

Mutually dependent secretion of proteins required for mycobacterial virulence

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The ESX-1 locus is a region critical for full virulence in *Mycobacterium tuberculosis*, which encodes two secreted proteins as well as other genes involved in their secretion. The mechanism of secretion of the two proteins, ESAT-6 and CFP-10, and their function remain unknown. Using proteomic methods to search for additional proteins secreted by the ESX-1 locus, we discovered that a protein encoded by a chromosomally unlinked gene, *espA*, is also secreted by strains that contain the ESX-1 locus but not by strains with ESX-1 deletions. Mutations in individual ESX-1 genes, including those that encode ESAT-6 and CFP-10, were found to block *EspA* secretion. Surprisingly, mutants that lack *espA* reciprocally failed to secrete ESAT-6 and CFP-10 and were as attenuated as ESX-1 mutants in virulence assays. The results indicate that secretion of these proteins, which are each critical for virulence of pathogenic mycobacteria, is mutually dependent. The results further suggest that discerning the nature of the interaction and the structure of macromolecular complexes will provide insights into both an alternative mechanism of protein secretion and mycobacterial virulence.

ESAT-6 protein | *Mycobacterium tuberculosis* | virulence factor

Interactions between bacterial pathogens and their hosts occur at the surface of cells. Many pathogenic bacteria secrete proteins involved in virulence (1). These proteins often interfere with normal host defenses and allow bacteria to survive and multiply. Strikingly, many of these virulence factors are secreted by nonclassical secretory systems that, in some cases, facilitate translocation of proteins across host cell membranes (2, 3).

During the evolution of *Mycobacterium bovis* Bacille Calmette–Guérin (BCG), the attenuated bacillus widely used as vaccine against tuberculosis, a major deletion removed a set of nine ORFs (4). Similar deletions are found in *Mycobacterium microti* (5) and the Dassie bacillus (6), other relatively avirulent mycobacteria. The BCG deletion, known as RD1, includes two genes that encode the abundantly secreted proteins, ESAT-6 and CFP-10. The RD1 deletion encompasses most but not all of a larger, 15-gene region known as ESX-1 (7), which is hypothesized to encode an alternative secretory system (8, 9).

The proteins encoded by the ESX-1 locus clearly play an important role in virulence. The secreted proteins encoded within the locus are potent T cell antigens (7, 10, 11). More significantly, *Mycobacterium tuberculosis* strains with RD1 deletions are far less virulent in mice than strains that are otherwise isogenic (12–14). They replicate poorly and cause less pathology in both immunocompetent and immunocompromised mice, although the latter ultimately succumb to infection. RD1-deleted strains of *Mycobacterium marinum* elicit only very modest macrophage aggregation and granuloma formation in zebrafish (15). Additional work suggests the RD1-encoded proteins may disrupt host cell membranes. In *M. tuberculosis*, these proteins may be required for lysis of infected cells (13) and cell-to-cell spread (16), whereas, in *M. marinum*, RD1 is needed for efficient bacterial escape from the phagosome (17).

Several lines of experimental evidence support the idea that the genes in the ESX-1 locus are functionally related. First, several of the ESX-1-encoded proteins have been shown to interact through yeast two-hybrid analysis (14). More strikingly, inactivation of a number of the individual ESX-1 genes disrupts ESAT-6 and CFP-10 secretion and attenuates the bacterium to a similar extent as deletion of the whole locus (13, 16). These data are consistent with the model that ESX-1 encodes an alternative protein secretory apparatus required for the export of ESAT-6 and CFP-10.

However, the specialized type III and IV secretory systems present in Gram-negative pathogens mediate the export of many proteins encoded by genes unlinked to the secretory apparatus (2, 3). These “effector” proteins often play complementary roles in altering host resistance. Here we describe a proteomic search for proteins secreted by the same machinery used for ESAT-6 and CFP-10 secretion. We find that the product of an unlinked gene, Rv3616c, here renamed *espA* for ESX-1 secretion-associated protein A, has a critical interaction with the genes of the ESX-1 region.

