A Duplicated ESAT-6 Region of ESX-5 Is Involved in Protein Export and Virulence of Mycobacteria

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The ESX-5 secretion system of *Mycobacterium tuberculosis* is important for bacterial virulence and for the secretion of the large PE/PPE protein family, whose genes constitute 10% of the *M. tuberculosis* genome. A four-gene region of the ESX-5 system is duplicated three times in the *M. tuberculosis* genome, but the functions of these duplicates are unknown. Here we investigated one of these duplicates: the region carrying the *esxI*, *esxJ*, *ppe15*, and *pe8* genes (ESX-5a). An ESX-5a deletion mutant in the model system *M. marinum* background was deficient in the secretion of some members of the PE/PPE family of proteins. Surprisingly, we also identified other proteins that are not members of this family, thus expanding the range of ESX-5 secretion substrates. In addition, we demonstrated that ESX-5a is important for the virulence of *M. marinum* in the zebrafish model. Furthermore, we showed the role of the *M. tuberculosis* ESX-5a region in inflammasome activation but not host cell death induction, which is different from the case for the *M. tuberculosis* ESX-5 system. In conclusion, the ESX-5a region is nonredundant with its ESX-5 paralog and is necessary for secretion of a specific subset of proteins in *M. tuberculosis* and *M. marinum* that are important for bacterial virulence of *M. marinum*. Our findings point to a role for the three ESX-5 duplicate regions in the selection of substrates for secretion via ESX-5, and hence, they provide the basis for a refined model of the molecular mechanism of this type VII secretion system.

Mycobacterium tuberculosis is an extremely successful pathogen that employs various strategies to evade immune responses and persist within the host (1, 2). Integral to this manipulation of the host by *M. tuberculosis* is the secretion of virulence factors by various secretion systems (3). There are five different type VII secretion systems (T7SS) in *M. tuberculosis* (ESX-1 to ESX-5) (4, 5). The different ESX systems were most likely generated via duplications of the ancestral system ESX-4 in the chronological order ESX-1, ESX-3, ESX-2, and ESX-5 (6). All the ESX secretion systems have a set of common genes which encode the core components of their secretion machinery, among which are genes encoding two members of the ESAT-6-like family (Esx proteins) (5, 7).

The region of difference 1 (RD1) is a 9.5-kbp stretch comprising 9 genes of ESX-1 that is deleted in all strains of the live tuberculosis vaccine *M. bovis* BCG (8). The importance of ESX-1 for virulence was first demonstrated by expressing the entire RD1 locus in the BCG vaccine strain and restoring virulence in the mouse model of tuberculosis (9, 10). ESX-1 is essential for full virulence of *M. tuberculosis* in mice (11–13). In *M. marinum*, it is required for the growth of bacteria within macrophages, cell-to-cell spread, and virulence in the zebrafish model (14). ESX-1 is required for the cosecretion of two ESAT-6 family proteins, EsxA and EsxB (13). The other known substrates are Rv3881c (15) and Rv3864 (16).

The ESX-5 system is the most recently evolved of all the T7SSs and is found only in slow-growing mycobacterial species (17). It is important for the secretion of the majority of members of the PE and PPE families of proteins (18, 19). The PE/PPE families are unique to mycobacteria and are found mostly in pathogenic and slow-growing mycobacteria, in which their genes expanded over time to now occupy about 10% of the *M. tuberculosis* genome (17, 20). ESX-5 mediates the suppression of secretion of host cell proinflammatory cytokines, such as tumor necrosis factor (TNF), in-

terleukin-6 (IL-6), and IL-12, after infection of macrophages with *M. marinum* (21). After bacteria escape from the phagosome into the host cell cytosol, ESX-5 is required for the induction of caspase-independent cell death in macrophages, which allows the bacteria to exit the host cell and infect new cells (22). Hence, it is not surprising that *M. tuberculosis* ESX-5-deficient mutants are attenuated in SCID mice (23) and that *M. tuberculosis* lacking the ESX-5 PE-PPE proteins is strongly attenuated in immunocompetent mice (24). However, it has been reported that an ESX-5 mutant of *M. marinum* was found to be hypervirulent in adult zebrafish (25).

In addition to their isolated location in the *M. tuberculosis* genome, one PE protein gene and one PPE protein gene are also found flanking the genes for the ESAT-6-like proteins in each ESX locus, except for the ESX-4 system (5, 17). This shows that they were most likely associated with the ESX-1 system first and then coduplicated until they further expanded independently of the ESX secretion systems (17). The genes encoding the two ESAT-6-like proteins and their flanking PE/PPE protein genes from the ESX-5 system underwent three more duplication events (17). One of the loci is found in the *M. tuberculosis* genome, from Rv1037c to Rv1040 (ESX-5a), and contains the *esxI*, *esxJ*, *ppe15*, and *pe8* genes.

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EsxI and EsxJ are themselves secreted into the extracellular medium (26). Interestingly, an EsxJ-derived peptide constitutes a CD8⁺ T-cell epitope found in human individuals with latent or active tuberculosis infections (27). The biological functions of none of the three ESX-5 duplication regions have been investigated to date.

In the present study, we characterized the role of ESX-5a (Rv1037c to Rv1040) in protein secretion, inflammasome activation, host cell death induction, and virulence during *in vivo* infections. *M. marinum* and *M. tuberculosis* ESX-5a deletion mutants did not secrete any alanine-L-dehydrogenase (ALD). The *M. marinum* ESX-5a mutant showed a partial reduction in secretion of the PE_PGRS family of proteins, which are ESX-5 substrates. Surprisingly, the mutant was also defective in the secretion of superoxide dismutase A (SodA). Furthermore, we demonstrate that the ESX-5a-dependent substrates ALD and SodA are secreted via core components of the parent ESX-5 system. The *M. tuberculosis* Δ ESX-5a mutant, like the ESX-5 mutant, showed reduced activation of the host cell inflammasome. However, unlike the ESX-5 mutant, the *M. tuberculosis* Δ ESX-5a mutant showed no difference in apoptotic and necrotic cell death induction. Finally, the *M. marinum* ESX-5a mutant showed reduced virulence in the adult zebrafish model.

MATERIALS AND METHODS

Mice. C57BL/6 wild-type (WT) mice were obtained from The Jackson Laboratory. Mice were maintained under pathogen-free conditions and used between 6 and 12 weeks of age. All studies were approved by the Institutional Care and Use Committee of The University of Maryland at College Park and were conducted in accordance with the IACUC guidelines and the National Research Council's *Guide for the Care and Use of Laboratory Animals* (28).

