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MINIREVIEW

Mycobacterium smegmatis: The Vanguard of Mycobacterial Research

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ABSTRACT The genus *Mycobacterium* contains several slow-growing human pathogens, including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium avium*. *Mycobacterium smegmatis* is a nonpathogenic and fast growing species within this genus. In 1990, a mutant of *M. smegmatis*, designated mc^{2}155, that could be transformed with episomal plasmids was isolated, elevating *M. smegmatis* to model status as the ideal surrogate for mycobacterial research. Classical bacterial models, such as *Escherichia coli*, were inadequate for mycobacteria research because they have low genetic conservation, different physiology, and lack the novel envelope structure that distinguishes the *Mycobacterium* genus. By contrast, *M. smegmatis* encodes thousands of conserved mycobacterial gene orthologs and has the same cell architecture and physiology. Dissection and characterization of conserved genes, structures, and processes in genetically tractable *M. smegmatis* mc^{2}155 have since provided previously unattainable insights on these same features in its slow-growing relatives. Notably, tuberculosis (TB) drugs, including the first-line drugs isoniazid and ethambutol, are active against *M. smegmatis*, but not against *E. coli*, allowing the identification of their physiological targets. Furthermore, Bedaquiline, the first new TB drug in 40 years, was discovered through an *M. smegmatis* screen. *M. smegmatis* has become a model bacterium, not only for *M. tuberculosis*, but for all other *Mycobacterium* species and related genera. With a repertoire of bioinformatic and physical resources, including the recently established Mycobacterial Systems Resource, *M. smegmatis* will continue to accelerate mycobacterial research and advance the field of microbiology.

KEYWORDS distributive conjugal transfer, mycobacterial systems resource, cell envelope, drug discovery, efficient plasmid transformation, leaderless mRNA, mycobacteriophage, mycolic acid, phasmid, virulence factors

Despite the discovery of *Mycobacterium tuberculosis* in 1882 by Robert Koch (1) and its global health burden (2, 3), it is only in the last 30 years that the secrets of its genetic make-up have begun to be revealed and characterized. For example, in 1990, we did not know: (i) the targets of tuberculosis (TB)-specific drugs, (ii) the molecular genetic basis of attenuation of the vaccine strain, *Mycobacterium bovis* BCG, (iii) which genes were essential for *in vitro* and *in vivo* growth, and if these genes were similar to those of other bacteria. The pathogenicity and slow growth − 3 to 4 weeks to form colonies – make *M. tuberculosis* extremely difficult to work with in the laboratory. The seminal breakthrough toward developing genetic approaches to study pathogenic mycobacteria was the isolation of a transformable derivative of *Mycobacterium smegmatis*, designated mc^{2}155. *M. smegmatis* is a nonpathogenic and fast-growing species (colonies in 3 days) and was historically used as a mycobacteriophage host. The development of mc^{2}155 made *M. smegmatis* the model for studying properties of all mycobacteria, including pathogens, such as *M. tuberculosis*

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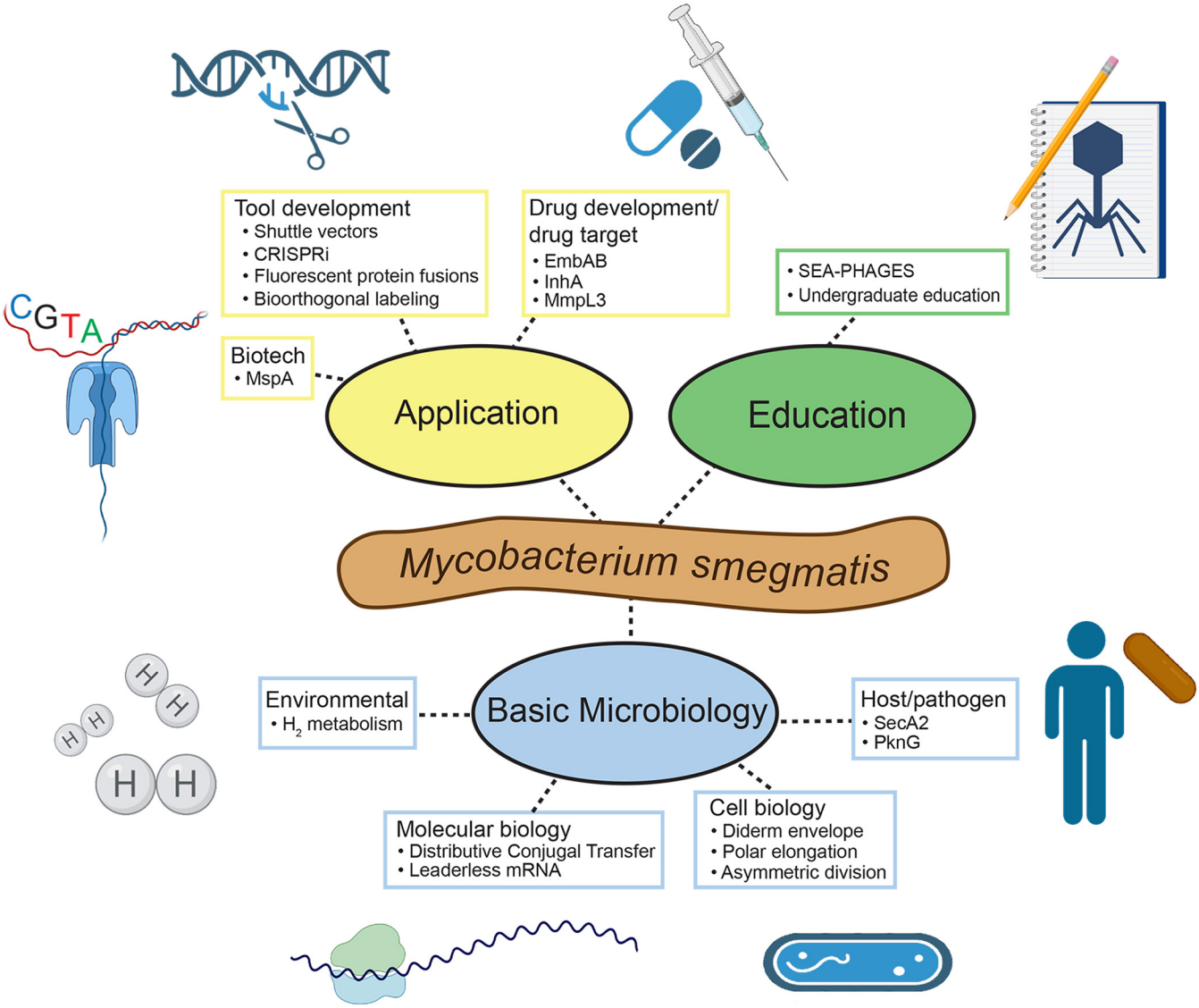

