 50 µg ml^{-1} hygromycin B.

Table 1. Mycobacterial strains and *E. coli* strain used in this study

Strain	Origin / Description	Reference / Source
M. avium 104	HIV Patient	NRCM*, Borstel, Germany
M. avium 2721/04	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 10091/06	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 10203/06	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 4557/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 4023/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 3646/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 3449/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 3269/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 2630/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 2014/08	Child with lymphadenitis	NRCM*, Borstel, Germany

M. avium 772/08	Child with lymphadenitis	NRC M*, Borstel, Germany
M. avium 709/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 528/08	Child with lymphadenitis	NRCM*, Borstel, Germany
M. avium 128	Soil (environment)	FLI+, Jena, Germany
M. avium 129	Soil (environment)	FLI+, Jena, Germany
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Stratagene, Agilent Technologies, Waldbronn, Germany.

* National Reference Center for Mycobacteria, Borstel, Germany.
+ Friedrich Löffler Institute, Jena, Germany.

All clinical isolates of mycobacteria were kindly provided by Dr. Elvira Richter, from National Reference Center for Mycobacteria, Borstel, Germany. Dr. Petra Möbius, from Friedrich Löffler Institute, Jena, Germany generously provided the two environmental strains of mycobacteria.

2.2 Cell lines and culture conditions

The human acute monocytic leukemia cell line THP-1 (DSMZ no. ACC16, Braunschweig, Germany) and human lung epithelial cell line A549 (DSMZ no. ACC107, Braunschweig, Germany) were maintained by passaging twice weekly in RPMI 1640 (GIBCO® Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany). Cells were cultured in BD Falcon™ 75 cm^2 trays (BD Biosciences, Heidelberg, Germany) at 37°C and 5% CO_2 [128].

2.3 Isolation and infection of human monocytes

Human monocytes were isolated from buffy coats from different healthy donors using Ficoll-Paque™ Plus (GE Healthcare, München, Germany) and Percoll™ (GE Healthcare, München, Germany) gradient centrifugation according to the manufacturer's recommendations and as described in Sharbati *et al.* [129]. Infection and washing were performed in Iscove's Modified Dulbecco's Media (IMDM; PAA

laboratories, Pasching, Austria) with 3% human AB-serum (PAA laboratories, Pasching, Austria).

2.4 Amoeba and culture conditions

The *Acanthamoeba castellanii* strain 1BU group II [130] was cultivated in BD Falcon™ 75 cm^2 trays (BD Biosciences, Heidelberg, Germany) with Proteose-peptone yeast-extract glucose (PYG) medium ([131] in dark at 28°C and passaged once per week.

2.5 Molecular biology techniques

All molecular biology techniques were carried out according to standard protocols [132] or according to the recommendations of the manufacturer's kits and enzymes. Primers were purchased from Metabion (Martinsried, Germany). Plasmid DNA was isolated with the QIAGEN Plasmid Mini prep kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed with the DreamTaq kit from Fermentas (St. Leon-Rot, Germany) and quantitative real-time PCR with Stratagene MX3000P (Agilent Technologies, Waldbronn, Germany). Restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). For elution of DNA fragments from agarose gels, the QIAquick Gel Extraction kit (Qiagen) was used. Ligation reactions were performed with the T4 DNA Ligase kit from Fermentas (St. Leon-Rot, Germany). Sequencing reactions were performed by using the Prism Big Dye Terminator 3.1 FS Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems 2500 (Darmstadt, Germany) in combination with an Applied Biosystems 3500xl Dx Genetic Analyser.

2.6 DNA isolation from Mycobacteria

When mycobacterial cultures reached an optical density ($OD_{600\ nm}$) 2, 3 ml were centrifuged to pellet at 6000x g at 4°C for 10 minutes. The pellet was re-suspended in 400 µl TE-8 buffer (0,01 M Tris-HCl, 0,001 M EDTA, pH 8) followed by inactivation of mycobacteria at 80°C for 30 minutes. After cooling samples to room temperature, 5 µl of lysozyme (150 mg ml^{-1}) was added to the suspension and incubated at 37°C over night. Next day 70 µl of 10% Sodium dodecyl sulfate (SDS) and 5 µl

Proteinase K (20 mg ml^{-1}) were added to the lysate, before incubating it at 65°C. After 2 hours incubation at 65°C, 100 µl 5 M NaCl and 100 µl Cetyl-trimethyl-ammonium-bromid (CTAB: 10% CTAB in 0.7 M NaCl: SGMA-ALDRICH, Taufkirchen, Germany) were added to the sample and incubation at 65°C was extended for another 10 minutes. First DNA was extracted by chloroform, then by phenol/chloroform, followed by another round of chloroform extraction and finally precipitated with 100% ethanol. The genomic DNA was utilized for different experiments, for example Southern blots, cloning or was applied as template in PCRs.

2.7 Confirmation of bacterial strains

M. avium 104 hereafter referred as wild-type strain was confirmed for *Mycobacterium* genus specificity by PCR with primers MT1 and MT2 (Table 2) [133]. Wild-type, mutants and the complemented mutant were confirmed for *M. avium* subspecies *hominissuis* specificity by triplex PCR with three sets of primer pairs: DnaJ-1 & DnaJ-2, IS*1245*-1 & IS*1245*-2 and IS*901*-1 & IS*901*-2 (Table 2) [134].

Table 2. Primer pairs used for confirmation of mycobacterial strains

Primers	Sequence	Ann. temp*	Product
MT1	5'-TTC CTG ACC AGC GAG CTG CCG-3'	62.7°C	506 bp
MT2	5'-CCC CAG TAC TCC CAG CTG TGC-3'		
DnaJ-1	5'-GAC TTC TAC AAG GAG CTG GG-3'	58°C	140 bp
DnaJ-2	5'-GAG ACC GCC TTG AAT CGT TC-3'		
IS*1245*-1	5'-GAG TTG ACC GCG TTC ATC G-3'		385 bp
IS*1245*-2	5'-CGT CGA GGA AGA CAT ACG G-3'		
IS*901*-1	5'-GGA TTG CTA ACC ACG TGG TG-3'		577 bp
IS*901*-2	5'-GCG AGT TGC TTG ATG AGC G-3'		

* Annealing temperature for the primer pairs

2.8 Ziehl-Neelsen staining of wild-type and mutants

Wild-type and all mutants (Table 5) were also confirmed for acid fastness by Ziehl-Neelsen staining. A 500 µl well grown culture of wild-type and mutants were centrifuged to pellet at 6000 x g for 10 minutes and re-suspended in 500 µl phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4). From this 10 µl was pipetted on to a soap washed, air dried, glass slide and heat fixed three times over a Bunsen burner with caution of avoiding excessive heating. The preparation was stained with carbol fuchsin solution (Merck, Darmstadt, Germany) and heated three times until steam formation within a time period of 5 minutes. The slides were carefully washed with de-ionized water, for removal of dye. De-staining was performed with HCl/Ethanol solution (Merck, Darmstadt, Germany) for 15 to 30 seconds and immediately rinsed with de-ionized water. For counter staining Löffler's methylene blue solution (Merck, Darmstadt, Germany) was used for 30 seconds. Finally the slides were washed with de-ionized water, air dried and for fixation a drop of Entellan (Merck, Darmstadt, Germany) was added before covering with a cover slip. The slides were examined under microscope with small amount of immersion oil.

2.9 Genetic manipulations

2.9.1 Isolation of recombination substrate for transformation

The *hygromycin resistance* gene (*hygR*) flanked by two multiple cloning sites in plasmid pYUB854 (Table 3 and section 7.2.1) [135] was selected as recombination substrate for illegitimate recombination. A total of 1 µg plasmid pYUB854 DNA was double digested with restriction enzymes Fast digest Stu**I** and Spe**I** at 37°C for 30 minutes (Figure 2). Folllowing gel electrophoresis, the 2030 bp linear DNA fragment, carrying the 999 bp *hygR* gene (Figure 2) flanked by adjacent non-coding regions was gel eluted and then used for transformation in mycobacteria.

Table 3. Plasmids used in this study

Plamids	Description	Reference
pGFP261	Plasmid pMV261 with gene *gfp* downstream of the promoter *hsp*60 from BCG inserted in restriction sites PstI and HindIII. (see section 7.2.5 for map)	[136]
pYUB854	*E. coli* cosmid with *hygR* gene flanked by multiple cloning sites and containing an *OriE*. (see section 7.2.1 for map)	[135]
pMN437	*E. coli* cosmid with *hygR* gene, COLE1_Ori, Ori\myc and mycgfp2+. (see section 7.2.2 for map)	[137]
pMV306	*E. coli* cosmid containing an OriE, *kanamycin resistance* gene, *aph* gene from transposon Tn903, an *integrase* gene *Int*, phage L5 attP site and a multiple cloning site. (see section 7.2.3 for map)	[138]
pFKaMAV3128	Plasmid pMV306 with gene *MAV_3128* from wild-type inserted in restriction sites XbaI and HpaI. (see section 7.2.4 for map)	This study

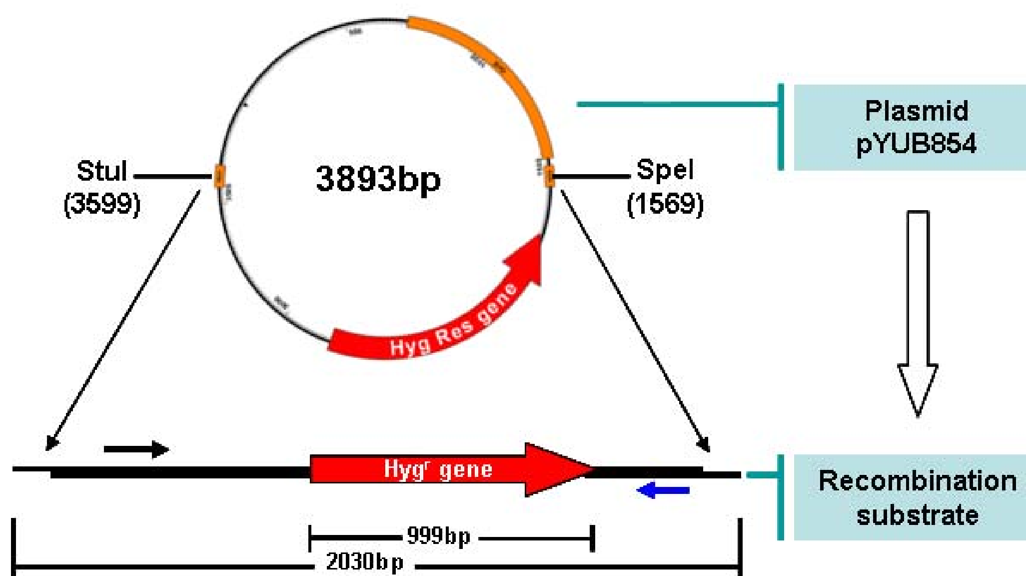


Figure 2. Diagram showing isolation of recombination substrate from plasmid pYUB854.

Plasmid pYUB854 was digested with restriction enzymes StuI and SpeI to yield the 2030 bp linear DNA fragment (recombination substrate) with *hygR* gene (999 bp).

2.9.2 Mutants generation by transformation

A total of 3-6 µg recombination substrate was transformed in wild-type strain by electroporation with the Biorad GenePulser apparatus applying 1000 Ω, 25 µF and 1.25 kV in 1 mm gap cuvettes (Peqlab, Erlangen, Germany). The preparation of electro-competent cells and electroporation were performed using standard protocols [139]. Plasmid pMN437 (see section 7.2.2) [137] donated by Prof. Dr. Michael Niederweis, University of Alabama, Birmingham, USA was used as positive control for transformation. Electroporated bacteria were incubated at 37°C for 24 hours before plating on Middlebrook agar, supplemented with ADC and hygromycin (50 µg ml^{-1}). Potential mutants were characterised by PCR amplifying a part of the *hygR* gene by using primers Hyg 2K FW and Hyg 2K BW (Table 4). Furthermore Southern blots were performed to confirm single copy insertion of selectable marker in the genome.

Table 4. Primer pairs used in cloning and quantitative real-time PCR

Primers	Sequence*	Ann. Temp$^+$	Product
Hyg 2K FW	5’-CAC CGT ACG TCT CGA GGA ATT CCT G-3’	64°C	1818 bp
Hyg 2K BW	5’-GCG TCG TGA AGA AGG TGT TGC TGA-3’		
Hyg LC FW	5’-AGT TCC TCC GGA TCG GTG AA-3’	62.4°C	445 bp
Hyg LC BW	5’-AGG TCG TCC CGG AAC TGC TGC G-3’		
Hyg mut_1	5’- AAC TGG CGC AGT TCC TCT G-3’	62°C	Variable
Hyg mut_2	5’- TCA GCA ACA CCT TCT TCA CGA -3’		
MAV3128_MV306_1	5’-CGG <u>TCT AGA</u> CTA TGC CTA CCT GCT CTC-3’	59°C	3907 bp
MAV3128_MV306_2	5’-GCA <u>GTT AAC</u> CTA ATG CGG CTT GGC		

	CAG-3’		
Myko_Gatt_Taq_FW	5’- GGG GTG TGG TGT TTG AG -3’		
Myko_Gatt_Taq_BW	5’- CTC CCA CGT CCT TCA TC -3’	55°C	176 bp
Myko_Gatt_Taq_SoFa	5’- Fam-TGG ATA GTG GTT GCG AGC ATC-Tamra -3’		

* Sequences of restriction enzymes were added to the primers for cloning purposes

+ Annealing Temperature for the primer pairs

2.9.3 Reverse PCR

In order to locate the insertion site of the *hygR* gene in the mutant genome, a reverse PCR approach was conducted (Figure 3). Only 2 µg of DNA from each mutant listed in Table 5 was digested with restriction enzyme Fast digest ApaI or SmaI (which do not cut in recombination substrate of 2030 bp) at 37°C during 30 minutes for complete digestion (Figure 3 A). Multiple sized DNA fragments (Figure 3 B) were ethanol precipitated and then self ligated by T4 DNA ligase enzyme at room temperature for 10 minutes, thus resulting in different sized circular DNA products (Figure 3 C). PCR amplification was done around the circle with Hyg mut_1 and Hyg mut_2 primers (Table 4) binding to the recombination substrate, but oriented in opposite direction unlike normal PCR. This allows primers amplifying DNA (Figure 3 D) from known region (recombination substrate sequences) towards known region (DNA sequences of the mutated gene). This amplified product was sequenced with the Hyg mut_1 and Hyg mut_2 primers. NCBI (BLAST) search was performed for finding regions of close similarities between the sequences.

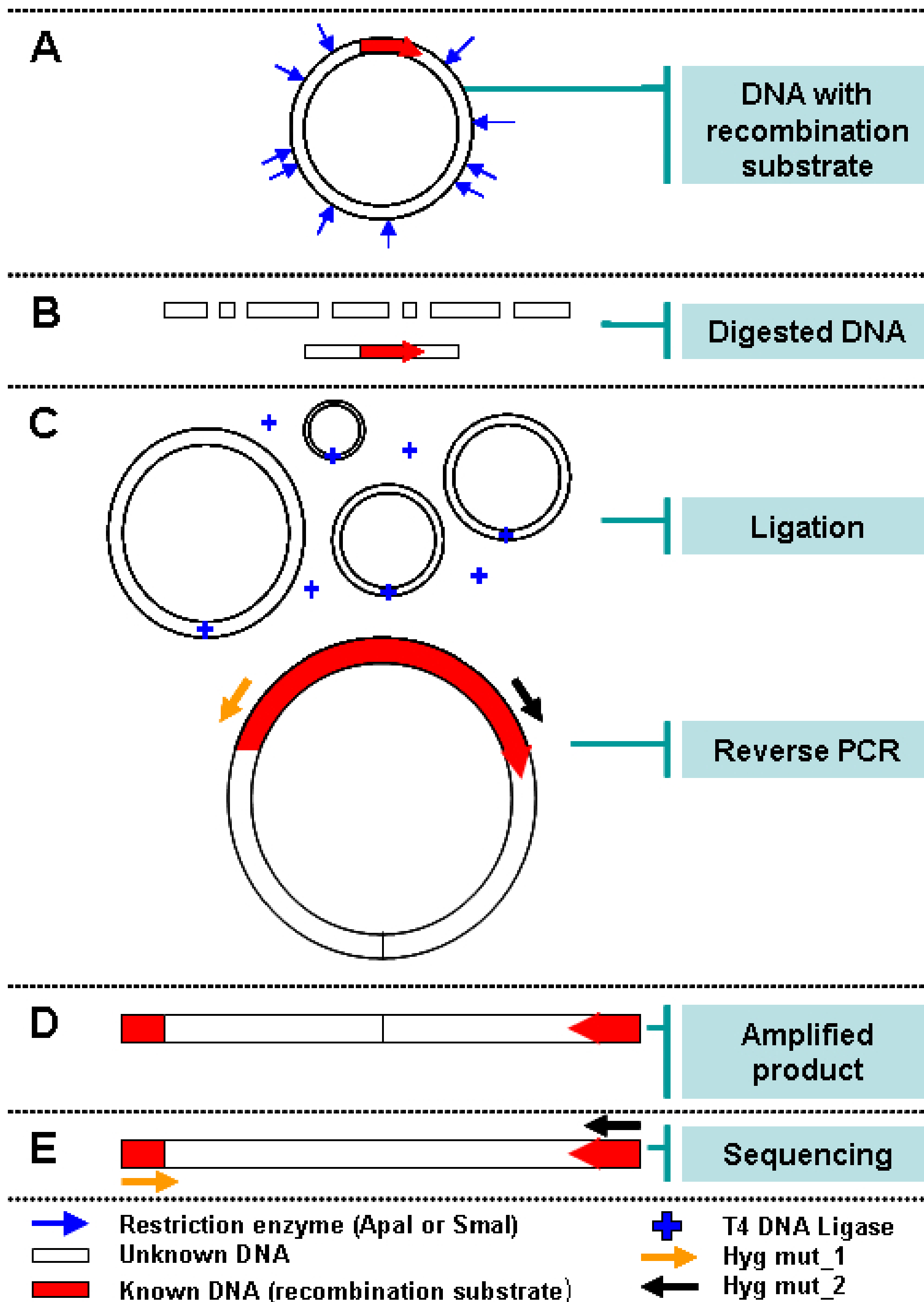


Figure 3. Schematic representation of reverse PCR method

A: Mutant DNA digested with restriction enzyme ApaI or SmaI. **B**: Digested DNA fragments, one having recombination substrate. **C**: DNA ligations followed by reverse PCR using Hyg mut_1 and Hyg mut_2 primers. **D**: the amplified region after reverse PCR was gel eluted. **E**: Sequencing was done with Hyg mut_1 and Hyg mut_2 primers.

