The ESX-5 Associated eccB5-eccC5 Locus Is Essential for Mycobacterium tuberculosis Viability

Mariagrazia Di Luca1,2,*, Daria Bottai1,2,*, Giovanna Batoni1,2, Mickael Orgeur3, Anna Aulicino1,2, Claudio Counoupas1ab, Mario Campa1,2, Roland Brosch3, Semih Esin1,2

1 Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, University of Pisa, Pisa, Italy, 2 Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, University of Pisa, Pisa, Italy, 3 Institut Pasteur, Unit for Integrated Mycobacterial Pathogenomics, Paris, France

Abstract

The recently described ESX-5 secretion system of Mycobacterium tuberculosis is one of the most important modulators of host-pathogen interactions due to its crucial impact on PPE protein secretion, cell wall stability and virulence. Although various components of the ESX-5 secretion machinery have been defined, other ESX-5 core components still remain to be characterized. In this study, we focused on EccB5 and EccC5, a transmembrane protein (EccB5) and a membrane-bound ATPase (EccC5), both predicted to be building blocks of the M. tuberculosis ESX-5 membrane-associated complex. In vitro expression studies demonstrated that EccB5 and EccC5 encoding genes constitute an operon. The expression of this operon is essential for M. tuberculosis, since the deletion of the eccB5-eccC5 genomic segment at the ESX-5 locus is possible only after the integration of a second functional copy of eccB5-eccC5 genes into the M. tuberculosis chromosome. The characterization of two M. tuberculosis conditional mutant strains (MtbΔeccB5 and MtbΔeccC5), in which the eccB5-eccC5 operon or the eccC5 gene, respectively, were expressed under the control of an anhydrotetracycline-repressible promoter, confirmed that the repression of eccB5-eccC5 genes is detrimental for growth of M. tuberculosis both in vitro and in THP-1 human macrophage cell line. Moreover, analysis of the secretome of Mtb eccB5-eccC5 and Mtb eccB5-eccC5 strains revealed that both EccB5 and EccC5 are required for secretion of ESX-5 specific substrates, thus confirming that they are indeed components of the ESX-5 secretion machinery. Taken together, these findings demonstrate the importance of an intact and functional ESX-5 system for viability of M. tuberculosis, thus opening new interesting options for alternative antimycobacterial control strategies.

Introduction

Throughout evolution, numerous bacterial pathogens have acquired specialized protein secretion pathways to deliver effector proteins to host cells. These pathways, which are distinct from the ubiquitous Sec pathway, are critical for mediating interactions during infection and for allowing pathogen survival in the hostile environment of the host. The recently described mycobacterial type VII secretion systems are specialized secretion systems of small, highly immunogenic proteins lacking a classical N-terminal signal sequence, belonging to the Esx or WXG-100 family [1,2], which are distantly related to protein transport systems of Gram-positive bacteria [3]. The genome of Mycobacterium tuberculosis, the causative agent of human tuberculosis, encodes five type VII secretion systems (ESX-1 – ESX-5), the genes of which are arranged in highly conserved clusters [4–6]. Each ESX cluster typically carries a pair of esx genes flanked by esx conserved components (ec) genes coding for predicted core components of the ESX secretion machineries responsible for the ATP-dependent transport of the corresponding ESX substrates outside the cell [5,7]. ESX-5 is the most recently evolved ESX cluster and is only present in the group of slow-growing mycobacteria that includes all major pathogenic species [8]. In the fish pathogen M. marinum, ESX-5 modulates host-pathogen interactions [9,10] and is responsible for secretion of several PPE and PE proteins, two of the most important classes of mycobacterial proteins involved in virulence/pathogenicity and implicated in immune evasion strategies used by pathogenic mycobacteria to survive in host tissues [11–15]. To date, PPE and PE proteins identified as being transported to the cell surface/secreted by the M. marinum ESX-5 are PE25-PPE41 [16,17], LipY [18], and members of the PPE_MPTR and PE_PGRS subgroups [19], the most recent subclasses of PE and PPE proteins, genes of which were suggested to have evolved by duplication/insertion events from ancestral genes encoded at the ESX-5 locus [8]. By the characterization of several M. tuberculosis knock-out mutants for ESX-5 components, we recently demon-
strated that ESX-5 plays a crucial role in host pathogen interaction also in *M. tuberculosis*. The *M. tuberculosis* ESX-5 system mediates PPE protein transport and secretion [20] with impact on T-cell immunogenicity of ESX-5- and non-ESX-5-encoded PPE and PE proteins. As such, ESX-5 is a major modulator of the host immune response [21] and a key virulence determinant of *M. tuberculosis*: inactivation of ESX-5 core components results in a strong attenuation of the corresponding mutant strains, which are unable to replicate both in immunodeficient and in immunocompetent mice [20,21]. Various components of the ESX-5 secretion machinery, such as EccD₅ (the predicted transmembrane channel) or EccA₅ (a cytosolic ATPase belonging to the AAA⁺ family) have been characterized, and their impact on secretion of ESX-5 specific substrates has been investigated [20]. However, other putative building blocks of the ESX-5 secretion apparatus in *M. tuberculosis* still remain to be characterized. In this study, we focused on EccB₅ and EccC₅, a transmembrane protein and an ATP-binding protein belonging to the FtsK/SpoIIE-like protein family, respectively [7,22]. Encoded at the ESX-5 locus upstream of the *ppb25-pe19* cluster, EccB₅ and EccC₅ are both predicted to be components of the *M. tuberculosis* ESX-5-membrane-associated complex [7,20,23]. We demonstrated that *eccB5-eccC5* genes constitute an operon, expression of which is required for an efficient secretion of ESX-5 specific substrates. Moreover, by constructing/characterizing multi-copy gene variants and conditional mutants, in which the *eccB5-eccC5* genes were deleted or expressed under the control of an anhydrotetracycline-repressible promoter, we demonstrate that an intact *eccB5-eccC5* locus is essential for *M. tuberculosis* and that disruption/repression of single core components of the ESX-5 secretion machinery strongly impacts the *M. tuberculosis* in vitro growth properties. Taken together, the results obtained demonstrated the importance of an intact and functional ESX-5 for *M. tuberculosis* viability, emphasizing the key role of this secretion system in the biology of this human pathogen.