FIG 1 Web diagram of *M. smegmatis'* role as a model organism. *M. smegmatis* serves applied, educational, and basic science in myriad ways. Colored ovals represent key fields that *M. smegmatis* contributes to, while terminal rectangular nodes represent examples of scientific contributions in each field, most of which were discussed in this review. Illustrations were created with BioRender.com.

and the nontuberculous mycobacteria (NTM) pathogens, *Mycobacterium abscessus* and *Mycobacterium avium* (4). This review describes the history of mc^{2}155, its use in developing genetic tools for pathogenic mycobacteria, and its application to characterizing biological mechanisms and targets of TB drugs (Fig. 1). Moreover, this review describes current "omic" approaches pioneered in *M. smegmatis* and now used for unbiased genome-wide discovery of complex multi-faceted processes in medically important mycobacteria.

Note on the proposal to rename *Mycobacterium smegmatis*. We disagree with the recent proposal to rename *Mycobacterium smegmatis* as *Mycolicibacterium smegmatis* (5). We support the editorial by Tortoli et al. (6): (i) the split of the *Mycobacterium* genus has caused unnecessary confusion in health care settings, compromising patient safety, and costing time and money on reeducating health care professionals; (ii) the proposed split was incomplete, not accounting for ~40 species; and (iii) all previously verified taxonomic names are considered valid for use in publication. In addition, the new taxonomic classification was based on conserved signature indels (CSIs) and conserved signature proteins (CSPs). Many CSIs and CSPs may be the result of horizontal gene transfer, which are poor traits for taxonomic classification. Most researchers have chosen to continue using the conventional genus

name after the new classification proposal, indicating that *Mycobacterium smegmatis* is still the preferred taxonomic name.

THE ORIGIN OF *M. SMEGMATIS* MC²155

While Crawford and Bates had isolated plasmids from mycobacteria in 1979 (7), subsequent attempts to transform mycobacteria had been unsuccessful (8). Foreign DNA was first introduced into mycobacteria in 1987 with a shuttle phasmid (9). This chimeric vector had an *E. coli* bacteriophage lambda cosmid inserted in a non-essential region of the lytic mycobacteriophage TM4 (9, 10). The shuttle phasmid replicates in *E. coli* as a plasmid and in mycobacteria as a phage (9, 11). The generation of viable phage that infected both *M. smegmatis* and *M. bovis* BCG demonstrated the feasibility of the approach and established that DNA from *E. coli* was not degraded by mycobacteria. A second shuttle phasmid based on the temperate mycobacteriophage, L1, was generated and shown to lysogenize *M. smegmatis* mc²6, the progenitor strain of *M. smegmatis* mc²155 (12). Subsequently, a gene encoding kanamycin-resistance (Km^R) was cloned into the L1-based shuttle phasmid and successfully transfected into mc²6, establishing kanamycin as an effective selection in mycobacteria (12). With this knowledge, a library of plasmids was made by cloning random fragments of the mycobacterial plasmid pAL5000 (13, 14), which replicates in *Mycobacterium fortuitum*, in an *E. coli* plasmid encoding Km^R (12). This library of chimeric episomal plasmids was electroporated into *M. smegmatis* mc²6 and a few rare transformants were isolated and thus, establishing a plasmid-transformation system (12). These rare transformants turned out to be a mutant of *M. smegmatis* that was defective in preventing plasmid establishment (15). The plasmid in the original transformant was cured and the resulting plasmid-free strain was designated mc²155. Whereas mc²6 remained virtually un-transformable, mc²155 routinely yielded up to a million transformants per microgram of plasmid DNA; it had acquired an efficient plasmid transformation (*ept-1*) phenotype. In 2014, whole-genome sequence comparisons between mc²155 and its parental strain revealed that the efficient plasmid transformation (ept) mutation mapped to *eptC*, encoding a structural-maintenance-of-the-chromosome (SMC) protein (16). A similar SMC protein-mediated plasmid restriction has been found in other bacteria as well (17). This *ept* mutant of *M. smegmatis* revolutionized approaches to mycobacterial biology and established mc²155 as the workhorse of mycobacterial genetics; fast-growing, nonpathogenic, and transformable.

