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Review

Advances and Perspectives on Mycobacterial Secretion Systems

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Abstract: Mycobacteria possess a uniquely complex cell envelope and rely on a diverse array of secretion systems to interact with their environment, ensure survival, and modulate host immune responses. This review provides a comprehensive overview of these secretion pathways, from the universally conserved Sec and Tat systems to the specialized ESX/type VII secretion systems, as well as lipid transporters of the MmpL family, with particular emphasis on *Mycobacterium tuberculosis* and other clinically relevant members of the *M. tuberculosis* complex and non-tuberculous mycobacteria. By integrating findings from historical literature and the most recent experimental and bioinformatic studies, we outline the genetic organization, structure, regulation, and functional interplay of these pathways. Emphasis is placed on how these systems are not isolated entities but form a highly interconnected network that coordinates protein and lipid export essential for virulence, immune modulation, and cell wall integrity. We also explore the translational potential of secreted effectors and their transport machineries, discussing their relevance as targets for therapeutic interventions, including novel inhibitors, diagnostic biomarkers, and vaccine candidates. We highlight critical knowledge gaps and propose avenues for future research, particularly those that leverage multidisciplinary approaches. By drawing connections across secretion systems and emphasizing their shared and distinct roles, this work aims to provide an integrated framework that supports both fundamental understanding and biomedical innovation in mycobacterial pathogenesis.

Keywords: mycobacteria; secretion system; Tat; Sec; ESX; MmpL

1. Overview of Mycobacterial Secretion Systems

Persistence in hostile intracellular niches and a sophisticated aptitude towards immune evasion are, in a broader sense, the roots of *Mycobacterium tuberculosis* everlasting success. Among the multiple mechanisms that detail the establishment of the pathogen's infective profile, secretion systems play crucial and very diverse roles, from skewing immune responses to preserving homeostasis under nutrient deprivation and oxidative bursts. Bridging transport across the structurally complex mycobacterial cell envelope requires the coordinated effort of a network of specialized systems, able to adapt and respond to ever-changing environmental stresses. Indeed, mycobacteria do not operate their secretion machineries in isolation; rather, a layering of regulatory overlap and even shared accessory factors integrates the different pathways to ensure efficient export and virulence. As apparent as their crosstalk, role diversification is especially marked even among members of the same family of transporters, arguably as an evolutionary strategy to oppose distinct immune defenses [1–3].

Though mainly involved in cell wall assembly and envelope maintenance, the wide array of proteins trafficked by the classical Sec and Tat pathways includes virulence factors and modulators of host-pathogen interactions [2,4–7]. Separated functions are also described for the almost exclusively mycobacterial Type VII Secretion Systems, T7SS, also known as ESX Systems. From phagosome escape and immune modulation, mediated by ESX-1 through *M. tuberculosis* major virulence factor ESAT-6 (EsxA) [8–10], to iron and zinc scavenging, delivered by the essential ESX-3 [11,12], again to ESX-5-dependent secretion of PE/PPE protein family members, influencing cell-surface integrity and intracellular survival [13]. Different yet, but not less diverse, are MmpL lipid transporters, vital for the unique structure of the mycobacterial cell wall and in the pathogen's ability to establish and maintain infection [14–16]. With so many export routes to deliver effectors, acquire nutrients, and shape host immunity, a deeper understanding of the regulatory mechanisms governing their coordination is central to exploiting these secretory machines as drug targets and vaccine components, and as such to devising novel interventions against tuberculosis.

Key structural, functional, and accessory features of *M. tuberculosis* Sec and Tat pathways, five ESX systems, and six main MmpL transporters are summarized in Table 1.

Table 1. Integrated overview of *Mycobacterium tuberculosis* secretion systems.

Locus	Key Substrates/Effectors	Core Components	Accessory Components	Functional Role	Notable Features/Regulatory Control
SecA1	RipA, PBs, LrpG, LppX, PknB, etc.	SecA1, SecY, SecE, SecG	SecB, SRP, LepB	Cell wall assembly and maintenance, virulence	Ubiquitary and essential, secretes unfolded proteins
SecA2	LipO, SodA, KatG, PknG, etc.	SecA2, SecY, SecE, SecG	SecB, SRP, LepB	Virulence, resistance to stress	Restricted to pathogenic Mycobacteria, nonessential, secretes unfolded proteins
Tat	PlcB, BlaC, MmcO, Rv2525c, Ag85A/B/C, etc.	TatA, TatB, TatC	Undefined	Cell wall assembly and maintenance, redox homeostasis, nutrient acquisition, virulence	Ubiquitary and essential, secretes folded proteins that carry N-terminal RR motif
ESX-1	EsxA/B, EspA/EspC, EspB	EccA1, EccB1, EccC, EccD1, EccE1	EspG1, MycP1	Virulence, phagosomal escape, immune activation	RD1 locus, activates cGAS/STING via cytosolic DNA release
ESX-2	PE/PPE-like proteins (minimal)	EccA2, EccB2, EccC2, EccD2, EccE2	Putative MycP2	Immune modulation (putative)	Most recently acquired, poorly expressed, function and regulation remain speculative
ESX-3	EsxG/H, EsxR/S (putative), PE5/PPE4	EccA3, EccB3, EccC3, EccD3, EccE3	EspG3, MycP3	Metal acquisition (Fe^{3+}/Zn^{2+}), immune evasion	Essential for in vitro growth, interferes with autophagy and ESCRT
ESX-4	EsxU/T	EccA4, EccB4, EccC4, EccD4, EccE4	Putative MycP4	Stress response, envelope integrity, heme uptake	Simplest and ancestral system, conserved in non-pathogenic mycobacteria
ESX-5	PE/PPE family, EsxM/N	EccA5, EccB5, EccC5, EccD5, EccE5	EspG5, MycP5, MycP7	Secretion of PE/PPE proteins, immune modulation	Expanded in slow-growing mycobacteria, remodels cell envelope
MmpL3	TMM, PE, TDM	MmpL3	Wag31	General cell wall function, HPI	Essential and prominent drug target, TDM (cord factor) is produced from TMM
MmpL4	cMBT, MBT	MmpL4	MmpS4	Iron uptake	Works in tight coordination with ESX-3, doubles as drug efflux pump
MmpL5	cMBT, MBT	MmpL5	MmpS5	Iron uptake	Works in tight coordination with ESX-3, doubles as drug efflux pump
MmpL7	PDIM	MmpL7	PpsE	Phagosomal escape, immune modulation	PDIM are uniquely produced by virulent strains and enhance EsxA/B effect
MmpL8	SL-1	MmpL8	Undefined	Immune modulation, resistance to antibiotics, resistance to surface stress	SL-1 is uniquely produced by virulent strains
MmpL11	PIM, cardiolipin, LC-TAG, MWE	MmpL11	Undefined	General cell wall function, biofilm formation, persistence	Upregulated in hypoxia and nutrient deficit

Note: *M. tuberculosis* genome encodes two classical secretion systems, Sec (with two separate ATPases SecA1 and SecA2) and Tat, five paralogous Type VII Secretion Systems (named ESX-1 to ESX-5), and eleven MmpL transporters, whose main members are schematized. For each of these systems, key substrates, core and accessory components, functional roles, and main notable features are listed.

2. Classical Secretion Pathways: Sec and Tat Systems

The general secretory (Sec) and twin-arginine translocation (Tat) pathways represent the most highly conserved protein-export machineries, identified in all domains of life: Bacteria, Archaea and Eukarya [7]. Employed by both Gram-positive and Gram-negative bacteria, most of the proteins they deliver remain within the

cell envelope, in either the periplasm or embedded in the inner membrane [2,3,7]. However, in diderm microorganisms such as mycobacteria, cooperation with additional secretion systems allows to shuttle proteins beyond the outer membrane, enabling their specific extracellular functions [2,3]. Proper assembly and remodeling of the mycobacterial cell envelope is itself heavily dependent on enzymes and transporters delivered by Sec and Tat [3,4,17]. A schematic overview of the two systems in mycobacteria is provided in Figure 1.

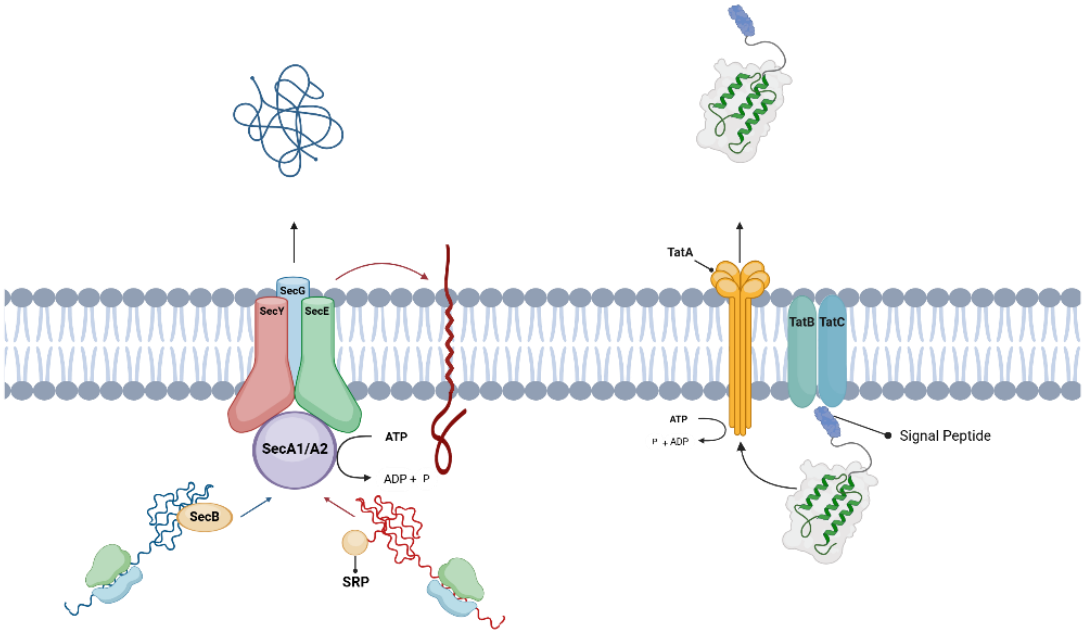

Figure 1. Classical protein secretion pathways in mycobacteria. The Sec pathway (left) primarily exports unfolded proteins via the SecYEG translocase, powered by the SecA1/A2 ATPase and guided by SecB chaperones or the SRP for membrane targeting. The Tat pathway (right) exports folded proteins via a TatA oligomeric pore, following substrate recognition by the TatBC receptor complex. Tat substrates are marked by N-terminal signal peptides with a conserved twin-arginine (RR) motif. Sec: secretion; Tat: twin-arginine translocation; SRP: signal recognition particle. Created with Biorender.com.