Cell culture. Bone marrow cells were obtained from the femurs and tibias of mice. Cells were then cultured in 100-mm dishes for 7 to 9 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), 2-mercaptoethanol (50 μ M) (all from Invitrogen), 10% heat-inactivated fetal calf serum (FCS) (HyClone), and either 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech) to generate bone marrow-derived dendritic cells (BMDcs) or 20% L929 cell supernatant (LCCM) to generate bone marrow-derived macrophages (BMDMs). The generation of BMDcs was performed as described previously (29). The human myelomonocytic cell line THP-1 (ATCC TIB-202) was cultured and differentiated using phorbol myristate acetate (PMA) (Sigma) as described previously (30).

Bacteria. Wild-type *M. marinum* strain M and an *M. marinum* Δ secA2 mutant, obtained from Lian Yong Gao (University of Maryland, College Park, MD), were cultured and maintained as described previously (31). *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv (ATCC 25618) were obtained from W. R. Jacobs, Jr. (AECOM). The *M. marinum* ESX-5 transposon (Tn) mutant, *eccA₅::Tn* (MMAR_2680/Rv1798), was kindly gifted to us by Lalita Ramakrishnan (Cambridge University, England). The gene length of *eccA₅* is 1,833 bp, while the Tn insertion site is at bp 670 (or the distance from the start site is 37%).

Generation of *M. marinum* and *M. tuberculosis* mutants. Regions flanking the *M. marinum* genes *esxP* and *esxN₂* (homologs of *M. tuberculosis* *esxI* and *esxJ*) were amplified by PCR and inserted on either side of the kanamycin resistance (Kan^r) cassette contained in a pBluescript plasmid. The left fragment was generated using primers LLFP_*esx* (GATGG TACCTCTAGACCCATCGCCGCTGGCATTGG) and LLRP_*esx* (GCA GTCGACGATTCTGTCTCTTATGTTGAAGTCCCG), and the right fragment was generated using RLFP_*esx* (GCCGACTAGTTACAAGAGC AGGACAACAGTGGC) and RLRP_*esx* (GAATGAGCTCTCTAGAGGT

AATACCGACCACCCACAC). This entire sequence was cut out from the plasmid and ligated into pLYG304.zeo. This knockout plasmid was electroporated into WT *M. marinum*, and the resulting double-crossover mutants, Δ *esxIJ* mutants, were selected as described earlier (31).

The *M. tuberculosis* Δ ESX-5a mutant was generated using a recombining approach followed by specialized transduction (32). About 400- to 600-bp flanking sequences on either side of the Rv1037c-to-Rv1040c gene region were amplified using PCR. The left fragment primers were Rvesx_LLFP (GCAACTCGAGCCGCCGTCAGGTGAT CGAATCAG) and Rvesx_LLRP (GGAGAAGCTTGCTGGCTTAAGG CCCGCGCC), and the right fragment primers were Rvesx_RLFP (GGCATCTAGAGCTGCTGTCTCTCTTGTCTCGAAGTCG) and Rvesx_RLRP (GTGAGGTACCATCCCCACCGCATATTCTAGC). The fragments were then ligated on either side of a hygromycin resistance gene on the pMSG360 plasmid. This recombinant plasmid was then linearized and electroporated into a specialized strain of *Escherichia coli* (EL350/phAE87). A recombination event between the phasmid phAE87 and the linearized plasmid created a new phasmid containing the hygromycin marker and the flanking sequences. This phasmid DNA was extracted and transformed into *M. smegmatis* to generate recombinant phage that were then used for *M. tuberculosis* transduction. The hygromycin-resistant *M. tuberculosis* colonies were picked and screened by Southern blotting to confirm the knockout. EL350/phAE87 and pMSG360 were kind gifts from Michael S. Glickman, Memorial Sloan-Kettering Cancer Center (32).

Southern blotting. Confirmation of gene deletion in the mutants was done using Southern blotting. The DNA probes for both *M. marinum* (FP, GGCAAACGCTCTCGATACC; and RP, GCCGAATCGCGGATAGAT TAC) and *M. tuberculosis* (FP, CCGGACCGAACGGGATGAAC; and RP, GTGCGTCTGGCGGCAGAAAC) mutants were biotinylated using a BrightStar psoralen-biotin nonisotopic labeling kit (Ambion) per the manufacturer's instructions. Genomic DNAs were digested using the restriction enzymes (Fermentas) PstI (Δ *esxIJ*) and BamHI (Δ ESX-5a) and run in an agarose gel. The DNAs from the gel were transferred to a nylon membrane and exposed to the biotinylated hybridization probes. The bands were detected using a BrightStar BioDetect kit (Ambion).

Complementation of mutants. Complementation of the *M. marinum* Δ *esxIJ* mutant was done by amplifying a gene fragment containing both the genes and ligating it into the plasmid pLYG206.zeo (31). This recombinant plasmid was electroporated into the Δ *esxIJ* mutant to generate the complemented strain.

Similarly, an *M. tuberculosis* fragment containing the *esxI*, *esxJ*, *ppe15*, and *ppe8* genes was amplified by PCR using the primers FP (GCACGAAT TCAGCGGCCGGCAGGTTTCAC) and RP (GACCATCGATACGGCG CGGGCCTTAAGC) and ligated into pMV261. This recombinant plasmid was electroporated into the Δ ESX-5a mutant to obtain a complemented strain.

In vitro superoxide assay. The *in vitro* superoxide assay was performed using a hypoxanthine/xanthine oxidase system (Sigma) to generate superoxides. Mid-log-phase bacteria grown in 7H9 medium were washed and adjusted to a density of 10⁶ CFU/ml. The bacteria were then exposed to superoxide, generated by combining 250 μ M hypoxanthine with 0.1 U of xanthine oxidase per ml in phosphate-buffered saline (PBS). Cell lysates (CLs) were made at 0, 1.5, and 3 h postexposure.

