

A specific secretion system mediates PPE41 transport in pathogenic mycobacteria

Abdallah M. Abdallah,¹ Theo Verboom,¹
Fredericke Hannes,¹ Mohamad Safi,¹
Michael Strong,² David Eisenberg,²
René J. P. Musters,³
Christina M. J. E. Vandembroucke-Grauls,¹
Ben J. Appelmek,¹ Joen Luirink⁴ and Wilbert Bitter^{1*}

Departments of ¹Medical Microbiology and Infection Control and ³Physiology, VU Medical Centre, Amsterdam, the Netherlands.

²UCLA-DOE Institute for Genomics and Proteomics, University of California, Los Angeles, CA, USA.

⁴Department of Molecular Microbiology, Vrije Universiteit, Amsterdam, the Netherlands.

Summary

Mycobacterial genomes contain two unique gene families, the so-called PE and PPE gene families, which are highly expanded in the pathogenic members of this genus. Here we report that one of the PPE proteins, i.e. PPE41, is secreted by pathogenic mycobacteria, both in culture and in infected macrophages. As PPE41 lacks a signal sequence a dedicated secretion system must be involved. A single gene was identified in *Mycobacterium marinum* that showed strongly reduced PPE41 secretion. This gene was located in a gene cluster whose predicted proteins encode components of an ESAT-6-like secretion system. This cluster, designated ESX-5, is conserved in various pathogenic mycobacteria, but not in the saprophytic species *Mycobacterium smegmatis*. Therefore, different regions of this cluster were introduced in *M. smegmatis*. Only introduction of the complete ESX-5 locus resulted in efficient secretion of heterologously expressed PPE41. This PPE secretion system is also involved in the virulence of pathogenic mycobacteria, as the ESX-5 mutant of *M. marinum* was affected in spreading to uninfected macrophages.

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis, a chronic infectious disease that is respon-

sible for the death of over two million people each year. One of the major surprises of the *M. tuberculosis* genome sequence was that almost 10% of its coding capacity (167 genes) is devoted to two new gene families, the PE and PPE genes, named for the Proline and Glutamic acid (PE) and Pro–Pro–Glu (PPE) motifs near the N terminus of their gene-products (Cole *et al.*, 1998). In addition to these PE and PPE motifs, the family members share homologous N-terminal domains of approximately 110 amino acids for PE proteins and 180 amino acids for PPE proteins. Many PE and PPE proteins are composed only of these homologous domains, whereas other members have an additional C-terminal segment of variable length. These additional segments are often composed of multiple copies of polymorphic repetitive sequences, which led to the hypothesis that these proteins have a structural role (Cole *et al.*, 1998; Brennan and Delogu, 2002). PE and PPE genes have not been identified yet in any non-mycobacterial species, not even in closely related bacteria such as *Nocardia farcinica* (Ishikawa *et al.*, 2004).

Surprisingly, although PPE genes are widely present in pathogenic mycobacteria, such as *Mycobacterium avium* and *Mycobacterium marinum*, the non-pathogenic species *Mycobacterium smegmatis* shows a conspicuous lack of these genes. The *M. smegmatis* genome, which is more than 50% larger than that of *M. tuberculosis*, contains only two putative PPE genes (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>). Apparently, there is a strong selection for PPE proteins in pathogenic mycobacteria. The function of these proteins is still an enigma, but the available data suggest that PPE proteins are located at the cell surface (Sampson *et al.*, 2001; Pym *et al.*, 2002) and that they are involved in virulence (Li *et al.*, 2005). As PPE proteins lack a distinguishable signal sequence we reasoned that, if these proteins are indeed surface exposed, a dedicated secretion system must be present. Therefore, we analysed the expression and localization of PPE41, a small and soluble PPE protein. This hydrophilic protein, which is encoded by the *M. tuberculosis* Rv2430c gene, has been shown to induce a strong B-cell response in humans (Choudhary *et al.*, 2003). Rv2430c forms together with the PE25-encoding gene Rv2431c an operon (Tundup *et al.*, 2006) and recently the structure of the heterodimeric complex formed by PPE41 and PE25 has been determined (Strong *et al.*, 2006). In this study we show that PPE41 is secreted by the pathogenic species *M. marinum* and

Accepted 28 August, 2006. *For correspondence. E-mail w.bitter@vumc.nl; Tel. (+31) 20 4448319; Fax (+31) 20 4448318.

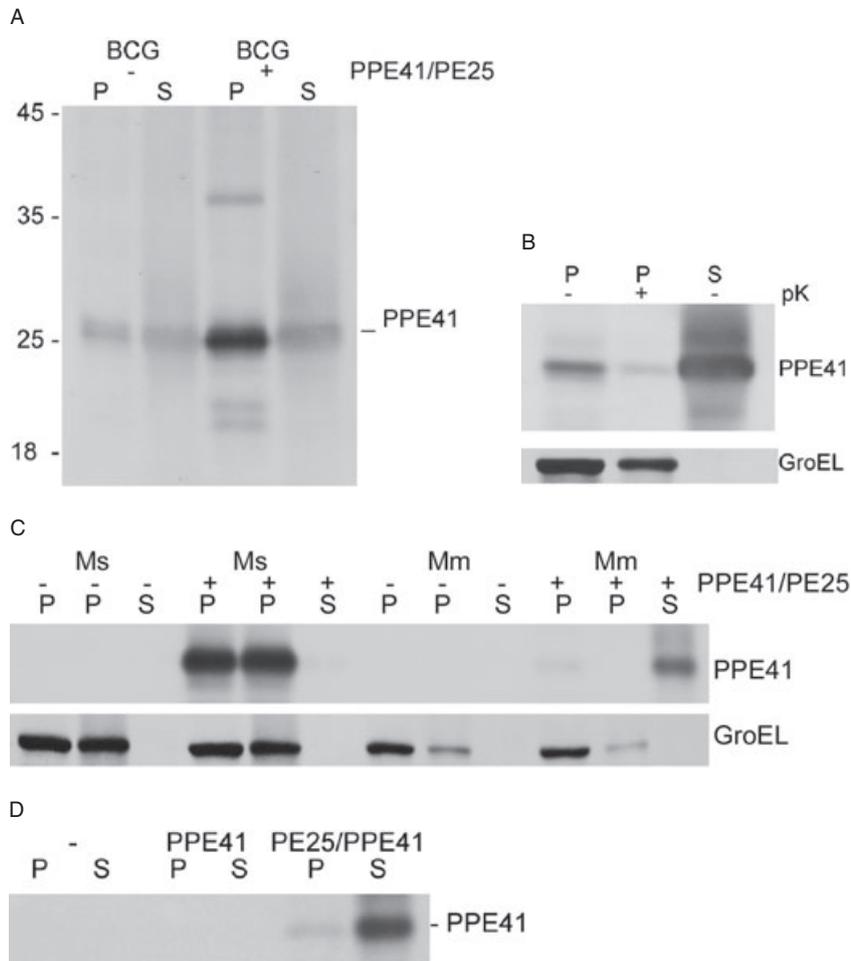


Fig. 1. Secretion of PPE41 by *M. bovis* BCG (A and B) and *M. smegmatis* (C) and *M. marinum* (C and D). Equivalent OD units of the cell pellet (P) and the supernatant (S) fractions were analysed for the presence of PPE41 or GroEL by immunoblot. When indicated, bacterial cell pellet fractions were treated with proteinase K (pK). A. PPE41 secretion of *M. bovis* BCG and *M. bovis* BCG complemented with the PE25/PPE41 operon under control of the *hsp60* promoter. Molecular size markers are shown in kDa on the left. B. PPE41 secretion in *M. bovis* BCG using GroEL as intracellular control and analysing the protease sensitivity of cell-associated PPE41. C. Introduction of the PPE41/PE25 operon in *M. smegmatis* (Ms) does not result in PPE41 secretion, whereas introduction in *M. marinum* (Mm) results in efficient secretion. D. In *M. marinum* the expression and/or stability of PPE41 is dependent on PE25.

Mycobacterium bovis, but not by *M. smegmatis*. Furthermore, the secretion system involved in this process has been identified and belongs to the class of ESAT-6-like secretion systems.