Materials and Methods

Culture of *M. tuberculosis* and Preparation of Culture Filtrates and Cell Lysates. *M. tuberculosis* strains were maintained in Middlebrook 7H9 medium supplemented with oleic acid–albumin–dextrose–catalase (Difco) and 0.05% Tween 80. The generation of H37Rv Δ RD1 and H37Rv Δ esat6 was described in refs. 12 and 16, and identification and confirmation of the transposon mutants were described in ref. 16.

For analysis of protein expression and secretion, strains were cultured in Sauton’s medium supplemented with 0.05% Tween 80 instead of 7H9 medium, in which the large quantity of albumin confounded analysis of secreted bacterial proteins. To analyze bacterial proteins by SDS/PAGE, mid-log phase bacteria were resuspended in fresh medium and cultured overnight at 37°C. Cell pellets were resuspended in protein extraction buffer (50 mM Tris·HCl, pH 7.5/5 mM EDTA/1 mM 2-mercaptoethanol) and disrupted by bead beating. Protein loading buffer was added to cell lysates, and samples were boiled for 20 min before removal from the biosafety level 3 facility and analysis by SDS/PAGE and Western blotting. Culture supernatants were sterilized by double filtration through 0.2- μ m filters. Filtrates were concentrated \approx 20-fold by using Centricon (Millipore) and Vivacell (Vivascience, Hannover, Germany) filter units and analyzed by SDS/PAGE and Western blotting. The volume of culture filtrate

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Abbreviations: MS/MS, tandem MS; SCID, immunocompromised severe combined immunodeficient.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ086480 and DQ068700).

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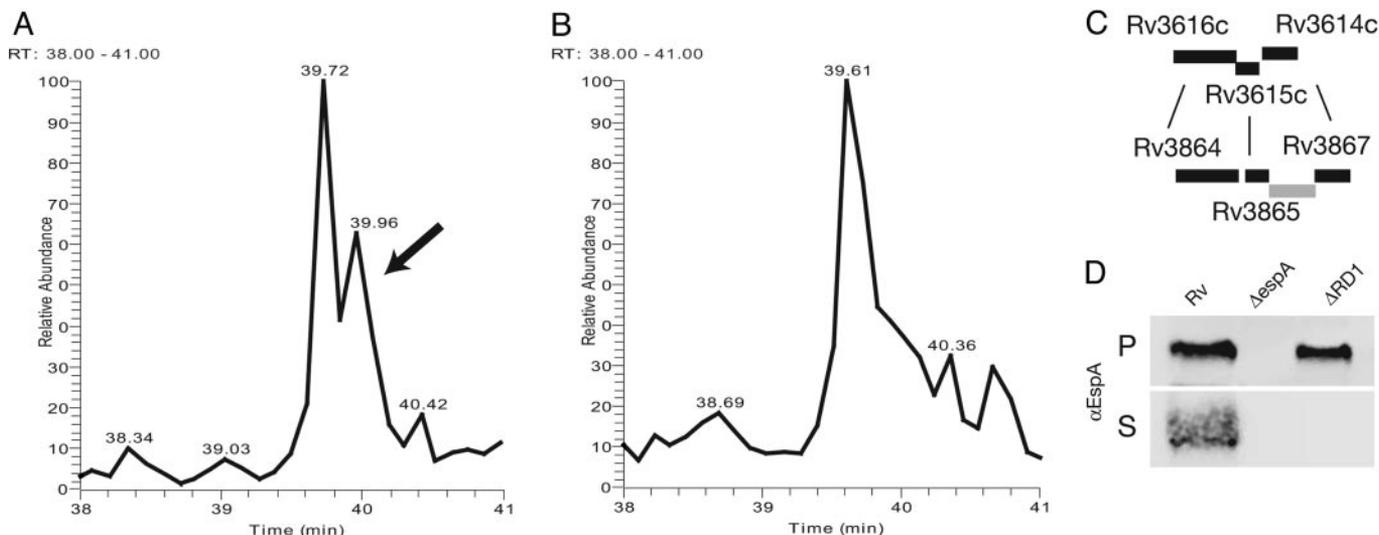


