Biosynthesis and Roles of Virulence Conferring Cell Wall Associated Dimycocerosate Esters in \textit{Mycobacterium marinum}

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BIOSYNTHESIS AND ROLES OF VIRULENCE CONFERRING CELL WALL ASSOCIATED
DIMYOCEROSATE ESTERS IN *MYCOBACTERIUM MARINUM*

by

POORNIMA MOHANDAS

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

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Advisor: Dr. Luis E.N. Quadri

Mycobacterial species include a variety of obligate and opportunistic pathogens that cause several important diseases affecting mankind such as tuberculosis and leprosy. The most unique feature of these bacteria is their intricate cell wall that poses a permeability barrier to antibiotics and contributes to their pathogenicity and persistence within the host. The cell wall hosts several complex lipids such as dimycocerosate esters (DIMs), which are found in many clinically relevant pathogenic species of mycobacteria. DIMs have been implicated in the virulence of mycobacteria and play a major role in helping the bacteria evade host immune responses. It is therefore crucial to define the biosynthesis and role of DIMs in mycobacteria, to better understand these organisms and identify new drug target candidates. DIMs consist of two structurally related groups: phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs). PDIMs and PGLs share part of a biosynthetic pathway that consists of two enzyme families: polyketide synthases (PKSs) and fatty acyl AMP ligases (FAALs).

This dissertation has investigated the roles of PKSs and FAALs during PGL biosynthesis in the pathogenic nontuberculous mycobacterium; *Mycobacterium marinum*. More specifically, it is focused on mutational studies that probed the mechanism by which intermediates
synthesized by an iterative PKS, Pks15/1 are transferred to a non-iterative PKS, PpsA during PGL biosynthesis. Our findings specified the role of the loading acyl carrier protein domain of PpsA, in the capture of intermediates from Pks15/1 during PGL biosynthesis. We also provided the first evidence supporting a model in which the transfer of intermediates during PGL biosynthesis is dependent on a novel FAAL enzyme (FadD29) that acts as an intermediary between Pks15/1 and PpsA, within a nontuberculous mycobacterial species.

This dissertation has also explored the hypothesis that different gene knockouts that render the same PDIM and/or PGL deficiency phenotypes lead to strains with equivalent pleiotropic profiles. The availability of six M. marinum mutants, each with a different gene knockout in the PDIM/PGL biosynthetic pathway, provided an opportunity to probe for the pleiotropic consequences of gene knockouts leading to PDIM− PGL−, PDIM+ PGL−, or PDIM− PGL+ phenotypes. We evaluated the mutants for changes in cell surface properties, cell envelope permeability, antimicrobial drug susceptibility, biofilm formation virulence in an amoeba model system, sliding motility and in vitro growth assays. Our results revealed that the pleiotropic patterns emerging from the different gene knockouts lead to: altered cell surface properties, weakened cell envelope permeability barrier, increased antibiotic susceptibility, reduced biofilm formation and different attenuation levels in an amoeba model. No notable differences were observed in sliding motility and in vitro growth of the different mutants. Our findings also advocate that, different enzymes of the pathway whose elimination equally leads to PDIM and PGL deficiency might not be equivalent drug target candidates.
ACKNOWLEDGEMENTS

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<tbody>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi drug resistant</td>
</tr>
<tr>
<td>XDR</td>
<td>Extremely drug resistant</td>
</tr>
<tr>
<td>MTBC</td>
<td>Mycobacterium tuberculosis complex</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus of Calmette and Guerin</td>
</tr>
<tr>
<td>MOTT</td>
<td>Mycobacteria other than TB</td>
</tr>
<tr>
<td>NTM</td>
<td>Non tuberculous mycobacteria</td>
</tr>
<tr>
<td>PIM</td>
<td>Phosphotidylinositol mannosides</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LM</td>
<td>Lipomannan</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>NAM</td>
<td>N-acetyl muramic acid</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose dimycocerosate</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharides</td>
</tr>
<tr>
<td>PGL</td>
<td>Phenolic glycolipid</td>
</tr>
<tr>
<td>PDIM</td>
<td>Phthiocerol dimycocerosate</td>
</tr>
<tr>
<td>SGL</td>
<td>Sulfoglycolipids</td>
</tr>
<tr>
<td>DAT</td>
<td>Diacyl trehalose</td>
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<tr>
<td>PAT</td>
<td>Penta acyl trehalose</td>
</tr>
<tr>
<td>GPL</td>
<td>Glycopeptidolipids</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide synthase</td>
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<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<td>KS</td>
<td>Ketosynthase</td>
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<tr>
<td>AT</td>
<td>Acyltransferase</td>
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<td>DH</td>
<td>Dehydratase</td>
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<tr>
<td>ER</td>
<td>Enoylreductase</td>
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<tr>
<td>KR</td>
<td>Ketoreductase</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
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**ACP<sub>L</sub>** Loading acyl carrier protein

**TE** Thioesterase

**MPM** Mannosyl-β-1-phosphomycoketides

**FAAL** Fatty acyl AMP ligase

**FACL** Fatty acyl CoA ligase

**AAE** Acyl activating enzymes

**p-HBA** p-hydroxybenzoic acid

**PHPA** p-hydroxyphenylalkanoate

**iNOS** inducible nitric oxide synthase

**PAMP** Pathogen associated molecular patterns

**WT** Wild type

**Mm** *M. marinum*
CHAPTER 1: INTRODUCTION

“The act of making an addition to the overwhelming literature on acid-fast bacteria needs an excuse, if not an apology. No other bacterial group has received quite the same flattering attention or yielded in return so rich a harvest of chemical knowledge; yet no other group has more stubbornly resisted all efforts to expose the intimate secrets of its metabolism.”

-N.L. Edson (3)

MYCOBACTERIAL SPECIES: CLASSIFICATION AND CHARACTERISTICS

Mycobacteria are a genus of over 150 species in the phylum Actinobacteria (4-6). They are a group of aerobic, rod shaped bacteria with a high G+C content (50-60%) (6). One of the most unique features of this group of bacteria is their acid fast cell wall that shares characteristics with both gram positive and gram negative bacteria. Mycobacteria have been shown by cryo-electron microscopy studies to possess a periplasm similar to gram positive bacteria, along with an outer membrane composed of unique esoteric lipids as seen in gram negative bacteria, (7,8). Their habitat includes both terrestrial and aquatic environments in addition to intracellular environments where they continue to hone their excellent pathogenic skills. Based on their pathogenic behavior, mycobacteria are most commonly grouped into obligate pathogens, opportunistic pathogens or non-pathogenic saprophytes (9,10).

Mycobacteria were first identified by Dr. Armauer Hansen in 1873 with the discovery of Mycobacterium leprae, the causative agent of leprosy (9,11,12). However it was the discovery of Mycobacterium tuberculosis by Dr. Robert Koch, nine years later that truly kick-started the study of mycobacteria while simultaneously launching microbiology as a science. By isolating the bacterium from infected tissue, staining it, culturing it, inoculating it into healthy laboratory animals, and reproducing the disease, Koch had not only provided strong support for the germ
theory of disease which was widely contested at the time, he had also indisputably and very elegantly unveiled the sinister causal agent of mankind’s deadliest disease, known over time as the white plague, phthisis, consumption or tuberculosis as we call it today (13,14).

**TUBERCULOSIS**

“If the number of victims is a measure of the significance of a disease, even the most dreaded infectious diseases such as plague or cholera must rank far behind tuberculosis.”

-Robert Koch (15)

**CHARACTERISTICS AND GLOBAL HEALTH PARADIGM**

*M. tuberculosis* is one of the deadliest pathogens known to man. It is the causative agent of tuberculosis or TB, an air borne disease characterized by the presence of tubercles or granuloma in the lungs or other tissues in the body (16,17) TB is a major global health problem and along with HIV has been declared as a leading cause of death from an infectious disease (18). Approximately one third of the global human population has asymptomatic latent tuberculosis which comes with the risk of developing into active TB disease that typically presents itself as a pulmonary or extra pulmonary disseminated disease. The World Health Organization in 2014 reported that, 9.6 million people around the world were diagnosed with active TB disease while ~ 1.5 million TB-related deaths were reported worldwide. In the United States alone a total of 9,421 TB cases were reported at the rate of 2.96 cases per 100,000 persons in 2014 (18). TB is also a leading killer of HIV-positive individuals, who are 30 times more susceptible to tuberculosis than individuals who do not harbor HIV. With the advances in the diagnosis and treatment of TB, it might be encouraging to realize that globally, the mortality rate of TB has decreased in the last decade, and the incidence of active disease is steadily but slowly declining by ~1 - 3% per year). However, the burden of disease remains substantial, especially in
low-income countries an issue compounded by the rise of tuberculosis caused by multi drug resistant (MDR) and extensively drug (XDR) strains of \textit{M. tuberculosis}. This is a phenomenon that is expected to occur considering the fact that \textit{M. tuberculosis} is a pathogen that has coevolved with the modern human affecting their morbidity and mortality for at least 20,000 years.

**MYCOBACTERIUM TUBERCULOSIS COMPLEX: EVOLUTION AND PHYLOGENY**

Tuberculosis is an ancient disease that has been with mankind for thousands of years, and our relationship with its causative agent, \textit{M. tuberculosis} has revealed a remarkably parallel demographic evolution \cite{19,20}. The agents of tuberculosis in mammals have been identified as obligate intracellular pathogens and grouped as the \textit{Mycobacterium tuberculosis} Complex (MTBC) in which the predominantly human pathogens include \textit{M. tuberculosis}, \textit{M. africanum}, \textit{M. canettii}, and members that largely infect animals include \textit{M. bovis}, \textit{M. microti}, \textit{M. caprae}, \textit{M. orygis}, and \textit{M. pinnipedii}. MTBC also encompasses the \textit{M. bovis} bacillus Calmette - Guérin (BCG) strains used in the TB vaccine \cite{9,10,19}.

\textit{M. tuberculosis} was originally believed to have evolved from \textit{M. bovis}, a pathogen of cattle that spread to humans upon domestication of animals in the neolithic era. \cite{21,22}. The sequencing of the entire \textit{M. tuberculosis} genome has since exposed evidence suggesting its evolution approximately 40,000 years ago, from a pool of ancestral tubercle bacilli called \textit{M. prototuberculosis} \cite{19,23,24}. This ancient precursor strain is believed to have given rise to two lineages some 20-30,000 years ago; one that exclusively spread among humans and the other that is thought to have moved through both humans and animals \cite{19}. While the whole genome sequencing of the strains in the MTBC provided valuable insight into the origin of the disease and its pathogen, it also offers the promise of providing important information that will guide the
diagnosis, prevention and treatment of the disease especially with the rise in mutations of the strains that have led to MDR and XDR stains of MTBC (10, 20).

**TUBERCULOSIS: DIAGNOSIS**

The first step in the identification and diagnosis of *M. tuberculosis* and TB is sputum smear microscopy where the presence of *M. tuberculosis* is revealed by the acid fast nature of the bacillus. This is usually done in combination with chest X-rays and PCR methods to quickly diagnose active tuberculosis. Tuberculin or the Mantoux test and IFNγ release assays are used to diagnose latent TB depending on the patient’s immunization status and affordability (17, 25). Detection of the bacilli in the sputum smear requires the presence of a large load of the bacteria in the sputum (10^5 CFU/ml) whereas the X-ray and PCR methods are not exactly cheap or reliable enough to be made available to countries with limited resources. These methods while being quick do not offer the best diagnosis of TB (10). The true gold standard method for the identification of the bacilli continues to be culturing of the mycobacteria in a selective media. This technique is harder to implement and manage because of the slow growth rate of the bacteria (18-24 hour doubling time) as well as safety requirements surrounding the maintenance of a biosafety level 3 pathogen (10). Efforts are being made to develop and implement whole genome sequencing of MTBC isolates from infected candidates to overcome the existing shortcomings and better aid the diagnosis and treatment of the disease. Nevertheless a 130 year old sputum smear microscopy test used from the days of Robert Koch continues to be the most widely used method to detect TB.

**TUBERCULOSIS: PREVENTION**

Vaccines against the disease are extremely limited and have proven to have at best a variable efficacy against pulmonary TB. The vaccine used against TB is the BCG (bacillus of
Calmette and Guerin) vaccine named after Albert Calmette and Camille Guérin who derived the attenuated strain by serially passaging *M. bovis* which caused the disease in various animal models. It was first used as a vaccine for prevention of TB in 1921 and continues to be the single most effective vaccine against TB albeit with variable efficacies (26,27). Studies have shown that the BCG vaccine is 80% effective against the disseminated forms of TB such as miliary TB and meningitis in children but offering little to no protection in adults (26).

**TUBERCULOSIS: TREATMENT**

Tackling *M. tuberculosis* seemed like an impossible task because of its complex lipid rich cell wall which fortified the bacterium against most of the antibiotics of the 19th century such as penicillin and sulfonamides. The treatment options for eradicating tuberculosis were welcomed with great expectation in the early part of the 20th century with the discovery of an aminoglycoside, streptomycin by Albert Schatz and Selman Waksman in 1943 (27-29). However it was observed that *M. tuberculosis* quickly developed resistance to streptomycin which necessitated the administration of a cocktail of antibiotics which was strengthened by the discovery of isoniazid a drug that blocks the biosynthesis of mycolic acid, a key component of the mycobacterial cell wall and rifamycin, a lipophilic drug that blocks bacterial RNA synthesis (27,29-31). The current treatment recommendation for active tuberculosis by the WHO warrants a long and complicated regimen that lasts a minimum of six months and includes a cocktail of at least four first line drugs – isoniazid, rifampin, pyrazinamide, and ethambutol (18,29). This treatment plan is expensive and difficult to track leading to the emergence of MDR and XDR strains of *M. tuberculosis* that has confounded the scientific community and contributed to the tragedy of tuberculosis. MDR-TB occurs as a result of *in vitro* resistance to the two first-line drugs, isoniazid and rifampin while XDR-TB is resistant not only to isoniazid and rifampin, but also to other antibiotics like kanamycin, amikacin, or capreomycin, that are used in drug resistant
TB therapy (32) The emergence of these strains emphasizes the urgency to investigate the ins and outs of mycobacterial pathogenesis in order to curb the alarming rise of the TB epidemic and death in millions of humans every year.

**NONTUBERCULOUS MYCOBACTERIA**

All mycobacteria that are indigenous to various environmental niches and are not obligate pathogens like their counterparts in the MTBC and *M. leprae*, are classified as nontuberculous mycobacteria or NTMs (33-35). These mycobacteria are referred to by different names - anonymous or atypical mycobacteria, mycobacteria other than tuberculosis (MOTT) and nontuberculous mycobacteria (NTM) (36). NTMs include opportunistic mycobacterial pathogens like *Mycobacterium avium*, *Mycobacterium kansasii* which cause tuberculosis like infections in patients with immune or respiratory defects (33,37) as well as nonpathogenic saprophytes such as *Mycobacterium smegmatis* and *Mycobacterium phlei* (38). They were classified into four groups by Dr. Ernest Runyon based on their growth properties (slow or rapid) and their ability to produce pigment (photochromogens: pigmented in the presence of light; scotochromogen: pigmented in the presence or absence of light; non-chromogens: non-pigmented) (9,34,39). While the clinical relevance of NTMs is indisputable, their phylogenetic closeness to the MTBC adds to their applicability in the context of serving as model systems to understand the evolution and pathogenicity of *M. tuberculosis* (21,23,40-42).

