Direct Detection of Bacterial Protein Secretion Using Whole Colony Proteomics*[S]

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Bacteria use a variety of secretion systems to transport proteins beyond their cell membrane to interact with their environment. For bacterial pathogens, these systems are key virulence determinants that transport bacterial proteins into host cells. Genetic screens to identify bacterial genes required for export have relied on enzymatic or fluorescent reporters fused to known substrates to monitor secretion. However, they cannot be used in analysis of all secretion systems, limiting the implementation across bacteria. Here, we introduce the first application of a modified form of whole colony MALDI-TOF MS to directly detect protein secretion from intact bacterial colonies. We show that this method is able to specifically monitor the ESX-1 system protein secretion system, a major virulence determinant in both mycobacterial and Gram-positive pathogens that is refractory to reporter analysis. We validate the use of this technology as a high throughput screening tool by identifying an ESAT-6 system 1-deficient mutant from a *Mycobacterium marinum* transposon insertion library. Furthermore, we also demonstrate detection of secreted proteins of the prevalent type III secretion system from the Gram-negative pathogen, *Pseudomonas aeruginosa.* This method will be broadly applicable to study other bacterial protein export systems and for the identification of compounds that inhibit bacterial protein secretion. *Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.017533, 596–604, 2012.*

Bacterial secretion systems play important roles in protein localization, bacterial motility conjugation, and pathogenesis (1–5). For pathogens these systems export proteins that mediate virulence in the host. Genetic screens are a powerful tool for understanding the molecular details of bacterial protein secretion. Traditionally, screens have relied on enzymatic or fluorescent reporters fused to known substrates to monitor secretion from cells grown on solid media. However, some proteins that are exported by bacterial protein secretion systems are not amenable to fusion with reporter constructs, making the power of genetic screens inaccessible for these systems.

Whole colony MALDI-TOF is a proteomics application that has been used previously for microorganism speciation and identification (6–10). Hettick *et al.* (11, 12) used this approach to identify mycobacterial species from crude colony lysates. The application of "top-down" (the identification of intact small proteins without proteolytic digestion) processing to whole colony mass spectrometry demonstrated that bacterial proteins are detectable from whole bacterial colony lysates (13). We sought to design an assay to directly measure bacterial protein secretion that would not result in bacterial lysis and would be adaptable to high throughput applications. Here, we report the first application of a modified form of whole colony MALDI-TOF MS to directly and specifically detect protein secretion from whole bacterial colonies.

The ESX-1[1] secretion system (also known as type VII secretion) is a major bacterial virulence determinant and is conserved and functional in important mycobacterial and Gram-positive human pathogens including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Bacillus anthracis* (14–19). In these pathogens, ESX-1 has been shown to export virulence factors that modulate the macrophage response to infection (15, 16, 19, 20). Indeed, ESX-1 mutants are attenuated in macrophages, mice, and zebrafish (15–17, 19, 21, 22). The ESX-1 system is also conserved in nonpathogenic mycobacteria and is thought to be involved in conjugation (23–25).

The mycobacterial ESX-1 system is the most well defined system at the molecular level. In pathogenic mycobacteria, eight ESX-1 substrates have been identified, including the two major substrates ESAT-6 (early secreted antigen, 6 kDa) and CFP-10 (culture filtrate protein, 10 kDa) (17, 18, 26–30). How these substrates contribute to virulence is unknown. However, ESAT-6 is thought to promote membrane lysis, pointing to a potential role for ESX-1 in signaling from the phagosome (18, 31, 32). There are 12 designated components of the ESX-1 machine that are required for export of ESAT-6, CFP-10, or additional substrates (17, 19, 21, 26–28, 30, 33–35).

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[1] The abbreviations used are: ESX-1, ESAT-6 system 1; ESAT-6, early secreted antigen, 6 kDa; CFP-10, culture filtrate protein, 10 kDa; Tn, transposon; WT, wild type; RD1, Region of Difference 1; sRBC, sheep red blood cell; FDR, false discovery rate.

Although the ESX-1 substrates are exported from the bacterial cytosol through the cell envelope, it is unclear which components are involved in translocation across the bacterial membrane and mycolate layer.

Type VII secretion systems are not amenable to fusion with reporter constructs. ESX-1 substrates fused to reporter enzymes or fluorescent proteins are not generally exported. A recent example demonstrating this using the Tn′bla_{TEM-1} (transposon bearing a β-lactamase reporter) selection was reported for proteins secreted by *M. tuberculosis* (36). Although this study identified 111 secreted proteins from *M. tuberculosis*, ESX-1 substrates were notably missing. Direct fusion of this reporter to ESAT-6 resulted in retention of ESAT-6 in the mycobacterial cell (36). As such, there has never been a direct saturating screen to identify components of the ESX-1 secretion system, and there are likely additional components that remain undiscovered. *Mycobacterium marinum* is a mycobacterial species that is closely related to *M. tuberculosis.* The ESX-1 system is conserved and functional in *M. marinum* (21, 22, 29). Top-down whole colony mass spectrometry methods preferentially identify small, abundant proteins, properties characteristic of the two major ESX-1 substrates, ESAT-6 and CFP-10. We therefore applied MALDI-TOF mass spectrometry to directly monitor ESX-1 protein secretion from whole intact *M. marinum* colonies and tested its utility in studying protein secretion from other bacterial pathogens.

EXPERIMENTAL PROCEDURES

Growth of Bacterial Strains—*M. marinum* were grown as described (27). The *M. marinum* M strain, the *EccCb*::TnKan, *EspB*::TnKan, and ΔRD1 strains (21, 22, 26) were obtained from Dr. Eric J. Brown. Strains were grown at 30 °C and maintained on Middlebrook 7H11 agar (Accumedia, Lansing, MI), supplemented when appropriate with kanamycin (20 μg/ml; IBI, Poesta, IL). Sauton's medium was previously demonstrated to induce ESX-1 export *in vitro* (19). Sauton's agar was made by adding 10 g of agar (Accumedia) to 1 liter of Sauton's medium, which was made as previously described (19). The 6B10 strain bears a transposon insertion in the *eccB1* gene (MMAR_5444) between residues 6584232 and 6584233 in the *M. marinum* genome, in reverse orientation.

