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GENE EXPRESSION PROFILES OF PATHOGENIC MYCOBACTERIA REVEAL ESPJ AS A NOVEL SUBSTRATE AND WHIB6 AS A TRANSCRIPTIONAL REGULATOR OF ESX-1

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SUMMARY

The mycobacterial type VII secretion system ESX-1 is responsible for secretion of a number of proteins that play an important role during infection of the host. Regulation of expression of secreted proteins is often essential to establish a successful infection. Using transcriptome sequencing (RNA-seq), we found that abrogation of ESX-1 function in *M. marinum* leads to a pronounced increase in gene expression levels of the *espA* operon during infection of macrophages, suggesting an important role in ESX-1-mediated virulence during the early phase of infection. In addition, we found that disruption of ESX-1-mediated protein secretion leads to a specific down-regulation of substrates, but not the structural components of the transport machinery, in both *M. marinum* and *M. tuberculosis* during growth in culture medium. Based on these gene expression data, we could identify EspJ as a novel ESX-1 substrate that is cleaved upon transport. We established that down-regulation of ESX-1 substrates is the result of a regulatory process, which is influenced by the transcriptional regulatory gene *whib6*, located adjacent to the *esx-1* locus. In addition, overexpression of the ESX-1-associated PE35/PPE68 protein pair resulted in significantly increased secretion of the ESX-1 substrate EsxA, demonstrating a functional link between these proteins. Together, these data show that ESX-1 substrates are regulated independently from the structural components of the transport machinery, both during infection and as a result of active secretion.

INTRODUCTION

Mycobacteria use several different type VII secretion systems (T7S) to transport proteins across their thick and waxy cell envelope. One of these T7S systems, ESX-1, is responsible for the transport of a number of important virulence factors. Disruption of the *esx-1* gene cluster severely reduces virulence of *M. tuberculosis* [1], whereas restoration of *esx-1* in the *Mycobacterium bovis*-derived vaccine strain BCG, which has lost part of the *esx-1* region as a result of continuous passaging, leads to an increase in virulence [2]. Many studies have tried to elucidate the function of ESX-1 substrates in virulence. In pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and the fish pathogen *Mycobacterium marinum*, ESX-1 is responsible for the translocation of these bacteria from the phagolysosomal compartment to the cytosol of macrophages [3,4]. This translocation activity has been attributed to the membrane-lysing capacity of the secreted protein EsxA (also called ESAT-6) [5,6]. Interestingly, a close homologue of this protein is also secreted by non-pathogenic and non-translocating mycobacteria such as *Mycobacterium smegmatis*. A recent report indicated that, although EsxA of *M. smegmatis* and *M. tuberculosis* are highly homologous, their membrane-lysing potential is different [7]. In *M. smegmatis*, ESX-1 is involved in a completely different process, i.e. conjugative DNA transfer [8]. The proposed functions of ESX-1 in pathogenic mycobacterial species include host cell entry and intercellular spread [9].

The ESX-1 substrates identified so far are mostly encoded by genes of the *esx-1* locus, such as EsxA, EsxB, EspE and EspB, and by genes of the *espA* operon, located elsewhere in the genome. This *espA* operon is homologous to genes of the *esx-1* locus and encodes two ESX-1 substrates, EspA and EspC [10,11]. A peculiar characteristic of ESX-1 substrates is that they are mutually dependent, meaning that their secretion is dependent on secretion of the other substrates [10]. The small secreted Esx proteins contain a conserved WxG amino acid motif located between two α-helices [12]. Recently, another conserved secretion signal present in all secreted protein pairs was identified. This C-terminal YxxxD/E motif can target proteins for secretion, but does not determine specificity to a particular type VII system [13]. Therefore, it is still difficult to predict novel ESX-1 substrates.

In order to establish a successful infection, mycobacteria need regulatory mechanisms to express the right proteins at the right time. Different environments require specific transcriptional responses to successfully deal with the stress conditions encountered. During the first stages of infection, ESX-1-mediated protein secretion is one of the most important virulence mechanisms of pathogenic mycobacteria [4,9,10,14,15]. Consequently, transcriptional regulation of *esx-1* and associated genes needs to be orchestrated tightly. The transcriptional regulator PhoP of the two-component system PhoPR positively regulates transcription of many *esx-1* associated genes, including the *espA* operon [16,17]. It has been proposed that PhoP regulation is dependent on environmental pH [18], which could indicate that the acidic environment of the phagosome induces *esx-1* gene transcription via PhoP leading to bacterial escape from this compartment. Other studies have shown that the *espA* operon is, in addition to PhoP, also regulated by the

transcription factors EspR, MprAB and the repressors CRP and Lsr2, indicating that tight regulation of this operon is essential and, furthermore, suggesting that the *espA* operon may be regulated separately from the other ESX-1 substrates [19-22].

Since ESX-1 is crucial for virulence and determines intracellular localization, inactivation of this secretion system would be expected to have a large impact on gene regulation processes in mycobacteria. In this study, we have determined gene expression profiles of the pathogenic mycobacteria *M. marinum* and *M. tuberculosis* in absence of a functional ESX-1 secretion system. During short-term infection of macrophages, we observed highly increased transcript levels of the *espA* operon. In contrast, during *in vitro* growth in culture medium, transcription of most ESX-1 substrates and some putative new substrates was decreased. Based on these gene transcription levels, we could establish a regulatory role for the putative transcriptional regulator WhiB6 in gene expression of ESX-1 substrates. In addition, we identified EspJ as a novel ESX-1 substrate, indicating that gene expression profiles can be of predictive value to identify new substrates.

RESULTS

Transcription of genes encoding ESX-1 substrates is decreased in ESX-1 defective *M. marinum* and *M. tuberculosis* strains

Since ESX-1 is crucial for virulence of pathogenic mycobacteria, abrogation of this secretion system would be expected to affect gene expression. To study these effects, we determined gene expression profiles of *M. marinum* and *M. tuberculosis* strains by RNA sequencing and compared them with their isogenic *esx-1*-mutants. We found that a substantial number of *esx-1* associated genes were down-regulated in the mutant strains during growth in culture medium. Of the 70 significantly down-regulated genes in the *M. marinum esx-1*-mutant strain, 11 were located within or directly adjacent to the *esx-1* gene cluster (Table 1 and S2). Among these down-regulated genes were those coding for known ESX-1 substrates, such as EsxA, EspE and EspB. Remarkably, mRNA levels of core components of the ESX-1 secretion system, i.e. encoding members of the type VII secretion complex such as $EccB_1$, $EccD_1$, $EccE_1$ and $MycP_1$, remained unchanged, even though their respective genes are interspersed with genes encoding ESX-1 substrates. For *M. tuberculosis*, a similar trend was observed. In fact, of all genes, the ESX-1 substrates EsxA and EsxB were the most significantly down-regulated in the mutant strain (Table 2 and S4). Although gene expression levels of *eccD1* were also decreased, none of the other 32 significantly down-regulated genes encode ESX-1 core components, whereas 10 of them encode (putative) ESX-1 substrates or auxiliary proteins. We next determined the effect of ESX-1 abrogation in *M. marinum* on gene transcription during infection of macrophages. Upon 6 hours infection of THP-1 cells, none of the genes within the *esx-1* region were differentially expressed in the *esx-1*-mutant compared to the wild-type strain. However, we did find a specific and pronounced increase in transcript levels of the *espA* operon (Table 3 and S3). During growth in culture medium, mRNA levels of *espA* did not differ between the

wild-type and ESX-1-deficient *M. marinum*, which was confirmed by qPCR (Figure 1A). Therefore, these data suggest that proteins encoded by the *espA* operon, i.e. EspA, EspC and EspD, play an important role in ESX-1-specific processes during the first stages of macrophage infection.