Results

PPE41 is secreted by *M. marinum* and *M. bovis*

PPE41 is a small hydrophilic protein, which is encoded by the *M. tuberculosis* Rv2430c gene. First, the expression and localization of PPE41 was examined in *M. bovis* BCG, which contains an operon encoding a PPE protein with 100% identity to PPE41 of *M. tuberculosis*. Analysis of the culture supernatant showed that *M. bovis* BCG secretes a protein that is recognized by the antiserum directed against PPE41 (Fig. 1A). Introduction of the PE25/PPE41 operon placed under the control of the Hsp60 promoter into *M. bovis* BCG resulted in the overproduction of a protein with an identical apparent molecular weight, indicating that this protein is PPE41 (Fig. 1A). Introduction of this operon also resulted in the presence of

bands at higher and lower molecular weight, which probably represent partially degraded, modified or complexed PPE41 (Fig. 1A). Because not all of PPE41 is secreted in the culture supernatant by *M. bovis* BCG (in Fig. 1B 63% is secreted), we also examined whether the cell-associated PPE41 molecules are intracellular or located on the surface using a protease sensitivity assay (proteinase K). The cytoplasmic control protein GroEL was not affected by proteinase K treatment (Fig. 1B), although this protein was degraded completely if lysed bacteria were used (Fig. S1). However, PPE41 present in the cell fraction could be efficiently removed by proteinase K (Fig. 1B), which indicates that the cell-associated form of PPE41 is located on the surface.

To determine whether PPE41 is also secreted by other mycobacterial species, the PE25/PPE41 operon placed under control of the Hsp60 promoter was introduced in *M. marinum* and *M. smegmatis*. These two species do not contain an endogenous copy of this operon, although *M. marinum* does contain a large number of other PPE genes. Upon introduction of the PE25/PPE41 operon in *M. marinum* this bacterium efficiently secreted PPE41;

73% of the protein could be detected in the culture supernatant (Fig. 1C). Also in *M. marinum* leakage of the cytoplasmic protein GroEL in the culture supernatant was minimal (Fig. 1C). The non-pathogenic species *M. smegmatis* efficiently expressed the PPE41 protein (Fig. 1C); however, only minimal amounts of PPE41 (< 5%) were observed in the culture supernatant. In this case, the cell-associated PPE41 material was resistant to proteinase K (Fig. 1C). Apparently, *M. smegmatis* does not secrete PPE41 efficiently.

The PPE41-encoding gene Rv2430c forms an operon together with the PE25-encoding gene Rv2431c. These two proteins form a stable heterodimeric complex (Strong *et al.*, 2006), and therefore the secretion of PPE41 might be dependent on the presence of PE25. Introduction in *M. marinum* of the Rv2430c gene, under control of the Hsp60 promoter, did not result in the presence of significant amounts of PPE41 (Fig. 1D), which indicates that PE25 is needed for secretion and/or stability of PPE41.

PPE41 is secreted by M. marinum inside macrophages

Next, we determined whether PPE41 was also secreted by mycobacteria inside macrophages. For these experiments we used human THP-1 cells, differentiated into macrophage-like cells in the presence of PMA, infected with *M. marinum*-expressing PE25/PPE41. The infected macrophages were differentially disrupted (*Experimental procedures*) and the various fractions were analysed by immunoblot for the presence of PPE41. In addition, immunoblots containing these fractions were also incubated with antibodies directed against the cytoplasmic protein GroEL and the secreted protein PknG (Walburger *et al.*, 2004) as controls. About half of the total amount of PPE41 was found associated with the bacteria in a protease-resistant form, which could represent intracellular PPE41. However, a similar amount of PPE41 was located in the Triton X-100-soluble fraction, representing the contents of macrophage-derived vesicles (Fig. 2). As the cytoplasmic protein GroEL was exclusively present in a protease-resistant form in the bacteria-containing fraction, we concluded that bacterial lysis inside macrophages was not significant. This shows that PPE41 is indeed secreted inside macrophages. The other control protein, i.e. PknG, was identified in small amounts in the fraction representing the macrophage cytosol and mainly in the Triton X-100-soluble fraction, as expected (Fig. 2). Intracellular secretion of PPE41 was confirmed with immunofluorescence microscopy. For this approach, macrophages were infected with either live or heat-killed *M. marinum*-expressing PE25/PPE41. Using antibodies directed against PPE41 and against lipoarabinomannan (LAM), a surface glycolipid of *M. marinum*, PPE41, was found colocalized with phagocytosed bacteria (Fig. 3A, Z-stack

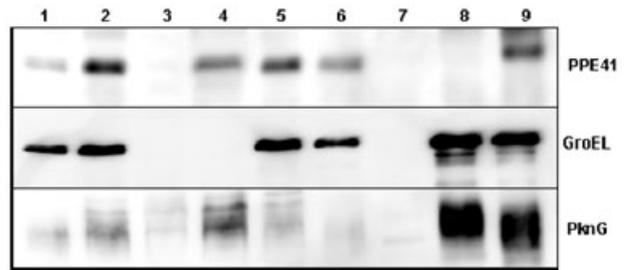


Fig. 2. Intracellular secretion of PPE41 by *M. marinum*. Immunoblot analysis of THP-1 cells infected for 48 h with *M. marinum* supplemented with PE25/PPE41. Infected cells were homogenized and cell debris was collected by low-speed sedimentation (lane 1). Subsequently, mycobacterial phagosomes (lane 2) were separated from the cytosol (lane 3) by sedimentation. Phagosomes were isolated and lysed by Triton X-100 treatment (lane 4) and bacteria were separated by sedimentation (lane 5) and treated with trypsin (lane 6). As a control we also analysed uninfected THP-1 cells (lane 7), THP-1 cells infected with *M. marinum* wild type (lane 8) and *in vitro* grown *M. marinum*-expressing PE25/PPE41 (lane 9). Immunoblots were incubated with antisera directed against PPE41, GroEL (cytoplasmic mycobacterial protein) and PknG (secreted mycobacterial protein).

shown in Movie S1). However, PPE41 was also present in vesicles devoid of bacteria (Fig. 3B, Z-stack shown in Movie S2), which indicates the release of PPE41 from mycobacterial phagosomes into the endocytic network of the host cell. To analyse further the intracellular localization of PPE41, infected cells were stained for LAMP-1, a late-endosome/lysosome marker. As PPE41-stained vesicles were usually associated with LAMP-1 (Fig. 3C) extracellular PPE41 probably accumulates in late endosomal vesicles. Both heat-killed *M. marinum* cells complemented with the PE25/PPE41 operon (Fig. 3D) and *M. marinum* cells devoid of this operon (results not shown) showed no extracellular PPE41 in macrophages, which indicates that this effect is specific and requires live bacteria. Next, we also tested whether PPE41 was secreted by *M. bovis* BCG. Although this species secretes lower amounts of PPE41, intracellular secretion of PPE41 could be observed (Fig. S2).

Identification of the PPE41 secretion system

PPE41 lacks a distinguishable signal sequence and is therefore probably not secreted via the Sec-secretion machinery. This means that a dedicated secretion system must be present in *M. marinum* and *M. bovis*. To identify this secretion system we first tested the only known Sec-independent secretion system in mycobacteria: the so-called ESAT-6 secretion system or ESX-1. ESAT-6 is a small protein, which is secreted together with CFP-10 by pathogenic and non-pathogenic mycobacteria. The secretion system involved has recently been identified and is (partially) encoded by the RD1 region (also called ESX-1)

