Chapter 2

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PPE AND PE_PGRS PROTEINS OF *MYCOBACTERIUM MARINUM* ARE TRANSPORTED VIA THE TYPE VII SECRETION SYSTEM ESX-5

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SUMMARY

ESX-5 is one of the five type VII secretion systems found in mycobacteria. These secretion systems are also known as ESAT-6-like secretion systems. Here, we have determined the secretome of ESX-5 by a proteomic approach in two different strains of *Mycobacterium marinum*. Comparison of the secretion profile of wild-type strains and their ESX-5 mutants showed that a number of PE_PGRS and PPE-MPTR proteins are dependent on ESX-5 for transport. The PE and PPE protein families are unique to mycobacteria, are highly expanded in several pathogenic species, such as *Mycobacterium tuberculosis* and *M. marinum,* and certain family members are cell surface antigens associated with virulence. Using a monoclonal antibody directed against the PGRS domain we showed that nearly all PE_PGRS proteins that are recognized by this antibody are missing in the supernatant of ESX-5 mutants. In addition to PE_PGRS and PPE proteins, the ESX-5 secretion system is responsible for the secretion of a ESAT-6-like proteins. Together, these data show that ESX-5 is probably a major secretion pathway for mycobacteria and that this system is responsible for the secretion of recently evolved PE_PGRS and PPE proteins.

INTRODUCTION

Many pathogenic bacteria rely on specialized protein secretion pathways to secrete effector proteins that are important for virulence [1-3]. An important group of pathogens that have only recently been shown to secrete virulence factors are the mycobacteria [4]. This genus includes the fish pathogen *Mycobacterium marinum* and *Mycobacterium tuberculosis*, which is, with an estimate of 2 million deaths per year, the most deadly bacterial pathogen worldwide. Most pathogenic mycobacteria are intracellular pathogens that survive and replicate within cells of the host immune system, primarily macrophages, by inhibiting the acidification and maturation of the phagosome [5-7]. As in other bacterial pathogens, the exported proteins of pathogenic mycobacteria also play an important role in pathogenesis of the disease, by altering host resistance and by modifying the macrophage environment [8-12]. Proteomic studies have shown that *M. tuberculosis* secretes a number of different proteins [13,14], although many of these proteins are produced without a classical signal sequence [14,15]. This fact, combined with the highly complex architecture of the mycobacterial cell wall implies that (a) specialized secretion pathway(s) must be present in these bacteria.

Recently, a novel type of protein secretion system has been identified in mycobacteria [16-20], the type VII secretion pathway [21], which is also known as the ESAT-6 secretion pathway. The ESX-1 system [22] is the archetype of type VII, this system is responsible for the secretion of 5 proteins, including the important T-cell antigens ESAT-6 and CFP-10 [20,23-26]. The ESX-1 secretion system is affected by a spontaneous deletion, called RD1, in the vaccine strain *Mycobacterium bovis* BCG [27-29] and this deletion is (partially) responsible for the attenuated phenotype of BCG [26,30,31]. Genome analysis has shown that there are four gene clusters homologous to the ESX-1 cluster, designated ESX-2 to ESX-5 [32,33]. Interestingly, whereas ESX-1 is present in most mycobacteria, the ESX-5 system is confined to the subclass of slow-growing mycobacteria, which contains most pathogenic species [16,34]. The appearance of the ESX-5 system in slow-growing mycobacteria predates the recent expansion of two gene families, *i.e.* PE and PPE [34]. These two gene families are unique for the mycobacteria and were one of the major surprises of the *M. tuberculosis* genome sequence [35]: together they cover almost 10% of the coding capacity of the genome (167 genes). The PE and PPE families are named after the conserved Proline and Glutamic acid (PE) and Pro–Pro–Glu (PPE) motifs near the N terminus of their gene-products [35,36]. In fact, PE and PPE protein family members share homologous N-terminal domains of approximately 110 amino acids and 180 amino acids, respectively. PE and PPE proteins have been shown to be secreted or located on the cell surface. Although their exact function is generally unknown, they are associated with virulence and have been hypothesized to show antigenic variation. The evolutionary link between the expansion of PE/PPEs in slow-growing mycobacteria and the appearance of ESX-5 led to the hypothesis that ESX-5 is involved in the functioning of these proteins [34].

Previously, we have shown that an ESX-5 mutant of *M. marinum* is defective in the secretion of the heterologously expressed PPE41 protein [16]. In this study we

determined which other proteins are secreted by the ESX-5 pathway and show that ESX-5 is a major secretion pathway responsible for the transport of a number of PPE and PE proteins, including members of the largest subfamily of PE proteins, *i.e.* PE_PGRS. These PE proteins contain multiple tandem repeats of Gly-Gly-Ala or Gly-Gly-Asn and are encoded by genes with a polymorphic GC-rich sequence (PGRS) motif [37].

RESULTS

Isolation of an ESX-5 mutant in *M. marinum* E11

M. marinum strains can be divided into two clusters, cluster I contains strains isolated from both cold-blooded animals and humans and these strains cause an acute disease in zebrafish, whereas cluster II isolates are predominantly isolated from poikilothermic species and cause a chronic infection in zebrafish [38]. Previously, we identified ESX-5 as the secretion system responsible for the transport of heterologously expressed PPE41 in the *M. marinum* M strain, which belongs to cluster I [16]. To study the effect of the ESX-5 mutation in both clusters, we also isolated PPE41 secretion mutants in the cluster II strain E11 by genetic screening as described previously [16]. Eleven mutants with undetectable quantities of PPE41 were isolated, but analysis showed that all but one of these mutants contained deletions in the PPE41 encoding plasmid. This instability was not observed in the previous screen using the M strain [16] and indicates differences in recombination frequencies. The last mutant, named 7C1, contained an intact PPE41 gene and was unable to secrete PPE41 in the culture supernatant (Fig. 1A). This mutant contained a transposon insertion in a gene of the ESX-5 cluster, namely MMAR_2676, which is the *M. marinum* orthologue of *M. tuberculosis* Rv1794 (96% identity). A mutation in the ESX-5 cluster was expected, based on our previous results obtained with the M strain [16]. Interestingly however, although in both screens we identified only a single gene in the ESX-5 cluster to be affected, both these genes are different, *i.e.* MMAR_2676 for E11 and MMAR_2680 for the M strain.