Pseudomonas aeruginosa PA103, *esxA*::Ω, and Δ*exsD*, *pscC*::Ω strains (37) were obtained from Dr. Timothy Yahr. Strains were grown at 30 °C on either lysogeny broth agar (Fisher) or lysogeny broth agar containing 5 mM EGTA (Fisher) and 20 mM $MgCl_2$ (Alfa Aesar, Ward Hill, MA) for induction of the type III secretion system as reported in McCaw *et al.* (37). The strains bear a P*exsD-lacZ*$^+$ transcriptional reporter integrated at a phage attachment site. Induction was confirmed by growth of the *P. aeruginosa* strains on lysogeny broth agar or lysogeny broth agar with EGTA and $MgCl_2$ in the presence of 5-bromo-4-chloro-indolyl-galactopyranoside (X-gal; RPI, Mount Prospect, IL) (data not shown).

Construction of M. marinum Transposon Library—The *M. marinum* transposon library was constructed using pMB272 (38) as described previously with the following modifications. First, the incubation temperature for selection against the plasmid (because of the presence of a temperature-sensitive origin) was increased to 32 °C. Second, we included a second round of purification on 7H11 agar plates supplemented with 10% sucrose and kanamycin. Finally, we tested transposon-bearing strains for sensitivity to hygromycin (50 μg/ml; InvivoGen, San Diego, CA) and resistance to kanamycin, which demonstrates loss of the plasmid and retention of the transposon. Approximately 400 colonies were screened using the MALDI assay described below from a 96-well arrayed nonsaturating *M. marinum* library.

Development of MALDI-TOF Assay—Individual *M. marinum* colonies were removed from Sauton's agar plates at 8 days post inoculation. The colonies were either deposited into a 1.5-ml tube (Eppendorf, Westbury, NY) (see Fig. 1) or a 300-μl 96-well 0.22-μm filter plate (Millipore, Temecula, CA). 75 μl of MS grade H_2O (Fisher) with 0.04% Tween 80 (Fisher) was added, sealed, and vortexed for 6 s. The plates/tubes were centrifuged at 1200/6000 rpm for 12 min, and filtrates were collected into a clean 96-well plate (see Fig. 2). The top row of each plate series was WT, ΔRD1, and *espB*::TnKan for normalization of ESAT-6 and CFP-10 signal yield. Several blank (no growth) colonies were selected per plate to serve as cross-well/plate contamination controls. Following centrifugation, the plates were stored at 4 °C until analysis (typically $<$1 h).

Colonies of *Pseudomonas* were picked from lysogeny broth plates under inducing or noninducing conditions for type III secretion as described above. The colonies were washed in 100 μl of PBS, pH 7.5, and filtered to remove cells. The filtrate was precipitated and desalted with 700 μl of ice-cold acetone (Sigma-Aldrich). Colony precipitate was resuspended into 20 μl of water and spotted onto a MALDI target as described.

MALDI-TOF Analysis—1 μl of each colony filtrate was spotted onto a 384-well Gold MALDI target (Bruker Daltronics, Billerica, MA) using the dried overlay method and allowed to evaporate under vacuum. 1 μl of matrix, saturated sinapinic acid (Sigma-Aldrich) in 50% acetonitrile, 0.1% TFA was deposited onto each spot and allowed to evaporate. Plate calibration was provided externally using a cal-mix of myoglobin and insulin spotted periodically on the target. MALDI-TOF spectra were acquired on an Autoflex III MALDI-rTOF instrument running in linear mode. Linear calibration was performed on the insulin $[M + H]^+$ at M_{av} = 5734.5 *m/z* and the myoglobin $[M + H]^+$ $[M + 2H]^{2+}$ ions at M_{av} = 16,952.3 and 8476.6 *m/z*, respectively. Source voltage 1 (IS1) was set to 20kV, (IS2) was 18 kV, Lens voltage was 7.3 kV. Pulsed ion extraction delay was 300 ns, a 4000 *m/z* deflection gate was used and a mass range of 4000–20000 *m/z* was acquired at a digitizer setting of 1GS/s. 50 mV was used as the full scale bias on the detector. Laser fluence varied with sample quality and age but was generally ~25% of full-scale. An offset of 73% and a range of 20% were used. 1000–2000 shots at 100 Hz were summed for each spot acquired; one sample was analyzed approximately every 15 s. Automatic spot acquisition was performed using Flex Analysis (Bruker Daltronics) using a random walk pattern with no criteria to pass, and all of the spectra were saved to disk. Colony MS from the *P. aeruginosa* were acquired in linear mode with the following modifications. Linear Calibration was performed using a mixture of BSA and Myoglobin. BSA $[M + H]^+$ M_{av} = 66,382*m/z*, $[M + 2H]2^+$ M_{av} = 33,191 *m/z*. A pulsed ion extraction delay was 60 nS, and 10,000 *m/z* deflection gate was used. 2000–3000 shots were summed per spectra at 25Hz; one sample was analyzed approximately every 2 min.