FEATURES OF *M. SMEGMATIS* THAT MAKE IT A GREAT MODEL

Shuttle phasmids and plasmids not only enabled the isolation of mc²155, but were used to generate key molecular genetic tools, including integration-proficient vectors (18), expression vectors (19), luciferase-reporter phages (20), and suicide- (21), and recombineering-vectors (22). Furthermore, the ability of *M. smegmatis* to grow in temperature ranges up to 55°C allowed the isolation of temperature-sensitive TM4 phage mutants (23, 24), providing for both efficient delivery and counter-selection for specialized transduction and Tn- and targeted-mutagenesis (20, 25–27). These early technologies allowed the generation of insertion mutant libraries and the identification of essential genes using Tn-site hybridization and Tn-seq technologies (28–31). As these genetic tools flourished, mc²155 became broadly accepted as the genetically tractable model *Mycobacterium*.

Its non-pathogenicity was a great entry point for researchers not equipped with a biosafety level (BSL)-3 facility and for novice microbiologists, such as undergraduate students, who cannot work with pathogens. Fast growth is not only ideal for genetics and biochemistry, but also for studying single cells, their growth, and cell architecture. In particular, high-throughput screenings involving single-cell analyses, such as microscopy, are nearly impossible with the pathogens because of the slow growth and need for specialized equipment in BSL-3 facilities. In this section, we highlight the notable features of *M. smegmatis*, and how newer, shared resources have further enhanced mycobacterial research.

Comparative genomics validate the choice of *M. smegmatis* as the model *Mycobacterium*. Of the ~4,000 protein-coding genes in the genome of *M. tuberculosis*, > 2,800 have orthologs in *M. smegmatis* with >50% amino acid identity (32). The average protein identities between protein orthologs in *M. smegmatis* and their pathogenic cousins

are >70% in most cases (33). Most importantly, the similarities extend beyond the *M. tuberculosis* complex (MTBC). As a conservative estimate, there is a core set of ~1,150 *M. smegmatis* proteins that share >50% amino acid identity, which are encoded by many species including the MTBC, *M. abscessus*, *Mycobacterium marinum*, *M. avium*, and *M. leprae* (32). The core proteins almost certainly perform the same functional role in each species. This level of conserved function is also highlighted by transposon mutagenesis studies: 96% of the genes identified as essential in *M. smegmatis* have orthologs in *M. tuberculosis*, and 90% of which are essential in *M. tuberculosis* (34). The genetic organization of each species is also well conserved, with similar patterns of gene co-localization around the chromosome, suggesting conserved mechanisms of gene regulation.

Centralized resources for the study of mycobacteria. A good model organism is accompanied by community-shared resources of strains, defined knockouts, and plasmids expressing individual genes (e.g., https://biocyc.org/, https://bgsc.org/). These physical resources, integrated with data sets (protein-protein interactions, phenotypic profiling, *etc.*), permit a systems biology approach to gene function and provide insights on cellular processes. While still in their infancy, similar resources are becoming available for mycobacteria, most importantly in *M. smegmatis*. For example, bioinformatic resources such as Mycobrowser (https://mycobrowser.epfl.ch/) and BioCyc (https://mycobacterium.biocyc.org/) provide an interactive space to analyze genes of interest, which are cross-referenced to orthologs in other sequenced genomes, with links to key sites describing BLAST searches, putative gene functions, and structures. The Wadsworth Center's Interactive Genomics browser displays RNA-seq, Ribo-seq, and transcription start site data for *M. smegmatis* and *M. tuberculosis*, which allow the user to accurately determine gene boundaries, identify novel genes, leadered and leaderless transcripts, and non-coding RNAs (https://www.wadsworth.org/research/scientific-resources/interactive-genomics). MSRdb (https://msrdb.org/) describes a collection of *M. smegmatis* gene knockouts, knockdowns, and strains expressing genes fused to a fluorescent reporter for protein localization. It was created as part of the Mycobacterial Systems Resource (MSR) (32), which is based on the 1,153 core proteins described above, most having no assigned function. The physical resource contains: a total of 569 precise gene knockouts of non-essential genes; a collection of plasmids that encode small guide RNAs for CRISPRi-mediated targeted suppression of 843 genes; and 1,116 genes cloned in a vector to express a fluorescent protein (Dendra2)-tagged fusion protein. Like other method breakthroughs, the CRISPRi technology was developed and optimized in *M. smegmatis* (35) and its application to fine-tune gene repression elegantly demonstrated in *M. tuberculosis* (36). The pooled collection of CRISPRi plasmid libraries targeting genes and ncRNAs in both *M. smegmatis* and *M. tuberculosis* are available through Addgene (https://www.addgene.org/) (36).

We highlight 2 recent studies that exemplify the benefit of using *M. smegmatis* for microscopy-based approaches. The first example is high-throughput imaging on the library of conserved, fluorescently tagged, core proteins generated in the MSR (37). The spatiotemporal analysis of over 700 proteins demonstrated that sub-cellular protein localization often correlates with function. For example, ribosomal proteins were clustered, but excluded from the nucleoid and the polar and subpolar regions of the cell. The co-localization of proteins with both known and unknown functions provides immediate insight (guilty by association) on the possible function of many hypothetical core proteins, which can be directly translated to other species.