**MYCBACTERIUM MARINUM**

*M. marinum* is an environmental mycobacteria with a wide range of host preferences. It is typically found in aquatic environments where it is able to survive as a pathogen of poikilotherms such as frogs and fish (40,43,44). The bacterium was first isolated by Joseph D. Aronson in 1926 from the tubercle lesions of diseased fish. It was later isolated from swimming
pool associated human granulomatous skin lesions called fish tank or aquarium tank granuloma in the 1950s which established *M. marinum* as a relevant human pathogen albeit opportunistic (40,44). The disease is typically limited to the skin and soft tissues extremities and is pathologically indistinguishable from dermal infections caused by *M. tuberculosis*. This *M. marinum* disease necessitates treatment with anti-tuberculosis drugs such as rifampin, ethambutol, and quinolones, as well as other agents such as doxycycline and clarithromycin and like tuberculosis long-term antibiotic treatment is required due to the development of antibiotic resistance in the infecting bacteria (35,40).

*M. marinum* shares most of its growth and physiological properties with *M. tuberculosis*. However, compared to the ~20 hour generation time of *M. tuberculosis*, *M. marinum* is a relatively rapid growing NTM with a division time of 6-8 hours during log phase. It is a photochromogen that produces bright yellow pigment when exposed to light (43) and grows at an optimum temperature of 25°C - 35°C unlike *M. tuberculosis* which grows optimally at 37°C (45). *M. marinum* has a large genome of ~6.6 MB, about 50% more than the size of the genome of *M. tuberculosis* which is ~4.4 MB and smaller due to its exclusive host niche preferences (40). The *M. marinum* genome shares 3000 orthologous genes with *M. tuberculosis* with amino acid identity averages of ~85% between the orthologs (40,46). Phylogenetic studies have shown that *M. marinum* is most closely related to *Mycobacterium ulcerans* with a >97% nucleotide identity and then to *M. tuberculosis* with a >85% nucleotide identity (38,40,47). Genome comparison data have indicated that *M. marinum* and *M. tuberculosis* deviated from a common environmental mycobacterium with *M. marinum* retaining genes required for its dual lifestyle and *M. tuberculosis* undergoing reductive evolution compatible with its intracellular, parasitic lifestyle (40). These studies also advocate a common prototype for both their pathogenesis approaches. Due to its phylogenetic similarity to *M. tuberculosis* and its ease of use as a
biologically safer and faster growing organism, *M. marinum* has consequently emerged as a model for *M. tuberculosis* pathogenesis (40,46,48).

**CELL ENVELOPE OF MYCOBACTERIA**

Mycobacteria are surrounded by a very complex and unique cell envelope layer. Its uniqueness stems from its unusually esoteric and high lipid composition which constitutes about 40% of the dry weight of the cell (49,50). The cell envelope not only contributes to the rigidity and shape of the bacterium but also plays an active role in its interaction with the host during infection. It has long been thought of as the permeability barrier conferring intracellular resilience to the bacterium. It is also responsible for the resistance of the bacterium towards many antibiotics while simultaneously being the target of the antibiotics that are effective in the treatment of mycobacterial infections (5).

Several models have been proposed to describe the organization and architecture of the mycobacterial cell envelope. The model proposed by Minnikin in 1982 which was later supported with cryo-electron microscopy studies are in consensus that the cell envelope includes the plasma membrane, the cell wall and an additional electron transparent capsule layer (8,49,51). These studies also revealed a periplasm layer separating the plasma membrane from the outer membrane, a conformation similar to that observed in gram negative bacteria (7,8,50) (Figure 1).

**PLASMA MEMBRANE**

Mycobacteria have a typical plasma membrane which is a bilayer composed of polar lipids such as phosphatidylinositol, phosphatidylethanolamine and diphosphatidylglycerol. In addition to the conventional lipids, the mycobacterial plasma membrane also supports various glycosyl phosphopolyprenols and phosphotidylinositol mannosides (PIMs) and its variants which
are extractable phospholipids unevenly distributed throughout the membrane. PIMs extend into the outer layers of the mycobacterial cell envelope, reinforcing its selective permeability (5,41). PIMs are involved in anchoring polysaccharides like lipoarabinomannan (LAM) and lipomannan (LM) in the cell wall (43,52-54). The plasma membrane also hosts carotenoids, compounds known to contribute towards the photochromogenic properties of some mycobacteria and menaquinones which are compounds thought to be active in electron transport (51).

**CELL WALL**

Mycobacteria along with Corynebacteria and Nocardia produce a unique a chemotype IV cell wall that is essential for their viability (5,31,55). The cell wall of mycobacteria is an asymmetric lipid bilayer composed of an inner leaflet containing mycolic acids and an outer leaflet composed of extractable lipids (49,56) (Figure 1).

**INNER LEAFLET**

The inner leaflet also known as the cell wall core is responsible for the basic structural integrity of mycobacteria and their resilience towards antibiotics due to extremely low permeability (5,49,57). It is composed of three covalently linked molecules; peptidoglycan, arabinogalactan and mycolic acid (Figure 1). The peptidoglycan layer surrounds the plasma membrane and lays the foundation and framework of the cell wall core. Unlike the peptidoglycan layer in most eubacteria which is composed of N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM), mycobacteria possess a distinct layer in which the muramic acid is N-glycosylated which confers resistance to typical antibacterial agents like lysozyme that target the peptidoglycan (5,58). Mycobacterial peptidoglycan is covalently linked via a phosphodiester linkage to the arabinogalactan layer. Arabinogalactan is a complex polysaccharide composed of arabinan and galactan units in the furanose form that is unique to mycobacteria. One end of the
arabinogalactan layer is covalently linked to the peptidoglycan layer while the other end is intermingled with mycolic acid residues (5,31).

Mycolic acids are the most exceptional lipid of the cell wall core of all mycobacteria. They are α-branched β-hydroxy acids composed of up to 90 carbon atoms. They are made up of a long branch known as the meromycolate that is more versatile and a shorter branch known as the alpha-branch which is conserved among the family of mycobacteria (5,57,59). There are three classes of mycolic acids; the α-mycolate, methoxymycolate and the ketomycolate. Each mycobacterial species has a distinct ratio of these classes of mycolic acids which contributes to the differences in hydrophobicity of their cell wall (41). The mycolic acids are proposed to be packed side by side, perpendicular to the plane of the cell surface (50,54). They exist in the cell wall in two basic forms: covalently bound to arabinogalactan in the cell wall core, and as loosely-associated entities that are esterified to a variety of carbohydrate-containing molecules and distributed throughout the cell wall (59,60).

**OUTER LEAFLET**

The outer leaflet of the mycobacterial cell wall is composed of extractable lipids that contribute to the asymmetry of the lipid bilayer. This layer is highly fluid/permeable in contrast to the inner leaflet. While the outer leaflet hosts most of the extractable lipids, these extractable lipids are also distributed throughout the cell envelope. Triacylglycerols (TAGs) and the mycolic acid derived lipids such as trehalose dimycolates (TDM) are some of these extractable lipids that are ubiquitous to all mycobacteria. Lipoooligosaccharides (LOS), phenolic glycolipids (PGLs), phthiocerol dimycocerosates (PDIMs), Diacyl trehalose (DATs), Pentaacyl trehalose (PATs), Sulfoglycolipids (SGLs) and glycopeptidolipids (GPLs) are extractable lipids that are found in the outer leaflet of the mycobacterial cell wall in a species specific manner (5,61) (Figure 1). These lipids also range in polarity from the hydrophilic LOSs, through PGLs, DATs, SGLs and
PATs, to the highly apolar hydrophobic PDIMs (54). This spectrum in the polarity and the species specific diversity of the outer membrane lipids adds to the fascinating complexity of the mycobacterial cell envelope.

CAPSULE

The outermost compartment of the mycobacterial cell envelope consists of an electron transparent layer called the capsule (Figure 1). The capsule hosts a wide variety of a readily soluble mixture of polysaccharides, proteins and lipids. The major capsular polysaccharides are glucan, arabinomannan and mannan. Glucan is the most abundant polysaccharide in the M. tuberculosis capsule (62). The components of the capsule are believed to act as effector/signaling molecules actively involved in the pathogenic behavior of the bacterium and play a crucial role in host pathogen interactions (49,56).

BIOSYNTHESIS OF CELL WALL LIPIDS

Due to its known impact on the resilience and pathogenesis of mycobacteria, biosynthesis of cell wall associated mycobacterial lipids has been extensively investigated over time. Apart from fatty acid synthases (FASs), the most significant group of enzymes associated with the biosynthesis of mycobacterial lipids are polyketide synthases (PKSs). Other pathway associated enzymes such as fatty acyl AMP ligases are also critical to the biosynthesis of cell wall lipids.

POLYKETIDE SYNTHASES

Most of the unique lipids and glycolipids of the mycobacterial cell envelope are synthesized by enzyme systems called polyketide synthases or PKSs. PKSs are large multi modular enzymes similar to fatty acid synthases (FASs) that are responsible for the synthesis of secondary metabolites or polyketides. In a process reminiscent of fatty acid biosynthesis, PKSs implement a succession of decarboxylative claisen-like condensations of activated two-carbon
starter units of acetyl CoA or malonyl CoA. PKS elongates the polyketide chain either by repetitively using a single active site to perform multiple condensation reactions or by using a modular assembly line mechanism (63,64). Based on their biosynthetic strategies, PKSs are classified into three types i.e., type I, type II, or type III. Type I PKSs are further categorized as modular and iterative. A typical modular type I PKS has distinct functional domains or catalytic units on a single protein that is organized in a module, and each domain in a module is utilized only once during polyketide product formation. In contrast, in a typical iterative type I PKS, each functional domain of those clustered on a single protein is used repeatedly during the assembly of the polyketide product. Type II PKSs are multienzyme complexes that catalyze a single set of iteratively acting reactions during formation of the polyketide product. Type III PKSs, also known as chalcone synthases has been recently discovered in bacteria and belongs to the plant chalcone synthases superfamily of condensing enzymes. They are homodimeric enzymes that are structurally and mechanistically distinct from type I and type II PKSs (65-67).

The catalytic domains incorporated within PKSs are covalently linked in very long polypeptides and typically include a β-ketosynthase (KS), acyl transferase (AT), dehydratase (DH), enoylreductase (ER), β-ketoreductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (68). The AT domains are responsible for maintaining the substrate selectivity and specificity while the KS domain carries out the key claisen condensation reaction. The AT, KS and ACP are the essential domains present in all PKS modules. Additional domains like the ER, KR and DH domains all carry out various specific reductive processing leading to the complex structure of the resulting polyketide. The discretionary TE domain which is present at the end of the assembly line, is responsible for chain termination and release of the final polyketide (68-70). All through the synthesis process, the incomplete polyketide chains are tethered to the ACP domain through long ‘swinging arms’ or the phosphopantetheinylnyl group, which ensures that the
polyketide intermediate is correctly steered to the various domains within a given PKS. The product synthesized by a PKS is very often only an intermediate on the way to becoming a bioactive compound. Once it is released from the PKS, it is processed by a host of other ‘post-PKS’ enzymes which add functional groups (methyls, hydroxyls and carbonyls, among others) or which decorate the polyketide base structure with sugars as seen in the case of some mycobacterial lipids (70).

Until recently PKSs were assumed to be mostly limited to the biosynthesis of secondary metabolites; however PKSs identified in mycobacteria have provided a new dimension to their functional versatility (66). Ahead of the genome sequence revelation, Kolattukudy and colleagues were the first to report the occurrence of PKSs in mycobacteria. They suggested that the biosynthesis of mycocerosic acids a key structural component of PDIMs and PGLs, required the catalytic activity of a modular PKS similar to that seen in erythromycin biosynthesis (71,72). Since then a number of PKSs have been implicated in the biosynthesis of various mycobacterial cell wall lipids. The genome sequence of \textit{M. tuberculosis} exposed the presence of a large number of PKS-like genes-explaining in part the presence of a number of lipid metabolites unique to \textit{M. tuberculosis} (73). Data from various genome projects indicate that several gene clusters homologous to all three PKS systems are present in the mycobacterial genome and their differential distribution across species can be correlated with type specific variations in their lipid profile. There are 24 genes encoding PKS homologs in the genome of \textit{M. tuberculosis} strain H37Rv (65,73). These PKS-encoding genes total about 103,644 bp, which is 2.6% of the total protein coding bases (4,027,296 bp) of the \textit{M. tuberculosis} chromosome (65,73).

PKSs in \textit{M. tuberculosis} work in conjunction with FASs to produce complex lipids such as mycolic acids, PGLs, PDIMs, sulfolipids and mannosyl-β-1-phosphomycoketides (MPMs) many of which are essential for its survival. This association between FASs and PKSs in
mycobacteria is made possible by a family of enzymes called fatty acyl-AMP ligases (FAALs), which activate fatty acids as acyl-adenylates. The mycobacterial PKSs then use various biochemical mechanisms to extend these fatty acyl precursors to produce novel lipid compounds that decorate the cell wall.

**FATTY ACYL AMP LIGASES**

Quite a few non-PKS genes have also been implicated in the biosynthesis of the mycobacterial cell wall lipids. Among them the *fadD* genes deserve special mention since they encode important acyl activating adenylating enzymes that are essential for lipid metabolism (73). They encode two specific groups of enzymes called fatty acyl-AMP ligases (FAALs) and fatty acyl-coA ligases (FACLs) based on their ability to convert fatty acids into fatty acyl-adenylates or fatty acyl CoA intermediates respectively. This mode of activation is analogous to the adenylation domains of nonribosomal peptide synthetases (NRPSs), which along with FAAL and FACL proteins constitute a large superfamily of acyl-activating enzymes (AAEs) (74). Within the context of lipid metabolism, the FAALs are involved in lipid biosynthesis, while the FACLs play a predominant role in the breakdown of lipids. Therefore, the metabolic fate of the activated fatty acid is dictated by whether it is activated as an acyl-CoA thioester, for energy generation, phospholipid biosynthesis etc., or as an acyl-AMP, for complex lipid synthesis (74-76). The *M. tuberculosis* genome has sequences encoding 12 FAALs and 22 FACLs (77). *M. marinum* being an environmental mycobacteria that is equipped to persist intracellularly, has a larger genome that encodes for 32 FACLs and 14 FAALs, six of which are shared with *M. tuberculosis* (FadD25, FadD26, FadD28, FadD29, FadD30, FadD32), that were predicted by sequence analysis (40). Interestingly several FAAL members are located next to PKS and NRPS gene clusters in the *M. tuberculosis* genome. To establish a functional crosstalk with PKS enzymes in generating complex cell wall associated lipids, it has been shown that the FAAL
proteins transfer activated fatty acids to the neighboring PKSs for further chain extension. The discovery of FAALs in mycobacteria has provided a novel take on fatty acid activation. By activating fatty acids as acyl adenylates, these proteins redirect the metabolic flux toward biosynthesis of complex lipid metabolites and are therefore crucial nodes in the biosynthetic network of virulent lipids (75).

**DIMYOCEROSATE ESTERS (DIMs)**

Dimycocerosate esters (DIMs) consist of an entire family of methyl branched lipids that are among the most studied cell wall associated lipids of mycobacteria. They are non-covalently bound within the outer leaflet of the mycobacterial cell wall and occur only in pathogenic mycobacteria like *M. tuberculosis, M. marinum, M. ulcerans, M. kansasii, M. bovis, M. leprae, M. africanum, M. microti, M. haemophilium* (78). They consist of two structurally-related groups of lipid diesters of long-chain multi methyl-branched fatty acids (mycocerosic acids), called PDIMs and PGLs. PDIMs or phthiocerol dimycocerosates are the aliphatic non glycosylated derivatives of β-diols called phthiocerols whereas PGLs or phenolic glycolipids are glycosylated lipids that share the same conserved β-diol core as PDIMs with a variable carbohydrate moiety (79) (Figure 3). Within various strains of *M. tuberculosis*, PDIMs are thought to be produced by all strains of *M. tuberculosis*, except those that have lost its production capacity due to spontaneous mutations from *in vitro* culturing (65). Unlike PDIMs, PGLs are produced only by a subset of *M. tuberculosis* strains (e.g., strains of the W-Beijing family). This trait is speculated to contribute to the characteristic epidemic spread of *M. tuberculosis* and its increased likelihood of developing drug resistance (65,78).