MALDI-TOF Normalization—MALDI-TOF/MS spectra were screened using a two-pass protocol. First, the presence and absence of ESAT-6/CFP-10 peaks for secretion was used to score colonies. A second pass comparing the normalized ratio of ESAT-6/CFP-10 peaks to ubiquitous background peaks ~6–8000 *m/z versus* those observed in WT and known ESX-1 mutants. Spectra passing these criteria were manually evaluated and used to direct further molecular analysis (see "ESX-1 secretion assays/sRBC lysis assay" below). The MALDI response of each plate is normalized to the average of several

WT, ΔRD1, and *espB*::*TnKan* colonies picked per plate to correct for instrument performance, laser fluence etc. Our data are then normalized per spectrum to the peaks present in Fig. 1 at 8261 and 8490 *m/z*, respectively. These peaks appear to be present in all spectra, and their intensity is in general proportional to total protein. We further tested normalization against other peaks, matrix adducts, total protein (Micro BCA see "Protein Quantification" below), and myoglobin doped into each spot (data not shown). For these data, all gave a similar response. Because we were only scoring for the presence or absence of ESAT-6 and CFP-10, this experimentally works to elucidate mutations in ESX-1, with sufficient correction of common sample, and MALDI spot-spot variability.

LC/MS/MS—Colony washates from *M. marinum* or *P. aeruginosa* were digested and analyzed as previously described (27). Briefly, the samples were denatured with 2,2,2 trifluoroethanol, reduced with DTT, alkylated with iodoacetamide (Sigma-Aldrich), and digested with sequencing grade trypsin (Promega, Fitchburg, WI). The samples were cleaned with a C18 ZipTip (Millipore), dried, and resuspended in 12 μl of 1% formic acid. LC/MS/MS was performed on a 100-μm × 100-mm C18 BEH column (Waters, Milford, MA) coupled to a two-dimensional Nano Ultra High-Performance Liquid Chromatography (Eksigent, Dublin, CA) system running at 600 nl/min. A 60-min gradient to 30% ACN was used. MS and MS/MS analysis was performed on a QTrap5500 (AB Sciex, Foster City, CA) running a TOP8 data-dependent method. Peak lists were generated with Protein Pilot (AB-Sciex) 4.0.8085 Rev 148085 and searched using the Paragon algorithm within the software (4.0.0.0 148083). Enzyme specificity was trypsin, two missed cleavages are allowed, and search was performed with carboxyamidomethylation of Cys as the only fixed modification with variable modification of carboxyamidomethylation at Lys and peptide/protein N termini. Further variable modifications not utilized in this study are searched automatically by Paragon, the details of which are outlined in Ref. 39. Mass tolerance of precursors was set to those of an ion trap instrument, which typically includes ±0.3 atomic mass unit including the possibility of precursors falling within the isolation range (~0.7 atomic mass unit). A current FASTA (June 1, 2011) of *M. marinum* download from Marinolist (45) or NCBI, respectively (November 1, 2011) (combined with an in-house contaminant list; approximately 500 entries), was searched. This combined protein list contained 11,158 protein entries. *P. aeruginosa* PA01 FASTA was downloaded November 20, 2011 from NCBI (ftp://ftp.ncbi.nlm.nih-.gov/genomes/Bacteria) combined with the in-house contaminant list and contained 11,460 protein entries. Individual MS/MS spectra (supplemental materials) were considered if their individual confidence values exceeded 95%. This threshold is actually substantially more strict than the 1% false discovery rate (FDR) for peptides which included 2322 peptides at 1% FDR for the *P. aeruginosa* WT induced cells. The only post-translational modification of note was the determination of acetylation of the protein N terminus of ESAT-6. This was made by enabling Paragon to consider N-terminal acetylation as a potential N-terminal protein modification at a low frequency (~2–3%). This is consistent with previous reports and our published work (27, 40–42). False positive estimation was made using the Elias method (43, 44) and all described proteins (ESAT6, CFP10, and PopD) were below the 1% FDR (all at 0% measured FDR). Because we were primarily concerned with just three proteins: ESAT6, CFP10, and PopD, the annotated spectra are included in the supplemental materials. The description of these protein identifications are shown in Table I (also in the supplemental materials). Raw data files from these analyses are available through the Peptide Atlas http://www-.peptideatlas.org/PASS/PASS00047 (49), FDR calculations, other parameters, and protein alignments are included. These were generated using the "Protein Alignment Template" v2.0000p (ABSciex), the results of which are in the supplemental materials.

Protein Quantification—MicroBCA (Pierce) was performed according to the manufacturer's instructions, on filter eluate used in the MALDI assays. The BCA reaction measures protein concentration via a polypeptide assisted reaction of Cu^{+} with bicinchoninic acid read as a shift in $\lambda_{562\ nm}$. Absorbance at $\lambda_{562\ nm}$ was read on a SpectraMax Plate reader (Molecular Devices, Sunnyvale, CA) using built-in protocols. Calibration was performed against a linear curve of a serial dilution of bovine albumin in the same buffer used for MALDI extraction.

ESX-1 Secretion Assays—*M. marinum* secretion assays were performed as described previously (27). Briefly, strains were grown to stationary phase (A_{600} 1.5–2.0) in 50 ml of 7H9 liquid medium and diluted to an A_{600} of 0.8 in 50 ml of Sauton's liquid medium with 0.005% Tween 80. After 48 h of growth at 30 °C, the pellet and supernatant fractions were prepared as previously described (27). Western blot analysis of ESX-1 secretion was performed as described previously (27, 40), with the following modifications (see Fig. 3). Following incubation with primary antibody, the nitrocellulose was washed with PBS with 0.1% Tween 20 (PBS-T) five times. The blots were incubated with antibodies against either ESAT-6, CFP-10, Mpt32, or GroEL as described previously (27, 40). The membranes were incubated with DyLight anti-rabbit IgG (680 Conjugate) and anti-mouse IgG (800 conjugate) (Cell Signaling Technologies, Danvers, MA). Following incubation with secondary antibody, the blots were washed four times in PBS-T, followed by a single wash in PBS. The blots were imaged using a LI-COR Odyssey Imager and analyzed with LI-COR Odyssey Software. The Western blot in Fig. 1*a* was developed using chemiluminescence as described previously (27, 40).

