Supplementary Information for:

Esx paralogs are functionally equivalent to ESX-1 proteins but are dispensable for virulence in *M. marinum*

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Running Head: Gene duplications of ESX-1 virulence factors

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Supplementary Methods

Crystal violet assay. $5x10^5$ RAW 264.7 cells were seeded overnight in a 24 well TC treated plate (CELLSTAR, Greiner Bio-one, Monroe, NC). Bacteria were grown in 7H9 broth for 3 days, washed three times with sterile PBS and syringe passaged to break up clumps. RAW 264.7 cells were incubated with $1x10^6$ bacteria (MOI 2) for 2 hours at 37°C. Each infection was performed in technical triplicate. Wells were treated with 100µg/mL gentamycin for 1 hour at 37°C to kill extracellular bacteria. Each well was washed three times with PBS and then replenished with DMEM media containing FBS. After 2 days, the media was removed and the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min. at room temperature. The wells were stained with 0.1% crystal violet (Alfa Aesar) in dH_2O for 30 min. at room temperature, with gentle rocking. The plate was washed by carefully submerging in a tray of distilled water, under running water until the rinses were clear. The plate was allowed to dry completely and then imaged with a camera.

Table S1. *M. marinum* strains used in this study.

Strain	Description	Reference
M	Wild-type strain (WT)	ATCC BAA-535
ΔRD1	Unmarked deletion of *eccCb$_1$'-espK'*	(1)
ΔRD1 Δ*esxB_1*	ΔRD1, unmarked deletion of *esxB_1* (*MMAR_0187*)	This study
Δ*esxBA*	Δ*esxBA*, Kan^r	(2)
Δ*esxBA*/p$_{Mops}$*esxBA*	Δ*esxBA,* p$_{Mops}$*esxBA* integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/ p$_{Mops}$*esxB_1-esxA_3*	Δ*esxBA,* p$_{Mops}$*esxB_1-esxA_3* integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/ p$_{Mops}$*esxB_1* M98A -*esxA_3*	Δ*esxBA*, p$_{Mops}$*esxB_1* M98A -*esxA_3* integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/ p$_{Mops}$*esxB_2-esxA_2*	Δ*esxBA*, p$_{Mops}$*esxB_2-esxA_2* integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406	Δ*esxBA*, pMH406 integrated at the *attB* site; Kan^r, Hyg^r. The pMH406 plasmid includes the *esxBA* genes from *M. tb* behind the mycobacterial optimal promoter.	Gift from Jeff Cox, and (3)
Δ*esxBA*/pMH406 *esxB* L94A	Δ*esxBA,* pMH406 *esxB* L94A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* S95A	Δ*esxBA,* pMH406 *esxB* S95A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* S96A	Δ*esxBA,* pMH406 *esxB* S96A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* Q97A	Δ*esxBA,* pMH406 *esxB* Q97A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* M98A	Δ*esxBA,* pMH406 *esxB* M98A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* G99A	Δ*esxBA,* pMH406 *esxB* G99A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxBA*/pMH406 *esxB* F100A	Δ*esxBA,* pMH406 *esxB* F100A integrated at the *attB* site; Kan^r, Hyg^r	This study
Δ*esxB_1*	Unmarked deletion of *esxB_1* (*MMAR_0187*)	This study
Δ*esxB_1*/p$_{Mops}$*esxB_1-esxA_3*	Δ*esxB_1*, p$_{Mops}$*esxB_1-esxA_3* integrated at the *attB* site; Hyg^r	This study
Δ*MMAR_0184-esxA_2* (ΔextESX-6)	Unmarked deletion of *MMAR_0184-MMAR_0196*	This study