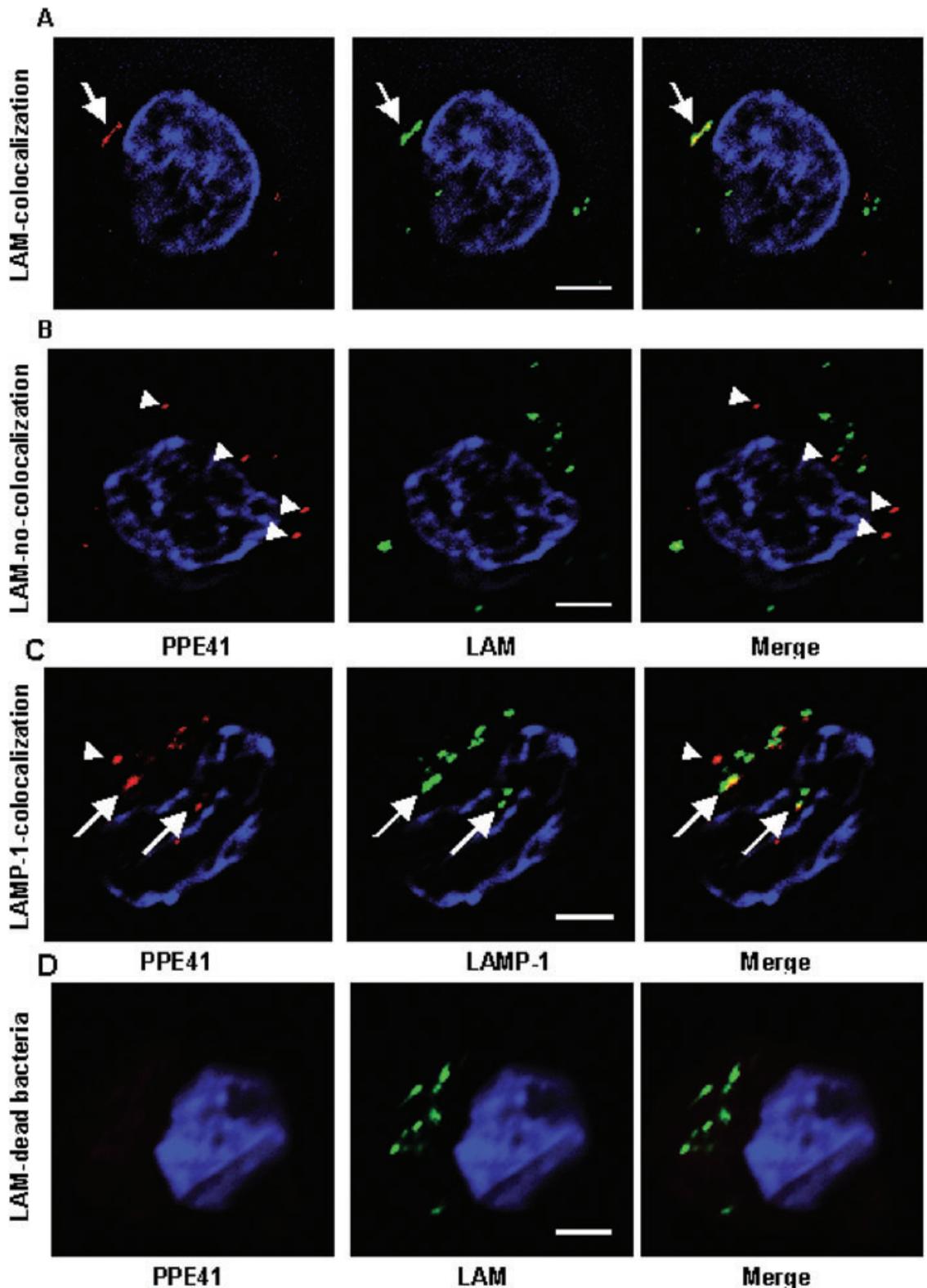


Fig. 3. Intracellular secretion of PPE41 by *M. marinum*. THP-1 cells were seeded on glass coverslips and infected with Mm::PE25/PPE41 (A–C) or heat-killed Mm::PE25/PPE41 (D) for 24 h. Infected cells were fixed, permeabilized and incubated with antisera directed against PPE41 or against LAM and examined by 3D digital imaging fluorescence microscopy. Secreted PPE41 is shown in red and intracellular bacteria (LAM) or the lysosomal marker protein (LAMP-1) in green, the nuclei are stained with DAPI (blue). Arrows show colocalization of PPE41 and mycobacteria, whereas arrowheads show PPE41 not colocalized with bacteria. Bar represents 10 μ M.

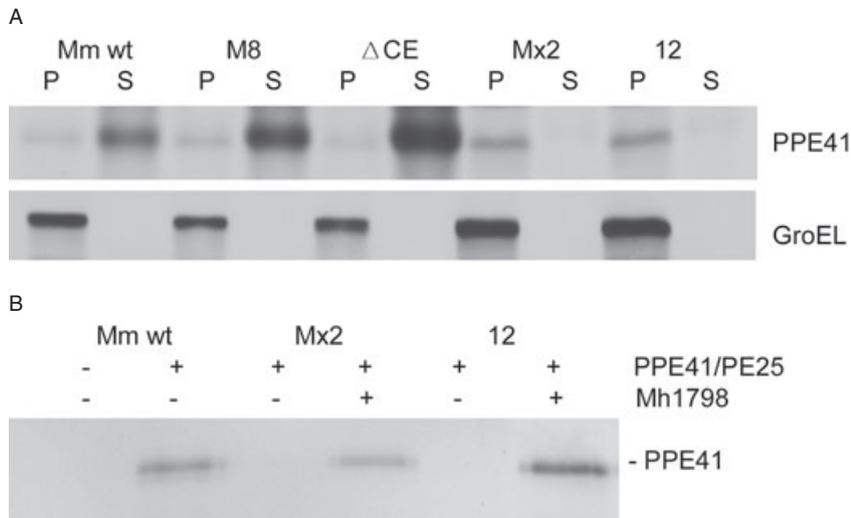


Fig. 4. Isolation and characterization of the ESX-5 secretion mutants.

A. Equivalent OD units of cell pellets (P) and culture supernatant (S) of *M. marinum* wild type (Mm wt), the ESX-1 mutants M8 and Δ CE and the isolated secretion mutants Mx2 and 12 were separated on SDS-PAGE, immunoblotted and incubated with polyclonal antiserum against PPE41 or GroEL.

B. Complementation of PPE41 secretion mutants. Equivalent amounts of culture supernatant of *M. marinum* wild type, with or without PE25/PPE41, the secretion mutants Mx2 and 12, supplemented with PE25/PPE41 and with the empty vector pSMT3 or with pSMT3 containing the Mh1798 gene, were separated on SDS-PAGE and analysed for the presence of PPE41 by immunoblot.

(Hsu *et al.*, 2003; Stanley *et al.*, 2003; Gao *et al.*, 2004; Guinn *et al.*, 2004; Converse and Cox, 2005), which is deleted in the vaccine strain *M. bovis* BCG (Mahairas *et al.*, 1996). Although we already know that *M. bovis* BCG is able to secrete PPE41, we still wanted to compare the level of PPE41 secretion in wild-type and mutant strains. Therefore, two RD1 mutants of *M. marinum* were analysed for PPE41 secretion, i.e. M8, which is mutated in one of the components of ESX-1, and Δ CE, which contains a deletion for the *cfp10/esat-6* operon (Gao *et al.*, 2004). The M8 secreted normal amounts of PPE41 in the culture supernatant (Fig. 4A), which shows that ESX-1 is indeed not required for PPE41 secretion. However, surprisingly the mutant devoid of the ESX-1 substrates (Δ CE) showed significantly increased secretion of PPE41 (Fig. 4A), indicating a link between the two secretion systems. The intracellular levels of PPE41 were comparable for these strains (Fig. 4A).

A genetic screen was used to identify the PPE41 secretion system in *M. marinum*. *M. marinum* strain M-expressing PE25/PPE41 was subjected to transposon mutagenesis, using the mycobacterial specific phage phiMycMarT7 containing the mariner-like transposon Himar1 (*Experimental procedures*). Subsequently, the mutants were grown on nitrocellulose filters and screened for PPE41 secretion using a double-filter colony blot approach (*Experimental procedures*, Fig. S3). Screening of 10 000 transposon mutants resulted in the isolation of two mutants with strongly reduced amounts of extracellular PPE41 (Fig. 4A). The transposon insertion sites of both mutants with reduced secretion, i.e. mutant Mx2 and Mx12, were determined by ligation-mediated PCR and sequence analysis. Comparison of these sequences with the complete genome of *M. marinum* M (http://www.sanger.ac.uk/Projects/M_marinum/) showed that both mutants contained the

mariner transposon at different positions in a single coding sequence (CDS). The gene product of this CDS shows high homology (93% identity) to Rv1798 of *M. tuberculosis* (Fig. S4) and was therefore designated Mh1798. Complementation of Mx2 and Mx12 with the corresponding gene on a mycobacterial shuttle plasmid resulted in restoration of PPE41 secretion (Fig. 4B). Mh1798 and Rv1798 belong to the AAA+ protein family of ATPases. Members of this family are chaperones that assist in the assembly and/or operation of protein complexes. Therefore, Mh1798 probably does not form the secretion channel, but is part of a multiprotein secretion machinery. Alternatively, as Mh1798 belongs to a family of chaperones, the transposon insertion could affect the folding and/or stability of the PPE41 protein. To discriminate between these two possibilities we used the non-secreting species *M. smegmatis*.