Red Blood Cell Lysis Assay—sRBC (Fisher) lysis assays were performed as described previously (21), with the following modifications. Following three washes with PBS, *M. marinum* pellets were resuspended in 200 μl of PBS, mixed with 100 μl of sRBCs, centrifuged, and incubated for 2 h at 32 °C. All of the readings were performed in duplicate. Each assay was repeated with three biological replicates. The data provided here are representative of the three replicates. The error bars represent standard deviation.

Identification of Transposon Insertion—Genomic DNA was isolated from transposon bearing strains as described previously (19) and digested with restriction enzymes following manufacturer's recommendations (New England Biolabs, Ipswich, MA). The resulting digestion mixture was then introduced into pBluescript SK+ (Stratagene/Agilent, Wilmington, DE) and transformed into *Escherichia coli* DH5α. Transformants were selected for growth on agar containing ampicillin (200 μg/ml; Fisher) and then replica-plated to agar containing kanamycin (50 μg/ml). Resulting colonies were purified on agar containing kanamycin, and plasmids were extracted using the *AccuPrep* DNA extraction kit (Bioneer, Alameda, CA). Sequencing was performed using transposon-specific primers (38). Capillary sequencing was performed using Big Dye Terminator V3 on an ABI 3730xl in the Notre Dame Genomics Core Facility following the manufacturer's instructions. The point of insertion of each transposon was identified by analyzing the resulting sequences using BLAST and MarinoList (45).

RESULTS

Specific Detection of ESX-1 Substrate Secretion from Whole Mycobacterial Colonies—*M. marinum* is a Biosafety Level 2 pathogenic mycobacterial species that is closely related to the *M. tuberculosis* complex (46). It is well established that the ESX-1 system is conserved and functional in *M. marinum* (21, 22, 29). ESX-1 genes from *M. tuberculosis* complement *M. marinum* strains mutant for ESX-1 function, and vice versa (29). Previous studies using whole colony MALDI-TOF

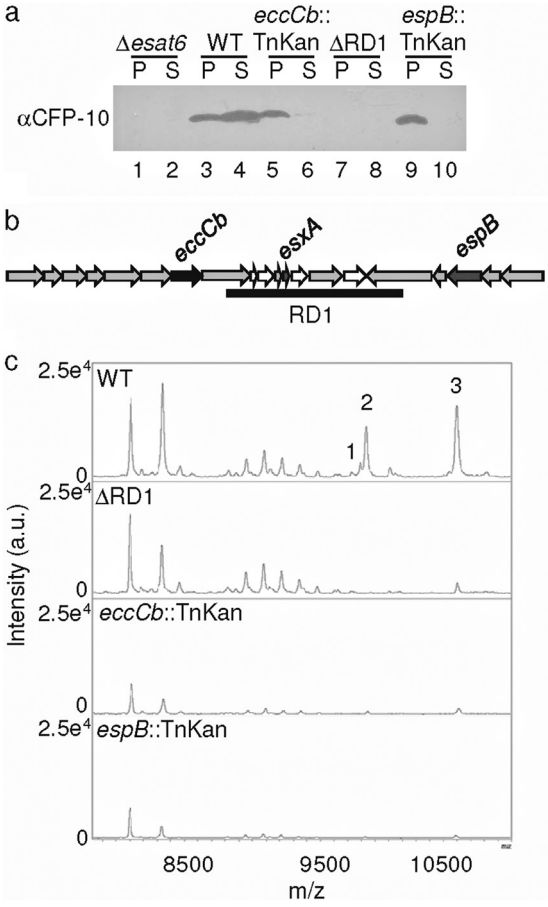

FIG. 1. **Direct detection of ESX-1 protein secretion.** *a*, Western blot analysis demonstrating the production (*P*, pellet) and secretion (*S*, supernatant) of CFP-10 in the wild-type *M. marinum* strain (*lanes 3* and *4*). CFP-10 is produced in but not secreted from strains bearing mutations in *eccCb* (*lanes 5* and *6*) and *espB* (*lanes 9* and *10*). CFP-10 is not detectable in strains bearing a deletion in *esxA* (*lanes 1* and *2*) or in RD1 (*lanes 5* and *6*). *b*, schematic of the ESX-1 locus in *M. marinum*. The genes covered by the RD1 deletion are indicated by the *line* below the genes. The labeled genes (*black*) specify strains used in this investigation. *c*, MALDI-TOF spectra obtained from wild-type *M. marinum* M (*WT*), *M. marinum* bearing a deletion in RD1 (Δ*RD1*), *eccCb*::TnKan, and *espB*::TnKan. Peaks labeled *1*, *2*, and *3* correspond to ESAT-6 (9913 Da), acetylated ESAT-6 (9960 Da), and CFP-10 (10609 Da).

mass spectrometry in mycobacteria demonstrated that these methods do not always kill the mycobacterial colonies, making application of this method to Biosafety Level 3 organisms difficult (11). We therefore used *M. marinum* to establish this method for studying ESX-1 protein secretion.