Different *M. marinum* esx-1 transposon mutants have similar gene transcription profiles

The ESX-1-deficient strain of *M. marinum* used for RNA sequencing contains a transposon in the *eccCb*$_1$ gene. To confirm that the gene transcription effects we observed were due to a defective ESX-1 system and not an unfortunate side-effect of this particular mutation, we analyzed several mutants containing transposon insertions

Top 10 most significantly down-regulated genes of ***M. marinum*** grown in culture medium			
Gene nr	Gene product	Fold decrease	p-value
MMAR_3490	Pyruvate dehydrogenase E1 component (beta subunit)	29,5	8,30E-11
MMAR_3491	Pyruvate dehydrogenase (E2 component)	12,5	7,32E-10
MMAR_1928	Secreted L-alanine dehydrogenase Ald (40 kDa antigen)	9,9	0
MMAR_0299	PE_PGRS1	8,9	0
MMAR_4786	PE-PGRS family protein	8,6	1,35E-14
MMAR_4784	Dehydrogenase	7,4	0
MMAR_5447	PE35	7,0	0
MMAR_4785	Integral membrane protein	6,9	6,66E-16
MMAR_5448	PPE68	6,7	0
MMAR_5450	EsxA	6,1	0
Significantly down-regulated *esx-1*-associated genes			
Gene nr	Gene product	Fold decrease	p-value
MMAR_5437	WhiB6	5,4	3,28E-11
MMAR_5439	EspE	3,7	2,58E-11
MMAR_5455	EspK	3,4	1,53E-11
MMAR_5453	EspJ	2,7	1,92E-07
MMAR_5441	EspI	2,4	8,51E-06
MMAR_5457	EspB	2,0	7,44E-04
MMAR_5442	EspH	1,9	3,36E-04
MMAR_5443	EccA1	1,9	9,33E-04

Table 1. Most significantly down-regulated genes of M. marinum grown in culture medium.
Top-10 list of genes that show the most significantly decreased transcript levels in the *M. marinum eccCb*$_1$ transposon mutant compared to its isogenic wild-type strain E11 during growth in 7H9 culture medium. Significantly down-regulated *esx-1*-associated genes are shown in grey and those that were not included in the top-10 are listed below. Representative results of one out of three biological replicates are shown (Figure S1).

Top 10 most significantly down-regulated genes of ***M. tuberculosis*** grown in culture medium			
Gene nr	Gene product	Fold decrease	p-value
Rv3289c	Possible transmembrane protein	25,8	3,26E-40
Rv3879c	EspK	22,2	4,92E-86
Rv3290c	Lat	22,2	1,10E-84
Rv3288c	UsfY	5,6	3,23E-14
Rv3862c	WhiB6	4,8	7,95E-19
Rv1986	Conserved integral membrane protein	4,7	1,44E-57
Rv3861	Hypothetical protein	3,9	4,62E-14
Rv3291c	LrpA	3,6	8,66E-04
Rv3292	Conserved hypothetical protein	3,1	3,62E-12
Rv3293	Pcd	2,7	3,41E-23

Table 2. Most significantly decreased genes of M. tuberculosis grown in culture medium.
Top-10 list of genes that show the most significantly decreased transcript levels in the auxotrophic *M. tuberculosis* RD1 deletion mutant strain mc^{2}6030 compared to its isogenic control strain mc^{2}6020 during growth in 7H9 culture medium. Significantly down-regulated *esx-1*-associated genes are indicated in grey. Rv3872-Rv3878 are not included as these genes are deleted in the RD1 mutant strain. Values result from the analysis of three biological replicates.

Top 10 most significantly up-regulated genes of ***M. marinum*** during infection			
Gene nr	Gene product	Fold increase	p-value
MMAR_4166	EspA	23,4	2,78E-14
MMAR_4167	EspC	20,3	0
MMAR_4168	EspD	16,6	2,00E-14
MMAR_1846	Conserved hypothetical membrane protein	15,1	2,22E-15
MMAR_2517	PPE23	13,2	8,61E-12
MMAR_1132	WhiB3	12,5	2,00E-11
MMAR_2905	Hypothetical protein	12,4	3,38E-13
MMAR_2214	Methyltransferase	11,1	3,91E-13
MMAR_5286	PE34	10,9	6,03E-04
MMAR_2516	PPE family protein	10,8	8,81E-13

Table 3. Most significantly increased genes of M. marinum during infection.
Top-10 list of genes that show significantly increased transcription levels in the *M. marinum eccCb*$_1$ transposon mutant compared to the wild-type strain E11 during infection of human THP-1 macrophages. Representative results of one out of two biological replicates are shown.

in different genes from the *esx-1* gene cluster and compared mRNA levels of selected genes by quantitative RT-PCR. Our results show decreased transcript levels of the known ESX-1 substrate *esxA* and other *esx-1* secretion associated (*esp*) genes *espL*,

espK and *espJ* for all tested *esx-1* mutants, whereas transcript levels of $eccD_1$, which encodes a structural component of the ESX-1 system, did not differ from wild-type *M. marinum* (Figure 1B). These gene expression patterns in the $eccB_1$, $eccCa_1$, $eccD_1$ and $eccE_1$ transposon mutants are similar to the RNA sequencing results obtained for the $eccCb_1$ mutant. The only exception was that for the mutant containing a transposon