### Materials and Methods

#### Bacterial Strains, Media and Growth Conditions

*Escherichia coli* strain DH10B (Stratagene), used for cloning procedures, was grown in LB broth (Sigma) or LB agar (Sigma). *Mycobacterium tuberculosis* H₃₇Rv (stocks obtained from the Institut Pasteur) [22] was used as reference strain and for construction of deletion/conditional mutants. Mycobacterial strains were grown in Middlebrook 7H₉ broth (Difco) supplemented with 10% (v/v) albumin-dextrose-catalase (ADC), 0.2% (v/v) glycerol, and 0.05% (v/v) Tween80 (Sigma) or on solid Middlebrook 7H₁₁ medium (Difco) supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC). When required, the media were supplemented with 50 μg/ml kanamycin, 100 μg/ml hygromycin or 20 μg/ml gentamycin for *E. coli*, and 20 μg/ml kanamycin, 50 μg/ml hygromycin or 20 μg/ml streptomycin for *M. tuberculosis*.

For analysis of in vitro growth of conditional mutants, mycobacterial strains in exponential growth phase were diluted to an optical density of 600 nm (OD₆₀₀) of 0.5, and 5 μl of 10-fold serial dilutions were spotted on Middlebrook 7H₁₁ medium, added or not with different concentrations of anhydrotetracycline (ATc) (100, 200 and 400 μg/ml). Alternatively, bacterial strains were grown in Middlebrook 7H₉ medium containing or not 400 ng/ml ATc. After 3 days, cultures were diluted to OD₆₀₀ = 0.04 in fresh medium containing or not 400 ng/ml ATc, and the bacterial growth was monitored by daily OD₆₀₀ measurements.

#### RNA Extraction and Reverse Transcription RT-PCR/5′ RACE Assays

RNA extraction was performed from *M. tuberculosis* H₃₇Rv cultures in exponential growth phase as previously described [24]. Briefly, bacteria were recovered by centrifugation and broken in 1 ml of TRIZol (Applied Biosystems) in presence of zirconia beads (0.1 mm diameter) in a MM300 apparatus (Qiagen) (30 sec at maximum speed). RNA was obtained by extraction with 0.2 vol of chloroform and precipitation for 1 h at −80°C with 0.1 vol of 3 M sodium acetate and 0.45 vol of isopropanol. Removal of contaminating DNA was performed using DNAfree kit (Applied Biosystems/Ambion), according to the manufacturer’s instructions. RT-PCR reactions were performed using Superscript one-step RT-PCR kit (Applied Biosystems) as recommended by the producer. Sequences of primers used in amplification reactions are listed in Table S1. 5′ Rapid Amplification of cDNA Ends (RACE) was performed using the 5′/3′ RACE kit (Roche Molecular Biochemicals). One microgram of RNA and *eccB5*-specific primer (Table S1) were incubated at 70°C for 5 min. Denatured RNA and primer were then incubated at 55°C for 1 h in the presence of 1X cDNA synthesis buffer, 1 mM dNTPs, 40 U Promoter RNase Inhibitor and 25 U Transcriptor Reverse Transcripase. The cDNA obtained was purified by using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals), and used in poly(A) tailing reaction (30 min at 37°C in the presence of 0.2 mM dATP and 80 U Terminal Transferase). Nested PCR amplification on poly(A)-tailed cDNA was performed using an oligo dT-anchor primer and an *eccB5* specific primer (Table S1). The single amplification product obtained was directly sequenced.

#### Construction of eccB5-eccC5 Deletion and Conditional Mutants in *M. tuberculosis*

Deletion of the *eccB5-eccC5* segment in *M. tuberculosis* H₃₇Rv was performed by allelic exchange using the ts-satB technology [25]. Briefly, a 2445 bp and a 1227 bp fragment encompassing the *eccB5*-upstream region and the *eccC5*-downstream region, respectively, were amplified by PCR from *M. tuberculosis* H₃₇Rv genomic DNA (See Table S1 for primer details). The amplicons were digested with *SpeI/XbaI* and *XbaI/VsoI*, respectively, and cloned into the *SpeI/VsoI*-digested pPR27 vector to obtain pMDL92. The *aph* cassette, conferring resistance to kanamycin, was amplified by PCR from the pUC4K plasmid, digested by *XbaI* and inserted into the *XbaI*-digested pMDL92 plasmid. The resulting plasmid, pMDL92-aph, was used in allelic exchange experiments. Kanamycin-resistant/sucrese-resistant (*Kana'/Sac⁺*) transformants were screened by PCR using primers specific for the *aph* cassette and for the *rv1780* or *rv1786* genes (see Table S1 for primer sequences).

The Mtb::*eccB5-eccC5* merodiploid strain was constructed by using the integrative vector pRBeXint [26]. The genomic segment *eccB5-eccC5* was amplified by PCR (see Table S1 for primer details), digested with *SpeI/HpaI* and cloned into the *SpeI/HpaI*-digested pRBeXint. The resulting plasmid (pExistecB5-eccC5) was used to transform *M. tuberculosis*, and hygromycin resistant clones were selected. To construct *eccB5* and *eccC5* conditional mutants the recently developed TetR/Pip OFF mycobacterial repressible system was used [27,28]. Briefly, a recombinant *M. tuberculosis* strain (Mtb::*tetR-pip*) was constructed, in which the genes encoding the tetracycline-sensitive repressor TetR and the *Streptomycetes pristinaeae* Pip repressor, as well as the reporter gene *lacZ*, were integrated into the genome at the *attB* site. Such a genomic organization allows the transcriptional repression of genes expressed under the control of the *P_tac* promoter as a consequence of the addition of ATc to the medium [27,28]. The functionality of
the PpON/Tet OFF regulatory circuit was confirmed by β-galactosidase activity assays performed on the Mtb::tetR-pip strain, demonstrating that the addition of ATc to the culture medium results in a dose-dependent reduction of the expression of the lacZ gene reporter (Figure S1). A 550-bp and 870-bp fragment encompassing the 5′-portions of eccB5 and eccC5 genes, respectively, were amplified by PCR on M. tuberculosis genomic DNA (see Table S1 for primer details), digested by NotI and cloned in frame with the S. pyrogenesuis promoter Pₚ into the NotI site of suicide vector pFRA50 [27]. The two obtained plasmids, pMDL-eccB5 and pMDL-eccC5 were then electroporated in the Mtb::tetR-pip strain, and recombinant clones were selected for resistance to hygromycin. Genomic DNAs from hygromycin resistant clones were analyzed by PCR for the correct integration of the Pₚ promoter immediately upstream the eccB5 or eccC5 coding regions, respectively. Sequences of primers used in screening PCR reactions are reported in Table S1.

