Control and manipulation of bacterial populations requires an understanding of the factors that govern growth, division, and antibiotic action. Fluorescent and chemically reactive small molecule probes of cell envelope components can visualize these processes and advance our knowledge of cell envelope biosynthesis (e.g., peptidoglycan production). Still, fundamental gaps remain in our understanding of the spatial and temporal dynamics of cell envelope assembly. Previously described reporters require steps that limit their use to static imaging. Probes that can be used for real-time imaging would advance our understanding of cell envelope construction. To this end, we synthesized a fluorogenic probe that enables continuous live cell imaging in mycobacteria and related genera. This probe reports on the mycolyltransferases that assemble the mycolic acid membrane. This peptidoglycan-anchored bilayer-like assembly functions to protect these cells from antibiotics and host defenses. Our probe, quencher-trehalose-fluorophore (QTF), is an analog of the natural mycolyltransferase substrate. Mycolyltransferases process QTF by diverting their normal transesterification activity to hydrolysis, a process that unleashes fluorescence. QTF enables high contrast continuous imaging and the visualization of mycolyltransferase activity in cells. QTF revealed that mycolyltransferase activity is augmented before cell division and localized to the septa and cell poles, especially at the old pole. This observed localization suggests that mycolyltransferases are components of extracellular cell envelope assemblies, in analogy to the intracellular divisomes and polar elongation complexes. We anticipate QTF can be exploited to detect and monitor mycobacteria in physiologically relevant environments.

**Significance**

Mycobacteria, including the notorious pathogen *Mycobacterium tuberculosis*, possess a mycolic acid membrane that is a barrier to antibiotics. Although key enzymes that generate this structure are known, a full understanding of cell envelope assembly is lacking. We synthesized a fluorogenic analog of trehalose monomycoclylate, the building block used by mycolyltransferases to construct the mycolic acid membrane. When this analog, termed QTF, is processed in cells by mycolyltransferases, fluorescence is generated. Thus, mycolic acid membrane biosynthesis can be monitored in real time over several genera. Although mycolyltransferases are the most abundantly secreted proteins, we found that mycolyltransferase activity is localized. Finally, we show QTF has diagnostic features in that its processing by mycolyltransferases allows it to selectively detect mycobacteria.
processed by mycolyltransferases to construct the mycolic acid membrane. All bacteria known to generate a mycolic acid membrane produce multiple mycolyltransferases (up to 11); *M. tuberculosis* possesses three, Ag85A–C mycolyltransferases (17). Mycolyltransferases carry out transesterification of TMM through an acyl-enzyme intermediate. This mycolyl-enzyme adduct persists until transfer to a mycolyl acceptor (e.g., trehalose, TMM, or AG), a reaction that can afford three different products: TDM, mAG, or TMM (*SI Appendix*, Fig. S1A). Trehalose analogs bearing fluorophores (12, 13) or possessing intermediary reactive handles (e.g., an azide or alkyne group) can be used to label mycobacteria (14–16). Such fluorophore or chemically reactive trehalose analogs are of limited use for real-time monitoring of bacterial growth. Fixing and washing steps are required because the trehalose analog precursor must be removed before visualization of the labeled metabolic products. The conversion of these probes can require the action of intracellular enzymes of the mycolic acid production pathway; thus, they do not report directly on Ag85 activity. In all cases, the timing and location of mycolyltransferase activity is lost. As a result, fluorescent probes have been lacking that report on mycolyltransferase activity during cell growth.

To address this deficiency, we developed quencher-trehalose-fluorophore (QTF), a fluorogenic probe for imaging mycolyltransferase activity. QTF, an analog of the mycolyltransferase donor TMM, is hydrolyzed by mycolyltransferases to provide a real-time fluorescent readout of mycolyltransferase activity and mycolic acid metabolic products. The conversion of these probes can require QTF hydrolysis and thereby avoid covalent cell envelope modification. A reported kinetic study using the artificial substrate 6,6'-dihexanoyl trehalose revealed Ag85 exhibits substantial acylhydrolyase activity when acting on simple lipid linkages, a reactivity orthogonal to its primary role as an acyltransferase (23). Therefore, we replaced the native α-branched, β-hydroxylated lipid head group of TMM with an unbranched lipid in QTF to promote hydrolysis of the acyl-enzyme intermediate. The active site homology evident in the X-ray crystal structures of Ag85A–C and in alignments of mycolyltransferase sequences suggests the design of QTF should allow broad utilization by mycolyltransferases across genera (30–32). Thus, our modified mycolyltransferase substrate should avoid covalent modification of the cell wall with a nonnatural lipid, minimize any perturbation of Ag85 function, and improve substrate turnover. In this way, QTF’s features should simplify the interpretation of fluorescence imaging data.