Fig. 1. EspA is present in H37Rv culture filtrates but not in RvΔRD1 culture filtrates. (A and B) EspA identified by MS as present in H37Rv culture filtrates but not in RvΔRD1 culture filtrates. Culture filtrate proteins from H37Rv (A) and H37RvΔRD1 (B) were tryptically digested. In parallel runs, the resultant peptide fragments were resolved through reverse-phase chromatography and analyzed by LCQ-MS/MS. The tracing indicated by the arrow was identified as peptide KYSEGAAAGTEDAERAPVEADAGGGQK from EspA, which was one of four unique peptides identified from EspA. (C) The *espA* locus shares significant homology to genes upstream of the ESX-1 locus. Rv3616c, here renamed EspA, shares 31.75% identity in a 400-aa overlap with Rv3864. Rv3615c shares 36.25% identity in a 102-aa overlap with Rv3865. Rv3614c shares 54.9% identity in a 173-aa overlap with Rv3867. (D) Presence of EspA in the pellet (P) and supernatant (S) of H37Rv, H37RvΔ*espA* (Δ*espA*), and H37RvΔRD1 (ΔRD1). Results are representative of more than three experiments.

identified in the H37RvΔRD1 culture filtrates (178 proteins were identified in three independently obtained samples). In ion-trap MS, abundant peptides can suppress the detection of less abundant species. Peptides from CFP-10 were the most frequently identified peptides in our analyses of wild-type culture filtrates. Thus, we may have identified more proteins in the H37RvΔRD1 culture filtrates because the absence of peptides from CFP-10 and, to a lesser extent, ESAT-6 allowed detection of many peptides present in lower quantities.

The greater coverage of proteins secreted by the H37RvΔRD1 strain biased our screen against the identification of proteins secreted by wild-type *M. tuberculosis* but not by the RD1 mutant. Because comparative quantitation by MS is difficult where samples are significantly different (i.e., in the presence or absence of CFP-10 and ESAT-6), we required that proteins be identified in multiple independent samples of the wild-type culture filtrates and not be detected in any of the H37RvΔRD1 culture filtrates. Thus, this screen was specific for differences in proteins that are relatively abundant in

wild-type culture filtrates, but it was not comprehensive. By these stringent criteria, we identified four proteins present in wild-type cultures but absent from H37RvΔRD1 culture filtrates. These included three genes encoded within the ESX-1 region: ESAT-6, CFP-10, and PE35, which has not previously been identified in culture filtrates from *M. tuberculosis*. A single unlinked gene product, encoded by Rv3616c and here named *espA*, was identified as missing from the mutant culture filtrates (Fig. 1 A and B).

EspA is a 40-kDa protein without predicted signal sequence or putative transmembrane domains. It is a conserved hypothetical protein of unknown function, but a global transposon analysis of mycobacterial genes required for infection predicted that *espA* is necessary for the virulence of *M. tuberculosis* (19). Moreover, the predicted amino acid sequence of EspA is 31% identical to Rv3864, which lies immediately 5' of the ESX-1 region (Fig. 1C). *EspA* appears to be in a three-gene operon with Rv3615c and Rv3614c, which show significant homology to Rv3865 and Rv3867, respectively. The homology of the *espA* operon to genes