2.9.4 Construction of complemented strain of mutant MAV_3128

Primers MAV3128_MV306_1 and MAV3128_MV306_2 (see Table 4) were designed to amplify the gene *MAV_3128* (3228 bp) along with 680 bp upstream sequence of wild-type with *pfu* Fermentas (St. Leon-Rot, Germany) PCR (Figure 4 A). The amplified PCR product was digested with restriction enzymes Fast digest XbaI and HpaI at 37°C for 10 minutes (Figure 4 B). The amplified and double digested gene *MAV_3128* along with upstream region of wild-type was cloned into pMV306 (see Table 3 and section 7.2.3) [138] already double digested with the same restriction enzymes (Figure 4 C). The recombinant plasmid pFKaMAV3128 (see Table 3 and section 7.2.4) was transformed into *E. coli* by a method already described by Hanahan, [140].

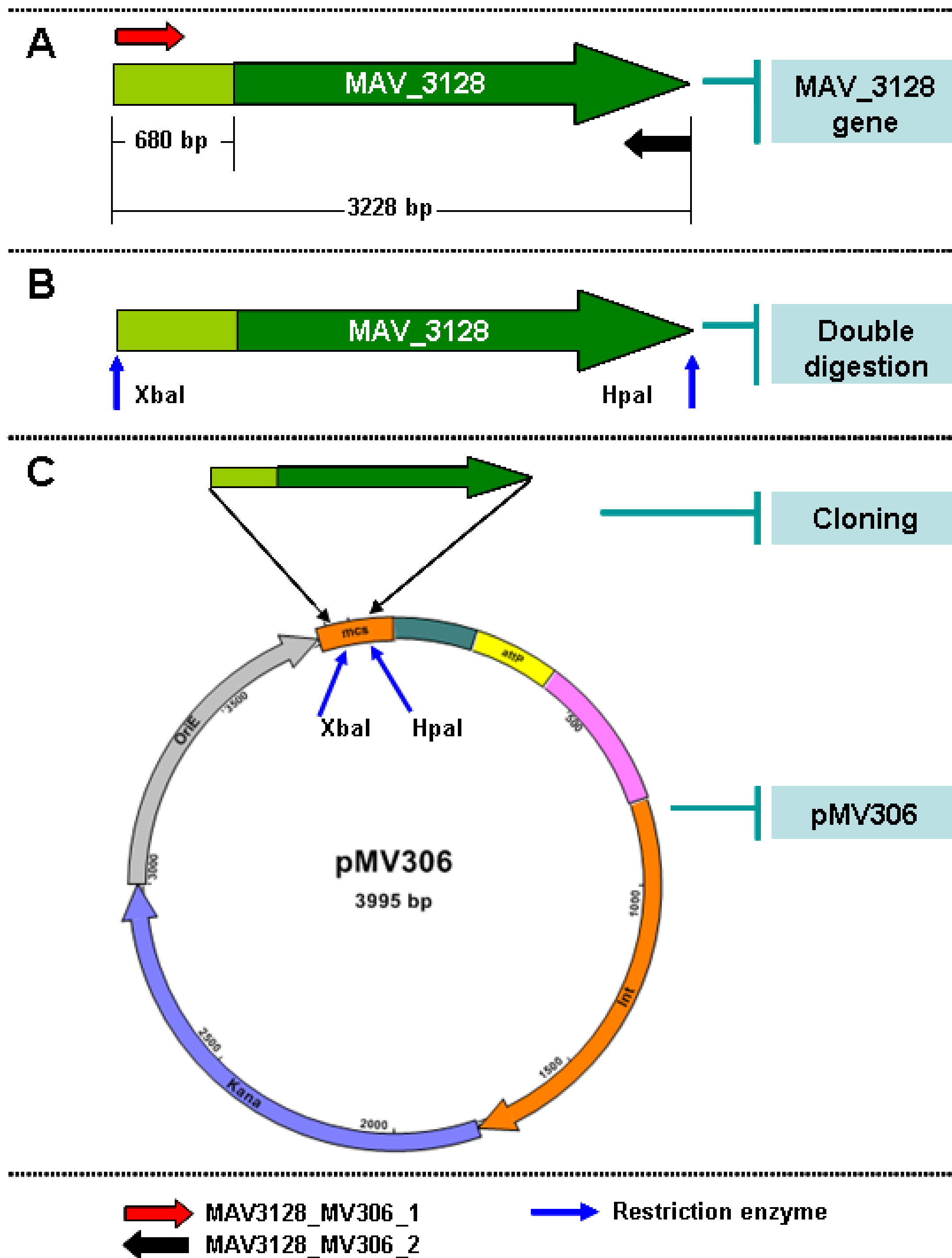


Figure 4. Schematic representation of gene *MAV_3128* cloning in plasmid pMV306.

A: Gene *MAV_3128* (3228 bp) along with upstream region of 680 bp was amplified with primer pair MAV3128_MV306_1 (Red arrow) & 2 (Black arrow). **B**: Gene *MAV3128* ends were digested with restriction enzymes XbaI and HpaI. **C**: Cloning of digested gene *MAV3128* in plasmid pMV306 at restriction sites XbaI and HpaI.

The recombinant plasmid pFKaMAV3128 isolated from overnight culture of recombinant *E. coli*, was electroporated in competent cells made from the mutant MAV_3128. Transformed bacteria were selected by addition of kanamycin (50 µg ml^{-1}) into the Middlebrook agar, supplemented with ADC. The screening of the complemented clones was performed by PCR using primer pair MAV3128_MV306_1 and MAV3128_MV306_2 (see Table 4).

2.10 Southern blot

For Southern blot 2 µg of genomic DNA from wild-type and all mutants were digested with ApaI or SmaI, separated by electrophoresis in a 1% agarose gel and capillary transferred to positively charged nylon membranes (GE Healthcare, Buckinghamshire, UK) by following a standard protocol [132]. An 1818 bp region of the plasmid pYUB854 carrying the *hygR* gene was amplified using the primer pair Hyg 2K FW and Hyg 2K BW (see Table 4) and the digoxigenin labelling kit (Roche, Mannheim Germany). The labelled PCR-product was used as a probe and detection was carried out using anti-digoxigenin-AP conjugate and CDP-star (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.11 Screening for virulence-mutants

2.11.1 Amoeba plate test (APT)

The APT was previously described [141] and was modified to fit the growth conditions (medium, temperature, duration) of *M. avium*. In short, 3 million *Acanthamoeba castellanii* diluted in 1.5 ml PYG medium were spread on Middlebrook agar plates, supplemented with ADC and these plates served as test plates. For control plates only 1.5 ml PYG medium without amoeba was spread on Middlebrook agar plates, supplemented with ADC. Then these control and test plates were dried under the safety hood for 30 minutes and then incubated at room temperature. The next day series of tenfold dilutions (1:10, 1:100, and 1:1000) in de-ionized water were prepared from cultures of the mutants and the wild-type. Only 3 µl of undiluted culture and of each dilution were spotted onto the test and control plates. After one week of incubation at 28°C, plates were analysed for results. Mutants showing reduced

growth on test plates compared to the control plates were selected for further molecular characterisation.

2.11.2 Growth rate in broth cultures under pH stress

The growth rates of all the transformed mycobacteria and wild-type were compared in Middlebrook 7H9 broth, supplemented with ADC, with neutral pH (7) and under pH stress (pH 5). Cultures were inoculated to an initial $OD_{600\ nm}$ 0.02 to 0.03 and allowed to grow at 37°C without shaking for two weeks. Three cultures per strain were inoculated. Growth of cultures was determined by measurement of OD and by ATP quantification with the luminescence-based kit BacTiter-GloTM Microbial cell viability Assay (Promega Corporation, WI, USA) according to manufacturer instructions. The luminescence was recorded as relative light units (RLU) with the microplate luminometer LB96V (Microlumat Plus, EG & G Berthold, Bad Wild, Germany).
Mutants showing differences of growth pattern compared to the wild-type strain in both normal medium or under pH stress conditions were considered for further molecular characterisation.

2.11.3 Characterisation of colony morphology by Congo-red plating

Only 100 µl of $1:10^5$ and $1:10^6$ dilutions in de-ionized water of mutants and wild-type were spread in triplicate on Middlebrook agar plates, supplemented with OADC and 100 µg ml^{-1} Congo-red. Plates were incubated for 2 to 3 weeks and observed for colony morphology. Mutants showing differences in colony morphology (white vs. red staining, transparent vs. opaque colonies, smooth vs. rough colonies) compared to the wild-type were considered for further molecular characterisation.

2.11.4 Induction of cytokine expression in THP-1 cells

Infection of the cell line THP-1 was performed in 24-well cell culture plates (TPP, Trasadingen, Switzerland) with three to five wells per sample. A total of 200,000 cells per well of THP-1 were grown along with addition of Phorbol-12-myristat-13-acetate (PMA: 10 ng ml^{-1}) and allowed to adhere to the well surface overnight at 37°C and in 5% CO_2. Cells were then infected with mutants and wild-type at a multiplicity of infection (MOI) of 50 colony forming units (CFU). The supernatants were removed

after 24 hours and cytokines were quantified in appropriate dilutions of the supernatants by ELISA using the Human ELISA Ready Set Go kits (Natutec, Frankfurt, Germany). Statistical analysis was done using GraphPad Prism 5.

2.11.5 Infection of cell lines and intracellular growth measurement

THP-1 cells were seeded, treated with PMA and infected as described above. The supernatants were removed after 4 hours, cells were washed twice with RPMI 1640. The cells were then treated with 200 µg ml^{-1} amikacin for 2 hours to kill the mycobacteria in the supernatant. After washing twice with RPMI media, 1 ml medium supplemented with 5 µg ml^{-1} amikacin was added to each well. Samples for quantification of intracellular bacteria were taken at the end of the 4 hours infection time after removal and killing of extracellular bacteria and then after 1, 2, and 4 days. For this, the cells were lysed in 1 ml sterile water at 37°C for 20 minutes.

The intracellular survival was ascertained by quantifying the amount of mycobacterial DNA in sample lysates by quantitative real-time PCR with Stratagene MX3000P Sequence Detection System (PE Applied Biosystems). For DNA extraction, 200 µl of the sample lysate was mixed with 100 µl TE-9 2 x buffer (1 M Tris, 40 mM EDTA, 20 mM NaCl pH 9 and 2% SDS) [142] and 6 µl Proteinase K (50 mg ml^{-1}). The mixture was incubated at 58°C for 60 minutes and then at 97°C for 30 minutes. After cooling the mixture to room temperature, DNA was extracted by first phenol, then chloroform and precipitated with 12.5 µl 3 M sodium acetate pH 5 and 300 µl ice cold 100% ethanol. The pellet was air dried and resuspended in 50 µl de-ionized water. The *M. avium* DNA was quantified by amplifying an amplicon in the internal transcribed spacer between the 16S and the 23S rRNA genes of Mycobacteria using primers Myko_Gatt_Tag_FW and Myko_Gatt_Taq_BW (see Table 4) [143] and a FAM-labelled detector probe Myko_Gatt_Taq_SoFa (see Table 4). Fermentas Dream Taq PCR kit (St. Leon-Rot, Germany) was used for mycobacterial DNA amplification. The PCR reaction (final volume of 25 µl) was conducted by running the first cycle at 97°C for 3 minutes, followed by 40 cycles: 95°C for 30 seconds, primers annealing at 55°C for 40 seconds and an elongation at 72°C for 30 seconds. A standard consisting of five dilutions of genomic *M. avium* DNA of already known concentrations was included in the real-time PCR to determine the amount of DNA in samples.

Additionally, 100 µl of 1:1000 dilution in de-ionized water of samples were plated in triplicate on agar plates supplemented with ADC for counting of CFU.
A549 cells were seeded in 24-well cell culture plates (TPP, Trasadingen, Switzerland) with 75,000 cells per well, three to five wells per sample and allowed to adhere to the surface of the plate well overnight at 37°C and in 5% CO_2. Infection and sampling were done as described for THP-1 cells. Intracellular survival was measured only by plating in triplicate 100 µl of 1:100 dilution in de-ionized water of samples on agar plates supplemented with ADC for counting of CFU.

2.11.6 Infection of human monocytes and intracellular growth measurement

One million monocyte-derived macrophages were seeded per well in 24-well cell culture plates, with three to five wells per sample per sampling point. Infection with mutants and wild-type, amikacin treatment and sampling were done as described above for THP-1 cells infection. Except that human monocytes were pre-activated with 100 U ml^{-1} INF-γ and 10 ng ml^{-1} LPS, IMDM was used for washing, the MOI for infection was 10 and the dilution of the samples for plating and counting of CFU was 1:500.

2.12 Statistical analysis

Each experiment was performed at least in triplicate, means ± standard deviations were determined and the whole experiment was repeated at least 3 times. The significance of the results between control (wild-type) and experimental groups (mutants) was determined by Student's t test. $P<0.05$ was considered to be significant (*).

2.13 Computer software and programs used

Nucleotide sequence analysis was performed using the software packages MacVector™ 7.2.3 (Accelrys, Cambridge, UK) and Lasergene (DNASTAR, Inc., Madison, WI, USA). A NCBI (BLAST) search was performed for finding regions of close similarities between the biological sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Drawing graphs and statistical analysis was done using GraphPad Prism v5.01 (Graph pad software, Inc.).

3 Results

In the last decade increasing incidence of *Mycobacterium avium* complex infections were reported. Due to little knowledge on virulence associated genes and difficulties in generating site-directed mutants, a method of random mutagenesis in *Mycobacterium avium* subsp *hominissuis* was established in this study. Secondly certain phenotypic tests were devised in order to identify virulence markers.

Illegitimate recombination mutagenesis approach was conducted to generate mutants and to ascertain the mutations influence on the bacterium, (A) Congo-red plating test, (B) pH stress test, (C) test for interaction with amoeba, (D) measurement of induction of cytokines secretion, and (E) intracellular survival experiments in both cell lines and human monocytes were performed.

Most of the experiments performed simulated conditions occurring in the natural infection process, first from the uptake of bacteria by macrophages (pH stress, intracellular survival) and then the response (induction of cytokine expression) from the immune system especially THP-1 macrophages. Investigation for interaction with amoeba was also done. Lastly, one of the mutants was complemented to investigate the reversal of mutation.

Chapter 3 is sub-divided in three sections; the first section deals with the mutagenesis and characterisation of the generated mutant strains (section 3.1), the second with phenotypic characterisation of the mutants (section 3.2) and the last with complementation of mutant MAV_3128 (section 3.3).