**QTF Synthesis and Chemical Properties.** We synthesized QTF from a hexaoylated trehalose derivative by adapting a known route to maradolipid, a naturally occurring substituted trehalose derivative (*SI Appendix*, Fig. S2A) (33). Similarly, we prepared the predicted cleavage products (*SI Appendix*, Scheme S2B) as standards for analysis. QTF is freely soluble on dilution into aqueous buffer at all tested concentrations and maximally soluble up to 100 μM (in 2% DMSO). As expected, an equimolar mixture of the cleavage standards gave rise to a 1,500-fold increase in relative fluorescence emission corresponding to BODIPY-FL (λmax (excitation = 503 nm), λmax (emission = 515 nm)), while QTF lacks a measurable emission across these wavelengths (*SI Appendix*, Fig. S3). Solutions of QTF retained quenched fluorescence emission for extended periods at elevated temperatures (*SI Appendix*, Fig. S4). Lack of QTF fluorescence is presumably due to intramolecular fluorescence quenching by DABCYL, and we calculated a quenching efficiency of 99.9% between 10 nM and 10 μM (*SI Appendix*, Fig. S5). These observations confirm QTF has the spectral properties required for a “turn-on” probe.

**Results**

**Fluorogenic Probe Design.** To create a fluorogenic reporter of Ag85 activity, we modified the natural donor TMM with a fluorophore-quencher pair using the available structural data and postulated mycolyl transfer mechanism. The substrate TMM is composed of the disaccharide trehalose whose 6-hydroxyl bears a mycolate substituent, which is a branched lipid with an α-chain and a longer β-chain (Fig. 1C). The structure of Ag85B bound to trehalose determined by X-ray crystallography [Protein Data Bank (PDB) ID code 1F0P] indicates the 6’-hydroxyl of the disaccharide points out of the sugar binding pocket and should tolerate chemical modification (*SI Appendix*, Fig. S1B) (12, 23). Similarly, TMM is thought to be oriented with the shorter α-branched of the mycolic acid residing in a hydrophobic tunnel, while the longer β-chain is outside of the active site (*SI Appendix*, Fig. S1C). We reasoned that mycolyltransferases would process substrates bearing a range of nonnatural lipids, by placing any bulky lipid groups in the binding groove occupied by the substrate β-chain. Accordingly, we designed a reporter in which the hydrophobic fluorescent dye BODIPY-FL was appended to the terminus of a simplified lipid chain and the fluorescence quencher DABCYL linked to the 6’-hydroxyl (*SI Appendix*, Fig. S1D). We reasoned that if this potential Ag85 substrate turnover, as DABCYL efficiently quenches the fluorescence emission of BODIPY-FL (24, 25). If QTF undergoes processing with nucleophilic attack by the catalytic serine residue, the release of DABCYL–6’-trehalose would separate the FRET pair and trigger fluorescence emission (26–29).

We sought a fluorogenic probe that mycolyltransferases would hydrolyze and thereby avoid covalent cell envelope modification. A reported kinetic study using the artificial substrate 6,6'-dihexanoyl trehalose revealed Ag85 exhibits substantial acylhydrolysis activity when acting on simple lipid linkages, a reactivity orthogonal to its primary role as an acyltransferase (23). We therefore replaced the native α-branched, β-hydroxylated lipid head group of TMM with an unbranched lipid in QTF to promote hydrolysis of the acyl-enzyme intermediate. The active site homology evident in the X-ray crystal structures of Ag85A–C and in alignments of mycolyltransferase sequences suggests the design of QTF should allow broad utilization by mycolyltransferases across genera (30–32). Thus, our modified mycolyltransferase substrate should avoid covalent modification of the cell wall with a nonnatural lipid, minimize any perturbation of Ag85 function, and improve substrate turnover. In this way, QTF’s features should simplify the interpretation of fluorescence imaging data.