Cell culture and infection. Culture medium containing differentiated BMDcs in suspension was collected and centrifuged. Cell pellets were then resuspended in infection medium containing GM-CSF and seeded in 24-well plates. Bacteria were grown to optical densities at 600 nm (OD₆₀₀s) ranging from 0.5 to 0.8 and centrifuged. Each pellet was resuspended in PBS (Cellgro) supplemented with 0.05% Tween 80 and centrifuged at 80 $\times$ g to remove clumps. Infections were carried out at a multiplicity of infection (MOI) of 10:1 for 4 h in infection medium containing 10% non-heat-inactivated FCS. The extracellular bacteria were then removed by washing twice with PBS, and the cells were incubated in chase medium containing GM-CSF and 100 μ g/ml of gentamicin (Invitrogen)

for 24 h. In the case of BMDMs, the cells were seeded at least 16 h before infection in complete medium containing 20% LCCM. The cells were then washed, infection medium (without LCCM) was added, and the cells were infected at 3 h post-LCCM starvation under conditions similar to those for BMDCs, with the exception of the GM-CSF addition. THP-1 cells were seeded in 24-well plates with PMA for 18 to 22 h and then washed twice with PBS to remove the PMA. Infection medium containing 5% pooled human serum (Sigma) was added to the cells and infected with bacteria at an MOI of 3:1 for 4 h. The extracellular bacteria were washed off, and cells were incubated with chase medium containing gentamicin for 3 days.

Cell death assays. The adenylate kinase (AK) release assay Toxilight-BioAssay (Lonza) was used to quantify necrotic cell death. The assay was performed according to the manufacturer's instructions, using cell-free supernatants harvested at 1 or 3 days postinfection. The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed to reveal apoptosis-induced DNA fragmentation in THP-1 cells, using an *in situ* cell death detection kit with fluorescein (Roche Applied Science). The assay was carried out as described by the manufacturer, and the percentage of stained cells was analyzed using flow cytometry. For the detection of apoptosis in BMDMs and BMDCs, a hypodiploid stain was used. Cells were collected after infection, pelleted, and resuspended in propidium iodide (PI)-RNase buffer (BD Pharmingen) for 20 min, and the percentage of hypodiploid-positive cells was determined by flow cytometry.

Cytokine measurement. An enzyme-linked immunosorbent assay (ELISA) was used to measure secreted interleukin-1 β (IL-1 β) in cell supernatants collected from infected cells at the indicated time points, using a BD OptEIA set mouse IL-1 β kit (BD Biosciences) and a BD OptEIA set human IL-1 β kit (BD Biosciences).

Preparation of CFs and CLs. *M. marinum* culture filtrate (CF) and cell lysate (CL) preparation was done as previously described (15). *M. tuberculosis* cultures were grown in 7H9 medium to log phase, washed, and then transferred to Sauton's medium (with 0.05% Tween 80) at a starting OD of 0.05. These cultures were grown at 37°C in a shaker (~100 rpm) for 4 days (OD, ~0.8) and then passaged again in 50 ml of fresh Sauton's medium (with 0.005% Tween 80) to an initial OD of 0.05. The bacteria were allowed to grow for another 4 days and then spun down to separate the pellet and supernatant. The CF was filtered and then concentrated using Amicon Ultra centrifugal filter units (Millipore). The CL was generated by resuspending the bacterial pellet in 20 mM Tris-HCl (pH 7.6) and breaking up the cells by bead beating as described previously (15).

BMDC lysate preparation was performed as described earlier (29). To detect cleavage fragments of caspase-1 and IL-1 β , supernatants of infected cells were collected at the indicated time points and subjected to methanol-chloroform extraction. The precipitated proteins were pelleted, resuspended in reducing sample buffer, and boiled.

Western blotting. Ten-microgram aliquots of BMDC lysates were loaded onto Any KD TGX SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked using 5% dry milk for 1 h and then incubated for 1 h or overnight with the primary antibody. This was followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. The membrane was then developed using the Pico chemiluminescence substrate (Pierce) and exposed on films (GeneMate). The primary antibodies used were anti-IL-1 β (R&D Systems; AF401NA), used at 0.15 μ g/ml in 0.1% bovine serum albumin (BSA); anti-caspase-1 (Santa Cruz; sc-514), used at 1:300; and anti-tubulin (Cell Signaling; 9774), used at 1:1,000. The last two antibodies were diluted in 5% dry milk with Tris-buffered saline containing Tween 20 (TBST). The secondary antibodies used were donkey anti-goat (Jackson; 705-035-003), used at 1:25,000; goat anti-rabbit (Jackson; 111-035-003), used at 1:50,000; and goat anti-mouse (Jackson; 115-035-174), used at 1:50,000.

Bacterial CF and CL Western blotting was done as mentioned above.

The primary antibodies used were against ALD (NR-13651), Soda (NR-13810), and GroEL2 (NR-13813) (obtained from BEI Resources and used at the recommended dilutions). Rabbit polyclonal serum against fibronectin attachment protein (FAP) (33) was used at 1:8,000. The anti-PGRS antibody was kindly provided by Wilbert Bitter, VU University Medical Centre, Amsterdam, Netherlands, and used at a 1:2,000 dilution (19).

2D gel electrophoresis. One hundred micrograms of CF proteins was precipitated using a ReadyPrep 2-D cleanup kit (Bio-Rad) and dissolved in 200 μ l of rehydration buffer. This solution was loaded onto an isoelectric focusing (IEF) tray, and the first dimension was run on an 11-cm IPG strip (pH 4 to 7) placed in an IEF cell (Bio-Rad). The running conditions were as follows: rehydration for 12 h, 250 V for 20 min with a linear ramp, 8,000 V for 2.5 h with a linear ramp, and 8,000 V with a rapid ramp at 20°C. Following this, the IPG strip was placed onto a 4 to 20% gel, and the second dimension was run at 150 V for 1 h. The gel was removed, washed, stained with Bio-Safe Coomassie blue for 90 min, and destained overnight in deionized water. A GS-800 densitometer (Bio-Rad) was used to image the two-dimensional (2D) gel.

Mass spectrometry analysis. Coomassie blue-stained gel spots were analyzed according to the method of Shevchenko et al. (34), with minor modifications. Briefly, gel spots were excised with a transfer pipette and destained via three consecutive 30-min incubations at room temperature in 50:50 (vol/vol) 100 mM NH₄HCO₃-acetonitrile. After the stain was removed, the excised gel pieces were dehydrated in 100% acetonitrile and subsequently rehydrated on ice in 100 mM NH₄HCO₃ containing trypsin (Promega) at 20 ng/ μ l. The gel bands were allowed to fully rehydrate for 2 h on ice, and then buffer was added to keep the gel pieces hydrated over the course of the digestion. Proteins were digested at 37°C overnight. The resulting peptides were extracted by adding an equal volume of 5% formic acid in acetonitrile (vol/vol) and incubating them for 30 min on a shaker. The peptides were removed and lyophilized to near dryness.