3.1 Mutagenesis and characterisation of *M. avium* mutants

3.1.1 Recombination substrate isolation

The *hygromycin resistance* gene flanked by multiple cloning sites in plasmid pYUB854 was selected as recombination substrate for transformation in order to generate random mutants. Plasmid pYUB854 DNA was double digested with restriction enzymes StuI and SpeI. The 2030 bp linear DNA fragment representing the recombination substrate was gel eluted and later used for electroporation. The *hygR* gene is flanked by plasmid DNA of 793 bp and 238 bp on each side in the recombination substrate (Figure 5). These flanking regions served as substrates for the illegitimate recombination.

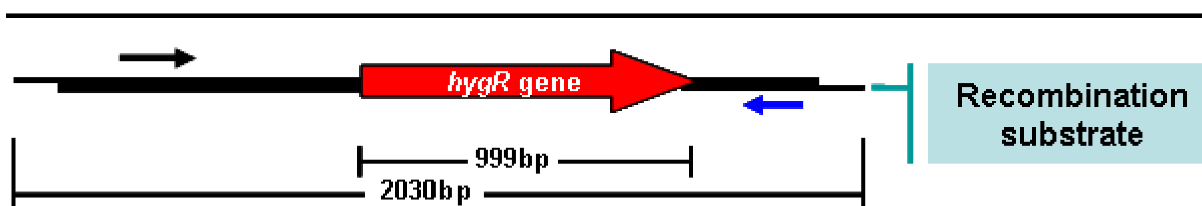


Figure 5. Diagram representing recombination substrate.

The 2030 bp plasmid DNA fragment having the *hygR* gene flanked with 793 bp and 238 bp of plasmid DNA on each side. The blue arrow represents the binding site for Hyg 2K FW primer and black arrow shows the binding site for Hyg 2K BW primer. A PCR using these primers generated a 1818 bp product.

3.1.2 Selection of *M. avium* strains applicable for transformation

A total of 16 *M. avium* strains were tested (Table 1: 14 clinical isolates and 2 environmental strains) for transformation with plasmid pGFP261 (see Table 3 and section 7.2.5). Out of these 16 *M. avium* strains only five strains (104, 2721/04, 2014/08, 4023/08 and 528/08) could be transformed (data not shown). Since the genome sequence from *M. avium* strain 104 is available in the genome data bases, simplifying a precise mutant description, it was decided to concentrate on this strain for further analysis. This strain was confirmed as *Mycobacterium* by a genus-specific PCR (data not shown) using the primer pair MT1 and MT2 and by Ziehl-Neelsen staining (data not shown) before using it for transformation.

3.1.3 Electroporation of recombination substrate

In order to generate random mutants, transformation of the 2030 bp recombination substrate into *M. avium* 104 strain was conducted. After electroporation of 3 to 6 µg of recombination substrate and selection on Middlebrook plates, supplemented with ADC and hygromycin (50 µg ml^{-1}), about 50 to 100 colonies appeared, while 250 to 300 colonies appeared by plating the positive controls containing plasmid pMN437. Five to ten colonies appeared when the non-transformed negative controls were plated. A total of 50 randomly chosen colonies from the sample plates were tested for insertion of the *hygR* gene by performing a PCR (Figure 6A) using the primer pair Hyg 2K FW and Hyg 2K BW (Figure 5). The PCR result showed that 30 colonies out of 50 randomly chosen colonies (Figure 6 A: data for all not shown) were positive for the recombination substrate. This shows overall 60% success rate in electroporation of recombination substrate in *M. avium*. Nevertheless the product size of one colony

(Figure 6 A: lane 13) was not equal to 1818 bp, indicating deletions in the recombination substrate. Another PCR was conducted with primer pair Hyg LC FW and Hyg LC BW (Table 4), binding within *hygR* gene generating a 445 bp product at an annealing temperature of 62.4°C (Figure 6 B). A total of 49 out of 50 colonies proved out to be positive with this PCR (Figure 6 B: data for all not shown), which showed a 98% success rate for transformation. All the positive mutants were grown in Middlebrook broth, supplemented with ADC and hygromycin (50 µg ml^{-1}) until OD_{600nm} 2 for genomic DNA isolation and also served as pre-cultures for other experiments.

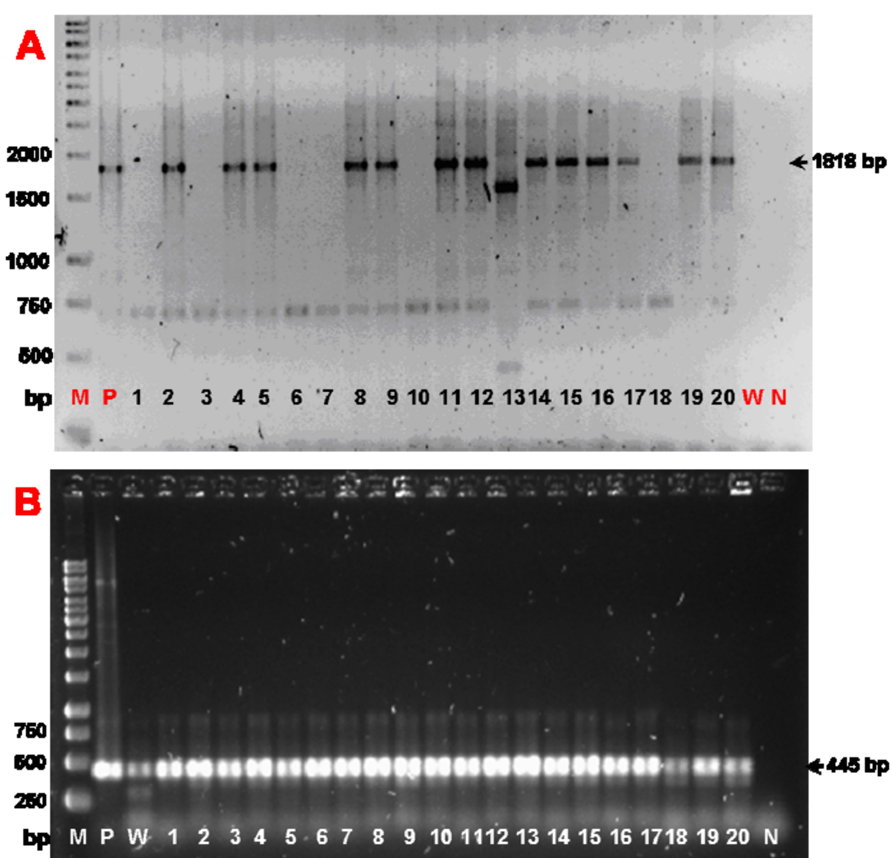


Figure 6. PCR results for *hygR* gene insertion in selected colonies

(**A**) PCR result using primer pair Hyg 2K FW and BW. A 1818 bp product of PCR obtained for different colonies to confirm the insertion of recombination substrate. Colony 13 has a deletion in recombination substrate resulting in showing a band lower than 1818 bp. (**B**) PCR result using primer pair Hyg LC FW and BW. A 445 bp product of PCR obtained for colonies to confirm the insertion of recombination substrate. **bp**: base pair; **M**: 1kb marker; **P**: Positive control (pYUB854); **W**: Wild-type; **N**: Negative control.

Furthermore Southern blots were performed to confirm the PCR results using a PCR fragment generated with Hyg 2K FW and Hyg 2K BW primers as probe. By this procedure 20 colonies (Figure 7) could be confirmed to carry an insertion of the *hygR* gene in the genome. The different sizes proved that the insertions occurred at different sites and with the exception of one mutant (Figure 7: black arrow) only one strong band appeared indicating single insertion in each mutant. This result is in conjunction with PCR result, as less positive clones were obtained by using the same Hyg 2K FW and Hyg 2K BW primer for PCR. Nonetheless all 49 PCR positive clones were used for further analysis.

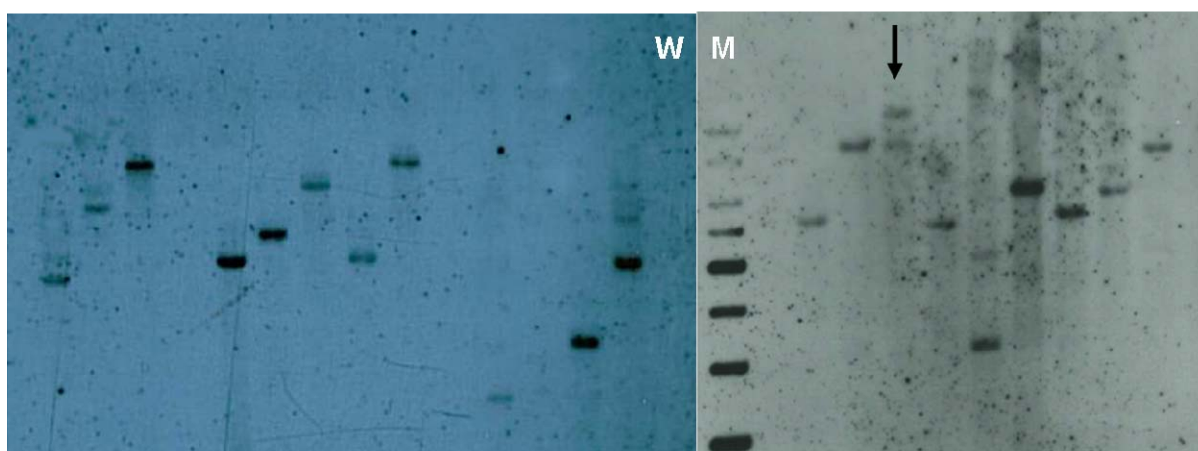


Figure 7. Confirmation of *M. avium* mutants by Southern blot

Two Southern blots showing mutants with single insertion of recombination substrate confirmed by hybridisation with a 1818 bp probe generated with primer pair Hyg 2K FW and Hyg 2K BW. The different sizes indicate different sites of insertion in the genome of each mutant. **W**: Wild-type; **M**: 1kb marker. Arrow indicate a mutant with double insertion.

Reverse PCR was conducted in order to identify the insertion site of the recombination substrate in genomes of all 49 PCR confirmed mutants. DNA from mutants was digested by either ApaI or SmaI. These DNA fragments were self ligated and a reverse PCR was performed using the primers Hyg mut_1 and Hyg mut_2 (Figure 3). The figure 8 represents one of the reverse PCR results. Lanes 4, 13 and 14 shows mutants generating products after independent digest with either of the two enzymes. Lanes 1, 5 and 11 represent mutants showing only positive results when digested with one of the restriction enzymes and not when digested with the other restriction enzyme.

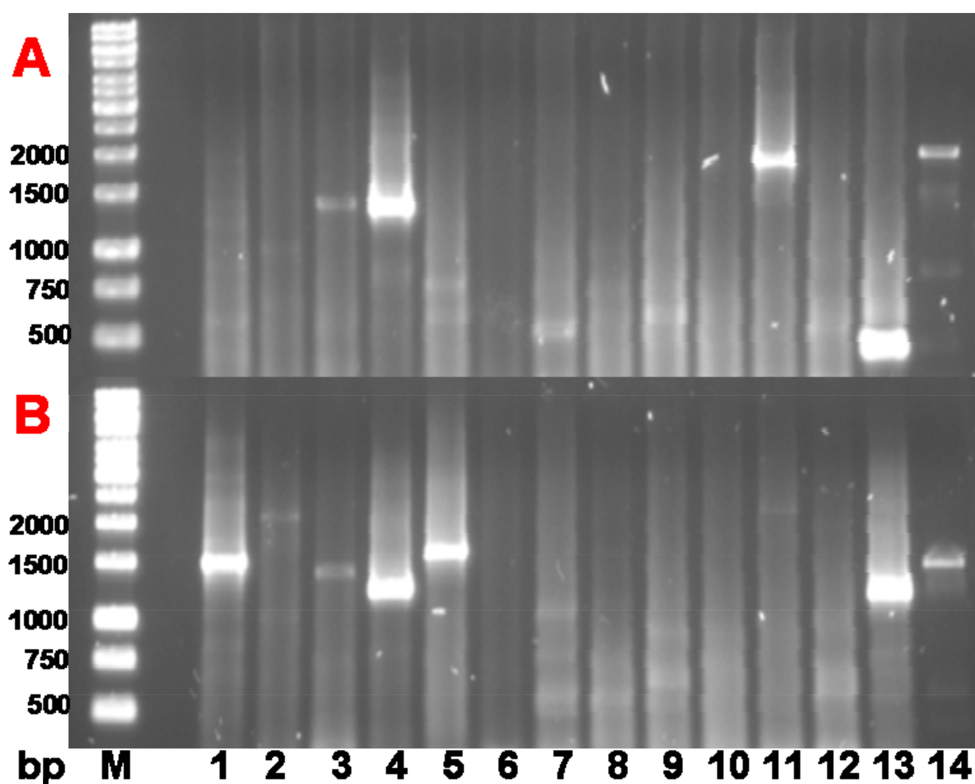


Figure 8. Reverse PCR for the localisation of insertion sites

Amplified products of reverse PCR using Hyg mut_1 and Hyg mut_2 primers for (**A**) ApaI or (**B**) SmaI digested and ligated DNA. Bands of unequal size indicate variable size of the circular DNA molecules. **bp**: base pair; **M**: 1 kb marker.

In 49 PCR confirmed mutants, many mutants had no product at all for reverse PCR, due to either too large or too small DNA circular molecules. Reverse PCR products were gel eluted and sequenced using Hyg mut_1 and Hyg mut_2 primers. A NCBI blast search enabled the identification of the site/gene with insertion of the recombination substrate in only thirteen mutants (Figure 9).

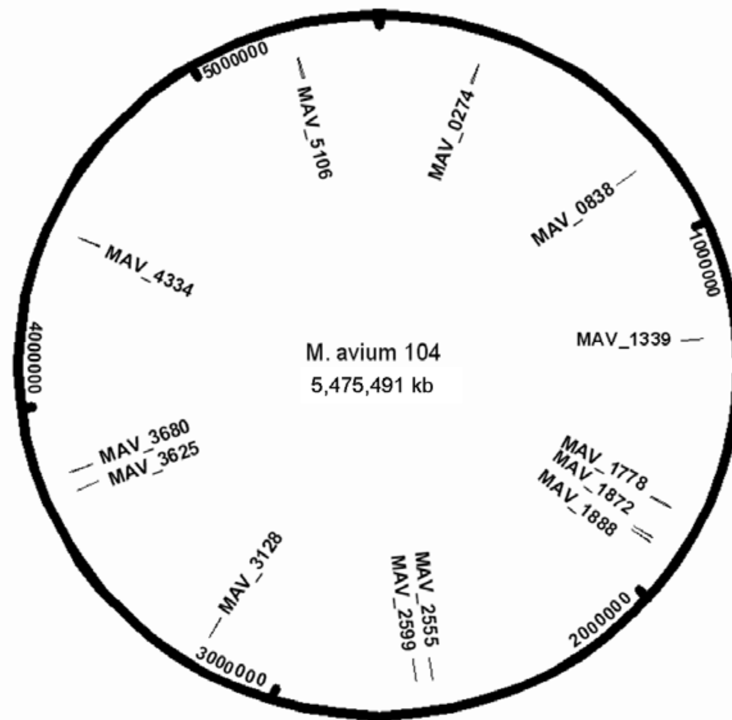


Figure 9. Sketch to show randomly mutated genes distributed within the *M. avium* genome

The mutated genes from 13 mutants mapped on the genome after sequencing of the reverse PCR products.

As an example, the genetic characterisation of the mutant MAV_1778 is shown in Figure 10. The insertion lies within the coding sequence of gene *MAV_1778* encoding the GTP-binding protein LepA. The integration was accompanied by a loss of 669 bp (1771120 to 1771789) of the coding region of *lepA* gene.

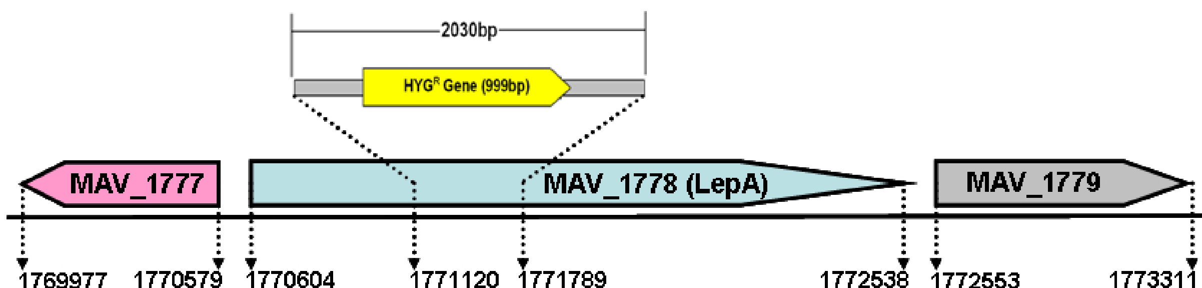


Figure 10. Sketch illustrating the genetic characterisation of the mutant MAV_1778

Position of the recombination substrate (*hygR* gene) identified as a result of sequencing of the eluted fragment isolated after reverse PCR.

