AN UNEXPECTED LINK BETWEEN LIPOOLIGOSACCHARIDE BIOSYNTHESIS AND SURFACE PROTEIN RELEASE IN MYCOBACTERIUM MARINUM

Aniek D. van der Woude1,2, Debasmita Sarkar3, Apoorva Bhatt1, Marion Sparrius1, Susanne A. Raadsen1, Louis Boon4, Jeroen Geurtsen1, Astrid M. van der Sar1, Joen Luirink2, Edith N.G. Houben1, Gurdyal S. Besra3 and Wilbert Bitter1,2

From: 1Department of Medical Microbiology and Infection Control, VU university medical center, Amsterdam, The Netherlands; 2Department of Molecular Microbiology, Institute of Molecular Cell Biology, VU University, Amsterdam, The Netherlands; 3School of Biosciences, University of Birmingham, Birmingham, United Kingdom; 4Bioceros BV, Utrecht, The Netherlands

Running title: Lipooligosaccharide biosynthesis and surface protein release

Address correspondence to: Prof. Dr. Wilbert Bitter, Department of Molecular Microbiology, VU University, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, (Tel.) +31205987177, (Email) w.bitter@vumc.nl

Background: Various cell surface proteins of pathogenic mycobacteria have been implicated in virulence.

Results: A screen for secretion defects of specific cell surface proteins in Mycobacterium marinum identified predominantly lipooligosaccharide (LOS) biosynthesis mutants.

Conclusion: Defects in LOS biosynthesis alter the release of cell surface proteins.

Significance: Ten novel genes are described for LOS biosynthesis and increased virulence is observed for a LOS-IV mutant.

The mycobacterial cell envelope is characterized by the presence of a highly impermeable second membrane, which is composed of mycolic acids intercalated with different unusual free lipids, such as lipooligosaccharides (LOS). Transport across this cell envelope requires a dedicated secretion system for extracellular proteins, such as PE_PGRS proteins, which are specific mycobacterial proteins with polymorphic GC-rich sequence (PGRS). In this study, we set out to identify novel components involved in the secretion of PE_PGRS proteins by screening Mycobacterium marinum transposon mutants for secretion defects. Interestingly, most mutants were not affected in secretion, but in the release of PE_PGRS proteins from the cell surface. These mutants had insertions in a gene cluster associated with LOS biosynthesis. Lipid analysis of these mutants revealed a role at different stages of LOS biosynthesis for ten novel genes. Furthermore, we show that regulatory protein WhiB4 is involved in LOS biosynthesis. The absence of the most extended LOS molecule, i.e. LOS-IV, and a concomitant accumulation of LOS-III was already sufficient to reduce the release of PE_PGRS proteins from the mycobacterial cell surface. A similar effect was observed for major surface protein EspE. These results show that the attachment of surface proteins is strongly influenced by the glycolipid composition of the mycobacterial cell envelope. Finally, we tested the virulence of a LOS-IV deficient mutant in our zebrafish embryo infection model. This mutant showed a marked increase in virulence as compared to the wild-type strain, suggesting that LOS-IV plays a role in the modulation of mycobacterial virulence.

Mycobacterium tuberculosis is the causative agent of tuberculosis, one of the world’s major infectious diseases that is responsible for an estimated 1.4 million deaths annually (1). One of the major problems in controlling this disease is that the classical antibiotic treatment regimens for tuberculosis are lengthy and require four
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different drugs. These long treatments are needed due to the high intrinsic resistance of *M. tuberculosis* to antibiotics and the occurrence of highly resistant persister forms. The natural antibiotic resistance of *M. tuberculosis* is partially due to its exceptionally hydrophobic and complex cell envelope structure. Although mycobacteria are classified as high GC Gram-positives, recent cryo-transmission electron microscopy unequivocally showed that the mycobacterial cell envelope consists of two membranes: a cytoplasmic membrane and a unique outer membrane (2-4). The inner leaflet of this atypical outer membrane consists of long-chain fatty acids called mycolic acids, which are covalently linked to a periplasmic arabinogalactan polymer. The outer leaflet of the outer membrane is formed by a range of different and mostly unique (glyco)lipids, which are extractable with organic solvents. This diverse group of extractable glycolipids includes lipoarabinomannan (LAM), phosphatidylinositol mannoside (PIM), trehalose dimycolate (TDM), phenolic glycolipid (PGL) and lipooligosaccharide (LOS) (5). Together with the mycolic acids they form a thick permeability barrier.

To facilitate transport of virulence factors and other surface molecules over the mycobacterial cell envelope dedicated secretion systems are required. Recently, a group of such secretion systems has been identified in mycobacteria, which was classified as type VII secretion systems (T7SS) (6). *M. tuberculosis* contains five different T7SS, each of which is encoded by a specific gene cluster, ESX-1 to ESX-5. Of these T7SS, the ESX-5 secretion system is predicted to be the most recently evolved system and is restricted to the slow-growing mycobacteria, including all major pathogens (7). Several T7SS were found to mediate the secretion of small proteins belonging to the WXG100 family (8), such as EsxN, which is secreted by ESX-5. In addition, ESX-5 has been shown to be responsible for the secretion of different members of the PE and PPE protein families (9,10). These enigmatic protein families are unique to mycobacteria and highly expanded among slow-growing mycobacteria, especially *Mycobacterium kansasii*, *Mycobacterium marinum* and species of the *M. tuberculosis* complex (11). Their names are derived from a conserved N-terminal motif of respectively Pro-Glu (PE) and Pro-Pro-Glu (PPE) residues, although the homology regions are in fact considerably larger (12).

The PE and PPE families can be subdivided into different subfamilies, of which the PE_PGRS family is the largest, consisting of 67 members in *M. tuberculosis* and 148 members in *M. marinum* (12,13). PE_PGRS proteins are characterized by multiple tandem repeats of glycine and alanine residues (14). Due to this highly specific amino acid composition the encoding genes have a very high percentage of GC residues, which is reflected in their name (polymorphic GC rich sequence). The large expansion of PE_PGRS genes on the genome of *M. tuberculosis* suggests a role in virulence or antigenic variation. However, although PE_PGRS proteins indeed seem to be located at the cell surface, their function remains largely unknown. Secretion and surface localization of PE_PGRS proteins have recently been shown to depend on an intact ESX-5 secretion system (10).

In search for additional essential components of the ESX-5 system, we conducted a transposon mutant screen in *M. marinum* for impaired PE_PGRS secretion. Surprisingly, most PE_PGRS secretion mutants were located in the genomic region described to be involved in LOS biosynthesis (15). LOS is a trehalose-based glycolipid present in the mycobacterial outer membrane. This glycolipid has been described for several mycobacterial species, including *M. marinum* (16) and *Mycobacterium canettii* of the *M. tuberculosis* complex (17). Interestingly, other species of the *M. tuberculosis* complex have lost the ability to produce LOS. In contrast to several other mycobacterial glycolipids, the length and composition of LOS is highly variable between different species. *M. marinum* produces under laboratory conditions four different LOS structures of increasing size, in which different unusual sugar moieties are sequentially added to a core of acylated trehalose (15). The genes responsible for LOS biosynthesis of *M. marinum* have been proposed to comprise mmar_2302 through mmar_2344 (15,18). So far, only six mutants affected in this genomic region have been characterized and
were demonstrated to be involved in LOS biosynthesis (15,16,18-20). In this study we have identified ten novel genes, both expected and unexpected, with a role at different stages of LOS biosynthesis. We also show that transcriptional regulatory protein WhiB4 affects LOS biosynthesis gene expression. Interestingly, PE_PGRS proteins appear to be more firmly attached to the cell surface of these LOS mutants. This, together with the diversity and high number of the LOS biosynthesis mutants found, indicates an intriguing relation between PE_PGRS release and cell wall composition.