2.1. Sec Pathway: Translocation of Unfolded Proteins

The general secretory pathway primarily translocates proteins in their unfolded state. The system consists of three parts: a protein-targeting chaperone component (SecB), a motor protein (SecA ATPase), and a membrane-integrated conducting channel (SecYEG translocase), along with several accessory proteins [2,3,7].

In this system, proteins destined for secretion into the periplasm or outside the cell carry removable SecB-specific N-terminal signal sequences and are post-translationally delivered to the SecYEG channel through SecB chaperoning [7,18]. Translocation is actively driven by SecA ATPase and supported by the SecD/F complex on the periplasmic side of the inner membrane [18]. Direct, co-translational transfer to the SecYEG channel for insertion in the inner membrane is instead mediated by the signal recognition particle SRP, a ribonucleoprotein which recognizes nascent polypeptides intended for transmembrane localization [7], as illustrated.

In *M. tuberculosis*, two SecA paralogs exist: SecA1, the canonical exporter, committed to the secretion of mainly peptidoglycan polymerization and turnover factors (e.g., RipA, penicillin binding proteins PBs) and thus essential for bacterial viability [2–4,7]; and SecA2, a supplementary pathway specific for a distinct subset of mostly stress-related enzymes (e.g., putative lipase/esterase LipO, kinase PknG) and glycoproteins, contributing to the pathogen's resilience in hostile environments, along with virulence-linked proteins (e.g., superoxide dismutase A SodA; catalase/peroxidase KatG) [2,3,5]. Recent in silico docking studies performed on the yet uncharacterized secretory protein Rv0398c, however, suggest that a distinct set of substrates may be inherently accessible for recognition and translocated in a non-specific manner by either of the two alternative paralogs, or that still undefined conditions could drive interchangeable use of both SecA1 and SecA2 [19].

2.2. Tat Pathway: Transport of Folded Proteins

In contrast to the Sec system, the twin-arginine translocation pathway primarily secretes folded proteins, and as such, it is particularly suited for cofactors or metal-dependent enzymes that must fold in the reducing

environment of the cytosol, and fundamental for all other proteins that undergo posttranslational modifications for which the substrates or machineries are not available extracellularly [2,3,6,7].

As visible in Figure 1**,** the system involves two functional elements: ATP-hydrolyzing TatA oligomers, that assemble to form the transient channel through which the folded polypeptides cross the inner membrane; and the TatBC receptor complex, that recognizes and binds substrates bearing the conserved twin-arginine (R-R) motif in the N-terminal signaling peptide S/T-R-R-X-F-L-K [2,3,7].

Experimentally demonstrated Tat substrates in *M. tuberculosis* include virulence factors such as phospholipase C PlcB, and the class A β-lactamase BlaC. Potential roles in nutrient acquisition (e.g., putative sugar-binding proteins, glucanase precursors), cell wall maintenance and fluidity (e.g., carbohydrate/lipid modifying enzymes), and redox homeostasis (e.g., oxidoreductases, multicopper oxidase MmcO) have also been predicted and identified [6,20], together highlighting the pathway's functional implications in general viability and pathogenesis.

2.3. Mapping the Sec/Tat Exportomes: Recent Advances and Computational Approaches

As supported by multiple lines of investigation, both the canonical Sec pathway and the Tat pathway are essential for *M. tuberculosis* survival and general physiology, hindering all attempts at generating full null mutant strains to be exploited for functional studies and target identification [4,6]. In fact, no naturally occurring *secA1*-defective [4] or *tatA/B/C*-defective clinical isolates have been documented, and no transposon insertions have been recovered in *tat* loci through analysis of high-density transposon libraries [17]. Additionally, *secY/E/G* genes conservation across *M. tuberculosis* strains has been profiled through in silico comparative genomics surveys, further confirming the non-redundant activity of these export machineries [15]. By contrast, export routes primarily deputed to pathogenicity and virulence, such as accessory SecA2, are not required for in vitro growth of *M. tuberculosis* [5]. Interestingly, a *tat*-defective mutant of the non-pathogenic environmental species *M. smegmatis*, often used as a surrogate model for the tubercular bacterium, remains viable despite defects in the export of several Tat substrates [6].

For these reasons, bioinformatic tools have been extensively leveraged for the identification of Sec/Tat-exported proteins and have enabled subsequent comprehensive mapping of Sec/Tat secretion routes, allowing for functional and evolutionary studies in *M. tuberculosis*.

Signal-peptide predictors such as SignalP 5.0 [21] or TatP [22] are widely used computational tools that combine hidden Markov models (HMMs) and neural network algorithms to flag the distinctive N-terminal signal peptides of respectively Sec- and Tat-secreted substrates among prokaryotic proteins and reveal lineage-specific patterns. Conserved machinery components are instead profiled through manually curated HMM protein library collections such as TIGRFAMs, while complete export pathways are inferred from TIGRFAM hits with the Genome Properties framework, allowing automated function annotation across mycobacterial genomes [23]. Building on these approaches, later developed all-encompassing bioinformatic resources, such as the SecretoMyc database, now provide a comprehensive view of secretion system exportomes and enable structure-based identification of secreted homologs across mycobacterial species [24].

By coupling these in silico predictions with quantitative mass spectrometry, pathway-specific repertoires such as the SecA2-dependent exportome have recently been mapped, allowing to identify numerous cell wall hydrolases, lipoglycan synthases, and immunodominant antigens as SecA2-exclusive cargo [4,5], while the evolutionary origin of the system, and its structural components and virulence-related functions have been elucidated by correlating recurring peptide motifs to protein functions, through extensive phylogenetic profiling and comparative genomics approaches [7,20,22].

Similarly, on the Tat side, combinations of in silico predictions with targeted signal-peptide mutagenesis have allowed for downscaling the investigation to single candidate substrates, as in the case of protein Rv2525c, whose inactivation causes increased sensitivity to β-lactams, and its subsequent appointment as a Tat substrate [17]. In a different approach, experimental validation of over a dozen other Tat substrates, including many host-derived stress resistance factors, has been performed on proteins predicted to contain twin-arginine signal peptides, by fusing them with a β-lactamase reporter [6].

Additionally, innovations in cryo-electronic and live-cell fluorescence microscopy have recently allowed us to gain detailed resolution on the finer architecture and molecular dynamics of both systems, from clarifying the initiation of SecYEG channel opening after SecA-bound signal peptide insertion, to revealing how TatA oligomerization cycles are dependent on substrate flux [18].

3. Type VII Secretion Systems: A Multifaceted Machinery

3.1. Origin of ESX Systems: From Fast- to Slow-Growing Mycobacteria

Beyond the generalist Sec and Tat pathways, which maintain cell envelope integrity and general protein export, the T7SS family of export machines constitutes a finely orchestrated network, channeling specific effectors that drive virulence, modulate host immunity, and tailor the pathogen's adaptation to its environment. Their relatively recent discovery has marked a turning point in understanding mycobacterial specialized protein secretion, highlighting its pivotal roles in nutrient acquisition, cell–cell communication, and pathogenesis.

Early insights into this specialized modality came from the description of the *RD1* (Region of Difference 1) locus in *M. tuberculosis*, shown to encode a novel secretion apparatus critical for virulence and pathogenesis, originally defined as ESX-1 [25–28], and later yet formalized into the official nomenclature as a "Type VII Secretion System" (T7SS) [29,30].

The collective and simultaneous designation of ESX-1 as the archetypal T7SS ultimately led to the discovery of four additional ESX paralogs within the *M. tuberculosis* complex (ESX-2 to ESX-5), and of their species-dependent homologues across and beyond the mycobacterial genus. Subsequent studies have thus used ESX-1 as a reference for practical and historical reasons, rather than its position in an evolutionary timeline, which was later explored through synteny mapping and compositional-bias matrix analyses, which have instead appointed ESX-4 as the primordial cluster. Leader of a stepwise, plasmid-driven cascade of duplication events and horizontal gene transfers, ESX-4 is present in fast-growing environmental species (e.g., *Mycobacterium smegmatis*), where it likely mediates basic cellular functions unrelated to virulence, and closely resembles the WXG–FtsK/SpoIIIE-type ATPase modules found among non-mycomembrane-containing actinobacteria (e.g., *Streptomyces*) [30], implying an origin that predates the acquisition of the mycolic-acid outer barrier [31]. Furthermore, the reported presence of ESX-like proteins in firmicutes (e.g., *Bacillus, Streptococcus*) indicates a broader evolutionary distribution of simplified T7SS modules and adaptation for distinct biological roles [32].

Ecological transition, from nutrient-deprived environmental habitats for fast growers to intracellular niches in pathogens, was indeed shown to be a powerful driver for the plasmid-to-chromosome dynamics in T7SS radial evolution. As demonstrated by the phylogenetic profiling of a pan-genomic dataset of roughly 100 entries among chromosomes and plasmids, horizontal transfer of conjugative plasmid-borne ESX-3 modules equipped emerging lineages with a specialized metal-scavenging machinery essential for iron and zinc homeostasis. Similar events gave rise to ESX-1 loci on plasmid precursors that later integrated into slow-growing lineages such as *M. tuberculosis* and *Mycobacterium marinum*, marking the shift from environmental commensalism to pathogenicity. Further specialization produced ESX-2 and ESX-5, both confined to pathogenic, slow-growing mycobacteria, and underscoring the trend of incremental complexity and finer host adaptation in disease-causing species [31,33].