3.1.4 Selection and verification of mutants

The integration events were accompanied by deletions in the genomes of all thirteen mutants. The smallest deletion had a size of 2 bp, the largest one of 669 bp. All insertions were located within coding regions. Only in one mutant more than one gene was affected by the insertion. In eleven of the thirteen mutants the linear recombination substrate had been completely inserted and in two mutants the inserted fragment had been shortened at both ends. In favour of a straight forward procedure, further efforts were concentrated on those mutants, which fulfilled the following requirements:

1. An insertion in the middle of the coding region of a gene.
2. Mutation of only one gene.
3. Mutation of a single copy gene.

Subsequently, after applying these criteria, eight mutants (see Table 5 for mutated genes and their functions) were selected for phenotypic analysis. Mutants and wild-type were first confirmed by Ziehl-Neelsen staining for mycobacteria-specificity and for subspecies-specificity by PCR using three primer pairs DnaJ-1 & 2, IS1245-1 & 2 and IS901-1 & 2 (data not shown).

Table 5. Mutants, mutated genes and their functions

Mutants	Function of the mutated gene
Mutant MAV_2555	Short-chain dehydrogenase/reductase SDR
Mutant MAV_1888	Hypothetical protein
Mutant MAV_4334	Nitroreductase family protein
Mutant MAV_5106	Phosphoenolpyruvate carboxykinase
Mutant MAV_1778	GTP-Binding protein LepA
Mutant MAV_3128	lysl-tRNA Synthetase (LysS)
Mutant MAV_3625	Hypothetical protein
Mutant MAV_2599	Hypothetical protein

3.2 Phenotypic characterisation of selected *M. avium* mutants

A search for phenotypic assays was done, allowing a fast screening of generated mutants, in order to identify genes possibly involved in virulence. The selected tests should monitor changes in (i) cell wall composition (plating on Congo-red agar), (ii) resistance towards low pH, (iii) intra-amoeba survival, (iv) induction of cytokine secretion by infected THP-1 macrophages and (v) intracellular survival and growth in human macrophages. All phenotypic tests were performed for the eight selected mutants. The following results are only described for those mutants, which have shown significantly different results in comparison to wild-type.

3.2.1 Colony morphology and Congo-red staining characteristics

The colony morphology of the wild-type and the selected mutants upon plating on Congo-red agar is shown in Figure 11. The wild-type (Figure 11 A) mainly formed smooth-domed-opaque colonies along with smooth-transparent colonies. Mutant MAV_2555 showed the same morphologies, but additionally smooth-flat-red colonies were visible (Figure 11 B). Relatively few smooth-transparent and rough colonies occurred in mutant MAV_1888 (Figure 11 C), MAV_4334 (Figure 11 D) and MAV_5106 (Figure 11 E). Mutant MAV_4334 (Figure 11 D) showed a higher variation with respect to the intensity of red colour of smooth-domed-opaque colonies. Mutant MAV_1778 showed a very high degree of variability displaying red-rough and smooth-flat-red colonies additionally to the smooth-domed-opaque, smooth-transparent and rough-white colonies (Figure 11 F). The colonies generated by mutant MAV_3128 (Figure 11 G) were in average larger in size and the smooth-opaque colonies appeared paler than in the wild-type. Also, the edges of these colonies were more irregular. Some red-rough colonies were also visible. The most multifaceted image was displayed by mutant MAV_3625. This strain generated smooth-domed-opaque, smooth-domed-red, smooth-flat-red, smooth-transparent and rough-transparent and red-rough colonies (Figure 11 H). A high proportion of red colonies (smooth-domed-red, smooth-flat-red, red-rough) was generated by mutant MAV_2599 (Figure 11 I) additionally to smooth-opaque and smooth-transparent colonies. This mutant produced only few rough (rough-transparent, rough-red) colonies.

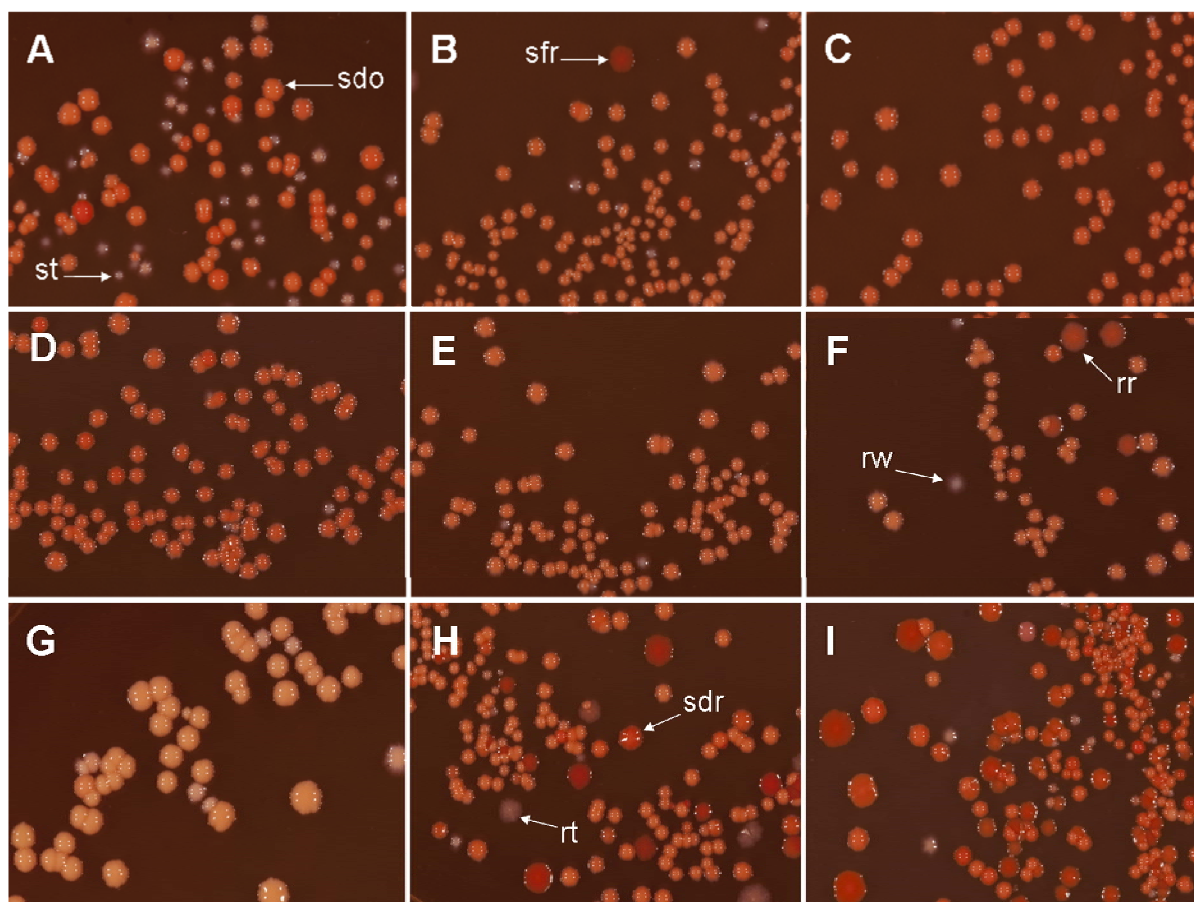


Figure 11. Colony morphology upon plating on Congo-red agar plates

Well-grown broth cultures of all strains were diluted 1:10^6 and 100 µl plated in triplicate onto Middlebrook agar, supplemented with OADC and 100 µg ml^{-1} Congo-red. Plates were incubated on average for 3 weeks. The arrows point to smooth-domed-opaque (sdo), smooth-flat-red (sfr), smooth transparent (st), rough red (rr) and rough transparent (rt) colonies. **A**: wild-type; **B**: mutant MAV_2555; **C**: mutant MAV_1888; **D**: mutant MAV_4334; **E**: mutant MAV_5106; **F**: mutant MAV_1778; **G**: mutant MAV_3128; **H**: mutant MAV_3625; **I**: mutant MAV_2599.

3.2.2 pH-resistance

A resistance towards low pH was investigated for the eight mutants compared to the wild-type, by inoculating them into Middlebrook broth, supplemented with ADC at pH 5 and pH 7. The growth was measured during 11 days at 37°C by means of OD measurement and ATP quantification. As shown in Figure 12, the wild-type grew better at neutral pH than at low pH. After 11 days of growth in neutral medium, it generated 722,491 RLU (Relative light unit), while in medium with acidic pH only 143,082 RLU were achieved. The mutants MAV_2555, MAV_1888, MAV_4334 and

MAV_5106 showed a similar growth pattern as the wild-type, both in neutral and acidic pH (data not shown). The mutants MAV_1778 and MAV_3128 grew similar as the wild-type at neutral pH; however, at low pH these strains enhanced their growth rate even above the level reached at neutral pH. (Figure 12 A and B). While the mutant MAV_3128 showed enhanced growth in comparison to the wild-type at low pH already at day 1, the mutant MAV_1778 showed an identical growth rate as the wild-type at low pH until day 5 and then showed strongly enhanced growth. The mutants MAV_3625 and MAV_2599 grew better than the wild-type at pH 7 and were able to maintain this growth rate at pH 5 (Figure 12 C and D). Results of mutants with no significant difference in comparison to wild type data are not shown.

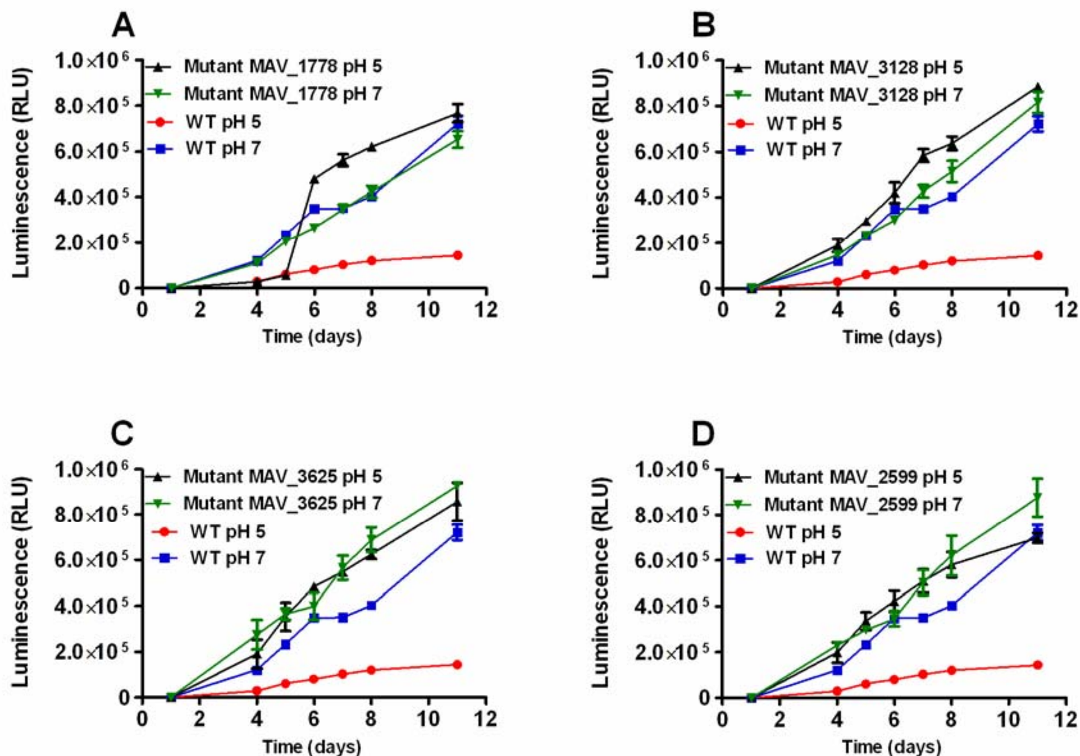


Figure 12. Resistance towards low pH of mutants as compared to wild-type

The bacteria were grown in Middlebrook broth, supplemented with OADC at pH 5 and pH 7 during 11 days; the ATP content was recorded by quantification of the amount of ATP in the cultures. **A**: wild-type and mutant MAV_1778; **B**: wild-type and mutant MAV_3128; **C**: wild-type and mutant MAV_3625; **D**: wild-type and mutant MAV_2599. Each value represents the mean of three cultures with the standard deviation. **RLU**: relative light unit.

3.2.3 Amoeba plating test

The amoeba plate test [141] was modified to fit the growth conditions (medium, temperature, duration) of *M. avium.* The wild-type and mutants were tested control (without amoeba) and test (with amoeba) plates. After incubation for seven days at 28°C, the wild-type formed colonies even if the cultures were diluted $1:10^3$ before being dropped on the lawn of amoebae. The growth of some mutants was more strongly affected by the amoebae and mutant MAV_3128 grew only if undiluted before addition to the amoebae (Figure 13). Mutants MAV_1778, MAV_3625 and MAV_2599 (Figure 13: lane 4, 6 & 7) reacted as wild-type (Figure 13: lane 1). Mutants MAV_4334 and MAV_5106 (Figure 13: lane 2 & 3) were similar to each other but were less affected than mutant MAV_3128 (Figure 13: lane 5). The results of mutants with no significant difference in comparison to wild-type data are not shown.

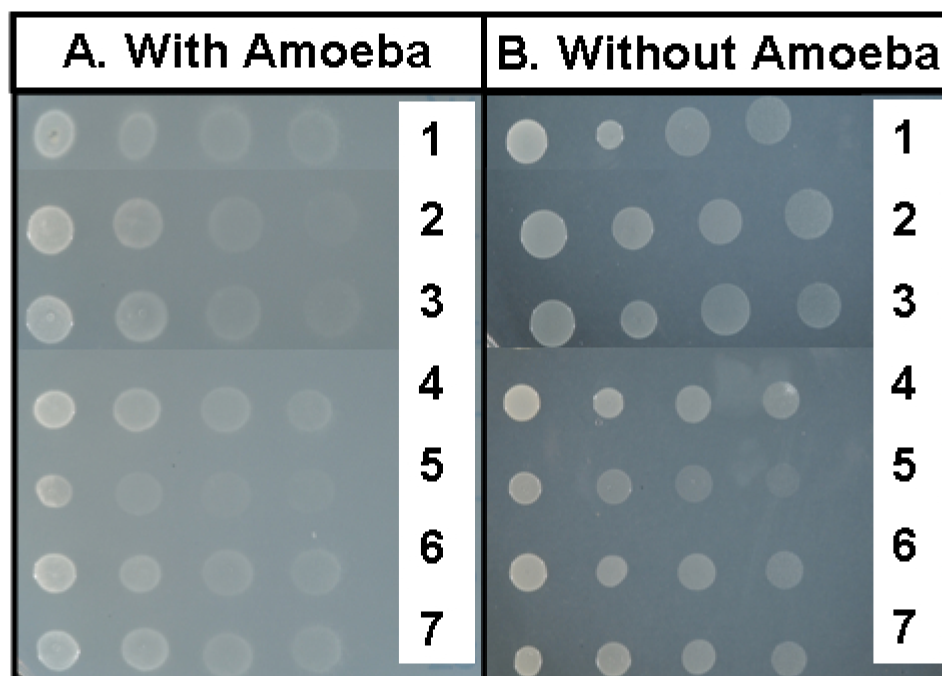


Figure 13. Growth of mutants and wild-type in the amoeba plate test

Only 3 µl of undiluted and of each (1:10, $1:10^2$, $1:10^3$) dilution from the mutants and wild-type were spotted onto a lawn of *Acanthamoeba castellanii* on test plates (**A**) and on Middlebrook with ADC plates without amoeba as control plates (**B**). Growth was observed after one week incubation at 28°C. **1**: wild-type; **2**: Mutant MAV_4334; **3**: Mutant MAV_5106; **4**: Mutant MAV_1778; **5**: Mutant MAV_3128; **6**: Mutant MAV_3625; **7**: Mutant MAV_2599.

3.2.4 Induction of cytokine secretion

In order to evaluate the ability of wild-type and eight mutants to stimulate the immune signalling in THP-1 macrophages, the secretion of the pro-inflammatory cytokines TNF-α, IL-1β and the anti-inflammatory cytokine IL-10 were quantified after 24 hours. Five independent experiments were normalised for wild-type (expression ratio 1) to determine the expression ratio for the mutants in comparison to wild-type. While results for TNF-α (Figure 14 A) and IL-1β (Figure 14 B) were not significantly different for all the mutants compared to wild-type, IL-10 was significantly ($P<0.007$) up-regulated only for mutant MAV_4334 (Figure 14). Results of mutants with no significant difference in comparison to wild-type data are not shown.