Infection of THP-1 Human Macrophage Cell Line

THP-1 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Euroclone) and 2 mM L-glutamine (Euroclone). Before infection, cells were seeded in 96-well plates at a density of 7.5 x 10⁴ and differentiated into macrophages by incubation with 50 nM PMA for 1 day. The two obtained plasmids, pMDL-eccB5 and pMDL-eccC5 were then electroporated in the M. tuberculosis control strain were pre-grown for 48 h in ATc-containing medium, and used in cell infection assays at a multiplicity of infection (m.o.i.) of 1:20 (bactericidal). After phagocytosis (90 min), infected cells were washed twice with PBS to remove extracellular bacteria, and incubated for 6 days in culture media supplemented or not with 400 ng/ml ATc. Culture media, containing or not ATc, were replaced every 48 h as described elsewhere [27]. At different time points (immediately after phagocytosis, and 2, 4 and 6 days post infection), cells were lysed in PBS 0.01% Triton X-100 and the number intracellular bacteria was determined by plating 10-fold serial dilutions of cell lysates on solid medium.

Preparation of Culture Supernatants and Total Lysates, SDS-PAGE and Immunoblotting

Conditional mutants and M. tuberculosis control strain were grown in Middlebrook 7H9 medium as described above. Primers in exponential growth phase were diluted to OD₆₀₀ 0.04 in Middlebrook 7H9 fresh medium added with 0.1% (w/v) ADC, and containing or not 400 ng/ml ATc. After 3 and 6 days, culture supernatants were recovered and proteins were precipitated with 10% (w/v) TCA as previously described [19]. To obtain total lysates, mycobacterial pellets were washed twice and resuspended in 20 mM TrisCl. Bacterial cells were broken by shaking with acid washed-glass beads (106 µm diameter) for 8 min, in a Tissue Lyser apparatus (Qiagen). Suspensions were centrifuged at 5,000 rpm at 4°C for 30 min and the supernatant fraction obtained represented the total-cell lysate. Immunoblot analyses were performed with rabbit anti-EscN [20] and anti-PP141 [16] polyclonal sera, or anti-GroEL2 monoclonal antibody (Colorado State University, NIH, NIAID contract NO AI75320) as previously reported [20].

Results

Characterisation of the eccB5-eccC5 Locus in M. tuberculosis H37Rv

EccB5 and EccC5 are encoded by genes eccB5 (rv1782) and eccC5 (rv1783-rv1784) located in the 5′ region of the ESX-5 locus of M. tuberculosis (Figure 1A). As for the ESX-1-associated EccC1, which is encoded by the two adjacent genes eccC1 (rv3870) and eccC2 (rv3871), EccC5 was thought to be encoded by two separate genes, eccCa5 (rv1783) and eccCb5 (rv1784) in the M. tuberculosis H37Rv reference strain [7,22]. Conversely, it was reported to be encoded by a single non-divided eccC5 gene in several mycobacterial species such as M. leprae, M. leprae biovar Tuscan and Mycobacterium marinum, as well as in other M. tuberculosis strains (Figure 1B) [29–32]. Sequence verification of the M. tuberculosis H37Rv strain used in our study revealed that in the frame stop codon separating eccCa5 gene into two genes, previously reported in the H37Rv reference genome sequence [7,22], seems to be due to a sequencing error (A instead of T at position 2020563) (Figure S2). Thus, in agreement with sequence data reported for other M. tuberculosis H37Rv variants [32], a single eccC5 is also present in the M. tuberculosis H37Rv strain used in this study (Figure 1A).

The genomic organisation of eccB5-eccC5 locus (Figure 1A) suggested that the two genes might be cotranscribed and form a transcriptional unit independent from their flanking genes. Analysis of eccB5-eccC5 transcripts performed by RT-PCR using different combinations of eccB5-eccC5 gene-specific primers revealed a 670-bp amplicon when a combination of primers specific for eccB5 and eccC5 was used (Figure 1C, lane 4), thus indicating that these two genes are indeed cotranscribed. Transcription data were confirmed by mapping the 5′ end of the eccB5-eccC5 transcript using 5′ RACE, which identified only one specific transcript starting 139 bases upstream of the eccB5 start codon (Figure 1E).

The eccB5-eccC5 Operon is Essential for In Vitro Growth of M. tuberculosis

Insertional mutagenesis and transposon site hybridization (TraSH) studies performed in M. tuberculosis identified transposon insertion mutants in various genes coding for different components of the ESX-5 secretion apparatus, such as eccA5 or eccDa5, but no consistent data were available for eccB5-eccC5 [33,34]. To assess the potential essentiality of the eccB5-eccC5 operon, an attempt to delete both genes was performed by using a classical knockout strategy based approach, which employs the replicative thermostable vector pPR27, carrying the sacB counterselectable marker [25]. A recombinant pPR27 plasmid containing the aph cassette encoding resistance to kanamycin flanked by eccB5-upstream and eccC5-downstream regions was constructed and used in the allelic replacement experiments. According to this strategy, at the end of the procedure putative M. tuberculosis double cross over recombinants with a kan’/sac’ phenotype were expected to carry the aph cassette replacing the eccB5-eccC5 operon. However, PCR analysis of genomic DNAs from 150 clones, using primers specific for the aph cassette and rv1780 or rv1786 genes, revealed that none of them showed the expected pattern for an allelic exchange mutant (Figure 2 A and B). While eccB5 and aph specific amplification products were detected in PCR control reactions (Figure 2B, lanes 1–2 and 5–6, respectively), no aph-rv1786 specific amplification products were obtained (Figure 2B, lanes 3–4), demonstrating the presence of an intact eccB5-eccC5 operon at the ESX-5 locus, as well as the non homologous integration of the aph cassette into the genomes of the tested kan’/sac’ clones. As in a previous work the same technique has allowed the deletion of genomic segments up to ~ 20 kb (in size) in M. tuberculosis [24], the inability to obtain eccB5-eccC5 knock-out mutants in this study strongly suggests that the deletion of the eccB5-eccC5 operon might be lethal for M. tuberculosis.