Reconstitution of the PPE41 secretion system in *M. smegmatis*

The genomic organization of Rv1798 and Mh1798 is highly conserved between *M. tuberculosis* and *M. marinum* (Fig. 5A). Furthermore, both regions contain CDSs homologous to the *M. tuberculosis* ESX-1 cluster (Table S1). Four gene clusters homologous to the ESAT-6 secretion system ESX-1 have been identified in *M. tuberculosis*, including the Rv1798-containing region that is called ESX-5 (Gey Van Pittius *et al.*, 2001). This could mean that ESX-5 encodes an ESAT-6-like secretion machinery involved in PPE41 transport. Interestingly, ESX-5 is conserved in *M. marinum*, but is not present in the non-secreting *M. smegmatis* (Gey Van Pittius *et al.*, 2001). To determine whether cluster ESX-5 is directly involved in PPE41 secretion, different regions (Fig. 5A) of the ESX-5 cluster of *M. marinum* were isolated by long-

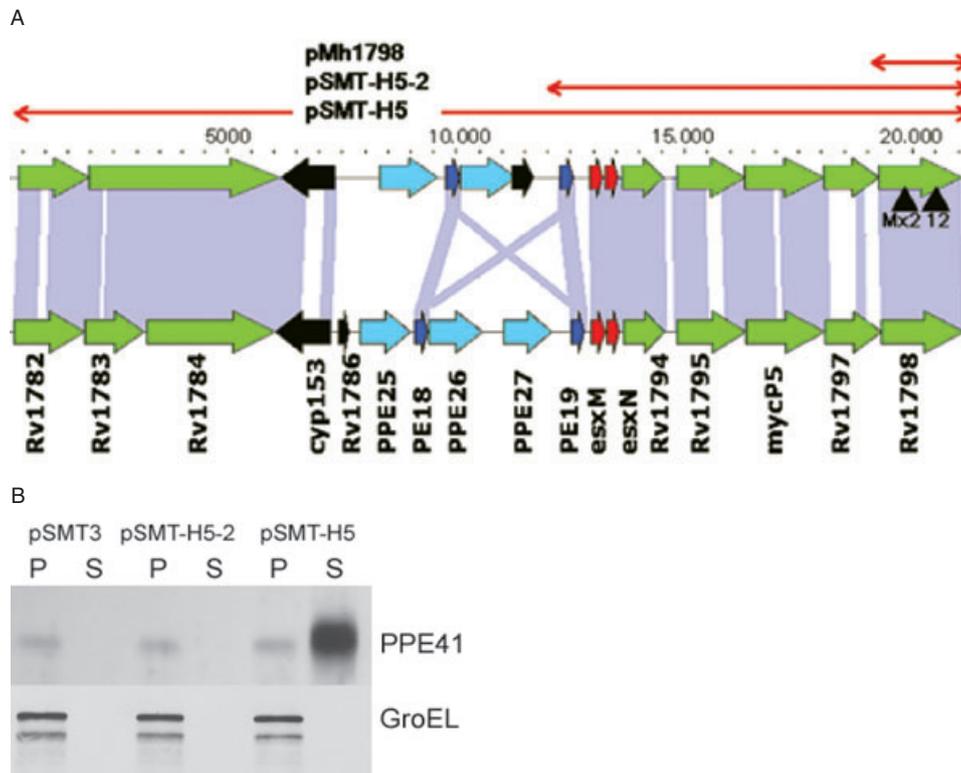


Fig. 5. Reconstitution of the ESX-5 secretion system in *M. smegmatis*.

A. ESX-5 cluster of *M. marinum* M compared with the corresponding region of *M. tuberculosis* H37Rv. Grey blue areas indicate regions with more than 65% identity (200 bp). Genes predicted to be involved in the secretion system (Gey Van Pittius *et al.*, 2001) are shown in green, CFP-10/ESAT-6-like genes in red, PE in dark blue and PPE in light blue. Genes not present in other ESX systems are shown in black. Arrowheads indicate the transposon insertion sites and the regions cloned for reconstitution in *M. smegmatis* are indicated in red. Size is shown in base pairs.

B. Reconstitution of the PPE41 secretion system in *M. smegmatis*. Equivalent amounts of pellet (P) and supernatant fractions (S) of *M. smegmatis* supplemented with the PE25/PPE41 plasmid and the empty control plasmid (pSMT3), pSMT3-H5-2 or pSMT3-H5 (part of ESX-5 present in these plasmids is shown in A) were analysed for the presence of PPE41 and GroEL, as an intracellular control, as described in Fig. 4.

range PCR, cloned on a mycobacterial shuttle vector and introduced in *M. smegmatis*. The *M. smegmatis* strain used already contained the Rv2430c/Rv2431c operon integrated in the genome. Introduction of the *mh1798* gene alone or half of the ESX-5 cluster (PE19-*mh1798*) did not result in increased amounts of extracellular PPE41 (Fig. 5B). However, the presence of the entire ESX-5 cluster (*mh1782*–*mh1798*) enabled *M. smegmatis* to secrete PPE41 efficiently (Fig. 5B). To verify that this secretion was not due to the lysis of *M. smegmatis* cells, the different fractions were also analysed for GroEL. As expected, GroEL was only present in the cell pellet (Fig. 5B). Together, these data show that the ESX-5 cluster is necessary for PPE41 secretion. The secretion of PPE41 by *M. smegmatis* through the introduction of ESX-5 did not result in reduced intracellular amounts of PPE41 (Fig. 5B), which indicates that intracellular levels of PPE41 are tightly regulated, probably by proteolytic degradation.

Proteins secreted by ESX-5 are involved in macrophage escape

ESX-5 is specific for pathogenic mycobacteria, which could mean that the ESX-5-secreted substrates are involved in virulence. To test this possibility, we studied the interaction of the *M. marinum* secretion mutant Mx2 with human macrophages and fish leukocytes, as described previously (van der Sar *et al.*, 2004). For this experiment, Mx2 was cured for the Rv2430c/Rv2431c plasmid to study the effect of ESX-5 independent of heterologous PPE expression. Both the wild type and the Mx2 mutant were supplemented with a DsRed encoding plasmid, which results in red fluorescent bacteria. First the surface characteristics of these two strains were examined by determining the hydrophobicity and binding to human macrophages. Although the secretion mutant showed a decreased surface hydrophobicity as compared with the wild type (Fig. S5), the binding and uptake of both

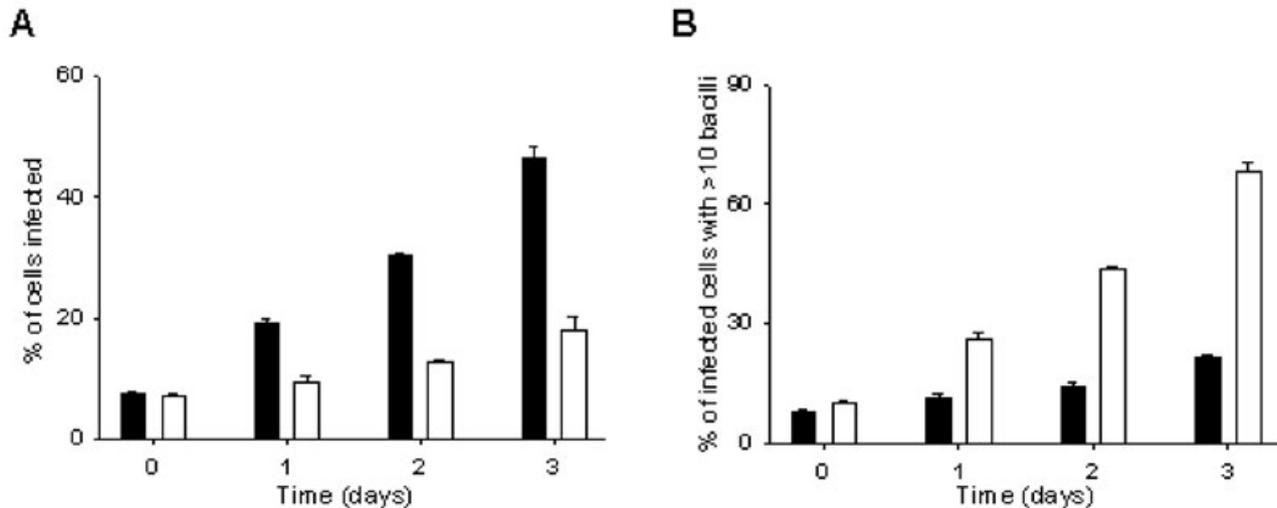


Fig. 6. Effect of ESX-5 on intracellular replication and spreading of *M. marinum*. THP-1 macrophage-like cells were seeded on glass coverslips and infected with the wild type (closed bars) and Mx2 mutant (open bars) expressing DsRed. At each time point the percentage of infected cells (A) and the percentage of the infected cells with more than 10 bacteria (B) were determined. The results are the average of three separate experiments and the standard deviation is shown. Enumeration of bacilli within THP-1 cells was visualized by microscopy, with a minimal of 50 infected cells per time point.

cells by macrophages were similar (results not shown). Subsequent infection experiments showed that ESX-5 mutants survived and multiplied in both types of leukocytes. However, a clear difference was observed when the percentage of infected cells was determined (Fig. 6A). With *M. marinum* wild type, the percentage of infected cells increased throughout the assay, reaching a value of 53% by day 3. In contrast, the Mx2 mutant infected only 17% of the host cells by day 3. This could indicate that the secretion mutant was impaired in spreading to new cells. To examine this phenotype in more detail, also the number of bacteria per infected cell was determined (Fig. 6B). Throughout the assay, most of *M. marinum* wild-type infected cells contain less than 10 bacteria, although the number of *M. marinum* wild-type infected cells increased (Fig. 6B). For the secretion mutant Mx2 the total number of infected cells remained relatively constant, whereas the percentage of cells containing more than 10 bacteria progressively increased (Fig. 6B). This result indicates that Mx2 mutant is capable of intracellular growth, but is impaired in macrophage escape. Together our data show that ESX-5 plays an important role in the infection cycle of *M. marinum*, possibly by facilitating host cell lysis.