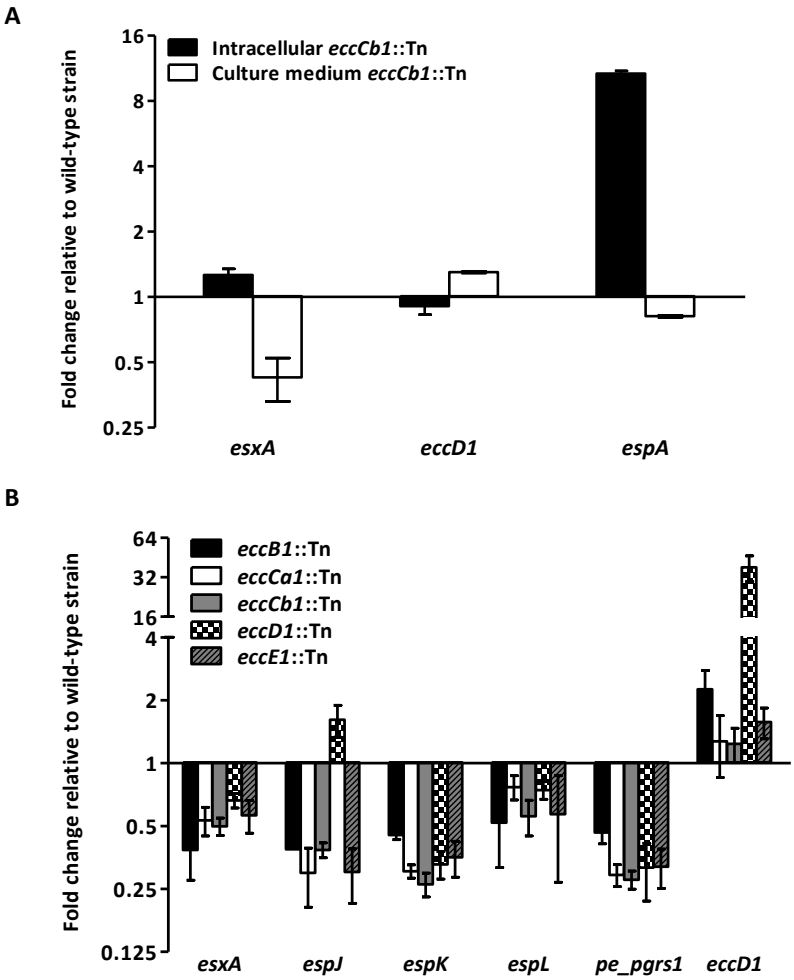

Figure 1. ESX-1 affects gene transcription during infection and growth in culture medium. A. Gene expression levels, as measured by qRT-PCR, in an *M. marinum* $eccCb_1$ transposon mutant grown in 7H9 culture medium and during 6 hours of infection in human THP-1 macrophages. Gene expression levels were compared to those of the wild-type strain E11 grown in similar conditions. Values represent mean ± standard error of two biological replicates. B. Gene expression levels for *M. marinum* $eccB_1$, $eccCa_1$, $eccCb_1$, $eccD_1$ and $eccE_1$ transposon mutants as measured by qRT-PCR. All strains were grown in 7H9 culture medium and gene expression levels were compared to those of the wild-type strain E11. Values represent mean ± standard error of at least three biological replicates.

insertion in *eccD*$_1$, we observed an increase of *eccD*$_1$ transcription itself and to a lesser extent of the adjacent gene *espJ* (Figure 1B). However, this increase is most likely due to the action of the strong promoter on the transposon that transcribes the kanamycin resistance cassette, as the measured mRNA is transcribed from sequences directly downstream of the transposon. Altogether, our results demonstrate that inactivation of the ESX-1 secretion system leads to a down-regulation in transcription of ESX-1 substrates and associated proteins.

Based on gene transcription, novel potential ESX-1 substrates can be identified

In addition to the known ESX-1 substrates, several other genes were also down-regulated in ESX-1-deficient *M. marinum* and *M. tuberculosis* (Table 1 and 2). Based on this observed co-regulation and on their genomic location or homology, we reasoned that some of these other genes could also encode ESX-1 substrates. For example, transcript levels of several *esp* genes within the *esx-1* gene cluster were decreased in the *esx-1* mutant strains. One of these genes, *espJ* was recently predicted to encode a putative T7S substrate as it contains the general secretion motif YxxxD/E that is conserved among known T7S substrates [13]. EspL does not contain this motif but might be secreted via complex formation with a partner protein containing such a motif. In order to determine whether the products of these genes are secreted and if so, via which route, we expressed HA-tagged versions of these proteins in *M. marinum*. We found that EspJ but not EspL is secreted by the bacteria (Figure 2A). Expression of *espJ* in the ESX-1-deficient *eccCb*$_1$ transposon mutant revealed that secretion of its gene product is dependent on the ESX-1 secretion system. In addition, we found that the secreted form of EspJ is probably processed. Full length HA-tagged EspJ has a calculated mass of 28 kDa. However, on SDS-PAGE this protein runs at ~38 kDa. Western blot analysis of the culture supernatant fraction showed both the full length protein and an additional protein band of ~20 kDa, indicating post-translational modification and cleavage of the full length protein (Figure 2A). This would mean that EspJ is, in addition to EspB, the second ESX-1 substrate that is processed upon transport.

In addition to the decreased transcript levels of the *esp* genes in ESX-1-deficient *M. marinum*, we also found decreased transcript levels of some genes from the *pe_pgrs* gene family. Although most PE and PPE proteins (including the PE_PGRS proteins) are secreted via the ESX-5 secretion system [23], we recently have shown that ESX-1 is also able to secrete certain PE and PPE proteins [24]. Therefore, we reasoned that these down-regulated *pe_pgrs* genes might encode ESX-1 substrates. To investigate this, we expressed an HA-tagged version of one of the most down-regulated genes, *pe_pgrs1* (Mmar_0299, Table 1), and analyzed secretion of its gene product by the *M. marinum* wild-type strain and the isogenic *esx-1* and *esx-5* mutants. By qPCR we first confirmed down-regulation of *pe_pgrs1* transcripts in all *esx-1* mutants (Figure 1B). Protein secretion analysis indicated that PE_PGRS1 is indeed a secreted protein, as it is present in moderate amounts in the culture supernatant and abundantly present on the cell surface, where it can be released using a Genapol treatment (Figure 2B), indicative

of a capsular localization [25]. However, protein analysis of *M. marinum* mutant strains showed that, just like most other PE-PGRS proteins, PE_PGRS1 is mainly dependent on ESX-5 for transport to the cell surface (Figure 2B). As the amount of protein that is present on the cell surface and in the secreted fraction does not majorly differ between wild-type and ESX-1-deficient *M. marinum*, it is not an ESX-1 substrate. Furthermore, we also noticed that despite placing the *pe_pgrs1* gene under control of a strong promoter, protein expression levels were quite low in the *esx-5* mutant strain. This indicates that *M. marinum* actively degrades (transcripts of) ESX-5 substrates that cannot be secreted.

ESX-1 substrate gene transcription is reduced by a regulatory mechanism

We next sought to determine the mechanistic process underlying down-regulation of specific transcripts in *esx-1* mutant strains of *M. marinum*. It is possible that the decrease in mRNA levels is due to a decrease in transcription and therefore a regulatory effect. Alternatively, mRNA derived from specific sequences may be degraded via a post-transcriptional mechanism. To investigate this possibility, we expressed an extra copy of the *espL* gene under control of a constitutively active promoter in the *M. marinum* wild-type and *eccCb*$_1$ mutant strains and determined *espL* gene transcript levels. We found a similar increase in *espL* transcripts in both wild-type and *eccCb*$_1$ mutant strains, indicating that degradation of specific mRNA is probably not the cause of the decreased mRNA levels in the mutant strain (Figure 3). Expression levels of the downstream gene *espK* were not affected by the introduction of *espL*. These results indicate that there is a regulatory mechanism that prevents transcription of genes encoding ESX-1 substrates and associated proteins in absence of a functional ESX-1 system.