To confirm the requirement of an intact eccB5-eccC5 operon for growth of M. tuberculosis an Mtb::eccB5-eccC5 merodiploid strain, carrying an additional wild-type copy of eccB5-eccC5 genes
integrated into the attB site of the genome, was constructed. Allelic exchange experiments were then repeated in the Mtb::eccB5-eccC5 merodiploid genetic background. Again, genomic DNAs from resultant kan'/suc' clones were tested by PCR, using combinations of primers specific for the aph cassette and rv1780 or rv1786 genes. As depicted in Figure 2D for a representative mutant (Mtb400), the presence of 2706-bp aph-rv1780-specific and 1585-bp aph-rv1786-specific amplification products confirmed the replacement of the wild-type eccB5-eccC5 genomic segment at the ESX-5 locus with the aph cassette. PCR analysis revealed that the deletion of eccB5-eccC5 genes at the ESX-5 locus had occurred in 5% of the recombinants, thus demonstrating that only the presence of an additional copy of eccB5-eccC5 genes allowed the deletion of the chromosomal eccB5-eccC5 segment to take place.

Construction of Mtb<sub>Ppt</sub>eccB5 and Mtb<sub>Ppt</sub>eccC5 Conditional Mutant Strains

To investigate more in depth the effect of inactivation of each gene of the eccB5-eccC5 locus on growth of M. tuberculosis, two conditional mutant strains were constructed using the Pip ON/Tet OFF repressible system [27]. This system, which has been successfully used to construct and characterize an M. tuberculosis conditional mutant for the ESX-3 secretion system [28], is based on the expression of the gene of interest under the control of the anhydrotetracycline-repressible P<sub>Ptr</sub> promoter [27]. As schematically depicted in Figure 3A, in the first mutant, the P<sub>Pt</sub> promoter is placed immediately upstream of the eccB5 coding sequence, replacing the physiological eccB5 promoter. As eccB5 and eccC5 genes are cotranscribed from the same promoter (Figure 1 C and E), this mutant is actually a conditional mutant for the eccB5-eccC5 operon and was thus referred as Mtb<sub>Ppt</sub>eccB5-eccC5. In the second mutant (Mtb<sub>Ppt</sub>eccB5-eccC5), the P<sub>Pt</sub> promoter is placed immediately upstream of the eccC5 coding region, so that only the eccC5 is regulated by P<sub>Pt</sub>, while the eccB5 gene is expressed from its own promoter. Both conditional mutants are derivatives of the Mtb<sub>Ppt</sub>attB-<i>Ppt</i> strain and carry the tetracycline-sensitive repressor TetR and the Pip repressor encoding genes integrated into the chromosome at the attB site. A 950-bp and 876-bp fragment encompassing the 5'-portions of eccB5 and eccC5 coding regions, respectively, were cloned in frame with the P<sub>Pt</sub> promoter into the suicide vector.

Figure 1. The eccB<sub>5</sub>-eccC<sub>5</sub> operon in M. tuberculosis H37Rv. (A) Schematic representation of chromosomal organization of ESX-5 locus in M. tuberculosis H37Rv. Black arrows represent eccB<sub>5</sub> and eccC<sub>5</sub> genes, while gray arrows indicate other ESX-5 genes coding for components of the corresponding secretory apparatus. White arrows represent ESX-5-flanking genes or region-associated genes coding for proteins not thought to be involved in the ESX-5 secretion machinery. Amplification fragments obtained in RT-PCR reactions are also depicted. (B) Comparison of eccC<sub>5</sub> sequence in different mycobacterial species and in the M. tuberculosis CDC1551 strain. The eccC<sub>5</sub> gene segment that includes the T (in bold) corresponding to the A at position 2020563 in the previously reported H37Rv reference genome sequence is represented. For each mycobacterial species or strain, numbers indicate the nucleotidic position in the corresponding annotated genome. (C) Analysis of eccB<sub>5</sub> and eccC<sub>5</sub> transcripts performed by RT-PCR on total RNA using various combinations of gene specific primers: primers specific for eccB<sub>5</sub> (lane 1); primers specific for the 5' terminus of eccC<sub>5</sub> (lane 2); primers specific for the internal portion of eccC<sub>5</sub> (lane 3); primer specific for eccB<sub>5</sub> and eccC<sub>5</sub> (lane 4) M: molecular weight markers. (D) Control PCR reactions performed on RNA samples using the same combinations of eccB<sub>5</sub> and eccC<sub>5</sub> specific primers. No amplification products were detected, thus confirming the absence of contaminating genomic DNA in RNA preparations. M: molecular weight markers. (E) DNA sequence of the eccB<sub>5</sub> upstream region. The 5' end of the eccB<sub>5</sub>-eccC<sub>5</sub> transcript is indicated by the arrow. The eccB<sub>5</sub>-eccC<sub>5</sub> translational start site is indicated in bold. Numbers indicate the nucleotidic position in the annotated genome of M. tuberculosis H37Rv strain.

doi:10.1371/journal.pone.0052059.g001
pFRA50, and recombinant plasmids obtained were used to transform the Mtb::tetR-pip strain. Analysis by PCR on genomic DNA from selected hygromycin resistant clones confirmed the correct replacement of the eccB5-eccC5 promoter with the Pptr promoter in the MtbPptreccB5-eccC5 strain, and the insertion of the Pptr promoter upstream the eccC5 gene in the MtbPptreccC5 mutant (Figure 3B). In these strains, the expression of eccB5-eccC5 operon or the eccC5 gene is exclusively regulated by the activity of the Pptr promoter, which in turn is strictly dependent on the absence/presence of ATc in the culture medium (Figure S3). In the absence of ATc, TetR binds to its operators, turning off the pip transcription, and thus allowing the expression of the eccB5-eccC5 or the eccC5 genes from the Pptr promoter (Figure S3 A). In the presence of ATc, the transcription of pip is allowed. The Pip production results in the block of the Pptr promoter activity, and finally in the repression of eccB5-eccC5 or eccC5 gene expression (Figure S3 B).