ESX-1 protein secretion is typically measured during growth of bacteria in broth cultures using fractionation to isolate the secreted proteins and Western blot analysis or proteomics to identify the presence of secreted proteins (Fig. 1*a*) (19, 27). In *M. marinum*, this requires growth to near saturation in 7H9 liquid broth, followed by subculturing to Sauton's liquid broth for 48 h. Under these growth conditions, we are able to detect secretion of the known ESX-1 substrate, CFP-10, by Western blot analysis (Fig. 1*a*, *lanes 3* and *4*). Detection of CFP-10 in the supernatant is abrogated when genes associated with protein secretion (components or substrates) are disrupted or deleted (Fig. 1a, *lanes 1*, *2*, and *5–10*). For example, strains bearing transposon insertions in the genes encoding for either an ATPase component of the secretion system, EccCb, or a substrate of the machine, EspB, result in retention of ESAT-6 and CFP-10 in the bacterial cell (Fig. 1, *a*, *lanes 5*, *6*, *9*, and *10*, and *b*) (19, 29). Moreover, CFP-10 is absent from the *M. marinum* strain lacking the RD1 region. RD1 includes the *esxB* gene, which encodes for CFP-10, as well as additional genes required for ESX-1 secretion (Fig. 1, *a*, *lanes 7* and *8*, and *b*) (17–19). However, this method is not easily adapted to a rapid high throughput screen for the identification of novel ESX-1 associated genes.

We sought to devise a method that can be translated to a high throughput assay to allow for genetic screens. To validate this approach, we applied this method to the direct detection of the two most abundant ESX-1 substrates, ESAT-6 and CFP-10. We evaluated whether we could use MALDI-TOF MS to detect ESAT-6 and CFP-10 from the surface of wild-type *M. marinum* colonies grown on agar permissive for ESX-1 protein secretion. As shown in Fig. 1*c*, we specifically detected the secretion of ESAT-6 and CFP-10 from wild-type *M. marinum* colonies using this method (Fig. 1*c*, *WT*). The peaks labeled 1 and 2 correspond to ESAT-6 and acetylated ESAT-6, respectively. This is consistent with the observed N-terminal acetylation of ESAT-6 in both *M. tuberculosis* and *M. marinum* (40, 42). Peak 3 corresponds to CFP-10. The identities of these peaks were established by first confirming that the peaks are at the correct molecular weight for ESAT-6, acetylated ESAT-6, and CFP-10. Moreover, these three peaks were absent (peaks 1 and 2) or greatly diminished (peak 3, see below) from the spectra obtained from the *M. marinum* strain lacking the RD1 region, which includes genes required for ESX-1 secretion (17–19). We expected the loss of ESAT-6 and CFP-10 in this strain (Fig. 1, *c*, Δ*RD1*, and *a*, *lanes 7* and *8*) because the genes encoding for ESAT-6 and CFP-10 (*esxA* and *esxB*, respectively) are deleted. We independently confirmed the identity of these peaks by LC/MS/MS analysis (Table I, supplemental materials, and supplemental Fig. 3). Whole colony washates of WT and ESX-1 mutants were subjected to digestion with trypsin as described (27, 40) and analyzed by LC/MS/MS. The two major proteins missing from mutant LC/MS/MS experiments were ESAT-6 and CFP-10 (supplemental materials). In the spectra obtained from the ΔRD1 strain, we observed a minor residual peak isobaric with CFP-10. Although the protein contributing to this peak is unknown, it is likely due to ESX-1-independent secretion of additional Esx proteins, which are similar in mass to charge as CFP-10 (40, 50). Therefore, we conclude that we have, for the first time, directly detected ESAT-6 and CFP-10 on the surface of wild-type *M. marinum* colonies grown on agar using MALDI-TOF mass spectrometry.

TABLE I
LC/MS/MS confirmation of relevant MALDI-MS peaks from M. marinum and P. aeruginosa

N	Strain	Accession number	Name	Gene name	No. of unique peptides	Sequence coverage (%)[a]	Confidence of identification[b]	Local FDR for protein (%)
M. marinum								
1	WT	gi 183981941	Secreted AlaDH	*ald*	29	64	~>1e-46	1.5e-128
2	WT	gi 183985421	ESAT6	*esxA*	4	77	~>1e-8	1.4e-6
3	WT	gi 183985420	CFP10	*esxB*	10	100	~>1e-20	3.3e-31
1	ΔRD1	gi 183981941	Secreted AlaDH	*ald*	34	75	~>1e-51	5e-12
2	ΔRD1	gi 183985421	ESAT6	*esxA*	None observed	0	0	NA[c]
3	ΔRD1	gi 183985420	CFP10	*esxB*	None observed	0	0	NA
1	Δ*esxA*, Δ*esxB*	gi 183981941	Secreted AlaDH	*ald*	14	52	~>5e-25	0.005
2	Δ*esxA*, Δ*esxB*	gi 183985421	ESAT6	*esxA*	None observed	0	0	NA
3	Δ*esxA*, Δ*esxB*	gi 183985420	CFP10	*esxB*	None observed	0	0	NA
P. aeruginosa (*PA01*)								
1	WT-PA01	gi 15596906	PopD	*popD*	4	25	~>1.1e-7	0.083
2	WT-PA01	gi 15596905	PopB	*popB*	5	25	~>4.1e-8	0.08
1	Δ*exsA*	gi 15596906	PopD	*popD*	None Observed	0	0	NA
2	Δ*exsA*	gi 15596905	PopB	*popB*	None Observed	0	0	NA
PopD in *Pseudomonas* cell pellet (cytosol)								
1	Δ*exsD*, *pscC*::Ω	gi 15596906	PopD	*popD*	8	30	~>7.4e-11	7.4e-09

[a] Assuming initiator Met residue is removed.
[b] This is the confidence of the identification based on the algorithm, which is the log conversion of the ProtScore (39). Metadata for this table can be found in the supplemental materials.
[c] NA, not applicable.