The 7C1 mutant showed normal growth in cultures, but reduced growth on plates, accompanied by a more smooth colony morphology. Complementation of 7C1 with the corresponding MMAR_2676 gene using various DNA fragments or with the entire ESX-5 region of *M. marinum* [16] on a mycobacterial low copy number shuttle plasmid did not result in complementation of PPE41 secretion (results not shown), but showed some restoration of EsxN secretion (Fig. 1B). This result probably means that there is a secondary mutation in the 7C1 mutant or that expression of MMAR_2676 needs to be tightly regulated to avoid stoichiometry problems affecting protein secretion. Additional experiments showed that the latter situation was probably true, since complementation of PE_PGRS secretion was achieved when a chromosomal integration construct was used containing both the MMAR_2676 gene and *esxMN* genes (see below).

ESX-5 mediates the secretion of EsxN

To study which proteins are secreted via ESX-5 we first focused on an obvious candidate, namely EsxN, the ESAT-6 homologue that is encoded by the ESX-5 gene cluster. A problem

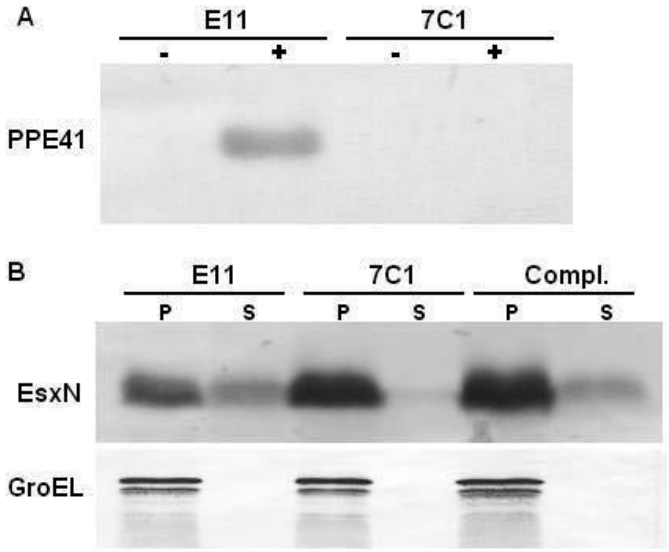

Figure 1. Isolation and characterization of the ESX-5 secretion mutant 7C1 of *M. marinum* E11. A. Immunoblot analysis of equivalent amounts of culture supernatant of *M. marinum* wild type E11 and the secretion mutant 7C1, with (+) or without (-) the PE25/PPE41-containing plasmid, for the presence of PPE41. B. Immunoblot analysis of the ESX-5 secretion mutant 7C1, and complemented 7C1 mutant showing cell pellets (P) and the culture supernatant (S) fraction using polyclonal antiserum directed against EsxN-like proteins (Mtb9.9) or monoclonal antibody against GroEL2.

with detecting EsxN is that both *M. tuberculosis* and *M. marinum* are coding for several (3-6) proteins that are highly homologous to EsxN (more than 90% identity) [39,40]. Thus, when antibodies are used, it cannot be concluded with confidence which of these EsxN-like proteins is seen on immunoblot. These proteins are collectively known as Mtb9.9 [39].

First, we analyzed culture supernatant of *M. marinum* and showed that indeed a small protein is recognized by antiserum specific for EsxN and that this protein is partially secreted by both *M. marinum* strains (69% and 49% is secreted by E11 and M strains, respectively) (Fig. 2A,B). Since the intracellular control protein GroEL2 was not detected in the culture supernatant (Fig. 2A,B), the extracellular presence of this EsxN-like protein was not due to cell leakage or lysis. Analysis of both ESX-5 mutants showed that, although EsxN was efficiently expressed in these mutants (Fig. 2A,B), only a minimal amount of EsxN-like protein (<3% and <9% respectively) was detected in the culture supernatant, indicating ESX-5 dependence.

To determine if EsxN can also be located to the cell surface, intact bacteria were subjected to proteinase K treatment (Fig 2A,B). This protease treatment efficiently removed the majority of the EsxN-like molecules of the wild-type E11 strain (Fig. 2B), whereas there was no significant removal of EsxN from the cell fraction of the M strain (Fig. 2A). In contrast, both ESX-5 mutants contained protease resistant EsxN molecules (Fig. 2A,B). Together, these results show that the localization of EsxN-like protein(s) in

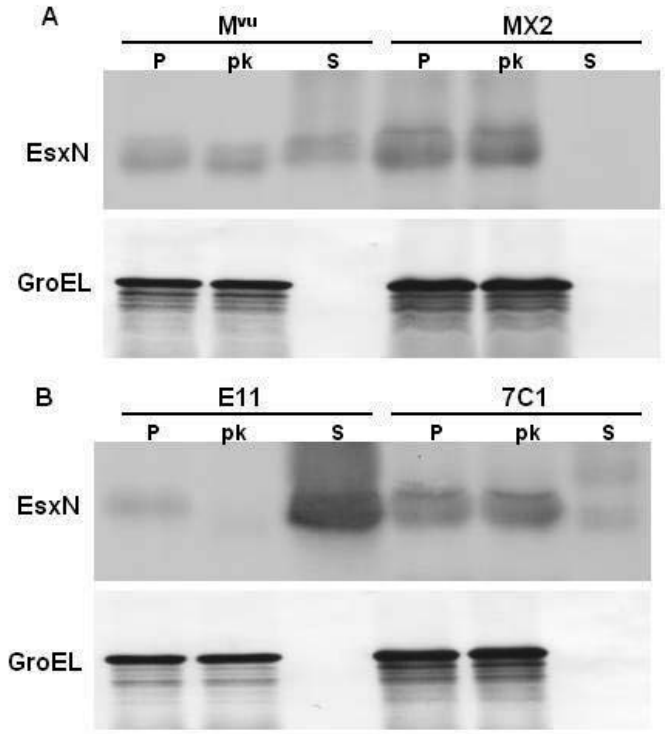

Figure 2. Secretion and surface accessibility of EsxN-like proteins in *M. marinum* M strain and its ESX-5 mutant Mx2 (A) or the E11 strain and its mutant 7C1 (B). Immunoblot of *M. marinum* M and the Mx2 mutant showing cell pellet (P), cells treated with proteinase K (pk) and the culture supernatant (S) fraction using either antiserum directed against EsxN or against GroEL2 as a control for cell death/leakage.

the culture supernatant and on the cell surface of strain E11 is dependent on ESX-5. Secretion of EsxN-like proteins in E11 could be partially restored by the introduction of the MMAR_2676 gene on a plasmid (Fig. 1B).