Discussion

Most pathogenic bacteria are dependent on specialized secretion systems for the export of crucial virulence factors. Over the last decade a number of these secretion systems have been identified, ranging from the type I to the type V secretion pathway. Until recently, patho-

genic mycobacteria were thought to be an exception to the rule. *M. tuberculosis* contains, apart from the omnipresent Sec-dependent transport system, no homologue of any known specialized secretion system and only relatively small amounts of extracellular proteins could be identified as compared with other bacterial species. However, 3 years ago this idea changed when it was shown that *M. tuberculosis* possesses a Sec-independent secretion system to transport two small virulence proteins, ESAT-6 and CFP-10 (Hsu *et al.*, 2003; Stanley *et al.*, 2003; Gao *et al.*, 2004; Guinn *et al.*, 2004; Converse and Cox, 2005). This secretion pathway was called the ESAT-6 secretion pathway or ESX-1 and represented a new class of secretion systems. Genome analysis showed that ESAT-6-like secretion systems are in fact also present in other Gram-positive bacteria (Gey Van Pittius *et al.*, 2001; Pallen, 2002) and a homologue of this secretion pathway was found to be involved in the secretion of two small proteins of *Staphylococcus aureus* and necessary for pathogenesis of this bacterium (Burts *et al.*, 2005). *M. tuberculosis* itself contains the genetic information for five of these ESAT-6-like secretion machineries (Gey Van Pittius *et al.*, 2001), which indicates that Sec-independent protein secretion is an important feature of this pathogen. These ESAT-6-like gene clusters are conserved in the genomes of other mycobacteria, although not all species contain the full extent of these gene clusters (Gey Van Pittius *et al.*, 2001). In this study, we show that the *M. marinum* orthologue of cluster ESX-5 encodes a protein secretion system, which is specific for pathogenic mycobacteria and is involved in the secretion of PPE41.

Introduction of the entire ESX-5 gene cluster in *M. smegmatis* resulted in the secretion of heterologously expressed PPE41. Smaller genome fragments did not result in the reconstitution of this ESAT-6-like secretion system, which shows that the predictions of Gey Van Pittius *et al.* (2001), based on genome comparisons, were very accurate. This ESX-5 cluster encodes several proteins that are likely to be directly associated with a secretion apparatus, such as a membrane protein with a putative ATP binding site (Mh1783/84) and various other putative membrane proteins (Mh1782, Mh1794, Mh1795 and Mh1797), but also proteins that are perhaps not involved, such as several PEs and PPEs, a putative cytochrome P450 and a mycobacteriophage protein (Table S1). Future experiments will have to show which of these genes are actually involved in PPE41 secretion. But, if most genes of the ESX-5 gene cluster are needed for secretion, why are only transposon insertion mutants in a single gene (Mh1798) isolated in the genetic screen in *M. marinum*? The results from two separate high-density transposon mutagenesis studies in *M. tuberculosis* provide a possible explanation for this apparent discrepancy. In both these studies transposon insertions in the Mh1798 homologue Rv1798 were isolated, but mutations in the other genes of ESX-5 encoding structural components of the secretion system, i.e. the homologues of Rv1782–Rv1784 and Rv1794–Rv1797 (CDSs shown in green in Fig. 5A), were not detected (Lamichhane *et al.*, 2003; Sasseti *et al.*, 2003). This indicates that these transposon insertions are, in contrast to the ESX-1 secretion system, lethal. The Mh1798 mutants isolated in our screen showed only a strongly reduced secretion of PPE41, and perhaps mutations in the other ESX-5 genes could block secretion completely. Interestingly, we isolated one mutant that showed no PPE41 secretion at all on colony blot. Unfortunately, this mutant grew very slowly and could not be resuscitated after two passages and was therefore not further analysed. Future experiments will be directed to prove the essential nature of ESX-5.

The saprophytic species *M. smegmatis* does not contain an ESX-5 cluster and because this bacterium produces a cell wall similar to that of *M. tuberculosis* and *M. marinum*, it is not likely that ESX-5-secreted substrates play an important role in cell wall biosynthesis. Therefore, the lethality of ESX-5 mutations is probably due to the accumulation of normally secreted proteins. As ESX-5 secretes PPE41, the logical candidates for these toxic proteins are PPE proteins. *M. smegmatis* contains only two genes encoding putative PPEs (<http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi>) and these are located in the ESX-1 and ESX-3 region respectively (Gey Van Pittius *et al.*, 2001). Apart from ESX-4, all ESX regions have one or more PPE genes. Comparison of the conserved N-terminal PPE motif of all PPE proteins of

M. tuberculosis shows that the majority of PPEs cluster together with PPE41 and the PPEs of ESX-5. This clustering indicates that the expansion of PPEs is correlated with ESX-5 (N.C. Gey Van Pittius, in preparation). Therefore, we hypothesize that more PPEs are secreted via the ESX-5 system. The PPEs encoded by ESX-1, -2 or -3 are more distantly related and could have a different function. Thus far, the PPE protein of the ESX-1 region (PPE68) has been most extensively studied. This protein is not involved in the secretion of ESAT-6 and CFP-10 (Demangel *et al.*, 2004). Overlay experiments showed that PPE68 specifically interacts with both of these secreted proteins (Okkels and Andersen, 2004), which indicates that PPE68 is secreted, together with ESAT-6/CFP-10, via the ESX-1 secretion system. However, proteomic analysis of ESX-1 secretion mutants failed to show secretion of PPE68, although the secretion of the ESX-1-encoded PE35 could be determined (Fortune *et al.*, 2005). Another report showed that PPE68 is in fact located in the cell wall (Demangel *et al.*, 2004). Therefore, the exact role and fate of PPEs in the various ESX systems need to be examined in more detail.

An interesting observation was that the Δ CE mutant strain in fact secreted significantly higher amounts of PPE41, whereas a deletion of a gene encoding a structural component of the ESX-1 system did not affect the level of PPE41 secretion. These data show that there is some level of cross-talk between the two secretion systems ESX-1 and ESX-5. Deletion of the ESAT-6- and CFP-10-encoding genes could perhaps result in upregulation of the ESX-5 secretion system. Alternatively, the absence of the ESX-1-secreted substrates ESAT-6 and CFP-10 could result in cross-secretion of ESX-5 substrates via ESX-1.

The ESX-5 secretion system seems to be specific for pathogenic mycobacteria and our data show that ESX-5 plays an important role in the macrophage infection cycle. Interestingly, the observed effects are highly similar to those described for ESX-1 mutations in *M. tuberculosis*/*M. bovis* (Hsu *et al.*, 2003; Stanley *et al.*, 2003; Gao *et al.*, 2004; Guinn *et al.*, 2004). This probably means that ESAT-6 secretion itself is not sufficient to perturb the macrophage cell membrane and complete the macrophage infection cycle. A recent study of Li *et al.* (2005) identified a PPE gene of *M. avium* that was associated with virulence and the ability to grow in macrophages. Interestingly, this PPE gene is highly homologous to the Rv1787 gene of *M. tuberculosis*, which is located within the ESX-5 cluster and encodes PPE25. Therefore, this protein could very well be one of the virulence-associated ESX-5-secreted substrates needed for the macrophage infection cycle. Future experiments will be directed to unravel the role of extracellular PPE proteins within the host cell.