DIMs were identified from the lipid extracts of mycobacteria in the early part of the 20th century (78) and were believed to play a predominant role in the “public relations” of mycobacteria due to their peripheral location on the cell wall (80). The role of DIMs as
significant cell wall associated virulence factors was established when two different groups simultaneously applied the signature-tagged mutagenesis system to select for mutants unable to grow in a high-dose intravenous challenge mouse model of tuberculosis \( (81,82) \). These studies conclusively demonstrated DIMs as one of the key players in the interaction of \( M. \) \( \text{tuberculosis} \) with its host, providing the much needed impetus to better understand the virulence strategies of this exceptional pathogen. Since then a combination of both genetic and biochemical studies have contributed to the assembly of a comprehensive portrait of DIM biosynthesis thus cementing its distinctive relevance in the biology of \( M. \) \( \text{tuberculosis} \).

**DIM BIOSYNTHESIS: OVERVIEW**

The biosynthesis of DIMs is a complex process that requires the coordinated activity of several PKSs and associated enzymes like FAALs (Figure 2). The DIM locus in \( M. \) \( \text{tuberculosis} \) contains seven different type I PKSs; PpsA, PpsB, PpsC, PpsD, PpsE, Pks15/1 and Mas \( (52,65) \). PpsA-E are modular type I PKSs that add the precursors in a sequential assembly line-like manner, while Mas is a type I PKS that uses an iterative mechanism where the same active sites are used repetitively to produce the final product \( (52,72,75) \). PpsA-E and Mas are shared by both PDIMs and PGLs during biosynthesis. Pks15/1 on the other hand is also a type I iterative PKS similar to Mas, but it is implicated specifically in PGL biosynthesis \( (83) \). These PKSs act in conjunction with FAALs whose genes are located nearby within the DIM locus. The DIM locus hosts four ORFs that encode FAALs, more commonly known as FadD proteins, which activate and transfer biosynthetic intermediates onto the various PKSs. Among the FAALs involved in DIM biosynthesis FadD28 has been implicated in the biosynthesis of the mycocerosic acid group shared by both PDIMs and PGLs, while FadD26 has been implicated specifically in the biosynthesis of PDIMs \( (81,84-86) \). The FadD22 and FadD29 on the other hand are nonredundant enzymes that are specifically relevant in PGL biosynthesis \( (87,88) \). The available data on the
biosynthesis of DIMs have led to the proposal of a model that can be summarized in the following steps (Figure 2):

1. Priming of PpsA with appropriate PGL or PDIM-specific fatty acyl starter unit.
2. Extension of the primer unit by the PKS, PpsABCDE, leading to the generation of the phthiocerol/phenolphthiocerol entity.
3. A thioesterase (TesA)-dependent release of the polyketide products thioesterified to PpsE (89-91).
4. Priming of Mas with acyl adenylates of long chain fatty acids by FadD28.
5. Extension of the fatty acyl adenylate and subsequent biosynthesis of mycocerosic acids by Mas.
6. Trans-esterification of mycocerosic acids with the diol component of phthiocerol/phenolphthiocerol by the polyketide-associated protein A5 (PapA5) and final assembly (92,93).

The priming of PpsA during phenolphthiocerol synthesis is preceded by reactions that emphasize the significance of the Pks15/1 enzyme as well as the FadD22 and FadD29 that are exclusively utilized during PGL biosynthesis. PGL biosynthesis is initiated by the metabolic intermediate called chorismate, which is converted to p-hydroxybenzoic acid (p-HBA) by a chorismate pyruvate-lyase, Rv2949c (52,78,94). p-HBA is then activated by FadD22 and transferred to the Pks15/1 enzyme which extends p-HBA to form the p-hydroxyphenylalkanoate (PHPA) intermediate (95). The PHPA chain is then transferred to PpsA for PGL biosynthesis (88). During PDIM biosynthesis, FadD26 utilizes a novel acyl adenylation activation mechanism to prime PpsA (86). Subsequent to the priming of PpsA, the biosynthesis of PGLs and PDIMs follow the same biosynthetic route. In addition to the enzymes mentioned above, DIM biosynthesis also necessitates the involvement of accessory enzymes that further decorate the
DIM structure, creating an intricately designed pathway that is responsible for the production of a whole array of the DIM family of lipids.

**POLYKETIDE SYNTHASES IN DIM BIOSYNTHESIS**

**PpsABCDE**

The PKSs PpsA-E are implicated in the production of phthiocerol and phenolphthiocerol components of PDIMs and PGLs respectively \((65,96)\). The pioneering studies of Kolattukudy and colleagues established the first genetic evidence for the association of \(ppsABCDE\) gene cluster with the production of DIMs \((97)\). They demonstrated in \(M. bovis\) Bacille-Calmette-Guerin (BCG), a PDIM- and PGL-producing species, that replacement of the \(ppsB-ppsC\) segment from the \(ppsABCDE\) cluster, with a hygromycin-resistance gene cassette renders a mutant incapable of producing both PDIMs and PGLs \((97)\). *In vitro* enzymology studies probing the function of \(M. tuberculosis\) PpsA, PpsB and PpsE by Trivedi and coworkers concluded that, a) PpsA is necessary for the initiation of (phenol) phthiocerols synthesis through the extension of long-chain fatty acids, b) PpsA and PpsB are necessary for the biosynthesis of the \(\beta\)-diol functionality of (phenol) phthiocerols, and c) PpsE is required in the final acyl chain extension step of (phenol) phthiocerol biosynthesis \((96)\). Since then the domain arrangement and function of each protein in the PpsABCDE system, has been predicted and progressively refined to the present-day model \((65)\).

**Mas**

The PKS Mas synthesizes mycocerosic acid which is esterified onto the phthiocerol and phenolphthiocerol units to generate PDIMs and PGLs respectively. Kolattukudy and colleagues in their series of iconic papers uncovering DIM biosynthesis, also established *mas* as the mycocerosic synthase encoding gene \((71,98)\). They showed that in \(M. bovis\) BCG, replacement
of the mas gene with a hygromycin resistance marker produces a mutant lacking mycocerosic acids as well as PDIMs and PGLs. The group also delineated the enzymatic role of Mas, in the biosynthesis of mycocerosic acids (99). They showed that Mas can extend n-fatty acyl primers to produce mycocerosic acids. This study was corroborated more recently by Trivedi et al who characterized the mycocerosic acid synthase activity of Mas from M. tuberculosis H37Rv generated recombinantly in E. coli (96). Overall, these studies have effectively validated Mas as an iterative type I PKS involved in DIM biosynthesis, a finding consistent with the domain organization predicted for the protein.

Pks15-1

The initial link between the pks15/1 gene locus and production of PGLs was established by Constant and colleagues in M. bovis (83). However due to a frameshift mutation in this gene, the H37Rv type strain of M. tuberculosis is devoid of PGLs. Constant et al demonstrated that this PGL-deficiency arises from natural sequence polymorphisms that split the parental pks15/1 gene, found in the PGL-producing mycobacterium species like M. bovis strain BCG, into the individual genes pks1 and pks15 found in certain PGL-deficient M. tuberculosis strains. Quadri and colleagues established that, Pks15/1 is a 6-domain reducing iterative type I PKS. It uses malonyl-CoA as donor to extend a p-HBA starter unit to produce long-chain PHPA biosynthetic intermediates required for the biosynthesis of the phenolphthiocerol component of PGLs (95).

FATTY ACYL AMP LIGASES IN DIM BIOSYNTHESIS

FadD26, FadD28, and FadD29

The fadD genes with the mycobacterial DIM locus have been proposed to encode FAALs based on genetic and biochemical studies. Overall, these studies indicated that insertions in fadD26, fadD28 abrogate PDIM production (81,85,100), and that FadD29 is required for
biosynthesis of PGLs, yet dispensable for PDIM production (88). Kolattukudy and colleagues initiated the enzymology studies on FAALs and their role in DIM biosynthesis (84). They reported the homology of the FadD28 ortholog in M. bovis BCG to acyl adenylate-forming enzymes and concluded that the enzyme was an acyl-CoA synthase-like protein that catalyzes formation of CoA thioesters of fatty acids. Subsequently Gokhale and colleagues systematically delineated the biochemical function of FadD26, FadD28 and FadD29 proteins from M. tuberculosis H37Rv (86). The proteins were identified as acyl adenylate-forming enzymes and not acyl-CoA synthetases, as suggested in the previous studies. They also reported the inhibition of FadD28 by acylsulfamoyl (acyl-AMS) analogs and demonstrated that FadD26 specifically transfers the activated fatty acid to the PpsA for further carbon chain extension during PDIM synthesis (74). Overall, FadD26, FadD28, and FadD29 are proposed to belong to a new class of adenylation enzymes (FAALs) that activate long-chain fatty acids and transfer them to their cognate PKS partners for chain extension.

**FadD22**

FadD22 unlike the other FadD enzymes in the DIM biosynthesis pathway, is an unusual stand alone, didomain initiation module that loads a p-HBA intermediates instead of fatty acid units. It is comprised of a p-HBA adenylation domain and an aroyl carrier protein domain (ArCP) that loads the p-HBA intermediate to the iterative type I PKS Pks15/1 for the formation of long-chain PHPA products as noted above(87). Mutational analysis of the fadD22 gene in M. bovis implicated the exclusive role of the gene in PGL production (87). Quadri and colleagues developed the first specific small-molecule inhibitor of PGL biosynthesis, a p-hydroxybenzoyl AMP analog called 5′-O-[N-(4-hydroxybenzoyl) sulfamoyl]-adenosine (PHB-AMS) that has potent and specific activity in PGL producing mycobacterial pathogens. As expected, it does not inhibit PDIM biosynthesis (for which FadD22 is not required) or mycobacterial growth *in vitro*.
The inhibitor work from Quadri and colleagues established the possibility of selectively targeting DIM biosynthesis and probing the biological relevance of PGLs in mycobacterial biology.

ROLE OF DIMYCOCEROSATE ESTERS IN MYCOBACTERIAL PATHOPHYSIOLOGY

DIMs play an important role in the virulence of mycobacteria. Their production is modulated throughout the infection process. Numerous often varied conditions affect DIM production in mycobacteria, including availability of carbon source, phosphorylation by serine/threonine kinases, reductive stress and transcriptional regulation upon infection of macrophages (101, 102). Analysis of the global lipid profile of *M. tuberculosis* shows that the size and abundance of PDIMs are controlled by the availability of their precursor methyl malonyl CoA and that this regulation occurs during infection (103, 104). During infection, DIMs are thought to moderate the host innate immune response by modifying the biophysical properties of the mycobacterial cell wall. They subsequently act as direct effectors of pathogenesis by mediating cross talk between the infecting mycobacteria and the host immune cells like macrophages and dendritic cells. They may physically interfere with host membranes in order to impair immune response-related signaling pathways. It is therefore important to consider the specific structural features of these lipids like the hydrophobic nature of PDIMs and the glycosylated groups of PGL in exploring their functional role (105). As a result mutant strains with deletion of the genes involved in PDIM/PGL biosynthesis have a reduced capacity to infect human macrophages (105-109).

Cambier et al. investigated the role of PDIMs and PGLs in a *M. marinum*-infected zebrafish larvae model system. They proposed that PDIMs physically mask mycobacterial pathogen associated molecular patterns (PAMPs) thereby preventing their recognition by toll like receptors. This subsequently led to their evasion of inducible nitric oxide synthase (iNOS) -
expressing macrophages, which were effective in killing the PDIM mutant through production of reactive nitrogen species. Cambier et al. proposed that pathogenic mycobacteria, in a strain specific manner, recruit PDIMs for averting macrophages from their natural function and establishing infection. In addition to the masking effect of PDIMs, they also reported that, PGLs are involved in selectively recruiting pathogen permissive macrophages to increase the fitness of the pathogenic *M. marinum* within the host (105,110).

PDIM deficient mutants have also been shown to be sensitive to reactive nitrogen species generated in murine macrophages pre-treated with interferon-gamma and tumor necrosis factor-alpha (111). Whereas loss of PGLs produced by the *M. tuberculosis* W-Beijing isolate was found to correlate with an increase in the release of the pro-inflammatory cytokines; tumor necrosis factor and interleukins 6 and 12 *in vitro* causing a ‘hyperlethality’ profile of the mycobacterium in murine disease models (109). Uncovering the means by which WT *M. tuberculosis* avoids killing after infection will lead to a better understanding of its mechanisms of persistence and may lead to the identification of possible targets for novel therapeutics. It also remains to be determined how these lipids have an impact throughout the disease as a consequence of their pleiotropic effects.
RESEARCH OBJECTIVES

Despite knowing a great deal about the contributions of the cell wall associated DIMs to the pathophysiology of mycobacteria, there are a lot of gaps that preclude a thorough understanding of their biosynthesis and role in mycobacteria. This dissertation seeks to unravel some of the intricacies surrounding DIMs in *M. marinum*, a nontuberculous mycobacterial pathogen that is a close genetic relative of the *M. tuberculosis* complex. The research reported in this dissertation investigates:

I. The mechanism by which *p*-hydroxyphenylalkanoate (PHPA) intermediates synthesized by the iterative FadD22-Pks15/1 system are transferred to the non-iterative PpsABCDE system for elongation of the phenol phthiocerol moiety during PGL synthesis.

II. The various pleiotropic consequences resulting from the loss of PDIMs and/or PGLs to further elucidate their relevance in the biology of mycobacteria.
CHAPTER 2: BIOSYNTHESIS OF MYCOBACTERIAL CELL-ENVELOPE-ASSOCIATED PHENOLIC GLYCOLIPIDS IN MYCOBACTERIUM MARINUM

INTRODUCTION

Multiple lines of investigation have provided considerable support for the idea that PGLs and PDIMs are implicated in virulence via complex mechanisms of action that are not fully elucidated (81,85,100,107-122). These (glyco)lipids are also believed to strengthen the cell envelope permeability barrier (82,100) and to increase the bacterium’s intrinsic resistance to antimicrobial drugs (82,89,100,115). The relevance of PGLs in mycobacterial biology underscores the importance of developing a comprehensive knowledge of its biosynthetic pathway, which remains incompletely understood.

Previous studies of the PGL biosynthetic pathway have revealed a functional cooperation between the *M. marinum* proteins FadD22 (p-HBA ligase/initiation module) and Pks15/1 (iterative type I PKS) for production of PHPA intermediates required for PGL biosynthesis (87,95). The PHPA intermediates synthesized by the *M. marinum* FadD22-Pks15/1 iterative system, which is conserved in PGL producers, are thought to be further extended to form the phenolphthiocerol moiety of PGLs (Figure 3). The extension of the PHPA intermediates has been proposed to be carried out by PpsABCDE, a conserved, modular, non-iterative type I PKS that is known to extend fatty acids to form the phthiocerol moiety of PDIMs in *M. tuberculosis* (78,81,85,96,97) (Figure 3). During PDIM biosynthesis, the fatty acids are activated and loaded by the conserved fatty acyl-AMP ligase FadD26 (Figure 4) onto the loading acyl carrier protein (ACPₜ) domain of PpsA, the first acting enzyme of the PpsABCDE system (86,96). However, the mechanism by which the PHPA intermediates assembled by the FadD22-Pks15/1 system would be transferred to the PpsABCDE system for acyl chain extension during PGL biosynthesis remains to be experimentally interrogated.
Herein, we report a mutational study that interrogates the mechanism by which the PHPA intermediates are transferred to PpsA, the first acting enzyme of the PpsABCDE system. The findings of this study support a mechanistic model in which the PHPA intermediates are activated and loaded onto the loading acyl carrier protein (ACP_L) domain of *M. marinum* PpsA by a dedicated PHPA-AMP ligase. The conservation of the PGL biosynthetic genes across species (65,78) (Figure 4) and studies in the *M. tuberculosis* complex (88) strongly suggests that the mechanistic insights into PGL biosynthesis gained herein are applicable to other PGL producers. Overall, these studies advance our understanding of the biosynthesis of an important group of mycobacterial cell envelope-associated glycolipids.