Repression of eccB5-eccC5 Shows Stronger Impact on in vitro Growth of M. tuberculosis than Repression of eccC5

The impact of EccB5 and EccC5 on M. tuberculosis viability was determined by evaluating the growth properties of MtbPptreccB5-eccC5 and MtbPptreccC5 conditional mutants both on solid and liquid media. Ten-fold dilutions of MtbPptreccB5-eccC5 and MtbPptreccC5 mutants or M. tuberculosis control strain were spotted onto Middlebrook 7H11 medium added with different concentrations of ATc, ranging from 100 to 400 ng/ml. As control, bacterial dilutions were also spotted on the same medium without the antibiotic. While the growth of the control strain was not affected by the presence of ATc even at the highest concentration of the antibiotic, the growth of the MtbPptreccB5-eccC5 mutant was strongly affected by the presence of the antibiotic in an ATc-concentration dependent manner (Figure 4). After 2 weeks of incubation, no growth was indeed observed for the MtbPptreccB5-eccC5 strain on plates containing the highest concentration of ATc, and some colonies were detectable for the mutant only on agar plates.
containing lower ATc concentrations. After 3 weeks of incubation, whereas bacterial growth was detected for the control strain at higher concentrations of antibiotic and less concentrated bacterial dilutions, some growth was detectable for the Mtb\_PptreccB5-eccC5 mutant, but only at the most concentrated bacterial dilutions. Similar results were obtained when the growth kinetics of the Mtb\_PptreccB5-eccC5 conditional mutant and control strain were analyzed in liquid medium (Figure 5). Again, the growth of the M. tuberculosis control strain was not affected by the addition of ATc into the medium (Figure 5C). In contrast, the growth of Mtb\_PptreccB5-eccC5 mutant was impaired in the presence of 400 ng/ml ATc (Figure 5A), a growth defect that was detectable after 3 days from the addition of the ATc. As EccB5 and EccC5 are predicted to be structural components of the M. tuberculosis ESX-5 membrane complex it is possible that EccB5 and EccC5 proteins already produced by bacterial strains before the exposure to ATc might compensate the effect of eccB5-eccC5 gene repression immediately after the addition of the antibiotic. These results further confirm that the repression of the eccB5-eccC5 operon strongly inhibits the M. tuberculosis in vitro growth.

**Figure 3. Construction of Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 conditional mutants.** (A) Schematic representation of genomic organization of Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 strains. White boxes represent the TetR and the Pip encoding genes integrated at the attB site of the genomes of Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 conditional mutants; white arrows indicated the ESX-5 region-associated genes flanking the eccB5-eccC5 operon; dotted boxes represent the hygromycin resistance marker; black arrows indicate the eccB5 and the eccC5 genes at ESX-5 locus; gray boxes represent the P\_Ppp promoter integrated immediately upstream the eccB5 or the eccC5 coding region in the Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 strain, respectively. Arrows represent primers used for PCR analysis. (B) Analysis of genomic DNA from Mtb\_PptreccB5-eccC5 (lane 1) and Mtb\_PptreccC5 (lane 3) by PCR using primers specific for the P\_Ppp promoter and an internal region of eccB5 or eccC5 genes, respectively. PCR reactions were also performed on genomic DNA form wild-type M. tuberculosis (lane 2 and 4), which was used as negative control. Amplification profiles obtained demonstrated the correct integration of the P\_Ppp promoter upstream the eccB5 or eccC5 coding regions, in the Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 mutants, respectively. doi:10.1371/journal.pone.0052059.g003

**Figure 4. Growth of Mtb\_PptreccB5-eccC5 and Mtb\_PptreccC5 strains on solid medium.** Ten-fold serial dilutions of cultures from Mtb\_PptreccB5-eccC5, Mtb\_PptreccC5 and M. tuberculosis control strain (Mtb\_::tetR-pip) were plated on Middlebrook 7H11, containing different concentrations of ATc, ranging from 0 to 400 ng/ml. Bacterial growth was checked after 2 and 3 weeks of incubation at 37°C. doi:10.1371/journal.pone.0052059.g004
The Mtb<sub>Ppr</sub><sub>eccC<sub>5</sub></sub> strain, in which only the ecc<sub>5</sub> gene is repressed in presence of ATc, showed a different phenotype. This mutant displayed impaired growth as compared to the control strain on agar plates containing ATc (Figure 4), indicating that the repression of ecc<sub>5</sub> affects the ability of M. tuberculosis to grow on solid medium. In contrast, no difference between the Mtb<sub>Ppr</sub><sub>eccC<sub>5</sub></sub> mutant and the control strain was detected when the growth was evaluated in liquid medium (Figure 5). In this case, similarly to the M. tuberculosis control strain, the growth of Ecc<sub>C5</sub> conditional mutant was not affected by the addition of ATc, and comparable growth kinetics were observed for the Mtb<sub>Ppr</sub><sub>eccC<sub>5</sub></sub> strain in Middlebrook 7H9 medium added or not with the antibiotic (Figure 4), indicating that the repression of ecc<sub>C5</sub> affects the ability of Ecc<sub>B5</sub> and Ecc<sub>C5</sub> to grow on M. tuberculosis substrates, the presence of EsxN and PPE41 in culture supernatants recovered at day 6 from all mycobacterial strains was obtained when the secretion of PPE41 was analyzed. These data demonstrate that expression of ecc<sub>B5</sub> and ecc<sub>C5</sub> is required for optimal replication of M. tuberculosis in macrophages.

Ecc<sub>B5</sub> and Ecc<sub>C5</sub> are Required for Secretion of ESX-5 specific Substrates

ESX-5 is responsible for secretion/transport of EsxN, the Esx protein encoded by the ESX-5 locus, and PPE41, a representative member of the large PPE protein family [20]. To determine the impact of Ecc<sub>B5</sub> and Ecc<sub>C5</sub> on secretion of ESX-5 specific substrates, the presence of EsxN and PPE41 in culture supernatants from Mtb<sub>Ppr</sub><sub>eccB<sub>5</sub>-eccC<sub>5</sub></sub> and Mtb<sub>Ppr</sub><sub>eccC<sub>5</sub></sub> strains grown in the presence or not of ATc was investigated. As expected, similar amounts of EsxN were detected in the culture supernatants from Ecc<sub>B5</sub> and Ecc<sub>C5</sub> mutants grown in the absence of ATc, while the addition of ATc did not affect the EsxN secretion in the control strain, the presence of the antibiotic abolished the secretion of the protein in Ecc<sub>B5</sub> and Ecc<sub>C5</sub> conditional mutants. Three and six days after addition of ATc to the medium, no EsxN was detectable in the culture supernatants from Mtb<sub>Ppr</sub><sub>eccB<sub>5</sub>-eccC<sub>5</sub></sub> and Mtb<sub>Ppr</sub><sub>eccC<sub>5</sub></sub> strains, despite the presence of an EsxN-specific band in the corresponding total lysate samples (Figure 7). Similar results were obtained when the secretion of PPE41 was analyzed. Comparable amounts of the protein were detected in the culture supernatants recovered at day 6 from all mycobacterial strains grown in the absence of ATc (Figure 7). Again, while the addition of the antibiotic did not impact the secretion of PPE41 in the control strain, the presence of ATc into the medium resulted in a significant reduction of the amount of PPE41 exported in culture.
supernatants form the \( \text{Mtb}_{\text{PptreccB5-eccC5}} \) and \( \text{Mtb}_{\text{PptreccC5}} \) mutants (Figure 7).