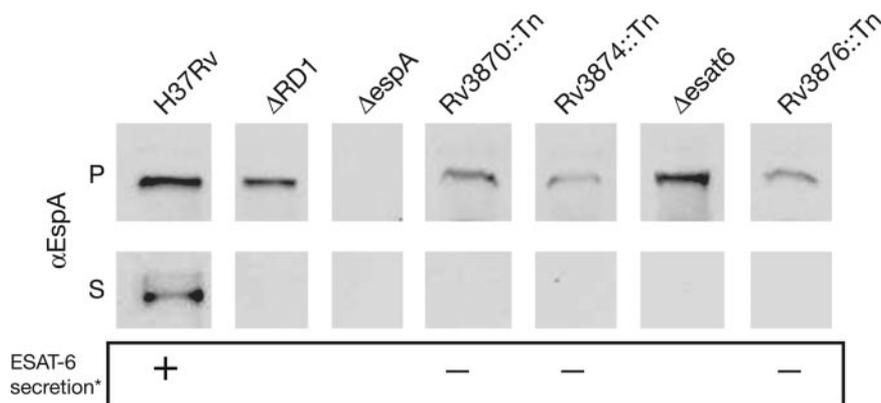


Fig. 2. Localization of EspA in wild-type *M. tuberculosis* and strains carrying deletions of RD1 (ΔRD1), of *esat-6* (Δ*esat-6*), and transposon insertions in the indicated genes. Cell pellet (P) and culture supernatant (S) proteins were resolved through SDS/PAGE. EspA was identified by immunoblotting with protein-specific antibody. Results are representative of at least two experiments. *, ESAT-6 secretion as published in ref. 16.

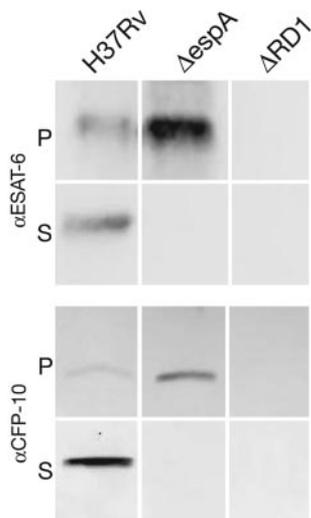


Fig. 3. Localization of ESAT-6 and CFP-10 in the presence or absence of *espA*. Cell pellet (P) and culture supernatant (S) proteins from H37Rv, H37Rv Δ RD1 (Δ RD1), and H37Rv Δ espA (Δ espA) were resolved through SDS/PAGE. ESAT-6 and CFP-10 were identified by immunoblotting with protein-specific antibody. Results are representative of more than three experiments.

flanking the ESX-1 locus suggested that EspA might indeed be secreted by or associate with the ESX-1 apparatus.

We confirmed that EspA is not present in H37Rv Δ RD1 culture filtrates using both epitope-tagged EspA (data not shown) and antibody to native EspA protein. EspA was present in both the cell lysates and supernatants of wild-type mycobacteria. However, the protein was completely absent from the supernatants of the H37Rv Δ RD1 strain, confirming the LCQ-MS/MS findings (Fig. 1D). In addition, EspA appeared to accumulate in the cell-associated fraction of the H37Rv Δ RD1 strain, indicating that the protein is produced normally but is retained in the bacterial cell cytoplasm.

Individual Genes in the ESX-1 Region Are Required for Export of EspA.

Mutations in individual genes of the ESX-1 region block secretion of ESAT-6 and CFP-10 and attenuate *M. tuberculosis* (13, 14, 16). Similarly, mutations in several of the putative structural genes of the ESX-1 apparatus abolish export EspA (Fig. 2). These results are consistent with several models of EspA, ESAT-6, and CFP-10 secretion. EspA and the ESAT-6/CFP-10 complex could be secreted independently by the ESX-1 apparatus. Alternatively, either EspA or ESAT-6 and CFP-10 could be secreted by the apparatus and then form a distal part of the structure, promoting secretion of the other protein(s). To test these models, we assessed the secretion of EspA, ESAT-6, and CFP-10 in the absence of each other.