3.2. Genetic Organization: Conserved and Variable Components

Having expanded from the same ancestral locus through subsequent duplication events and horizontal gene transfers, as described, mycobacterial ESX systems share core structural elements and substrate configurations, encoded in gene clusters which are scattered throughout the chromosome. Highly conserved components (Ecc) include the membrane ATPase EccC and soluble ATPase EccA, three pore-forming integral membrane proteins EccB, D, and E, and a substrate dimer of EsxA/B-like proteins. [1,3,30,34]. Across systems, dedicated EspG chaperones selectively escort system-specific substrates to their respective translocons, ensuring fidelity of secretion under ever-changing stressful conditions [1,35]. Of note, T7SS secretome consists of folded proteins which lack classical Tat signal-peptides but contain a typical WXG (Trp-variable-Gly) internal motif, and are commonly about 100 amino acids long, and are thus referred to as "WXG100" family. A schematic illustration of the main ESX machinery elements is represented in Figure 2.

Downstream of the conserved genetic scaffold, each ESX locus incorporates specific accessory modules, comprising regulatory genes and variable effectors, such as EspM/EspN regulatory circuits, that fine-tune ESX-1 expression during macrophage infection [36], or iron/zinc-binding effectors in ESX-3, whose coding genes flank the essential ESX-3 locus [11,12,37]. Mycosins, subtilisin-like serine-proteases shown to be required for proper assembly and stabilization of core complex Ecc proteins, and removal of misassembled subunits, have been described as ESX accessory elements [2,38–40], and further supplementary secretion factors and lipoprotein chaperones have been reported for ESX-5, which specializes in the secretion of PE/PPE proteins that modulate host immunogenicity and cell-surface architecture [13,35,41]. Coupled by the limited size of their respective secretomes, the most recent ESX-2 and most ancestral ESX-4 widely diverge in their functional specializations. Highly induced upon dendritic cell phagocytosis, ESX-2 effectors have been shown to correlate with disease

severity [42]. By contrast, ESX-4 secreted WXG100/PPE proteins drive envelope-stress adaptation and sustain heme uptake [43].

Together, the mosaic of conserved core machinery and variable accessory factors equips *M. tuberculosis* with a modular toolkit to adapt its secretome for nutrient acquisition, immune modulation, and intercellular interactions, pivotal elements to its success as a persistent pathogen.

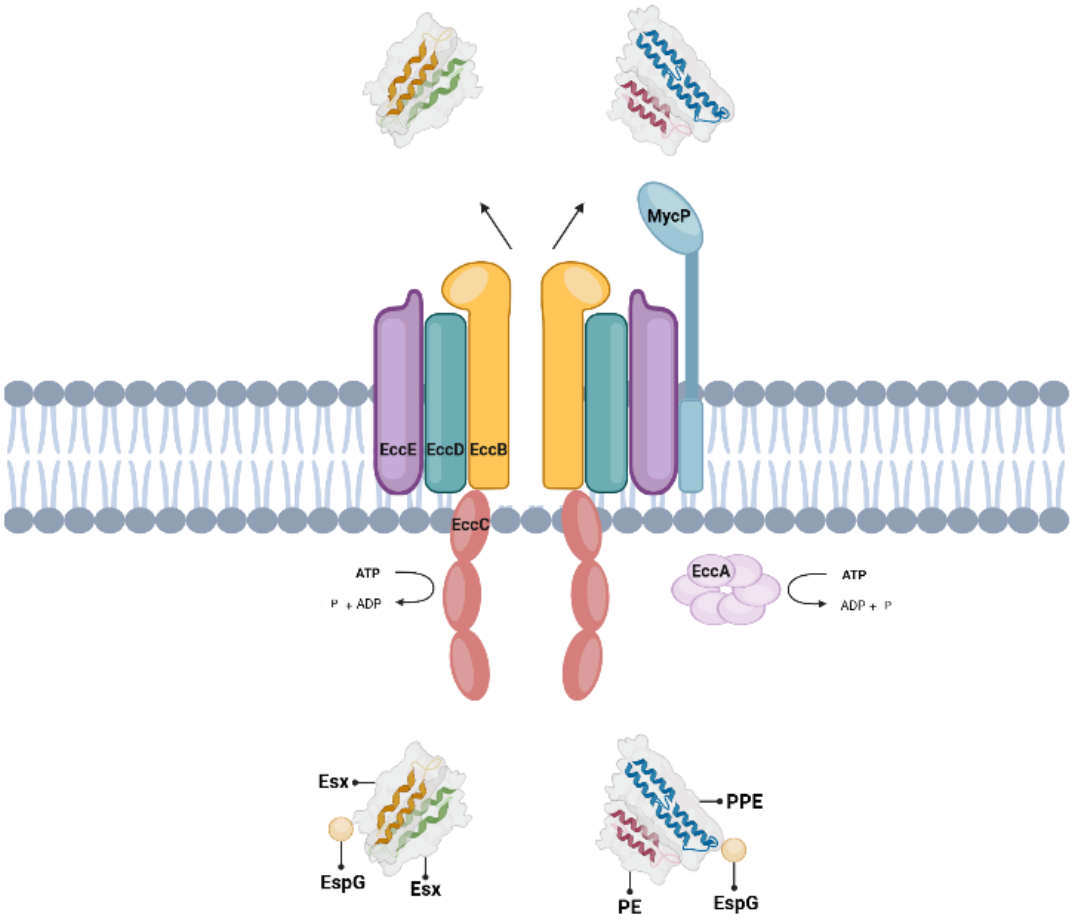

Figure 2. Type VII Secretion systems in mycobacteria. Type VII Secretion systems (ESX) include three pore-forming proteins, EccB, EccD, and EccE, one membrane ATPase EccC and one soluble ATPase, EccA. Substrate proteins contain a WXG domain and are usually exported in pairs (ESX or PE/PPE pairs), chaperoned in the cytosol by EspG and often cleaved by MycP proteases. ESX: ESAT-6 Secretion; Ecc: ESX core component; Esp: ESX secretion-associated protein; MycP: mycosin protease; PE/PPE: conserved Pro-Glu/Pro-Pro-Glu motif. Created with Biorender.com.

3.3. ESX-1: Phagosome Escape and Host-Cell Lysis

The ESX-1 locus of *M. tuberculosis* is organized as a single operon of approximately 6 kb, encoded, as mentioned, within the *RD1* genomic region, and encompassing four integral membrane proteins (EccB1 to EccE1), a dedicated cytosolic ATPase (EccA1), the secreted WXG100 heterodimer EsxA/EsxB, and the chaperone EspG1 [30]. Historically, ablation of the *RD1* homologue in *M. bovis* BCG was shown to be directly linked to the vaccine strain attenuation, as to the collateral deficiency of ESX-1 secreted heterodimeric effectors, originally named ESAT-6 (Early Secreted Antigen 6 kDa) and CFP-10 (Culture Filtrate Protein 10 kDa) [25,27]. Correspondingly, reintroduction of the two protein-coding genes in BCG had proven to elicit stronger antigen-specific responses and offer better protection in murine tuberculosis models than the wildtype strain [28]. Application of *RD1*-null mutants of *M. tuberculosis* to murine macrophage infection models had eventually provided functional proof of ESAT-6/CFP-10 role in phagosome permeabilization and cytolysis [26].

In the span of the following twenty years, experimental dissection of the prototype T7SS has uncovered novel essential and accessory components, suggested functional parallels to other bacterial secretion systems, and offered continuous insights into ESX-1 finely "macrophage-tuned" regulation. High-resolution crystal structures of inner

membrane components are now available that reveal the conserved dimerization interfaces and extracellular loops of EccB1 and EccD1, which likely function as gating elements, controlling passage of effector proteins [44].

Similarly, X-ray crystallography and molecular dynamics simulations of EccC internal domains support a model in which the protein forms a channel-like structure within the inner mycobacterial membrane, thereby facilitating EsxA/B translocation [45]. These findings align with previous electron-tomographic reconstructions, proposing that the tandem ATPase domains of EccC1 assemble into a filamentous conduit spanning the periplasmic space, resembling the Type III secretion needles found in classical diderm bacteria [46]. Simulations further indicate that substrate binding triggers coordinated conformational changes and controlled flexibility, emphasizing the dynamic plasticity of the secretion machinery [45]. Collectively, these complementary approaches reinforce the view of ESX-1 as a tightly regulated, ATP-driven translocation system. In parallel, the role of EccE1 in nucleating translocon assembly has been clarified, determining how its loss leads to mislocalization and degradation of core EccB1-EccD1 and ultimately abolishes substrate export [47].

At the transcriptional level, the expression of key genes within the ESX-1 locus, such as *espA*, *espC*, and *espD*, is driven by dedicated transcription factors, such as WhiB6 or EspR, whose activity can be modulated by environmental cues or chromatin structure, indirectly affecting the cellular abundance of ESX-1 proteins [48,49]. Interestingly, mutations in a homopolymeric region upstream of *espR* have recently been linked to its increased expression and subsequent enhanced ESX-1 secretion. These mutations confer isoniazid resistance and improve bacterial fitness in a murine infection model, suggesting that *M. tuberculosis* exploits such phase variations to rapidly adapt to diverse environmental conditions [50]. In an opposite manner, the ATP-sensing regulator EspI has been shown to silence ESX-1 operon under steady-state conditions, a fail-safe measure to prevent premature secretion. ATP-depletion, cueing on the nutrient limitation and oxidative stress that *M. tuberculosis* encounters within the phagosome compartment, relieves EspI-derived repression, setting off the pathogen's emergency response [51]. Additional transcriptomics studies conducted in *M. marinum* have mapped a second regulatory circuit, centered on the antagonistic transcription factors EspM and EspN, which dynamically control ESX-1 gene expression during macrophage infection, ensuring balanced effector production in synchrony with phagocytosis [36].