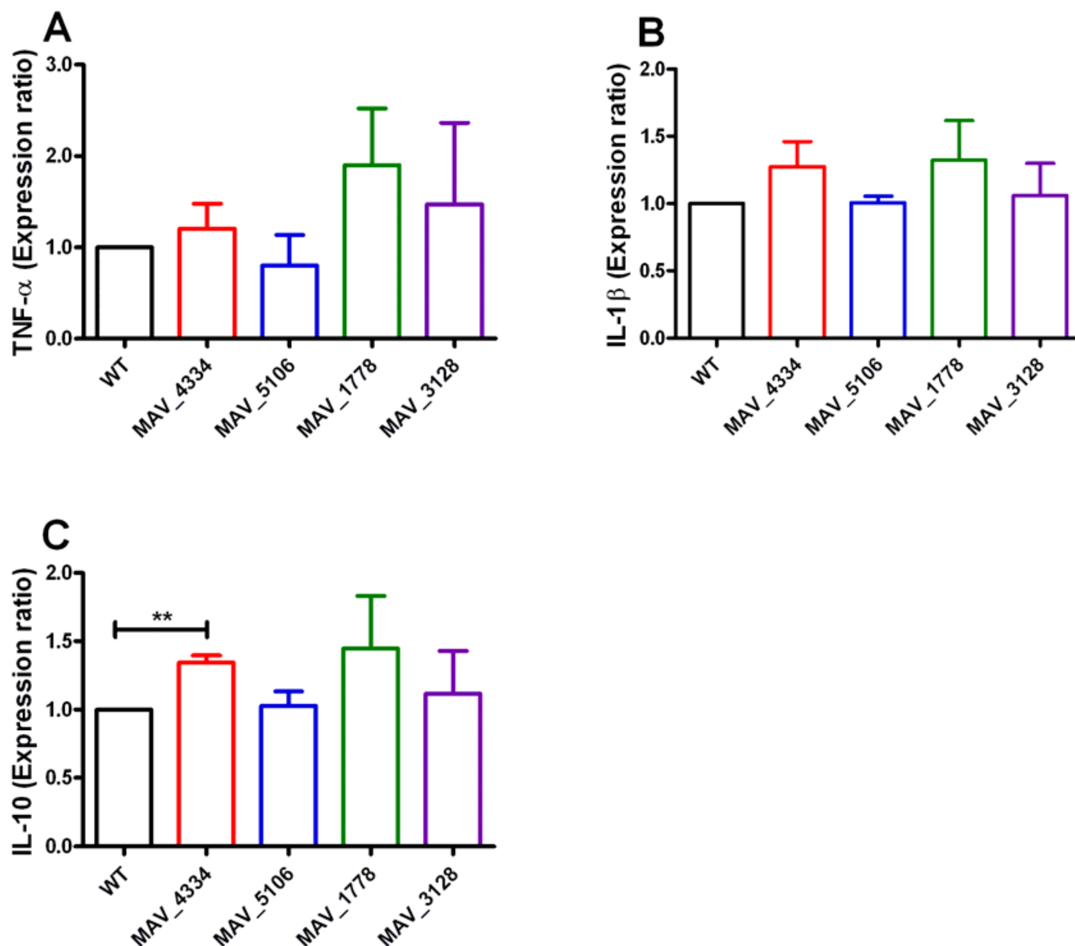


Figure 14. Induction of cytokine secretion by infected macrophages

THP-1 cells ($2.0x10^5$) were infected (MOI 1:50) with mutants and wild-type. After 24 hours TNF-α (**A**), IL-1β (**B**) and IL-10 (**C**) cytokines from supernatants

were measured by ELISA. The values represent the median of five independent cultures with standard deviation. The expression profile of measured cytokines was calculated by normalising to the wild-type and then measuring the percentile deviation. Statistical analysis was done using a two-tailed, unpaired Mann Whitney test. When compared to wild-type a P<0.01 was considered very significant (**).

3.2.5 Intracellular survival in cell lines

The THP-1 and A549 cell lines were independently infected with the wild-type and the eight mutants. Intracellular mycobacteria were measured by quantitative real-time PCR and CFU by plating. Survival of all eight mutants in both THP-1 and A549 cell lines was not consistently different if compared to the wild-type in three repeated experiments (result not given). Thus the experiment was shifted to more natural course of proceedings i.e. intracellular survival in human blood monocytes isolated from different individuals.

3.2.6 Intracellular survival in human blood monocytes

Wild-type and eight mutants intracellular survival was measured by using human blood monocytes for the infection experiments. The growth of mutant MAV_4334, MAV_1778 and MAV_3128 was affected the most in human monocytes (Figure 15). In comparison to wild-type all the three mutants number were reduced significantly (P<0.05 to P<0.01) for the first two days. Mutant MAV_4334 and MAV_1778 (Figure 15 A and C) were almost reduced to half during first two days. As shown in Figure 15 D, mutant MAV_3128 had the highest significant (P<0.001) difference in growth as compared to wild-type, which had survived better during this time period. The mutant MAV_5106 largely differed from the other mutants and during four days of infection had shown constant survival (Figure 15 B).

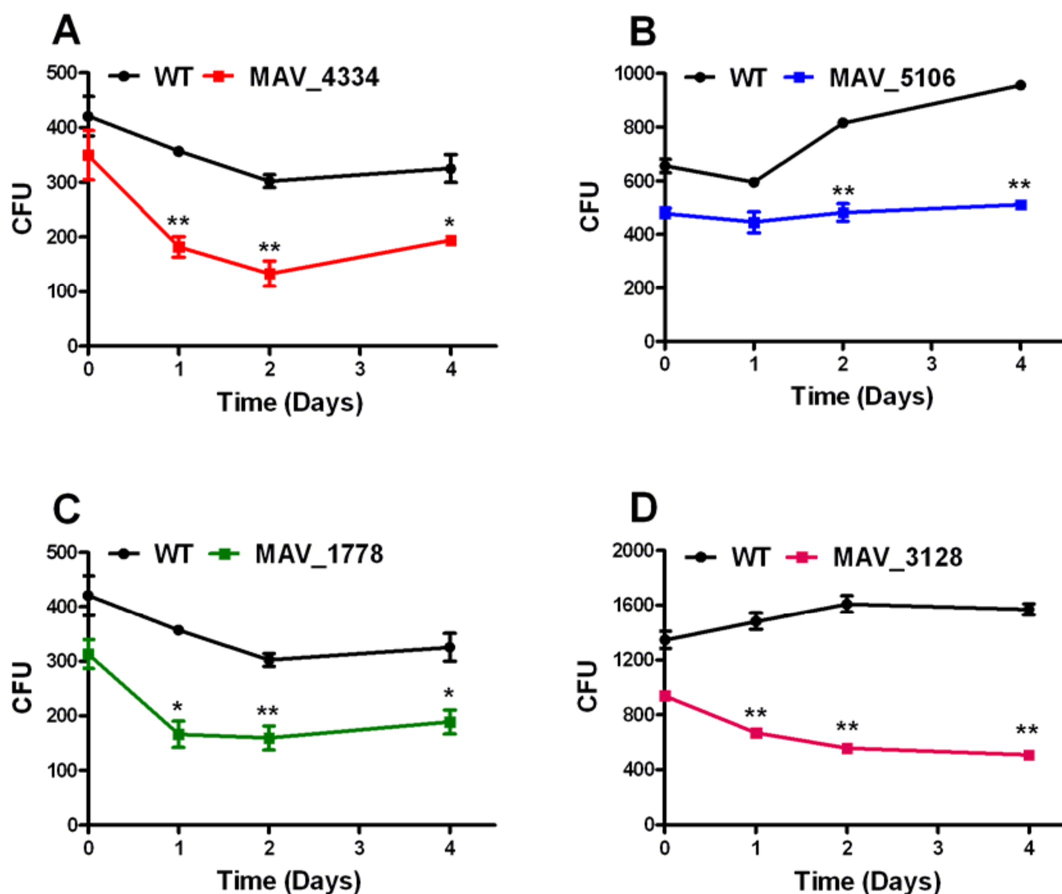


Figure 15. Intracellular survival of mutants compared to wild-type in human monocytes

Human blood monocytes ($1.0x10^6$) from healthy volunteers were infected (MOI 1:10) with mutants and wild-type. Intracellular bacteria were quantified after 4 hour of infection, and after 1, 2, & 4 days. The monocytes were lysed in 1000 µl sterile water and 100 µl of 1:500 dilution in sterile water were plated on agar plates supplemented with ADC for CFU counting. **A**: wild-type and mutant MAV_4334; **B**: wild-type and mutant MAV_5106; **C**: wild-type and mutant MAV_1778; **D**: wild-type and mutant MAV_3128. Statistical analysis was done using a two tailed, paired Student's t test. When compared to wild-type a P<0.01 was considered very significant (**). **WT**: wild-type; **CFU**: colony forming units.

3.3 Complementation of mutant MAV_3128

Mutant MAV_3128 behaved differently compared to the wild-type in four out of five phenotypic tests and therefore was among the eight selected mutants with displaying

strongest phenotype. For this reason this mutant was selected for complementation to prove that the phenotypic changes of the mutant were in fact caused by the inactivation of the gene. The gene *MAV_3128* (3907 bp) was amplified by PCR using wild-type DNA as template and the primer pair MAV3128_MV306_1 and MAV3128_MV306_2. This amplified DNA fragment and plasmid pMV306 (3995 bp) DNA were double digested with the restriction enzymes XbaI and HpaI. *M. avium* gene *MAV_3128* DNA (Figure 16 A; lane 1) and the digested plasmid DNA (Figure 16 A; lane 2) were separated in a 1% agarose gel.

The recombinant plasmid pFKaMAV3128 was constructed by cloning the 3907 bp DNA fragment containing the *MAV_3128* gene along with upstream region from *M. avium* wild-type in an integrative vector pMV306. First this recombinant plasmid pFKaMAV306 (7855 bp) was transformed in *E. coli*. The recombinant *E. coli* colonies were selected on LB plates supplemented with antibiotic kanamycin (50 µg ml^{-1}). Subsequently plasmid was isolated and for confirmation digested with XbaI, HpaI and NheI restriction enzymes (Figure 16 C). This resulted in three DNA fragments (3907 bp, 2150 bp and 1800 bp; Figure 16 B; lane 3) when separated in 1% agarose gel, which confirmed the size and complete integration of the cloned gene *MAV_3128* together with the upstream region.

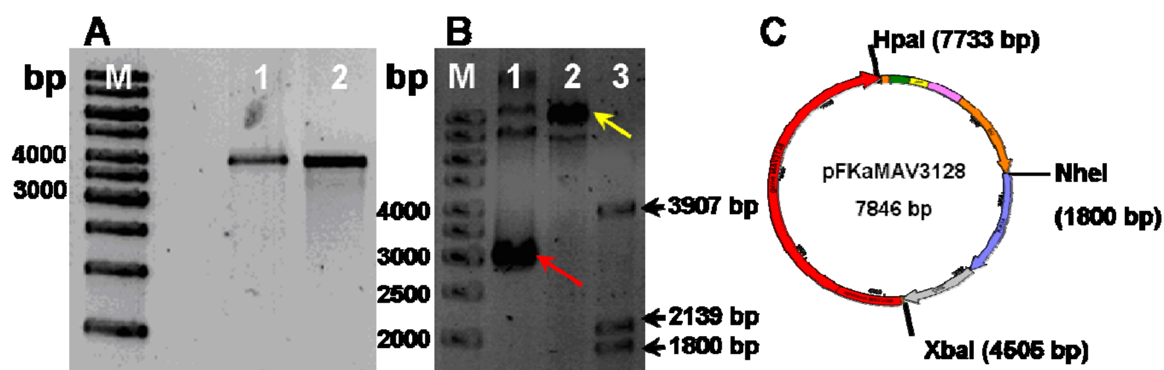


Figure 16. Confirmation of plasmid pFKaMAV3128 by PCR and restriction digestion

A: Gel electrophoresis for double digested *MAV_3128* gene **(lane 1)** and double digested plasmid pMV306 **(lane 2)**. **B**: Gel electrophoresis for **(1)** circular plasmid pMV306 (lane 1: red arrow); **(2)** circular recombinant plasmid pFKaMAV3128 (Lane 2: yellow arrow); (**3)** recombinant plasmid pFKaMAV3128 digested with the restriction enzymes XbaI, HpaI and NheI (Lane 3: black arrows). **C**: Map of pFKaMAV3128 showing the restriction sites for HpaI, NheI and XbaI. **bp**: base pair; **M**: 1 kb marker.

Complementation was achieved by transforming the mutant MAV_3128 with plasmid pFKaMAV3128. The complemented strain *MAV3128Comp* was selected on Middlebrook agar plates, supplemented with ADC, kanamycin (50 µg ml^{-1}) and hygromycin (50 µg ml^{-1}). The complemented strain was confirmed for the presence of the mutation by PCR for *hygR* gene (Figure 17; lane 1) using Hyg 2K FW and BW primers. The *MAV_3128* gene (to confirm the complementation) insertion was confirmed by PCR using primer pair MAV3128_MV306_1 and MAV3128_MV306_2 (Figure 17; lane 2). The 3907 bp product indicate the complete insertion of the gene along with upstream region.

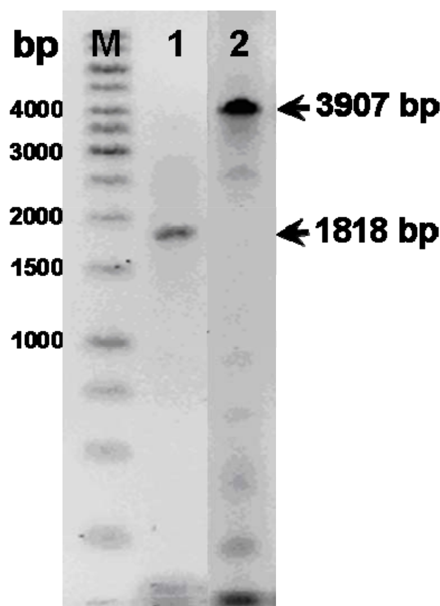


Figure 17. Confirmation of the complemented *MAV3128Comp* strain by PCR

PCR products obtained after using *MAV3128Comp* strain DNA as template. **Lane 1:** 1818 bp product obtained by PCR with primer pair Hyg 2K FW and Hyg 2K BW. **Lane 2**: 3907 bp product obtained by PCR with primer pair MAV3128_MV306_1 and MAV3128_MV306_2. **bp**: base pair; **M**: 1kb marker.

Once the complemented strain was generated and confirmed, test for colony morphology (Congo-red plating), cytokine expression and intracellular survival were performed. These tests gave quick and clear results to confirm the reversal of the mutation in complemented strain *MAV3128Comp*.

3.3.1 Colony morphology changes in response to complementation

The colony morphology results obtained by plating wild-type, mutant MAV_3128 and the complemented strain *MAV3128Comp* on Cong-red agar plates are shown in Figure 18. The wild-type (Figure 18 A) mainly formed smooth-domed-opaque colonies, whereas mutant MAV_3128 (Figure 18 B) colonies appeared paler, smooth-opaque and larger in size. The complemented strain *MAV3128Comp* (Figure 18 C) had a similar appearance as the wild-type in terms of colour and smoothness, but had a larger size similar to the mutant MAV_3128. Unique pale corner were observed in some colonies of *MAV3128Comp*, which corresponded to the colour of mutant MAV_3128 colonies.

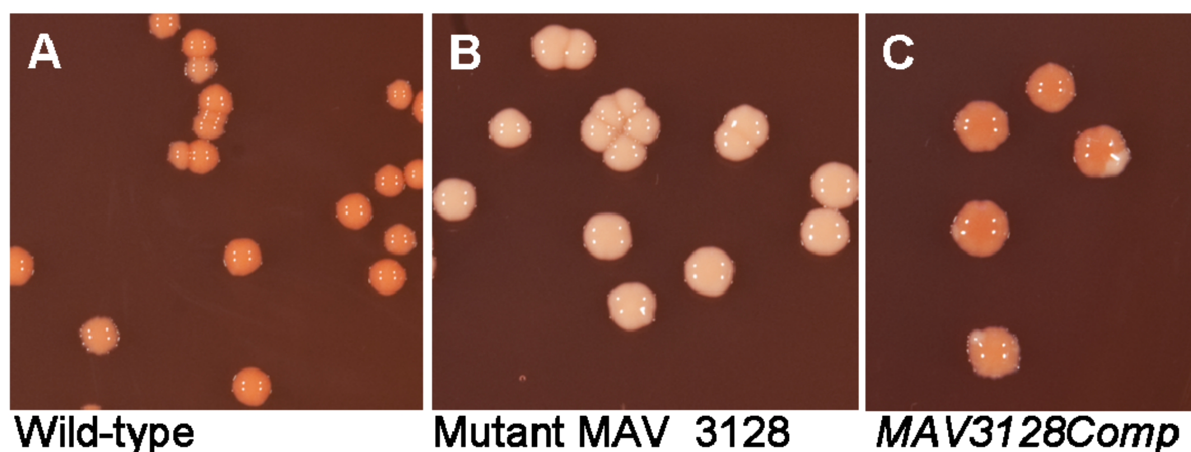


Figure 18. Congo-red plate result for the complemented strain in comparison to the wild-type and the mutant

Well-grown broth cultures of wild-type, mutant MAV_3128 and *MAV3128Comp* strains were diluted 1:10^6 and 100 µl plated in triplicate onto Middlebrook agar, supplemented with OADC and 100 µg ml^{-1} Congo-red. Plates were incubated on average for 3 weeks. **A**: Wild-type; **B**: Mutant MAV_3128; **C**: *MAV3128Comp*.