Peptides were analyzed using an LTQ-Orbitrap XL (ThermoFisher) mass spectrometer coupled to a Prominence nano-liquid chromatograph (nLC) (Shimadzu). As peptides eluted from the nLC precursor, mass analysis was performed at high resolution while simultaneously fragmenting the 5 most abundant multiply charged precursors by using collision-induced dissociation. The resulting spectra were searched against a mycobacterial database by using the Mascot search engine (Matrix Science, London, United Kingdom). Precursor masses were searched with a mass tolerance of 10 ppm, while product ion masses were searched within 0.8 Da. Peptides were considered significant if they were identified with E values of <0.5.

Real-time PCR. BMDCs were harvested at 0 h and 8 h postinfection by using TRIzol (Invitrogen). Total RNAs were extracted from these fractions by using chloroform and were precipitated with isopropyl alcohol. The residual DNA was digested using Ambion Turbo DNase per the manufacturer's instructions. Real-time PCR was done using SYBR green PCR master mix (Roche), with *GAPDH* as the housekeeping gene. The samples were run on a Bio-Rad CFX96 Touch real-time PCR detection system, and the data were analyzed using CFX software (version 3.0). The primers used were as follows: for IL-1 β , 5'-AGCCTCGTGCTGTCGGACCC-3' and 5'-TGAGGCCCAAGGCCACAGGT-3'; and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-ATGGGATTTCATTGATGACA-3' and 5'-CCACCATGGCAAATTC-3'.

Survival studies using zebrafish. Zebrafish were obtained from Shaojun Du (University of Maryland, Baltimore, MD). All studies were approved by the Institutional Care and Use Committee of The University of Maryland at College Park and were conducted in accordance with the IACUC guidelines and the *Guide for the Care and Use of Laboratory Animals* (28). Prior to infection, the fish were exposed to 0.02% tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) for 2 to 3 min and then injected intraperitoneally (i.p.) with 5 to 10 μ l of an *M. marinum* suspension in PBS containing 1×10^4 bacilli. The control fish were in-

FIG 1 Confirmation of *M. marinum* Δ *esxIJ* mutant (Δ *esx-i/j*) and *M. tuberculosis* Δ ESX-5a mutant. (A) Genome organization of the ESX-5a region in *M. tuberculosis* (Mtb) and *M. marinum* (Mm) compared to the parent ESX-5 region. (B) Schematic diagram of the ESX-5a gene region deleted in *M. marinum* (left) and a Southern blot to confirm the deletion of the *esxI* and *esxJ* genes (right). (C) Schematic diagram of the ESX-5a gene region deleted in *M. tuberculosis* (left) and a Southern blot to confirm the deletion of the *esxI*, *esxJ*, *ppe15*, and *pep8* genes (right).

jected with PBS only. For survival studies, 10 fish were infected with each *M. marinum* strain.

Statistical analysis. Statistical analysis was performed on at least three independent experiments, using GraphPad Prism 5.0 software and one-way analysis of variance (ANOVA) with Tukey's posttest unless otherwise noted in the figure legends. Data shown are representative results for triplicate values, with standard deviations. The ranges of *P* values are indicated as follows: *, $0.01 < P < 0.5$; **, $0.001 < P < 0.01$; and ***, $0.0001 < P < 0.001$.

RESULTS

Generation of deletion mutants and complemented strains in the ESX-5a region of *M. marinum* and *M. tuberculosis*. The *M. marinum* homolog of the *M. tuberculosis* ESX-5a region consists of the *pep8*, *ppe15*, *esxP*, and *esxN_1* genes (*MMAR_4451* to

MMAR_4454) (Fig. 1A). The Δ *esxIJ* mutant was generated using homologous recombination by deleting both *esxP* and *esxN_1* (homologs of *M. tuberculosis* *esxI* and *esxJ*, respectively). The resulting two-gene deletion in the Δ *esxIJ* mutant was confirmed using Southern blotting (Fig. 1B) (see Materials and Methods for details). To complement the mutant, we cloned the full-length *M. marinum* *esxI* and *esxJ* genes into the pLG206.zeo plasmid. This construct was transformed into the Δ *esxIJ* mutant by electroporation to obtain the complemented strain. However, in *M. tuberculosis*, the entire ESX-5a region was deleted as described in Materials and Methods, and the deletion was confirmed by Southern blotting (Fig. 1C). The *M. tuberculosis* Δ ESX-5a mutant was complemented by expressing all four genes of the ESX-5a region in the pMV261 plasmid.

FIG 2 No *in vitro* growth defects due to ESX-5 deletion were seen. OD₆₀₀ values for *M. marinum* WT, Δ esxIJ mutant (Δ esx-ij/j), and complemented mutant (Δ esx-ij/jC) cultures grown in 7H9 growth medium (A) and Sauton's minimal medium (B) were measured over time. OD₆₀₀ values for *M. tuberculosis* WT, Δ ESX-5a mutant, and complemented mutant (Δ ESX-5aC) cultures grown in 7H9 growth medium (C) and Sauton's minimal medium (D) were measured over time. Data shown are for one representative experiment out of three.

***M. marinum* Δ esxIJ and *M. tuberculosis* Δ ESX-5a mutants do not show *in vitro* growth defects.** The wild-type (WT), Δ esxIJ, and complemented Δ esxIJ *M. marinum* cultures were grown in both 7H9 growth medium and Sauton's minimal medium, with an initial OD₆₀₀ of 0.025. The OD₆₀₀ was measured at regular intervals until the cultures reached the stationary phase at day 5. All cultures grew at similar rates, with no differences seen in either the enriched or minimal medium (Fig. 2A and B). WT *M. tuberculosis*, the Δ ESX-5a mutant, and the complemented strain were grown in 7H9 and Sauton's media. The OD₆₀₀ was measured every 24 h until the cultures reached stationary phase. There was no difference in the *in vitro* growth rates of all three *M. tuberculosis* cultures in either medium (Fig. 2C and D).