PPE10, PPE13 and PE_PGRS45 are secreted via ESX-5

Since the known substrate of ESX-5 is a PPE protein, two other PPE proteins as well as a PE_PGRS protein were tested as potential substrates [16]. For this approach we have chosen the PPE10, PPE13 and PE_PGRS45 genes of *M. tuberculosis*, which all have close homologues or orthologues in *M. marinum*. PPE10 and PPE13 both belong to the major polymorphic tandem repeat (MPTR) PPE subfamily. These three genes were individually cloned under control of the promoter of the 19kDa antigen gene and modified to express an HA-tag at the C-terminus. Subsequently, these constructs were introduced into *M. marinum* M, E11 strains and their ESX-5 mutants. Upon introduction of the different constructs in *M. marinum* wild-type strains, 39%, <5% and 35% of PPE10, PPE13 or PE_PGRS45 could be detected in the culture supernatant, respectively by immunoblots (Fig. 3A-C, data shown for

the M strain). The supernatant of PPE10-expressing wild-type strains also showed a major band with lower molecular weight, probably representing processed or partially degraded PPE10 (Fig. 3A). Bacterial lysis did not contribute significantly to the protein profiles in the culture supernatant, as GroEL2 was exclusively present in the cell fraction (Fig. 3A-C). Since the culture supernatants of the ESX-5 mutants did not show bands reacting with the HA antisera, these results show that PPE10 and PE_PGRS45 are partially secreted via ESX-5, whereas PPE13 was not secreted in substantial amounts.

To study the potential surface localization of PPE13 proteinase K assay was performed. Protease treatment of intact cells showed that the majority of cell-associated PPE13 material could be removed (Fig. 3B), indicating a location at the cell surface. Since PPE13 expressed in ESX-5 mutant cells was not affected by proteinase K we conclude that surface localization of this protein is also due to ESX-5. Also substantial amounts of PPE10 and PE_PGRS45 could be removed from the cell fraction by protease treatment in wild-type cells but not in the ESX-5 mutants (Fig. 3A,C). Together, these results show that these randomly chosen PE_PGRS and PPE-MPTR proteins are all secreted across the mycobacterial cell envelope via the ESX-5 pathway.

Proteomics of the ESX-5 secretome

To study ESX-5 pathway secretion in an unbiased manner, we analyzed the short-term culture filtrates of both wild-type M and E11 strains and their ESX-5 mutants using a

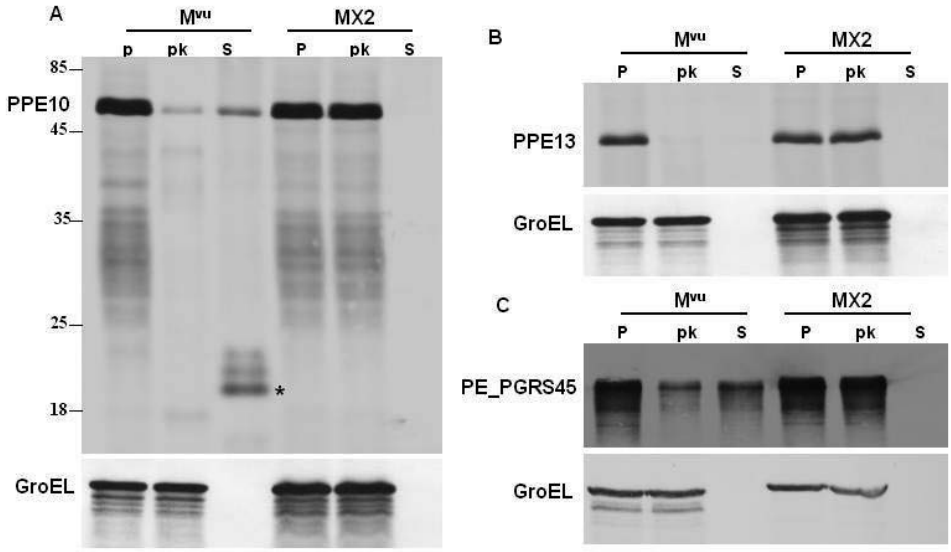

Figure 3. Secretion of PPE10 (A), PPE31 (B) and PE_PGRS45 (C) by *M. marinum* M strain and its ESX-5 mutant (Mx2). Immunoblot analysis of *M. marinum* strains supplemented with the various plasmids showing cell pellet (P), cells treated with proteinase K (pk) and the culture supernatant (S) fraction using either antibody directed against the HA-tag or GroEL2. * indicates processed or degraded PPE10. Similar results were obtained for strain E11 (data not shown)

proteomic approach. Because the addition of BSA is essential for efficient (induction of) secretion via ESX-5 [16], we first cultivated *M. marinum* in the presence of BSA, washed the cells extensively and incubated for an additional 24 hours in medium without BSA (experimental procedures). The culture supernatants of three independent cultures were analyzed and only the protein spots that were present in significantly increased amounts in all three gels were analyzed by MALDI-TOF/TOF mass spectrometry.

Although the protein secretion profiles of the two wild-type strains E11 and M showed many similarities (Fig. 4 and Supplemental Fig. S1, respectively), there were also major differences. In particular, the absence of several major spots at the 10kD range of the M strain was apparent. Mass spectrometry (MS) showed that these spots were ESAT-6 and CFP-10 (Table 1). This means that E11 secretes high amounts of these proteins whereas M does not. These results were confirmed by immunoblot using antibodies directed against *M. tuberculosis* ESAT-6 (Supplemental Fig. S2), indicating an ESX-1 secretion defect in the M strain. However, from the literature it is known that the M strain does secrete ESAT-6 efficiently [18]. Analysis of a different M isolate obtained from the Brown lab also confirmed these results (results not shown), which means that our first *M. marinum* M isolate, now designated M^{vu}, somehow acquired a mutation affecting ESAT-6 expression and/or secretion. Because the extended RD1 region is, together with the