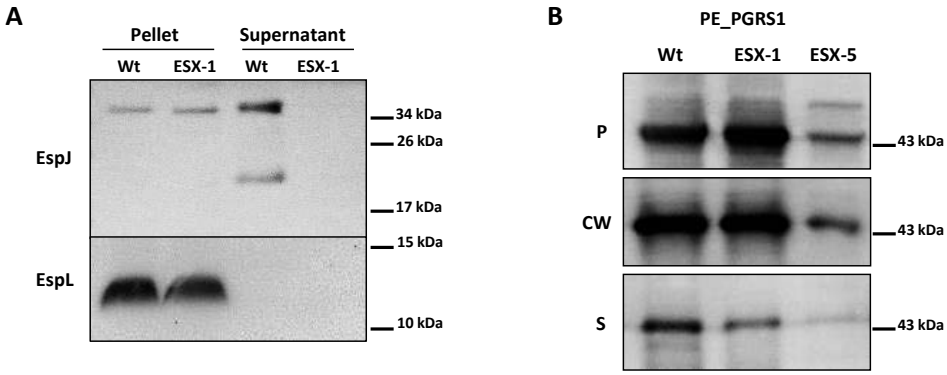

Figure 2. EspJ is secreted via ESX-1. A. HA-tagged EspJ or EspL were introduced in *M. marinum* wild-type E11 and *eccCb*$_1$ mutant strains. Cell pellet and supernatant fractions were analyzed for EspJ and EspL expression and secretion by Western blot and α-HA-antibody staining. Fractions represent either 0.5 or 2 OD units of original culture, respectively. **B.** HA-tagged PE_PGRS1 was introduced in *M. marinum* wild-type E11, *espG*$_5$ and *eccCb*$_1$ mutant strains. Protein expression in the cell pellet (p), localization on the cell wall as determined by genapol extraction (cw), and expression in the secreted fraction (s) was analyzed by Western blot and α-HA-antibody staining. Fractions represent 0.5, 1 or 2 OD units of original culture, respectively.

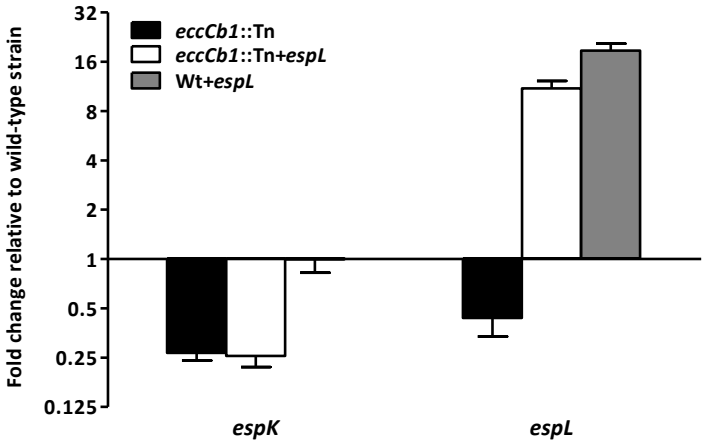

Figure 3. Down-regulation of *espL* is the result of a regulatory process. EspL was introduced in *M. marinum* wild-type and *eccCb*$_1$ mutant strains and *espK* and *espL* gene expression levels were measured by qRT-PCR. Gene expression levels were compared to those of the wild-type strain E11. Values represent mean ± standard error of two biological replicates.

PE35 and PPE68 play an important role in ESX-1 secretion but not in gene regulation

Previously, PE35, which is located within the *esx-1* gene cluster, has been implicated in the regulation of *esxA/esxB* gene expression in *M. tuberculosis* [26]. In contrast to this proposed function, the PE35/PPE68_1 protein pair of *M. marinum*, which coding genes have been duplicated from the *esx-1* cluster, is secreted via ESX-1 [24,25]. To determine whether PE35 plays a role in regulation of ESX-1 substrates, we overexpressed the *pe35/ppe68_1* operon in *M. marinum*. Interestingly, although there was no effect on gene transcription (Figure 5A), we did notice a substantial increase of EsxA secretion in the wild-type strain (Figure 4). This increased EsxA secretion does not seem to represent a general increase in ESX-1 secretion, as protein levels of the cell surface localized EspE remained similar (Figure 4B). To study this effect in more detail, we introduced PE35 with a truncated version of PPE68_1 that only contained the PPE domain and was devoid of the C-terminal part. Although the introduced PE35 protein was expressed and secreted efficiently by ESX-1 (Figure 4), levels of secreted EsxA were not increased, indicating that the C-terminal part of PPE68_1 plays a role in EsxA secretion. To determine if secretion of the PE35/PPE68_1 protein pair itself was important for this process, we also determined the effect of removing the last 15 amino acids of the PE protein, containing the general YxxxD/E secretion signal [13]. This small deletion not only abolished secretion of the introduced PE35 protein, it also abolished EsxA secretion completely, despite the presence of an intact chromosomal copy of the *pe35/ppe68_1* operon. This suggests that the truncated form of PE35 somehow interferes with EsxA secretion. Together these data show that, although PE35 and PPE68_1 do not seem to regulate the transcription of genes encoding ESX-1 substrates, they have a strong effect on EsxA.