Experimental procedures

Bacterial strains and growth conditions

Three different mycobacterial species were used in this study: *M. marinum* strain M (Ramakrishnan and Falkow, 1994), *M. smegmatis* strain mc²155 (Snapper *et al.*, 1990) and *M. bovis* BCG Copenhagen. Mycobacteria were grown in shaking cultures in (i) Middlebrook 7H9 liquid medium, supplemented either with Middlebrook ADC (BD, Biosciences) and 0.2% Tween, or (ii) modified Sauton's medium, enriched with 0.5% sodium pyruvate and 0.5% glucose. For secretion in *M. bovis* BCG and *M. marinum* 7H9 medium was used, supplemented with 0.2% (w/v) dextrose, 0.2% Tween and 0.1% or 0.01% of the advised amount of ADC supplement respectively. The presence of BSA in the medium (part of the ADC supplement) is essential for secretion in these two species. For secretion in *M. smegmatis* we used the pre-culture method described by Converse and Cox (2005). As a solid medium Middlebrook 7H10 plates supplemented with OADC (BD, Biosciences) were used. Both *M. smegmatis* and BCG were grown at 37°C, whereas *M. marinum* was grown at 30°C. If *M. marinum* cells were cultured for electroporation experiments, 2.5 mg ml⁻¹ glycine was added to the media once the culture reached an optical density at 600 nm (OD₆₀₀) of 0.5, to increase the electroporation efficiency (D. Lakey, pers. comm.). For cloning experiments, *Escherichia coli* strain DH5 α was used. Hygromycin, chloramphenicol and kanamycin were used at final concentrations of 50 μ g ml⁻¹, 30 μ g ml⁻¹ and 25 μ g ml⁻¹, respectively, both for *E. coli* and for mycobacteria, and gentamicin was used at a final concentration of 5 μ g ml⁻¹ for *M. smegmatis* and 10 μ g ml⁻¹ for *E. coli*.

Expression of Rv2430c/Rv2431c in M. smegmatis and M. marinum

H37Rv genomic DNA was used as a template to amplify the Rv2431c/Rv2430c genes with Expand polymerase (Boehringer) using the primers Rv2430cR (GACACGAAATCCG CAGGTAT) and Rv2431cF (CTCATCTGTACGAGCCGTA). The resulting PCR product was cloned in pUC18 digested with SmaI, which resulted in pUC18-30c/31cL and pUC18-30c/31cR. Subsequently, the operon was cloned in pSMT3-eGFP (Hayward *et al.*, 1999) and placed under the control of the Hsp60 promoter, resulting in pSMT30/31c. The PPE41-encoding gene Rv2430c was also inserted without the Rv2431c gene in pSMT3eGFP by digesting the pUC18-30c/31cL plasmid with HpaI, which overlaps the stop codon of Rv2431c and HindIII. As a second construction vector pBH10 was used, which is compatible with pAL5000 derivatives such as pSMT3. pBH10 is a derivative of pBP10 (Bachrach *et al.*, 2000) that contains the Hsp60 promoter of pSMT3-eGFP by cloning a XbaI and BamHI fragment of pSMT3 in SpeI and BamHI-digested pBP10. Subsequently, the putative Rv2431c/30c operon was cloned in pBH10 using BamHI/PstI, resulting in plasmid pBH30c/31c. A chloramphenicol-resistant version of this plasmid, designated pBH30/31cat, was produced using pEMCat. pEMCat contains the chloramphenicol acetyl transferase-encoding gene (*cat*) of pACYC184, cloned as a Sau3A fragment in BamHI-digested pEM37 (Pashley

and Parish, 2003). This places the *cat* gene under control of the mycobacterial Ag85 promoter. pBH30/31cat was created by cloning the *cat*-containing Ecl136II–EcoRV fragment of pEMCat in pBH30/31c restricted with EcoRV. For reconstitution of the secretion system in *M. smegmatis* an integration construct expressing Rv2430c/Rv2431c was used. This construct was created by isolating the integration region of plasmid pUC-Gm-Int plasmid (Lee *et al.*, 1991) as a HindIII fragment and clone it into pBH30c/31c, thereby deleting the OriM. The resulting plasmid is designated pBH30c/31c-Int. Gentamicin-resistant colonies of *M. smegmatis* were analysed for correct genome insertion by PCR, using primers specific for the Rv2431c/Rv2430c operon and primers surrounding the integration site, i.e. wbINTgen (CTACCAAGCTGCGCTACACC) and wbINTpls (TCGTTTGTCAGCATCGAAAG).

Polyclonal antiserum directed against Rv2430c

Rv2431c and Rv2430c were expressed together in *E. coli* with an optimized translation initiation site and a His-tag at the C-terminal end of Rv2430c (Strong *et al.*, 2006). Rv2430c was purified from disrupted *E. coli* cells and the Rv2431c protein co-purified. This purified material was used to raise polyclonal antibodies. Two rabbits were immunized subcutaneously with 0.4 ml of Rv2430c/Rv2431c preparation (containing 100 μ g of protein) 4:5 diluted in the mineral oil-based adjuvant Stimune (Cedi Diagnostics BV, Lelystad NL) and subcutaneously booster-immunized after 4 weeks with 0.2 ml of plain antigen. The resulting antisera were analysed for activity against the purified proteins, which showed only a response against PPE41.

SDS-PAGE and immunoblot

Mycobacteria were grown to mid-logarithmic phase. Subsequently, secreted proteins were precipitated from cell-free supernatant with 5% TCA (w/v). The cell pellets were resuspended in PBS and disrupted with a mini-BeadBeater (BioSpec Products) using 0.1 mm zirconia/silica beads. Cell lysates and supernatants were separated by SDS/PAGE on 12% polyacrylamide gels. For the treatment of intact cells with protease bacteria were grown to mid-log phase and isolated by centrifugation. Cells were resuspended in water and left untreated or incubated with 0.1 mg ml⁻¹ proteinase K (Qiagen). After a 30 min incubation at room temperature, sample buffer was added to all samples, samples were boiled and separated on 12% polyacrylamide gels. Proteins were visualized by immunoblotting using antibodies directed against PPE41 (see above), antibodies against PknG (kindly provided by Y. Avenue-Gay, Vancouver, Canada), and antibodies against GroEL and GroES. The GroEL monoclonal antibody used is CS44, obtained from J. Belisle (Colorado State University, Fort Collins, CO, and the National Institutes of Health, Bethesda, MD, contract NO1 AI-75320). The presence of the second antibody, GARpo or GAMpo, was visualized using 4-chloronaphtol/3,3-diaminobenzidine staining or using ECL detection (PIERCE). For protein quantification nitrocellulose membranes were probed with a-PPE41 polyclonal antibody and detected by Lumi-Light Western Blotting

Substrate (Roche Applied Science). Quantification was performed on a Fluor-S Multilmager (Bio-Rad) using Bio-Rad multianalyst software, version 1.0.2.

Transposon mutagenesis

Transposon mutagenesis was performed using the mycobacterial specific phage phiMycoMarT7 containing the mariner-like transposon Himar1 (Sasseti *et al.*, 2003). Transductants of *M. marinum* strain M supplemented with the Rv2430c containing vector pSMT3-30/31c were plated on a nitrocellulose filter, which was placed on 7H10 agar plates supplemented with kanamycin (to select for the presence of the transposon) and hygromycin (pSMT3-30/31c). After visible colonies had appeared, the filter was removed and placed on a new filter, on top of another 7H10 plate. This sandwich was incubated for 5–6 h at 30°C and subsequently the top filter with the colonies was preserved at 4°C, whereas the bottom filter with the secreted proteins was incubated with antibodies directed against PPE41. Colonies of interest, i.e. those that showed reduced mounts of PPE41, were first checked on a similar colony blot assay and subsequently checked in culture for PPE41 secretion. To establish the chromosomal location of the transposon insertion, ligation-mediated PCR was used essentially as described by Prod'homme *et al.* (1998), with the following modifications. Chromosomal DNA (50 ng), isolated from plate-grown mutants with the DNA tissue kit (Qiagen), was digested with BamHI and BglII instead of Sall. These two restriction enzymes together have approximately the same digestion frequency in mycobacterial DNA as Sall, but in addition are functional in a buffer also suited for ligation. In the same incubation reaction (overnight at room temperature) as the digestion, an adaptor, consisting of the SalgD primer together with the Bampt primer (GATCGCTCGTGCC), is ligated to the digested DNA. This adaptor does not restore the BamHI/BglII restriction sites. Subsequently, this ligation mixture is used as a template in a PCR reaction with the pSalg primer (GCTTATTCCTCAAGGCACGA), which is identical to the single-stranded part of the adaptor, and the pMyco primer (CCGGGGACTTATCAGCCAAC), which binds to both sides of the Himar1 transposon. PCR fragments were sequenced using an ABIprism300 (Applied biosystems) and analysed using the sequence information of the *M. marinum* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/M_marinum/). In both mutants the MycoMar transposon inserted into the dinucleotide TA, as can be expected for this mariner-based transposon.