ESAT-6 and CFP-10 form a tight 1:1 complex *in vitro* and are thought to be secreted as a heterodimer by the ESX-1 apparatus (22). Therefore, we looked at EspA expression and secretion in *M. tuberculosis* strains carrying a deletion of *esat-6* (H37Rv Δ esat6) or a transposon insertion in *cfp-10* (Rv3874::Tn). In H37Rv Δ esat6, EspA accumulated within the cell pellet but was not found in the culture filtrate (Fig. 2). Likewise, Rv3874::Tn, which expresses but does not secrete ESAT-6, failed to secrete EspA (Fig. 2). Thus, both ESAT-6 and CFP-10 are required for the export of EspA.

EspA Is Required for Secretion of ESAT-6 and CFP-10. To determine whether EspA is required for ESX-1 function as assessed by ESAT-6 and CFP-10 secretion, we constructed a deletion mutant of *espA*. In wild-type *M. tuberculosis*, ESAT-6 was found in both

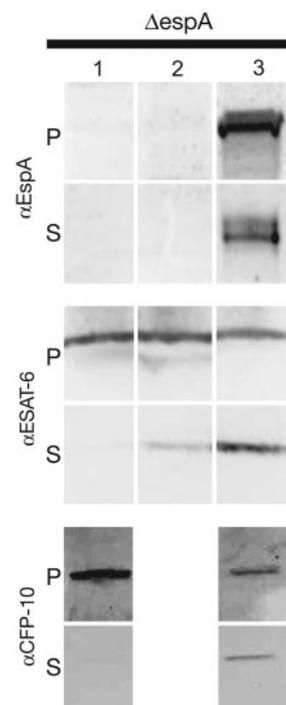


Fig. 4. Complementation of H37Rv Δ espA for ESAT-6 and CFP-10 secretion. H37Rv Δ espA was complemented by integration of a single copy of a control vector (lane 1), epitope-tagged *espA* under a synthetic MOP promoter (pALE2) (lane 2) or epitope-tagged *espA* under its native promoter (pALE4) (lane 3) into the chromosomal *attB* site. Cell pellet (P) and culture supernatant (S) proteins from the complemented strain or a strain carrying the backbone vector were resolved through SDS/PAGE. EspA, ESAT-6, and CFP-10 were identified by immunoblotting with protein-specific antibody. Pellet-associated EspA expressed from pALE2 could be visualized only by overexposing the blot. Results are representative of two experiments.

the cell pellet and culture filtrate. However, in H37Rv Δ espA, ESAT-6 was absent from the culture filtrate and consistently accumulated in the cell pellet (Fig. 3). Thus, EspA is required for ESAT-6 secretion. Likewise, wild-type *M. tuberculosis* expressed and secreted CFP-10. In H37Rv Δ espA, secretion of CFP-10 was inhibited and the protein accumulated in the cell pellet (Fig. 3).

Complementation of H37Rv Δ espA by integration of a single copy of *espA* restored ESAT-6 and CFP-10 secretion (Fig. 4). Several complementing constructs were made that expressed and secreted EspA at varying levels. In the presence of these constructs, ESAT-6 secretion was proportional to the amount of EspA expression (Fig. 4).

Rv Δ espA Is Attenuated in Mice to a Similar Extent as H37Rv Δ RD1.

Previous work has shown that the ESX-1 region is required for virulence in mice. Deletion of RD1 has been shown to attenuate mycobacterial replication and virulence in immunocompetent mice and to delay mortality in immunocompromised severe combined immunodeficient (SCID) mice. Because our data indicate that EspA is both secreted by the ESX-1 apparatus and necessary for ESX-1 function as measured by ESAT-6 and CFP-10 secretion, it was important to explore whether EspA is required for mycobacterial virulence in these mouse models.