Post translocation, folded EsxA/B were shown to be stabilized by the non-core but essential EspD factor, suggesting a further regulatory step for quality control in the periplasmic environment [52]. Concurrently, full length EspB is translocated and extracellularly cleaved by MycP1 protease in a negative feedback loop that balances substrate flux for virulence optimization [53]. Processed EspB has been demonstrated to oligomerize and insert into the phagosome membrane alongside the EsxA/B heterodimer following selective binding to phosphatidic acid (PA) and phosphatidylserine (PE) [54,55], leading to cytosolic escape, and subsequent mitochondrial damage and Caspase-3 activation in infected macrophages [8,9]. The TLR2/Caspase pathway was shown to be furthermore exploited by EsxA to inhibit macrophage apoptosis, reduce phagocytosis and bactericidal activity, and broadly modulate host immune responses, including in dendritic cells [56]. In this scenario, membrane PDIM lipids co-localization with ESX-1 effectors has been proven to bolster host cell apoptosis, bridging the collaborative effort of proteinaceous and non-proteinaceous secreted effectors [57]. Additional evidence comes from recent time-lapse fluorescence microscopy experiments showing that surface contact between mycobacterial aggregates and macrophages is sufficient to trigger pyroptotic cell death, independently of phagocytosis. In this scenario, the proteins responsible for plasma membrane perturbation at the site of contact appear to be EsxA/B acting in concert with PDIM, or, in their absence, heptameric EspB [58]. Consistently, recent single-cell and spatial transcriptomic analyses in mouse lungs have further demonstrated that ESX-1 actively promotes the recruitment and differentiation of permissive macrophages, which harbor higher bacterial loads and exhibit transcriptional programs that suppress both innate and adaptive immunity, thereby creating a favorable microenvironment for the pathogen's survival [59].

3.4. ESX-2: Minimalist Cargo, Elusive Roles

The ESX-2 locus in *M. tuberculosis* spans roughly 5 kb and mirrors the canonical Type VII secretion scaffold, yet the role of the putative accessory protease MycP2 and the existence of other associated regulators remain mainly unexplored [1,30]. Comparative genomics studies mark it as one of the most recently acquired systems, restricted to slow-growing, pathogenic mycobacteria, and often co-localized with *PE/PPE* genes [31,33]; however, contrasting evidence of its conservation in environmental species like *Mycobacterium paragordonae* has recently been obtained, suggesting an early environmental role for ESX-2 prior to its restriction to pathogens [60]. Arguably, the limited secretome would point toward a specialized, perhaps regulatory or signaling function for ESX-2, operative in specific niches and under narrow activation cues, rather than broad virulence mediation.

Nonetheless, a putative ESX-1-supporting function has been proposed for ESX-2, in driving phagosome rupture and protein trafficking to the macrophage cytosol [39], and confirming previously suggested roles in antigen presentation and immune modulation, as inferred by detection of upregulated ESX-2 transcripts upon pathogen uptake by dendritic cells, rather than macrophages [31,42]. Additionally, a possible clinical role for ESX-2 in non-tuberculous mycobacterial infections has been suggested with genome-wide correlations between ESX-2 strain-specific variants and disease severity in *Mycobacterium intracellulare* infections [61].

Despite these recent findings, no targeted complete ESX-2 knockouts exist in *M. tuberculosis*, leaving the precise substrates, regulatory signals, and phenotypic outcomes still undefined, and highlighting a critical knowledge gap.

3.5. ESX-3: Nutrient Uptake and Immune Subversion

Among the five T7SS of *M. tuberculosis*, ESX-3 is uniquely indispensable for in vitro growth in standard laboratory conditions [11], emphasizing the role of its primary secreted heterodimer EsxG/H as a key player in metal homeostasis under host-imposed nutritional immunity. A broader activity spectrum is gradually emerging, however, supported by genetic and structural findings that position ESX-3 as a dual-function system, integrating metabolic adaptation with immune interference. Indeed, a separate, host-modulatory effect of EsxH in sequestering ESCRT (Endosomal Sorting Complex Required for Transport) components has been detailed, which ultimately results in preventing phagosome maturation and $CD4^+$ T-lymphocytes priming, dampening adaptive immunity [62,63]. Concurrently, by hijacking ESCRT-mediated repair pathways, the ESX-3 effector has been shown to hamper membrane lesion mending and ensure *M. tuberculosis* replicative niche within damaged phagosomes [64].

Other secreted effectors of ESX-3 have mostly been connected to full virulence expression, such as EspG3-chaperoned PE5/PPE4 pairs, with depleted mutants retaining partial iron uptake capabilities, but showing significant attenuation in murine infection models [37,65]. Moreover, a putative second pair of secreted WXG100 effectors (EsxR/S) has been catalogued from Actinobacteria to several mycobacterial species, and shown to be variably retained in the *M. tuberculosis* complex (MTBC) [66]. Possibly specialized under niche-specific conditions, as suggested, the effective recognition of these proteins and their translocation modalities by the ESX-3 machinery remains to be fully defined [12,32]. However, recent studies indicate relevant induction of the heterodimer coding genes *esxR/S* in strains lacking *esxG* and/or *esxH*, suggesting possible complementation of the essential metal uptake function [67].

Regulation of ESX-3 core-encoding genes *eccB3* to *eccE3*, as well as *esxG* and *esxH*, encoding its small effectors, is governed by metal-sensitive bidirectional promoters, the iron-dependent repressor IdeR and the zinc-responsive regulator Zur, in a tightly modulated response to metal availability [34,68–70]. Indeed, while not directly involved in encoding the high-affinity siderophores (mycobactins) of *M. tuberculosis* MbtA–J or their cytosolic importers IrtA/B, ESX-3 activity results essential for their correct deployment [71,72]. In this context, system dysregulation caused by bacterial pre-growth in metal-rich medium leads to a generalized downregulation of the whole locus, which in turn affects iron homeostasis and reduces survival in macrophages [73]. On the contrary, transposon-mediated loss of ESX-3 effectors is documented to be bypassed through the compensatory amplification of proper iron-uptake genes [72]. Further modulation of ESX-3 effector function is most likely mediated by the MycP3 protease [2,74], but a functional characterization of the protein is still missing.

Additional studies on EsxG/H extracellular dynamics have shown the heterodimer to further oligomerize, generating a Zinc-binding pocket at the interface of two EsxH domains [75]. Yet, no evidence has been reported of ESX-3 secreted effectors functioning as zinc chelators, actively scavenging the metal from the environment, rather than it being more than a structural feature to stabilize the tetramer.

Despite these advances, the precise molecular mechanisms coordinating the hierarchy and spatiotemporal organization of ESX-3 components during host infection remain largely unexplored. Only very recently, cryo-EM studies of *M. smegmatis* ESX-3 homologues have revealed a dimeric architecture of core complex proteins (EccB3 to E3), with EccC3 harboring a flexible array of ATPase domains spanning the membrane, and connected EccB3 being prevalently periplasmic, which suggests that conformational shifts triggered by ATP hydrolysis and substrate engagement are necessary for secretion [76]. Models of how these dynamics evolve under infection-relevant conditions, which would be fundamental to clarify how *M. tuberculosis* balances nutritional demands with immune evasion strategies, are however, still lacking.

3.6. ESX-4: Ancestral Module for Core Physiology

ESX-4 represents the primordial T7SS, identified by phylogenomic surveys to be the first "WXG–FtsK" cluster to traverse the inner membrane, and thus predating the emergence of the mycomembrane complexity

[31,33]. It is in fact preserved across environmental Actinobacteria, non-tuberculous mycobacteria (NTM) where it represents the only universally retained system [77–79], and the MTBC. Findings emerging from the genome-scale analysis of 108 geographically and clinically diverse *M. tuberculosis* isolates show that ESX-4 presents the least sequence variation of all five ESX loci, underscoring its evolutionary constraint and core importance, while higher level of diversity and recombination signatures were detected for its derived paralogues, supporting the idea that maintenance of critical functional motifs across systems happens under diversifying pressures related to host-pathogen interactions [80].

In terms of immune engagement, integrated secretome predictions and cell-surface proteomics indicate the extracellular exposure and antigenic potential of ESX-4 secreted heterodimer EsxU/T, characterized as a stable four-helix structure, revealed to elicit specific T-cell responses in murine immunization assays [81]. In the NTM pathogen *Mycobacterium abscessus,* the initial analysis of a transposon insertion library of mutants provided further proof of the essential roles for core protein EccB4 and EsxU/T in mediating survival, and profile indirect signaling roles in governing global changes in stress-response genes, later confirmed with subsequent zebrafish infection studies [78,82]. Recent reports have further determined that deletion of ESX-4 in *M. abscessus* impairs phagosome escape and immune activation in macrophages, and attenuates bacterial survival and virulence in mice, albeit to a lesser extent than ESX-3 deletion [83].

Although ESX-4 role in *M. tuberculosis* has recently been expanded to nutrient acquisition, with the discovery of its implication in heme-iron uptake via PPE pores [43], and connected to the secretion of the CpnT protein, also known as TNT (Tuberculosis Necrotizing Toxin) [39], no full ESX-4 knockouts exist in *M. tuberculosis*, leaving its full range of functions, regulation and substrate repertoire undefined.

3.7. ESX-5: Bulk and Heterogeneity

The ESX-5 secretion system represents the largest and most functionally varied Type VII machinery in *M. tuberculosis*, supporting both fundamental physiology and virulence. Genetically, the ESX-5 locus spans approximately 15 kb, flanking the canonical core components e*ccB5–eccE5* and *eccC5* ATPase with two dedicated mycosins (*mycP5* and *mycP7*) and a remarkable extension of accessory factors and duplicated clusters that regulate assembly and substrate selection [84]. At the transcriptional level, strong induction of the system is driven by phosphate starvation, with the SenX3–RegX3 two-component system binding to promoter regions upstream of the cluster [85,86]. Excessive activation of the export machinery, due to flawed phosphate sensing, was shown to dampen *M. tuberculosis* virulence in mouse infection models, indicating the importance of accurate secretion for pathogenesis [87].

ESX-5 hallmark is the massive repertoire of exported PE and PPE proteins, whose N-terminal domains serve as modular secretion signals, and are recognized by the cytosolic linker-2 segment of EccC5 in a species-specific way, as demonstrated by bioinformatics analyses [88,89]. PPE helices are then clamped by the EspG5 protein [90], and peptide-chaperone complexes are bound by the ATPase N-terminal pocket and translocated through the inner membrane channel [91].

Proteomic surveys and genetic-fusion experiments demonstrate that these short Gly-X-Gly-containing PE/PPE tags direct lipases (e.g., LipY), cell wall remodeling enzymes, and heterologous reporters to the cell surface, where they mediate outer-membrane permeability and nutrient acquisition via lipolysis [92,93], as well as cytokine production and antigen presentation [94]. Additionally, reconstruction of minimal ESX-5 complexes in synthetic liposomes has revealed that PPE proteins directly contribute to the pore assembly, redeeming them as active structural components rather than mere cargo [95].