The second study applied CRISPRi gene suppression in *M. smegmatis* to specifically inhibit 263 essential genes with direct orthologs in *M. tuberculosis* (38). Single cells were visualized following induction of the CRISPRi system and the impact of essential gene suppression on cell morphotypes captured by high-throughput quantitative imaging, which generated a morphological atlas of phenotypes (cell length, curvature, bulging). Strikingly, suppression of genes with related biological function (e.g., cell division, cell wall synthesis) clustered together according to their morphology, allowing an educated prediction of gene function of hypothetical proteins. The conservation of these essential genes throughout the genus allows their characterization in *M. smegmatis* to provide a more directed experimental approach in the less experimentally amenable mycobacteria.

NOTABLE DISCOVERIES DRIVEN BY *M. SMEGMATIS*

Studies with *M. smegmatis* have revealed many unusual features of the genus. In this section, we highlight a few examples of notable discoveries, while acknowledging the countless other remarkable contributions, which cannot be included due to space limitations.

Distributive conjugal transfer and genome evolution. Distributive conjugal transfer (DCT) is a novel form of horizontal gene transfer (HGT) first described in *M. smegmatis* (39, 40). DCT is conceptually equivalent to conjugation in that it requires direct contact between a donor and recipient cell, but the products of DCT are distinct from that of any other form of HGT; the progeny genomes are mosaic blends of the parental genomes (41). Mosaicism results from a single DCT event, dramatically shortening the time to combine parental traits that could confer a competitive evolutionary advantage (42). DCT is not limited to laboratory strains and conditions, as the genomes of independent environmental isolates of *M. smegmatis* also exhibit the hallmark genome mosaicism (43).

In the past, the concept of HGT within the MTBC was considered almost heretical. While there are abundant mycobacteriophages, no plasmids have been described within the MTBC, and given the pathogen's intracellular "solitary" lifestyle, it was not surprising there was little evidence for HGT (44). Members of the MTBC are very closely related (>99.9% genome identity), with speciation reflecting host adaptations (e.g., *M. bovis* causes TB in cattle) (45–47). That concept was challenged when the comparative analysis of multiple genome sequences of *Mycobacterium canettii* (a smooth colony outlier of the MTBC) revealed thousands of SNPs: the striking similarity to the experimentally produced mosaic genomes of *M. smegmatis* provided the first indication that HGT had occurred in the MTBC (48–51). Subsequently, direct evidence for DCT between 2 isolates of *M. canettii* was demonstrated by isolating transconjugant progeny that contained the hallmark, blended parental genomes (52). These studies provided experimental support for the proposal that DCT was a major evolutionary driving force among *M. canettii*, which ultimately led to the evolution of a progenitor *M. tuberculosis* species and subsequent speciation into the animal-adapted MTBC (41). Thus, DCT in *M. smegmatis* has forced a rethinking on HGT and genome evolution among mycobacteria and provided a novel mechanism of bacterial HGT (42).

Type VII (ESX) secretion systems are found in all mycobacteria and are encoded, primarily, in a large, multi-gene locus, *esx* (53–57). Mycobacteria can contain multiple *esx* loci (up to 5), which are functionally non-redundant. Functions for most ESX systems and their secreted substrates remain poorly defined, but ESX-1- and ESX-5-mediated secretion is essential for pathogenesis and survival during infection. Remarkably, DCT has provided alternative roles for ESX secretion systems. RNA-seq analysis demonstrated that donor and recipient cells have specific transcriptional responses to direct contact with the opposite mating type. Notably, the ESX-4 secretion system is transcriptionally activated in the recipient on donor contact and is essential for DCT (58). This contact-dependent activation of ESX-4 in the recipient is regulated by ESX-1 activity in the donor providing the first direct evidence for mycobacterial cell-cell communication (58). ESX-4 has since been shown to be important for macrophage infection in *M. abscessus* (59) and contributes to phagosome permeabilization and protein trafficking in *M. tuberculosis* (60). Thus, DCT in *M. smegmatis* has provided the first model system for mycobacterial communication and indicates that ESX systems are likely to mediate unexpected functions beyond virulence.

Leaderless gene expression and small proteins in mycobacteria. The application of RNA-profiling techniques (RNA-seq and Ribo-seq) in *M. smegmatis*, *M. tuberculosis*, and *M. abscessus* (61–64) has emphasized a startling difference in the gene architecture of mycobacteria compared with the textbook model organisms; the presence of leaderless mRNAs (LLmRNA). LLmRNAs lack a 5′-UTR; the 5′ end of the mRNA begins at the start codon of the gene and, thus, the mRNA lacks a ribosome-binding site (RBS) (65). In *E. coli* LLmRNAs are extremely rare (3 have been characterized) and poorly expressed (66), but they are abundant in Actinobacteria (25 to 30% of mRNAs in mycobacteria) and Archaea (65, 67–69). *M. smegmatis* was used in its "model" role, using *lacZ* and luciferase-gene reporters to demonstrate that: (i) these LLmRNAs were robustly expressed in *M. smegmatis* but not *E. coli*; (ii) the level of gene expression was comparable to that of leadered mRNA (LDmRNA); and (iii) any