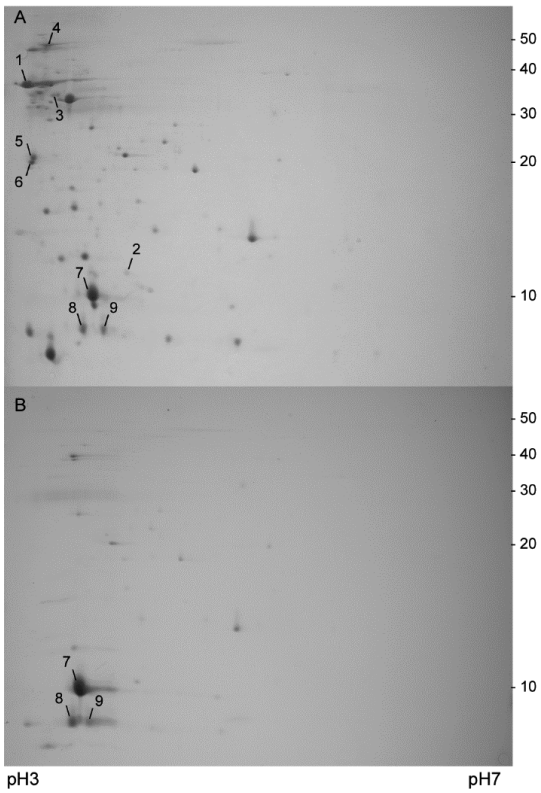

Figure 4. 2D gels of culture filtrate proteins from *M. marinum* wild-type E11 (upper panel) and its ESX-5 mutant 7C1 (lower panel). 150 µg protein of short-term culture filtrates was hydrated into 3-10 pH strips in the first dimension. In the second dimension, proteins were separated on a 10% polyacrylamide gel and visualized with coomassie brilliant blue. This 2D gel is a representative of three independent experiments. Numbers indicate the protein spots that were present ion all three experiments, excised and successfully identified by MS analysis (Table 1).

espA locus (Rv3614c-Rv3616c), required for ESAT-6 secretion [18,23,24], it seems likely that the M^{vu} strain was disturbed in ESAT-6 secretion by a spontaneous mutation in this region. However, our efforts to determine the precise component responsible for this defect have thus far been unsuccessful (results not shown).

2D gel analysis showed many differences between the culture filtrates of wild-types and their ESX-5 mutants (Fig. 4 for E11, Supplemental Fig. S1 for M). MALDI-TOF/TOF mass spectrometry showed that the wild-type-specific spots were corresponding to PE_PGRS and PPE-MPTR proteins (Table 1). Two of these proteins, encoded by MMAR_1402 (a PPE-MPTR protein) and MMAR_3570 (a PE_PGRS protein), are present in culture filtrates of both wild-type strains. Both proteins are present at multiple locations, indicating proteolytic processing and/or degradation (Table 1). A number of (major) spots in the highly acidic region (pH~3,5) could not be determined by trypsin cleavage. Subsequent digestion with endoGluC resulted in a positive identification for one of these spots in the supernatant of strain E11 as the C-terminal domain of PPE protein MMAR_1402 (Table 1). *In silico* analysis of the C-terminal sequence of MMAR_1402 indeed showed a complete absence of Lys/Arg residues needed for trypsin cleavage. In fact, difficulties in identifying PE_PGRS and PPE-MPTR proteins by a proteomic approach seems to be a general characteristic of these proteins, they all have limited numbers of charged amino acid residues and therefore only few peptides can be recovered for MS analysis with either

Spot	MMAR_ gene number	Mt orthologue	Gene product	No of peptides	Protein coverage	Protein Score C. I. %	Protein MW (KDa)	Protein PI
Wild-type strain E11 (Figure 4)								
1*	MMAR_1402	-	PPE-MPTR	1	2%	100	61.1	3.93
2	MMAR_1402	-	PPE-MPTR	4	9%	100	61.1	3.93
3	MMAR_2933	-	PE_PGRS	1	2%	99.99	53.2	3.7
4	MMAR_3316	-	PE_PGRS	1	2%	100	79.9	3.84
5	MMAR_3570	-	PE_PGRS	3	5%	100	81.9	3.78
6	MMAR_3570	-	PE_PGRS	3	5%	100	81.9	3.78
7	MMAR_5449	esxB	CFP-10	3	46%	100	10.7	4.56
8	MMAR_5450	esxA	ESAT-6	2	38%	100	9.9	4.67
9	MMAR_5450	esxA	ESAT-6	2	38%	100	9.9	4.67
Wild-type strain M (Figure S1)								
10	MMAR_1402	-	PPE-MPTR	4	9%	100	61.1	3.93
11	MMAR_3570	-	PE_PGRS	3	5%	100	81.9	3.78
12	MMAR_3570	-	PE_PGRS	3	5%	100	81.9	3.78
13	MMAR_3728	-	PE_PGRS	1	3%	100	33.9	4.74

Table 1. List of secreted proteins identified by 2-DE combined with MALDI-TOF MS from culture filtrates of M. marinum wild-types and ESX-5 mutants.
* this protein was identified by EndoGlu digestion.

trypsin or EndoGlu. PPE or PE_PGRS proteins were never identified in the culture filtrates of the ESX-5 mutants. Together, these findings confirm that an intact ESX-5 cluster is required for extracellular presence of PPE-MPTR and PE_PGRS proteins.

PE_PGRS proteins are secreted via ESX-5

The proteomics analysis described above showed that PE_PGRS proteins are secreted via ESX-5. PE_PGRS is the major subclass of PE proteins, with 69 members in *M. tuberculosis* H37Rv [34,35,41] and 148 family members in *M. marinum* M (http://genolist.pasteur.fr/MarinoList/; [40]). This family is characterized by a variable number of (imperfect) glycine-rich repeats (GGAGGX) in their C-terminal domain. To study the effect of the ESX-5 mutation on PE_PGRS proteins we used a novel monoclonal antibody raised against the PGRS domain of PE_PGRS33 (Rv1818c) of *M. tuberculosis* (Fig. 5A). To determine if this antibody is able to recognize also other PE_PGRS proteins three randomly chosen PE_PGRS genes were heterologously expressed in *E. coli* and tested. As can be seen in Fig. 5B, all three PE_PGRS proteins were recognized by this antiserum. To determine the specificity of the antibody, total cell fractions of a number of different mycobacterial species were tested on immunoblot. *M. tuberculosis, M. bovis* and *M. marinum* all have a large amount of PE_PGRS genes [34], *M. avium* contains ESX-5 and a number of PE genes, but no PE_PGRS genes, while fast-growing species like *M. smegmatis* contains only two PE genes and no PE_PGRS genes or ESX-5 [34]. As can be seen in Fig. 5C only species containing PE_PGRS genes produce proteins that react with the antiserum, indicating specificity for PE_PGRS proteins.

Subsequently, this monoclonal antibody was used to study the supernatant of our two different *M. marinum* strains, their ESX-5 mutants and the complemented strains. The supernatant of both wild-type strains E11 and M and the complemented strains contained various bands cross-reacting with the antibody, whereas only a faint band was seen in the supernatant of the ESX-5 mutants (Fig. 6). This reduction in extracellular PE_PGRS protein is not due to a general secretion defect since the secreted APA protein was present in equal amounts in the supernatant of all strains (shown in Figure 6A for strain M). To confirm that different PE_PGRS proteins are visualized by this antiserum we also performed these experiments on 2D gels (Supplemental Fig. S3) and analyzed the antibody-reacting spots by MS analysis (Table 2). All spots that could be identified by tandem MS contained PE_PGRS proteins and in total 6 different PE_PGRS proteins were identified in the culture filtrates. Together, these results indicate that all PE_PGRS proteins secreted in culture medium of *M. marinum* are mainly dependent on an active ESX-5 system.