Identification of a novel secreted substrate of ESX-5a. The secretomes of *M. marinum* (Fig. 3A, top panel) and the ESX-5a mutant (the Δ esxIJ mutant) (Fig. 3A, bottom panel) were analyzed by subjecting their short-term culture filtrates (CFs) to 2D gel electrophoresis (pH 4 to 7 and 6 to 70 kDa). Based on a visual inspection, one major spot (circled in the figure) was found to be missing from the 2D gel of the mutant CF. Between multiple repeats, this spot, among others that were sometimes missing, was the only one found to be consistently absent in the *M. marinum* Δ esxIJ CF. The circled spot was subsequently identified as alanine-L-dehydrogenase (ALD) by mass spectrometry analysis, and this was further confirmed by Western blotting (Fig. 3B). To see if this

phenotype was conserved in *M. tuberculosis*, short-term CFs and CLs were collected from both WT *M. tuberculosis* and the Δ ESX-5a mutant and run on an SDS-PAGE gel. When the blots were probed with anti-ALD, no protein could be detected in the CL itself (data not shown). It has previously been shown that ALD is upregulated under various stress conditions (35, 36). Thus, the *M. tuberculosis* cultures were exposed to reactive oxygen species (ROS) for 0, 1.5, or 3 h, and cell lysates were prepared. Upon probing of the CL blots with anti-ALD, we could see some expression at 3 h in the *M. tuberculosis* lysate and, to a lesser extent, in the mutant lysate (Fig. 3C). Therefore, to conclusively show that there is an ALD secretion defect in the mutant, we overexpressed ALD in both the WT and Δ ESX-5a strains. Analysis of the Western blot shows that only WT *M. tuberculosis* secreted ALD, even though both WT *M. tuberculosis* and the Δ ESX-5a mutant expressed comparable amounts of the protein (Fig. 3D). The *M. marinum* Δ esxIJ strain is missing only the *esxI* and *esxJ* genes, while in the *M. tuberculosis* mutant, the entire ESX-5a region is deleted. However, despite our leaving the *ppe15* and *pe8* genes intact in *M. marinum*, the secretion of ALD seemed to be affected in the mutants of both species, indicating that only the two Esx proteins are involved in the actual export of ALD. Immunodetection of the cytosolic chaperone GroEL was used as a lysis control for the quality of the CF preparation, and the fibronectin attachment protein (FAP), a SecA1-dependent secreted substrate, served as a loading control

FIG 3 Secretion defects in *M. marinum* $\Delta esxIJ$ and *M. tuberculosis* $\Delta ESX-5a$ strains. (A) 2D gel electrophoresis was performed on secreted proteins from *M. marinum* (top) and the ESX-5a-deficient $\Delta esxIJ$ mutant (bottom). The encircled protein spot was consistently absent in the mutant proteome. It was collected from the *M. marinum* gel and identified as alanine-L-dehydrogenase (ALD) via mass spectrometry analysis. The 2D gel image is one representative of two independent experiments. (B) Western blot of WT *M. marinum* and the ESX-5a-deficient $\Delta esxIJ$. Culture filtrates (CFs) and bacterial cell lysates (CLs) were probed for ALD. (C) WT *M. tuberculosis* and $\Delta ESX-5a$ mutant cultures were exposed to superoxide for different times, as indicated, and the corresponding CLs were probed for ALD (top) and FAP (bottom) by Western blotting. For this particular image, an empty lane between the 1st and 2nd lanes

FIG 4 ESX-5a contributes to the secretion of PE_PGRS proteins and SodA. Western blots of the CFs and CLs of the *M. marinum* WT, $\Delta esxIJ$, and complemented strains were probed for PGRS-containing proteins, which are a subgroup of the PE family, and for SodA. Detection of GroEL was used as a quality control for the CFs, while the FAP signal served as a loading control. Images are for samples run on the same gel and developed for the same length of time, but the lane between the $\Delta esxIJ$ and $\Delta esxIJ-C$ lanes was cropped. Data shown are for one representative experiment out of three.

for the CFs. This confirmed that ESX-5a is necessary for secretion of ALD in *M. tuberculosis* and *M. marinum*.

Surprisingly, the *M. marinum* $\Delta esxIJ$ mutant was also found to be defective in the secretion of superoxide dismutase A (SodA), which was previously shown to be secreted via the SecA2 system in *M. tuberculosis* (37). We were unable to detect this secretion defect in the 2D gel, presumably because we used Coomassie blue to stain the gels and the SodA signal may have been below the detection limit. WT *M. marinum* secreted small amounts of SodA, which was completely abolished in the *M. marinum* $\Delta esxIJ$ CF. The secretion of SodA was restored in the complemented strain, confirming the requirement of ESX-5a for SodA secretion. A larger amount of secreted SodA was seen for the complemented strain than for WT *M. marinum* CF, possibly because the *esxI* and *esxJ* genes were constitutively expressed (Fig. 4).

ESX-5a contributes to secretion of ESX-5 substrates. Since the ESX-5a region has been duplicated from the ESX-5 locus, we wanted to see if the secretion of any other ESX-5 substrates was affected in the *M. marinum* $\Delta esxIJ$ mutant. When probed for the PE_PGRS proteins, the *M. marinum* mutant was partially defective in the secretion of some of these proteins (Fig. 4). The antibody used here recognizes the PGRS motif and thus identifies multiple proteins from the family. The above data indicate that the

was cropped. (D) The secreted proteins (CFs) and CLs from WT *M. tuberculosis* and the $\Delta ESX-5a$ strain, constitutively expressing ALD, were blotted for ALD, FAP, and GroEL. Detection of GroEL was used as a quality control for the CFs, while the FAP signal served as a loading control. Data shown are for one representative experiment out of three.

FIG 5 ALD is secreted via the ESX-5 system. (A) CLs and CFs were collected from WT *M. marinum* and a transposon insertion mutant of the ESX-5 ATPase (*eccA5::Tn*). The Western blots were probed for ALD, GroEL, FAP, and PE_PGRS proteins. Images are for samples run on the same gel and developed for the same length of time, but the lane between the WT and *eccA5::Tn* lanes was cropped. (B) CFs and CLs were collected from the *M. marinum* WT, *eccA5::Tn*, and Δ *secA2* strains and run on a gel. The Western blot was then probed for SodA, GroEL, and FAP. Data shown are for one representative experiment out of three.

accessory region could play a role in the secretion of ESX-5 substrates, which points to cross talk between the two loci.