**EXPERIMENTAL PROCEDURES**

_Bacterial strains and culture conditions_- Wild-type and mutant _M. marinum_ strains _M USA_ (21) and E11 (22,23) were routinely grown at 30°C in Middlebrook 7H9 (Difco) liquid medium and on Middlebrook 7H10 plates (Difco) supplemented with 10% Middlebrook ADC and 0.05% Tween-80 (BD, Biosciences) or only OADC, respectively. _Escherichia coli_ strain DH5α was used for amplification and manipulation of plasmid DNA. Antibiotics concentrations used: kanamycin 25µg/ml, hygromycin 50µg/ml and chloramphenicol 30µg/ml and 10µg/ml for mycobacteria and _E. coli_, respectively.

_Construction and screening of _M. marinum_ transposon library_- Transposon mutagenesis was performed on _M. marinum_ E11 or _M USA_ using the mycobacterial specific phage φMycobMarT7 containing the mariner-like transposon Himar1 (24). The resulting transposon insertion mutant library was plated on nitrocellulose filters (Millipore HATF08250) that were placed on 7H10 agar plates for a double filter assay as described previously (9). After colonies appeared on the nitrocellulose filters, these filters were placed on top of a second nitrocellulose filter on a fresh plate and incubated overnight at 30°C. Subsequently, the bottom filter containing the secreted proteins was stained for the presence of PE_PGRS proteins using a mouse monoclonal antibody that recognizes the PGRS domain of PE_PGRS proteins (mAb 7C4.1F7) (10). To obtain large amounts of purified antibody, clone mAb 7C4.1F7 was cultured in IMDM supplemented with 1% FCS. Subsequently, the antibodies were purified using a gradient of ammonium sulphate on thiophilic agarose (AFFI-T, KemEnTec) and dialyzed against PBS. After incubation with the PGRS antibody and a conjugate (Goat- anti Mouse horse radish peroxidase (HRP)), the presence of PE_PGRS proteins on the filter was visualized by staining with 4-chloronaphthol/3,3-diaminobenzidine. Colonies with reduced amounts of secreted PE_PGRS proteins were selected and re-checked for PE_PGRS secretion defects in a second double filter assay. In a similar assay, a selection of mutants was also tested with antiserum directed against the surface protein EspE (4,25).

_Identification of transposon insertion site_- To identify the location of the transposon, DNA was isolated and ligation-mediated PCR was performed using primers bampt2, salgD, pSalg and pMyc01 (Table S1) as described previously (9). The resulting DNA fragments were sequenced and compared to the _M. marinum_ genome sequence (http://genolist.pasteur.fr/MarinoList/). To determine the orientation of the transposon, genomic DNA of the mutants was used in a PCR reaction with one of the primers that anneal to either ends of the transposon, T7_1 or T7_2, together with a gene specific primer (Table S1).

_SDS-PAGE and immunoblotting_- Mycobacteria were grown routinely to mid-logarithmic phase. Subsequently, the bacteria were washed twice in Middlebrook 7H9 medium without supplement to remove all traces of bovine serum albumin (BSA) and grown overnight in Middlebrook 7H9 liquid medium supplemented with 0.2% dextrose and optionally 0.05% Tween-80. Secreted proteins present in cell-free supernatants were obtained by centrifugation and subsequent passage through a 0.45 µm filter, and concentrated by precipitation with 10% trichloroacetic acid in 10% acetone. Bacterial pellets were disrupted with 425-600 µm glass beads (Sigma) in PBS for 3 minutes using a Mini-beadbeater (BioSpec Products). Subsequently, the protein concentration of these samples was measured using the BCA protein assay (Pierce) to assure equal loading. Based on wild-type OD600 measurements, an equivalent of
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0.1 OD pellet and 0.6 OD supernatant were loaded on 10 or 15% SDS-PAGE gels (Biorad). Proteins were visualized by immunoblotting using mouse monoclonal antiserum against GroEL2 (CS44, Colorado State University), rabbit antiserum reactive to EsxN (rMt9.9A (26)), the ESAT-6 specific mouse monoclonal antiserum Hyb76-8 (27) and the antibody against PE_PGRS that was described above. The presence of bound HRP-conjugated secondary antibodies was detected via chemiluminescence (Pierce) using a CCD camera (Biorad).

For the extraction of surface proteins from plate-grown bacteria, a loop-full of bacteria grown on 7H10 plates was resuspended in PBS, spun down and washed again in PBS. Subsequently, bacteria were incubated with 0.5% Genapol X-080 (Sigma-Aldrich) for 30 minutes at room temperature and centrifuged to separate bacterial cells from extracted fractions. Both fractions were analyzed by SDS-PAGE and immunoblotting using anti-PGRS, anti-GroEL and anti-EspE as described above.

Molecular cloning - M. marinum E11 chromosomal DNA was used as a template to amplify the papA3 gene using Pfu polymerase (Fermentas) with gene specific primers (Table S1). The resulting PCR product was ligated into the cloning vector pJet1.2 (Fermentas), subsequently isolated by BglII restriction and cloned in BamHI-digested shuttle vector pSMT3-eGFP (28) under control of the hsp60 promoter, resulting in pSMT3-papA3-eGFP. Both the whiB4 gene and the operon mmar_2319-2322 were amplified from M. marinum E11 chromosomal DNA using Phusion polymerase (Finnzymes) and specific primers containing anchoring restriction sites (Table S1). The resulting PCR products and integration vector pUC-Int-cat (10) were digested with XbaI and either EcoRV or Stul for the mmar_2319-2322 operon. Subsequent ligation resulted in plasmids pUC-Int-cat-whiB4 and pUC-Int-cat-mmar_2319-22. All resulting complementation constructs were introduced by electroporation in the M. marinum wecE (A3 and A8), papA3 (A5) and whiB4 (A9) transposon mutants as described previously (9).

RNA extraction and quantitative real-time PCR - Bacterial cultures of 15ml were pelleted at OD600 of 0.8-2.0 and immediately dissolved in 1ml cold TRIZol (Invitrogen). Subsequently, cells were lysed with using a Mini-beadbeater for 2 min, followed by 10 min incubation at 60°C. After centrifugation, the lysate was used to extract RNA after incubation with 200µl chloroform and centrifugation. Next, the RNA in the aqueous phase was precipitated with 2-propanol and, after resuspension, treated with DNase (Fermentas). cDNA was generated from ~200ng RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR (RT-PCR) was performed using the SYBR GreenER qPCR kit (Invitrogen) and the Lightcycler 480 (Roche) to amplify parts of the LOS biosynthesis region with specific primers (Table S1). Ct values were normalized to values obtained for the mycobacterial household gene sigA.