C57BL/6 mice were infected with H37Rv, H37Rv Δ RD1, H37Rv Δ espA, or H37Rv Δ RD1 Δ espA. The mycobacterial organ burden in lung and spleen was measured at 1, 2, 4, and 8 weeks. Consistent with data published in ref. 12, H37Rv Δ RD1 was highly attenuated relative to wild type in both lung and spleen at all time points (Fig. 5A and B). At all time points, H37Rv Δ espA or H37Rv Δ RD1 Δ espA was also highly attenuated in both lung

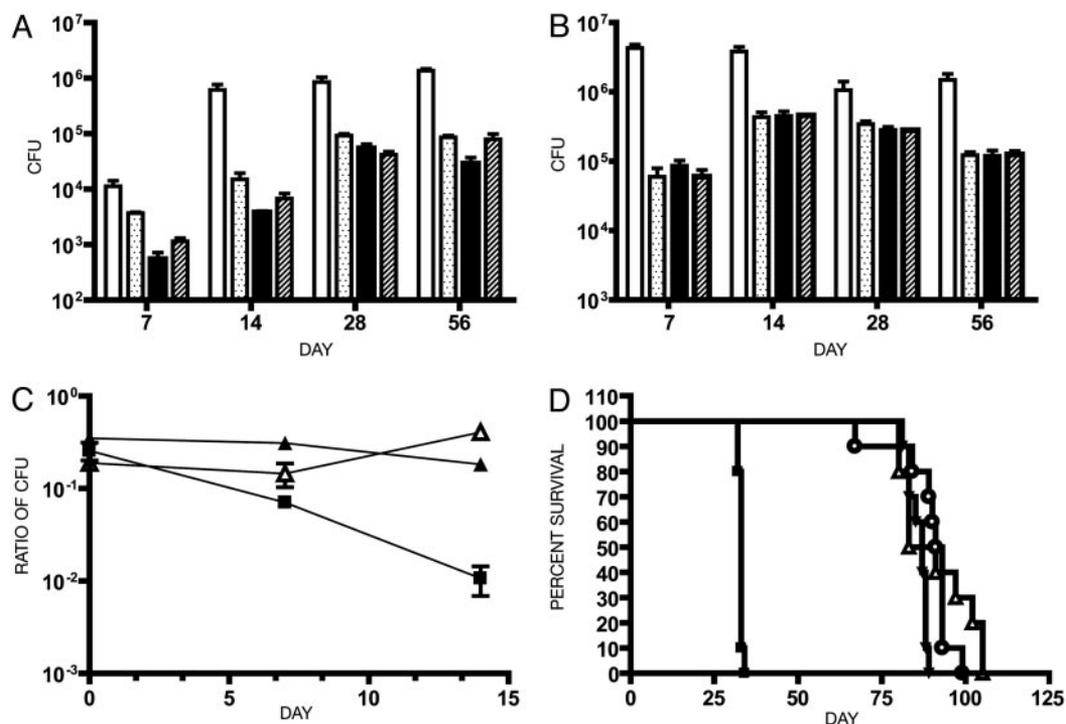


Fig. 5. Deletion of *espA* attenuates *M. tuberculosis* to a similar extent as deletion of RD1. Lungs (A) and spleen (B) colony-forming units from C57BL/6 mice infected with the indicated mycobacterial strains. The infecting inocula were determined by plating serial dilutions as follows: open bars, H37Rv, 1.9×10^5 ; dotted bars, H37RvΔRD1, 1.6×10^5 ; filled bars, H37RvΔespA, 1.2×10^5 ; hashed bars, H37RvΔRD1ΔespA, 2.1×10^5 . Numbers represent the mean and standard deviation of four mice per time point. Each of the mutants was significantly attenuated compared to H37Rv in both organs at all time points except in the spleen colony-forming units at 28 days ($P \leq 0.01$). In addition, the lung burden of RvΔRD1 was slightly but significantly higher than that of H37RvΔespA and H37RvΔRD1ΔespA at 7 days and H37RvΔespA at 14 days ($P < 0.05$). (C) C57BL/6 mice were infected with two strains of *M. tuberculosis* in competition. Mice were infected with a 1:3 mixture of kanamycin marked to unmarked strains as indicated: ■, H37RvΔRD1:H37Rv; ▲, H37RvΔRD1:H37RvΔespA; △, H37RvΔRD1ΔespA:H37RvΔespA. The organ burden of each strain was determined at the indicated times by plating in the presence and absence of kanamycin. Numbers represent the mean and standard deviation of three mice per time point. H37RvΔRD1 was significantly attenuated relative to H37Rv over time as determined by linear regression analysis ($P < 0.05$). In the other two competitions, the ratios of the strains did not change significantly over time. (D) SCID mice were infected with a goal inoculum of 300 organisms. The infecting inocula, determined by plating serial dilutions, were as follows: ■, H37Rv, 3×10^2 ; △, H37RvΔRD1, 7×10^2 ; ▼, H37RvΔespA, 23×10^2 ; ○, H37RvΔRD1ΔespA, 7×10^2 . Ten mice were infected with each strain. The difference in survival between wild-type and mutant strains was highly statistically significant ($P < 0.0001$), and the times to death of RvΔespA and RvΔRD1ΔespA were not significantly different from that of RvΔRD1.

and spleen. In both organs they appeared attenuated to an extent similar to or greater than H37RvΔRD1.