Together these results confirmed that EccB5 and EccC5 are building blocks of the ESX-5 secretion machinery, both required for transport of ESX-5 specific substrates outside the cell.

**Discussion**

In previous studies we demonstrated that selected ESX-5 encoded genes had an impact on secretion of ESX-5 specific substrates, cell wall integrity and virulence of \( \text{M. tuberculosis} \) [20,21]. However, for some other ESX-5 associated genes, i.e. \( \text{eccB5/C5} \), initial gene-inactivation attempts were unsuccessful, which made us think that these genes might be essential for \( \text{M. tuberculosis} \). As results from TraSH analysis were inconclusive for this genomic locus [34], the ability to construct an \( \text{eccB5-eccC5} \) deletion mutant only in the presence of a second functional copy of \( \text{eccB5-eccC5} \) genes, as well as the inability of \( \text{Mtb}_{\text{PptreccB5-eccC5}} \) (in which the \( \text{eccB5-eccC5} \) operon is repressed) to grow on solid medium consistently demonstrate that an intact \( \text{eccB5-eccC5} \) locus is essential for viability of \( \text{M. tuberculosis} \).

In a recent study by Griffin and colleagues, in which \( \text{M. tuberculosis} \) essential genes were identified by using high density transposon mutagenesis and next generation sequencing techniques, no transposon insertions were found in \( \text{rv1783} \) gene (encoding the N-terminal part of EccC5), whereas only a few insertions were detected in \( \text{rv1784} \) (encoding the C-terminal part of EccC5) or in \( \text{rv1782} \) (\( \text{eccB5} \)) [35], further indicating that, in accordance with our data, an intact \( \text{eccB5-eccC5} \) locus is indispensable for growth of \( \text{M. tuberculosis} \). The finding that the only ESX-5 \( \text{M. marinum} \) mutants identified in transposon mutagenesis studies were inactivated for \( \text{MMAR}_{2676} \) and \( \text{MMAR}_{2680}/\text{eccA5Mm} \) genes (orthologous to the \( \text{M. tuberculosis} \) \( \text{eccB5} \) and \( \text{eccC5} \)) is consistent with these findings.

**Figure 6. Intracellular growth kinetics of \( \text{Mtb}_{\text{PptreccB5-eccC5}} \) and \( \text{Mtb}_{\text{PptreccC5}} \) strains in THP-1 derived macrophages.** THP-1-derived macrophages were infected with \( \text{Mtb}_{\text{PptreccB5-eccC5}} \) and \( \text{Mtb}_{\text{PptreccC5}} \) conditional mutants as well as with the \( \text{M. tuberculosis} \) control strain at a m.o.i of 1:20 (bactericells), and cultured in the presence or not of ATc. Immediately after phagocytosis and 2, 4 and 6 days after infection, the number of viable intracellular bacteria was determined. The figure reports the means of CFU number (A, B, and C) and CFU ratio values (CFU/CFU at day 0) (D, E and F) obtained in a representative experiment performed in triplicate.

doi:10.1371/journal.pone.0052059.g006
rv1794 and rv1798/eccA5Mt genes, respectively) [16,19], suggests that the ESX-5 locus in M. marinum might also encode some ESX-5 components that are essential for its in vitro growth. It seems clear that the inactivation of ESX-5 via the simultaneous deletion/repression of various genes encoding building blocks of the ESX-5 secretion machinery strongly affects the viability of M. tuberculosis, and might explain the reason why in this study it was not possible to obtain M. tuberculosis mutants deleted for the eccB5-eccC5 operon. EccB5 and EccC5 are required for secretion of ESX-5 specific substrates (Figure 7) and are both predicted to encode membrane-bound ESX-5 components. During the preparation of this manuscript, the characterization of the composition of the ESX-5 membrane-bound complex in M. marinum and M. bovis BCG was reported [23]. Although the impact of eccB5-eccC5 inactivation on mycobacterial in vitro growth properties was not investigated, secretion and/or biochemical data confirmed the involvement of EccB5 and EccC5 as core components of the ESX-5 secretion apparatus.

ESX-5 has a strong impact in maintaining the mycobacterial cell wall integrity, and inactivation of a single core component of the ESX-5-transmembrane complex results in an increased sensitivity to detergents and hydrophilic antibiotics to which mycobacteria are naturally resistant [20]. It is possible that the disruption of large portions of the ESX-5 secretion apparatus, as is the case for the eccB5-eccC5 deletion/repression, causes more extensive damage to the cell wall or more profound alterations to cell wall stability, resulting in the inability of the corresponding mutant strain to grow both on solid and liquid media. We also observed the intriguing phenomenon that inactivation of a single ESX-5 core component, such as EccC5, has an impact on the mutant’s growth characteristics on solid medium, whereas it has not any discernable inhibitory effect on growth in liquid medium. Interestingly, eccC5 repression strongly affects the intracellular growth properties of M. tuberculosis, further confirming that repression of single genes encoding ESX-5 structural components is sufficient to strongly impair the growth of M. tuberculosis in a restrictive environment such as the macrophage. A similar phenotype has indeed been previously observed for a different ESX-5 mutant, in which the eccD5 gene encoding the predicted ESX-5 transmembrane channel was disrupted [20]. The EccD5 mutant is unable to replicate in murine macrophages, displayed an impaired growth on solid medium, as revealed by a small colony morphotype on Middlebrook 7H11 agar plates, but showed no difference in growth as compared to the control strain in liquid medium, thus suggesting a potential role for the ESX-5 system in modeling the bacterial surface during colony formation on solid medium. A functional link between ESX secretion systems and mycobacterial cell wall has been demonstrated in M. marinum, where the ESX-1-encoded EccA1 ATPase, involved in secretion of ESX-1 substrates, was also found to be required for optimal synthesis of mycolic acids [36]. The recent finding that a plethora of genes encoding enzymes involved in cell-wall synthesis or

![Figure 7. EsxN and PPE41 secretion in MtbPp eccB5-eccC5 and MtbPp eccC5.](image-url)
functioning as well as various ESX loci (ESX-1, ESX-2 and ESX-5) are regulated by the nucleoid-associated EspR regulator [37], provides further evidence of the link between two mycobacterial virulence hallmarks such as ESX-mediated protein secretion/transport and cell envelope biogenesis.