Infection of leukocytes

For intracellular experiments the human acute monocytic leukaemia cell line THP-1 and the carp leukocyte cell line CLC were used as described previously (van der Sar *et al.*, 2004). THP-1 cells were cultured in RPMI-1640 with glutamax-1 media (Gibco, BRL) supplemented with 10% fetal calf serum (FCS), and differentiated into macrophage-like cells in the presence of PMA (phorbol 12-myristate 13-acetate, 1 ng ml⁻¹, Sigma). For phagosome isolation, cells were seeded in 75 cm diameter flasks whereas 24-well plates (Nunc) were used for survival assays and microscopy. For the infection,

the mycobacteria were grown to logarithmic phase (OD₆₀₀ = 0.5–0.8) in 7H9 media, washed and diluted in RPMI-1640 with 10% FCS and used at a multiplicity of infection (moi) of 10 bacteria per cell. After 1 h of uptake at 33°C, infected cells were washed three times with PBS to remove extracellular bacteria and incubated in fresh medium plus amikacin (200 µg ml⁻¹; Sigma Chemicals, St Louis, MO) for 2 h. The cells were then washed once with PBS and incubated in fresh medium plus amikacin (30 µg ml⁻¹) for indicated time periods. Mycobacterial survival was analysed by lysing the infected macrophages in 0.1% Triton X-100 and plating serial dilutions on 7H10 agar (Difco) supplemented with 10% OADC. For microscopic examination, cells were differentiated with coverslips in wells and infection was performed with a pre-treatment of amikacin as described, but incubated in the absence of amikacin.

Mycobacterial phagosomes isolation

Isolation of mycobacterial phagosomes was performed as described previously (Schuller *et al.*, 2001). Briefly, infected THP-1 cells (7–10 × 10⁷ with moi of 25 for 48 h) were homogenized in DGE buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4) by passaging through a 23-gauge needle. After removal of the cell nuclei by low-speed centrifugation (250 g, 10 min), the resulting post-nuclear supernatant (PNS) was transferred to a fresh Eppendorf tube and sedimented at 35 000 g for 30 min. The resulting supernatant corresponds to the macrophage cytosol whereas the pellet corresponds to the mycobacterial phagosomes. The bacteria-free phagosomes were recovered, by treating them with 1% Triton X-100 for 15 min at room temperature and sediment the intact mycobacteria at 35 000 g for 30 min. The resulting supernatant corresponds to the phagosomes and the pellet to the mycobacteria.

Digital imaging fluorescence microscopy

THP-1 cells (7.5 × 10⁵ per well) were seeded on glass coverslips in 24-well plates in the presence of PMA and infected with different strains of mycobacteria as described in the infection procedures for 24 h. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and blocked in 2% BSA (Sigma) in PBS for 30 min. This blocking solution was also used for dilution of antibodies. The slides were incubated with antibodies directed against PPE41, monoclonal antibody F30-5 (Kolk *et al.*, 1984) directed against mycobacterial lipoarabinomannan (LAM), obtained from Arend Kolk (KIT, Amsterdam), or against LAMP-1 (H4A3, obtained from the Hybridoma Bank of the University of Iowa, Iowa City). After overnight incubation with primary antibodies at 4°C, coverslips were washed with PBST and incubated with Alexa fluor 488 or 546-conjugated secondary antibodies (Molecular Probes). Coverslips were mounted in Vectashield (Vector Laboratories) and analysed using a ZEISS Axiovert 200 Marianas™ digital imaging microscopy workstation as described previously (van der Sar *et al.*, 2003). The microscope, camera and data viewing/processing were conducted/controlled by Slidebook™ software [Slide-

book version 4.1.0.4 (Intelligent Imaging Innovations, Denver, CO)]. The data acquisition protocol included confocal optical planes to obtain three-dimensional (3D) definition.

Cloning and expression of the M. marinum ESX-5 cluster

Mycobacterium marinum genomic DNA was used as a template to amplify the ESX-5 region in two fragments with Extensor Hi-Fidelity PCR Master Mix (ABgene) using the primers AbMmfront (CAAAGTGTCTGAGAGACGGGTA) and AbMmMiddle-H5-R (ACCTAGCTTCCCCTCAGCAAAC) for the first part and AbMmMiddle-H5-F (AAGGGAGCACCGAAAATGTTAAA) and AbMmEnd (CGAGTTGATCTCAATCCATCCAC) for the second part. The resulting PCR products were cloned in pCRII-TOPO (Invitrogen). To transfer these constructs into *M. smegmatis* mc2155, the pAL5000 replicon and the hygromycin resistance gene was cloned from pSMT3-eGFP using the restriction enzymes DraI/EcoRV for pSMT3-eGFP and DraI for the TOPO-PCR constructs, resulting in pSMT-H5-1 and pSMT-H5-2 respectively. Subsequently, a complete ESX-5 region was constructed by digesting both plasmids with the restriction enzymes DraI/MluI and re-ligation. The resulting plasmid was designated pSMT-H5. Also the *mh1798* gene alone was amplified by PCR, using chromosomal DNA of *M. marinum* M as a template and the primers 1798R (TGTTAACTGATGCCAATTCCGATTTC) and 1798F (CGTTAACTCGATGAGGTCTGGCTCTC). This fragment was cloned in pCRII-Topo (Invitrogen) and checked by sequence analysis. Subsequently, *mh1798* was cloned downstream of the mycobacterial *hsp60* promoter and the gene encoding DsRed, by ligating the HindIII/NotI fragment of pTOPO-Mh1798 with the NotI/BamHI fragment of pSMT3DsRed and the BamHI/HindIII vector fragment of pSMT3eGFP. This tripartite ligation was checked by restriction digestion and designated pMh1798.

Hydrophobicity

Cell surface hydrophobicity was assessed in three separate experiments as described previously (Rosenberg *et al.*, 1980). Briefly, *M. marinum* wild-type and Mx2 strains were grown on 7H9 broth for 1 week. Cells were harvested by centrifugation at 4°C and 2000 *g* and washed twice with PBS. Bacterial suspensions were prepared in PBS to an OD₆₀₀ of 1.0. Duplicate (3 ml) samples of each suspension were placed in test tubes. Xylene was added to each suspension at concentrations of 0%, 0.5%, 1% and 2% (v/v). The tube contents were mixed for 45 s with a vortex mixer at the maximum setting. The aqueous and organic phases were allowed to separate for 30 min, after which the aqueous phase was carefully removed with a Pasteur pipette and transferred to cuvettes. Contaminating xylene was allowed to evaporate for 60 min. After this, each cuvette was vortexed for 1 s to resuspend the remaining bacteria. The level of absorption of the cells to the xylene droplets was calculated as the loss in OD₆₀₀ of the aqueous phase compared with the initial cell suspension, expressed as a percentage. Similar results were obtained if hexadecane (0%, 5%, 10% and 15%) was used instead of xylene.

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References

- Bachrach, G., Colston, M.J., Bercovier, H., Bar-Nir, D., Anderson, C., and Papavinasundaram, K.G. (2000) A new single-copy mycobacterial plasmid, pMF1, from *Mycobacterium fortuitum* which is compatible with the pAL5000 replicon. *Microbiology* **146**: 297–303.
- Brennan, M.J., and Delogu, G. (2002) The PE multigene family: a 'molecular mantra' for mycobacteria. *Trends Microbiol* **10**: 246–249.
- Burts, M.L., Williams, W.A., DeBord, K., and Missiakas, D.M. (2005) EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc Natl Acad Sci USA* **102**: 1169–1174.
- Choudhary, R.K., Mukhopadhyay, S., Chakhaiyar, P., Sharma, N., Murthy, K.J., Katoch, V.M., and Hasnain, S.E. (2003) PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. *Infect Immun* **71**: 6338–6343.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537–544.
- Converse, S.E., and Cox, J.S. (2005) A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*. *J Bacteriol* **187**: 1238–1245.
- Demangel, C., Brodin, P., Cockle, P.J., Brosch, R., Majlessi, L., Leclerc, C., and Cole, S.T. (2004) Cell envelope protein PPE68 contributes to *Mycobacterium tuberculosis* RD1 immunogenicity independently of a 10-kilodalton culture filtrate protein and ESAT-6. *Infect Immun* **72**: 2170–2176.
- Fortune, S.M., Jaeger, A., Sarracino, D.A., Chase, M.R., Sasseti, C.M., Sherman, D.R., *et al.* (2005) Mutually dependent secretion of proteins required for mycobacterial virulence. *Proc Natl Acad Sci USA* **102**: 10676–10681.
- Gao, L.Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J.N., and Brown, E.J. (2004) A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Mol Microbiol* **53**: 1677–1693.
- Gey Van Pittius, N.C., Gamielien, J., Hide, W., Brown, G.D., Siezen, R.J., and Beyers, A.D. (2001) The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol* **2**: RESEARCH0044.
- Guinn, K.M., Hickey, M.J., Mathur, S.K., Zakei, K.L., Grotzke, J.E., Lewinsohn, D.M., *et al.* (2004) Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* **51**: 359–370.