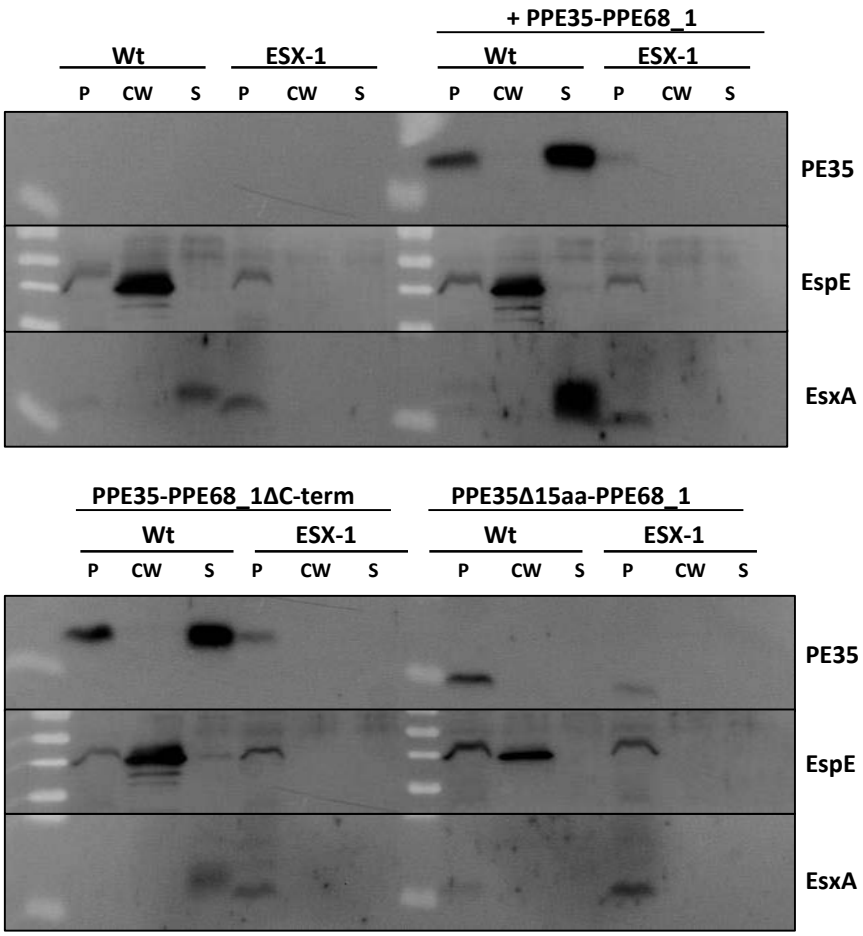

Figure 4. Introduction of PE35/PPE68_1 results in increased EsxA secretion. Pellet (p), cell wall extract (cw) and supernatant (s) fractions of *M. marinum* wild-type and *eccCb*$_1$ mutant strains expressing PE35/PPE68_1, PE35/PPE68 containing a C-terminal deletion of PPE68_1, or PE35/PPE68_1 containing a 15 amino acid C-terminal deletion of PE35, were analyzed for the presence of EspE, EsxA and the introduced PE35 by Western blot. Fractions represent 0.5, 1 or 2 OD units of original culture, respectively. In all cases, PE35 contained a C-terminal HA-tag.

Increasing EspI and EspG$_1$ does not lead to altered esx-1 gene expression

A second candidate protein that might regulate gene expression levels of ESX-1 substrates is EspI. The gene encoding this *esx-1* secretion associated protein of unknown function is located within the *esx-1* region and is down-regulated in *esx-1* mutants of both *M. marinum* and *M. tuberculosis* (Table 1 and 2). In contrast to the other Esp proteins, EspI contains a putative nucleotide-binding domain. However, when we overproduced this protein, we did not observe a change in down-regulation of *esx-1* associated gene transcription in the *M. marinum eccCb*$_1$ transposon mutant,

suggesting that EspI does not regulate this process (Figure 5A). We next focused on $EspG_1$ as a candidate *esx-1* gene regulator. $EspG_1$, which is a cytosolic protein that is not part of the membrane-bound secretion machinery, has recently been shown to interact specifically with PE35/PPE68_1 in *M. marinum* [24]. It is conceivable that $EspG_1$ might function as a sensor that measures protein levels of intracellular ESX-1 substrates. When substrate levels are low, unbound $EspG_1$ may signal to induce gene expression. In absence of a functional ESX-1 system, accumulated PE35/PPE68_1 or other substrates may occupy $EspG_1$ leading to reduced transcription of *esx-1* associated genes. In order to investigate the effect of $EspG_1$ on *esx-1* associated gene expression and protein levels, we increased $EspG_1$ levels by overexpressing the *espG*$_1$ gene in wild-type and ESX-1-deficient *M. marinum*. However, this did not result in altered gene transcription (Figure 5A), nor ESX-1 protein secretion (not shown). Together, our data shows that EspI and $EspG_1$ do not seem to play a key role in *esx-1* associated gene regulation.

WhiB6 plays a role in transcription of ESX-1 substrates

In addition to *espI*, *whiB6*, another gene encoding a putative regulatory protein, was also down-regulated in *esx-1* mutant strains of both *M. marinum* and *M. tuberculosis* (Table 1 and 2). WhiB proteins are actinobacteria-specific regulators that contain iron-sulfur clusters and are thought to act as redox-sensing transcription factors that can result in both gene activation and repression [27]. For WhiB6 there is no specific function known. The *whib6* gene is located less than 2 kb upstream of the *esx-1*

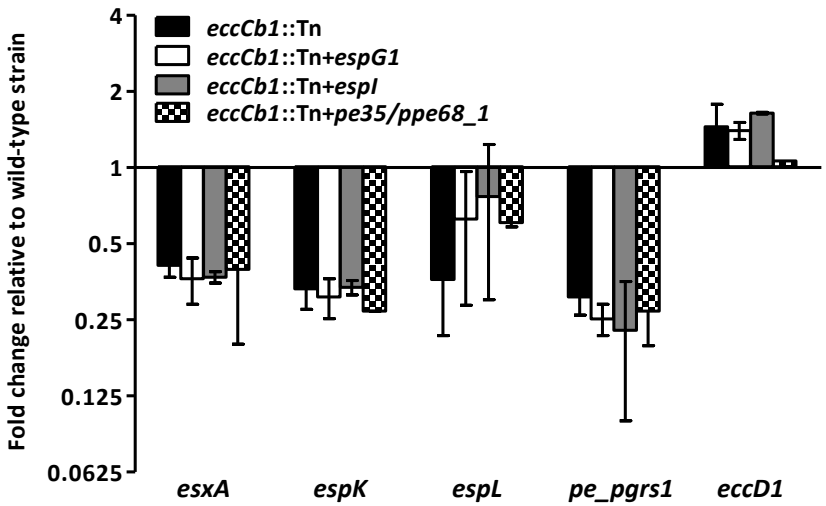

Figure 5. $EspG_1$, EspI and PE35/PPE68_1 do not regulate transcription of selected *esx-1* (associated) genes. $EspG_1$, EspI or PE35/PPE68_1 were overexpressed in the *M. marinum eccCb*$_1$ mutant strain and expression levels of *espK*, *espL*, *esxA*, *pe_pgrs1* and *eccD1* were measured by qRT-PCR. Gene expression levels were compared to those of the wild-type strain E11. Values represent mean ± standard error of at least two biological replicates.

region, which could indicate a regulatory function. A previous computational study also suggested a link between WhiB6 and the *esx-1* locus, based on its phylogenetic profile [28]. In order to determine whether Whib6 had an effect on expression levels of *esx-1* associated genes, we overexpressed this protein in the ESX-1-deficient *M. marinum eccCb*$_1$ transposon mutant strain. We found that specifically those genes that were already down-regulated in the mutant strain, such as *esxA* and *espK*, showed an even further transcriptional inhibition when *whib6* levels were increased (Figure 6). Furthermore, expression of *eccD1* was unaltered by *whib6* overexpression, indicating that *whib6* is involved in transcription of ESX-1 substrates and associated genes, but not of the system components. Surprisingly, *whiB6* is one of the genes that is down-regulated upon abrogation of ESX-1-mediated protein secretion. Possibly, WhiB6 is activated when the ESX-1 machinery is jammed and represses genes encoding ESX-1 substrates as well as its own gene. Together, our data suggests that ESX-1 regulation is even more complex than previously anticipated.