DISCUSSION

In our previous study we have shown that *M. marinum* contains a functional ESX-5 type VII secretion system, which is responsible for the secretion of heterologously expressed PPE41 [16]. Based on comparative genomics and cluster analysis it has been proposed that ESX-5 is recently evolved in the mycobacteria, and that its appearance

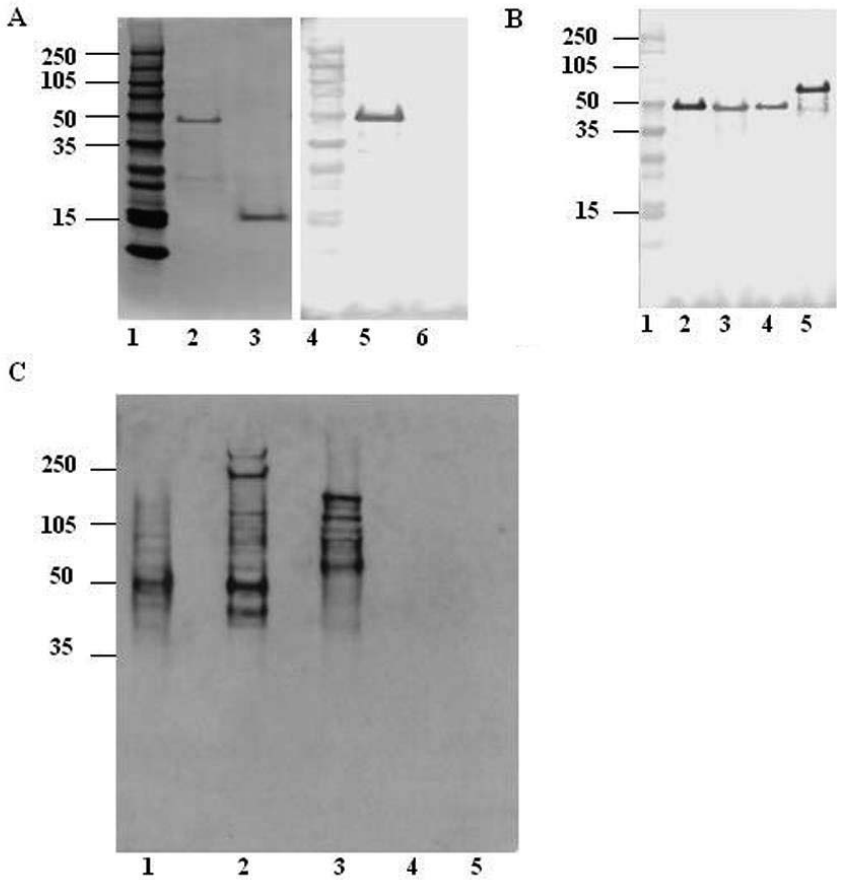

Figure 5. Characterization of the PGRS-specific monoclonal antibody. (A) PGRS monoclonal antibody is directed against the PGRS domain and not against the PE domain. Purified His-tagged recombinant PE_PGRS33 (lanes 2 and 5) or the PE domain of PE_PGRS33 (lanes 3 and 6) was separated on SDS-PAGE and analyzed by coomassie stain (lanes 1-3) and immunoblot (lanes 4-6). (B) The PGRS monoclonal recognizes different, randomly chosen, and heterologously expressed PGRS proteins. Immunoblot of purified His-tagged recombinant PE_PGRS33 (lane 2), PE_PGRS1 (lane 3), PE_PGRS18 (lane 4) and PE_PGRS24 (lane 5). (C) The PGRS monoclonal is specific, it recognizes (PE_PGRS) proteins in the total cell fraction of *M. bovis* BCG (lane 1), *M. tuberculosis* CDC 1551 (lane 2), *M. marinum* (lane 3), but it does not recognize any protein of *M. avium* 104 (lane 4) and *M. smegmatis* mc^2155 (lane 5), which both do not contain PE_PGRS genes.

in evolution predates the expansion of the PE and PPE genes [34]. This finding led us to hypothesize that ESX-5 is a specialized protein secretion pathway that is devoted to the transport of the recently evolved subclasses of PE and PPE proteins, *i.e.* PE_PGRS and PPE-MPTR. By comparing the profile of proteins secreted by *M. marinum* wild-type and ESX-5 mutants we now show that a number of PE_PGRS and PPE-MPTR proteins are indeed dependent on ESX-5. With 69 members in *M. tuberculosis* and 148 members in *M. marinum*, the PE_PGRS proteins form by far the largest subfamily of

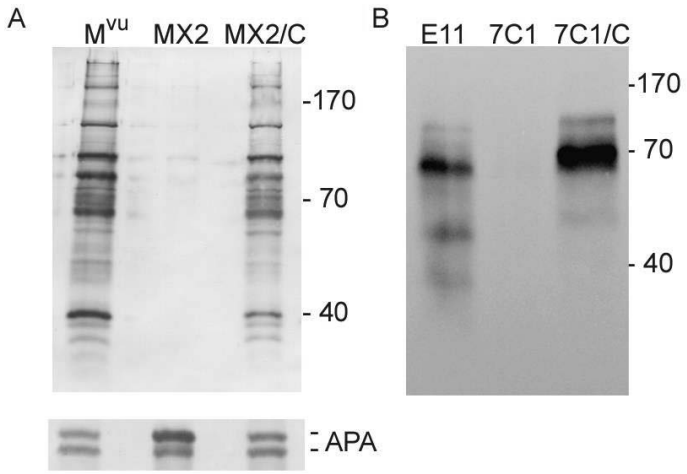

Figure 6. Secreted PE_PGRS proteins are dependent on ESX-5. Immunoblot analysis of equivalent amounts of collected short-term culture filtrates of *M. marinum* wild-type M and E11 strains, their ESX5-mutants and the complemented strains. Immunoblots are analyzed using monoclonal antiserum directed against PE-PGRS proteins (A,B) or polyclonal serum directed against the ESX-5-independent secreted protein (A, lower panel).

spot	gene number	Number of peptides	Protein coverage	Protein Score C. I. %	Protein MW (KDa)	Protein PI
1	MMAR_2933	1	2%	100	53.2	3.7
2	MMAR_2933	1	2%	100	53.2	3.7
3	MMAR_3316	1	2%	100	79.9	3.84
4	MMAR_3316	2	6%	100	79.9	3.84
4	MMAR_5135	1	3%	100	56.6	4.04
5	MMAR_3316	1	2%	100	79.9	3.84
6	MMAR_3105	2	7%	100	64.4	3.97
6	MMAR_3316	1	2%	100	79.9	3.84
6	MMAR_3400	3	7%	100	68.5	4.02

Table 2. PE_PGRS proteins in the culture filtrate of M. marinum wild-type E11 strain as identified by immunoblot using PGRS antiserum combined with MALDI-TOF MS.