mRNA beginning with a 5′ AUG or GUG (RUG) is translated (64, 70). The consequences of this fundamental difference in gene architecture are many. First, the rationale behind genome annotations in mycobacteria had to be reevaluated. The assumed requirement for a gene to have a promoter and RBS resulted in many mis-annotations. For example, of the 1,285 genes transcribed as LLmRNAs in *M. tuberculosis*, 338 were incorrectly annotated and required (in-frame) modifications of the start sites, and a further 370 were not annotated (64, 70). In addition, most of the novel genes identified by transcriptional profiling (from both LDmRNAs and LLmRNAs) encoded small proteins (sproteins, $<$50 amino acids). In the past, small ORFs (sORFs) encoding sproteins had been overlooked by genome annotation pipelines, which use "50 codons" as a cut off to ensure confidence in gene prediction. However, growing experimental evidence in mycobacteria and other prokaryotes has shown that many sproteins are functional, requiring a rethinking of gene definitions (71–75). The addition of hundreds of novel sproteins to the mycobacterial proteome will demand new genetic, biochemical, and proteomic studies to determine their function and *M. smegmatis* is the ideal organism for those studies, especially for conserved sproteins. As an example, a subclass of conserved sproteins encode 2 to 8 consecutive cysteine residues. These poly-cysteine sORFs act as *cis*-acting attenuators of downstream genes required for cysteine uptake and biosynthesis (76). Finally, ribosomes must recognize LLmRNAs by a mechanism fundamentally different from that of LDmRNAs. The abundance of LLmRNA indicates that efficient translation mechanisms for both LDmRNAs and LLmRNAs have evolved in-parallel in mycobacteria. Thus, the best bacterial model for the mechanistic study of leaderless translation is *M. smegmatis*.

Virulence factor secretion. One prominent example of an *M. smegmatis* discovery enlightening host-pathogen interactions involves virulence factor secretion. Secretion analyses in *M. smegmatis* revealed a non-essential, but non-redundant SecA2 pathway, in addition to the housekeeping SecA1 (77). SecA2 is found in all species of *Mycobacterium*, other Actinobacteria, and also in several Gram-positive pathogens (78, 79). SecA1 interacts with the SecYEG complex, the membrane-spanning channel that translocates nascent unfolded polypeptides into the periplasm. Mycobacteria do not encode an additional *secY* paralog, in contrast to other bacteria with a second SecA. Instead, structural studies in *M. tuberculosis* have revealed that SecA2 likely interacts with the canonical SecYEG channel to mediate secretion of SecA2-specific proteins (80, 81).

M. tuberculosis Δ*secA2* is attenuated in SCID and C57BL/6 mice (82, 83). Attenuation of the Δ*secA2* strain stemmed from its inability to export two proteins involved in blocking phagosome maturation: PknG and SapM (84–86). PknG is one of 11 serine-threonine protein kinases (STPK) encoded by *M. tuberculosis* (87); most of the SPTKs are membrane proteins, are not SecA2 substrates, and function as mediators of trans-membrane signaling. PknG is an exception; it is secreted in a SecA2-dependent manner (88, 89), in addition to having cytoplasmic roles regulating metabolism, redox balance, and biofilm formation (90–94). The first evidence suggesting PknG was a secreted virulence factor was obtained by heterologously expressing *M. tuberculosis* PknG in *M. smegmatis*. This *M. smegmatis* strain secreted PknG and was more resistant to macrophage killing than the wild-type parent (95). This was subsequently validated in *M. tuberculosis* where PknG, in conjunction with the SapM phosphatase (86), blocks host phagosome maturation and modulates the host immune response (88, 96–98), establishing PknG and SapM as bona fide virulence factors.

Cell envelope, polar elongation, and asymmetric division. Mycobacterial envelopes are unlike those of any other bacteria. The unusually lipid-rich diderm structure of the mycobacterial cell envelope, initially proposed by Minnikin in 1982 (99), was visualized in 2008 by cryo-electron microscopy of *M. smegmatis* and *M. bovis* BCG, indicating a conserved outer membrane (termed the mycomembrane) (100, 101). While some early studies used *Mycobacterium phlei*, mc^{2}155 inevitably became the preferred model to determine the structure, biosynthesis, and function of the mycobacterial cell envelope (see reviews [102–105]). Here, we discuss the roles *M. smegmatis* has played in uncovering the spatiotemporal coordination of mycobacterial cell envelope elongation and division, which are distinct from other rod-shaped model bacteria.

Pathogenic mycobacteria persist in the host for decades in chronic infections. Thus, it is important to understand how growth and division are regulated and how cell envelope integrity

is maintained in hostile host environments. Early genome analyses revealed that mycobacteria lack conserved cell division proteins, such as MreB and the Min-family proteins, implying fundamental differences in the mechanisms of cell envelope elongation and division compared with other bacteria (106). Using fluorescein-labeled vancomycin, it was shown that *de novo* growth of the mycobacterial cell occurred at the cell poles and not along its length, unlike other rod-shaped bacteria (107). While mycobacteria lack MreB, they do encode a bacterial homolog of the eukaryotic cytoskeletal protein tropomyosin, named DivIVA (Wag31). DivIVA is localized to the poles where it has been shown to promote peptidoglycan biosynthesis and cell elongation in *M. smegmatis* (108–111). Intriguingly, the *M. smegmatis* plasma membrane partitions into two domains. One, termed the intracellular membrane domain (IMD), contains many envelope synthases that localize to sub-polar regions (i.e., regions directly adjacent to the growing poles) (112, 113). There is a positive-feedback loop between IMD formation and peptidoglycan biosynthesis: IMD formation promotes polar-peptidoglycan synthesis, and the synthesized peptidoglycan maintains the IMD (114, 115). These cytoskeletal proteins, envelope synthases, and specialized membrane domains are proposed to form a mycobacterial "elongasome" that coordinates polar growth. As expected for a process fundamental to cell growth, the elongasome is conserved in *M. tuberculosis*, as evidenced by the polar localization of DivIVA (109), peptidoglycan biosynthesis (109, 116), and the subpolar enrichment of the IMD (117). Proteomic analysis of the *M. tuberculosis* IMD indicates that it serves as the biosynthetic site of pathogen-specific lipid virulence factors, including phthiocerol dimycolate (117).