Secretion of ALD and SodA requires the ESX-5 system. The ESX-5a region lacks its own secretion machinery, and since we found evidence of cross talk with its parent system, we hypothesized that the ESX-5a substrates were also exported via the ESX-5 secretion system. Cultures of the WT *M. marinum* strain and a mutant with a transposon insertion in the ATPase gene (*eccA5*) of the ESX-5 system were grown in Sauton's medium. Their short-term CFs and CLs were collected, run on an SDS-PAGE gel, and subjected to Western blotting. The blot was then probed with anti-ALD. The CL of the *eccA5::Tn* strain showed ALD protein concentrations comparable to those of the WT, yet the strain did not secrete any of it, unlike the WT (Fig. 5A), indicating the requirement of an intact ESX-5 system for ALD secretion. The absence of GroEL in the CF indicated that the supernatants were devoid of any cytosolic contamination. The blots were also probed with the anti-PGRS antibody to show that the *eccA5::Tn* strain is a functional ESX-5 mutant, as it was found to be defective in the secretion of multiple proteins from the PE_PGRS family, even though the effect was less pronounced than in the earlier reported ESX-5 mutant (19). It has also been mentioned previously (23) that *EccA5* is not thought to be a part of the core ESX-5 secretion complex, and this could explain the relatively minor defect in PE_PGRS secretion by the *eccA5::Tn* strain. It is also possible that

the *EccA5* transporter may not be involved in the export of all PE_PGRS proteins, while the mutation in MMAR_2676 (used in reference 19) might have a greater impact on the secretion of the protein family as a whole. Next, we checked for SodA secretion in the *M. marinum eccA5::Tn* and Δ *secA2* CFs. Since it has already been documented that SodA is secreted via the SecA2 system, we hypothesized that either the ESX-5a region is also able to export substrates through other secretion systems or the SecA2 accessory protein can shuttle substrates through both the ESX-5 secretion system and the Sec translocon. However, to our surprise, we were able to detect SodA in the Δ *secA2* CF but not the *eccA5::Tn* CF. Nevertheless, these results thus confirm our hypothesis that the ESX-5a substrates are secreted through the core ESX-5 secretion system.

Deletion of ESX-5a does not affect host cell death induction.

The interaction of *M. tuberculosis* with its host cell in regard to cell death induction is complex (38). It is most likely that *M. tuberculosis* inhibits cell death induction during the initial phase of replication but induces cell death later on in order to exit the host cell (2). Esx-5-deficient *M. tuberculosis* and *M. marinum* strains show less induction of host cell death than the WT strains (22). To assess the effect of ESX-5a on apoptotic cell death in macrophages, THP-1 cells were infected with *M. tuberculosis*, the Δ ESX-5a mutant, or the complemented strain for 3 days. The apoptosis rate was calculated as the percent TUNEL-positive cells by flow cytometry and expressed as the fold change with respect to WT-infected cells. Both *M. tuberculosis* and the mutant induced very similar rates of apoptotic death, while the complemented mutant induced slightly higher levels of cell death (Fig. 6A). This small increase could be attributed to the fact that the ESX-5a region is constitutively expressed in the complemented strain, which may lead to higher protein levels than those in WT *M. tuberculosis*. Death by necrosis was measured by the release of cytosolic adenylate kinase (AK) into the supernatant. Here again, the mutant or the complemented strain showed no difference in necrotic cell death compared to WT *M. tuberculosis* (Fig. 6A). Primary murine BMDMs infected with the same three strains also showed no difference in cell death induction (Fig. 6B). In this case, the apoptotic cell death rate was measured by hypodiploid staining and expressed as the fold change with respect to WT *M. tuberculosis*. In primary mouse BMDCs infected with the bacterial strains, the complemented strain induced a slightly higher apoptotic rate than those of both the WT and Δ ESX-5a strains, but there was no difference in the amounts of necrosis induced (Fig. 6C).

Inflammasome activation is reduced in bacteria lacking functional ESX-5a. The activation of the inflammasome and the secretion of mature IL-1 β by BMDMs and BMDCs after *M. tuberculosis* infection are dependent on a functional ESX-1 secretion system (29, 39, 40). In *M. marinum*, both the ESX-1 and ESX-5 secretion systems have been shown to be required for IL-1 β secretion (22). Thus, we investigated the effect of ESX-5a deletion on IL-1 β secretion in THP-1 cells, BMDMs, and BMDCs. THP-1 cells were infected with *M. tuberculosis*, the Δ ESX-5a mutant, and the complemented strain at an MOI of 3, and the supernatants were assayed for IL-1 β secretion by ELISA at 3 days postinfection. The mutant showed around a 50% reduction in cytokine secretion compared to both WT *M. tuberculosis* and the complemented strain (Fig. 7A). BMDMs and BMDCs were infected at an MOI of 10, and the cell-free supernatants were analyzed for IL-1 β secretion at 24 h postinfection. BMDCs infected with the mutant also

FIG 6 ESX-5a deletion has no effect on host cell death induction. Apoptotic cell death (blue bars; left y axis) induction by the *M. tuberculosis* WT, ΔESX-5a, and complemented (ΔESX-5aC) strains was measured in THP-1 macrophages by TUNEL assay at day 3 (A), in BMDMs by hypodiploid staining at day 1 (B), and in BMDCs by hypodiploid staining at day 1 (C), while the release of adenylate kinase into the supernatant was used as a measure of necrosis (red bars; right y axis). The data are shown as fold changes over the WT levels. Data shown are the means $\pm$ standard deviations (SD) for triplicate measurements from one representative experiment out of three. ns, not significant.

showed a 50% reduction in secretion of IL-1 β (Fig. 7C), while in BMDMs, secretion of this cytokine was completely abrogated (Fig. 7B). All supernatants were tested for release of AK as a control for contamination with cytosolic contents due to necrosis, and we found minimal induction after infection for all three strains and no differences between the strains (data not shown). Inflammasome activation is a two-signal process ultimately leading to

the production of mature inflammatory cytokines, such as IL-18 and IL-1 β . The first signal leads to the induction of pro-IL-1 β synthesis. The second is required for caspase-1 activation via the inflammasome. In order to identify at what point the ΔESX-5a mutant resulted in reduced activation, the mRNAs and cell lysates of infected BMDCs were collected to check for expression of pro-IL-1 β at both the transcriptional and translational levels. We observed that there was no difference in expression of pro-IL-1 β mRNA between the mutant and WT *M. tuberculosis* after 4 h of infection (Fig. 7D). The protein levels of pro-IL-1 β were also found to be similar (Fig. 7E). We next analyzed caspase-1 activation by blotting for activated, cleaved caspase-1 (p10) in the supernatants of infected BMDCs. The signal for activated caspase-1 was clearly visible in supernatants of both WT *M. tuberculosis*- and complemented mutant-infected cells but was reduced in the mutant-infected cell supernatants (Fig. 7E). In conclusion, the absence of ESX-5a proteins reduces the generation of mature IL-1 β via reduced activation of the inflammasome, leading to the generation of less active caspase-1.