3.3.2 Cytokine induction in response to complementation

Cytokine secretion of THP-1 macrophages was measured after infection with wild-type, mutant MAV_3128 and *MAV3128Comp* to assess the role of *MAV_3128* gene for the immune response. Secretion was measured for TNF-α, IL-1β and IL-10. Three independent experiments were normalised to wild-type expression level (expression ratio 1) to determine the expression ratio for the mutant and complemented strain in comparison to wild-type (Figure 19). Here TNF-α (Figure 19 A) was significantly down-regulated after infection with mutant MAV_3128 in

comparison to wild-type, otherwise IL-1β (Figure 19 B) and IL-10 (Figure 19 C) had not shown significant differences. The reduced secretion of TNF-α upon infection with the mutant compared to the wild-type was reverted if the cells were infected with the complemented strain.

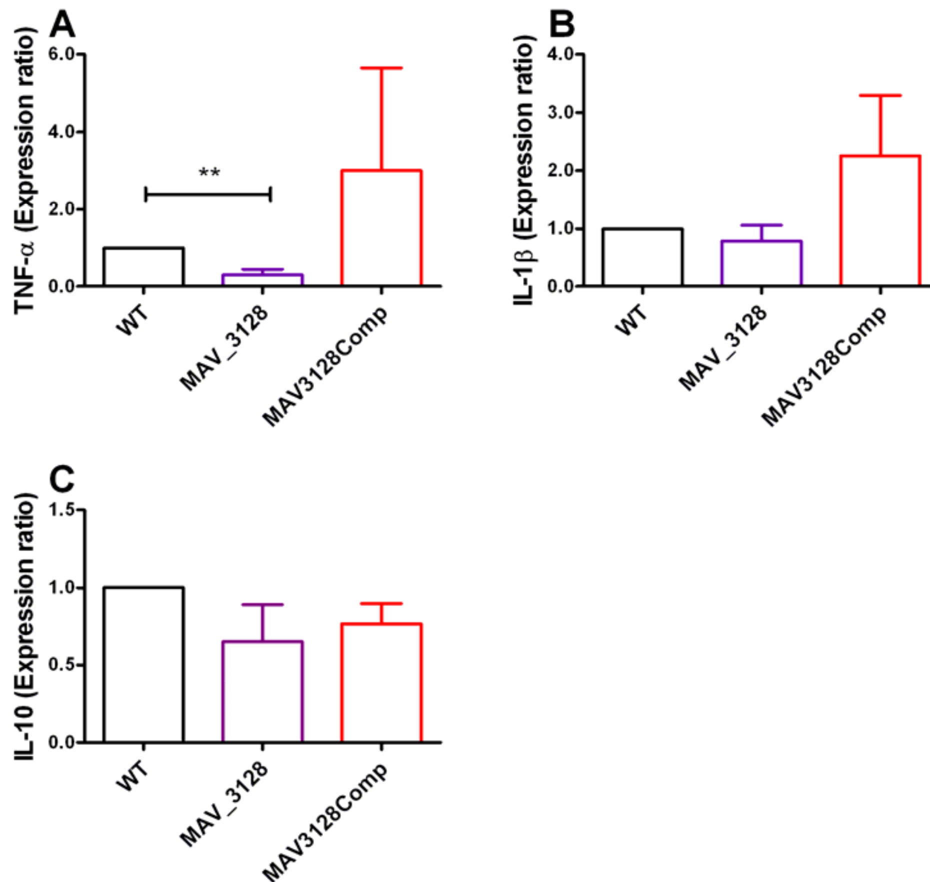


Figure 19. Cytokines secretion by macrophages after infection with wild-type, mutant MAV_3128 and the complemented strain *MAV3128Comp*

THP-1 cells ($2.0x10^5$) were infected (MOI 1:50) with *MAV3128comp*, mutant MAV3128 and wild-type. After 24 hours TNF-α (**A**), IL-1β (**B**) and IL-10 (**C**) in supernatants were measured by ELISA. The values represent the median of three independent cultures with standard deviation. The expression profile of measured cytokines was calculated by normalising to the wild-type and then measuring the percentile deviation. Statistical analysis was done using a two tailed, unpaired Mann Whitney test. When compared to wild-type a $P<0.01$ was considered very significant (**). **WT**: wild-type.

3.3.3 Intracellular survival in response to complementation

Human monocytes were infected with wild-type, mutant MAV_3128 and complemented strain *MAV3128Comp*. Infection and measurement for survival of

mycobacteria, was done as previously described (section 2.11.6). The result described in Figure 20 is one out of three independent experiments conducted, but the tendency was the same in all experiments. The mutant after being phagocytosed was reduced rapidly and within the first 24 hours reduced significantly to almost half of its original number while slight growth was observed for wild-type and complemented strain during the same period. From day 1 until day 4 the killing of the mutant strain continued, at a time when the bacterial load of the wild-type and the complemented strain were least affected. This indicated the remediation of the mutation for *MAV3128Comp* strain and supports that the complemented strain to some extent survived similar to the wild-type inside human monocytes during the whole measured period of infection.

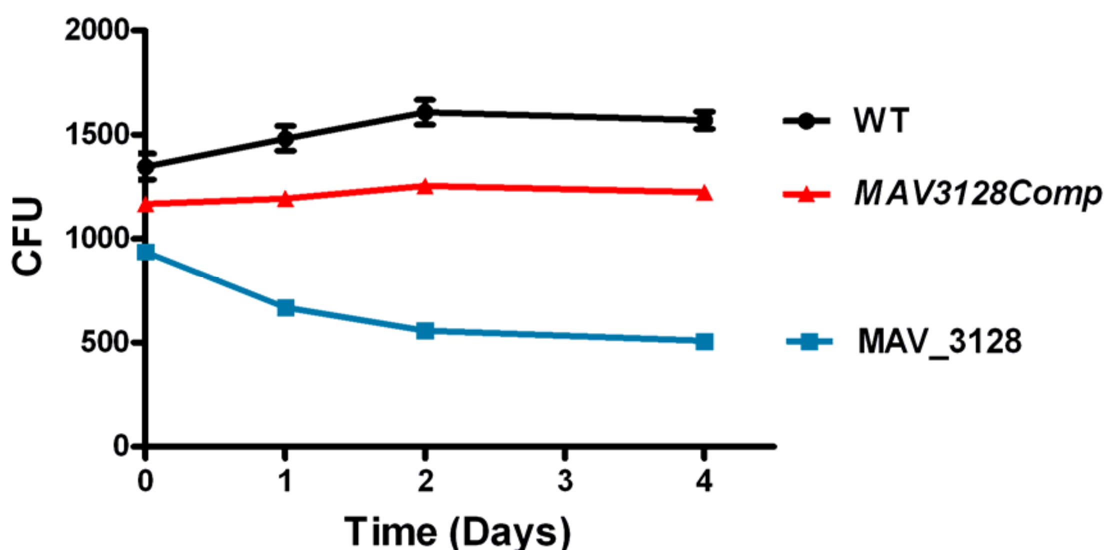


Figure 20. Intracellular survival of the complemented strain

Human blood monocytes (1.0×10^6) from healthy volunteers were infected (MOI 10) with mutant MAV_3128, *MAV3128Comp* and wild-type. Intracellular bacteria were quantified after 4 hour of infection, and after 1, 2, & 4 days. The monocytes were lysed in 1000 µl sterile water and 100 µl of 1:500 dilution in sterile water were plated on agar plates supplemented with ADC for CFU counting. This figure represents one result out of three independent experiments. **WT**: wild-type; **CFU**: colony forming units.

After assessment of the above results for the complemented strain, it was assumed that the mutation reversal was achieved at least to some extent.

4 Discussion

Transformation of mycobacteria is not an easy task. Since they are slow growing bacteria, transformation requires a lot of time and special care. With the limited genetic tools, it is very difficult to understand the pathogenesis of *M. avium*. Thick cell wall, marker selection, growth medium, transformation setting [82], and strains selection [144] are limiting factors of transformation in mycobacteria. In this study random mutants from *M. avium* subsp. *hominissuis* were generated. The mutagenesis approach involved transformation of a recombination substrate by electroporation into *M. avium* subsp. *hominissuis*, therefore first different *M. avium* strains were tested for electroporation competence. This prior investigation of transformability was considered to be necessary, because other authors had reported some clinical *M. avium* strains to be inaccessible to electroporation [82]. As proposed by Lee *et al.,* [82], a *gfp*-containing plasmid (pGFP261: *gfp* cloned in vector pMV261) [136] was chosen for pre-selection transformation assays. Out of 16 tested *M. avium* strains five could be transformed. *M. avium* 104 strain for its availability of genome data base was selected for generation of mutants and analysis.
More efficient transformation frequencies were achieved by using hygromycin resistance as a selectable marker in comparison to kanamycin resistance in *M. smegmatis* and *M. bovis* [145]. Therefore in this study a linear DNA fragment (the recombination substrate) containing a *hygromycin resistance* gene flanked by plasmid DNA on each side was isolated by double digestion from plasmid pYUB854 and used for transformation of *M. avium* 104. The mutagenesis approach employed in this study took advantage of the high rate of illegitimate recombination in slow growing mycobacteria [70, 83] and their ability to take up linear DNA [146].

Generated mutants were tested for the selective marker by PCR. Additionally to confirm the insertion Southern blots were performed, for which genomic DNA from mutants was digested, separated in a gel and transferred onto nylon membrane by capillary transfer method. Hybridisation of a *hygromycin resistance* gene labelled probe conclusively confirmed the insertion of the recombination substrate for twenty mutants. A reverse PCR approach was employed to identify the insertion position in the genome of the twenty mutants. But for some mutants it was difficult to obtain a PCR product, because the ligated circular DNA containing the *hygromycin resistance*

gene were either too large or too small. Williams and colleagues [147] also came across the same problems in obtaining products in inverse PCR for some *hprt* mutant T-cell clones. Therefore as a result only 13 mutants out of 20 mutants generated products in reverse PCR. Sequencing of these products facilitated the identification of the insertion sites for marker in the genomes of these mutants. The sequences next to the inserted fragment showed no special structure or nucleotide sequences. The positions of the mutated genes showed that the mutagenesis by this method had generated random mutations in the genome.

Both, the random distribution of insertion sites and the low rate of large deletions affecting more than one gene are benefits of this method. Contrary to this experience with *M. avium* subsp. *hominissuis*, Collins and colleagues [148] observed more clustered insertions and deletions up to 12 genes by mutagenising *M. bovis* with a DNA fragment carrying a *kanamycin resistance* gene by illegitimate recombination. It would be interesting to find out the reasons for these differing outcomes. Are the specific parameters of the illegitimate recombination events species-specific or does the composition of the recombination substrate play a more important role?

In order to simplify things criteria were set to select mutants for further analysis, which included 1) the insertion within the coding region of a gene, 2) mutation of not more than one gene, and 3) mutation in a single copy gene. Since mutations in non-coding regions of a genome, mutations of several genes and mutation of one copy in multiple copy of a genes, would have little significance only and would reveal little information on virulence markers, these mutants were eliminated from further phenotype analysis.

The second objective of this study was to adapt certain phenotypic tests to quickly screen mutations and recognise candidate genes. The resulting strains were then tested under experimental conditions following the course of natural infection stages implicated in virulence. Possible changes in the cell wall composition resulting from mutagenesis were observed by Congo-red plating test. As the phagosomes constitute an acidic environment, pH stress experiments were conducted akin to the natural environment. To follow the immune response, intercellular communication was investigated by cytokine expression from supernatants of the infected cells. The

ability of ubiquitous mycobacteria for intracellular survival was investigated by two different methods i.e. amoeba plate tests and infection experiments with human blood monocytes.

The occurrence of different colony morphotypes is an eye-catching feature of *M. avium* and has attracted attention also because it is associated to virulence. The colony morphology is influenced by the composition of the cell wall, which is a major determinant of mycobacterial virulence [149, 150]. Congo-red, a planar hydrophobic molecule can bind to diverse lipids and lipoproteins and is thus applicable for the detection of changes in cell wall composition. Upon plating of *M. avium* on Congo-red agar plates, smooth transparent, smooth opaque and rough colonies as well as red and white colonies can be distinguished. Appearance of the three different morphotypes smooth opaque, smooth transparent and rough was also confirmed for strain 104 [54]. While the opaque-transparent switch is reversible, the rough phenotype results from irreversible deletion of cell envelope glycopeptidolipid genes. The transparent colony variant grows better in macrophages compared to the opaque variant. Moreover, white transparent colonies survived better in macrophages than red transparent colonies [151, 152]. These differences in intracellular survival may be caused by variations in the cytokine response towards infection by different morphotypes. The smooth opaque morphotype has been shown to induce higher levels of secretion of IL-10, IL-1β and TNF-α by human blood monocytes compared to the smooth-transparent morphotype [153]. Variations in cytokine response upon infection with either smooth-opaque or smooth-transparent *M. avium* was also reported upon infection of human microglia cultures [154].

In this investigation mainly smooth-domed-opaque and smooth-transparent colonies were dominant colonies in *M. avium* 104 wild-type and in some of the mutants. In addition, mutant MAV_2555 had smooth-flat-red and mutant MAV_2599 had smooth-domed-red, smooth-flat-red and red-rough colonies. While mutant MAV_1888, mutant MAV_5106 and mutant MAV_4334 showed rather fewer smooth-transparent and rough colonies. Mutant MAV_4334 showed variable intensity of red colour colonies on Congo-red plates. Rough white colonies, inconsistent red-rough and smooth-flat-red colonies were recorded for the mutant MAV_1778. Striking results were obtained for mutant MAV_3128, where larger and paler colonies were represented on Congo-red plates in addition to red-rough colonies. Another unique

result was obtained for mutant MAV_3625 on plates, where smooth forms included smooth-domed-opaque, smooth-domed red, smooth-flat-red and smooth-transparent colonies, and red form constituted red-transparent and red-rough colonies. Altogether, a high frequency and intensity of morphological changes were observed in the mutants generated in this study pointing to involvement of the mutated genes in the composition of cell wall structure, which may have impact on the virulence of the strains.

The intra-phagosomal pH of *M. avium*-containing phagosomes decreases to pH 5.2 in activated macrophages [155] and a number of pathogens like *Legionella pneumophila* [156] and *Coxiella burnetii* [157, 158] have been reported to survive in low pH environment of phagosomes. Mutants and wild-type growth in low (pH 5) and neutral (pH 7) media was recorded by OD measurement and ATP quantification in this study. ATP measurement represented a much more sensitive method than the OD measurement, since the OD of a culture not only depends on cell number but also on the size of the cells, their morphology and the degree of clumping of the cells. For these reasons, ATP measurement was reported to be a more reliable method for quantification of mycobacteria in broth culture [128]. In this experiment the wild-type grew better in neutral pH media as compared to low pH media. The growth of mutant MAV_2555, MAV_1888, MAV_4334 and MAV_5106 were akin to the wild-type results. Interestingly mutant MAV_1778 and mutant MAV_3128 growth were by far higher in pH 5 as compared to pH 7, whereas they showed similar growth patterns as that of wild-type at neutral pH. Surprisingly mutant MAV_3625 and mutant MAV_2599 showed greater growth than wild-type in either of pH conditions. In summary, the mutations either had no influence on the survival under pH stress conditions or improved resistance towards pH stress.

Free living amoebae are known to host environmental mycobacteria including *M. avium*, which are able to survive in *Acanthamoeba* trophozoites as well as in the exocysts [116, 120, 121]. Growth in *Acanthamoeba* was associated with subsequently enhanced virulence in infection experiments with mice [123]. Since some virulence mechanisms are employed by amoeba-resistant bacteria to survive in amoebae as well as in macrophages [117, 120, 159], amoebae have been used as test systems for determination of bacterial virulence factors [141, 159, 160].