Importantly, while Tet-induced conditional knockdown of *eccB5–eccC5* in *M. tuberculosis* causes cell wall defects that abrogate growth, confirming ESX-5 as essential for viability [96], lower porosity of the outer membrane is demonstrated with cryo-EM structures in *M. marinum* and associated with attenuated virulence in ESX-5-defective mutants [13]. In this species, particularly, ESX-5-dependent secretion has been shown to orchestrate immune interactions by driving inflammasome activation and pyroptotic death in infected macrophages, a divergent response to ESX-1-mediated apoptosis [1,97]. Importantly, a recently optimized proteomic approach combining AspN protease and trypsin digestion has enabled comprehensive profiling of *M. marinum* PE/PPE proteome and general ESX-5 secretome, substantially increasing coverage and revealing potential PE/PPE interactions and novel functions, thus providing a robust strategy for systematic effector identification [98].

4. MmpL Transporters: Beyond Protein Secretion

Alongside classical and specialized protein export pathways, *M. tuberculosis* relies on the Mycobacterial Membrane Protein Large (MmpL) family of transporters, dedicated to the export of lipids and small molecules

critical for cell envelope remodeling, immune evasion and survival, thereby shaping the pathogen's surface architecture and pathogenic potential.

MmpLs constitute a unique subclass of the Resistance-Nodulation-Division (RND) superfamily, a group of integral membrane permeases widely conserved among diderm bacteria. Characterized by twelve transmembrane helices and large periplasmic loops, these complexes couple proton pumping to substrate export across complex cell envelopes [14,16]. In mycobacteria, MmpLs have diverged from the broader RND machineries (e.g., *E. coli* AcrB multidrug efflux system) to specialize in the translocation of large hydrophobic molecules such as cell wall building blocks (e.g., mycolic acid precursors, phthiocerol dimycocerosates (PDIM), phenolic glycolipids (PGL), sulfolipids) and siderophores, in an evolutionary innovative path that tailored RND structures to the unique demands of mycobacterial lipid physiology [14–16,99]. More precisely, maximum likelihood trees built from core transmembrane and periplasmic segments sequence alignments demonstrate that MmpLs form a monophyletic branch originating from a single ancestral RND, with sub-branching corresponding to functional specializations. For instance, the prototypical MmpL3 retains a tight channel for trehalose monomycolate (TMM) flipping, MmpL4 and MmpL5 have evolved enlarged periplasmic domains for (carboxy)mycobactin handling, and MmpL7 has acquired a specialized "lid" domain to shuffle bulky PDIMs [14–16].

4.1. Genetic Architecture and Transcriptional Control

The eleven *mmpL* genes of *M. tuberculosis* are scattered across the chromosome, often co-localized with cognate accessory lipoprotein-encoding *mmpS* partners, serving both as chaperones to stabilize extracellular loops and as adaptors, ensuring proper transporter folding and oligomerization, and linking export activity to cell-envelope assembly [14,16]. Indeed, co-expression of the *mmpL5/S5* tandem within the mycobactin-export locus is essential for MmpL5 trimerization and drug-efflux activity [100]. Furthermore, cognate *mmpL/S* genes are often flanked by lipid biosynthesis clusters, unlike typical drug-efflux RND pumps, highlighting syntenic rearrangements that coupled transporter evolution to lipid-synthesis functions [14,15].

At the evolutionary level, early comparative studies established that while core MmpL3 remains highly conserved from NTM species to specific *M. tuberculosis* lineages, other family members display remarkable genomic plasticity, with variable gene content and sequence polymorphisms that correlate with strain-specific virulence factors [15]. Moreover, focused pan-genome analyses performed on drug-resistant MTBC isolates identified recurrent promoter variants and internal duplications, frameshifts, and polymorphisms that likely modulate substrate-binding pockets and contribute to variable drug responses, thus correlating the evolution of transporter specificity with separate antimicrobial pressures [101].

Concomitantly, transcriptional control of *mmpL* expression involves multiple stress-responsive systems and local regulators. For example, the PhoP/PhoR two-component system, a master coordinator of lipid metabolism and virulence, directly influences *mmpL8* expression under acidic and low magnesium conditions, linking sulfolipid export to host-adaptive envelope remodeling [102]. Additionally, the MarR-family transcription factor Rv0678 has been demonstrated to inhibit *mmpL5/S5* transcription, a mechanism derepressed in drug-resistant *M. tuberculosis* isolates, enabling inducible efflux activity against drugs like bedaquiline and clofazimine [103,104]. Similar regulatory pathways have been recently identified in *M. abscessus*, regarding MmpL4 and MmpL5 homologues, whose inactivation is reported to increase resistance to clofazimine and ethionamide [105,106].

4.2. Regulatory and Structural Modulation

Beyond transcriptional regulation, post-translational control of MmpLs activity is mediated allosterically through phosphorylation/dephosphorylation cycles, fine-tuning secretion to environmental cues and independently of direct gene expression changes. As evidence, MmpL3 action is significantly inhibited by phosphorylation by the Ser/Thr kinase PknB, with dephosphorylation by cognate phosphatase PstP restoring activity [107], and similarly, phosphorylation of specific residues in the cytoplasmic C-terminus of MmpL11 is reported to negatively impact the transporter stability. Interestingly, the same modification on the D1 periplasmic loop and conserved Tyrosine residue (Y610) in TM10 transmembrane helix has the opposite effect of allowing MmpL11 function [108].

Co-immunoprecipitation studies on *M. smegmatis* MmpL3 interactome, followed by mass spectrometry, allowed to map robust interactions between the transporter and critical cell-cycle regulators, such as the polar scaffold protein Wag31, which directs new cell-wall synthesis, and the cell division regulatory protein CrgA [109]. Specifically, MmpL3 had been previously shown to localize at the growing poles and septum, and participate dynamically to lipid transport in conjunction with cell wall *de novo* synthesis [110]. In the same context, specific functional assays revealed that MmpL3 loss leads simultaneously to mislocalization of Wag31 and a decrease in

peptidoglycan cross-linking, implying how TMM export coordinates with peptidoglycan deposition during cell elongation to ensure envelope integrity and proper cell division [109].

Described modalities of spatial organization and post-translational regulation of MmpLs transporter activity are schematized in Figure 3.

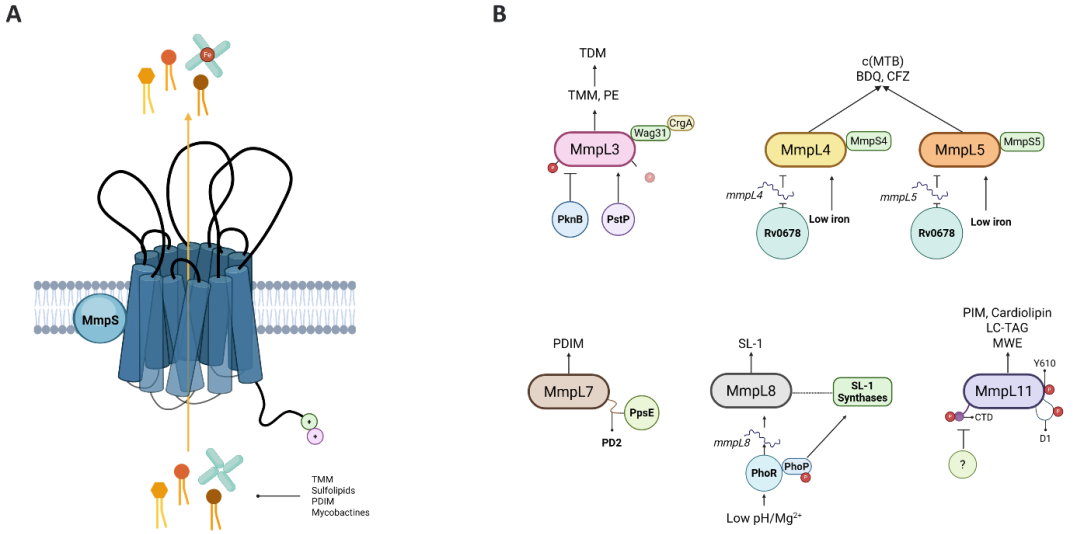

Figure 3. MmpL transporters in mycobacteria: spatial organization and regulatory control. MmpL transporters are integral membrane proteins of the RND superfamily that translocate diverse lipid substrates across the mycobacterial inner membrane. (**A**) Each monomer contains twelve transmembrane helices and large periplasmic loops that form substrate-binding chambers, with cytoplasmic C-terminal extensions modulating activity and interacting with accessory proteins. MmpL complexes typically function as trimers, often stabilized by cognate MmpS lipoproteins. Substrates are captured via alternating inward- and outward-facing conformations and released into the periplasm or cell wall. Specific periplasmic "lid" domains and gating loops regulate substrate access, coordinating lipid export with cell wall assembly and virulence factor display. (**B**) Distinct regulators modulate MmpL transporters' activity. Transcriptional control includes the low pH/low Mg^{2+} sensing two-component system PhoP-PhoR, which induces *mmpL8* and SL-1 synthases coding genes transcription, and the *mmpL4/5* inhibitory transcription factor Rv0678. Post-translational modifications include phosphorylation by Ser/Thr kinases, whose effect can be either stimulatory (MmpL11) or inhibitory (MmpL3, MmpL11). Direct protein-protein interactions (MmpS, Wag31, PpsE) are also involved in modulating transporter activity. MmpL/S: mycobacterial membrane protein large/small, P: phosphate group; PknB: protein kinase B; PstP: Ser/Thr phosphatase P; Wag31: cell division protein; CrgA: cell division protein partner; PhoP/R: two-component system (P: activator, R: sensor kinase); PpsE: phenolphthiocerol/phthiocerol polyketide synthase subunit E; DrrABC: ATP-binding cassette transporters; LppX: lipoprotein X; TMM: trehalose monomycolate; TDM: trehalose dimycolate; PE: phosphatidylethanolamine; c(MBT): carboxy(mycobactin); BDQ: bedaquiline; CFZ: clofazimine; PDIM: phthiocerol dimycocerosates; SL-1: sulfolipid-1; PIM: phosphatidylinositol mannosides; LG-TAG: long-chained triacyl-glycerols; LWE: mycolate wax esters. Created with Biorender.com.