**MATERIALS AND METHODS**

**Culturing conditions, recombinant DNA manipulations, and reagents.** *M. marinum* strain M (ATCC BAA-535) and its derivatives were cultured under standard conditions in Middlebrook 7H9 (Difco) supplemented with 10% ADN (5% BSA, 2% dextrose, 0.85% NaCl) and 0.05% Tween-80 (supplemented 7H9) or on Middlebrook 7H11 agar (Difco) with ADN (supplemented 7H11) (123). The strains used in this study are listed in (Table 1). *Escherichia coli* strains were cultured under standard conditions in Luria-Bertani (LB) media (124). When required, kanamycin (30 μg ml⁻¹), hygromycin (50 μg ml⁻¹), sucrose (2%) and/or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-gal, 70 μg ml⁻¹) were added to the growth media. DNA manipulations were carried out by standard methods and using *E. coli* DH5α (Invitrogen) as primary cloning host (124). All PCR-generated DNA fragments used in plasmid constructions were sequenced to verify fidelity. The plasmids used in this study are listed in Table 2. Genomic DNA isolation and plasmid electroporation into mycobacteria were carried out as reported (123). Molecular biology reagents were obtained from Sigma, Invitrogen, Novagen, or
Qiagen. The oligonucleotides used in this study are listed in Table 3 and Table 4, and they were purchased from Integrated DNA Technologies, Inc. Solvents and non-radiolabeled chemicals were purchased from Sigma, Acros Organics, or Fisher Scientific. [1-\textsuperscript{14}C] propionate (sp act = 54 mCi mmol\textsuperscript{-1}) and [carboxyl-\textsuperscript{14}C] \textit{p}-hydroxybenzoic acid (sp act = 55 mCi mmol\textsuperscript{-1}) were acquired from American Radiolabeled Chemicals, Inc.

**Construction of mycobacterial mutants.** The mutants were engineered using the p2NIL/pGOAL19-based flexible cassette method\textsuperscript{(125)} as reported \textsuperscript{(87,89,93)}. A gene-specific mutagenesis-cassette delivery vector (see below) was used to construct each mutant. Each vector was electroporated into \textit{Mm} and the transformants with a potential single-crossover (blue colonies) were selected on supplemented 7H11 containing hygromycin, kanamycin, and X-gal. Potential single crossover-bearing clones were grown in antibiotic-free, supplemented 7H9, and then plated for single colonies on supplemented 7H11 containing sucrose and X-gal. White colonies that grew on sucrose plates were re-streaked onto antibiotic-free and antibiotic-containing plates to identify drug sensitive clones, a trait indicating a possible double-crossover event with consequent allelic replacement or reversion to wild-type (wt). Gene deletions were confirmed by PCR. For PCR analysis, genomic DNA from mutant candidates was used as template along with two independent mutant-specific primer pairs (Table 3) to produce amplicons permitting differentiation between mutant and wt genotypes based on amplicon size (Figure 7). Nucleotide substitutions in the \textit{Mm} \textit{ppsA}\textsubscript{S-to-A} mutant were confirmed by DNA sequencing. The mutated region was PCR-amplified with specific primers (Table 4) and the resulting amplicon was sequenced (Figure 7 C)

**Construction of mutagenesis-cassette delivery vectors.** The following mutagenesis-cassette delivery vectors were construced: p2NILGOALc-\textit{ΔfadD}22c, carrying a \textit{fadD}22 (MMAR_1761) deletion cassette (\textit{ΔfadD}22c); p2NILGOALc-\textit{ΔfadD}26c, carrying a \textit{fadD}26
(MMAR_1777) deletion cassette (ΔfadD26c); p2NILGOALc-ΔfadD28c, carrying a fadD28 (MMAR_1765) deletion cassette (ΔfadD28c); p2NILGOALc-ΔfadD29c, carrying a fadD29 (MMAR_1759) deletion cassette (ΔfadD29c); and p2NILGOALc-ppsA, carrying a ppsA (MMAR_1776) mutagenesis cassette (ppsAc) (see Figure 6 and Table 1). Each cassette was constructed by joining a 5' arm and a 3' arm using splicing by overlap extension (SOE) PCR(126). The primers and amplicon sizes are shown in Table 3. Each PCR-generated cassette was first cloned into pCR2.1-TOPO (Invitrogen), verified for sequence fidelity, and then subcloned into p2NIL (125). The cassettes ΔfadD22c, ΔfadD26c, ΔfadD28c, ΔfadD29c, and ppsAc were cloned into p2NIL as Sall-NotI, HindIII-KpnI, BamHI-NotI, HindIII-PmlI, and HindIII-HpaII fragments, respectively. Each resulting p2NIL-mutagenesis cassette construct and the plasmid pGOAL19 (125) were digested with PacI, and then the PacI fragment with the marker cassette (GOALc) of pGOAL19 was ligated to the p2NIL construct backbones to generate the final delivery vectors. The configuration of each final cassette was as follows: ΔfadD22c = fadD22’s 1,000-bp upstream segment + fadD22’s first 2 codons + fadD22’s last 2 coding codons + stop codon + 983-bp downstream segment; ΔfadD26c = fadD26’s 962-bp upstream segment + fadD26’s first 5 codons + fadD26’s last coding 3 codons + stop codon + 929-bp downstream segment; ΔfadD28c = fadD28’s 947-bp upstream segment + fadD28’s first 5 codons + fadD28’s last 4 coding codons + stop codon + 935-bp downstream segment; ΔfadD29c = fadD29’s 947-bp upstream segment + fadD29’s first 4 codons + fadD29’s last 5 coding codons + stop codon + 976-bp downstream segment; ppsAc = 1,832-bp segment with Ser-to-Ala substitution mutations in the center.

**Construction of pCP0 derivatives.** Plasmids pCP0-FadD22, pCP0-FadD26, pCP0-FadD28, pCP0-FadD29, and pCP0-PpsA expressing FadD22, FadD26, FadD28, FadD29 and PpsA, respectively, were constructed using the vector pCP0 (89). DNA fragments each
encompassing a \( Mm \) gene and its predicted ribosome-binding site were PCR-generated using primer pairs shown in Table 4. The fragments were first cloned into pCR2.1-TOPO, verified for sequence fidelity, and then subcloned into pCP0. The \( fadD22, fadD26, fadD28, fadD29, \) and \( ppsA \) fragments were cloned into pCP0 as HindIII-HpaI, EcoRI-HindIII, NheI-HindIII, EcoRI-HindIII, and HpaI-NheI inserts, respectively.

**Analysis of PGLs and PDIMs.** Four day old cultures were diluted to an OD\(_{595\text{ nm}}\) of 0.6 in supplemented 7H9 and loaded into 12-well plates (1 ml per well). \([^{14}\text{C}]\)Propionate (labels both PDIMs and PGLs) or \([^{14}\text{C}]\) \( p \)-hydroxybenzoic acid (selectively labels PGLs) was added to each well (0.2 \( \mu \text{Ci ml}^{-1} \)) and the plates were incubated for 24 h (30\( ^\circ \text{C}, 170 \text{ rpm} \)). After incubation, the OD\(_{595\text{ nm}}\) of the cultures was measured in a plate reader (Beckman Coulter, Inc.) and the cells were harvested for apolar lipid fraction extraction with a biphasic mixture of methanolic saline and petroleum ether as reported (87,92). Lipid extracts were subjected to radio-TLC for analysis of \([^{14}\text{C}]\)-labeled PGLs and \([^{14}\text{C}]\)-labeled PDIMs as described earlier (87,92). Developed TLC plates were exposed to phosphor screens, which were scanned using a Cyclone Plus Storage Phosphor System (PerkinElmer, Inc.).

**RESULTS**

**A functional ACP\(_L\) domain in PpsA is required for production of both PGLs and PDIMs in \( M.\ marinum. \)**

The ACP\(_L\) domain of PpsA requires phosphopantetheinylation of the Ser residue embedded in the P-pant group attachment site motif (NCBI CDD pfam00550/smart00823) of the domain to become functional (127,128). Phosphopantetheinylation of the ACP\(_L\) domain introduces the P-pant group onto which the fatty acids are loaded with assistance of the fatty acyl-AMP ligase FadD26 to form the fatty acyl-ACP\(_L\) domain thioester intermediate required for PDIM biosynthesis in \( M.\ tuberculosi\) (86,96). Formation of the analogous PHPA-ACP\(_L\) domain
thioester intermediate would be required for PGL production in *M. marinum* if the Pks15/1-to-PpsA PHPA intermediate transfer takes place by the PHPA-AMP ligase-dependent pathway outlined in Figure 5 (pathway A). On the other hand, formation of the PHPA-ACP_L domain intermediate would not be needed for PGL production if the intermediate transfer proceeds via direct capture by the KS domain of PpsA as depicted in Figure 5 (pathway B). With these considerations in mind, we probed the essentiality of the P-pant group attachment site of the ACP_L domain of PpsA for PGL production by mutational analysis in *M. marinum*. To this end, we engineered an unmarked, site-directed mutant (*M. marinum ppsA_S-to-A*) with a Ser-to-Ala substitution that eliminated the phosphopantetheinylation site (Ser43) in the ACP_L domain of the synthase. We identified Ser43 as the phosphopantetheinylation target in the P-pant group attachment site sequence motif of the ACP_L domain of PpsA by sequence analysis. The *M. marinum ppsA_S-to-A* mutant carried also a Ser42-to-Ala substitution. Ser42 (adjacent to Ser43) was substituted in case it could become a surrogate phosphopantetheinylation target in the absence of Ser43, a potentially confounding scenario.

Evaluation of PGL production in the *M. marinum ppsA_S-to-A* mutant by radiometric-thin layer chromatography (radio-TLC) analysis revealed that the strain was PGL deficient (Figure 8; *cf.* lanes 1 and 2). Radio-TLC analysis revealed that the mutant strain was also unable to produce PDIMs (Figure 8; *cf.* lanes 1 and 2), a result in line with previous biochemical studies on PDIM biosynthesis in *M. tuberculosis* (86,96). Introduction of the plasmid pCP0-PpsA (expressing *M. marinum* PpsA) into *M. marinum ppsA_S-to-A* restored the capacity of the mutant to produce both PGLs (Figure 8; *cf.* lanes 2 and 3) and PDIMs (Figure 8; *cf.* lanes 2 and 3).

**Mutational analysis in *M. marinum* identifies FadD29 as a PHPA-AMP ligase candidate.**

To seek further support for the mechanistic model proposed in pathway A (Fig. 3), we undertook a systematic mutational analysis to conclusively establish the involvement of fadD22,
fadD26, fadD28 and fadD29 in PGL and PDIM production in M. marinum and inform the identification of a PHPA-AMP ligase candidate in a nontuberculous mycobacterial species. To our knowledge, the involvement of M. marinum fadD22 and M. marinum fadD29 in production of PDIMs and PGLs has not been probed by mutational analysis. M. marinum mutants with transposon insertions in fadD26 and fadD28 were recently shown to have defects in PGL and PDIM production, but potentially confounding polar effects produced by the transposon on other genes of the pathway (Figure 4) preclude unequivocal gene-to-function assignments (100). To conclusively probe the involvement of these M. marinum acyl-AMP ligases in PGL and PDIM production, we engineered four mutants (M. marinum ΔfadD22, M. marinum ΔfadD26, M. marinum ΔfadD28, and M. marinum ΔfadD29), each with an unmarked, in-frame deletion in each of the four acyl-AMP ligase genes, and then examined the ability of the mutants to produce PGLs and PDIMs by radio-TLC analysis.

The analysis of the PGL and PDIM production capacity of the fadD gene mutants revealed that deletion of fadD28 led to a PGLˉ PDIMˉ phenotype (Figure 9; cf. lanes 5a and 6a and cf. lanes 5b and 6b), deletion of fadD26 produced selective loss of PDIMs (Figure 9; cf. lanes 1a and 4a and cf. lanes 1b and 3b), and deletion of fadD22 and fadD29 led to selective loss of PGLs (Figure 9; cf. lanes 5a and 8a and cf. lanes 5b and 8b). We also constructed and analyzed four corresponding genetic complementation control strains (M. marinum ΔfadD22 + pCP0-fadD22, M. marinum ΔfadD26 + pCP0-fadD26, M. marinum ΔfadD28 + pCP0-fadD28, and M. marinum ΔfadD29 + pCP0-fadD29). Each of these control strains carried a pCP0-based plasmid expressing the specific fadD gene deleted from the genome of the host strain. Radio-TLC analysis demonstrated that episomal expression of the fadD gene reasonably restored (fully or partially) the PGL and/or PDIM production capacity of each of the M. marinum mutants.
The complementation controls indicate that none of the deletions exerted a confounding polar effect precluding \textit{fadD} gene functional assignments.

**CONCLUSIONS**

We hypothesized two possible pathways by which this PHPA intermediate transfer could take place in \textit{M. marinum} (Figure 5). In one of these pathways (Figure 5, pathway A, steps A1-A4), the intermediates would be first released from the phosphopantetheinyl (P-pant) group of the acyl carrier protein (ACP) domain of Pks15/1 (step A1), and subsequently activated (step A2) and loaded (step A3) by a dedicated PHPA-AMP ligase onto the P-pant group of the ACP\textsubscript{L} domain of PpsA. The PHPA intermediates bound to the ACP\textsubscript{L} would be subsequently captured by the ketosynthase (KS) domain (step A4) to generate the loaded PpsA (Figure 5, boxed acyl-PpsA species) ready for KS domain-dependent decarboxylation/condensation. The Pks15/1-to-PpsA PHPA intermediate transfer mechanism represented in pathway A (Figure 5) emerges from analogy to the fatty acyl-AMP ligase-dependent mechanism of fatty acid activation and loading onto the ACP\textsubscript{L} domain of PpsA during PDIM biosynthesis in \textit{M. tuberculosis} \cite{86}. Pathway A is further supported by recent studies in the \textit{M. tuberculosis} complex leading to the proposal that the conserved fatty acyl ligase FadD29 (Figure 4) loads PHPAs onto PpsA \cite{88}, yet this idea remains to be experimentally validated.

Notably, pathway A (Figure 5) requires free PHPA intermediates (step A1), yet sequence analysis of Pks15/1 orthologs does not reveal the presence of a possible thioesterase domain that would conveniently catalyze the release of the PHPA intermediates thioesterified to the P-pant group of the ACP domain of the synthase \cite{78,95}. This does not rule out, however, the possibility that the PHPA intermediates are released without the assistance of an external (self-standing) thioesterase or by the action of one. In the latter option, the thioesterase could be TesA,
which is encoded in the PGL/PDIM biosynthetic gene cluster (Figure 4) and was recently shown to be required for PGL and PDIM production in *M. marinum* (89,115).

In the second possible pathway for Pks15/1-to-PpsA PHPA intermediate transfer in *M. marinum* (Figure 5, pathway B), the PHPAs thioesterified to the P-pant group of the ACP domain of Pks15/1 would be directly captured by the KS domain of PpsA (step B1), thus bypassing the need for steps A1-A4. This direct Pks15/1-to-PpsA chain translocation would “skip” the ACP\_L domain of PpsA. Domain skipping has in fact been demonstrated in a few PKS systems (129,130). Moreover, this direct capture pathway would have adaptive value because it would not require ATP for PHPA intermediate activation, an essential step in pathway A (step A2). This pathway would also obviate the need to off-load the PHPA intermediates from the ACP domain of Pks15/1 (pathway A, step A1). Overall, the findings of the mutational analysis in *M. marinum* are in line with the idea that the Pks15/1-to-PpsA PHPA intermediate transfer takes place by the PHPA-AMP ligase-dependent pathway outlined in pathway A of Figure 5. The results also suggest that direct capture of the PHPA intermediates thioesterified to the ACP domain of Pks15/1 by the KS domain of PpsA (Figure 5, pathway B) is not a transfer mechanism of physiological relevance in *M. marinum*.