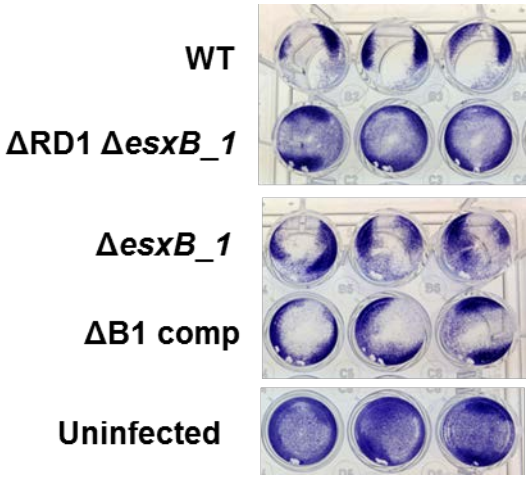
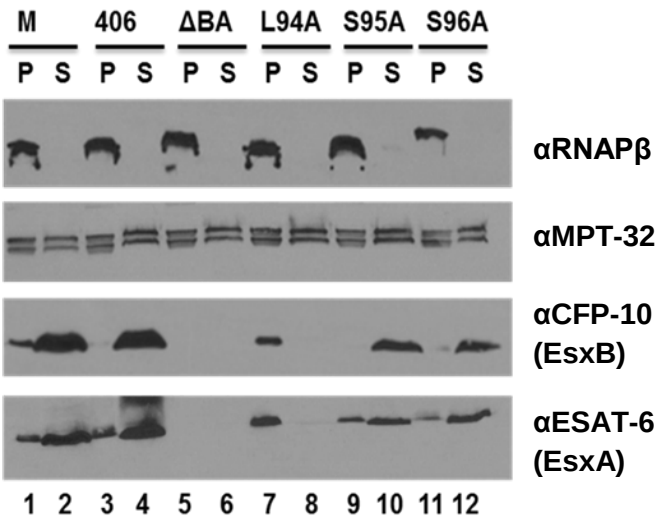


Figure S1. The *esxB_1* gene is dispensable for cytotoxicity to RAW cells. RAW264.7 cells were infected with M. marinum strains at an MOI of 2. 2 days post infection, the cells were fixed and stained with crystal violet. Clearing of the RAW cell monolayer indicates cytotoxicity. The figure is representative of 3 independent infections with each strain tested in triplicate.

A.



B.

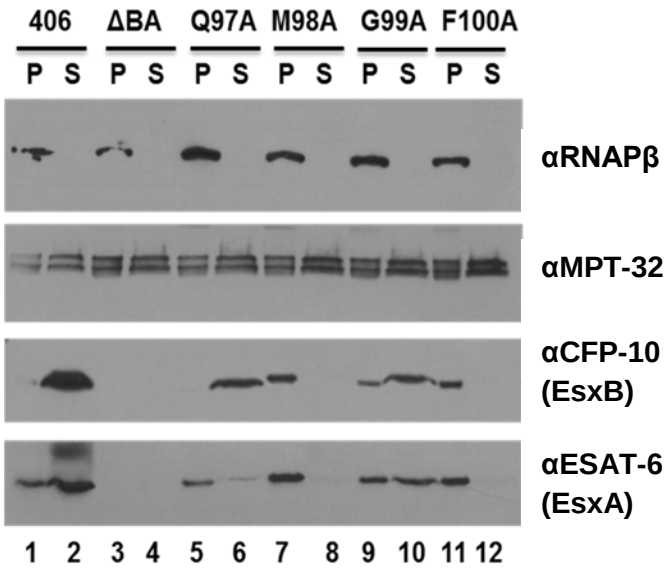
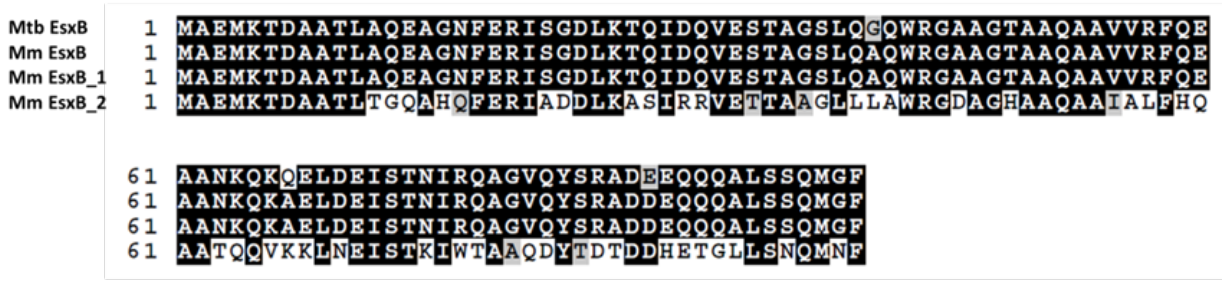