PE proteins [42]. This family is characterised by the presence of polymorphic GC-rich sequence motifs in their genes, which are coding for imperfect Gly-Gly-Ala or Gly-Gly-Asn repeats. Despite their abundance, very little is known about the function of the proteins encoded by these multigene families. Multiple laboratories have shown that some PE_PGRS and PPE proteins are located at the cell surface [26,36,41,43,44], although the secretion route was never established. Using a new monoclonal antibody we were able to show that in *M. marinum* secreted PE_PGRS proteins were dependent

on ESX-5. Apparently, ESX-5 is, directly or indirectly, involved in the appearance of PE_PGRS proteins in the culture supernatant. If PE_PGRS proteins are secreted directly through the ESX-5 system this would mean that ESX-5 is a major secretion pathway for extracellular proteins in *M. marinum* and possibly also in *M. tuberculosis*. However, it should be noted that all the extracellular PE_PGRS and PPE-MPTR proteins we have identified in this study do not have orthologues in *M. tuberculosis*, and therefore the situation for this species might be different.

Although we identified numerous PE_PGRS and PPE proteins among the ESX-5-dependent secreted proteins, we cannot rule out that other PE and PPE proteins may be secreted via other secretion pathways, such as via the other ESX secretion systems. For instance, the small amounts of PE_PGRS proteins in the culture supernatant of ESX-5 mutants could be secreted via other ESX systems. Furthermore, other studies have shown that the *M. tuberculosis* PE_PGRS33 protein is surface exposed when expressed heterologously in *M. smegmatis* [36,43]. Since *M. smegmatis* does not have an ESX-5 secretion system this would mean that this PE_PGRS protein is secreted via another secretion pathway. The ancestral PE and PPE proteins, which are either present within or duplicated from ESX clusters 1 to 3, could be secreted by their respective secretion apparatus [34]. Probably, the PE and PPE gene families have evolved in conjunction with the ESX secretion systems and the most recently duplicated members, *i.e.* the PE_PGRS and PPE-MPTR subfamilies, are specifically linked to ESX-5 [34].

Proteomic analysis also showed that in many cases, the same ESX-5 substrate was identified from different 2-DE spots with different molecular mass and acidic isoelectric point (pI) regions, possibly as a consequence of posttranslational modification or proteolytic processing. Similar processing was also observed for one of the HA-tagged PPE proteins, which could mean that the N-terminal PE and PPE domains are only present during transport and/or cell surface localization. Recent experiments have shown that the PE domain of PE_PGRS proteins is responsible for the transport of these proteins, indicating that this domain could function as a sort of leader peptide [45]. The recently identified ESX-1 substrate Rv3881c (EspB) was also shown to be processed [25], which could indicate that processing is common in ESX secretion pathways. The gene clusters encoding mycobacterial type VII secretion systems all contain a gene encoding a member of the subtilisin-like proteases, which could be involved in this process.

In addition to PE_PGRS and PPE proteins, we have also identified EsxN-like proteins as ESX-5-substrates. These proteins are ESAT-6-like proteins encoded by ESX-5 or paralogue of EsxN and it was therefore not surprising to detect them in the culture filtrate. Both EsxN-like proteins have been identified previously in culture filtrates of *M. tuberculosis* [24,39,46], although the transport pathway was never identified.

The ESX-5 secretion mutant in strain E11 was identified by screening a transposon mutant library. Similar as for our previous screen [16], we were able to identify very few non-secreting mutants (only 1 in approximately 10.000). The only non-secreting mutant was located in the ESX-5 cluster, which is consistent with our previous finding that the ESX-5 cluster is involved in this process [16]. Apparently, ESX-5 mutants are

difficult to obtain, either because of redundancy of other ESX systems present in mycobacteria or because these mutations are harmful. The gene identified in this screen was different form the previous screen in another *M. marinum* strain, which could indicate that there are strain differences. Similar situation seems to be present in *M. tuberculosis*, where most ESX-5 genes can not be mutagenized in a transposon screen in strain H37Rv [47], whereas these insertions are identified in strain CC1551 (http://webhost.nts.jhu.edu/target/).

In summary, we have provided experimental data that shows that ESX-5 is responsible for the transport and secretion of PE_PGRS and PPE-MPTR proteins. The ESX-5 substrates are secreted in the culture supernatant or present on the bacterial cell surface. Since disruption of ESX-5 in *M. marinum* has been shown to result in a dramatic effect on cytokine production by macrophages [48], these secreted PE-PGRS and PPE-MPTR proteins seem to be the main candidates involved in the modulation of the macrophage response. Studies are underway to determine the role of these individual proteins in this process.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Wild-type M. marinum strains M, E11 and their mutants Mx2 and 7C1 were routinely grown in Middlebrook 7H9 liquid medium or Middlebrook 7H10 agar supplemented with 10% Middlebrook ADC or OADC respectively (BD, Biosciences) and 0.05% Tween 80. Escherichia coli strain DH5α was used for DNA manipulation experiments and propagation of plasmid DNA. Antibiotics were added at the following concentration: kanamycin, 25 μg ml-1 and hygromycin, 50 μg ml-1 for both mycobacteria and E. coli.

Molecular cloning of PPE10, 13 and PE_PGRS45

Specific primer pairs (Table 3) bearing restriction sites were designed for the PE_PGRS Rv2615c and two PPE-MPTR genes, Rv0442c and Rv0878c. A hemaglutinin (HA) tag was incorporated in the C-terminal end of the primers. Rv2615c was amplified using nested-PCR with both the outer and inner primers (Table 3) and M. tuberculosis H37Rv chromosomal DNA as template. The PCR amplicons were cloned into the pGemT-Easy T vector (Promega) and sequenced. Subsequently, the cloned products were sub-cloned into the mycobacterial expression vector p19Kpro (Koen de Smet).