The mechanistic model proposed in pathway A (Figure 5) includes an acyl-AMP ligase competent to activate and load the PHPA intermediates onto the ACP\_L domain of *M. marinum* PpsA (steps A2 and A3, respectively). Aside from the p-hydroxybenzoic acid-specific adenylation domain of *M. marinum* FadD22 (87,95), there are three conserved acyl-AMP ligases encoded in the PGL/PDIM biosynthetic gene cluster, *i.e.*, FadD26, FadD28 and FadD29 (65,78,81,85,86,96) (Figure 4). Recent studies in the *M. tuberculosis* complex have led to the proposal that FadD29 adenylates and loads PHPAs onto PpsA in *M. tuberculosis* (88), but the idea has not been experimentally explored. Overall, the findings of the mutational analysis of
fadD genes conclusively demonstrate the specific roles of fadD22, fadD26, fadD28, and fadD29 in PGL and/or PDIM production in *M. marinum*. These studies in conjunction with the biochemical information available for *M. marinum* FadD22 (87,95) point at *M. marinum* FadD29 as the likely PHPA-specific AMP ligase involved in PHPA intermediate activation and loading onto PpsA in *M. marinum*. They also parallel the conclusions derived from experiments in the *M. tuberculosis* complex (84,87,88). To the best of our knowledge this is the first study in *M. marinum*, nontuberculous mycobacterium, which supports the role of FadD29 ligase in the transfer of the PHPA intermediates from the iterative polyketide synthase system (PKS 15/1) to the non-iterative system (PpsA) during PGL synthesis.

The mutational studies reported herein are supported by *in vitro* enzymatic studies conducted on the *M. marinum* FadD29-PpsA functional partnership (1), which represents the first acyl-AMP ligase and type I PKS partnership for acyl starter unit activation and PKS loading established in a nontuberculous mycobacteria. Three analogous partnerships have been demonstrated in *M. tuberculosis*. These are the FadD26-PpsA partnership noted above for PDIM production (86,96), a FadD32-Pks13 partnership that takes place during mycolic acid biosynthesis (86,131,132), and a FadD30-Pks6 partnership believed to be required for production of novel polar lipids (86), (91). To our knowledge, however, the partially overlapping PGL/PDIM biosynthetic pathways provide the first example of two distinct acyl-AMP ligases (i.e., FadD29 and FadD26) loading the same type I PKS (i.e., PpsA) with two alternate starter units (i.e., PHPAs and fatty acids). This bimodal loading strategy allows the bacterium to use the PpsABCDE megasynthase system to generate two structurally different products. Interestingly, recent host-pathogen interaction studies suggest that *M. marinum* PGLs and *M. marinum* PDIMs have different roles within a complex immune evasion mechanism (110). It will be interesting to investigate whether the FadD29-PpsA versus FadD26-PpsA alternative partnership is utilized by
the pathogen as a control point to modulate the relative abundance of PGLs and PDIMs in the cell.
CHAPTER 3: PLEIOTROPIC CONSEQUENCES OF GENE KNOCKOUTS IN THE
PHTHIOCEROL DIMYCOCEROSATE AND PHENOLIC GLYCOLIPID
BIOSYNTHETIC GENE CLUSTER OF *MYCOBACTERIUM MARINUM*

**INTRODUCTION**

Numerous studies support the view that the unique mycobacterial (glyco)lipids PDIMs and PGLs and their related congeners (Figure 3) are involved in pathogenicity through intricate and incompletely understood mechanisms (105). Notably, recent studies suggest complementary roles of PDIMs and PGLs in a complex immune evasion mechanism that leads to avoidance of microbicidal macrophages and preferential recruitment and infection of permissive ones (110). There is also evidence supporting the notion that, at least in some species, some of these lipids strengthen the cell envelope permeability barrier (82,100) and can increase the bacterium’s intrinsic resistance to antimicrobial drugs (89,100,115).

The relevance of PDIMs and PGLs in the biology of several pathogenic mycobacteria highlights the biosynthesis of these unique virulence factors as an attractive target for exploring the development of antivirulence drugs that might be useful in adjuvant chemotherapeutic approaches. This consideration has provided thrust for numerous studies directed towards the elucidation of the PDIM/PGL biosynthetic pathway. These studies have led to the identification of several conserved genes required for production of PDIMs and/or PGLs and to the elucidation of the specific roles that several of these genes play in the pathway (65). The knowledge gained from some of these studies has guided the rational design of the first inhibitor of PGL biosynthesis (87). The inhibitor specifically targets a conserved $p$-hydroxybenzoic acid adenylating enzyme (FadD22) essential for PGL synthesis and has potent activity in members of the *M. tuberculosis* complex and in nontuberculous mycobacterial pathogens, such as *M. marinum* (87,95). Studies with *M. marinum* mutants deficient in PDIM and/or PGL production
have revealed that both PDIMs and PGLs are required for virulence in the zebrafish (Danio rerio) model system (100,110,115).

We recently reported mutational studies with M. marinum that interrogated the involvement of the genes tesA, papA5, fadD22, fadD26, fadD28, and fadD29 in production of PDIMs and PGLs (1,89). These six genes are conserved in M. tuberculosis and other PDIM and PGL producers (Figure 4) (78). The studies based on gene-specific, unmarked deletion mutants, revealed that the ∆tesA, ∆papA5, and ∆fadD28 mutants are PDIM− PGL−, the ∆fadD22 and ∆fadD29 mutants are PDIM+ PGL−, and the ∆fadD26 mutant is PDIM− PGL+ Each (glyco)lipid deficiency was complemented by episomal expression of a wild-type (WT) copy of the deleted gene, thus permitting unambiguous genotype-to-phenotype assignments. Overall, these findings are in line with those from other studies (100,115). The proposed function of tesA, papA5, fadD22, fadD26, fadD28, and fadD29 in PDIM and/or PGL synthesis is outlined in Table 6 and (65).

In the current study, we utilized the available array of well-defined deletion mutants of M. marinum noted above to further interrogate the phenotypic consequence of each of the six gene knockouts. In particular, the parallel analysis of these mutants permitted us to begin exploring the hypothesis that different gene knockouts in the complex PDIM/PGL biosynthetic pathway that render the same PDIM and/or PGL deficiency lead to mutants with equivalent pleiotropic phenotype profiles. Exploration of this hypothesis is relevant in the context of considering specific enzymes of the pathway as potential targets for exploring the development of antivirulence drugs that might be useful in adjuvant chemotherapeutic approaches against mycobacterial infections.

We evaluated the M. marinum mutants in assays to probe for changes of cell surface properties, cell envelope permeability, drug susceptibility, biofilm formation, sliding motility or
growth *in vitro*. We also explored the relative virulence of the mutants using an assay developed with *Dictyostelium discoideum*, a phagocytic amoeba established as a model unicellular organism to study host-pathogen interactions with several pathogens, including *M. marinum* (133,134). Overall, the results presented herein illustrate the complexity of the phenotypic implications arising from different gene knockouts in the PDIM/PGL biosynthetic gene cluster and expose unexpected phenotypic inequalities, a finding with potential implications in the context of antivirulence drug target considerations.

**MATERIALS AND METHODS**

**Strains and routine culturing conditions.** Mycobacteria (Table 5) were cultured under standard conditions in Middlebrook 7H9 basal medium (Difco, BD Biosciences) supplemented with 10% ADN (5% BSA, 2% dextrose, 0.85% NaCl) (Difco, BD Biosciences), 0.05% Tween-80, and 0.2% glycerol (supplemented 7H9) or Middlebrook 7H11 basal medium (Difco, BD Biosciences) supplemented with 10% ADN (supplemented 7H11) (123). Kanamycin (30 μg/ml) was included in the growth media for maintenance of pCP0 plasmids. *M. marinum* WT and the *M. marinum* mutants carried the vector pCP0 so that they could be cultured in the same kanamycin-containing media used for the complementation control strains carrying pCP0 expressing a WT copy of the gene deleted from the chromosome. *D. discoideum* DH1-10 (135) was axenically cultured under standard conditions in supplemented HL5 medium (ForMedium Co. Ltd.) at 25°C with orbital shaking (170 rpm) (136).

**Congo red binding.** Plates of supplemented or nonsupplemented 7H11 medium without or with 100 μg/ml of Congo red (Sigma Aldrich Co.) as reported (137) were spot-inoculated with 2 μl of mycobacterial cultures grown to saturation in supplemented 7H9 medium. The inoculated plates were incubated at 30°C under a humidified atmosphere for 2 weeks (supplemented plates)
or 4 weeks (nonsupplemented plates). After incubation, mycobacterial colonies were imaged using an Olympus SZX7 stereo microscope (Olympus Corp.).

**Ethidium bromide accumulation.** Assay conditions were guided by previously published protocols (100,138,139). Mycobacterial cells from cultures grown to exponential phase in supplemented 7H9 medium were harvested by centrifugation, resuspended in of 50 mM phosphate-buffered solution, pH 7 (PBS, 1 × culture vol), and collected again by centrifugation. The PBS-washed cells were resuspended in PBS (1 × culture vol), and the suspension was loaded into black, flat-bottom, 96-well plates (Greiner Bio-One Co.) at 152 μl/well. Following loading, 8 μl of a 10% glucose solution in PBS were added to the wells and the plates were incubated for 5 min at 25°C. After incubation, 40 μl of an ethidium bromide (EtBr) solution in PBS (25 mg/l) were added to the wells and fluorescence intensity (535 nm excitation, 595 nm emission) was measure every 90 seconds for 30 min using a plate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc.). Initial EtBr accumulation rates were determined as the slope of the linear regression line fitted to datasets of normalized relative fluorescence units [nRFU = (RFU_{t=x}-RFU_{t=0})/OD_{595}] versus time using the program Microsoft Excel (Microsoft Corp.).

**Antibiotic susceptibility.** Dose-response studies were conducted using a standard microdilution method previously reported (89,140). Cultures grown to exponential growth phase in supplemented 7H9 medium were diluted in fresh medium to an OD_{595} of 0.005 and loaded into 96-well plates (200 μl/well). Rifampicin (EMD-Calbiochem), doxycycline (Sigma-Aldrich Co. LLC), and erythromycin (Sigma-Aldrich Co. LLC) were added to the medium from stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich Co. LLC). Cefuroxime (Sigma-Aldrich Co. LLC), ciprofloxacin (Fluka-Biochemika) and streptomycin (Sigma-Aldrich Co. LLC) were added from aqueous solutions. In the former case, DMSO was kept at 0.5% in both test and control wells. Plates were incubated at 30°C with orbital shaking (170 rpm) under a humidified
atmosphere for 8 days. After incubation, growth was assessed by measuring OD$_{595}$ using a plate reader. MIC values for fold-change determinations were calculated from sigmoidal curves fitted to dose-response datasets using the program GraphPad Prism (GraphPad Software, Inc.) as reported (141,142).

Biofilm formation. Mycobacterial cells from exponential growth phase cultures diluted to $1 \times 10^6$ CFU/ml in 7H9 basal medium supplemented with 0.2% glycerol were loaded (150 µl/well) into MBEC™ plates (143,144). After loading, the plates were covered with their 96-peg lids, and incubated at 30°C with gentle agitation (100 rpm) under a humidified atmosphere for 4 weeks. Biofilm formation on the pegs was quantified using a standard crystal violet-based colorimetric method (145,146). Briefly, the pegs were immersed in a 0.5% crystal violet solution (150 µl/well in a 96-well receiver plate) for 15 min, rinsed twice with distilled water, and allowed to air dry. Stained pegs were then immersed in 95% ethanol (150 µl) using a 96-well receiver plate and agitated (100 rpm) for 1 h for dye extraction. The 96-peg lids were then removed, allowed to air dry, and the biofilms on the pegs were imaged using an Olympus SZX7 stereo microscope and an Olympus BX41 compound microscope. Absorbance of the decolorizing ethanol solution was measured at 595 nm using a plate reader. Pellicle formation was investigated using a multiwell plate-based assay similar to those reported (137,147,148). Wells of 24-well plates preloaded with Sauton’s medium (without Tween 80; 1 ml/well) were inoculated at the liquid surface with 10 µl of saturated mycobacterial cultures and the plates were incubated at 30°C under stationary conditions and a humidified atmosphere for 5 weeks. After incubation, the pellicles were imaged using an Olympus SZX7 stereo microscope.

Sliding motility. Cultures grown to exponential growth phase in supplemented 7H9 medium were spotted (5 µl) on the center of sliding motility plates (7H9 basal medium, 0.3% or 0.5% high-grade agarose, 6 % glycerol; 25 ml of medium/Petri dish) (137,149,150). Inoculated
plates were incubated at 30°C under a humidified atmosphere (12 days and 14 days for 0.3% and 0.5% agarose plates, respectively). After incubation, sliding motility was determined as the average diameter of the spreading zone as reported (137).

**In vitro growth.** The experiments were conducted using a 96-well plate platform previously reported (89). The plates were loaded with supplemented 7H9 medium inoculated with late exponential growth phase cultures (200 μl/well; OD_{595} = 0.005) and incubated at 30°C with orbital shaking (170 rpm) under a humidified atmosphere for 14 days. Growth was assessed every 24 hours by measuring the OD_{595} of the cultures using a plate reader. Maximum specific growth rate (μ_m) values and the optical density plateau values reached at stationary phase were determined for each of three independent experiments from sigmoidal growth curves fitted with the modified Gompertz equation using the program GraphPad Prism (151).

**D. discoideum experiments.** The amoeba inhibition assay was a variation from an assay recently published (115). Briefly, a *Mycobacterium-Klebsiella aerogenes* mixture (50 μl) prepared as reported (115) was used to surface-inoculate wells of 24-well plates containing 2 ml of 7H10 agar (Difco, BD Biosciences) to generate bacterial lawns. After inoculation with the bacterial mixture, the wells were spot-inoculated (5 μl) in the center with 2-fold serial dilutions (2 × 10³ to 3×10⁷ cells/ml range) of a *D. discoideum* culture. Plates were then incubated for 14 days at 25°C under a humidified atmosphere. Starting on the third day of incubation, wells were visually inspected daily for formation of phagocytic plaques for 10 days. *D. discoideum* growth was defined for any given set of duplicates wells (same amoeba inoculum) that exhibited at least two phagocytic plaques in each of the wells.
RESULTS

Gene knockouts in the PDIM/PGL biosynthetic pathway alter cell surface properties.

Mutations producing changes in the composition of the (glyco)lipid-rich outer layer of the waxy mycobacterial cell envelope can potentially lead to differences in the binding of the hydrophobic dye Congo red (CR) (152). Guided by this precedent, we investigated the CR binding properties of the tesA, papA5, fadD22, fadD26, fadD28, and fadD29 M. marinum mutants (Figure 10). On supplemented 7H11 plates, the mutants and the WT developed the same reddish coloration in the presence of CR, thus indicating comparable CR binding properties. On the other hand, the mutants displayed a drastic increase in CR staining relative to the WT on nonsupplemented 7H11 plates, where mycobacteria grow under stress due to the lack of the protective and growth-stimulating effect of the ADN and glycerol supplements (153,154). Episomal expression in the mutants of a WT copy of the deleted gene led to clear complementation (reversion to WT CR staining) only in the tesA and fadD28 mutants, despite the fact that all the genetically complemented mutants regain the capacity for PDIM and/or PGL production (1,89,92). Overall, the results clearly indicate that the gene knockouts affect cell surface properties and highlight the fact that the outcome of a CR binding assay can be drastically influenced by growth medium composition.

Gene knockouts in the PDIM/PGL biosynthetic pathway weaken the cell envelope permeability barrier.