Figure S2. *M. tb* EsxB in *M. marinum* is recognized for ESX-1 export by its C-terminus. $EsxBA_{MT}$ are secreted from *M. marinum* as demonstrated by an ESX-1 secretion assay. 10µg of protein was loaded. "M" is the *M. marinum* WT strain. "406" refers to the Δ*esxBA*/pMH406 strain. "ΔBA" refers to the Δ*esxBA* strain. The remaining strains are Δ*esxBA*/pMH406 strains which encode a mutagenized $EsxB_{MT}$ protein from an integrated plasmid (e.g. Δ*esxBA*/pMH406 *esxB* L94A). RNAP-β serves as a lysis control. Mpt-32, a Sec-secreted protein, is the loading control. *In vitro* production (P, pellet) and secretion (S, supernatant) of ESX-1 substrates, CFP-10 (EsxB) and ESAT-6 (EsxA) were analyzed. The western blot is representative of 3 independent experiments.

A.



B.

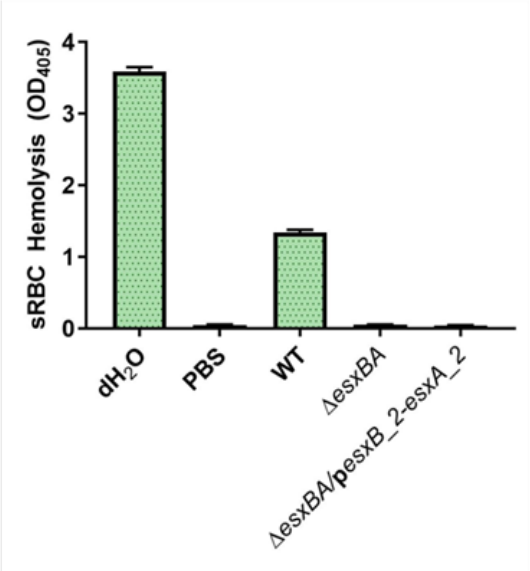


Figure S3. The EsxB_2 protein differs from and is not functionally equivalent to EsxB. (A) Alignment of the *M. tb* EsxB, *M. marinum* EsxB, *M. marinum* EsxB_1, and *M. marinum* EsxB_2 protein sequences. *M. marinum* EsxB and EsxB_1 are 100% identical at the protein level. The *M. marinum* EsxB and EsxB_1 proteins differ from *M. tb* EsxB by 3 amino acid residues. While the first 11 amino acids of the EsxB_2 protein are completely conserved among all EsxB proteins shown here, the remaining protein sequence shows variation. There is lack of conservation of the ESX-1 C-terminus (last 7 amino acids). **(B)** Introduction of an *esxB_2* expression plasmid does not restore hemolytic activity to the Δ*esxBA* strain, demonstrating the EsxB_2 and EsxA_2 proteins are not functionally equivalent to the EsxBA proteins. 3 isolates were tested. Error bars represent the standard deviation of three technical replicates.