4.3. The Essential Trehalose Monomycolate Flippase: MmpL3

The prototypical transporter, MmpL3, is the only member of the MmpL family to be designated as non-redundant and indispensable for *M. tuberculosis* viability, as verified by genetic inactivation attempts that failed to produce viable mutants [15].

It acts as a TMM flippase that translocates mycolic acid conjugates to the periplasmic leaflet, enabling subsequent incorporation into free-mycolate arabinogalactan-peptidoglycan layers by secreted mycolyltransferases (antigen 85 complex members, Ag85A/B/C) and the in situ generation of the virulence-associated free lipid trehalose dimycolate (TDM), also known as cord factor [14,16]. Substrate preference and accommodation in MmpL3 tight vestibule was confirmed with native mass spectrometry studies performed in *M. smegmatis*, demonstrating high-affinity binding of TMM and phosphatidylethanolamine (PE) [111]. Subsequently, the inward- and outward-facing states of the twelve-helix channel bundle were resolved through Cryo-EM and X-ray structures, revealing alternating conformations that mediate substrate capture and release, and supporting computational modelling analyses further elucidated gating helices and periplasmic exit pathways [112]. More

recently, biophysical elucidation of MmpL3 C-terminus revealed a flexible unstructured linker connecting the globular domain to the last transmembrane helix, possibly allowing its adjustment during lipid transport across the membrane. Moreover, the study identified positively charged residues, such as lysine and arginine, possibly interacting with polar membrane components or other associated proteins, to help stabilize the overall transporter structure. Similar features were also reported for MmpL11, hinting at a possible conserved structural arrangement trait that may be essential for transporter function and thus targeted for inhibition [113].

Initial evidence of MmpL3 inhibition came through the experimental screening of a series of non-tubercular-specific pharmacophores, reported as active against nutrient-starved and hypoxic nonreplicating *M. tuberculosis* [114], followed by the identification of more MmpL3-targeted molecules and their validation as antitubercular drugs [115,116]. Later analysis of the flippase-inhibitor complex structure through innovative near-atomic cryo-EM allowed to map hydrophobic pockets that, when occupied, lock MmpL3 in the inward-facing state, and to further define a focused TMM-mimicking compound library [117,118]. Seamlessly integrating biochemical assays and structural elucidation to computational drug design, the most recent in silico analyses eventually led to the validation of novel chemical scaffolds predicted to target the channel with sub-micromolar potency [119].

4.4. Siderophore Export: MmpL4 and MmpL5

Primarily responsible for the cross-membrane shuffling of lipophilic mycobactin (MBT) and hydrophilic carboxymycobactin (cMBT), MmpL4 and MmpL5 are positioned in a distinct sub-clade of exporters, separate from the TMM-flipping MmpL3 but closer to MmpL6/7 orthologs found in rapid growers, indicating functional conservation among pathogenic mycobacteria [14,16]. Their shared architecture is characterized by sizable periplasmic domains accommodating the amphipathic siderophores. In fact, Cryo-EM and MD simulations have recently highlighted conserved aromatic/polar residues and TM8 loop movements forming gating constrictions likely to regulate chamber access during transport [120]. Moreover, as mentioned, co-expression with accessory lipoprotein partners *mmpS4* and *mmpS5* is required for stable trimerization of the complexes and proper development of their periplasmic chambers, and is suggested to facilitate siderophore release [100,121]. Recent Cryo-EM studies have provided direct evidence for this model, revealing that MmpL5 associates with MmpS5 and the small cytoplasmic acyl carrier protein AcpM in a 3:3:3 stoichiometry, spanning the entire cell envelope [122]. As reported, AcpM stabilizes the transporter, and siderophore passage is then mediated through a narrow transmembrane channel lined by conserved aromatic and polar residues [123]. Loss of MmpS5, as demonstrated, leads to inefficient siderophore export and thus to higher susceptibility under iron-limiting conditions. Independently, increased sensitivity to bedaquiline and clofazimine is also recorded, confirming MmpL4/L5's additional role as efflux pumps and connecting their impairment to drug resistance phenotypes [14,100].

4.5. Virulence-Associated Transporters: MmpL7, MmpL8, MmpL11

MmpL7 serves as the dedicated transporter for phthiocerol dimycocerosates (PDIMs), long-chain, branched fatty esters uniquely found in the outer layer of virulent MTBC species, and plays valuable roles in phagosome escape and immune modulation [14,16,57,124]. While not essential for in vitro growth, MmpL7 knockouts result in significantly attenuated infection in murine models [15]. Evidence of the tight coordination between the MmpL transporter and its cargo is reported: genetically, the *mmpL7* locus is co-localized with PDIM biosynthesis genes (e.g., *pks1–15* and *papA5*), and transporters *drrABC* and *lppX*, thereby coordinating lipid assembly and export [16,125,126]; biochemically, the periplasmic PD2 insert has been demonstrated to directly interact with the PDIM synthase PpsE, again suggesting functional coupling between synthesis and transport [127]; and mechanistically, critical transmembrane loops TM4 and TM10 and periplasmic PD2 insert constitute a “lid” domain in MmpL7 that guides PDIM passage, supporting a model consistent with transfer from the inner leaflet where the lipids accumulate, and outer-membrane display [128].

Sulfolipid-1 (SL-1), whose precursors are exported by MmpL8, is a tetraacylated trehalose sulfoglycolipid uniquely produced by virulent *M. tuberculosis* strains. It was shown to dampen host immune responses by inhibiting both production of proinflammatory cytokines (e.g., TNF-α, IL-12) and TLR2 receptor signaling; and vice versa, enhance cell wall impermeability, improving resistance to antibiotics and other stress factors [14,124]. In confirmation, deletion of *mmpL8* directly reduces SL-1 surface levels, indicating biochemical correlation between substrate synthesis and transport [129], and is demonstrated to impair granuloma formation and alter cytokine responses in *M. abscessus*-infected zebrafish [130]. Linking SL-1 export to environmental cues, transcription of *mmpL8* and other genes involved in sulfolipid synthesis (e.g., *pks2*, *papA1*) is reportedly modulated by the PhoP/PhoR system under acidic and magnesium-limited conditions [102].

MmpL11 facilitates the export of specific phospholipid species, such as phosphatidylinositol mannosides (PIMs) and cardiolipin, that contribute to cell-wall fluidity and integrity under stress conditions, long-chained triacyl-glycerols (LC-TAG) and mycolate wax esters (MWE), important for biofilm formation and persistence of infection [14,15,99,131]. Notably, transcription of its coding gene is upregulated during hypoxia and nutrient limitation, specifically in non-replicating populations, suggesting a role for phospholipid redistribution in maintaining membrane homeostasis during persistence [131]. Moreover, deletion of *mmpL11* impairs biofilm integrity both in *M. tuberculosis* and in the model organism *M. smegmatis*, leading to thinner, fragile pellicles with reduced extracellular matrix [131,132]. From a structural perspective, conserved hydrophobic pockets and basic residues have been predicted on MmpL11 periplasmic loops, compatible with binding negatively charged phosphatidylinositol headgroups [133], though complete high-resolution structures remain unavailable.

5. Interplay Among Secretion Systems in *Mycobacterium tuberculosis*

5.1. Mechanisms of Functional Cooperation

The intricate network of secretion complexes employed by the tubercular pathogen to translocate proteins and lipids across its uniquely structured cell envelope represents a highly coordinated functional landscape, whereby the precisely regulated export of essential viability factors and virulence determinants is synchronized to promote adaptation to hostile microenvironments and facilitate survival during host immune engagement. Beyond the activity of individual systems, secretion is driven by the interplay between protein export machineries and MmpL transporters. While Sec, Tat, and ESX systems deliver protein effectors across the inner membrane, MmpLs mediate the export of lipids and cofactors that frequently provide the proper context for substrate localization and function at the cell surface. This interdependence extends to the transcriptional level, as summarized in Table 2: environmental cues such as nutrient limitation, metal availability, or host-imposed stresses activate overlapping regulatory networks that coordinate the expression of both groups of transporters. As schematized (Figure 4), such integrated regulation ensures that protein and lipid secretion are jointly tuned to optimize virulence, cell envelope remodeling, and immune evasion.

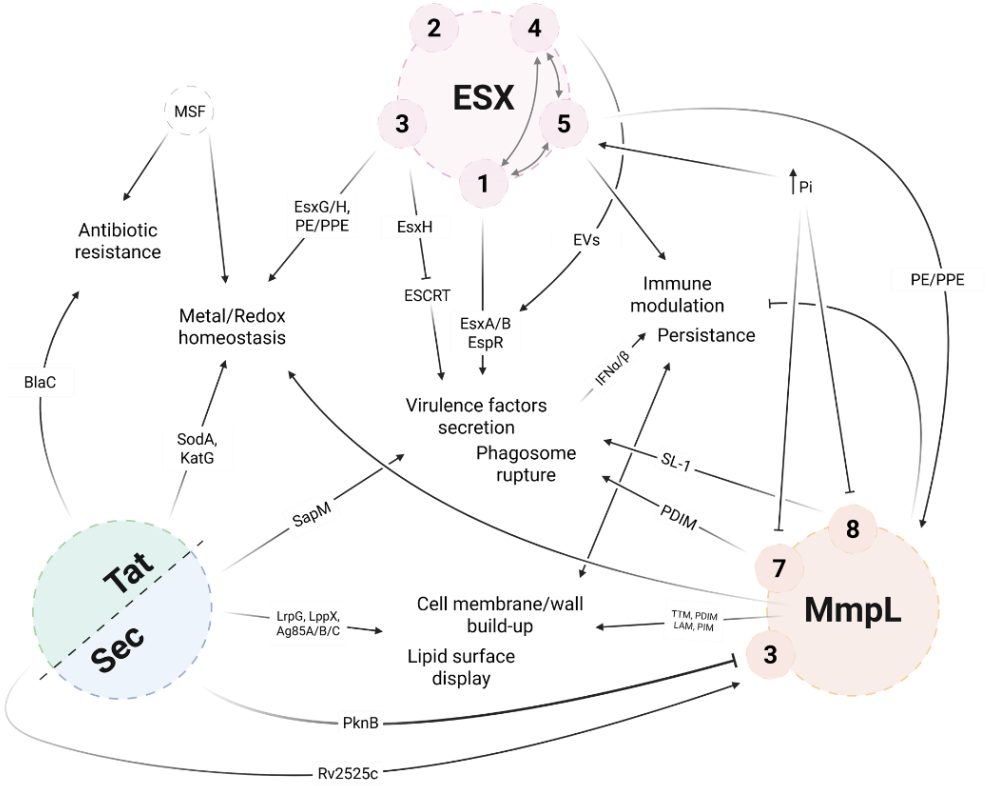

Figure 4. Functional interplay among the main secretion systems of *Mycobacterium tuberculosis*. The canonical Sec and Tat pathways cooperate with MmpL lipid transporters and Type VII secretion systems (ESX) to orchestrate the export of essential proteins and lipids. The concerted activity of these systems, through their secreted effectors, drives key pathogenic events, including phagosome rupture, suppression of immune responses, and establishment of chronic infection niches. Created with Biorender.com.