Mutations that alter cell surface properties may affect cell envelope permeability as well. In agreement with this view, previous studies revealed that a transposon insertion-based disruption of fadD28 or fadD26 in M. marinum leads to a comparable increase in cell envelope permeability (100). Disruption of fadD26 (leading to PDIM−) in the strain MT103 of M.
tuberculosis (a strain naturally PGL-) also results in an increase in cell envelope permeability (82). These previous studies are consistent with the notion that abrogation of PDIM production weakens the cell envelope permeability barrier. However, these studies provide no information as to whether gene knockouts leading to the selective loss of PGLs affect cell envelope permeability. To address this knowledge gap and to expand our overall understanding of the impact of inactivating different genes required for PDIM and/or PGL production on cell envelope permeability, we investigated the EtBr accumulation rates of the \textit{M. marinum} deletion mutants. EtBr accumulation assays are routinely used to probe cell envelope permeability in bacteria, including mycobacteria (100,138,139). Using this approach, we found that each gene knockout correlated with an increase in EtBr accumulation rate compared to \textit{M. marinum} WT and the corresponding complemented strain control (Figure 11). Interestingly, knockouts leading to concurrent loss of PDIMs and PGLs or selective loss of PDIMs roughly doubled the rate, whereas knockouts selectively eliminating PGLs produced a 25% increase. To the best of our knowledge, this is the first study linking loss of PGLs with a weakening of the cell envelope permeability barrier. Overall, these results are consistent with the view that PDIMs have a more relevant role in the integrity of the cell envelope permeability barrier of \textit{M. marinum} than PGLs.

Gene knockouts in the PDIM/PGL biosynthetic pathway increase antimicrobial drug susceptibility.

Mutations leading to a weakening of the cell envelope permeability barrier can potentially increase antimicrobial drug susceptibility by improving drug penetration. In agreement with this notion, we and others have recently shown that inactivation of \textit{tesA} in \textit{M. marinum} leads to hypersusceptibility to various antimicrobial drugs (89,115). To expand on this observation, we investigated the susceptibility of the \textit{M. marinum} mutants to antimicrobial drugs of different classes. The group of six drugs tested included rifampicin, doxycycline,
ciprofloxacin and streptomycin, which are four drugs with reported use in the treatment of *M. marinum* infections (155). In this analysis, a strain was defined as having an altered susceptibility when it displayed >2-fold change in the MIC value relative to the MIC value of the WT strain (Figure 12). As per this criterion, and in general agreement with previous studies (89,115), the *tesA* mutant showed hypersusceptibility to cefuroxime, doxycycline, rifampicin and erythromycin, but not to ciprofloxacin or streptomycin. This susceptibility profile was qualitatively and quantitatively recapitulated in the *papA5* mutant. On the other hand, the *fadD28, fadD26, fadD22* and *fadD29* mutants displayed hypersusceptibility profiles that, for the most part, were qualitatively comparable, yet quantitatively attenuated relative to that of the *tesA* and *papA5* mutants. The attenuated hypersusceptibility profile of the *fadD28* mutant is particularly unexpected because the mutant shares the PDIMˉ PGLˉ phenotype with the *tesA* and *papA5* mutants and has an increase in cell permeability comparable to that of these mutants. Episomal expression in the mutants of a WT copy of the corresponding deleted gene flattened the hypersusceptibility profiles and, in all, decreased or eliminated the hypersusceptibility of the mutants. In line with our findings, transposon insertion-based disruption of *fadD26* or *fadD28* in *M. marinum* correlates with increased drug susceptibility (100). Taken together, the results indicate that only two out of three knockouts producing concurrent loss of PDIMs and PGLs lead to a drastic increase in susceptibility to selective drugs and that mutations resulting in a selective loss of PDIMs or PGLs affect drug susceptibility to a lesser extent.

**Gene knockouts in the PDIM/PGL biosynthetic pathway are detrimental to biofilm formation.**

Recent studies implicated the *M. marinum* cell wall-associated glycolipids known as lipooligosaccharides (LOSs) in formation of biofilms (149). This raises the possibility that other cell wall (glyco)lipids of *M. marinum* could be relevant for biofilm formation. With this in
mind, we explored the capacity of the *M. marinum* mutants to form biofilms on solid and liquid surfaces. Biofilm formation on a solid surface was investigated using the MBEC™ plates (144), which consists of a standard 96-well plate with a lid containing 96 polystyrene pegs that fit into the wells and on which *M. marinum* forms biofilms (156,157). In line with previous observations, *M. marinum* WT formed biofilms on the pegs (Figure 13 A) and the biofilm microcolonies displayed the characteristic serpentine cords (Figure 13 B) (156,157). The mutants also formed biofilms, but they displayed smaller and/or less abundant microcolonies on the pegs (Figure 13 B) and less biofilm formation overall (32-80% reduction relative to WT) (Figure 13 C). Notably, the *tesA*, *papA5*, and *fadD26* knockouts led also to more compact cell aggregates with reduced cording (Figure 13). Episomal expression in the mutants of a WT copy of the deleted gene increased biofilm formation and corrected the cording deficiency.

Formation of biofilms on a liquid surface (pellicle formation) was evaluated using a multiwell plate-based assay, a set-up developed for evaluation of pellicle formation by other mycobacteria (137,147,148). We found an equally robust pellicle formation capacity for the WT and the mutants. No differences in pellicle surface topology between the strains that could consistently be maintained across experimental replicates were observed under our experimental conditions. Overall, all pellicles showed a comparably rich surface topology with folds, ridges, and creases (Figure 13).

Overall, the findings of the biofilm studies demonstrate that the knockouts leading to loss of PDIMs and/or PGLs produced a reduction in the capacity of *M. marinum* to form biofilms on a solid surface, but had no evident impact on pellicle formation. To the best of our knowledge, this is the first study indicating that loss of PDIM and/or PGL production affects biofilm formation.
Gene knockouts in the PDIM/PGL biosynthetic pathway have no evident impact on sliding motility.

Mycobacteria can spread on the surface of solid media by a sliding mechanism, a property that could have implications for surface colonization in the environment and in the host (158). In *M. marinum*, LOSs have been implicated in sliding motility (149), thus raising the possibility that other *M. marinum* cell wall (glyco)lipids could be relevant for this motility mechanism. We investigated the sliding motility capacity of the *M. marinum* mutants using a standard assay in which the diameter of the spreading zone provides a quantitative read-out of sliding (data not shown). We found that all the strains had sliding motility capacity, albeit with relatively high variability between experimental replicates. Overall, no significant differences were observed between the mutants and the WT (Student’s *t*-test *p* values > 0.05; data not shown). Thus, neither PDIMs nor PGLs play a critical role in sliding motility.

**Gene knockouts in the PDIM/PGL biosynthetic pathway do not compromise *in vitro* growth.**

To delineate the effect of different gene knockouts in the PDIM/PGL pathway on *M. marinum* growth *in vitro*, we compared the mutants and the WT strain on the basis of two quantitative parameters derived from sigmoidal growth curves: *i.e.* maximum specific growth rate (*µ*m) and optical density plateau reached at stationary phase. Under standard liquid culture conditions, no substantial differences (*i.e.* ≥10%) in *µ*m or optical density plateau between the mutants and the WT strain were observed (not shown). Overall, the results are in line with previous studies with *M. marinum ΔtesA* (89).
Gene knockouts in the PDIM/PGL biosynthetic pathway lead to attenuation in an amoeba inhibition assay.

*D. discoideum* is a phagocytic, bacterium-eating amoeba used as a model unicellular organism to study host-pathogen interaction and virulence of mycobacteria and other pathogens (133,134). *M. tuberculosis* complex members and non-tuberculous mycobacterial pathogens such as *M. marinum* are amoeba-resistant organisms that multiply intracellularly leading to amoeba growth arrest and cytotoxicity (159-161). These pathogens compromise amoebal phagolysosome maturation and replicate inside permissive endosomal compartments, a pathogenic strategy seen during macrophage infection (160,162).

A virulence assay based on *M. marinum*-dependent inhibition of *D. discoideum* was recently used to identify a *tesA* transposon mutant as an attenuated strain (115). In this assay, *D. discoideum* feeding and multiplying on a lawn of *K. aerogenes* (amoeba food) produce phagocytic plaques, i.e. *K. aerogenes* lawn clearing zones. Inclusion of *M. marinum* WT intermingled into the *K. aerogenes* lawn leads to *M. marinum* infection and consequent inhibition of *D. discoideum*, thus reducing phagocytic plaque formation. By comparison, the attenuated *tesA* mutant has a diminished plaque formation suppression capacity (115). We modified this assay to better differentiate virulence degrees between mycobacterial strains and used the modified assay to probe the virulence of the *M. marinum* mutants (Figure 14). We performed the assay in a 24-well plate platform, expanded the inoculum of amoeba cells to a 10-to-150,000 range from the 100-to-10,000 range reported, and escalated the amoeba inoculum using 2-fold increments, as opposed to the 10-fold increments used previously (115). The phagocytic plaque formation read-out was used to score wells in a binary fashion: amoeba growth versus amoeba inhibition. For each *M. marinum* strain, the highest amoeba inoculum inhibited was considered a measure of the amoeba inhibition capacity of the strain. Using
amoeba inhibition capacity as a quantitative measure of virulence, we found a drastic reduction in virulence (~300-fold to ~500-fold relative to WT) for the tesA, papA5, fadD28, and fadD26 mutants. On the other hand, the fadD22 and fadD29 mutants exhibited a modest virulence reduction (3-fold relative to WT). As expected, episomal expression of a WT copy of the deleted gene increased the virulence of the mutants. Interestingly, even the most attenuated mutants remained significantly more virulent than the nonpathogenic M. smegmatis species. Collectively, these results indicate that gene deletions producing concurrent loss of PDIMs and PGLs or selective loss of PDIMs lead to severe attenuation in the D. discoideum model system. In contrast, gene deletions producing selective loss of PGLs are relatively inconsequential in this amoeba model. These findings support the view that PDIMs have a more critical role in the pathogenicity of M. marinum against D. discoideum than PGLs.

**CONCLUSIONS**

The basic lipid cores of PDIMs and PGLs arise from esterification of phthiocerols and phenolphthiocerols, respectively, with mycocerosic acids (Figure 3). The current model for the biosynthesis of these esters includes the conserved enzymes TesA, PapA5, FadD22, FadD26, FadD28 and FadD29. The availability of a set of six M. marinum mutant strains that are isogenic except for a defined unmarked deletion of tesA, papA5, fadD22, fadD26, fadD28, or fadD29, respectively, provided an opportunity to comparatively probe for the pleiotropic consequences of mutations leading to PDIMˉ PGLˉ, PDIM⁺ PGL⁻, or PDIM⁻ PGL⁺ phenotypes in this opportunistic human pathogen. Furthermore, the tesA, papA5 and fadD28 mutant triad permitted us to explore whether M. marinum mutants with a PDIMˉ PGL⁻ phenotype arising from different genetic etiologies share other phenotypic traits. Similarly, the fadD22 and fadD29 mutant pair allowed us to investigate whether strains with a PDIM⁺ PGL⁻ phenotype emerging from different gene knockouts are equivalent with respect to other phenotypes as well. Thus, the parallel
analysis of the *M. marinum* mutants gave us an opportunity to begin exploring the hypothesis that different gene knockouts in the complex PDIM/PGL biosynthetic pathway that render the same PDIM and/or PGL deficiency lead to strains with equivalent pleiotropic profiles. Exploration of this hypothesis is relevant in the contexts of considering specific enzymes of the pathway as potential targets for exploring the development of antivirulence drugs that could be useful in adjuvant chemotherapeutic approaches against mycobacterial infections.

Our studies reveal that loss of production of PDIMs or PGLs leads to an alteration of cell envelope properties. In particular, our findings illustrate for the first time a link between loss of PGL production and a weakening of the cell envelope permeability barrier and show that PGLs are far less relevant to the integrity of such a barrier than PDIMs. The mutants also display changes in antimicrobial drug susceptibility. The increased susceptibility in the mutants might be due to better drug penetration arising from the weakening of the cell envelope permeability barrier; yet the magnitude of the change in susceptibility appears to be drug-specific and uncorrelated with the lipophilicity (*e.g.* log $P$ ([163])) of the drug. Our findings indicate that the *tesA* and *papA5* mutants (PDIM⁻ PGL⁻) have the same hypersusceptibility profile and are overall more susceptible than the *fadD26* mutant or the *fadD22* and *fadD29* mutants, in which only PDIMs or PGLs are lost, respectively. Intriguingly, the *fadD28* mutant (PDIM⁻ PGL⁻) shows a relatively attenuated hypersusceptibility profile compared with that of the *tesA* and *papA5* mutants. The *fadD28* mutant differs from the *tesA* and *papA5* mutants also in terms of biofilm microcolony phenotype. The microcolonies of the *fadD28* mutant retain the WT phenotype, whereas those of the *tesA* and *papA5* mutants form more compact cell aggregates with reduced cording. Interestingly, the appearance of anomalous PDIM-like lipid variants with shorter mycocerosic acid-like acyl chains has been documented in the mycocerosic acid-deficient mutant of *Mycobacterium bovis* BCG ([71,84,164]). Perhaps production of anomalous lipid variants with
surrogate acyl chains in the absence of mycocerosic acids due to deletion of *fadD28* in *M. marinum* Δ*fadD28* is responsible for the attenuated hypersusceptibility profile of the mutant. Overall, the findings of the antibiotic susceptibility studies support the exciting possibility that antivirulence drug therapy aimed at blocking PDIM and PGL production might increase the efficacy of some antimicrobial drug treatments and suggest that such an effect could be drastically influenced by the molecular target of the antivirulence drug, e.g. PapA5 versus FadD28.

Our studies also reveal that all the *M. marinum* mutants are attenuated in a *D. discoideum* inhibition assay, albeit with clear differences in the degree of attenuation. We found that concurrent loss of PDIMs and PGLs or selective loss of PDIMs has a far more drastic effect in virulence than selective loss of PGLs. Mycobacterial pathogens are known to compromise amoebal phagolysosome maturation to avoid killing and create niches for intracellular replication, a pathogenic strategy seen during macrophage infection (160,162). In *M. tuberculosis*, transposon-based disruption of *fadD28* impairs the ability of the pathogen to arrest phagolysosome maturation in macrophages (116), possibly by lacking PDIMs to elicit pathogen-directed reorganization of lipid in the host membrane (107). This precedent highlights the possibility that the attenuation of the *M. marinum* mutants might emerge from a reduced capacity to arrest the amoebal phagolysosome maturation pathway and create protective niches. Interestingly, even the most attenuated *M. marinum* mutant remains significantly more virulent than the nonpathogenic *M. smegmatis* species. This observation indicates that *M. marinum* has multiple virulence factors contributing to the interplay with the amoeba host. This notion is not surprising considering that *M. marinum* and amoebae share environmental niches and that amoebae are thought to act as evolutionary training grounds for intracellular bacterial pathogens (165).
Taken together, the results of the phenotypic characterization of *M. marinum* mutants presented herein reveal a complex picture of pleiotropic patterns emerging from different gene knockouts in the PDIM/PGL biosynthetic pathway. These pleiotropic patterns could be influenced by changes in metabolic flux redirecting surpluses of different biosynthetic precursors to production of other (glyco)lipids (87,103,104). Accumulation of different biosynthetic intermediates, some of which might be repurposed for production of unusual lipids with potential phenotypic impacts, might also contribute to define the pleiotropic patterns displayed by the different mutants (71,84,164). Ultimately, it is clear that (glyco)lipidomic profiling of the *M. marinum* mutants will be needed to investigate these possibilities. Information from such studies will be critical towards the challenging endeavor of unveiling the molecular mechanisms underlying the phenotypic profiles of the different mutants.