Lipid extraction and analysis - Polar lipids were extracted and analyzed as described in Burguiere et al. (16)

Zebrafish embryo infection - To visualize bacteria in zebrafish embryos, a vector containing a red fluorescent marker, pSMT3-mCherry (29), was introduced by electroporation into M. marinum E11 wt, wecE mutant A3 and A8 and A3 complemented with pUC-Int-cat-mmar_2322-19. For infections, a culture with an OD600 of approximately 1.0 was centrifuged at 3000xg, resuspended in PBS with 0.3% Tween and subsequently incubated for 10 minutes to obtain a single cell suspension. After another centrifugation step, the pellet was resuspended in 1 ml PBS and four times diluted in phenol red for the final injection solution. Zebrafish embryos were obtained and infected as described by van der Sar et al. (30). The equivalent of one injection was plated on 7H10 plates to determine the inoculum. At five days post infection (5dpi), the embryos were anesthetized in 0.02% buffered 3-aminobenzoic acid methyl ester (MS222, Sigma) and the infection was monitored by visualizing red fluorescence using a Leica MZ 16FA stereomicroscope. The relative fluorescence compared to wild-type infection was quantified by a specifically designed, dedicated analysis software program (31). In addition, a random selection of the infected...
zebrafish embryos were subsequently selected for whole embryo plating to determine bacterial counts (31).

RESULTS

Identification of PE_PGRS secretion mutants indicate link to LOS biosynthesis.- In a previous screen, three mutants deficient for PPE41 secretion were identified in M. marinum, all with a transposon insertion in the ESX-5 cluster, two in gene eccA5 and one in mmar_2676 (9,10). Further analysis demonstrated that these mutants were unable to secrete various other PPE proteins and also PE_PGRS proteins (10). To identify additional proteins involved in ESX-5-dependent secretion, we decided to perform a different transposon mutant screen, this time screening for impaired PE_PGRS secretion using a monoclonal antiserum raised against the PGRS domain of PE_PGRS33 (Rv1818c). This antibody specifically interacts with the PGRS domain of multiple proteins (10), and could therefore potentially recognize all 148 putative PE_PGRS proteins of M. marinum.

A mariner transposon library of approximately 12000 mutants of M. marinum strain E11 was plated on nitrocellulose filters and screened for PE_PGRS secretion in a double filter screening assay (see Methods). Mutants affected in PE_PGRS secretion were selected and retested in a second double filter assay. This resulted in twenty-six mutants that were consistently reduced or negative in PE_PGRS secretion. The mariner transposon insertion sites of all twenty-six mutants were determined by ligation-mediated PCR followed by sequencing (Table 1). Although the number of secretion deficient mutants was much higher than expected for ESX-5 mutants (9,10), it was still surprising that none of the PE_PGRS secretion mutants were found to be disrupted in the ESX-5 cluster. However, remarkably, for 23 out of the 26 mutants the transposon-insertion site was located in the same genomic region between genes mmar_2313 and mmar_2405, with several mutants containing a transposon at different positions in the same gene (Table 1 and Fig. 1). Part of this genomic region (mmar_2302 to mmar_2344) has been described previously to be involved in the biosynthesis of LOS (15,18).

After identification of the transposon insertion sites, the orientation of the inserted transposons was determined. This analysis showed that the transposons inserted in the same gene were commonly found in both orientations (Table 1), ruling out transposon-induced alterations of gene expression. Finally, to investigate strain-dependency, we have also performed a small screen for PE_PGRS secretion defects in M. marinum strain M_USA. The identified PE_PGRS secretion mutant M168 was also located in the LOS biosynthesis cluster (Table 1), which suggests that the link between LOS biosynthesis and PE_PGRS secretion is not strain-specific. In conclusion, the diversity and high number of detected mutants in the LOS biosynthesis region indicate a strong link between LOS production and PE_PGRS secretion.

All mutants within the LOS gene cluster show specific defects in LOS biosynthesis - M. marinum produces four different LOS structures, designated LOS-I to LOS-IV. The biosynthesis of these LOS variants is proposed to be sequential. The structure of LOS-I is 3-O-Me-Rhap-(1-3)-Glcp-(1-3)-Glc (16). The addition of xylose together with one or two molecules of the highly unusual sugar caryophyllose (19) produces LOS-II and LOS-III, respectively. The molecule added to produce LOS-IV has been characterized as a heterogenic group of mainly one acidic form of a N-acylated 4-amino-4,6-dideoxy-Galp residue (resulting in LOS-IVc) with a minor population of two neutral forms (LOS-IVa and b) (32). The biosynthetic pathway for LOS and the genes involved are largely unknown. Only two of the genes identified in this screen have been previously described to be involved in LOS production, i.e. losA (mmar_2313) was shown to have a role in LOS-IV production, whereas an ilvB1_3 (mmar_2332) mutant produces only LOS-I and a LOS-II intermediate, lacking caryophyllose (LOS-II*) (15,16). To determine whether the genes disrupted in the newly identified mutants have a role in LOS biosynthesis as well, their polar lipid profiles were examined by two-dimensional thin-layer chromatography (2D-TLC) (Fig. 2A/B and Table 2).
Both mutants A3 and A8, disrupted in a different location of \textit{mmar\_2320} with opposite directions of the transposon, showed a distinctive 2D-TLC pattern with a specific defect in the biosynthesis of LOS-IV and a concomitant accumulation of LOS-III (shown for A3 in Fig. 2A). Analysis of this product of mutant A8 by MALDI-MS (Fig. 2C) and NMR (not shown) confirmed that the accumulating intermediate was indeed LOS-III. \textit{Mmar\_2320} codes for WecE. A WecE homologue of \textit{E. coli} has been shown to be a sugar aminotransferase (33). Therefore, it probably has a function in the biosynthesis of the unusual sugar residue of LOS-IV. Complementation of the A3 mutant with integrative vector pUC-int-cat containing the intact \textit{mmar\_2319-2322} operon (A3c) restored LOS-IV biosynthesis (Fig. 2A). Similar to the \textit{wecE} mutants A3 and A8, disruption of downstream gene \textit{mmar\_2319} (mutant B10), encoding a hypothetical transmembrane protein, resulted in LOS-IV deficiency (Fig. 2A). To exclude the possibility that the LOS biosynthesis defect observed for the \textit{wecE} mutant A3 is caused by a polar effect on \textit{mmar\_2319}, the expression levels of this gene in mutant A3 were compared to wild-type levels by quantitative real-time PCR (qRT-PCR). This showed that \textit{mmar\_2319} expression was not decreased, but in fact elevated in the A3 mutant, possibly due to the promoter activity encoded by the transposon. Increased \textit{mmar\_2319} expression levels were equally high for the complemented mutant A3c with restored LOS biosynthesis (results not shown), suggesting that \textit{wecE} disruption is indeed responsible for the observed LOS-IV deficiency of the A3 mutant.