ESX-5a contributes to virulence of *M. marinum* in zebrafish.

Zebrafish are an *in vivo* model for *M. marinum* infections and have been used extensively to understand mycobacterial pathogenicity mechanisms that could be translated to the human pathogen *M. tuberculosis* (41, 42). To determine the effect of the ESX-5a region on virulence of mycobacteria as indicated by survival, zebrafish were injected i.p. with PBS, WT *M. marinum*, the Δ*esxIJ* mutant, and the complemented strain at a CFU of 10^4 /fish and monitored for up to 80 days. There were 10 fish per experimental group, and the PBS-injected fish were kept as a control group to negate the possibility of death due to needle injuries. Most of the WT *M. marinum*- and complemented mutant-infected fish died within 3 to 5 weeks, whereas the bulk of the Δ*esxIJ* mutant-infected fish started dying only after 5 weeks (Fig. 8). This shows that the absence of the ESX-5a proteins significantly reduced the virulence of the bacteria in zebrafish.

DISCUSSION

We demonstrate here that ESX-5a deletion partially affects the secretion of some ESX-5 system substrates, such as the PE_PGRS proteins (Fig. 4). Since the ESX-5a region does not include genes that code for a core secretory machinery, such as a channel protein, it must utilize another secretion system for protein export. This is supported by our findings that there is an overlap in some of the secretion substrates between the ESX-5 and ESX-5a systems. Additionally, it is indicated that members of the *M. tuberculosis* 9.9 family of proteins, which include EsxN (encoded in the parent ESX-5 region) and EsxI (encoded in the ESX-5a region), are secreted by the ESX-5 system (23). Our results showed that an *M. marinum* transposon mutant in the AAA ATPase (EccA₅) is deficient in the secretion of ALD and SodA (Fig. 5A and B), as is the *M. marinum* ESX-5a deletion mutant (Δ*esxIJ*) (Fig. 3A and B and 4). This was surprising, because SodA was previously shown to be a SecA2 system-dependent substrate in *M. tuberculosis* (37), but we found that the *M. marinum* Δ*secA2* strain was not defective in SodA secretion (Fig. 5B). This discrepancy could be due to inherent differences in protein secretion mechanisms between *M. marinum* and *M. tuberculosis* during adaptation to their respective hosts. Interestingly, it has been shown that complementing an *M. marinum* strain lacking the entire ESX-5 region with the *M. tuberculosis* ESX-5 region is not able to completely restore PE_PGRS

FIG 7 ESX-5a deletion in *M. tuberculosis* reduces host cell inflammasome activation and secretion of IL-1 β . IL-1 β ELISAs were performed on supernatants from cells infected with WT *M. tuberculosis*, the Δ ESX-5a mutant, or the complemented strain (Δ ESX-5aC) in THP-1 cells at day 3 postinfection (A) or in mouse BMDMs (B) or mouse BMDCs (C) at 24 h. (D) The mRNA levels of pro-IL-1 β were measured in BMDCs infected with *M. tuberculosis*, the ESX-5a mutant (Δ ESX-5a), or the complemented strain right after the 4-h infection period by using quantitative reverse transcription-PCR (RT-PCR). (E) Western blots of supernatants (SN) collected at 6 h postinfection, showing the active fragments of caspase-1 (10 kDa) and IL-1 β (17 kDa). The corresponding CLs were probed for pro-caspase-1 (45 kDa) and pro-IL-1 β (35 kDa). Tubulin (55 kDa) served as the loading control. Data are shown as means $\pm$ SD for triplicate measurements from one representative experiment out of three.

protein secretion. This indicates that there is a variation in substrate recognition between the ESX-5 regions of the two mycobacterial species (43). However, we demonstrated that the secretion of ALD in both *M. marinum* and *M. tuberculosis* is dependent on the ESX-5a region. We also demonstrated that deletion of the ESX-5a locus abrogates the secretion of SodA and that this defect

is reversed in the complemented strain (Fig. 4). The reduction of SodA secretion was not seen in our analysis of the *M. marinum* secretome via 2D gel electrophoresis (Fig. 3A), which is probably because the amount of secreted protein in wild-type *M. marinum* is minimal. Our results support the hypothesis that the ESX-5 gene region encodes the core secretion machinery, whereas the

FIG 8 Virulence defect of the *M. marinum* ESX-5a mutant. Adult zebrafish were injected i.p. with 10^4 bacteria per fish of WT *M. marinum* (WT Mm), the *M. marinum* ESX-5a-deficient $\Delta esx I/J$ mutant ($\Delta esx I/J$), or the complemented mutant strain ($\Delta esx I/J$ C) and monitored for survival. There were 10 fish per group ($n = 10$), with saline (PBS)-injected fish as a control group. Data are shown as means $\pm$ SD for triplicate measurements from one representative experiment out of three. Statistical analysis was performed using the log rank test.

ESX-5a region is important for proteins chaperoning a subset of ESX-5 secreted proteins. This model is also supported by recent findings that deletion of the *esxM* and *esxN* genes of the parental ESX-5 system did not affect secretion of PPE41, whereas deletion of the ESX-5 channel gene *eccD5* did (23). This is fundamentally different from the ESX-1 system, in which case the deletion of *esxA* and *esxB* abolished secretion of other ESX-1 substrates (5, 15, 44, 45). Hence, we propose a model in which the ESX-5 gene system encodes the core secretion machinery, whereas ESX-5a is important for chaperoning a subset of ESX-5 secreted proteins. Furthermore, this also suggests that all three ESX-5 duplicated regions, Rv1195 to Rv1198, Rv1037c to Rv1040c, and Rv3619c to Rv3622c, could potentially play roles in protein secretion, in conjunction with the parental ESX-5 core secretion components, and that all these different loci contribute to the overall secretory functioning of the ESX-5 system together with the EsxMN proteins encoded by the parental ESX-5 region.