Overall, the parallel analysis of the *M. marinum* mutants permitted us to uncover unexpected phenotypic inequalities, a finding with potential implications in the context of antivirulence drug target considerations. Most noticeably, the differences between the *tesA*, *papA5* and *fadD28* knockouts, three events leading to a PDIM⁺ PGL⁺ phenotype, suggest that the enzymes encoded by these genes might not be equivalent targets for development of antivirulence drugs. In more general terms, our results advocate that different enzymes for which elimination equally leads to PDIM and PGL deficiency might not be equivalent targets for adjuvant drug development.
CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

This thesis has provided cumulative evidence for the relevance of DIMs in the biology of mycobacteria by presenting new mechanistic insights into their biosynthetic pathway and elucidating their contribution to the phenotype of *M. marinum*. More specifically the work has helped establish the relevance of the ACP_L domain of PpsA in the extension of the PHPA intermediates as well as the vital role of FadD29, in the transfer of the PHPA intermediates from the iterative PKS Pks15/1 to the non-iterative PKS PpsA, during PGL biosynthesis. The results from the mutational studies and the supporting *in vitro* enzymatic studies has not only provided the first evidence of a PHPA-ligase candidate FadD29 within a nontuberculous mycobacterial species but has also provided valuable insight into the partnership of FadD29-PpsA versus FadD26-PpsA during PDIM and PGL biosynthesis, respectively. The conservation of the DIM biosynthetic genes across the species strongly suggests that the mechanistic insights into PGL biosynthesis gained herein are applicable to other PGL producers like members of the MTBC. Taken together these results provide vital information to build the framework of DIM biosynthesis in mycobacteria.

This thesis has also presented novel findings on the phenotypic consequences resulting from PDIM and/or PGL deficiency in *M. marinum* and suggested that the loss of genes in the same pathway alters the observed phenotypic output in different ways. The complex pleiotropic patterns emerging from the different knockouts might be influenced by changes in metabolic flux redirecting different biosynthetic precursor surpluses to the production of other (glyco)lipids ([87,103,104]). Accumulation of different biosynthetic intermediates, some of which might be repurposed for the production of unusual (glyco)lipids with potential phenotypic impacts ([71,84]), might also contribute to define the pleiotropic patterns of the mutants seen here. Overall the
pleiotropic changes reported here provide valuable information on the biology of mycobacteria and can possibly relate to adaptations of the bacterium within the host during infection.

While we have made considerable strides in understanding the biosynthesis of DIMs and their contributions to the phenotype of mycobacteria there are knowledge gaps that continue to challenge the scientific community. A natural progression of this work will be to further clarify the steps involved the biosynthesis of these lipids using genetic and biochemical studies. Another possible area of research will be to elucidate the molecular mechanisms underlying the phenotypic profiles of the DIM deficient mutants and the mechanisms surrounding the modulation of PDIMs and PGLs during infection.
Figure 1: Schematic representation of the *M. tuberculosis* cell envelope.

Adapted from (51). Many of the classes of lipids and glycolipids discussed in the text are represented schematically and are shown in probable locations in the cell envelope. The structures with light and dark green hexagons represent trehalose mono- and dimycolates respectively; the red lollipops represent phthiocerol dimycocerosates, and the gold ones represent sulfo-lipids, diacyltrehaloses and polyacyltrehaloses. Gray circles represent phospholipid headgroups; black circles, isoprenoids; light blue squares, NAG; white squares, NAM; white pentagons, arabinofuranose; yellow diamonds, galactofuranose; and blue hexagons, mannose.
Figure 2: Schematic representation of the proposed DIM biosynthesis pathway.

Adapted from (88). The proposed roles of the various FAAL and PKS proteins encoded by the DIM locus in *M. tuberculosis* are depicted. R = C2H5 or CH3. KS, ketosynthase; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein.
Figure 3: Representative structures of mycobacterial PGLs and PDIMs.

Adapted from (1). The carbon-chain variability and glycosyl unit represented are from main variants found in the opportunistic human pathogen *Mycobacterium marinum*. $m$ and $m'$: 16 – 20, $n$: 16 – 22, $p$: 14 – 22.
Figure 4: Conservation of *M. marinum* and *M. tuberculosis* chromosomal loci involved in PGL-PDIM production.

Adapted from (1). The five *M. marinum* genes targeted for mutational analysis (Δ, deletion; *, amino acid substitution) in this study and their respective orthologs in *M. tuberculosis* are highlighted with pattern-filled arrows. †, *pks15/1* is essential for PGL production. The gene *pks15/1* is disrupted (split into *pks1* and *pks15*) by natural mutations in *M. tuberculosis* H37Rv and other Euro-American lineage strains, thus leading to PGL-deficiency (83,109). Additional genes implicated in PGL and/or PDIM production located downstream *fadD29* are not depicted. Adapted from (1,78).
Figure 5: Two possible mechanisms for transfer of \( p \)-hydroxyphenylalkanoate (PHPA) intermediates to PpsA in \( M. \) marinum.

Adapted from (1). (A) PHPA-AMP ligase-dependent model. The model includes the release of PHPAs thioesterified to Pks15/1’s ACP domain (A1), activation of free PHPAs by a PHPA-AMP ligase (A2), PHPA-AMP ligase-dependent loading of PHPAs onto PpsA’s ACP\(_L\) domain (A3), and captured of the ACP\(_L\) domain-bound PHPAs by PpsA’s KS domain to yield the fully loaded PpsA (boxed) ready for KS domain-dependent decarboxylation/condensation.

(B) Direct KS domain capture model. In this model, the fully loaded PpsA (boxed) is generated directly by the capture of Pks15/1-bound PHPAs by PpsA’s KS domain (B1), thus bypassing the need for steps A1 through A4. Decarboxylation/condensation leads to extension of PHPAs by a 2-carbon unit via the first non-iterative extension cycle in the formation of phenolphthiocerols.

In the scheme, the depicted carbon-chain variability in the PHPA-S-Pks15/1 thioester intermediate is that expected during synthesis of \( M. \) marinum PGLs. Adenylated PHPAs are shown bound to the PHPA-AMP ligase via noncovalent linkages (by analogy to other acyl adenylating enzymes). PpsA’s C-terminal ACP domain is shown loaded (AT domain-dependent) with the malonyl-CoA-derived extender unit. Thiol groups of the phosphopantetheinyl group in the ACP domains and the catalytic Cys in the KS domain are depicted. The hydroxyl group in the extended PHPAs generated from the keto group by action of the KR domain of PpsA is shown. Sections of the PHPA intermediates are color-coded based on origin: blue, derived from a \( p \)-hydroxybenzoic acid starter unit; red, derived from malonyl.
extender units via iterative extension cycles; black, section derived from a malonyl extender unit via a non-iterative extension cycle.

Domain abbreviations: A, adenylation; ACP, acyl carrier protein; ACP_L, loading acyl carrier protein; AT, acyltransferase; DH dehydratase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase.
Figure 6: Mutagenesis cassette-delivery suicide vectors used for construction of *M. marinum* mutants.

Adapted from (1). The predicted translational products of the gene remnants or the amino acid substitutions in the mutagenesis cassette-delivery vectors are shown. The deletion/mutation point is flanked by ca. 1.0 kb of downstream and upstream wild-type sequence for homologous recombination with the chromosome.
Figure 7: Mutant verification analysis.

Adapted from (1). Each chromosomal deletion was confirmed by PCR using two independent primer pairs. One primer pair was used to verify the expected ~2-kb deletion at the deletion site (A). A second primer pair was used to confirm the elimination of the target gene from the chromosome (B). The nucleotide substitutions in Mm ppsAS-A introducing Ser-to-Ala substitutions and a BssHII restriction site were confirmed by sequence analysis (C).

Amplicon information for the agarose gel electrophoresis image shown in panel A: lane 2, Mm WT (4,110-bp amplicon expected with primers SalIFadD22OF and NotIFadD22OR); lane 3, Mm ΔfadD22 (2,016-bp amplicon expected with SalIFadD22OF and NotIFadD22OR); lane 4, Mm WT (3,658-bp amplicon expected with primers HindIIIMarFadD26OF and KpnIMarFadD26OR); lane 5, Mm ΔfadD26 (1,930-bp amplicon expected with HindIIIMarFadD26OF and KpnIMarFadD26OR); lane 6, Mm WT (3,639-bp amplicon expected with primers BamHIMarFadD28OF and NotIMarFadD28OR); lane 7, Mm ΔfadD28 (1,928-bp amplicon expected with BamHIMarFadD28OF and NotIMarFadD28OR); lane 8, Mm WT (3,804-bp amplicon expected with primers BamHIMarFadD28OF and NotIMarFadD28OR); lane 9, Mm ΔfadD29 (1,966-bp amplicon expected with HindIIIMarFadD29OF and PmlIMarFadD29OR).

Amplicon information for the agarose gel electrophoresis image shown in panel B: lane 11, Mm WT, 2,156-bp amplicon expected with primers HindIIIIfadD22For and HpaIFadD22Rev; lane 12, Mm ΔfadD22, no amplicon expected with HindIIIIfadD22For and HpaIFadD22Rev; lane 13, Mm WT, 1,794-bp amplicon expected with primers pCP0ForMarFadD26 and pCP0RevMarFadD26; lane 14, Mm ΔfadD26, no amplicon expected with pCP0ForMarFadD26 and pCP0RevMarFadD26; lane 15, Mm WT, 1,919-bp amplicon expected with primers pCP0ForMarFadD29 and pCP0RevMarFadD29; lane 16, Mm ΔfadD29, no amplicon expected with pCP0ForMarFadD29 and pCP0RevMarFadD29; lane 17, Mm WT, 1,779-bp amplicon expected with primers pCP0ForMarFadD28 and pCP0RevMarFadD28; lane 18, Mm WT, 1,779-bp amplicon expected with primers pCP0ForMarFadD28 and pCP0RevMarFadD28. DNA ladder marker: lanes 1, 10, and 17. The sizes of relevant DNA makers flanking the PCR product of of interests are indicated. WT, wild-type. Mm, M. marinum. See Table 3. PCR primer pairs and amplicon information used in mutant screening and verification.
Figure 8: Inactivation of PpsA’s ACP<sub>L</sub> domain leads to a PGL<sup>-</sup> PDIM<sup>-</sup> phenotype in *M. marinum*.

Adapted from (1). Radio-TLC analysis of [14C]-labeled PGLs (A) and [14C]-PDIMs (B) from *Mm* wt + pCP0 (WT), *Mm* ppsAS-A + pCP0 (ppsAS-A), and *Mm* ppsAS-A + pCP0-ppsA (ppsAS-A - c). The wild-type (WT) and mutant *M. marinum* (*Mm*) strains carried the vector pCP0 so they could be cultured in the same kanamycin-containing medium used for the complemented *Mm* ppsAS-A + pCP0-ppsA strain. TLC solvent systems used are indicated. CHCl<sub>3</sub>, chloroform; MeOH, methanol; PE, petroleum ether; Et<sub>2</sub>O, diethyl ether.
Figure 9: Mutational analysis points at *M. marinum* FadD29 as a PHPA-AMP ligase candidate.

Adapted from (1). Radio-TLC analysis of [14C]-labeled PGLs (A) and [14C]-PDIMs (B) from *Mm* wt + pCP0 (WT), *Mm* ΔfadD22 + pCP0 (22m), *Mm* ΔfadD22 + pCP0-fadD22 (22c), *Mm* ΔfadD26 + pCP0 (26m), *Mm* ΔfadD26 + pCP0-fadD26 (26c), *Mm* ΔfadD28 + pCP0 (28m), *Mm* ΔfadD28 + pCP0-fadD28 (28c), *Mm* ΔfadD29 + pCP0 (29m), and *Mm* ΔfadD29 + pCP0-fadD29 (29c). The wild-type (WT) and mutant *M. marinum* (*Mm*) strains carried the vector pCP0 so they could be cultured in the same kanamycin-containing medium used for the complemented strains. TLC solvent systems used are as depicted in Figure 8.
Figure 10: Congo red binding properties of *M. marinum* strains.

Adapted from (2). Plates of supplemented or nonsupplemented 7H11 medium without or with Congo red (CR) were spot-inoculated with *M. marinum* cultures, incubated for growth, and imaged. The results with the ΔtesA and ΔfadD22 mutants are equivalent to those seen with the rest of the mutants, except by the complementation differences noted in the text. The data is representative of three experiments. WT, *M. marinum* wild-type.
Figure 11: Ethidium bromide accumulation rate of *M. marinum* strains.

Adapted from (2). Rates of ethidium bromide (EtBr) accumulation were determined as the slopes of linear regression lines fitted to fluorescence-time datasets generated using a multiwell plate-based assay. The results correspond to means and SEM of five experiments. Student’s *t*-test *p* values versus wild-type (WT): *, < 0.05; **, < 0.01.
Figure 12: Antibiotic susceptibility of *M. marinum* strains.

Adapted from (2). Fold-change in MIC values between the wild-type (WT) strain and the mutants (A) or the mutants constitutively expressing an episomal WT copy of the deleted gene (B) are shown. Dose-response studies were conducted using a standard microdilution method. MIC values for fold-change determinations were derived from sigmoidal curves fitted to dose-response datasets. A strain was defined as having an altered antibiotic susceptibility when it displayed >2-fold change (dotted line) relative to WT. The results represent means and SEM of three experiments.
Figure 13: Biofilm formation by *M. marinum* strains.

Adapted from (2). Biofilm formation on a solid surface was investigated using the MBEC™ device, which consists of a standard 96-well plate with a 96-well plate lid containing 96 polystyrene pegs that fit into the wells and onto which wild-type (WT) *M. marinum* forms biofilms. Representative peg (A) and biofilm microcolony (B) images from *M. marinum* WT and from two mutants and their corresponding complemented strains are shown. The results with these mutants are equivalent of those seen with the rest of the mutants. Biofilm formation on the pegs was quantified using a standard colorimetric method (C). The results represent means and SEM of a minimum of six experiments. Student’s *t*-test *p* values versus WT: *, < 0.05; **, < 0.01. All *M. marinum* strains formed comparable pellicles on the surface of the liquid growth medium. The representative pellicle shown is for the WT strain (D).
Figure 14: Inhibition of *D. discoideum* by *M. marinum* strains.