Also other mutants in the LOS region showed various defects in LOS biosynthesis. Mutant B2, disrupted in \textit{mmar\_2327} encoding a multiple transmembrane protein, lost the ability to produce both LOS-III and LOS-IV and accumulated large amounts of LOS-II (Fig. 2A/D). The \textit{mmar\_2336} mutant B7, a gene encoding a putative UDP-glucose 4-epimerase, appeared to accumulate LOS-II* (Fig. 2A), a phenotype similar to the previously described mutant in \textit{ilvB1\_3} (also identified in our screen) (15). The \textit{M\textsuperscript{USX}} mutant M168 in \textit{mmar\_2307} appeared to accumulate LOS-I (Fig. 2B/E), similar as observed for a \textit{mmar\_2309} gene disruption (15).

The 2D-TLC patterns for polar lipids of the \textit{mmar\_2340} and \textit{mmar\_2341} mutants A2 and A4 revealed that they were both completely deficient in LOS production, as none of the four \textit{M. marinum} LOS structures were visible (Fig. 2A). This suggests that these genes have a role in the biosynthesis of the core acylated trehalose structure of LOS. Gene \textit{mmar\_2340} encodes a putative polyketide synthase, Pks5, which was already suggested to be involved in the synthesis of the acyl chains of LOS, based on lipid analysis of a \textit{M. smegmatis} mutant disrupted in the orthologue of \textit{pks5} (34). The downstream gene \textit{mmar\_2341} is annotated as a fatty acyl AMP ligase, FadD25, which has a proposed role in loading an adenylated metabolite onto the Pks5 multienzyme for extension. Therefore, it is not surprising that disruption of this gene resulted in a phenotype similar to the \textit{pks5} mutant.

The presence of LOS in mycobacteria has been associated with smooth colony morphology. For instance, \textit{M. kansasii} strains devoid of LOS have a rough colony morphology, whereas all tested strains with LOS were smooth (35). In \textit{M. marinum} defects in higher order LOS biosynthesis have also been correlated with altered colony morphology, described as a smooth-wrinkled phenotype (15,16). Our mutants with a shorter or modified LOS production also showed a smooth-wrinkled phenotype, whereas all isolates devoid of LOS exhibited characteristic a rough and dry colony morphology (Fig. 2F and Table 1) similar to the \textit{M. kansasii} strains.

Identification of unexpected genes involved in LOS biosynthesis - Interestingly, the rough-dry phenotype was also observed for some mutants located downstream of the proposed LOS biosynthesis region, \textit{i.e.} in genes \textit{mmar\_2353, mmar\_2355} and \textit{mmar\_2405}, and for one mutant located far from the LOS biosynthesis region, in gene \textit{mmar\_5170} (Table 1). The mutants disrupted in genes \textit{mmar\_1008} and \textit{mmar\_4419} exhibited wild-type colony morphology and were therefore not further analyzed. To examine whether these four aggregating mutants have a
role in LOS production, their polar lipid content was analyzed.

The 2D-TLC analysis of mutant A5 (mmar_2355) confirmed that this mutant did not produce LOS (Fig. 2A). This gene encodes the conserved polyketide synthase-associated protein PapA3. PapA3 might function as an acyltransferase associated with Pks5, explaining its role in the biosynthesis of the LOS core structure of acylated trehalose. LOS production of mutant A5 could be restored with a shuttle vector containing an intact copy of the papA3 gene (Fig. 2A). Mutants A7 and A1 with a disruption in the gene mmar_2353, located downstream of papA3 in the same operon, were both not visibly affected in LOS production as shown by 2D-TLC analysis, although the total amount of LOS may be reduced (shown for A1 in Fig. 2A), which would be in line with the observed colony phenotype and genomic location of this mutant.

Disruption of mmar_2405 (mutant A6) also resulted in a LOS-deficient phenotype (Fig. 2A). Surprisingly, this gene is annotated as a homologue of a cyanophycinase and, among the genus Mycobacterium, is unique to M. marinum. Cyanophycinase is a serine protease specialized in the degradation of cyanophycin, a large polymer of arginine and aspartic acid used as nitrogen storage by cyanobacteria (36). The M. marinum cyanophycinase is actually a fusion of two copies of cyanophycinase, positioned head-to-tail linked by a short stretch of amino acids, with one copy mutated in its active site residues (37). Apparently, this putative protease is necessary for biosynthesis or transport of the LOS core structure. Together, these results show that the LOS biosynthesis region is even more extended than proposed by Ren et al. (15)(Fig. 1).

*WhiB4 regulates expression of several LOS biosynthesis genes.* - Mutant A9 is disrupted in a gene located outside the LOS biosynthesis region, i.e. mmar_5170, coding for WhiB4. WhiB4 belongs to the family of WhiB-like proteins, putative transcriptional regulatory proteins characterized by an iron-sulfur cluster, of which six are annotated in M. marinum. In accordance with the colony morphology, 2D-TLC analysis of polar lipids of the whiB4 mutant showed that LOS production was affected in this mutant. LOS biosynthesis is highly diminished, although some traces of LOS, especially LOS-I, seem to be present (Fig. 3A). Reintroduction of an intact copy of the whiB4 gene restored LOS biosynthesis to levels almost comparable to wild-type (Fig. 3A). Quantification of the different LOS structures produced by wild-type, A9 and A9c show that total LOS biosynthesis of mutant A9 is roughly 50% percent diminished, most strongly observed for LOS-III and LOS-IV biosynthesis (Fig. 3B).

Recently, WhiB3, a WhiB4 paralogue, was shown to specifically control lipid biosynthesis by regulating the expression of pks2 and pks3 (38). Therefore, we hypothesized that WhiB4 regulates the production of LOS by controlling expression of genes in the LOS biosynthesis cluster. To examine this, we compared 3 biological replicates of wild-type, whiB4 mutant A9 and the complemented mutant A9c for their expression levels of different LOS biosynthesis genes in qRT-PCR (Fig. 3C). In our analysis we included genes necessary for the LOS core structure, pks5, fadD25 and papA3, but also genes involved in specific steps of higher order LOS biosynthesis, mmar_2319, wecE (LOS-IV) and mmar_2327 (LOS-III). As a negative control, pks12 was included, a gene which is involved in the biosynthesis of phosphoglycolipid mycoketide (39). Interestingly, the whiB4 mutant showed diminished expression for many of the tested LOS genes, but most apparent for mmar_2327 and papA3, which showed respectively 4% and 10% of wild-type expression levels (Fig. 3C). Other genes of these putative operons, namely mmar_2326 and mmar_2354, are likewise affected. The strong regulation effect on mmar_2327 might explain why the LOS biosynthesis defects are most evident for LOS-III and LOS-IV. These qRT-PCR data also show that complementation is only partial for many of the tested genes (Fig 3C), which correlates with the 2D-TLC data (Fig. 3A/B). In conclusion, WhiB4 has a role in LOS biosynthesis, which is likely by regulating expression of multiple genes of the LOS cluster.