SDS-PAGE and immunoblot

Mycobacteria were grown to mid-logarithmic phase in Middlebrook 7H9 liquid medium supplemented with 0.2% (w/v) dextrose, 0.05% Tween 80 and 0.1% of the advised amount of ADC supplement. The presence of BSA in the medium (part of the ADC supplement) is essential for PPE41 secretion in M. marinum [16]. Proteins in the cell free supernatants were precipitated with 5% TCA (w/v). Proteins were separated by SDS-PAGE on 10-15% polyacrylamide gels [49] and visualized by immunoblot using mouse monoclonal antibodies HA.11 (Eurogentec) and CS44, which is directed

Primer name	Sequence
SalgD	TAGCTTATTCCTCAAGGCACGAGC
Bampt	GATCGCTCGTGCC
PSalg	GCTTATTCCTCAAGGCACGA
pMyco	CCGGGGACTTATCAGCCAAC
Rv2615c outer f	CGTGGCGGTCAGGAGGATTT
Rv2615c outer r	GCTCGATGAGCCCAAAGGATGT
Rv2615c f	GGATCCATGTCGTTTGTCAACGTGGCCCCAC
Rv2615c r	AAGCTTTCAGGCGTAGTCCGGCACGTCGTACGGGTAGCCGTCGGCTCCGT TGG
Rv0442c f	GGATCCGTGACAAGCCCGCATTTTGCGTGGT
Rv0442c r	AAGCTTTCAGGCGTAGTCCGGCACGTCGTACGGGTACTCCGAACCGACCG GCTGCC
Rv0878c f	GGATCCATGAATTTCATGGTGCTGCCGCCGG
Rv0878c r	AAGCTTTCAGGCGTAGTCCGGCACGTCGTACGGGTACCCGCTGTTCCC TAC TTTTT

Table 3. Primers used in this study.
C-terminal HA tag is shown in italics and the introduced restriction sites (BamHI and HindIII) in bold.

against GroEL2 of M. tuberculosis (John Belisle, NIH, Bethesda, MD, contract NO1 AI-75320); rabbit antiserum reactive to EsxN (rMtb9.9A), [39], Rv3881c [50] and PPE41 [16]. The presence of peroxidase-conjugated secondary antibodies was detected via 4-chloronaphtol/3,3-diaminobenzidine staining or via chemiluminescence (Pierce). For the treatment of intact cells with protease, mycobacteria were resuspended in PBS and left untreated or treated with 0.1 mg ml-1 proteinase K (Qiagen). After 30 min incubation at room temperature, cells were harvested and sample buffer was added. For protein quantification of immunoblots the presence of the second antibody with the Lumi-Light Western Blotting Substrate (Roche Applied Science) on a Fluor-S MultiImager (Bio-Rad) using Bio-Rad multianalyst software, version 1.0.2.

Recombinant PE and PE_PGRS constructs

Recombinants of PE_PGRS and PE genes fused to a Histidine tag were constructed using the pET15b expression vector and proteins expressed in *E. coli* were purified as described in Brennan et al [36].

Monoclonal antibody directed against the PGRS domain— mAb 7C4.1F7 was produced after fusing spleen cells from BALB/c mice immunized with DNA coding for PE_PGRS 33 and boosted once with recombinant PE_PGRS protein with the P3-X63-Ag.8.653 myeloma cell line (ATCC), using the procedure of Margulies et al [51]. Ascites of mAb 7C4.1F7 were provided by Harlam, Bioproducts, Indianapolis, Indiana, USA.

Isolation, identification and complementation of *M. marinum* ESX-5 mutant

The mariner-based transposon system using the mycobacterial specific phage phiMycoMarT7 [47] was used to generate a transposon insertion mutant library of M. marinum strain E11 supplemented with the Rv2430c as described previously [16]. Ligation-mediated PCR was used to localize and identify the transposon-disrupted gene, as described previously [16]. The resulting DNA sequence was compared with the M. marinum genome sequence (http://www.sanger.ac.uk/Projects/M_marinum/). We tried to complement the M. marinum E11 MMAR_2676 mutant using various constructs. First, we used the low-copy pSMT3-GFP shuttle plasmid [52] as a vector for these constructs, utilising the HSP60 promoter to direct the expression of MMAR_2676. Different fragments containing the gene were amplified by PCR and cloned or cloned directly into this vector from the pSMT-H5 plasmid containing the entire ESX-5 cluster [16]. Similar version of these plasmids were constructed without the HSP60 promoter, with similar negative results. Finally, we also used an integration vector for complementation. For this, a derivate of pUC-Gm-Int [53] was used in which the gentamycin resistance gene was replaced by the chloramphenicol resistance gene of pACYC154 under control of the mycobacterial HSP60 promoter. This new integration vector is called pUC-Int-cat. Subsequently, the MMAR_2676 gene and the upstream genes esxMN were cloned as a 2.7 kb SpeI/XbaI fragment from pCR-H5-last [16] in two orientations in this vector and used to produce a chloramphenicol resistant co-integrate in the 7C1 mutant. Only one of orientations resulted in complementation of the PE_PGRS secretion.

Two-dimensional gel analysis of culture supernatants

Short-term culture filtrates were prepared as follows. Strains were grown under agitation at 30oC in 7H9 supplemented with 0,2% Dextrose and 0,002% BSA until the culture reached an OD600 of 0,8 - 1,0. Cultures were then washed 3 times with 50 mM Tris-HCl pH 8,0 in order to remove all BSA and the pellet was resuspended in 7H9 supplemented with 0,2% Dextrose and incubated overnight at 30oC. Culture filtrate was harvested by centrifugation and filtration, first through 0,45µm filter (Whatman) and then through 0,2 µm filter. This culture filtrate was concentrated by ultrafiltration (Millipore, PLBC, nmwl: 3000) and the proteins were precipitated with TCA and dialysed overnight (10 mM Tris-HCl pH 8,0). Equal amounts of protein (150 µg) from each strain were mixed with rehydration buffer (8M urea, 2% CHAPs, 40 mM DTT and 0,5% Pharmalyte 3-10). Protein was hydrated by performing isoelectric focusing (IEF) on 24-cm Immobiline dry strips (Amersham, Biosciences) with pH interval 3-10NL using Ettan IPGphor Isoelectric Focusing system (Amersham, Biosciences). Running conditions: 20oC, 50µa/strip, 30V 12.00 hr, 500V 5.00 hr, 1000V 1.00hr, and 8000V 4.00hr. Prior to the second dimension, the strips were incubated for 15 min in equilibration buffer (6M urea, 2% SDS, 50 mM Tris-HCl pH8, 8, 30% glycerol) with 65 mM DTT first and then with 135 mM iodoacetoamide. To resolve the second dimension, the strips were embedded into 10% SDS-PAGE gel. Gel was stained using colloidal Coomassie brilliant blue.