Adapted from (2). The virulence of the *M. marinum* strains was assessed using a *D. discoideum* inhibition assay. The highest amoeba inoculum inhibited by each strain was considered a measure of its amoeba inhibition capacity. The fold reduction in virulence of each strain relative to *M. marinum* wild-type (WT) is shown at the base of each bar for the mutants and above the bar for *M. smegmatis* (*Msm*). The results correspond to means and SEM of three experiments. **, Student’s *t*-test *p* < 0.01 (versus WT).
TABLES

Table 1: Bacterial strains

Adapted from (1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em> DH5α</td>
<td>Cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>M.marinum</em> strain M</td>
<td>Wild-type</td>
<td>American Type</td>
</tr>
<tr>
<td><em>M.marinum ΔfadD22</em></td>
<td>Carries an in-frame, unmarked <em>fadD22</em> deletion</td>
<td>(1)</td>
</tr>
<tr>
<td><em>M.marinum ΔfadD26</em></td>
<td>Carries an in-frame, unmarked <em>fadD26</em> deletion</td>
<td>(1)</td>
</tr>
<tr>
<td><em>M.marinum ΔfadD28</em></td>
<td>Carries an in-frame, unmarked <em>fadD28</em> deletion</td>
<td>(1)</td>
</tr>
<tr>
<td><em>M.marinum ΔfadD29</em></td>
<td>Carries an in-frame, unmarked <em>fadD29</em> deletion</td>
<td>(1)</td>
</tr>
<tr>
<td><em>M.marinum ppsAS-A</em></td>
<td>Carries a <em>ppsA</em> mutated allele with a Ser-to-Ala substitution</td>
<td>(1)</td>
</tr>
<tr>
<td><em>M.marinum ppsAS-A</em></td>
<td>Carries a mutated allele with a Ser-to-Ala substitution</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>Disrupting the phosphopantetheine attachment site of the <em>ACP-L</em> domain</td>
<td></td>
</tr>
</tbody>
</table>

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### Table 2: Plasmids

Adapted from (1)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1-TOPO</td>
<td>Cloning vector, kanamycin and ampicillin resistance</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>p2NIL</td>
<td>Vector with kanamycin resistance gene and OriE</td>
<td>(125)</td>
</tr>
<tr>
<td>pGOAL19</td>
<td>Vector with <em>sacB-lacZ</em> <em>PacI</em> cassette, hygromycin resistance, OriE</td>
<td>(125)</td>
</tr>
<tr>
<td>p2NILGOALc-ΔfadD22c</td>
<td><em>fadD22</em> deletion cassette delivery vector</td>
<td>(1)</td>
</tr>
<tr>
<td>p2NILGOALc-ΔfadD26c</td>
<td><em>fadD26</em> deletion cassette delivery vector</td>
<td>(1)</td>
</tr>
<tr>
<td>p2NILGOALc-ΔfadD28c</td>
<td><em>fadD28</em> deletion cassette delivery vector</td>
<td>(1)</td>
</tr>
<tr>
<td>p2NILGOALc-ΔfadD29c</td>
<td><em>fadD29</em> deletion cassette delivery vector</td>
<td>(1)</td>
</tr>
<tr>
<td>p2NILGOALc-ppsAe</td>
<td><em>ppsA</em> mutagenesis cassette delivery vector</td>
<td>(1)</td>
</tr>
<tr>
<td>pCP0</td>
<td>Mycobacterial expression vector, kanamycin resistance</td>
<td>(89)</td>
</tr>
<tr>
<td>pCP0-FadD22</td>
<td>pCP0 derivative expressing FadD22</td>
<td>(1)</td>
</tr>
<tr>
<td>pCP0-FadD26</td>
<td>pCP0 derivative expressing FadD26</td>
<td>(1)</td>
</tr>
<tr>
<td>pCP0-FadD28</td>
<td>pCP0 derivative expressing FadD28</td>
<td>(1)</td>
</tr>
<tr>
<td>pCP0-FadD29</td>
<td>pCP0 derivative expressing FadD29</td>
<td>(1)</td>
</tr>
<tr>
<td>pCP0-PpsA</td>
<td>pCP0 derivative expressing PpsA</td>
<td>(1)</td>
</tr>
</tbody>
</table>
Table 3. PCR primer pairs and amplicon information used in mutant screening and verification.

Adapted from (1)

<table>
<thead>
<tr>
<th>Mutant probed</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon (bp)</th>
<th>Mutant strain</th>
<th>Wild-type strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔfadD22</td>
<td>SalIFadD22OF</td>
<td>GTCGACTCACATCGTGGAGTTCGCCAGCTGG</td>
<td>2,016</td>
<td>4,110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotIFadD22OR</td>
<td>GCGGCCGCAAGCGCTGGGGCGAGCCCATTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HindIIIFadD22For</td>
<td>AAGCTTGCTAAGGCGGTATCCAAATAATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HpaIFadD22Rev</td>
<td>GTAAACCTCAGTTGCTCTGTTCATGATTC</td>
<td>0</td>
<td>2,156</td>
<td></td>
</tr>
<tr>
<td>ΔfadD26</td>
<td>HindIIIMarFadD26OF</td>
<td>AAGCTTATGAAAGAATTTCGAGTGGAGTCTGCTGCCGACATT</td>
<td>1,930</td>
<td>3,658</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KpnIMarFadD26OR</td>
<td>GGTACCTCCGCAACACCGCGGAGACACGCAAATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCP0ForMarFadD26</td>
<td>GAATTCAGTTGATGGGTATGCGATGCGG</td>
<td>0</td>
<td>1,794</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCP0RevMarFadD26</td>
<td>AAGCTTATGCTCTGTCATACCGTCACGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔfadD28</td>
<td>BamHIMarFadD28OF</td>
<td>GGATCCACCACCGCTCCAGTTGACGAAATGGGAAACTGAAAA</td>
<td>1,928</td>
<td>3,639</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotIMarFadD28OR</td>
<td>GCGGCCGCTTCCGCGGACAGTACGCGGGCGGTAGAT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCP0ForMarFadD28</td>
<td>AAGCTTCGTAACGTTGATGCCCATGAGTGT</td>
<td>0</td>
<td>1,779</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCP0RevMarFadD28</td>
<td>GCTAGCTTCAGTCAGCTAGCTCCAGGGCG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ΔfadD29</td>
<td>HindIIIMarFadD29OF</td>
<td>AAGCTTACCGAGCTCGGATCATCAGAGG</td>
<td>1,966</td>
<td>3,804</td>
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<tr>
<td></td>
<td>PmlIMarFadD29OR</td>
<td>CACGTGAAAACCTGCCAGCAGTCCGCGGATCCCGCATA</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pCP0ForMarFadD29</td>
<td>AAGCTTCCTCCTCCGGAGCACAGGAGATGAGT</td>
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<td>1,919</td>
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<tr>
<td></td>
<td>pCP0RevMarFadD29</td>
<td>AAGCTTTAAAGCGCCAACATGCCGCCGTCATG</td>
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</tr>
<tr>
<td>ΔppsA_{S,d}</td>
<td>ppsAACPMutOF</td>
<td>AAGCTTCGCTCCCTTCAACCTGACCCCCGCTGC</td>
<td>1,832</td>
<td>1,832</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ppsAACPMutOR</td>
<td>CCGGCACCTTCACCAGGAAACCGCCGTCG</td>
<td>(split by BssHII)</td>
<td>(no split by BssHII)</td>
<td></td>
</tr>
<tr>
<td>pm7</td>
<td></td>
<td>CGATCGAGGACTTGGCGGTACAG</td>
<td>526</td>
<td>526</td>
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</tr>
<tr>
<td>pm8</td>
<td></td>
<td>GGTGGCCGACGTCGTGGGTCAG</td>
<td>(Ser-to-Ala mutations)</td>
<td>(wild-type sequence)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. PCR primer pairs and amplicons pertaining to construction of mutagenesis cassettes & expression plasmids

Adapted from (1)

<table>
<thead>
<tr>
<th>Amplicon (bp)</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta \text{fadD22} ) 5’ arm (1,025)</td>
<td>Sall FadD22 OF</td>
<td>GTCGACTCACCATCGTGGAGTTCCGCCAGCTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FadD22 IR</td>
<td>TCCTGTTCATGATCCCCGCATATTATGGATGACCACCGCCTTA</td>
<td></td>
</tr>
<tr>
<td>( \Delta \text{fadD22} ) 3’ arm (1,018)</td>
<td>FadD22 IF</td>
<td>ATCCAAATAATGCGGGAATCATGAAACGGAGACACTGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotI FadD22 OR</td>
<td>GCGGCCGCACCGCTGGGCGAGGCCCATT</td>
<td>Full-length cloned into p2NIL as a Sall-NotI fragment.</td>
</tr>
<tr>
<td>( \Delta \text{fadD22} ) full-length (2,016)</td>
<td>Sall FadD22 OF</td>
<td>GTCGACTCACCATCGGAGTTCCGCCAGCTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotI FadD22 OR</td>
<td>GCGGCCGCACCGCTGGGCGAGGCCCATT</td>
<td></td>
</tr>
<tr>
<td>( \Delta \text{fadD26} ) 5’ arm (1029)</td>
<td>HindIII MarFadD26 OF</td>
<td>AAGCTTATGAAAGAATTTCGAGTGAGTAGCCTGCCGACATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FadD26 IR</td>
<td>TCATACCGTACGTGCCGATCCGACACTCACTAAGCTCCATTTC</td>
<td>Full-length cloned into p2NIL as a HindIII-KpnI fragment.</td>
</tr>
<tr>
<td>( \Delta \text{fadD26} ) 3’ arm (958)</td>
<td>FadD26 IF</td>
<td>ATGCCGGTGACCGACGTGACGTAGACACCAGCAGACATCGGTCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KpnI MarFadD26 OR</td>
<td>GCTACCTCGGCGAAACCCGGCGAGACACAGCAACTGAAAATT</td>
<td></td>
</tr>
<tr>
<td>( \Delta \text{fadD26} ) full-length (1952)</td>
<td>HindIII MarFadD26 OF</td>
<td>AAGCTTATGAAAGAATTTCGAGTGAGTAGCCTGCCGACATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KpnI MarFadD26 OR</td>
<td>GCTACCTCGGCGAAACCCGGCGAGACACAGCAACTGAAAATT</td>
<td></td>
</tr>
<tr>
<td>( \Delta \text{fadD28} ) 5’ arm (983)</td>
<td>BamHI MarFadD28 OF</td>
<td>GGATCCACCCCGCTTACCTTCTTGAGGAAAACTGAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FadD28 IR</td>
<td>CTAGACGTCCAGGCCGGGAAACCCGACACTCATGGGGCATCCGTTTA</td>
<td>Full-length cloned into p2NIL as a BamHI-NotI fragment.</td>
</tr>
<tr>
<td>( \Delta \text{fadD28} ) 3’ arm (972)</td>
<td>FadD28 IF</td>
<td>ATGAGTGTGCTTTCCGCCCTGAGCCTGACATCGGTGCAACCGAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotI MarFadD28 OR</td>
<td>GCGGCCGCTTCCGGGCCAGCAGTAGCGCGGGGTGAGTAG</td>
<td></td>
</tr>
<tr>
<td>( \Delta \text{fadD28} ) full-length (1922)</td>
<td>BamHI MarFadD28 OF</td>
<td>GGATCCACCCCGCTTACCTTCTTGAGGAAAACTGAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NotI MarFadD28 OR</td>
<td>GCGGCCGCTTCCGGGCCAGCAGTAGCGCGGGGTGAGTAG</td>
<td></td>
</tr>
<tr>
<td>Amplicon (bp)</td>
<td>Primer name</td>
<td>Primer sequence (5’-3’)</td>
<td>Comments</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>-------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>ΔfadD29c 5’ arm (982)</td>
<td>HindIII Mar FadD29 OF</td>
<td>AAGCTTACCACGGTGAGGATCTGCTGTGATCAAT</td>
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</tr>
<tr>
<td></td>
<td>Mar FadD29 IR</td>
<td>ATGC CGGACCGTCAAAGTCCATGATCATCCTTGCTCTCGACGAA</td>
<td></td>
</tr>
<tr>
<td>ΔfadD29c 3’ arm (1,015)</td>
<td>Mar FadD29 IF</td>
<td>AGGATGATCATGGGACATTGGACCGTGCGCATGACGGCATGTT</td>
<td>Full-length cloned into p2NIL as a HindIII-PmlI fragment.</td>
</tr>
<tr>
<td></td>
<td>PmlI Mar FadD29 OR</td>
<td>CACGTGAAAACCTCGACCAGCTCCGGCGGAATCCGGCATA</td>
<td></td>
</tr>
<tr>
<td>ΔfadD29c full-length (1,966)</td>
<td>HindIII Mar FadD29 OF</td>
<td>AAGCTTACCACGGTGAGGATCTGCTGTGATCAAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PmlI Mar FadD29 OR</td>
<td>CACGTGAAAACCTCGACCAGCTCCGGCGGAATCCGGCATA</td>
<td></td>
</tr>
<tr>
<td>ΔppsA 5’ arm (950)</td>
<td>ppsA ACP Mut OF</td>
<td>AAGCTTCGCTCCCTTACACCTACCCCGCTGCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ppsA ACP Mut IR</td>
<td>AGCACAACGGCGTGCGCGCGGCGCCACGCGGAGGCTA</td>
<td></td>
</tr>
<tr>
<td>ΔppsA 3’ arm (912)</td>
<td>ppsA ACP Mut IF</td>
<td>TGACCTCGGCGTGCGCGCGCGACGGGTTGCTGCT</td>
<td>Full-length cloned into p2NIL as a HindIII-HpaII insert.</td>
</tr>
<tr>
<td></td>
<td>ppsA ACP Mut OR</td>
<td>CCGGCACCTTCACCACGAGAAACCCTGCG</td>
<td></td>
</tr>
<tr>
<td>ΔppsA full-length (1,832)</td>
<td>ppsA ACP Mut OF</td>
<td>AAGCTTCGCTCCCTTACACCTACCCCGCTGCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ppsA ACP Mut OR</td>
<td>CCGGCACCTTCACCACGAGAAACCCTGCG</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Characteristics</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> M (ATCC BAA-535)</td>
<td>human clinical isolate, wild-type, PDIM⁺ PGL⁻</td>
<td>(166)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD22</td>
<td><em>fadD22</em> (MMAR_1761 / MMAR_RS08725)* deletion, PDIM⁺ PGL⁻</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD22-c</td>
<td><em>fadD22</em> deletion, carries a pCP0 vector derivative expressing <em>fadD22</em>, PDIM⁺ PGL⁻ (complemented strain)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD26</td>
<td><em>fadD26</em> (MMAR_1777 / MMAR_RS08805) deletion, PDIM⁻ PGL⁺</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD26-c</td>
<td><em>fadD26</em> deletion, carries a pCP0 derivative expressing <em>fadD26</em>, PDIM⁺ PGL⁻ (complemented strain)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD28</td>
<td><em>fadD28</em> (MMAR_1765 / MMAR_RS08745) deletion, PDIM⁻ PGL⁺</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD28-c</td>
<td><em>fadD28</em> deletion, carries a pCP0 derivative expressing <em>fadD28</em>, PDIM⁺ PGL⁻ (complemented strain)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD29</td>
<td><em>fadD29</em> (MMAR_1759 / MMAR_RS08715) deletion, PDIM⁺ PGL⁻</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔfadD29-c</td>
<td><em>fadD29</em> deletion, carries a pCP0 derivative expressing <em>fadD29</em>, PDIM⁺ PGL⁻ (complemented strain)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔtesA</td>
<td><em>tesA</em> (MMAR_1778 / MMAR_RS08810) deletion, PDIM⁻ PGL⁻</td>
<td>(89)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔtesA-c</td>
<td><em>tesA</em> deletion, carries a pCP0 derivative expressing <em>tesA</em>, PDIM⁻ PGL⁻ (complemented strain)</td>
<td>(89)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔpapA5</td>
<td><em>papA5</em> (MMAR_1768 / MMAR_RS08760) deletion, PDIM⁻ PGL⁺</td>
<td>(92)</td>
<td></td>
</tr>
<tr>
<td><em>M. marinum</em> ΔpapA5-c</td>
<td><em>papA5</em> deletion, carries a pCP0 derivative expressing <em>papA5</em>, PDIM⁻ PGL⁻ (complemented strain)</td>
<td>(92)</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> MC²155 (ATCC 700084)</td>
<td>nonpathogenic species, lacks the PDIM/PGL biosynthetic pathway</td>
<td>(166)</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (2).* Original and re-annotated locus tag designations as per National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) are provided for each gene.
Table 6: Roles of the gene products and the previously determined phenotypes of each deletion mutant and corresponding complemented strain.

Adapted from (2).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
<th>M. marinum deletion mutant</th>
<th>Complementation (Lipid Production)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PDIM Production</td>
<td>PGL Production</td>
<td></td>
</tr>
<tr>
<td>TesA</td>
<td>Thioesterase required for release of polyketide synthase bound products and/or acyl intermediates</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>PapA5</td>
<td>Acyltransferase required for esterification of phthiocerols and phenolphthiocerols with mycocerosic acids</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>FadD28</td>
<td>Activation and loading of fatty acids required for mycocerosic acid synthesis</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>FadD22</td>
<td>Activation and loading of p-hydroxybenzoic acid precursor required for phenolphthiocerols synthesis</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>FadD29</td>
<td>Activation and loading of p-hydroxyphenylalkanoates intermediates precursors required for phenolphthiocerol synthesis</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>FadD26</td>
<td>Activation and loading of fatty acids required for phthiocerol synthesis</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>
REFERENCES


63. Weissman, K. J. (2009) Chapter 1 Introduction to Polyketide Biosynthesis. *Methods in enzymology* 459, 3-16