An *Acanthamoeba castellanii* agar plate assay was developed and successfully employed for screening of mutants of *Legionella pneumophila* [141]. In the present study this test was modified due to differences of optimal growth conditions of *M. avium* (optimal growth temperature 37°C) and *Acanthamoeba castellanii* (optimal growth at room temperature). Several different growth media were tried for this test before selecting Middlebrook agar, supplemented with ADC media for amoeba plate test. This Middlebrook media supports *M. avium* growth only, and not of *Acanthamoeba castellanii*, so that the bacteria are the only nutrition for the amoeba. The cultures of *M. avium* wild-type and mutants were diluted and then pipetted on Middlebrook agar plates, supplemented with ADC and having a lawn of *Acanthamoeba castellanii*. After seven days of incubation at compromised temperature of 28°C, the growth of mutants was compared to that of wild-type. Mutant MAV_3128 growth was mostly affected and it survived only if applied undiluted. Although the effect of the presence of the amoebae had a higher impact on the growth of some mutants compared to the wild-type, a differentiated evaluation of the impact of the various mutations on survival in the amoebae was not possible. The amoeba plate test thus was not sensitive enough to reveal differences in the capacity of the mutants to survive within the amoebae. This was surprising, because the amoeba plate test has proven to be an efficient tool for the identification of virulence genes in *L. pneumophilae* [141]. There are several possible explanations for this discrepancy. Amoebae are the most important habitat of *Legionella*, while *M. avium* is not dependant on the presence of amoebae for survival and distribution. As a consequence, *Legionella* might have evolved more important virulence factors interacting with amoebae. Another possible explanation may result from the differences in the generation times of *L. pneumophilae* and *M. avium*. *L. pneumophilae* is a fast-growing bacterium forming clearly visible colonies few days after plating, while the slow-growing *M. avium* 104 requires two weeks to generate colonies of comparable size. This time span may be too long to maintain the amoebae as trophozoites actively interacting with the mycobacteria. In conclusion, the amoeba plate test was estimated to be of only little value for the detection of virulence genes of slow-growing mycobacteria.

In infection the cytokines attributed for intercellular co-ordination play a very important role and therefore important cytokines expressed by THP-1 cells infected

with wild-type and mutants were investigated. The innate immune recognition by phagocytic cells mediates cellular activation enabling killing of the bacteria and the production of pro- and anti-inflammatory cytokines. The signalling cascade is mainly initiated by binding of *M. avium* components to TLR2 followed by recruitment of the MyD88 adaptor molecule and the activation of NFκB and MAP kinases. This chain of events ends with the induction of inflammatory cytokines [110] controlling macrophage activation and granuloma formation. The selected cytokines are important in activation of adaptive immune system and thus on one side control the infectious agent and on other side protect the host cell from harms of immune response. The induction of cytokines in the THP-cells infected with mutants was in most cases not significantly different than after infection with wild-type. This may indicate that the mutated genes have no or little influence on the synthesis of components binding to TLR2 such as certain cell wall components. Another reason could be that the THP-1 cells used for the experiments were not well suited for cytokine testing, e.g. due to old age and too many passages. Out of the three tested cytokines only IL-10 was significantly up-regulated after infection with mutant MAV_4334. It is known that IL-10 can inhibit the production of inflammatory cytokines TNF-α and other cytokines in monocytes pre-activated by INF-γ and LPS [107, 161] and therefore plays an important role in the immune response.

The ability to survive and even replicate inside the phagosomes of macrophages is an important virulence factor of mycobacteria [125] and was therefore included in the screening options. Infection experiments with macrophages give information on the early host response to mycobacterial infections [112]. Different types of macrophages or monocytic cells have been employed to assess mycobacterial virulence and among these the human macrophage-like cell line THP-1 has proven a suitable system for virulence testing [112, 113]. It was shown that THP-1 cells are similar to primary human monocyte-derived macrophages with respect to their ability to take up mycobacteria and limit their growth [114]. In the present study, THP-1 cells were infected with mutants and wild-type strain and intracellular mycobacterial growth was measured by real-time PCR and CFU counting after plating of cell lysates. No significant differences with respect to intracellular survival in THP-1 cells were shown between mutants and wild-type. Therefore the A549 lung epithelial cell line was then used. In normal course of mycobacterial infections, bacteria are inhaled into human

lungs, they penetrate the epithelial cell lining of the lungs and are then phagocytosed by lung alveolar macrophages [162]. So the first contact between mycobacteria and the human body comes across through lung epithelial cells. Results obtained by using this cell line also showed no distinguishing growth patterns between the mutants and wild-type. Although cell lines are considered as suitable alternative to blood monocytes [163] and proven systems for virulence testing [113] they did not provide promising results in this investigation.

So lastly experiments were performed by using human blood monocytes, isolated from different healthy donors. These experiments gave more reliable results. In blood monocytes mutants MAV_4334, MAV_1778, and MAV_3128 growth was significantly reduced in comparison to wild-type. Only the mutant MAV_5106 was shown to survive better than the other mutant in the same conditions.

To prove that the phenotype of mutant was indeed a cause of the inactivation of the mutated gene, one mutant was complemented. Mutant MAV_3128 had shown the strongest and most different phenotypical changes in comparison to wild-type among the eight tested mutants in almost all the phenotypic tests. For this reason, the gene *MAV_3128* along with its upstream region was amplified from wild-type DNA. A complementation is best performed if the copy number of gene transcripts generated by the complementing gene narrows the copy number in the wild-type. We therefore used a plasmid for cloning that integrates once in the genome of the mutant and included the upstream region of *MAV_3128* to most likely cover the promoter of the gene. This upstream region had a size of about 680 bp and the gene *MAV_3127*, which is located upstream of *MAV_3128*, has an orientation in opposite direction of *MAV_3128*. Therefore it was expected that the upstream region will contain promoter sequence of the *MAV_3128* gene. Thus a 3907 bp DNA fragment was cloned into the integrative vector pMV306. The resulting recombinant plasmid pFKaMAV3128 was first transformed in *E. coli,* for generation of high copy number of recombinant plasmid. Later it was electroporated in competent cells of mutant MAV_3128 to generate the complemented strain.

The phenotypic tests performed with wild-type, mutant MAV_3128 and the complemented strain *MAV3128Comp* revealed significant results. In colony morphology, the pale colour of mutant MAV_3128 could no longer be seen in *MAV3128Comp*, except some pale corners in colonies. This may indicate the loss of

the plasmid and thus reversal of mutant phenotype is partially exhibited. Whereas the mutant induced less TNF-α in infected blood monocytes compared to the wild-type, this difference was not observed if the cells were infected with the complemented strain suggesting reversal of the mutation. Furthermore, the intracellular survival experiment has also conclusively indicated a reversal of the mutation. The complemented strain showed more similar growth tendency towards wild-type strain than the mutant. In conclusion we successfully complemented the mutant MAV_3128 by introducing the intact gene proving that the phenotype of mutant MAV_3128 was indeed caused by the inactivation of gene *MAV_3128* and not by a second line mutation.

A total of five screening methods (colony morphology, pH stress resistance, amoeba resistance, cytokine induction, intracellular survival) were employed to select mutants affected in virulence-related traits. Out of eight mutants selected for analysis two mutants (MAV_4334 and MAV_3128) responded differently from the wild-type in four of these five screening tests and two mutants (MAV_5106 and MAV_1778) reacted differently in three screening tests. The other mutants either did not show any differences compared to the wild-type or reacted differently in only one or two tests.

The insertions in mutants MAV_4334, MAV_5106, MAV_1778 and MAV_3128 have been mapped and the structure of the mutated regions has been analysed on nucleotide level. In all cases only one gene has been mutagenised. The insertions are located in the genes *MAV_4334* (nitrogenase reductase family), *MAV_5106* (phosphoenolpyruvate carboxykinase), *MAV_1778* (GTP-binding protein LepA) and *MAV_3128* (lysyl-tRNA synthestase LysS).

Phosphoenolpyruvate caboxykinases (PEPCK) catalyse the reversible decarboxylation and phosphorylation of oxaloacetate to from phosphoenolpyruvate. Mutations of the PEPCK gene from *M. bovis* BCG are characterised by attenuated virulence and reduced survival in macrophages [164]. The PEPCK gene from *M. tuberculosis* was shown to be required for replication in murine bone marrow macrophages and mice [165].

The LepA protein from *M. tuberculosis* possess GTPase activity. Bacterial GTP-binding proteins play a role in regulation of ribosomal function and cell cycle,

modulation of DNA partitioning and DNA segregation [166]. In *Helicobacter pylori* LepA is important for growth at low pH and may play a role in infection [167].

The *lysS* gene from *M. avium* is 81% homologous to the *lysX* gene from *M. tuberculosis*. LysX from *M. tuberculosis* is required for synthesis of lysinylated phosphatidylglycerol. A LysX mutant was shown to be sensitive to cationic antibiotics and peptides, to be more lysosome-associated and to display defective growth in mouse and guinea pig lungs [168].

So far, nothing is known about the role of nitrogenase reductase family protein MAV_4334 for growth and pathogenicity and answering this question will be one of the future aims.

In summary, by analysing 50 random mutants, four genes from *M. avium* were uncovered to play a role in the interaction with host cells and thus in virulence. The homologues of three of the four genes were shown to contribute to virulence in other bacterial species, which supports the significance of this screening procedure.

In conclusion this study proposes a well-functioning method to randomly mutagenise *M. avium* by illegitimate recombination, genetically characterise the mutations to the nucleotide level and screen the mutants with simple phenotypic tests providing information about virulence-associated features.

5 Summary

The genus *Mycobacterium* (*M.*) comprises highly pathogenic bacteria such as *M. tuberculosis* as well as environmental opportunistic bacteria called non tuberculous mycobacteria (NTM). While the incidence of tuberculosis is declining in the developed world, infection rates by NTM are increasing. NTM are ubiquitous and have been isolated from soil, natural water sources, tap water, biofilms, aerosols, dust and sawdust. Lung infections as well as lymphadenitis are most often caused by *M. avium*, which is considered to be among the clinically most important NTM. Only few virulence genes from *M. avium* have been defined due to difficulties in generating site-directed *M. avium* mutants. More efforts in developing new methods for mutagenesis of *M. avium* and identification of virulence-associated genes are therefore needed.

A random mutagenesis method was developed based on illegitimate recombination and integration of a hygromycin resistance marker. Screening for mutations possibly affecting virulence was performed by monitoring of low pH resistance, colony morphology, cytokine induction in infected macrophages and intracellular persistence. Out of 50 randomly chosen hygromycin resistant colonies, four revealed to be affected in virulence-related traits. The mutated genes were *MAV_4334* (nitroreductase family protein), *MAV_5106* (phosphoenolpyruvate carboxykinase), *MAV_1778* (GTP-binding protein LepA) and *MAV_3128* (lysyl-tRNA synthetase LysS). The complemented strain *MAV3128Comp* showed reversal of the mutant phenotype in selected phenotypic tests.

This study proposes a well-functioning method to randomly mutagenise *M. avium* by illegitimate recombination, genetically characterise the mutations to the nucleotide level and screen the mutants with simple phenotypic tests providing information about virulence-associated features. By this method, four *M. avium* genes were identified that may be involved in virulence.

6 Zusammenfassung

Die Gattung *Mycobacterium (M.)* umfasst hochpathogene Spezies, wie beispielsweise *M. tuberculosis,* aber auch opportunistische Umwelterreger, die zu den nicht tuberkulösen Mykobakterien (NTM) gezählt werden. Während die Inzidenzraten für Tuberkulose in den Industrieländern zurückgehen, steigen die Infektionsraten durch NTM. Ubiquitäre NTM wie das in dieser Arbeit untersuchte *M. avium* wurden schon aus dem Erdreich, natürlichen Wasserquellen, Leitungswasser, Biofilmen, Aerosolen, Staub und Sägemehl isoliert. Die am häufigsten von *M. avium* verursachten Krankheiten sind Lungeninfektionen und Lymphadenitis, *M. avium* zählt in vielen Ländern zu dem am häufigsten aus Patienten isolierten NTM, weswegen dieser Spezies unter den NTM die größte klinische Bedeutung zugeschrieben wird. Aufgrund von Schwierigkeiten bei der gezielten Mutagenese wurden bisher nur wenige Virulenzgene von *M. avium* charakterisiert. Es ist daher notwendig, neue Methoden zur Mutagenese und Identifizierung weiterer Virulenzgene zu entwickeln.

Aus diesem Grund wurde in dieser Arbeit eine neue Methode der Zufallsmutagenese, basierend auf illegitimer Rekombination und Integration eines Hygromycinresistenzmarkers entwickelt. Die anschließende Identifizierung möglicher, die Virulenz beeinflussender Mutationen, erfolgte anhand von Untersuchungen zur Resistenz gegenüber niedrigem pH-Wert, zur Koloniemorphologie, zur Amöben-Resistenz, zur Induktion der Zytokinsynthese infizierter Makrophagen sowie zum intrazellulärem Überleben in infizierten Makrophagen. Von 50 zufällig ausgewählten, hygromycinresistenten Kolonien, waren vier in virulenzassoziierten Eigenschaften verändert. Die mutagenisierten Gene waren *MAV_4334* (Proteinfamilie der Nitroreduktasen), *MAV_5106* (Phosphoenolpyruvat-Carboxykinase), *MAV_1778* (GTP-bindendes Protein LepA) und *MAV_3128* (Lysyl-tRNA Synthetase LysS). Der komplementierte Stamm *MAV3128Comp* zeigte in ausgewählten phänotypischen Tests eine Reversion des durch die Mutation in dem Gen MAV_3128 verursachten Phänotyps.

Diese Studie beschreibt eine für *M. avium* gut funktionierende Methode der Zufallsmutagenese, der molekularen Charakterisierung der Mutanten und des

phänotypischen Screenings zur Identifizierung von Mutanten, die in virulenzassoziierten Eigenschaften verändert sind. Mit Hilfe dieser Methode wurden vier Gene bei *M. avium* identifiziert, die an der Virulenz beteiligt sein könnten.

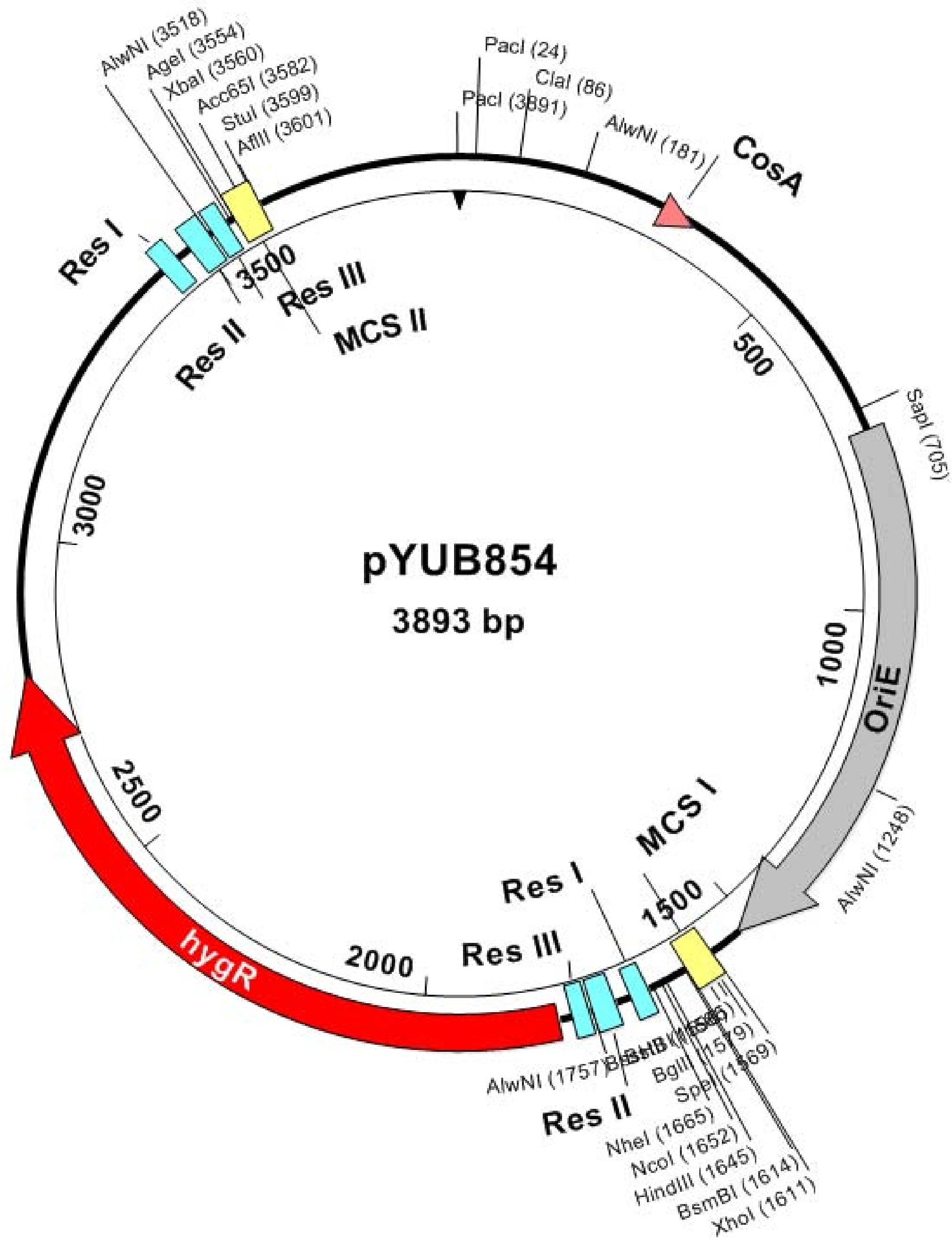
7 Appendix

7.1 Abbreviations

A.	*Acanthamoeba*
ADC	Albumin-dextrose-catalase
AIDS	Acquired Immuno deficiency Syndrome
BCG	Bacillus Calmette-Guérin
bp	Base pair
CFU	Colony forming unit
CTAB	Cetyl-trimethyl-ammonium-bromid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E.	*Escherichia*
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alia*
FLA	Free living amoeba
GPL	Glycopeptidolipid
HIV	Human immunodeficiency virus
hygR	*hygromycin resistance*
IS	Insertions sequence
IMDM	Iscove's Modified Dulbecco's Medium
LPS	Lipopolysaccharid
M.	*Mycobacterium*
MAA	*Mycobacterium avium* subsp. *avium*
MAC	*Mycobacterium avium* complex
MAH	*Mycobacterium avium* subsp. *hominissuis*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MOI	Multiplicity of infection
MTBC	*Mycobacteria tuberculosis* complex
NTM	Non tuberculous mycobacteria
OADC	Oleic acid albumin dextrose
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol-12-myristat-13-acetate
PYG	Proteose-peptone yeast-extract glucose
RFLP	Restriction fragment length polymorphism
RLU	Relative light unit
SDS	Sodium dodecyl sulfate
taq	Thermus aquaticus
tb	*tuberculosis*
TE	Tris-EDTA