DISCUSSION

In this study, we have determined the transcriptome of the *M. marinum* E11 wild-type and the double auxotrophic *M. tuberculosis* mc^{2}6020 mutant strains, and compared them with their isogenic *esx-1* mutants. We found that during growth in 7H9 culture medium, genes encoding ESX-1 substrates such as EsxA and other ESX-1-associated proteins were down-regulated in the mutant strains, whereas transcription of genes

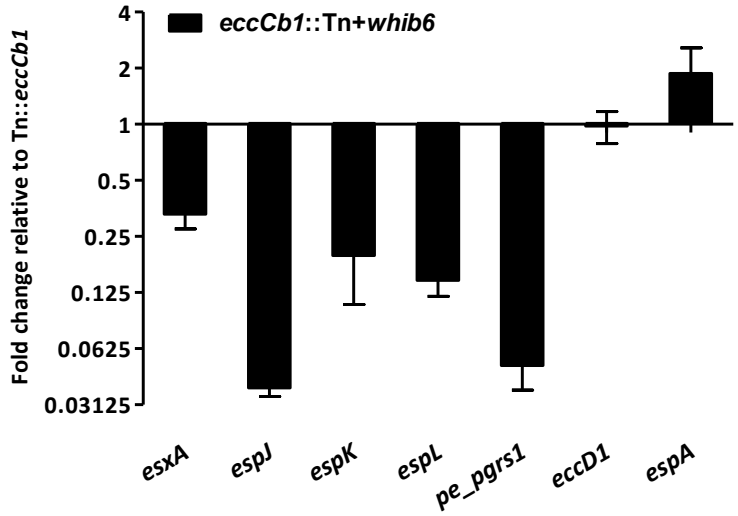

Figure 6. WhiB6 is involved in transcriptional regulation of ESX-1 substrates and associated genes. The *whib6* gene was overexpressed in the *M. marinum eccCb*$_1$ mutant strain and transcript levels of *espK, espL, esxA, pe_pgrs1* and *eccD1* were measured by qRT-PCR. Gene expression levels were compared to those of the *eccCb*$_1$ mutant strain. Values represent mean ± standard error of two biological replicates.

encoding structural components of the ESX-1 system remained unaffected. This specific decrease in transcription might function as a mechanism to avoid toxic accumulation of substrates. The list of down-regulated genes led us to investigate whether other genes could encode more, previously unidentified, ESX-1 substrates. By overexpression of candidate substrates, we could identify EspJ as a novel ESX-1-secreted protein. Additionally, we found that the secreted form of this protein is processed and probably post-translationally modified, as it runs with an apparent molecular weight of 10 kDa higher than expected. Recently, the ESX-1 substrate EspB has been shown to be C-terminally processed by the subtilisin-like serine protease MycP1, which is a membrane-bound component of the ESX-1 secretion system [29]. Similarly, EspJ may also be a target for MycP1-mediated processing. Using the recently published structure of MycP1 and the analysis of its active site cleft [30], we could identify a putative MycP1 cleavage site at amino acid 168, which would result in a C-terminal product of 10 kDa. If the observed post-translational modification of 10 kDa is linked to the C-terminal domain of EspJ, this would explain the length of the processed fragment. Processing of EspJ and EspB may serve as a mechanism to activate these proteins once they are outside the bacterium, similar to what is observed for the ESX-5 substrate LipY [31,32].

The down-regulated protein EspK, which is encoded within the *esx-1* gene cluster, contains a WxG motif and could therefore be another novel ESX-1 substrate. Indeed, EspK has been found on the cell surface of wild-type but not ESX-1-deficient bacteria [25]. In contrast to EspJ and EspK, we found that EspL does not seem to be secreted, despite the fact that the *espL* gene is also down-regulated in ESX-1-deficient *M. marinum*. Because of the observed co-regulation, EspL may have an auxiliary role in transport and/or functioning of other Esp proteins, such as that of a substrate-specific chaperone.

Transcription levels of several *pe_pgrs* genes such as *pe_pgrs1* were also strongly down-regulated in ESX-1-deficient *M. marinum*. However, secretion of HA-tagged PE_PGRS1 was found to be dependent on ESX-5 and not on ESX-1. Most of the PE_PGRS1 protein was localized on the bacterial cell surface, which is characteristic for PE_PGRS family proteins [25,33]. Upstream of *pe_pgrs1* are no genes with the same orientation and the gene is located far from the extended *esx-1* gene locus, suggesting that its down-regulation is specific and not a by-effect of a decrease in transcription from other genes. Possibly, the specifically down-regulated PE_PGRS family proteins such as PE_PGRS1 are functionally linked to the ESX-1 substrates. It has been shown previously that, although ESX-1-mediated secretion is essential for phagosome rupture and bacterial translocation [4,34], ESX-5 mutants show a delay in phagosomal escape [35]. This indicates that there could be a functional link between substrates of ESX-1 and ESX-5. Alternatively, the down-regulation of the often cell surface exposed PE_PGRS family proteins may be a response to an altered cell wall composition that occurs in absence of a functional ESX-1 system [36].

During *M. marinum* infection of human macrophages, we found that transcription of many *pe_pgrs* and *ppe* family genes was strongly down-regulated when ESX-1 function was abrogated. As much as 50% of all genes with decreased transcript levels

in the *esx-1* mutant strain, belong to one of these gene families (Table S3). It has to be noted that already in the wild-type strain transcription of *pe_pgrs* and *ppe* genes was decreased during infection, in comparison to growth in 7H9 medium (Table S5). As part of an adaptive response to the macrophage environment, expression of these cell wall localized proteins may be tuned down in order to evade immune recognition. The fact that, in absence of a functional ESX-1 secretion system, these genes are even further down-regulated, again suggests that there might be a functional link or shared transcriptional pathways between ESX-1 and PE_PGRS and PPE proteins.

The most prominent change in gene expression that was observed upon host cell infection by the *M. marinum esx-1* mutant strain, was the increase in transcription of the *espA* operon. The specific and pronounced transcriptional increase of this operon, and not of any other *esx-1* associated gene, indicates that transcription of the *espA* operon is regulated independently of the other substrates during infection. It also suggests that EspA, EspC and EspD are highly important for the bacteria during the early phase of infection. Since ESX-1 has been shown to be responsible for mycobacterial escape from the phagosome, which occurs within the first few hours of infection with *M. marinum* [5], the *espA* operon may play an important role in this process. Consequently, the avirulent phenotype of ESX-1-deficient mycobacteria might be partly attributable to the inability to secrete EspA, EspC and possibly the stabilizing protein EspD early in infection, despite the bacteria's attempts to increase transcription of their coding genes.

In order to determine how ESX-1 substrate regulation is mediated, we overexpressed proteins that may have a regulatory function. Overexpression of the *esx-1* encoded EspI and $EspG_1$ did not have an effect on the lowered transcription of ESX-1 substrates in ESX-1-deficient *M. marinum*. The putative regulatory protein WhiB6 however, did affect transcription of those genes. While transcript levels of *whib6* itself were decreased in *esx-1* mutants of *M. marinum* and *M. tuberculosis*, increasing WhiB6 by overexpression resulted in a further decrease in ESX-1 substrate transcription in ESX-1-deficient *M. marinum*. This clearly indicates that WhiB6 is involved in ESX-1 associated gene regulation. Indeed, there is (circumstantial) evidence that the WhiB-like proteins function as transcription factors which may play a role in survival within the host (reviewed in [37]). Further studies are required to elucidate the exact role of WhiB6 in ESX-1 gene regulation and virulence.