*LOS deficiency decreases PE_PGRS release from the cell surface.* - The ESX-5 mutant 7C1 is
highly affected in both secretion and expression of PE_PGRS proteins (shown in ref. 10 and Fig. 4A). To determine whether the identified LOS mutants are similarly affected in expression and/or secretion of specific PE_PGRS proteins, a selection of these mutants was grown in liquid cultures and secretion was analyzed by immunoblotting. Surprisingly, this analysis showed that production of PE_PGRS proteins was not affected and the extracellular levels of PE_PGRS were comparable to wild-type levels for all tested LOS mutants (not shown). This suggests that PE_PGRS production and secretion were normal under these conditions. In these experiments, we used culture medium supplemented with the detergent Tween-80, which is not present in 7H10 agar. Mycobacterial culture medium is routinely supplemented with this detergent to avoid bacterial aggregation (40). Since Tween-80 is known to (partially) remove mycobacterial surface structures, e.g. the capsule (4), we decided to re-test the mutants for PE_PGRS secretion in liquid cultures devoid of detergent. As PE_PGRS proteins are partially extracted by Tween-80 (4), their presence in the supernatant of wild-type M. marinum was reduced in culture conditions without detergent, but the overall pattern of secreted PE_PGRS was similar. The omission of Tween-80 however almost completely abolished the extracellular accumulation of PE_PGRS for the LOS-deficient mutants, shown in Fig. 4A for a selection of the mutants. This release effect could be restored by complementation (shown for the A5 mutant Fig. 4B). No such PE_PGRS release defects could be observed for mutants deficient in higher order LOS biosynthesis (Fig. 4A). To investigate the extent of the release or secretion deficiency of the LOS mutants, their culture filtrates were also analyzed for the presence of ESX-5 substrate EsxN and ESX-1 substrate ESAT-6. Interestingly, the LOS-deficient mutants also showed a reproducible reduction in EsxN secretion, whereas the secretion of ESAT-6 was unaffected in all conditions tested (Fig. 4A). Next, we also tested the extraction of surface proteins with the mild detergent Genapol X-080. This analysis further demonstrated that PE_PGRS proteins expressed by the mutants were normally surface localized (Fig. 4C), indicating that the lack of PE_PGRS secretion observed in the double filter assay was not caused by a transport defect. Taken together, these experiments demonstrate that LOS biosynthesis mutants are not disturbed in PE_PGRS translocation, but show, in the absence of detergent, a stronger attachment of PE_PGRS proteins to the cell surface.

This detergent-dependent increase in PE_PGRS cell surface attachment observed for LOS mutants indicates that in these mutants the capsule layer, which is also detergent labile and has shown to contain PE_PGRS proteins (4), may be affected. To further explore this possibility, we next studied the secretion of EspE, a major capsular protein (4). Using a double filter assay we found that release of EspE was clearly affected in all tested LOS biosynthesis mutants (Fig. 4D). This effect was not seen for the negative control transposon mutant in pks12, which was obtained in a different screen (Stoop et al., in preparation). Similar to PE_PGRS proteins, the level of EspE extraction with the mild detergent Genapol X-080 did not differ from that of wild-type bacteria (Fig. 4C), showing that EspE was still surface localized in the LOS mutants. Together, these results show that LOS biosynthesis defects result in a tighter surface attachment of capsular proteins, which presents itself as a secretion defect of these proteins in the double filter assay.

wecE mutant shows increased virulence in zebrafish embryo infection model. LOS has been shown to be antigenic (41), although its exact role in virulence has not been elucidated yet. Previous in vitro studies on the role of higher order LOS in M. marinum have suggested a role in macrophage cell entry and Tumor Necrosis Factor α (TNF-α) response (15,19). To study the effects of different levels of LOS deficiency in vivo, we decided to use the zebrafish embryo infection model. Infection of zebrafish embryos with (fluorescent) M. marinum leads to the formation of granuloma-like aggregates of (infected) macrophages, which can be monitored with fluorescence microscopy (42,43). Attempts to study the virulence of the LOS-deficient mutants in this infection model were unsuccessful due to the excessive aggregation of these mutants upon growth in

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both solid and liquid medium. Therefore, we limited our analysis to the wecE mutants A3 and A8 that do not show this clumping effect.

Zebrafish embryos were infected with an inoculum of roughly 100 colony forming units (cfu) of red fluorescent M. marinum E11, the LOS-IV deficient wecE mutants A3 and A8 and the complemented strain (A3c). The ESX-1 mutant eccC::tn was used as a negative control (31). Five days post infection (5dpi), the degree of infection and formation of early granulomas was monitored by measuring the relative red fluorescence. Remarkably, the embryos infected with the wecE mutants developed more and larger granulomatous structures than observed in wild-type infection (Fig. 5A). Close inspection of these aggregates showed that the localization of bacteria was similar to wild-type infection; i.e. all bacteria appeared intracellular (results not shown). This enhanced granuloma formation was quantified in three separate experiments as a ten-fold average increase for both mutants (Fig. 5B). Complementation of the wecE mutant A3, which was shown to restore LOS-IV biosynthesis (Fig. 2A), likewise restored infection to wild-type levels (Fig. 5B). In accordance with the fluorescence quantification, significantly more bacteria (3-fold) were recovered from embryos infected with the wecE mutant A8, as compared to a wild-type infection (Fig. 5B). The observed increased virulence in vivo is probably not due to a growth advantage, since the wecE mutants show no differences in growth rate when grown in culture medium (Fig. 5D). Our data demonstrate that disruption of wecE in M. marinum, which results in LOS-IV deficiency, causes hypervirulence in zebrafish embryos, which suggests that LOS-IV directly or indirectly plays a role in modulating innate immune response.

DISCUSSION

In M. marinum PE_PGRS proteins are substrates for the ESX-5 secretion system (10). Therefore, by screening transposon mutants for defects in the secretion of PE_PGRS proteins, we expected to find mutants in the ESX-5 secretion system. However, our screen of ~12000 clones surprisingly did not result in the identification of novel ESX-5 mutants. A possible explanation for this unanticipated result is that different transposon mutagenesis studies in both M. marinum and M. tuberculosis have shown that mutants in most ESX-5 genes cannot be isolated (10,24)(unpublished results), indicating that these mutations might be lethal. Perhaps, a more saturating transposon mutant screen for PE_PGRS secretion in M. marinum might lead to the isolation of ESX-5 mutants similar to the ones identified previously (9,10), for which indeed higher number of colonies were screened.