Table 2. Transcriptional regulation of *Mycobacterium tuberculosis* secretion systems.

Environmental Stimuli	Transcriptional Regulator(s)	Target System/Genes	Functional Outcomes
Macrophage uptake	EspM / EspN (antagonistic TFs)	ESX-1 locus	Dynamic regulation during phagocytosis; balanced effector production
Dendritic cell uptake	Undefined	ESX-2 locus	Induction upon dendritic cell, not macrophage phagocytosis; correlation with immune modulation and disease severity
ATP depletion, nutrient limitation, oxidative stress in phagosome, hypoxia	EspI (ATP-sensing repressor)	ESX-1 locus	Repression relieved under stress → induction of secretion, "emergency response"
	Undefined	MmpL11	Phospholipid redistribution, maintenance of membrane homeostasis, biofilm formation
Chromatin structure, promoter variation	WhiB6, EspR	*espA, espC, espD* (ESX-1 locus)	Activation of ESX-1 expression; increased secretion; EspR promoter mutations → enhanced secretion, isoniazid resistance, fitness gain
Iron/Zinc limitation	IdeR (iron-dependent repressor)	*eccB3–eccE3, esxG/H*	ESX-3 induction: → iron uptake via mycobactin/irtA/B system
	Zur (zinc-responsive regulator)		
Phosphate starvation	SenX3–RegX3 TCS	ESX-5 locus	Strong induction; promotes PE/PPE secretion and nutrient acquisition. Co-regulated secretion and lipid transport; influences ESX-1-mediated phagosome damage
		MmpL7/MmpL8	
Antibiotic pressure	Rv1258c (MFS transporter, transcriptional modulation)	*eccD3* (ESX-3 core)	Links iron metabolism, redox balance, and drug persistence
	Rv0678 (MarR family TF)	MmpL5/S5	Inducible efflux of drugs such as bedaquiline and clofazimine

Note: *M. tuberculosis* coordinately modulates the expression of protein and lipid secretion complexes, ensuring integration between effector secretion, cell wall remodeling, and host immune modulation. For the main environmental stimuli encountered by the pathogen during infection, the corresponding transcriptional regulators, target systems, and associated functional outputs are summarized.

The Sec pathway is responsible for the translocation of unfolded proteins, including many lipoproteins and enzymes involved in cell wall biosynthesis [2,3,7]. Sec-exported LrpG and LppX, for instance, work in tandem with MmpL transporters to translocate and properly arrange lipoarabinomannans (LAMs), phosphatidylinositol mannosides (PIMs), and PDIMs on the cell surface [3,14,126,134]. Cell wall integrity is further maintained by the membrane-embedded serine/threonine kinase PknB, secreted via the Sec system and suggested to be a negative modulator of MmpL3, as mentioned [107].

The Tat pathway, in contrast, mediates the export of a well-assorted cargo of fully folded proteins, covering disparate roles including redox homeostasis (e.g., KatG), antibiotic resistance (e.g., BlaC), and cell wall remodeling [2,3,6,7]. Importantly, the specific functions of many of these effectors can be influenced by the cell envelope state itself, which depends in part on Sec-dependent appropriate lipid modification and surface anchoring of Sec-secreted lipoproteins (e.g., LrpG, LppX, Ag85A/B/C) [3,14]. In a similar way, Tat putatively influences MmpL3-mediated translocation of TMM to the cell wall by maintaining a balanced peptidoglycan deposition, for which the Tat-secreted hydrolase Rv2525c is suggested to be indispensable [17].

As the most ancestral T7SS locus in mycobacteria, ESX-4 is proposed to play a foundational role in the evolution of ESX network interdependence [31,33]. Recently, evidence has been obtained in *M. marinum* and *M. abscessus* that *eccC4* deficiency results in a diminished or null export of ESX-4 substrate heterodimer EsxT/U, and conversely in a heightened export of ESX-1 and ESX-5 substrates [135,136]. Similarly, it was shown that ESX-4 directly cooperates with ESX-1 virulence by mediating the release of extracellular vesicles (EVs), which enhance host delivery of the EsxA/B pair [137,138]. While the management of PE/PPE substrates is classically partitioned among ESX systems, some overlap in substrate routing and chaperone specificity exists. Domain-swapping experiments in *M. marinum* have demonstrated that altering EspG binding regions can redirect PE/PPE substrate secretion, revealing a degree of chaperone plasticity within the systems [88,139]. However, structural studies confirm that native EspG chaperones exhibit high substrate specificity [65], and broad interchangeability

in natural settings has not been established. Additionally, the interaction between PE/PPE proteins and lipid transporters such as MmpLs appears to influence effector localization. In *M. marinum*, modifying the linker-2 region of the ESX-5-associated ATPase EccC5 altered membrane lipid composition and redirected secretion of the typically ESX-5-dependent PE31/PPE18 pair, suggesting that membrane environment can influence substrate routing [139]. Nevertheless, previous suggestions that perturbation of MmpL3 affects PE_PGRS33 localization [93] were based on analysis of PE domain deletions rather than direct manipulation of MmpL3 and thus do not establish a causal role for the transporter in periplasmic accumulation.

Phosphate deprivation represents an ulterior mechanism for co-regulation. It primarily acts on ESX-5 by enhancing secretion of PE/PPE couples that mediate host interaction and nutrient acquisition [1,2,85,86]. Upregulation of ESX-5 leads, however, to the altered translocation of SL-1 and PDIMs by MmpL7 and MmpL8, which may in turn impact ESX-1-mediated phagosome damage [57]. In general, it is reported how different environmental signals, such as hypoxia, nutrient depletion, and oxidative stress, dynamically alter the expression of both T7SS and MmpL genes, ensuring the fine adaptation of *M. tuberculosis* secretion landscape during infection [1,124]. Simultaneously, virulence factor secretion is closely coordinated with MmpL-mediated continuous envelope remodeling, necessary for both damage repair and the correct surface display of effectors [14,16].

In addition, ESX-3 functions as a central hub, integrating iron metabolism, redox homeostasis, and drug persistence in mycobacterial species [34]. In fact, beyond its canonical role in iron acquisition via PE-PPE substrates such as PPE4, ESX-3 interacts closely with efflux pumps like Rv1258c of the Major Facilitator Superfamily (MFS) and MmpL proteins [140]. Rv1258c was in fact shown to modulate the expression of core ESX-3 components (e.g., EccD3) and proposed to influence the formation of iron-specific channels, directly linking metal metabolism to intracellular redox balance. Concurrently, MmpL transporters such as MmpL6 contribute to oxidative stress responses and maintenance of membrane potential, suggesting that ESX-3, MFS/MmpL transporters, and antioxidant systems act synergistically to support bacterial survival under hostile conditions and enhance resistance to antibiotics such as bedaquiline [140,141].

5.2. Specific Impact on Immune Evasion

The concerted action of *M. tuberculosis* secretion systems exerts a broad influence on host immune functions, modulating cytokine production, antigen presentation, phagosome maturation, and leading eventually to macrophage apoptosis. Collectively, these systems orchestrate a multifaceted immune escape strategy that not only facilitates the establishment of the initial infection but also sustains long-term persistence.

As discussed, phagosome arrest and disruption exemplify systems crosstalk: ESX-1 principal secreted heterodimer EsxA/B mediates membrane rupture [8–10], an effect further enhanced by MmpL7-exported PDIMs [57] and complemented by SapM, a SecA2-translocated phosphatase [5] which disrupts the signaling pathway required for phagosome maturation and subsequent lysosome fusion [142]. In parallel, as mentioned, ESX-3 effector EsxH indirectly aggravates phagosome damage by inhibiting ESCRT-guided membrane mending [64].

Beyond this fulcrum of inter-system coordination, several more examples emerge in the context of immune evasion strategies. When inside the phagosome compartment, *M. tuberculosis* encounters harsh environmental conditions, marked by acidic pH, depletion of nutrients, reactive oxygen and nitrogen species, and diverse antimicrobial agents. In these conditions, iron-responsive ESX-3 secretion [11,12] and Sec-mediated export of SodA [4] are indispensable for metabolic adaptation and modulation of the oxidative burst.

Post cytosolic escape, MmpL-exported lipids such as SL-1 and trehalose polyphleates (TPPs) dampen TLR2-mediated responses by masking superficial PAMPs and reprogram macrophage metabolism [16,124], concurring with EsxA/B and ESX-5 secreted PE/PPE pairs in disrupting antigen presentation pathways [56,143]. Resulting in lower levels of proinflammatory cytokines, the effect is further sustained by type I interferon (IFNα/β) production, initiated by bacterial DNA sensing when it becomes available through ESX-1-mediated phagosome perforation [41].

Overall, the synergy between protein and lipid secretion systems enables the pathogen to actively remodel its envelope, interfere with innate and adaptive immune responses, and establish a chronic infection niche.

6. Therapeutic and Diagnostic Implications

The growing global burden of tuberculosis, bolstered by the rise in multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of the tubercular pathogen, has prompted intensified efforts to uncover novel therapeutic targets and diagnostic strategies. Central as they are to the bacterium's pathogenicity and adaptability, secretion systems and their effectors are increasingly recognized as promising targets for therapeutic intervention, biomarker discovery, and vaccine development.