In addition to the alterations of cell wall properties, it is plausible that such impairment in transport and secretion of ESX-5 specific substrates could contribute to the loss of viability observed after disruption of large portions of ESX-5. As a functional ESX-5 is required not only for secretion of PPE proteins [20,21] but also for their correct localization in the cell wall [20], it cannot be excluded that the massive intracellular accumulation of un-secreted or incorrectly localized ESX-5 substrates can exert a toxic, lethal effect for mycobacterial cells. ESX-5 systems of various mycobacterial species have been demonstrated or predicted to be involved in transport of PE_PGRS [19], and a number of PE/PPE proteins encoded in and outside the ESX-5 locus [20,21].

Consistent with previous data reported for another M. tuberculosis H37Rv ESX-5 knock-out strain [20], Western blot analyses of culture supernatants and cell lysates using a monoclonal antibody specific for the PGRS domain [19] did not reveal differences in the PE_PGRS profiles in samples from Mtb<sub>eccB5-eccC5</sub> or Mtb<sub>eccC5</sub> mutants strains, grown in the presence or not of ATc (data not shown). This finding thus argues against the possibility that the effects of eccB5-eccC5 and eccC5 repression on M. tuberculosis growth, as well as the phenotypic differences observed for eccB5-eccC5 and eccC5 mutants might be related to alterations/ differences in secretion or intracellular accumulation of PE_PGRS proteins. On the other hand, information on the presence of ESX-5 associated PE/PPE proteins and their homologs encoded outside the ESX-5 locus in samples from eccB5-eccC5 and eccC5 mutants are not available, due to the lack of antibodies specific for these proteins. Thus, at present it cannot be excluded that the defect in <i>in vitro</i> growth observed for eccB5-eccC5 or eccC5 mutants might also be caused in part by the lack of transport and/or by the intracellular accumulation of these substrates (or some of them).

Although an intact ESX-5 system is required for optimal growth of M. tuberculosis and M. marinum, the ESX-5 locus is absent in the genomes of fast growing, saprophytic mycobacterial species [8]. These findings suggest that during the evolution of the slowly growing, pathogenic mycobacteria, duplication-diversification events have led to the emergence of ESX-5 systems that were apparently linked to the expansion of the PE/PPE protein family. It might well be that fast-growing mycobacteria, which possess only a very limited set of PE/PPE proteins, have no need for an ESX-5 secretion system, while slow-growers that harbor a wide range of PE/PPE proteins require a fully functional ESX-5 system.

Despite the lack of antibodies specific for these proteins, we performed Western blot analysis on culture supernatants and cell lysates of Mtb<sub>eccB5-eccC5</sub> and Mtb<sub>eccC5</sub> mutants, respectively, in the absence or in presence of ATc. Sequence reads generated by next generation sequencing of M. tuberculosis H37Rv line up below the previously reported M. tuberculosis H37Rv reference sequence. From this alignment the presence of a T instead of an A at position 2020563 is clearly visible.

The eccB5-eccC5 locus in samples from Mtb<sub>eccB5-eccC5</sub> and Mtb<sub>eccC5</sub> mutants might also be caused in part by the lack of transport and/or by the intracellular accumulation of these substrates (or some of them).

Although an intact ESX-5 system is required for optimal growth of M. tuberculosis and M. marinum, the ESX-5 locus is absent in the genomes of fast growing, saprophytic mycobacterial species [8].

These findings suggest that during the evolution of the slowly growing, pathogenic mycobacteria, duplication-diversification events have led to the emergence of ESX-5 systems that were apparently linked to the expansion of the PE/PPE protein family.

It might well be that fast-growing mycobacteria, which possess only a very limited set of PE/PPE proteins, have no need for an ESX-5 secretion system, while slow-growers that harbor a wide range of PE/PPE proteins require a fully functional ESX-5 system that exports these proteins to the cell envelop and beyond for their viability. The described situation resembles observations made for the ESX-3 system involved in mycobactin-mediated iron uptake, which is essential in M. tuberculosis but dispensable for growth in the saprophytic species M. smegmatis [27,38]. Furthermore, ESX-1 is a key virulence determinant in pathogenic mycobacteria but seems to regulate the DNA transfer in M. smegmatis [2,39]. Such differences represent an intriguing and unexplored aspect of ESX secretion systems, leading to the requirement to study the individual ESX systems in the context of the mycobacterial species concerned.

Because of their crucial role in host-pathogen interactions as well as their involvement in basic biological processes of tubercle bacilli, ESX secretion systems of M. tuberculosis thus represent potential targets for new anti-tuberculosis drug directed against key mycobacterial factors required for viability and virulence.

**Supporting Information**

**Figure S1** Characterization of the TetR/Pip OFF system in the Mtb<sub>eccB5-eccC5</sub> strain. β-galactosidase assay performed on Mtb<sub>eccB5-eccC5</sub> strain grown on Middlebrook 7H11 plates containing X-gal (40 μg/ml) and different concentrations of ATc, ranging from 0 to 200 ng/ml. The β-galactosidase activity was clearly detected when bacteria were grown in the absence of ATc, and decreased in the presence of increasing concentrations of the antibiotic. The β-galactosidase activity was abolished when Mtb<sub>eccB5-eccC5</sub> was grown on Middlebrook 7H11 medium containing 200 ng/ml ATc.

**Figure S2** Sequence of the eccB5-eccC5 locus in the used M. tuberculosis H37Rv strain. Sequence reads generated by next generation sequencing of M. tuberculosis H37Rv lined up below the previously reported M. tuberculosis H37Rv reference sequence. From this alignment the presence of a T instead of an A at position 2020563 is clearly visible.

**Figure S3** Model of the Pip ON/Tet OFF repressible system in the Mtb<sub>eccB5-eccC5</sub> and Mtb<sub>eccC5</sub> mutants. Schematic representation of the Pip ON/Tet OFF mycobacterial repressible circuit regulating the expression of eccB5-eccC5 operon and eccC5 gene in the Mtb<sub>eccB5-eccC5</sub> and Mtb<sub>eccC5</sub> conditional mutants, respectively, in the absence or in presence of ATc.