The high number of identified secretion mutants with a disruption in the LOS biosynthesis region and the large variety of biosynthesis defects found, ranging from LOS-IV to complete LOS deficiency, show a clear link between PE_PGRS secretion and LOS biosynthesis in the double filter assay. Surprisingly, all LOS mutants still produce wild-type levels of PE_PGRS proteins and these PE_PGRS proteins are still (partially) surface exposed. PE_PGRS proteins seem to be more tightly attached to the surface of LOS mutants, resulting in an apparent secretion defect in a double filter blot assay. Although this effect is stronger for the LOS negative mutants, already the absence of LOS-IV resulted in an altered surface attachment of PE_PGRS proteins, indicating that the terminal sugar moiety plays a role in this process. For the mutants deficient in higher order LOS, this tighter attachment of PE_PGRS proteins can already be neutralized by growing them in liquid culture with agitation. The LOS negative mutants need the supplemental addition of Tween-80 for the release of PE_PGRS proteins. It was previously shown that the presence of Tween-80 in culture medium (partially) removes the mycobacterial capsule layer, including some PE_PGRS proteins (4), which suggests that in LOS-deficient mutants capsular proteins in general are more tightly attached to the cell surface. The observation that the surface attachment of the major capsular protein EspE is also affected in all LOS mutants confirms this change in capsular properties. One possibility is that LOS functions as a detergent for these capsular proteins. However, co-culturing of a LOS mutant mixed with an ESX-5 mutant, which expresses all LOS structures, did not show cross-complementation in the double filter assay (results not shown).
This suggests that the LOS of the ESX-5 mutant fails to function as a detergent for surface localized PE_PGRS proteins of the LOS mutant. Interestingly, for both M. bovis BCG and M. tuberculosis, which do not produce LOS, PE_PGRS release in the culture supernatant seems much lower than in M. marinum, while levels of secreted ESAT-6 are similar (unpublished results). The absence of LOS might explain these PE_PGRS secretion/release differences. Further studies of the capsule of the LOS mutants in M. marinum might give us more insight in the interaction of PE_PGRS proteins and the mycobacterial cell wall.

All M. marinum LOS-deficient mutants show a rough-dry colony morphology that resembles M. tuberculosis colony morphology, indicating a change in cell envelope structure. This rough-dry phenotype is also strongly associated with LOS deficiency in M. kansasii (35), but less clearly in M. gastri (44) and M. canetti (45). In M. marinum two mutants with rough-dry colony morphology, A1/A7 (mmar_2353) and A9 (whiB4), were still LOS producing. However, for the whiB4 mutant LOS production was clearly diminished, suggesting that a decrease in total LOS content could be responsible for the rough-dry colony morphology. Although the mmar_2353 mutant did not clearly show diminished LOS production, this gene is located within the extended LOS biosynthesis cluster and could therefore play a role in LOS production.

Apart from the observed link between PE_PGRS surface attachment and LOS production, this study also led to the identification of ten novel LOS biosynthesis mutants. We showed that the genes coding for Pks5 and FadD25 are both necessary for the synthesis of the core LOS structure, which supports their previously proposed role in the synthesis and activation of the fatty acids linked to the trehalose core (18). The gene disruption of papA3 also resulted in a LOS-deficient phenotype. Although this gene is located downstream of the putative LOS region, the role of an acyltransferase in the synthesis of the LOS core structure is not surprising. Interestingly, a papA4 mutant in M. marinum was also shown to be deficient in LOS biosynthesis, showing two different acyltransferases are necessary for the synthesis of the core LOS structure (18). A more unexpected finding is the involvement of the putative peptide protease CphB in LOS biosynthesis. Among the sequenced strains of the genus Mycobacterium this protease is unique to M. marinum (37) and its function is unclear. It is hard to imagine a role for this peptidase in LOS biosynthesis, as LOS does not contain peptides. Perhaps, CphB also plays a role in gene regulation. Further research is necessary to clarify the role of this gene in LOS biosynthesis.

The other five LOS mutants detected in this screen show a specific defect in higher order LOS production and a concomitant accumulation of the lower order LOS structures. The genes affected in these mutants also show a specific spatial genomic clustering, according to their role in LOS biosynthesis. Mutants that show LOS-I accumulation, indicating a deficiency to synthesize, attach or transport the xylose unit to the LOS structure, are found at the beginning of the LOS biosynthesis cluster, in genes mmar_2307 and mmar_2309. The region responsible for the synthesis and attachment of caryophyllose to produce LOS-II and LOS-III seems to be localized roughly between genes mmar_2327 and mmar_2336. Also a rather large part of the genome cluster, genes mmar_2313 to mmar_2320, seems to be reserved for the synthesis and transfer of the amino sugar necessary to produce LOS-IV. Together, these results show that the genomic locus involved in LOS biosynthesis is more extended than previously suggested.

The specific decrease in LOS production of the whiB4 mutant together with its predicted function in gene regulation suggests that this protein has a role in regulating LOS biosynthesis. In line with this hypothesis, we have shown that the expression of a number of LOS biosynthesis genes is strongly decreased in this mutant. Full complementation was only achieved for some genes, although we tested multiple complementing constructs (not shown). The effect of WhiB4 on LOS gene regulation could either be direct or indirect, through a general effect on metabolism. Because the whiB4 mutation had no effect on the pks12 gene involved in mycoketide biogenesis, we favor the first option. Correspondingly, WhiB3 has been shown to regulate biosynthesis of various
polyketides (38), which could indicate that WhiB proteins are important regulators of lipid biosynthesis in mycobacteria.

A number of microarray and qRT-PCR assays have shown that WhiB4 itself in *M. tuberculosis* is regulated in a variety of stress conditions such as macrophage infection and nutrient starvation (46-48), indicating it has an important role in infection. Biochemical and biophysical studies of recombinant *M. tuberculosis* WhiB4 showed that it has disulphide reductase activity upon removal of the iron sulfur cluster (49), but it is unclear how this observation relates to transcriptional regulatory activity. *M. tuberculosis* does not produce LOS, but does contain a large part of the LOS biosynthesis genome cluster, including a homologue of *mmar_2327* (15), which might be involved in biosynthesis of a different glycolipid.

A mutation in the *wecE* gene of *M. marinum*, resulting in LOS-IV deficiency and concomitant LOS-III accumulation, leads to significantly increased bacterial growth and early granuloma formation in zebrafish embryos. Increased virulence as a result of a single mutation is rarely observed. For instance, in an extensive screen for mycobacterial virulence of 1,000 transposon mutants of *M. marinum* in zebrafish embryos only a single hypervirulence mutant was found (31)(Stoop and van der Sar, personal communication). These *in vivo* data highlight the importance of WecE as a factor to suppress virulence in early mycobacterial infection. Whether the observed hypervirulence is actually caused by the loss of LOS-IV, the accumulation of LOS-III or the altered release of surface proteins, such as PE_PGRS, is not clear at this point. In contrast to these *in vivo* experiments, recent *in vitro* infections of murine macrophage cell lines with different *M. marinum* LOS mutants demonstrated impaired cell-entry efficiency (15). Also, the addition of purified LOS-IV to macrophages resulted in an altered pro-inflammatory response (19,32). However, from our studies it seems that LOS-IV deficiency actually results in a more pronounced infection, suggesting a different mechanism in the context of zebrafish embryos. Increased virulence was also observed in mouse infection studies with different *M. kansasii* strains. These studies showed that smooth strains producing LOS were rapidly cleared from the animal organs, whereas the rough LOS-deficient strains showed greater persistence (35,50). It has been proposed that the presence of LOS might act as a mask for other surface-associated factors, such as LAM and PGL (35); enhanced exposure of these glycolipids might result in a more virulent infection. The fact that LOS production is absent in most species of the *M. tuberculosis* complex, except for the ancient *M. canettii*, also fits with this hypothesis. Interestingly, in a screen for *M. tuberculosis* mutants with decreased ability to arrest phagosome maturation in human macrophages a mutant was found in the homologue of *wecE*. *M. tuberculosis* does not produce LOS and disruption of this gene was shown to be responsible for the increased biosynthesis of a sulfoglycolipid, which, in purified form, actually promotes phagosome acidification and therefore presumably mycobacterial killing (51). Further research is required to study the mechanism of the observed hypervirulence associated with LOS-IV deficiency, which could tell us more about the interaction of mycobacteria with its host.