- Hayward, C.M., O'Gaora, P., Young, D.B., Griffin, G.E., Thole, J., Hirst, T.R., et al. (1999) Construction and murine immunogenicity of recombinant Bacille Calmette Guerin vaccines expressing the B subunit of *Escherichia coli* heat labile enterotoxin. *Vaccine* **17**: 1272–1281.
- Hsu, T., Hingley-Wilson, S.M., Chen, B., Chen, M., Dai, A.Z., Morin, P.M., et al. (2003) The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc Natl Acad Sci USA* **100**: 12420–12425.
- Ishikawa, J., Yamashita, A., Mikami, Y., Hoshino, Y., Kurita, H., Hotta, K., et al. (2004) The complete genomic sequence of *Nocardia farcinica* IFM 10152. *Proc Natl Acad Sci USA* **101**: 14925–14930.
- Kolk, A.H., Ho, M.L., Klatser, P.R., Eggelte, T.A., Kuijper, S., de Jonge, S., and van Leeuwen, J. (1984) Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis*, *M. bovis* (BCG) and *M. leprae*. *Clin Exp Immunol* **58**: 511–521.
- Lamichhane, G., Zignol, M., Blades, N.J., Geiman, D.E., Dougherty, A., Grosset, J., et al. (2003) A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **100**: 7213–7218.
- Lee, M.H., Pascopella, L., Jacobs, W.R., and Hatfull, G.F. (1991) Site-specific integration of Mycobacteriophage-L5 – integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and Bacille Calmette-Guerin. *Proc Natl Acad Sci USA* **88**: 3111–3115.
- Li, Y., Miltner, E., Wu, M., Petrofsky, M., and Bermudez, L.E. (2005) A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cell Microbiol* **7**: 539–548.
- Mahairas, G.G., Sabo, P.J., Hickey, M.J., Singh, D.C., and Stover, C.K. (1996) Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* **178**: 1274–1282.
- Okkels, L.M., and Andersen, P. (2004) Protein–protein interactions of proteins from the ESAT-6 family of *Mycobacterium tuberculosis*. *J Bacteriol* **186**: 2487–2491.
- Pallen, M.J. (2002) The ESAT-6/WXG100 superfamily – and a new Gram-positive secretion system? *Trends Microbiol* **10**: 209–212.
- Pashley, C.A., and Parish, T. (2003) Efficient switching of mycobacteriophage L5-based integrating plasmids in *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* **229**: 211–215.
- Prod'hom, G., Lagier, B., Pelicic, V., Hance, A.J., Gicquel, B., and Guilhot, C. (1998) A reliable amplification technique for the characterization of genomic DNA sequences flanking insertion sequences. *FEMS Microbiol Lett* **158**: 75–81.
- Pym, A.S., Brodin, P., Brosch, R., Huerre, M., and Cole, S.T. (2002) Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* **4**: 709–717.
- Ramakrishnan, L., and Falkow, S. (1994) *Mycobacterium marinum* persists in cultured mammalian cells in a temperature-restricted fashion. *Infect Immun* **62**: 3222–3229.
- Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons – a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9**: 29–33.
- Sampson, S.L., Lukey, P., Warren, R.M., van Helden, P.D., Richardson, M., and Everett, M.J. (2001) Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. *Tuberculosis (Edinb)* **81**: 305–317.
- van der Sar, A.M., Musters, R.J., van Eeden, F.J., Appelmelk, B.J., Vandenbroucke-Grauls, C.M., and Bitter, W. (2003) Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. *Cell Microbiol* **5**: 601–611.
- van der Sar, A.M., Abdallah, A.M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C.M., and Bitter, W. (2004) *Mycobacterium marinum* strains can be divided into two distinct types based on genetic diversity and virulence. *Infect Immun* **72**: 6306–6312.
- Sassetti, C.M., Boyd, D.H., and Rubin, E.J. (2003) Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* **48**: 77–84.
- Schuller, S., Neefjes, J., Ottenhoff, T., Thole, J., and Young, D. (2001) Coronin is involved in uptake of *Mycobacterium bovis* BCG in human macrophages but not in phagosome maintenance. *Cell Microbiol* **3**: 785–793.
- Snapper, S.B., Melton, R.E., Mustafa, S., Kieser, T., and Jacobs, W.R., Jr (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* **4**: 1911–1919.
- Stanley, S.A., Raghavan, S., Hwang, W.W., and Cox, J.S. (2003) Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *Proc Natl Acad Sci USA* **100**: 13001–13006.
- Strong, M., Sawaya, M.R., Wang, S., Phillips, M., Cascio, D., and Eisenberg, D. (2006) Toward the structural genomics of complexes: crystal structure of a PE/PPE protein complex from *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **103**: 8060–8065.
- Tundup, S., Akhter, Y., Thiagarajan, D., and Hasnain, S.E. (2006) Clusters of PE and PPE genes of *Mycobacterium tuberculosis* are organized in operons: evidence that PE Rv2431c is co-transcribed with PPE Rv2430c and their gene products interact with each other. *FEBS Lett* **580**: 1285–1293.
- Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., et al. (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* **304**: 1800–1804.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Immunoblot showing that GroEL proteins of *M. bovis* BCG (BCG), *M. smegmatis* (Ms) and *M. marinum* (Mm) are sensitive to proteinase K (pK) if lysed cells are subjected to the protease.

Fig. S2. Intracellular secretion of PPE41 by *M. marinum* mutant Mx2 supplemented with PE25/PPE41 and *Mycobacterium bovis* BCG. THP-1 cells were seeded on glass coverslips and infected with *M. marinum* Mx2 (A) or *M. bovis* BCG

(B) for 24 h. Infected cells were fixed, permeabilized and incubated with antisera directed against PPE41 and LAM, and examined by 3D digital imaging fluorescence microscopy. Secreted PPE41 is shown in red and intracellular bacteria (LAM) in green; the nuclei are stained with DAPI (blue). Arrows indicate colocalization, whereas arrowheads indicate no colocalization. Bar represents 10 μ M.

Fig. S3. Quality of the two-filter colony blot screening procedure used in our experiments to isolate secretion mutants of *M. marinum*. The colony-containing filter (shown on the left) is placed for 5 h on a second filter after 9–10 days of incubation (time needed to grow the colonies). The second filter (shown on the right) is treated with antiserum directed against PPE41 and stained for the presence of peroxidase. Non-secreting colony is indicated.

Fig. S4. Alignment of Mh1798 and Rv1798. Amino acids corresponding with the Tn insertion sites of Mx2 and 12 are indicated with arrows, identical residues are shown in grey and amino acid numbers are indicated.

Fig. S5. Cell surface hydrophobicity of *M. marinum* wild type (●) and the Mx2 mutant (○).

Movie S1. Z-stack of macrophage infected with *M. marinum* supplemented with PE25/PPE41. Colocalization of PPE41 and *M. marinum* (identical cell shown in Fig. 2B). Infected cells were fixed, permeabilized and incubated with antisera directed against PPE41 (red) and against LAM (green) and examined by 3D digital imaging fluorescence microscopy. Nuclei are stained with DAPI (blue).

Movie S2. Z-stack of macrophage infected with *M. marinum* supplemented with PE25/PPE41. No colocalization of PPE41 and *M. marinum* (identical cell shown in Fig. 2C). Infected cells were fixed, permeabilized and incubated with antisera directed against PPE41 (red) and against LAM (green) and examined by 3D digital imaging fluorescence microscopy. Nuclei are stained with DAPI (blue).

Table S1. CDSs of the ESX-5 region of *M. marinum* M, their homologues in the EXS-5 and EXS-1 region of *M. tuberculosis* H37Rv and the proposed function of their gene products, localization or protein family they belong to.

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