6.1. Antimicrobial Discovery

Each secretion system offers unique structural and functional features that can be exploited pharmacologically. The Sec and Tat pathways are foundational protein export machineries, encompassing essential components whose limited redundancy marks them as promising therapeutic candidates. For instance, selective targeting of LepB, an indispensable Sec-associated type I signal peptidase, has allowed to identification of phenylhydrazones as novel compound scaffolds with minimal mammalian cytotoxicity [144], while characterization of Tat-exported BlaC [6] has informed novel therapeutic strategies leveraging first and second-generation β-lactamase inhibitors (BLI), such as clavulanate and diazabicyclooctane (DBO) derivatives aimed at restoring β-lactam efficacy against *M. tuberculosis* [145,146].

The T7SS family, particularly ESX-1, is deeply implicated in virulence through phagosome escape and immune modulation [26,34,147]. Inhibiting ESX-1 secretion attenuates virulence without exerting bactericidal pressure, a principle underlying recent drug discovery efforts. Examples are the small-molecule inhibitor BTP15, which promotes phagolysosome fusion in infected macrophages and effectively reduces uptake-independent killing of macrophages by mycobacterial aggregates [58], mainly preventing ESX-1 effectors secretion [148], or the benzofuran-based IMB-BZ, which specifically disrupts EsxA, EspA, EspB and EspC secretion [149]. In parallel, a new class of benzothiazole-derived ethionamide boosters has been described for their concurrent inhibitory activity on ESX-1 function [150]. Along these lines, new evidence has emerged from a recent study on the ESX-5 system of *M. marinum*, whose in vitro activity is only partially inhibited by treatment with novel 1,2,4-oxadiazole scaffolds, yet results in a significant reduction of bacterial burden in zebrafish infection [151].

ESX-3, crucial for metal and redox homeostasis and survival under host-induced nutritional immunity [11,12,34,140], also represents a druggable target, and has recently been explored for ivermectin repurposing, with molecular docking studies supporting the inhibitory binding of the antiparasitic drug to the essential core protein EccD3 [152]. Extending this perspective, *M. abscessus* growth under iron-limiting conditions is reported to be severely impaired but not lethal when ESX-3 is dysfunctional. Intriguingly, this state confers increased persistence to bedaquiline, likely due to reduced succinate dehydrogenase activity and consequent TCA cycle perturbation [141].

Among the MmpL family, MmpL3 is a prominent drug target due to its essential role as a TMM-flippase, crucial for mycolic acid incorporation and thus for viability [99,136]. Structural elucidation has allowed the screening, identification, and rational design of potent inhibitors, including thiazolidinones and indole derivatives, and their inclusion in drug discovery pipelines [115–117,119]. Complementing these efforts, recent studies suggest that modulating protein stability and oligomerization, such as the conserved C-terminal regions in MmpL3 and MmpL11 [113], or coiled-coil–driven assembly of MmpL10 [153], may provide an alternative therapeutic strategy.

Though direct experimental trials remain limited, synergistic approaches combining secretion systems-targeting drugs and host-directed therapies have been suggested as emerging strategies to bypass resistance and amplify immune responses. For instance, inhibition of ESX-1-driven type I IFN production, known to increase pathological inflammation [147], could be paired with immunomodulators targeting the same axis. Similarly, leveraging host immune mechanisms with phagolysosome fusion or autophagy booster candidates could synergize with direct inhibitors of ESX-1 or MmpL7 [154]. Additionally, inhibitors of cell envelope biogenesis-related systems, such as MmpL3, may also potentiate the efficacy of antibiotics characterized by low permeability, while inhibition of metal scavenging systems or effectors, such as ESX-3, could potentially synergize with iron chelating therapies [11,12,152], though directed co-treatment data remain limited.

6.2. Diagnostic and Treatment Efficacy Biomarkers

Several secreted proteins from the ESX and Sec pathways have been investigated for their diagnostic potential. EsxA and EsxB are already vastly used in Interferon Gamma Release Assays (IGRAs) to diagnose *M. tuberculosis* infection from blood samples [155], but other immunogenic proteins consistently expressed during infection, including Ag85 complex components, the CpnT toxin, and MPT53, could also be exploited as host-pathogen interaction (HPI) biomarkers [156]. Recent advances in rational antigen selection have been enabled through bioinformatics-guided profiling of secretion signatures coupled with mass spectrometry and immunoproteomic analyses that have further enabled the identification of novel extracellular candidates with high host-specific response rates [157], and with refined MHC epitope-binding predictions that have allowed the selection of antigenic candidates with diagnostic and vaccine relevance [158].

Exported antigen levels can moreover serve as biomarkers for treatment monitoring, facilitating early detection of therapeutic failure/success or emergence of resistance. For example, dynamic changes in EsxA/B levels in blood or sputum have been suggested to correlate with bacterial load reductions post-treatment [159,160], while monitoring the expression of drug efflux pumps such as MmpL5 could contribute to detecting cross-

resistance events [14,161]. Similarly, the identification of mutations within MmpL transporter coding genes may help predict potential resistance events, as recently demonstrated by whole genome sequence studies on *M. abscessus* strains resistant to oxazolidinones such as linezolid and tedizolid [161].

6.3. Vaccine Engineering

The BCG vaccine, first developed in the early 20th century, remains to date the only licensed vaccine against Tuberculosis, yet its limited efficacy has prompted extensive investigation into its underlying mechanisms and potential improvements. Deletion of the EsxA/B-encoding RD1 region, while central to BCG's safety, also compromises its immunogenicity, as evidenced by recombinant vaccine strains re-expressing ESX-1 major antigens, which showed enhanced protection in murine infection models [25,27,28].

A new generation of TB vaccine candidates, rationally designed to overcome BCG's limitations and exploiting the immunostimulatory capacity of ESX substrates and membrane-associated proteins, has since been described. Early studies demonstrated that multivalent DNA vaccines broadly targeting the ESX family elicit potent T-cell responses and better prevent immune escape in case of antigen mutation [162], an effect later reproduced with the Esx-5a subunit vaccine, composed of a fusion protein combining five ESX-5-associated antigens, able to elicit robust protection in challenge models [163].

In parallel, a significant advancement in rational TB vaccine design was achieved with the development of the live-attenuated *M. tuberculosis* strain MTBVAC [164], currently undergoing Phase 3 clinical trials [165], that retains an intact ESX-1 system but carries targeted deletions of major virulence genes *phoP* and *fadD26.* Further engineering of this model for the expression and secretion of heterologous non-tubercular antigens has not only confirmed it as a valid TB-specific vaccine candidate, but also as a flexible platform for next-generation combination vaccines [166].

Current additional strategies focus on incorporating soluble extracellular regions of MmpLs (SERoMs) as antigens in subunit vaccines, which show partial protection in mice preclinical models and suggest the feasibility of targeting transmembrane lipid transporters [167]. Interestingly, T and B cell multiepitope MmpL vaccine candidates have been recently designed against *Mycobacterium ulcerans*, showing stable in silico interactions with Toll-like receptors and theoretical immunogenic potential, though experimental validation as well as the potential translation to the tubercular pathogen are pending [168].

7. Research Gaps and Future Directions

Secretion systems are among the most complex and dynamic facets of mycobacterial physiology and pathogenesis, and yet, despite decades of investigation, critical knowledge gaps remain that greatly limit our capacity to predict, manipulate, and exploit them.

Interdependence among systems, promoted by an intricate regulatory crosstalk which is central to their ability to interact and compensate for each other in a holistic effort to deliver effectors, scavenge nutrients, and remodel host immunity, remains to date poorly characterized, and often inferred. In this context, time-resolved transcriptomic and proteomic profiling under defined stress conditions would help reveal the signal transduction networks governing Sec, Tat, T7SS, and MmpLs coordination, and highlight essential regulatory nodes (e.g., PknB-mediated MmpL3 regulation [107]) for targeted genetic and biochemical dissection. Direct manipulation of individual transporters could also be undertaken to define their causal roles in trafficking and cross-system synergies.

Similarly, no proteinaceous pore analogous to SecYEG, TatA, or Ecc-dependent channels has been demonstrated in the cell outer layer, specifically responsible for protein secretion in mycobacteria [2], representing a key gap in defining how proteins translocated to the periplasm ultimately exit the complex diderm envelope. Correspondingly, the dynamic choreography of MmpL substrate hand-off, from inner-membrane flippase to periplasmic carriers and outer-membrane assembly machineries, remains uncharted. Addressing these limitations will require integrating high-throughput proteomic/lipidomic studies with advanced cryo-EM visualization in native lipid environments, which in parallel would also refine our mechanistic knowledge on how these systems assemble and disassemble in accordance with *M. tuberculosis* developing needs during pathology progression.

Incidentally, the system's finer regulation in response to ever-changing environmental stimuli and during different stages of the infection process is also not entirely understood, and proper characterization of downstream signaling cascades or accessory regulatory effectors is often missing [1,124]. Nonetheless, these aspects remain very promising to appreciate mycobacterial adaptability and resilience, and their thorough assessment may shed light on alternative therapeutic targets.

In this perspective, the indispensable roles of many of these systems' components (e.g., SecA1 [4], TatA/B/C [17], ESX-3 [11], EccB5-D5 [96], MmpL3 [15]) as well as their high immunogenic potential or biomarker properties [41,156,163,167], or additional specific roles in drug tolerance (e.g., MmpL5 additional role as drug efflux pump [14]), hold promise for next-generation antimicrobials design, endorsed by continued integration of structural biology, computational discovery, and systems immunology. Notably, recent computational mapping of single-nucleotide variations (SNPs) from clinical *M. tuberculosis* isolates onto ESX-1-related protein structures has further highlighted conserved and functionally critical regions, revealing promising targets for both drug and vaccine development [169]. Such integrative structural-genomic approaches exemplify how computational analyses can effectively guide target prioritization, complement experimental efforts, and accelerate the rational design of next-generation antitubercular strategies.

In synthesis, meticulous examination of current understandings may eventually help outline the conceptual and technical challenges presently hindering research progress and highlight promising avenues for future investigations that could transform the field in both foundational and applied microbiology.

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