M. tuberculosis encodes 168 members of the PE/PPE family of proteins in its genome, which accounts to about 10% of the total genome. The functions of these proteins are not well understood. Their expression was hypothesized to provide antigenic variability in order to favor evasion of the host immune response. One problem with the study of individual members of a large protein family is the high chance of redundancy; thus, loss-of-function genetic approaches are often futile. Our results led to the hypothesis that the ESAT-6 duplicate regions of ESX-5 are important for selection of substrates to be secreted by ESX-5. Thus, by deleting each of the three duplicated regions and determining the phenotype of the mutant and its secretion defects, it should be possible to attribute a function to a smaller subset of the PE/PPE protein family. For example, the *M. tuberculosis* ESX-5a mutant has no cell death phenotype (Fig. 6), which is different from an ESX-5 mutant, and therefore we can conclude that the *M. tuberculosis* ESX-5a substrates are not involved in cell death induction. This approach may help in characterizing the functions of various PE/PPE proteins.

The molecular mechanism of substrate selection for type VII secretion systems is a matter of great interest and intense research efforts. Recently, a short peptide motif (YXXXD/E) in the C termini of proteins was identified as a general signal for type VII secretion (46). However, this signal does not determine ESX specificity, since exchanging it on ESX-1 and ESX-5 substrates did not

change their respective secretion pathways (46). Another characteristic of ESX and PE/PPE secretion substrates is the formation of heterodimers, which are essential for successful secretion and result in the generation of a characteristic four-helix bundle (5). These characteristics have been used to develop an algorithm to predict novel T7SS substrates (46). Interestingly, SodA has two YXXXD/E motifs in the N terminus, while ALD does not have the structural features or a YXXXD/E motif which would identify it as a T7SS substrate. Consequently, it is possible that the selection of secretion substrates by the accessory regions requires other distinguishing features that still remain to be identified. Therefore, by determining a specific feature or sequence motif, we may be able to identify other unknown T7SS substrates. However, this task may be complicated by the fact that these T7SS substrates may be highly expressed in *M. tuberculosis* only after infection of host cells, similar to ALD, which makes it impossible to identify them using standard *ex vivo* proteomic approaches.

M. tuberculosis infection activates the host cell inflammasome, which results in the generation of mature secreted IL-1 β . The active inflammasome consists of a variable sensing component (most often a nucleotide-binding and oligomerization domain, leucine-rich-repeat-containing protein, or NLR), the adaptor protein ASC, and pro-caspase-1 (47, 48). In the case of *M. tuberculosis* infections of macrophages or dendritic cells, only the NLRP3 inflammasome is activated (29, 49–55). There are several agonists that induce activation of the NLRP3 inflammasome, such as an increase in ROS, nigericin, ATP, bacterial toxins, and particulate matter (56–58). All of these agonists induce K⁺ efflux, which is the common trigger for the NLRP3 inflammasome (58). A central role was recently attributed to the mitochondria because, during mitochondrial dysfunction, cardiolipin is relocated to the outer mitochondrial membrane, where it can bind to and activate NLRP3 (57). Here we demonstrated that the *M. tuberculosis* Δ ESX-5a mutant does not induce NLRP3 inflammasome activation as efficiently as the wild-type bacterium does. We showed that this defect is not due to a lack of signaling to induce pro-IL-1 β or pro-caspase-1 transcription or translation (Fig. 7D and E) but because of the disruption of activation of the inflammasome (Fig. 7E). In the absence of either the ESX-1 or ESX-5 system, there is a reduction of mature IL-1 β secretion into the supernatant by the host cells (13, 22, 29, 40). It is thus somewhat surprising that deletion of the ESX-5a region also has such a strong effect on inflammasome activation. One interpretation of the results is that the reduced inflammasome activation observed with the ESX-5 mutant is actually due to a lack of secreted substrates dependent on the ESX-5a region, and hence, the deletion of ESX-5a has an effect similar to that of ESX-5 deletion. It seems unlikely that the inflammasome phenotype of the *M. tuberculosis* ESX-5a mutant is linked to cell death induction as reported for the *M. marinum* ESX-5 mutant (22), since cell death levels were not affected by the ESX-5a deletion in *M. tuberculosis* (Fig. 6).

The appearance of the ESX-5 system in the genomes of slow-growing mycobacteria demarcates them from the fast-growing mycobacterial species (17). Most of its secreted substrates are from the PE/PPE protein family, which is considerably expanded in the slow-growing species (17, 19, 23). Since all of the pathogenic mycobacterial species adapted to infection of a host are also slow-growing species, this correlation suggests that the ESX-5 system is involved in virulence. Indeed, the *M. tuberculosis* ESX-5 deletion mutants were attenuated upon *in vitro* infection of macrophages

and *in vivo* infections using the mouse model (23, 24). Our study showed that the *M. marinum* ESX-5a mutant was attenuated in the adult zebrafish model compared to both wild-type *M. marinum* and the complemented mutant strain (Fig. 8). Our results, however, contrast with a previous report showing an *M. marinum* ESX-5 deletion mutant to be slightly attenuated in zebrafish embryos but hypervirulent in adult zebrafish (25). This difference could be due to the type of *M. marinum* strain used. The authors of the previous report used the *M. marinum* strain E11, which causes a chronic infection in zebrafish, while our study was conducted using strain M, which causes an acute infection of fish (the differences in zebrafish pathology caused by the various *M. marinum* strains are demonstrated in reference 59). Another possibility is that the hypervirulence of the *M. marinum* ESX-5 mutant is caused by its inability to secrete certain proteins that could dampen the host immune responses and that these substrates are secreted independently of the ESX-5a region. Nonetheless, our result indicates that the substrates of this accessory ESX-5 secretion system are necessary for full virulence of *M. marinum*, although it remains to be seen what impact the ESX-5a region has on the virulence of *M. tuberculosis*. The transcription of two genes of the ESX-5a region (*esxI* and *esxJ*) is highly upregulated in bacteria isolated from human tuberculosis patients or from the lungs of infected mice (60), while SodA is required for detoxifying reactive oxygen species (37). Furthermore, it has been shown that *M. tuberculosis* ALD has a dual function, acting as both an alanine dehydrogenase and a glycine dehydrogenase (61). As a result, there have been wide-ranging suggestions about its actual function. Recently, it was reported that *M. tuberculosis* secretes an asparaginase, Ansa, to hydrolyze asparagine and generate ammonium ions, which can then block phagosomal acidification (62). This protein was shown to partially depend on the ESX-5 secretion system for export. It would be interesting to see if secreted ALD has a complementary role to play, particularly because the relative quantities of certain amino acids, such as alanine, are found to increase in granulomatous lesions (63, 64). Altogether, our findings point toward an important function of the three replicate ESAT-6 regions of ESX-5 in protein secretion and virulence of mycobacteria.

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