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**Fig. 1. Features of QTF, a fluorogenic probe of Ag85-mediated cell wall biogenesis.** (A) Components of the mycobacterial cell envelope include the peptidoglycan-anchored mycolyl-arabinogalactan (mAG) complex. The mycolyltransferases, including the antigen 85 complex (Ag85) of *M. tuberculosis*, construct the mycolic acid membrane by processing endogenous trehalose monomycolate (TMM) to afford trehalose dimycolate (TDM) or mycolyl-arabinogalactan (mAG). (B) QTF is a TMM mimic bearing a fluorophore and quencher. QTF processing by mycolyltransferases generates a fluorescent signal. (C) Chemical structure of TMM, QTF, and QTF products Q-Tre and lipid-FL generated by Ag85 cleavage.

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by mycobacteria, our data reveal their activity is spatially and temporally localized.
QTF Processing by Mycolyltransferases. A robust increase in fluorescence occurred upon QTF exposure to Ag85A, Ag85B, or Ag85C (Fig. 2, black bars). Changes in fluorescence emission were observed after 20 h at 37 °C. Probe turnover without a mycolyl acceptor present to intercept the acyl-enzyme intermediate suggested that the mycolyltransferases cleave QTF by hydrolysis. QTF processing could be accelerated in the presence of a mycolyl acceptor; therefore, we assessed fluorescence upon mixing QTF, an Ag85 homolog, and trehalose (Fig. 2, dark gray bars). No increase in fluorescence was observed with trehalose addition, a finding consistent with the proposed hydrolytic mechanism. To assess whether known Ag85 inhibitors block QTF turnover, we monitored fluorescence in the presence of tetrahydrolipstatin (THL) or ebselen. THL is a nonspecific lipase inhibitor that covalently modifies catalytic serine residues, whereas ebselen is reported to disrupt the Ag85 catalytic triad through allosteric covalent modification of a nonactive site cysteine (34). Addition of either THL (Fig. 2, light gray bars) or ebselen (Fig. 2, white bars) inhibited QTF processing, consistent with QTF hydrolysis mediated by the catalytic triad of Ag85.

We further examined mycolyltransferase processing of QTF by carrying out a Michaelis–Menten analysis of initial reaction velocities (SI Appendix, Fig. S6 and Table S1). The apparent $K_m$ values for QTF (Ag85A = 107 ± 27 μM, Ag85B = 25 ± 3 μM, and Ag85C = 44 ± 3 μM) are similar to the reported Michaelis constant for the natural mycolyl donor TMM ($K_m = 130 \mu M$) (35). Additionally, the apparent $V_{max}$ values reflect the relative activities measured by end-point analysis (Fig. 2 and SI Appendix, Fig. S6 and Table S1). Specifically, Ag85A cleaves QTF more efficiently than Ag85C, while the activity of Ag85B is lower; these relative rankings are consistent with the known mycolyltransferase activities of these enzymes (36). Thus, the kinetic parameters of mycolyltransferase-catalyzed QTF hydrolysis are consistent with those observed for mycolyltransferase activity.

QTF Is Selectively Processed by Bacteria with Mycolic Acid Membranes. When species that contain mycolyltransferases, including M. smegmatis and Corynebacterium glutamicum, were treated with QTF (2.5 μM), bacterial growth was unaffected (SI Appendix, Fig. S7).

![Fig. 2](image-url)

Fig. 2. Fluorescence resulting from QTF exposure to the native M. tuberculosis mycolyltransferases Ag85A, Ag85B, and Ag85C. Fluorescence emission was measured after incubation of QTF (1 μM) with purified M. tuberculosis Ag85A, Ag85B, or Ag85C (20 nM) for 20 h at 37 °C in 20 mM phosphate buffer (pH = 7.2) (black bars). Turnover was also assessed in the presence of a mycolyl acceptor (5 mM trehalose, dark gray bars) and generic mycolyltransferase inhibitors tetrahydrolipstatin (THL) (10 μM light gray) or ebselen (10 μM white). RFU, relative fluorescence units. Error bars depict SD.

but changes in bulk fluorescence emission of more than 10,000-fold were observed for each species (SI Appendix, Fig. S7A). We compared the fluorescence resulting from these mycolyltransferase-producing organisms to that from Escherichia coli and Bacillus subtilis, as the latter two species serve as representative Gram-negative and Gram-positive species that lack Ag85 orthologs. When cells were cultured with QTF (1 μM) for two to three doubling times and then analyzed using flow cytometry (Fig. 3A), either M. smegmatis and C. glutamicum became fluorescent. In contrast, neither E. coli nor B. subtilis cells lit up (SI Appendix, Fig. S8).