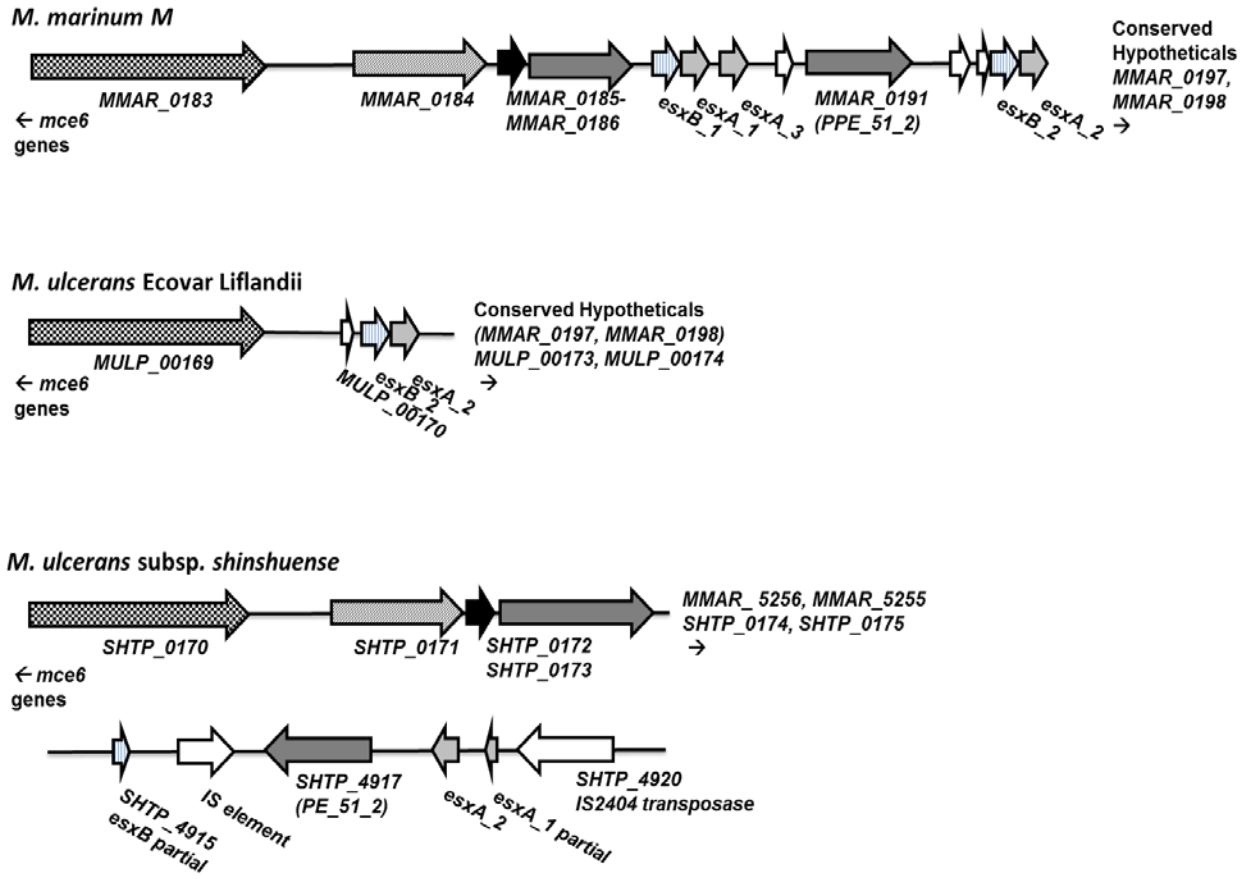


Figure S4. The *esx-6* locus is partially present in some *M. ulcerans* strains. Upstream of the Esx-6 region is *MMAR_0183*, which encodes a PE-PGRS family protein. *MMAR_0183* is found in both *M. ulcerans* strains depicted. The *M. ulcerans* ecovar Liflandii strain has the *esxB_2* locus. *MMAR_0184*, the *eccB* paralog and *pe35/ppe68_1* orthologs are present in *M. ulcerans* susp. *shinshuense*. The *esxB_1* and *esxB_2* loci are incomplete in this strain and found in a different area of the chromosome

References

1. **Volkman HE, Clay H, Beery D, Chang JC, Sherman DR, Ramakrishnan L.** 2004. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS Biol **2:**e367.
2. **Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, Brown EJ.** 2004. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol **53:**1677-1693.
3. **Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR.** 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.