ACKNOWLEDGEMENTS - We thank Ben Appelmelk for helpful discussions and advice. We also thank Ida Rosenkrands and Eric J. Brown for kindly providing the antiserum against ESAT-6 and EspE, respectively. We are grateful to Michael J. Brennan for providing the PGRS hybridoma clone and to Janneke Maaskant for her efforts in selection and subculture of the PGRS hybridoma clone. We also thank Gunny van den Brink-van Stempvoort and Esther Stoop for their help and suggestions in the zebrafish embryo infections, and Roy Ummels, Eveline Weerdenburg, Iris Braat and Wina Verlaat for their technical assistance.

REFERENCES
Lipoooligosaccharide biosynthesis and surface protein release

FOOTNOTES

* This work was supported by an ‘ECHO’ project grant from the Netherlands Organization for Scientific Research (NWO) for ADvdW, and a NWO-VENI grant for ENGH.

Abbreviations used are: LOS, lipooligosaccharide; PE_PGRS, group of proteins from PE family with polymorphic GC rich sequence; LAM, lipoarabinomannan; PIM, phosphatidylinositol mannoside; TDM, trehalose dimycolate; PGL, phenolic glycolipids; T7SS, type VII secretion system; Pks, polyketide synthase; 2D-TLC, two-dimensional thin-layer chromatography; qRT-PCR, quantitative real-time PCR;

FIGURE LEGENDS

Figure 1. Genetic locus involved in LOS biosynthesis.
Genes involved in LOS biosynthesis obtained from our screen are depicted in bold and underlined, with the location of individual mutants indicated beneath the dark grey arrows. LOS biosynthesis defects were shown previously for gene disruption of \textit{mm2309}, \textit{losA}, \textit{mm2332}, \textit{mm2333} and \textit{papA4} (15,16,18,20) and deletion of region \textit{mm2314} to \textit{mm2317} (19) (depicted in light grey as a single responsible gene was not assigned). Similar LOS biosynthesis defects are observed in disruption of adjacent genes or genes in close proximity to each other, suggesting genomic clustering. The defects are explained under grey arrows.

Figure 2. Secretion mutants show specific defects in LOS production.
\(^{14}\text{C}\)-labeled polar lipids were extracted from (A) \textit{M. marinum} E11 wild-type, mutants B10, A3, A3c (complemented with pUC-Int-cat-\textit{mmar} _2319-22), B2, B7, A2, A4, A1, A5, A5c (complemented with pSMT3-\textit{papA3}-eGFP), A6 and (B) M\(^{15}\text{SX}\) wild-type and mutant M168. Subsequently, they were separated on 2D-TLC using the solvent system chloroform/methanol/water (60:30:6, v/v/v) in the first direction and chloroform/acetic acid/methanol/water (40:25:3:6, v/v/v) in the second direction, and visualized by autoradiography. The LOS structures produced by the mutants are marked by arrows and the major biosynthesis defect is described for each mutant. (C-E) Per-O-methylated LOS-III, LOS-II and LOS-I isolated from mutant A8, B2 and M168, respectively, were analyzed by MALDI-MS. (C) A signal was obtained at 1915.9\text{m/}z (M+Na), which corresponds to the mass of the tetraglucose core, methylated rhamnose, xylose with 2 caryophyllose residues (i.e. LOS-III). (D) The signal of 1567.9 \text{m/}z corresponds to LOS-II, and (E) LOS-I is represented by a signal of 1059.3 \text{m/}z. (F) LOS deficiency results in a rough-dry colony morphology, as shown for mutant A5. Mutants such as A8, which have a defect in higher order LOS biosynthesis have an intermediate smooth-wrinkled phenotype.

Figure 3. WhiB4 has a role in LOS biosynthesis by regulation LOS gene expression.
(A) 2D-TLC of \(^{14}\text{C}\)-labeled polar lipids extracted from \textit{M. marinum} E11 wild-type, \textit{whiB4} mutant A9 and A9c (complemented with pUC-\textit{Int-cat-whiB4}) as described for Fig. 2A/B. (B) Quantification of LOS structures. Equal counts of polar lipids (20000 cpm) were loaded for 2D-TLC, and after development the density of each visible LOS spot was measured. (C) Relative expression levels of \textit{mmar} _2319, \textit{wecE}, \textit{mmar} _2325 to \textit{mmar} _2327, \textit{pks}5, \textit{fadD25},\textit{mmar} _2354, \textit{papA3} and \textit{pks12}, shown for A9 and A9c compared to wild-type expression levels. Total RNA was isolated from \textit{M. marinum} cultures (OD\(_{600}\): 0.8-2.0) from 3 independent replicates and subsequently qRT-PCR was performed. Ct values were normalized to expression of household gene \textit{sigA}. Expression of \textit{mmar} _2326-27 and \textit{papA3} is clearly diminished in the \textit{whiB4} mutant A9, while other genes involved in LOS biosynthesis are only slightly affected. Expression levels of \textit{mmar} _2325 and negative control \textit{pks12} are comparable to wild-type.
Figure 4. LOS biosynthesis mutants show reduced PE_PGRS and EspE release.

(A) Whole cells pellets and supernatant from *M. marinum* E11 wild-type, ESX-5 mutant 7C1 and PE_PGRS mutants A2, A5, A8 and B1 grown in 7H9 broth without Tween-80 were analyzed by immunoblot for expression and secretion of PE_PGRS, ESAT-6 and EsxN. GroEL2 was used as a negative control for lysis. LOS-deficient mutants A2 and A5 show a clear effect on the secretion of PE_PGRS and EsxN, while LOS-IV deficient mutants A8 and B1 are unaffected. Results are representative of three independent experiments. (B) Immunoblot of whole cells pellets and supernatant from *M. marinum* wt, mutants A5 and A5c shows that the secretion defect of mutant A5 can be complemented. (C) Genapol X-080 treated whole cell pellets (Gp) and cell surface extracts (Gs) of bacteria grown on solid agar. No reproducible differences between E11 wild-type and the tested mutants were observed for both PE_PGRS and EspE extractability. (D) Selected LOS mutants A2, A4, A5, A6, A8, A9, B2, B7 and B10 were plated on a nitrocellulose filter together with E11 wild-type, ESX-5 mutant 7C1 and a pks12 mutant. All LOS mutants are reproducibly negative for EspE secretion on double filter assay, whereas all the controls are positive for secretion.

Figure 5. LOS-IV biosynthesis mutant shows increased virulence in zebrafish embryo infection model.