Prior applications of whole colony MALDI-TOF MS have analyzed lysates of whole bacterial colonies (11–13). When investigating the process of bacterial protein secretion, it is imperative to distinguish between cell lysis and active protein export. Disrupting protein export often leads to accumulation of substrates in the bacterial cytosol. Therefore, in strains with a nonfunctional ESX-1 system, lysis and active export would be indistinguishable. We developed this approach to specifically detect proteins on the bacterial cell surface without colony lysis. To validate that we were not lysing the mycobacterial colonies, we examined the secretion of ESAT-6 and CFP-10 from the *eccCb*::TnKan and *espB*::TnKan (Fig. 1*b*) *M. marinum* strains (21, 26). Importantly, each of these strains produces but does not export ESAT-6 and CFP-10 (Fig. 1*a*, *lanes 5*, *6*, *9*, and *10*, and data not shown). In the spectra from these strains, the peaks corresponding to ESAT-6 are absent, and the CFP-10 peak is greatly diminished (Fig. 1*c*, compare *eccCb*::*TnKan* and *espB*::*TnKan*), demonstrating that our detection methods are capable of measuring active protein export. If colony lysis was responsible for the released proteins, we would have expected to detect ESAT-6 and CFP-10 peaks in these spectra. Together, these data demonstrate that we have established a proteomics-based method that allows direct, specific, and selective monitoring of ESX-1-mediated protein secretion from mycobacterial colonies without bacterial lysis.

Scaling Whole Colony MALDI-TOF MS for High Throughput Applications—To evaluate this assay for high throughout applications, we asked whether we could reproducibly discriminate between colonies that are positive or negative for ESX-1 secretion. We analyzed a single 384-well MALDI target with 160 wild-type *M. marinum* colonies and 160 ESX-1 mutant colonies. We collected and overlaid the spectra corresponding to ESAT-6 and CFP-10 for all 320 spots (supplemental Fig. 1). We did not observe any false-positive spectra (ESX-1 mutant colonies with peaks that correspond to ESAT-6) or false-negative spectra (wild-type colonies missing peaks that correspond to ESAT-6) for ESAT-6 (supplemental Fig. S1*a*). For CFP-10, because of the minor residual peak, there was slight overlap between some spectra from the wild-type and ESX-1 mutant colonies (Fig. 1*c*). However, when considered together with the ESAT-6 data, the assay is reproducibly discriminatory for high throughput analysis.

We next investigated the potential bias caused by colony morphology or size. It is probable that when using this application to screen a transposon library for colonies deficient in ESX-1 secretion, inconsistencies in colony spotting or growth rates caused by mutations in unrelated genes could lead to false-positive detection. As such, we constructed a transposon insertion library in *M. marinum* and conducted a pilot screen to identify genes required for ESX-1 protein secretion using whole colony MALDI-TOF. We obtained MALDI-TOF spectra from colonies spotted from our *M. marinum* transposon library in 96-colony format agar plates. We selected examples of large and small colonies, as well as agar without visible colonies from each plate. We observed that for both large and small colonies, detection of ESAT-6 and CFP-10 was not measurably different (supplemental Fig. 2). Importantly, in the absence of colony growth, no signal was observed, indicating there is no cross-contamination between wells or diffusion through the media.

We confirmed these findings by performing MicroBCA assays on the extracted protein from the colonies to determine

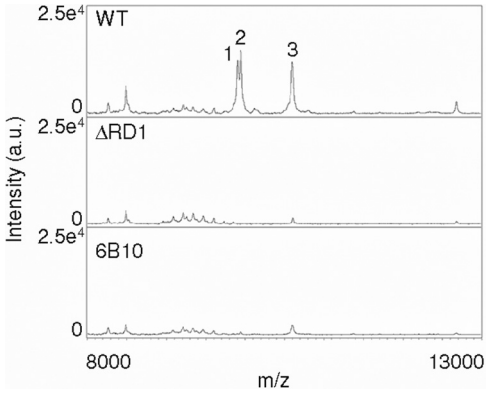

FIG. 2. **Identification of an ESX-1-deficient strain using whole colony MALDI-TOF.** Spectra generated by whole colony MALDI-TOF for wild-type *M. marinum* are shown. The panels show wild-type *M. marinum* (*WT*), *M. marinum* bearing a deletion in RD1 (Δ*RD1*), and the 6B10 strain of *M. marinum.* Peaks labeled *1*, *2*, and *3* correspond to ESAT-6 (9913 Da), acetylated ESAT-6 (9960 Da), and CFP-10 (10609 Da), respectively.

whether a normalization factor was needed or differences in protein level were conserved. Although protein levels from this approach varied, they were in general within 20% of one another (data not shown). Together, these data demonstrate that the MALDI-TOF MS assay is suitable for high throughput applications.

Identification of a Known ESX-1 Component Using Whole Colony MALDI-TOF—To validate this method for the identification of genes required for ESX-1 secretion in *M. marinum*, we conducted a pilot screen of ~400 colonies bearing independent transposon insertions. Although virtually all of colonies yielded spectra that were comparable with the wild-type strain (data not shown), we identified a single colony, 6B10, which produced spectra lacking the ESAT-6 and CFP-10 peaks (Fig. 2, compare *WT* and *6B10*). Importantly, the spectra from this colony resembled that generated from the RD1 deletion strain, which lacks a functional ESX-1 secretion machine (Fig. 2, compare Δ*RD1* and *6B10*).

To confirm that the 6B10 strain was truly defective for ESX-1 function, we tested whether this strain could lyse sRBCs. Previous studies demonstrated that *M. marinum* lyses sRBCs in an ESX-1-dependent fashion (21). Indeed, we found that although wild-type *M. marinum* lyses sRBCs, both the RD1 deletion strain and the 6B10 strain exhibited decreased sRBC lysis (Fig. 3*a*).

We also used Western blot analysis to test the secretion of ESAT-6 and CFP-10. As shown previously, ESAT-6 and CFP-10 are produced by and secreted from wild-type *M. marinum* (Fig. 3*b*, *lanes 1* and *2*). The strain bearing the RD1 deletion did not produce or secrete ESAT-6 or CFP-10 as expected, because the deletion covers the *esxBA* genes (Figs. 1*a* and 3*b*, *lanes 3* and *4*). We found that the 6B10 strain produced greatly decreased levels of ESAT-6, and we were unable to detect secretion of ESAT-6 into the culture supernatant. We were unable to detect CFP-10 in the cell lysate or the culture supernatant for this strain (Fig. 3*b*, *lanes 5* and *6*). Together, our data indicate that the 6B10 strain is likely defective in either the production or stability of ESAT-6 and CFP-10.