Mycobacteria divide asymmetrically by a fast, mechanical-snapping mechanism (107, 118–121). While the mechanism of asymmetric septal placement is unknown, studies largely performed in *M. smegmatis* have revealed that FtsZ and other cell division proteins assemble a protein complex at the new division site similar to other bacteria (for review [122, 123]). Strikingly, asymmetric division is an actively regulated process, not the result of random septal placement; a mutant of *M. smegmatis* lacking *lamA* elongates and divides symmetrically (124). LamA is a mycobacteria-specific, membrane protein of unknown function. Though the physiological significance of asymmetric cell division is not fully understood, loss of asymmetry in *lamA*-deficient *M. tuberculosis* was accompanied by increased sensitivity to rifampicin (RIF) (102). A second study that tracked single cells through multiple rounds of asymmetric cell divisions in a microfluidic system also demonstrated that long-birth length and mature-growth poles are associated with RIF tolerance (125), independently supporting the role of asymmetric cell division in producing heterogenous populations of cells with different drug susceptibilities. Stress and antibiotics were later shown to influence cell length heterogeneity in *M. tuberculosis* clinical isolates, reinforcing the evolutionarily conserved link between morphological heterogeneity and mycobacterial fitness first characterized in *M. smegmatis* (126).

APPLICATIONS OF *M. SMEGMATIS* FOR DRUG DISCOVERY AND DEVELOPMENT

Elucidating mechanisms of action for Isoniazid, Ethionamide, and Ethambutol. Chemotherapy of mycobacterial infections is a lengthy process requiring a cocktail of drugs, usually including Isoniazid (INH), RIF, Ethambutol (EMB), and Pyrazinamide. Of these, *M. smegmatis* has played a central role in determining the mechanisms of action for INH and EMB. An INH-resistant strain of mc^2155 was shown to become sensitive upon expression of the *M. tuberculosis* catalase-peroxidase gene, *katG*, suggesting that susceptibility to INH requires the expression of *katG* (127). KatG was subsequently discovered to modify INH into its active form, demonstrating that INH is a prodrug and explaining why *katG* mutations are a prevalent mechanism of INH resistance in *M. tuberculosis* (128). *inhA* was identified as the target of activated INH by an *M. smegmatis* missense mutation that conferred co-resistance to both INH and Ethionamide (ETH) (129). *inhA* encodes an NADH-specific enoyl-acyl carrier protein (ACP) reductase (130, 131). The ability of *M. smegmatis* to grow at 30°C enabled the discovery of mutants that elucidated the mechanisms of action of INH and ETH (reviewed in [132]). Briefly, when *M. smegmatis* mutants were isolated for co-resistance to INH and ETH on rich media, half of the mutants were temperature-sensitive. These mutations mapped to *ndh*, which encodes an NADH oxidase (133). Altered NADH/NAD^+ ratios of these mutants

were consistent with a model that INH- or ETH-NAD^+ adducts inhibit InhA. This model was later verified by X-ray crystallography of an INH-NAD^+ or ETH-NAD^+ adduct bound to the active site of InhA (134–136). To our knowledge, this is the first example of a prodrug that can be activated to form adducts with an enzyme co-factor (NADH).

The first attempts to determine the mechanism of EMB-mediated anti-mycobacterial activity were carried out using *M. smegmatis* as far back as the 1960s (e.g., [137, 138]), but more than 2 decades were needed to elucidate the correct mechanism of action. EMB-treated *M. smegmatis* cells were defective in incorporating radioactive D-glucose into arabinogalactan (139), and accumulated decaprenyl-P-arabinose (140), demonstrating that EMB blocks the transfer of arabinose from its lipid carrier to arabinogalactan in the cell wall. The targets of EMB were discovered through an overexpression screen using *M. smegmatis*. A plasmid library of *M. avium* chromosomal fragments were screened for clones exhibiting resistance to EMB. This screen identified 2 arabinosyltransferases, EmbA and B, as mediating resistance to EMB (141). Genetic studies in *M. tuberculosis* corroborated these results (142, 143). Cryo-EM and X-ray crystallography structures of *M. tuberculosis and M. smegmatis* EmbA and EmbB complexed with either substrate or EMB confirmed that EMB inhibits arabinosyltransferase activity by binding a region adjacent to the catalytic site (144). Mutations in genes such as *katG*, *inhA*, *embA*, and *embB*, that confer drug resistance are now rapidly detected by whole-genome sequencing, reducing time to diagnose multidrug-resistant and extensively drug-resistant TB (145, 146).