Mass spectrometry

For MS, protein spots from Coomassie stained 2-D gels were selected and excised manually. Protein spots were destained with ammonium bicarbonate, reduced with DTT, alkylated with iodoacetamide and digested for overnight in gel with modified porcine trypsin (Promega, Madison, USA). The extracted tryptic peptides were concentrated and desalted on reversed-phase ZipTips (Millipore) and eluted with 3 ml CHCA matrix (Sigma). A 0.8 µl sample from the peptide-matrix mixture was analyzed by MALDI-TOF/TOF mass spectrometry (4800 MALDI-TOF/TOF; Applied Biosystems). The obtained mass spectra were searched against M. marinum complex database using a MASCOT search engine (Matrix Science) with a mass tolerance set at 20 ppm for MS1 and 0.6 Da for MS/MS. The protonated trypsin autodigestion products at m/z 842.510 and 2,211.104 were used for internal calibration of the MALDI-TOF-MS spectra. All the proteins listed were identified with a confidence interval of at least 95% from the combined MS and MS/MS analysis.

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SUPPORTING INFORMATION

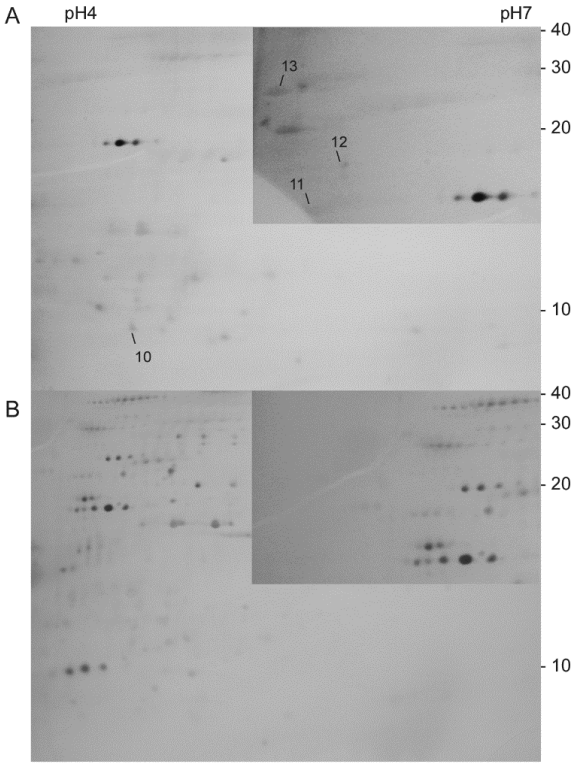

Figure S1. 2D gels of culture filtrate proteins from *M. marinum* wild-type M strain (A) and its ESX-5 mutant Mx2 (B) . 150 µg protein of short-term culture filtrates was used for this analysis. In the second dimension, proteins were separated on a 10% polyacrylamide gel and visualized with silver staining. The inset shows an enlargement of the top left part of the gel, which contains most extra spots in the wild-type strain. Numbers indicate the proteins that were excised and successfully identified by MS analysis (Table 1).

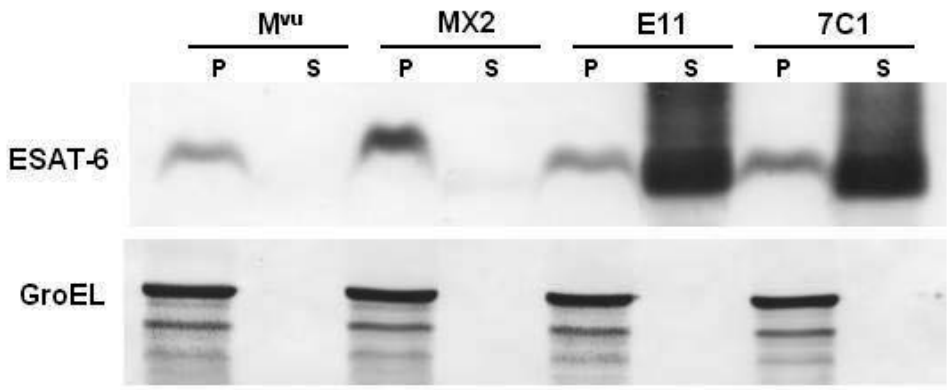

Figure S2. Secretion of ESAT-6 by *M. marinum* wild-type M and E11 strains and their mutants Mx2 and 7C1. Immunoblot analysis of equivalent amounts of *M. marinum* wild-types M and E11 strains and their mutants MX2 and 7C1 showing cell pellet (P) and the culture supernatant (S) fractions using either antiserum directed against ESAT-6 proteins or against GroEL2.

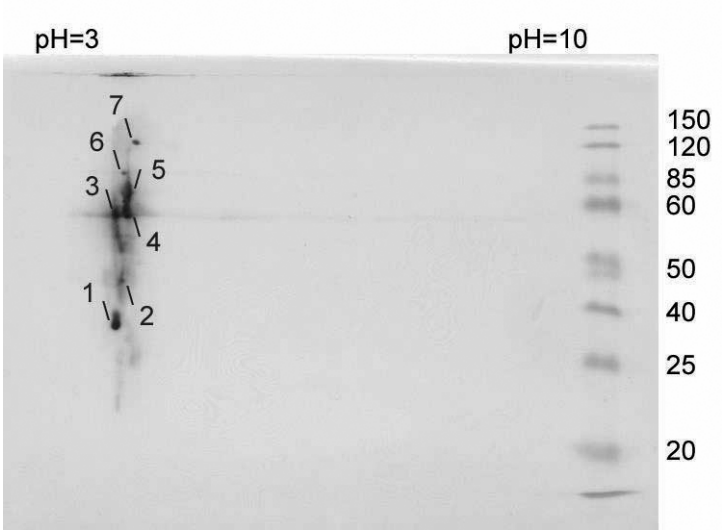

Figure S3. 2D gels of culture filtrate proteins from *M. marinum* wild-type E11 strain. 50 µg protein of short-term culture filtrates was hydrated into 3-10 pH strips in the first dimension. In the second dimension, proteins were separated on a 10% polyacrylamide gel, blotted onto nitrocellulose filter and incubated with monoclonal antiserum directed against PGRS proteins. In parallel another gel was run under identical conditions and stained with Coomassie Brilliant Blue. Spots appearing on immunoblot were excised at the similar location from the twin gels and subjected to MALDI-TOF/TOF mass spectrometry. Numbers indicate the proteins that were identified (Table 2), spot 7 did not give any results.