Infection of zebrafish embryos with red fluorescent wild-type *M. marinum* E11 (wt), mutant A3 and A8, A3 complemented with pUC-Int-cat-*mmar_2319-22* (A3c) and ESX-1 mutant eccCb1::tn (ESX1) monitored at 5 days post infection. (A) Representative overlay pictures of embryos infected with wt, A3, A3c and A8 with ~80 colony forming units (cfu). (B) Automated quantification of infection based on red pixel intensity (31). Represented are relative infection levels compared to wild-type infection as a log of the percentage. Results of three replicate experiments, with the average injected cfu below each group, shown with standard deviation (**p<0.001 by unpaired Student’s t-test). (C) Mean bacterial loads, measured as cfu, for zebrafish embryos infected for 5 days with wt and mutant A8 bacteria. Error bars represent standard error of the mean. (D) Growth curve of bacterial cultures in 7H9+0,05% Tween-80; mean OD₆₀₀ measurements of three independent cultures with standard deviation.
## TABLES

Table 1. Transposon insertion sites of mutants affected in PE_PGRS secretion.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annotation</th>
<th>(Putative) Function</th>
<th>Mutant</th>
<th>PE_PGRS secretion on filter&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Colony morphology&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Insertion in gene (bp 5')</th>
<th>Orientation transposon</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmar_1008</td>
<td>-</td>
<td>conserved hypothetical membrane protein</td>
<td>B5</td>
<td>+/-</td>
<td>S</td>
<td>437</td>
<td>NT</td>
</tr>
<tr>
<td>mmar_2307</td>
<td>-</td>
<td>Hypothetical transmembrane protein</td>
<td>M168</td>
<td>-</td>
<td>R</td>
<td>1000</td>
<td>NT</td>
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<tr>
<td>mmar_2313</td>
<td>losA</td>
<td>involved in assembly of the LOS-IV glycolipid</td>
<td>B12</td>
<td>+/-</td>
<td>S/W</td>
<td>88</td>
<td>NT</td>
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<tr>
<td>mmar_2319</td>
<td>-</td>
<td>conserved hypothetical transmembrane protein</td>
<td>B1</td>
<td>+/-</td>
<td>S/W</td>
<td>349</td>
<td>NT</td>
</tr>
<tr>
<td>mmar_2320</td>
<td>wecE</td>
<td>pyridoxal phosphate-dependent enzyme</td>
<td>B9</td>
<td>+/-</td>
<td>S/W</td>
<td>1548</td>
<td>+</td>
</tr>
<tr>
<td>mmar_2327</td>
<td>-</td>
<td>Conserved hypothetical transmembrane protein</td>
<td>B11</td>
<td>+/-</td>
<td>S</td>
<td>145</td>
<td>NT</td>
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<tr>
<td>mmar_2332</td>
<td>ilvB1_3</td>
<td>acetylactate synthase</td>
<td>B14</td>
<td>+/-</td>
<td>S/W</td>
<td>1683</td>
<td>+</td>
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<tr>
<td>mmar_2336</td>
<td>galE6</td>
<td>UDP-glucose 4-epimerase</td>
<td>B7</td>
<td>+/-</td>
<td>S</td>
<td>283</td>
<td>NT</td>
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<tr>
<td>mmar_2340</td>
<td>pks5</td>
<td>polyketide synthase</td>
<td>B16</td>
<td>-</td>
<td>R</td>
<td>1051</td>
<td>-</td>
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<tr>
<td>mmar_2341</td>
<td>fadD25</td>
<td>fatty acyl AMP ligase</td>
<td>B4</td>
<td>-</td>
<td>R</td>
<td>330</td>
<td>-</td>
</tr>
<tr>
<td>mmar_2353</td>
<td>-</td>
<td>UDP-glycosyltransferase</td>
<td>A1</td>
<td>-</td>
<td>R</td>
<td>751</td>
<td>NT</td>
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<tr>
<td>mmar_2355</td>
<td>papA3</td>
<td>conserved polyketide synthase associated protein</td>
<td>A5</td>
<td>-</td>
<td>R</td>
<td>691</td>
<td>+</td>
</tr>
<tr>
<td>mmar_2405</td>
<td>cphB</td>
<td>cyanophycinase</td>
<td>A6, A11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>R</td>
<td>496</td>
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<tr>
<td>mmar_4419</td>
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<td>+/-</td>
<td>S</td>
<td>373</td>
<td>NT</td>
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<tr>
<td>mmar_5170</td>
<td>whiB4</td>
<td>transcriptional regulatory protein Whib-like</td>
<td>A9</td>
<td>-</td>
<td>R</td>
<td>38</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> mutants detected with a transposon insertion at the exact same location in the gene  
<sup>b</sup> +/-: reduced, -: negative  
<sup>c</sup> S: smooth, R: rough, S/W: smooth-wrinkled  
<sup>d</sup> Orientation of the transposon: +:promoter activity in the same direction as the tested gene, -: promoter activity against the direction of the gene, NT: not tested
Table 2. LOS biosynthesis defects of selected mutants.

<table>
<thead>
<tr>
<th>Tested mutant</th>
<th>LOS biosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>M168 (mmar_2307)</td>
<td>No LOS-II, -III, -IV; Accumulation LOS-I</td>
</tr>
<tr>
<td>B10 (mmar_2319)</td>
<td>No LOS-IV; Accumulation LOS-II, -III</td>
</tr>
<tr>
<td>A3 / A8 (wecE)</td>
<td>No LOS-IV; Accumulation LOS-III</td>
</tr>
<tr>
<td>B2 (mmar_2327)</td>
<td>No LOS-IV; Accumulation LOS-II</td>
</tr>
<tr>
<td>B7 (galE6)</td>
<td>No LOS-III, -IV; Accumulation LOS-III*</td>
</tr>
<tr>
<td>A2 (pks5)</td>
<td>No LOS</td>
</tr>
<tr>
<td>A4 (fadD25)</td>
<td>No LOS</td>
</tr>
<tr>
<td>A1 / A7 (mmar_2353)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>A5 (papA3)</td>
<td>No LOS</td>
</tr>
<tr>
<td>A6 (cphB)</td>
<td>No LOS</td>
</tr>
<tr>
<td>A9 (whiB4)</td>
<td>Diminished LOS</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.

---

A) E11 wt vs A9: whiB4∆ in Diminished LOS

B) Density plot of LOS types

C) Bar graph showing percentage of protein release for each strain and role in biosynthesis:
   - LOS-IV
   - LOS-III
   - LOS-III
   - LOS
   - LOS
   - mycoketide
Figure 5.
An Unexpected link between lipooligosaccharide biosynthesis and surface protein release in *Mycobacterium marinum*

Aniek D. van der Woude, Debasmita Sarkar, Apoorva Bhatt, Marion Sparrius, Susanne A. Raadsen, Louis Boon, Jeroen Geurtsen, Astrid M. van der Sar, Joen Luijrinck, Edith N.G. Houben, Gurdyal S. Besra and Wilbert Bitter

*J. Biol. Chem.* *published online* April 13, 2012

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