Drug discovery and development. Early efforts to screen TB drugs were inefficient due to the use of slow-growing *M. tuberculosis*. In more recent years, the generalizability of most anti-TB drugs across all mycobacteria has led to the appreciation of *M. smegmatis* as a pragmatic model organism for initial drug screening and further drug optimization. *M. smegmatis* was used for the initial compound screening that identified diarylquinoline (later named Bedaquiline) as a specific inhibitor of mycobacterial ATP synthase in 2005, which ended a 40-year dearth in anti-TB drug discovery (147). Furthermore, a cryo-EM study determined the binding site of Bedaquiline in the *M. smegmatis* ATP synthase (148). WHO recommended the use of Bedaquiline for the treatment of RIF-resistant and multidrug-resistant adult TB in 2013 and, more recently, recommended replacement of injectable second-line drugs with Bedaquline for oral, short-course regimens (149). Bedaquiline has also been included in newer combination regimens in several ongoing clinical trials.

M. smegmatis has been instrumental in the development of inhibitors of the essential mycolic acid transporter protein, MmpL3. Knockdown of the *mmpL3* gene in *M. smegmatis* is lethal, and results in reduced levels of outer membrane mycolic acids and the accumulation of the mycolate carrier, trehalose monomycolate (TMM), suggesting a role in mycolic acid transport (150, 151). Early inhibitors of MmpL3 were discovered by screening chemical libraries for activity against *M. tuberculosis* growth and mapping resistance mutations back to the *mmpL3* gene (151). Using a novel *M. smegmatis* spheroplast assay, one inhibitor was used to show MmpL3 is a "floppase," that flips TMM from the cytoplasmic to the periplasmic leaflet of the inner membrane (152). While this study established the first, specific platform for validating other potential MmpL3 inhibitors, the technical difficulty involved with creating spheroplasts prompted development of a more high-throughput approach (153). *M. smegmatis* was used as a surrogate to express different drug-resistant *M. tuberculosis mmpL3* alleles. Unexpectedly, the *mmpL3* mutations conferred cross-resistance to different drugs, suggesting that most inhibitors bind a common active site, and predicting that undiscovered inhibitors would most likely bind this same site. Inspired by this insight, the authors developed fluorescent MmpL3 probes based on known inhibitors, which were successfully used in a competitive-binding assay in *M. smegmatis* to identify new MmpL3 inhibitors (153). This live *M. smegmatis*-based platform has extraordinary potential for both new drug discovery and drug optimization.

To gain a molecular view of the MmpL3 floppase and the mechanism of drug inhibition, X-ray crystallography structures of *M. smegmatis* MmpL3 alone and complexed with its ligand TMM and known inhibitors were solved (154, 155). These structures revealed the mechanism by which the protein utilizes a proton gradient to drive TMM translocation and how inhibitors block this proton translocation channel to arrest TMM translocation.

As expected, the cryo-EM structure of *M. tuberculosis* MmpL3 (61% amino acid identity) closely matched that of *M. smegmatis* MmpL3 (156). The recent discovery of 2 conserved accessory proteins in *M. smegmatis*, which stabilize MmpL3, add further insight on the mechanisms of MmpL3 function (157). Thus, our increased understanding of the MmpL3-drug mechanism of action promises to accelerate development of second-generation MmpL3 inhibitors, improving upon first-generation inhibitors such as SQ109, which is in phase 2 clinical trials (158). The application of *M. smegmatis* to isolate inhibitors of ATP synthase and MmpL3 demonstrates many of the key attributes *M. smegmatis* offers as a model for *M. tuberculosis* drug-target research.

M. SMEGMATIS WILL CONTINUE TO BE A MODEL FOR ALL MYCOBACTERIA

The development of genetic tools and resources over the past 3 decades has made *M. smegmatis* the model organism for mycobacterial pathogens, fast- and slow-growing. Here, we highlight the potential of some new tools and technologies, the limitations of *M. smegmatis* as a model and its creative use in education.

M. smegmatis will inevitably continue serving as a primary incubator space for mycobacterial tool development. Determining the functions of the many unannotated small (and large) proteins will be a priority. Identifying protein-protein interactions can provide enormous insight on protein function, as demonstrated by new *in vivo* proximity-labeling technologies. These use photo-cross-linking of unnatural amino acids incorporated into mycobacterial proteins (159, 160), or fuze the test protein to peroxidase to mediate biotinylation of nearby proteins (161). The interacting proteins are then identified by quantitative proteomics (160). The continued growth of these technologies requires improved specificity of cross-linking combined with robust proteome-wide MS approaches that encompass small proteins, protein modifications, and protein functions. The optimization of proteomics and metabolomics in *M. smegmatis* will establish systems-level approaches for high-throughput assignment of protein functions that can be subsequently extrapolated to less biochemically amenable mycobacteria (162).

Reporting metabolic reactions at the subcellular scale is crucial for correlating subcellular localizations of proteins with their activities. Genetic tools are less effective in manipulating and visualizing non-proteinaceous cellular structures such as lipids and glycans, but non-genetic approaches are challenging due to the low permeability and unusual architecture of the mycobacterial envelope. Nevertheless, there are emerging efforts to apply modern (click chemistry) approaches of bioorthogonal metabolic labeling in both *M. smegmatis* and *M. tuberculosis*, allowing the biosynthesis of trehalose-containing molecules, peptidoglycans, and arabinan to be visualized *in situ* (116, 163–165). These chemical reporters can document activities of different metabolic pathways in *M. smegmatis* (e.g., *de novo* peptidoglycan synthesis versus remodeling reactions [111]), which can be correlated with subcellular enzyme localizations. These chemical-biology reporters can also be used to determine the metabolic state of cells and have potential as diagnostic and therapeutic tools for mycobacterial infections.