**Table S1** Sequences of primers used in the study.

**Acknowledgments**

We are grateful to Riccardo Manganelli and Agnese Serafini for providing pFRA42 and pFRA50 plasmids and for helpful advice, Wilbert Bitter for providing the anti-PGRS monoclonal antibody, and Stewart Cole for fruitful discussion.

**Author Contributions**

Conceived and designed the experiments: MDL DB RB. Performed the experiments: MDL DB MO AA CC. Analyzed the data: MDL DB GB MC RB SE. Wrote the paper: DB MDL GB MC RB SE.

**References**

1. Stoop EJ, Bitter W, van der Sar AM (2012) Tubercle bacilli rely on a type VII army for pathogenicity. Trends Microbiol 20: 477–484.

2. Simoneau R, Botta D, Brosch R (2009) ESX-type VII secretion systems and their role in host-pathogen interaction. Curr Opin Microbiol 12: 4–10.

3. Schneewind O, Missiakas DM (2012) Protein secretion and surface display in Gram positive bacteria. Philos Trans R Soc Lond B Biol Sci 367: 1123–1139.

4. Brodin P, Rosenkranz I, Andersen P, Cole ST, Brosch R (2004) ESAT-6 protective antigens and virulence factors. Trends Microbiol 12: 500–508.

5. Grei Van Puitus NC, Gamieldien J, Hide W, Brown GD, Siezen RJ, et al. (2001) The ESAT-6 gene cluster of Mycobacterium tuberculosis and other high GC Gram positive bacteria. Genome Biol 2: RESEARCH0044.

6. Tekaia F, Gordon SV, Garnier T, Brosch R, Barrett RG, et al. (1999) Analysis of the proteome of Mycobacterium tuberculosis in silico. Tuberc Lung Dis 79: 329–342.

7. Bitter W, Houben EN, Botta D, Brodin P, Brown JD, et al. (2009) Systematic genetric nomenclature for type VII secretion systems. PLoS Pathog 5: e1000507.

8. Grei Van Puitus NC, Sampson SL, Lee H, Kim Y, van Holden PD, et al. (2006) Evolution and expansion of the Mycobacterium tuberculosis PE and PPE multigene families and their association with the duplication of the ESAT-6 (ecr) gene cluster regions. BMC Evol Biol 6: 95.
9. Abdallah AM, Savage ND, van Zon M, Wilson L, Vandenhoute-Grays CM, et al. (2008) The ESX-5 secretion system of Mycobacterium marinum modulates the macrophage response. J Immunol 181: 7166–7175.

10. Weerdenburg EM, Abdallah AM, Mitra S, de Punder K, van der Weel NN, et al. (2012) ESX-5-deficient Mycobacterium marinum is hypervirulent in adult zebrafish. Cell Microbiol 14: 721–739.

11. Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, et al. (2002) Are the PE-PGRS proteins of Mycobacterium tuberculosis variable surface antigens? Mol Microbiol 44: 9–19.

12. Bottai D, Boshch (2009) Mycobacterial PE, PPE and ESX clusters: novel insights into the secretion of these most unusual protein families. Mol Microbiol 71: 323–328.

13. Depoly G, Sanguinetti M, Pusceddu C, Bua A, Brennan MJ, et al. (2006) PE_PGRS proteins are differentially expressed by Mycobacterium tuberculosis in host tissues. Microbes Infect 8: 2061–2067.

14. Lustman R, Sali M, Cascioferro A, Paliucci I, Zambho A, et al. (2012) PE_PGRS80 is required for the full virulence of Mycobacterium tuberculosis. Cell Microbiol 14: 356–367.

15. Li Y, Milner E, Wu M, Petoyksy M, Bermudez LE (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.

16. Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, et al. (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 62: 667–679.

17. Daleke MH, Ummels R, Bawono P, Heringa J, Vandenbroucke-Grauls CM, et al. (2008) The ESX-5 system of Mycobacterium tuberculosis is essential for DNA transfer in pathogenic mycobacteria. Proc Natl Acad Sci USA 106: 18792–18797.

18. Boldrin F, Casemato S, Dianese F, Sala C, Dhar N, et al. (2010) Development of a repressive mycobacterial promoter system based on two transcriptional repressors. Nucl Acids Res 38: e134.

19. Seralini A, Boldrin F, Pini G, Manganelli R (2009) Characterization of a Mycobacterium smegmatis ESX-1 conditional mutant: essentiality and rescue by iron and zinc. J Bacteriol 191: 6340–6344.

20. Cole ST, Eighmeier K, Parkhill J, James KD, Thomson NR, et al. (2001) Massive gene decay in the leprosy bacillus. Nature 419: 1007–1011.

21. Garnier T, Eighmeier K, Campos JC, Medina N, Mansoor H, et al. (2003) The complete genome sequence of Mycobacterium haemophilum. Proc Natl Acad Sci USA 100: 7787–7782.

22. Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, et al. (2008) Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome Res 18: 729–741.

23. Joerger TR, Peng Y, Ganesula K, Chen X, Dobos KM, et al. (2010) Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J Bacteriol 192: 3645–3653.

24. Griffin JE, Gawronski JD, Dejesus MA, Joerger TR, Akerley BJ, et al. (2011) High-resolution phenotypic profiling defines genes essential for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48: 77–84.

25. Sassetti CM, Boyd DH, Rubin EJ (2003) Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48: 77–84.

26. Griffin JE, Gawronski JD, Dejesus MA, Joerger TR, Akerley BJ, et al. (2011) High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog 7: e1002251.

27. Houben EN, Bestebroer J, Ummels R, Wilson L, Vandenbroucke-Grauls CM, et al. (2008) The eccB5-eccC5 Locus of M. tuberculosis is a nucleoid-associated protein. PLoS Pathog 4: e1000261.

28. Siegrist MS, Unnikrishnan M, McConnell MJ, Borovsky M, Cheng TY, et al. (2009) Mycobacterial Ess-1 is required for mycobactin-mediated iron acquisition. Proc Natl Acad Sci USA 106: 18792–18797.

29. Coros A, Callahan B, Battaglioli E, Debyshire KM (2008) The specialized secretory apparatus ESX-1 is essential for DNA transfer in Mycobacterium smegmatis. Mol Microbiol 69: 794–808.