A remarkable finding in this study was that overproduction of PE35/PPE68_1 resulted in a large increase in EsxA secretion. Previously, deletion of *M. tuberculosis* PE35 was already shown to abolish *esxA* transcription and secretion of its gene product [26]. Now, we find that EsxA and PE35 secretion are linked, as an increase in PE35 secretion results in a concomitant increase in EsxA secretion. The fact that the C-terminus of PPE68_1 is required for this effect indicates that this is a specific process, which is supported by the fact that cell-surface localization of another ESX-1 substrate, EspE, is unaffected by overproduction of PE35/PPE68_1. Possibly, the PPE68 proteins serve as a chaperone to escort EsxA outside the bacterium, or it may be part of the secretion apparatus making secretion of specific substrates more efficient.

Taken together, our results show that gene expression levels in mycobacterial secretion mutants can be used to discover unknown secreted proteins. We identified EspJ as a novel ESX-1 substrate in *M. marinum* and showed that this protein is processed upon secretion, as was previously determined for EspB [29]. We also found that transcription of the *espA* locus plays an important role in ESX-1 mediated processes during the first hours of infection. Furthermore, we established a functional link between PE35 and EsxA secretion and lastly, we found that WhiB6 may play a regulatory role in transcription of ESX-1 substrates and associated genes.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The *esx-1* mutants of the *M. marinum* E11 wild-type strain used in this study contain transposon insertions in $eccB_1$, $eccCa_1$, $eccCb_1$, $eccD_1$ and $eccE_1$ [38] [Stoop *et al.*, in preparation]. The *M. marinum esx-5* mutant strain contains a transposon insertion in $espG_5$ [39]. For *M. tuberculosis,* the attenuated double deletion strains mc^26020 and mc^26030 of H37Rv were used, with deletions of *lysA* and *panCD* or *RD1* and *panCD*, respectively [40,41]. Bacterial strains were grown to log-phase, shaking at 30˚C in Middlebrook 7H9 culture medium supplemented with 10% ADC (Albumin-Dextrose-Catalase, BD Biosciences) and 0.05% Tween-80. Culture medium containing the auxotrophic *M. tuberculosis* deletion strains was supplemented with 50 µg/ml panthothenic acid and, for mc^26020, 100 µg/ml L-lysine.

Infection of human macrophages

THP-1 monocytes were cultured at 37˚C and 5% CO2 in RPMI-1640 with Glutamax-1 (Gibco) supplemented with 10% FBS, 100 µg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. Cells were seeded at a density of 3 x 10^7 cells per T175 flask and differentiated into macrophages by 48 hours of incubation with 25 ng/ml PMA (Sigma-Aldrich). 1,8 x 10^8 THP-1 cells were infected with *M. marinum* at an MOI of 20 for 2 hours, after which cells were washed with PBS to remove extracellular bacteria. After 4 additional hours of infection, THP-1 cells were lysed with 1% Triton X-100. After a low speed centrifugation step to remove cellular debris, mycobacteria were pelleted after which RNA was extracted.

RNA extraction and qRT-PCR

M. marinum and *M. tuberculosis* cultures were pelleted and bead-beated in 1 ml Trizol (Invitrogen) with 0.1 mm Zirconia/Silica beads (Biospec Products). After centrifugation, supernatants were extracted with chloroform and RNA was precipitated with isopropanol. RNA pellets were washed with 80% ethanol and dissolved in RNAse-free water. Contaminating DNA was removed by incubation with DNAse I (Fermentas). For RT-PCR, cDNA was generated using a SuperScript VILO cDNA synthesis kit (Invitrogen). An equivalent of 5 ng of RNA was used in the quantitative PCR reactions. qPCR was performed using SYBR GreenER (Invitrogen) and the LightCycler 480

(Roche). Transcript levels were normalized to the housekeeping gene *sigA* [42], using ΔΔCt analysis. All primer sequences used for qPCR are listed in Table S1.

RNA preparation for Illumina Sequencing

Total RNA was extracted with Trizol (Invitrogen) and then purified on RNeasy spin columns (Qiagen) according to the manufacturer's instructions. The RNA integrity (RNA Integrity Score≥6.8) and quantity was determined on the Agilent 2100 Bioanalyzer (Agilent; Palo Alto, CA, USA). As ribosomal RNA comprises the vast majority of the extracted RNA population, depletion of these molecules through RiboMinus-based rRNA depletion was used in efforts to increase the coverage of mRNA and to reduce rRNA reads. For this mRNA enrichment, the Invitrogen's RiboMinus™ Prokaryotic kit was used according to manufacturer's instructions. Briefly, 2μg of total RNA samples was hybridized with prokaryotic rRNA sequence-specific 5'-biotin labeled oligonucleotide probes to selectively deplete large rRNA molecules from total RNA. Then, these rRNA-hybridized, biotinylated probes were removed from the sample with streptavidin-coated magnetic beads. The resulting RNA sample was concentrated using the RiboMinus™ concentrate module according to the manufacturer's protocol. The final RiboMinus™ RNA sample was subjected to thermal mRNA fragmentation using Elute, Prime, and fragment Mix from the Illumina TruSeq™ RNA sample preparation kit (Low-Throughput protocol). The fragmented mRNA samples were subjected to cDNA synthesis using the Illumina TruSeq™ RNA sample preparation kit (Low-Throughput protocol) according to manufacturer's protocol. Briefly, cDNA was synthesized from enriched and fragmented RNA using SuperScript III Reverse Transcriptase (Invitrogen) and SRA RT primer (Illumina). The cDNA was further converted into double stranded DNA using the reagents supplied in the kit, and the resulting dsDNA was used for library preparation. To this end, cDNA fragments were end-repaired and phosphorylated, followed by adenylation of 3'ends and adapter ligation. Twelve cycles of PCR amplification were then performed, and the library was finally purified with AMPure beads (Beckman Coulter) as per the manufacturer's instructions. A small aliquot (1 μl) was analyzed on Invitrogen Qubit and Agilent Bioanalyzer. The bar-coded cDNA libraries were pooled together in equal concentrations in one pool before sequencing on Illumina HiSeq2000 using the TruSeq SR Cluster Generation Kit v3 and TruSeq SBS Kit v3. Data were processed with the Illumina Pipeline Software v1.82.