7.2 Maps of plasmids

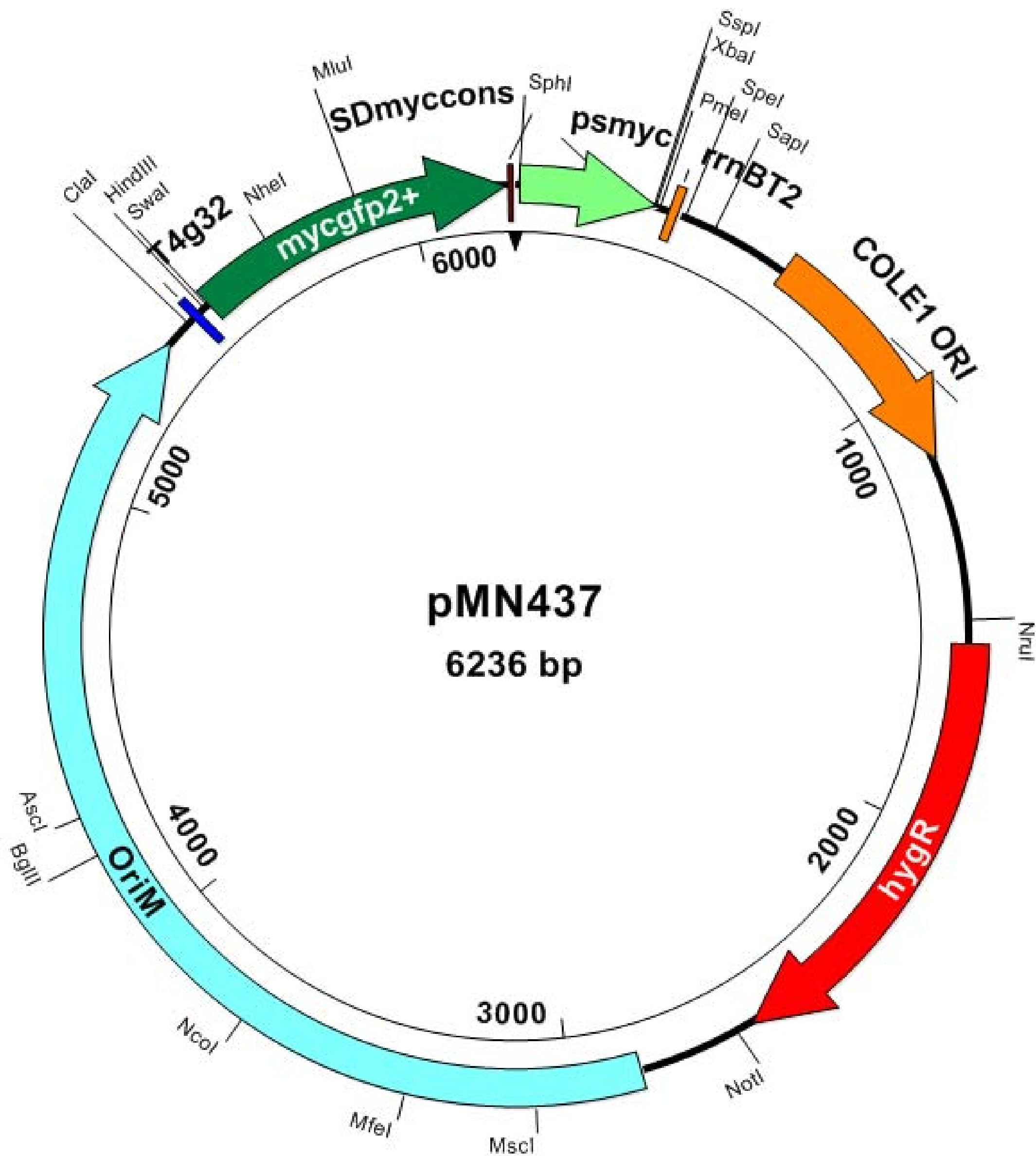
7.2.1 Plasmid pYUB854



Plasmid pYUB854 [135]

E. coli cosmid with an origin of replication in *E. coli* (OriE) and *hygromycin resistance* (hygR) gene flanked by multiple cloning site (MCS).

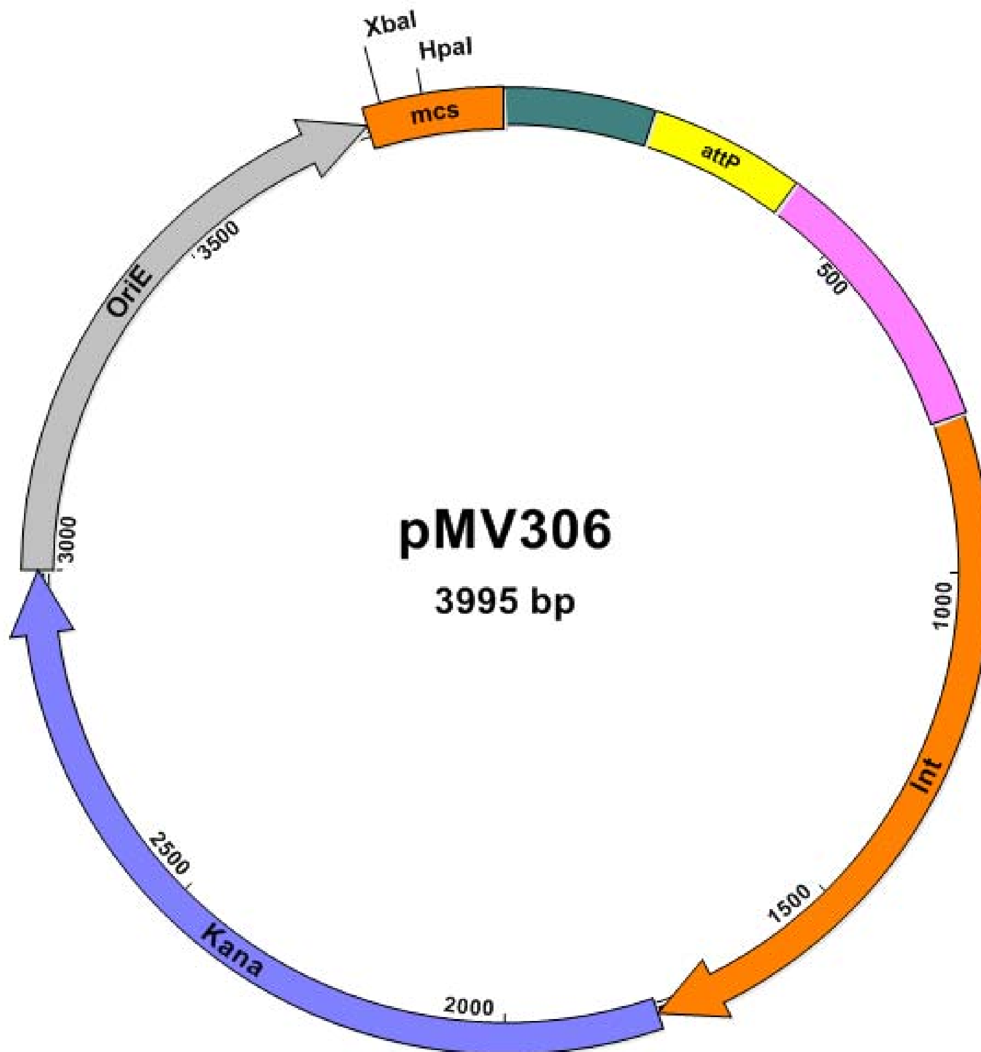
7.2.2 Plasmid pMN437



Plasmid pMN437 [137]

E. coli cosmid with COLE1 ORI, *hygromycin resistance* gene (hygR), origin of replication in mycobacteria (OriM) and *green fluorescent protein* gene (mycgfp2+).

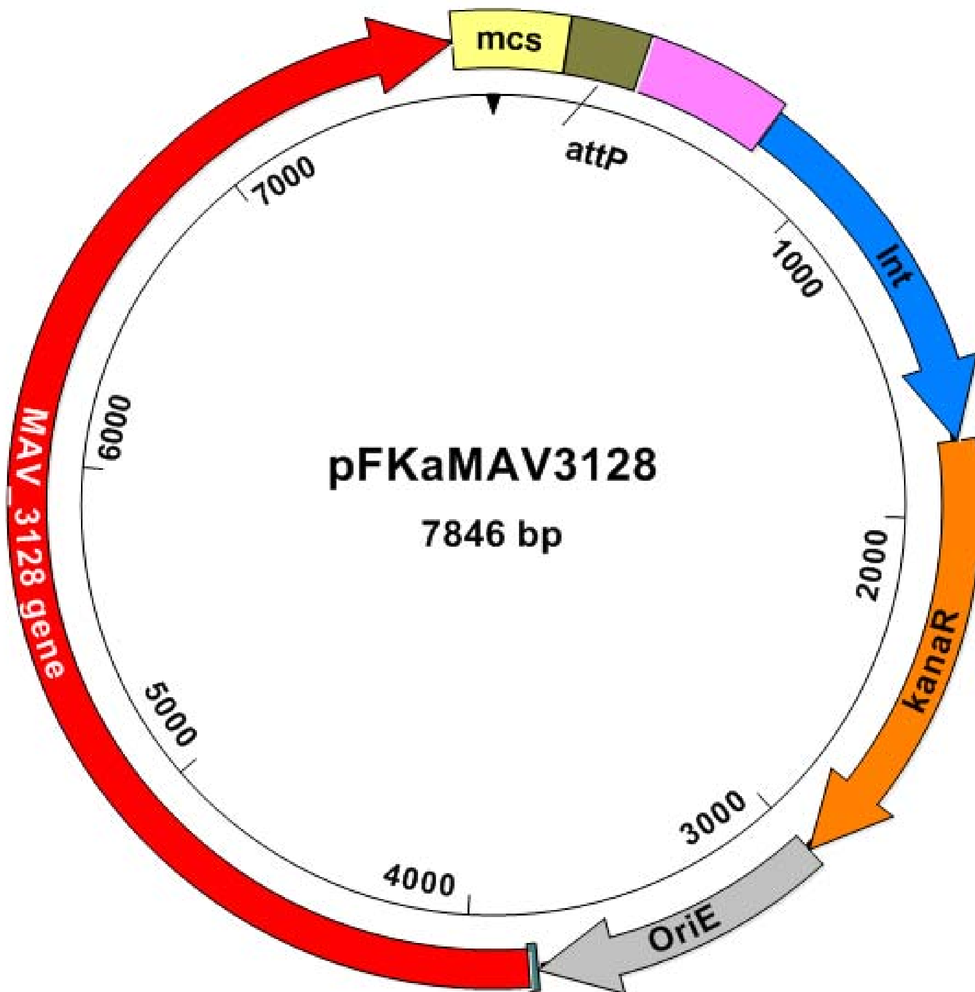
7.2.3 Plasmid pMV306



Plasmid pMV306 [138]

E. coli cosmid with phage L5 attP site, *aph* from transposon Tn903, an *integrase* gene (*Int)*, *kanamycin resistance* gene, origin of replication in *E. coli* (OriE) site and a multiple cloning site (mcs).

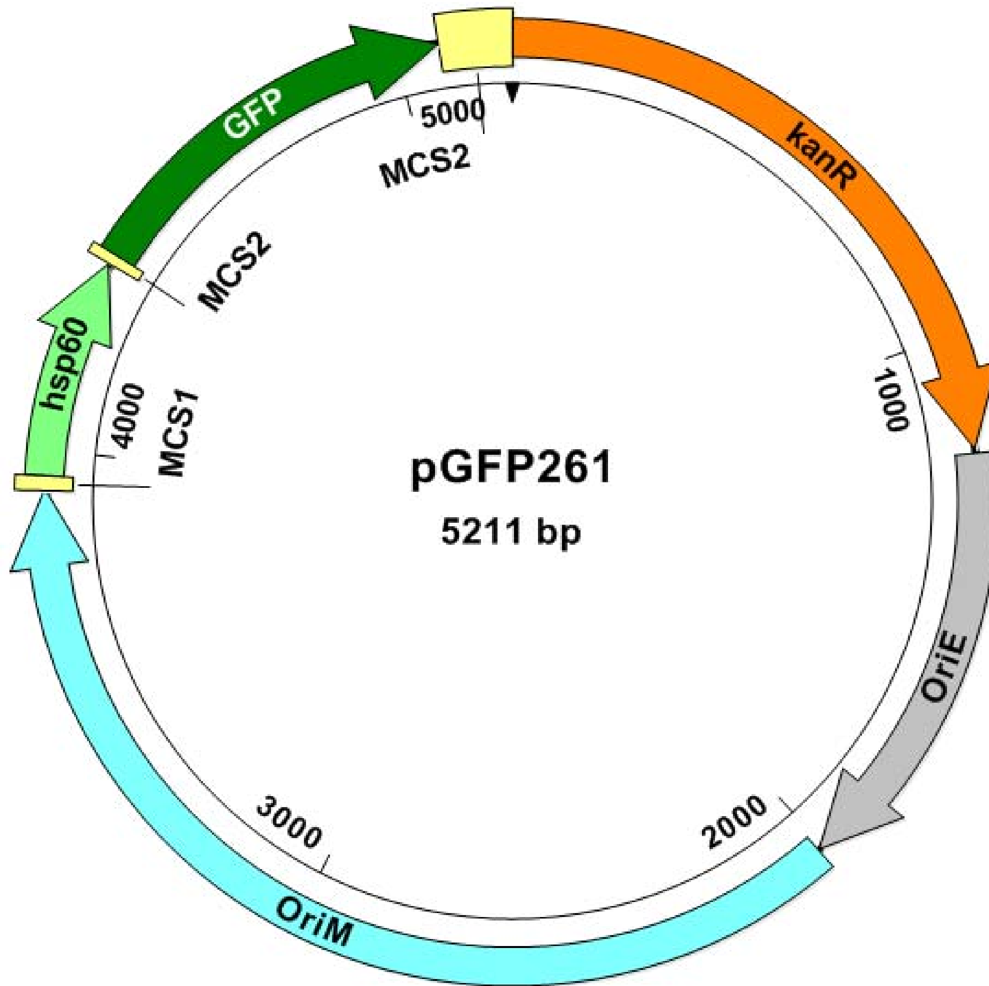
7.2.4 Plasmid pFKaMAV3128



Plasmid pFKa3128 used for the complementation of mutant MAV_3128 (prepared in this study)

Plasmid pMV306 with gene *MAV_3128* from wild-type inserted in restriction sites XbaI and HpaI of multiple cloning sites (mcs).

7.2.5 Plasmid pGFP261



Plasmid pGFP261 [136]

Plasmid pMV261 with gene *gfp* downstream of the promoter *hsp*60 from BCG inserted in restriction sites PstI and HindIII.

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