Lung histopathology was examined at all time points. At 8 weeks, wild-type *M. tuberculosis* caused significant disease with focal infiltrates containing foamy macrophages and lymphocytic aggregates (data not shown). In the sections examined, $\approx 50\%$ of the lung was diseased. In contrast, all of the mutants caused far less disease, although small infiltrates of similar cellular composition to the wild-type infection were evident. At most, 5–10% of the lung was involved in the multiple sections studied (data not shown). There was no clear difference in histopathology between the mutants.

There are several loci that encode ESX-1-related proteins within the mycobacterial genome, which are of unknown significance *in vivo*. We hypothesized that EspA might be required for the secretion of some of these proteins. If this were true, deletion of EspA might be more attenuating than mutations in the ESX-1 locus. In the infections described above, H37RvΔespA and H37RvΔRD1ΔespA appeared slightly more attenuated than H37RvΔRD1 in the lung at 7 and 14 days. However, it is difficult to make rigorous comparisons of the relative fitness of different strains in single-strain infections; therefore, we performed competition experiments to assess the relative fitness of wild-type H37Rv vs. H37RvΔRD1, H37RvΔRD1 vs. H37RvΔespA, and H37RvΔespA vs. H37RvΔRD1ΔespA (Fig. 5C). H37RvΔRD1

was ≈ 100 -fold less abundant than wild type in the lung at 2 weeks after infection. Deletion of *espA* attenuated *M. tuberculosis* to a similar degree as deletion of the RD1 locus. *M. tuberculosis* carrying both deletions was no more attenuated than the single mutants.

Finally, we examined the relative virulence of H37Rv, H37RvΔRD1, H37RvΔespA, or H37RvΔRD1ΔespA in a SCID mouse model of mycobacterial virulence. SCID mice infected with wild-type H37Rv died at 35 days after infection, whereas those infected with the mutant strains died at ≈ 90 days after infection. The difference between wild-type and mutant strains was highly statistically significant, whereas the times to death of H37RvΔespA and H37RvΔRD1ΔespA were not significantly different from H37RvΔRD1 (Fig. 5D).

Discussion

The secretion of ESAT-6 and CFP-10, critical to the virulence of *M. tuberculosis*, appears to be mediated by a secretion apparatus encoded by the surrounding genes (13, 14, 16). We have shown that the same genes are required to secrete an additional protein, EspA. Unexpectedly, the secretion of EspA also depends on the presence of ESAT-6 and CFP-10. Indeed, secretion of EspA, ESAT-6, and CFP-10 is codependent. Each member of the trio of proteins is secreted and is required for the others' secretion. The importance of the association between EspA and the ESX-1-encoded secretory