Plasmid construction

The *E.coli*/mycobacterial shuttle vector pSMT3 was used for construction of all plasmids. To overexpress PE35-PPE68_1 (mmar_0185-mmar_0186), we used a plasmid previously described [13]. For construction of the plasmid containing $espG_1$, this gene was amplified from the *M. marinum* E11 genome by PCR using primers containing NheI and EcoRV restriction sites and a 3' HA-epitope. The resulting PCR product and empty pSMT3 were digested with NheI and EcoRV followed by ligation of $espG_1$ into the vector by T4 ligase (Fermentas). For the construction of the plasmid containing *whib6*, this gene was amplified from the *M. marinum* E11 genome by PCR using primers containing NheI and BamHI restriction sites. For the other constructs, *espJ, espL,*

pe_pgrs1 and *espI* were amplified from the *M. marinum* E11 genome by PCR using primers containing NheI and BglII restriction site and for *espJ*, *espL* and *pe_pgrs1* a 3' HA-epitope. PCR products were digested with NheI and BglII or BamHI. Empty pSMT3 was digested with NheI and BamHI after which PCR products were ligated in the vector. All plasmids were introduced in the *M. marinum* wild-type E11 and its isogenic *eccCb*$_1$ mutant strain by electroporation. All primer sequences are listed in Table S1.

Analysis of protein expression and secretion

M. marinum cultures were grown to mid-logarithmic phase in 7H9 culture medium supplemented with 0.2% glycerol and 0.2% dextrose. Bacteria were pelleted, washed in PBS and incubated in 0.5% Genapol X-080 (Sigma-Aldrich) for 30 minutes to extract cell wall proteins. Genapol X-080-treated *M. marinum* cells were disrupted by sonication. Secreted proteins were precipitated from the culture supernatant by 10% trichloroacetic acid (TCA, Sigma-Aldrich). Proteins were separated by molecular weight on 15% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes (Amersham Hybond ECL, GE Healthcare Life Sciences). Immunostaining was performed with mouse monoclonal antibodies directed against the HA-epitope (HA.11, Covance), EsxA (Hyb76-8), or rabbit polyclonal sera recognizing EspE [43].

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qPCR primers			
Gene number	Gene product	Forward primer	Reverse primer
Mmar_0299	PE_PGRS1	TTCCACGATCAGTTTGTCCA	GTTTTGCCCCAGGTTCTGTA
Mmar_2011	SigA	GAAAAACCACCTGCTGGAAG	CGCGTAGGTGGAGAACTTGT
Mmar_4166	EspA	ACTCCGCACTGTCGTTCTTT	GGTTCTTGCCGGAGTAGTGA
Mmar_5450	EsxA	CACCAGCATTCATTCCCTTC	AGGTTCTGCAGCGAGTTGTT
Mmar_5452	EccD1	CTATGCCTGGCTGTTGTTGA	GTCATAGACGCCGGTAATCC
Mmar_5453	EspJ	GCGCAGACCATCAGTCAGT	GTCTTCCTCGCCCTCTTTG
Mmar_5455	EspK	CGACTACGGGTTCTTCTGGA	GCTGGCCATGTAGACCTTGT
Mmar_5456	EspL	CCTGCTGAAGGAAGTTGGTC	TCCCGTTGTTCATTGTTTGA

Cloning primers			
Gene number	Gene product	Forward primer	Restriction site
Mmar_0299	PE_PGRS1-Fw	CCCGCTAGCATGTCGTATGTATTCGCGACG	NheI
Mmar_0299	PE_PGRS1-Rv	CCCAGATCTTCACGCGTAGTCCGGCACGTCGTACGGGTAGCTGGGTCCGGCAGCGC	BglII
Mmar_5441	EspG1-Fw	GCACGCTAGCATGACCGGTCCGCTCGCTA	NheI
Mmar_5441	EspG1-Rv	TTGATATCACGCGTAGTCCGGCACGTCGTACGGGTAACCTCGGGCGGTGGCG	EcoRV
Mmar_5451	EspI-Fw	CCCGCTAGC ATGCCGGCGGACTACGAC	NheI
Mmar_5451	EspI-Rv	AGCAAGATCTCATCCGTCTGCCGGTAAG	BglII
Mmar_5453	EspJ-Fw	CCCGCTAGCATGGCTGAGCCTCTGGCC	NheI
Mmar_5453	EspJ-Rv	CCCAGATCTTCACGCGTAGTCCGGCACGTCGTACGGGTAGATCGGAGCTGAGACCGAA	BglII
Mmar_5456	EspL-Fw	CCCGCTAGCGTGACCAGCATGGAGATGG	NheI
Mmar_5456	EspL-Rv	CCCAGATCTTCACGCGTAGTCCGGCACGTCGTACGGGTAGACCATCCCGTTGTTCATTG	BglII

Table S1. Primers used in this study.

Supplementary tables S2-S6 are available upon request

Table S2. Complete list of genes that are significantly changed in the *M. marinum* *eccCb*$_1$ transposon mutant compared to its isogenic wild-type strain E11 during growth in 7H9 culture medium. P<0.001.

Table S3. Complete list of genes that are significantly changed in the *M. marinum* *eccCb*$_1$ transposon mutant compared to the wild-type strain E11 during infection of human THP-1 macrophages. P<0.001.

Table S4. Complete list of genes that are significantly changed in the auxotrophic *M. tuberculosis* RD1 deletion mutant strain mc^{2}6030 compared to its isogenic control strain mc^{2}6020 during growth in 7H9 culture medium. P<0.001

Table S5. Complete list of genes that are significantly changed in the *M. marinum* wild-type strain during infection of macrophages compared to growth in 7H9 culture medium. P<0.001

Table S6. Complete list of genes that are significantly changed in the *M. marinum* *eccCb*$_1$ transposon mutant strain during infection of macrophages compared to growth in 7H9 culture medium. P<0.001

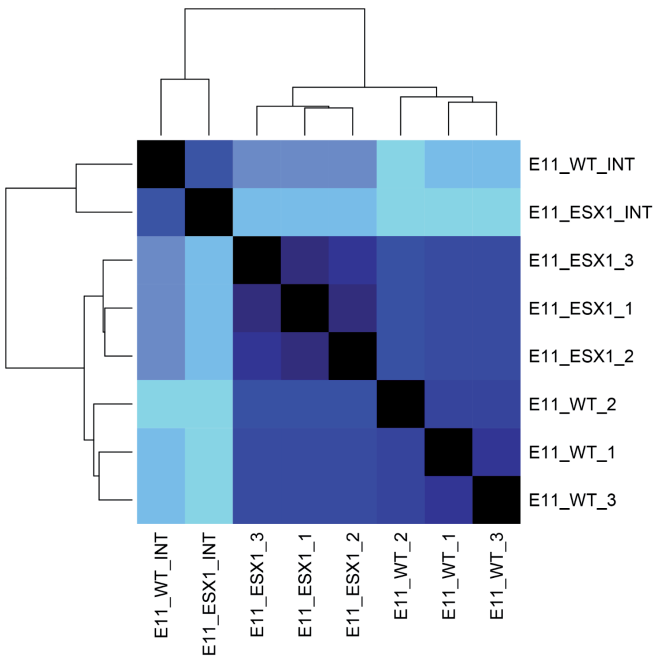

Figure S1. Heat-map showing clustering of *M. marinum* wild-type (E11) and eccCb$_1$ mutant samples (ESX-1) grown in culture medium (three biological replicates) or during infection of THP-1 cells (indicated as 'int'). Color gradient indicates high (dark colors) to low (light colors) similarity between samples.