Mycobacterium species are ubiquitous in the soil and include the disease-causing NTMs (e.g., *M. abscessus* and *M. avium*) (166). As many of these environmental mycobacteria are poorly characterized and difficult to culture, *M. smegmatis* provides the model system to characterize their gene products. For example, Tn-seq, recombineering and CRISPR tools applied to *M. abscessus* have begun to define those genes that make *M. abscessus* intrinsically resistant to many antibiotics (167–170). Not surprisingly as a soil saprophyte, *M. smegmatis* encodes novel enzymes, which allow it to survive environmental stress. Mycobacteria are obligate aerobes and, thus, need strategies to combat hypoxia, antimicrobials and toxins, such as carbon monoxide, that inhibit terminal oxidases. Studies using the *M. smegmatis* toolbox have shown that mycobacteria can metabolize hydrogen gas, detoxify carbon monoxide, and encode multiple flavin/deazaflavin oxidoreductases and hydride transferases to survive these stresses (171–174). These genes are conserved among many mycobacteria and other actinobacteria, indicating the usefulness of *M. smegmatis* as a model for environmental microbiology.

M. smegmatis has been a favorite host for isolating mycobacteriophages. Although it is beyond the scope of this review, isolated phages have provided many important genetic tools

such as site-specific integration systems, gene delivery by transduction, and recombineering (175, 176). Phages have also been explored as diagnostic tools, and successfully used to treat chronic, drug-resistant infections of *M. abscessus* (177–179). The genomes of bacteriophages are filled with genes of unknown functions, foretelling more surprises, and new tools. Notably, *M. smegmatis* has also become a prominent educational tool for the future of science; as a host for isolating mycobacteriophages. Through the Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program, high school and college students engage in isolating mycobacteriophages from their local environment, characterizing the phage, and sequencing and annotating its genome (180, 181). It is an inclusive, community-oriented, course-based research experience that has served the education of thousands of students worldwide, while also providing the research community with a cornucopia of novel genes and functions. The collective efforts of students around the world have demonstrated that mycobacteriophages and, thus, mycobacteria, are ubiquitous.

M. smegmatis, like other model organisms, has limitations; it is more distantly related to the slow-growing species and is a poor model for host-pathogen interactions and pathogenesis. Two alternative, slow-growing mycobacteria that are more closely related to *M. tuberculosis* and used in a BSL-2 laboratory are *M. bovis* BCG, the attenuated vaccine strain, and *M. marinum*, a fish pathogen, each with advantages for host infection studies over *M. smegmatis* (182, 183). Unfortunately, *M. bovis* BCG has a complex genealogy with lineage-specific mutations (184), making it a problematic model for systematic genetic studies. To address this drawback, genetically defined, attenuated mutants of virulent *M. tuberculosis* have been created by deleting 2 or more genes (185–187). These attenuated strains can be used safely in a BSL-2 lab, providing a more feasible alternative for *M. tuberculosis* genetics and biochemistry. Obviously, *in vivo* studies require virulent strains and here we rely on the *M. tuberculosis*-mouse and *M. marinum*-zebrafish models among others as the appropriate platforms to study the process of pathogenesis caused by mycobacterial pathogens (188). The *M. marinum*-zebrafish is particularly amenable to *in vivo* studies because of the translucent nature of the fish and the ability to genetically manipulate both the host and the bacterium (thanks to *M. smegmatis*!) (189–191).

M. smegmatis has been at the vanguard of mycobacterial research, not only revealing new strategies to tackle mycobacterial diseases but also contributing unexpected processes and methods that other model bacteria and textbooks fail to offer. Why and how do mycobacteria conjugate through DCT; translate proteins from leaderless transcripts; elongate from the polar ends; and divide asymmetrically? Mycobacteria rely on machineries and molecules that are more commonly found in eukaryotes, such as STPKs for signaling, proteasomes for protein degradation, and phosphatidylinositols as a major component of the plasma membrane. Why? These unique and unexpected characteristics will surely continue to spark the curiosities of microbiologists and inspire the mycobacterial research community and we foresee that mc^{2}155 will continue its place at the front of this research.

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William R. Jacobs, Jr. earned his MA in Mathematics from Edinboro State College in Pennsylvania (1977). As a graduate student with Josie Clark-Curtiss and Roy Curtiss III, he constructed the first genomic libraries of *Mycobacterium leprae* bacilli, which they had isolated from nine-banded armadillos with Charles Shepard. He embraced recombinant DNA developments by making *in vivo* cosmid packaging strains and graduated from the University of Alabama in Birmingham in 1985. As a postdoctoral fellow with Barry Bloom at the Albert Einstein College of Medicine (AECOM), he set out to develop genetic systems for *M. tuberculosis* and *M. bovis* BCG. In 1987, he successfully introduced foreign DNA into mycobacteria for the first time. Shuttle phasmids were used as tools that allowed his lab to isolate *M. smegmatis* mc^2155, the first plasmid-transformable mutant of *M. smegmatis*. He was an Investigator in the Howard Hughes Medical Institute for 30 years and was elected to the National Academy of Sciences in 2013. His lab, in the Department of Microbiology and Immunology at the AECOM, continues to use genetics to investigate Leprosy, Tuberculosis, and Herpes viruses.

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