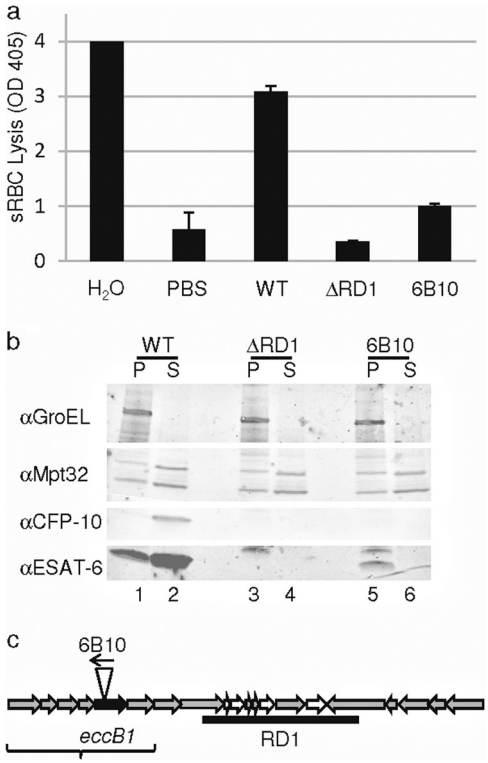

FIG. 3. **The 6B10 mutant is defective for ESX-1 secretion.** *a*, sRBC lysis assay. Although wild-type *M. marinum* lyses sRBC, strains bearing deletions in RD1 or the transposon insertion 6B10 fail to lyse sRBCs. The *error bars* represent standard deviation. *b*, Western blot analysis demonstrating that 6B10 fails to export ESAT-6 and CFP-10. GroEL was used as a lysis control, and Mpt32, a substrate of the Sec secretion system, was used as a loading control. CFP-10 is produced in the cell pellet (*P*) and secreted into the supernatant (*S*) by wild-type *M. marinum* (*lanes 1* and *2*) but not seen in the pellet or supernatant of ΔRD1 (*lanes 3* and *4*) and 6B10 (*lanes 5* and *6*). ESAT-6 is produced at reduced levels but is not secreted by 6B10. *c*, the point of transposon insertion was mapped (between bases 6584232 and 6584233 in the *M. marinum* genome, reverse orientation) and corresponds to 254 base pairs into the open reading frame of *MMAR_5444*, *eccB1.* The potential operon including *eccB1* is indicated by a *bracket* below the genes, including *MMAR_5440–5445* (*espF-eccCa1*).

To identify the site of transposon insertion, we isolated the DNA bearing the transposon and sequenced the junctions. The transposon in the 6B10 strain was inserted in reverse orientation to the gene between base 6584232 and 6584233 in the *M. marinum* genome. This corresponds to 254 base pairs from the start of *MMAR_5444* (*eccB1*, CDS 6583979–6585424). *eccB1* encodes EccB1, which has been previously shown to be required for ESX-1 protein secretion (25, 33, 47). Therefore, we conclude that this method can effectively be used to identify genes required for ESX-1 protein

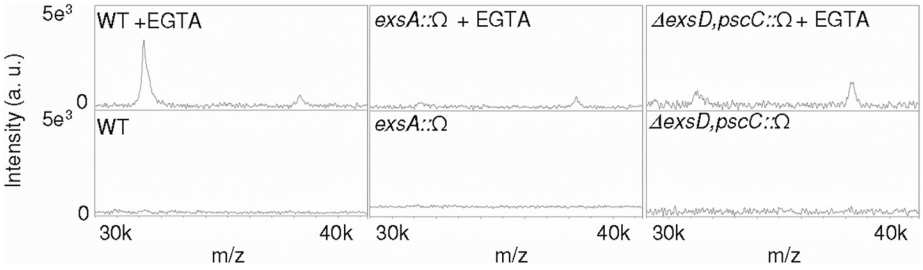

FIG. 4. **Direct detection of type III secretion from *P. aeruginosa* using whole colony MALDI-TOF.** Spectra generated from whole colony washates of wild-type PA103 (*WT*), *exsA*::Ω, and Δ*exsD*, *pscC*::Ω in the presence (*top*, + *EGTA*) or absence (*bottom*) of EGTA. The major peak at 31,198 *m/z* is specific to EGTA induction and absent in a type III mutant strains. This mass corresponds to PopD (±0.1%). LC/MS/MS was used to confirm the identity of this protein. PopD was not identified from LC/MS/MS analysis of uninduced PA103 or the *exsA*::Ω and Δ*exsD*, *pscC*::Ω strains under any condition.

secretion from a random transposon insertion library in *M. marinum.*

Specific Detection of Type III Secretion from Whole P. aeruginosa Colonies—To address the utility of this method for studying additional bacterial protein export systems, we applied this assay to the type III secretion system in *P. aeruginosa.* Type III secretion systems are highly conserved in a number of Gram-negative bacterial pathogens and mediate virulence by transporting effectors into the eukaryotic host cell. The type III secretome in *P. aeruginosa* includes four secreted effectors and a number of secreted components of the translocon that mediate transport of the effectors out of the bacterial cell and across the host cell membrane (48). We evaluated whether we could use MALDI-TOF MS to detect components of the type III translocon or effectors on the surface of wild-type *P. aeruginosa* colonies grown on agar permissive for type III protein secretion. As shown in Fig. 4, we specifically detected the secretion of a single protein from wild-type *P. aeruginosa* colonies using this method (Fig. 4, *WT* + *EGTA*). The peak was detected only in the presence of EGTA (Fig. 4, compare *WT* and *WT* + *EGTA*), which induces the type III system. Analysis of the *m/z* ratio and LC/MS/MS of the colony washates led us to identify this peak as PopD, a known secreted component of the type III translocon. The PopD peak was absent from the spectra obtained from the *exsA*::Ω *P. aeruginosa* strain (37) (Fig. 4, compare *exsA*::Ω and *exsA*::Ω + *EGTA*), which fails to produce or export type III substrates in the presence or absence of induction. To validate that we were not lysing the *P. aeruginosa* colonies, we examined the secretion of PopD from the Δ*exsD*, *pscC*::Ω strain (37), which produces (supplemental Fig. 4) but does not secrete type III substrates and components, including PopD (37). In the spectra from this strain, the peak corresponding to PopD is greatly diminished (Fig. 4, compare Δ*exsD*, *pscC*::Ω, and Δ*exsD*, *pscC*::Ω + *EGTA*), demonstrating that we measured active protein export. The small detectable peak likely represents minor colony lysis. Together, these data demonstrate that this method can be used to directly monitor type III secretion from Gram-negative bacterial pathogens.

DISCUSSION

Here, we report the first use of whole colony MALDI-TOF mass spectrometry, to our knowledge, to directly measure bacterial protein secretion from whole, intact bacterial colonies. Because the ESX-1 substrates are small, abundant, and not amenable to traditional reporter-based approaches, we applied this method to the direct detection of two major ESX-1 substrates, ESAT-6 and CFP-10 from whole *M. marinum* colonies (Fig. 1). Using *M. marinum* strains that produce but do not export ESAT-6 and CFP-10, we verified that the detected proteins were actively secreted and not as a result of bacterial lysis (Fig. 1).

This method is reproducible and scalable for high throughput applications. Importantly, this method can be readily applied to analyze libraries of mycobacterial mutants for strains that fail to secrete ESAT-6 and CFP-10, regardless of whether they produce ESAT-6/CFP-10 in the cytosol. We constructed a library of *M. marinum* strains bearing independent transposon insertions and conducted a small scale genetic screen. We demonstrated that this method can be applied to identify genes required for ESX-1 secretion. Importantly, we identified a transposon insertion in *eccB1* validating our approach (Figs. 2 and 3). Interestingly, *eccB1* is required for secretion of ESAT-6 and CFP-10 in both *Mycobacterium smegmatis* and the *Mycobacterium microti* complementation model (25, 33). This is the first demonstration that this gene is required for ESX-1 secretion in pathogenic mycobacteria with an intact ESX-1 secretion system.

Using multiple bioinformatic approaches, we found no predicted gene function for the *eccB1* gene, and there is no homology at either the primary amino acid sequence or at the structural level to any known protein. However, there are four paralogs of the *eccB* gene in the *M. marinum* genome (MMAR_0184, 0542, 2664, and 1009), and they are all associated with loci encoding ESX systems. Based on the annotations in Marinolist (45) and predictions by the TB database (www.tbdb.org), it is probable that the *eccB1* gene is the fifth gene in a six-gene operon (*espF-eccCa1*). Importantly, as we observed for the *eccB1*::TnKan strain, disruption of any of the

three genes immediately upstream of *eccB1*(*espG1*, *espH*, or *eccA1*) by the same transposon in *M. marinum* resulted in decreased levels of ESAT-6 and CFP-10 in the cell pellet, suggesting a role for these gene products in the production or stability of ESAT-6 and CFP-10 (21). Importantly, this highlights that the approach is sensitive to identifying strains that either fail to produce substrates or fail to export them but cannot distinguish between the two. However, both are interesting strains to pursue, because they may provide key insight into how the ESX-1 substrates are targeted and regulated.

Finally, we demonstrate the utility of this method in studying protein export from the type III system of the Gram-negative pathogen, *P. aeruginosa* (Fig. 4). Using this method, we specifically identified PopD, a component of the type III translocon required for transporting the effector proteins across the eukaryotic plasma membrane (48). Although additional type III substrates were identified in the LC/MS/MS, the MALDI-TOF assay was not optimized for their abundance or *m/z* ranges. This suggests widespread application of this approach in revealing new mechanisms of protein secretion across bacteria.

Direct measurement of protein secretion from whole bacterial colonies represents a great advance in our ability to apply analytical tools to agar-based bacterial genetic screens. Previous efforts using whole colony MALDI-TOF in bacteria have focused on principal component analysis to identify bacterial strains or to aid in clinical diagnosis. These methods require lysis of the bacteria to generate the data. We envision performing both the method described here alongside a duplicate lysed bacterial sample to identify both synthesis and secretion of proteins. Furthermore, this approach can be applied to identifying novel substrates from bacterial protein secretion system by comparing the spectra of wild-type and secretion-deficient bacterial strains. Novel peaks present in the wild-type but not deficient strains can be identified using protease digestion and LC/MS/MS or top-down methods. We applied this approach to confirm the identity of ESAT-6 and CFP-10 in *M. marinum* and to identify PopD from *P. aeruginosa*. Lastly, it has not escaped our attention that this assay can be readily employed on pathogens to screen libraries for compounds that inhibit secretion systems. This would be an important step in creating tools for probing molecular systems and potential drugs that selectively target pathogens within the body.

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Author Contributions—M. M. C. and P. A. C. conceived of the method and its application to bacterial protein secretion. M. M. C. and P. A. C. designed the experiments. M. M. C., P. A. C., E. A. W., and G. M. K. conducted the experiments and analyzed the data. M. M. C., E. A. W., G. M. K., and P. A. C. wrote the manuscript. All authors discussed the findings and edited the manuscript at all stages.

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