When QTF-treated bacteria were visualized by confocal fluorescence microscopy (Fig. 3B), fluorescence was observed at the cell surface of M. smegmatis and C. glutamicum, but not E. coli or B. subtilis. To test whether QTF could label mycolyltransferase-containing organisms within a community, we added the probe to mixed cultures of B. subtilis, E. coli, and C. glutamicum expressing cytoplasmic mCherry. The colocalization of QTF-derived and mCherry fluorescence (Fig. 3C) was observed only for C. glutamicum, a mycolyltransferase-producing species. These findings indicate that QTF is a fluorogenic probe of mycolyltransferase-producing bacteria.

QTF Processing in Live Cells. To determine whether mycolyltransferases are responsible for QTF cleavage in live cells, we harvested culture filtrate protein (CFP) from M. smegmatis, which contain secreted Ag85 proteins, and we evaluated QTF processing (Fig. 3 D–F). Exposure of the CFP to QTF afforded large increases in fluorescence emission (Fig. 3D). Treatment of M. smegmatis CFP with the Ag85 inhibitor ebselen reduced fluorescence by 63%. This reduction is consistent with mycolyltransferase-mediated probe turnover. The residual activity is likely due to inefficient inhibition of M. smegmatis mycolyltransferases by ebselen (13). To examine this possibility, we purified four M. smegmatis Ag85 homologs and measured their inhibition by ebselen (SI Appendix, Fig. S9). These data indicate ebselen is less potent against M. smegmatis mycolyltransferases (23–52% inhibition) than M. tuberculosis Ag85A–C (78–99% inhibition).

CFP from M. smegmatis was treated with QTF and the resulting mixture was analyzed by nondenaturing PAGE (Fig. 3E and SI Appendix, Fig. S10). Exposure of the gel to QTF followed by fluorescence imaging revealed only three fluorescent regions. Each band was excised and subjected to protein digest mass spectrometry analysis, which revealed the presence of at least one mycolyltransferase protein in each band (SI Appendix, Table S2). To determine the products of QTF processing, the probe (100 μM) was incubated with M. smegmatis CFP and the resulting sample was analyzed by high-resolution MS. We identified intact QTF and its predicted cleavage standards Q-Tre and lipid-FL (Fig. 3F), but no other cleavage products were detected (Fig. 3E and SI Appendix, Fig. S11). These findings are consistent with data from TLC analysis of membrane extracts and released cell wall mycolates from whole cells treated with QTF. In all cases, lipid-FL was the only fluorescent product recovered from M. smegmatis and C. glutamicum (SI Appendix, Figs. S12 and S13). These experiments establish that QTF is activated via mycolyltransferase-catalyzed hydrolysis in living cells.

QTF as a Reporter of Cell Envelope Assembly. To examine the spatial and temporal dynamics of mycolyltransferase activity, we performed time-lapse microscopy of M. smegmatis exposed to QTF. We developed a microfluidic device (SI Appendix) to maintain cells in a single focal plane for imaging and to facilitate long-term growth by perfusion of growth media containing QTF. As a result, cells could be monitored for three to four generations before crowding effects precluded distinguishing specific activity of individual cells. QTF fluorescence was readily observable during growth. Moreover, because of the probe’s sensitivity, even low
Our results using QTF suggested mycolyltransferases are concentrated at the growing poles. As the cellular location of these enzymes was unknown, we generated C-terminal fusions of mCherry to three *M. smegmatis* mycolyltransferases (Msmeg_3580, Msmeg_6398, and Msmeg_6399) and assessed the localization of each protein ([SI Appendix, Figs. S14–S17]). Fluorescence was localized to the cell envelope, but we observed differential subcellular localizations. Msmeg_3580 and Msmeg_6398 were concentrated at polar and septal regions, while Msmeg_6399 was preferentially located at the septum. The differences in localization indicate that the mycolyltransferases may have distinct functions. To confirm that the observed localization of the mCherry fusion proteins was derived from secreted, extracellular mycolyltransferases, we generated signal sequence variants of Msmeg_3580, Msmeg_6398, and Msmeg_6399. Secretion through the twin-arginine translocation (Tat) pathway can be tested by modifying the twin-arginine motif in the signal sequences. When these residues were substituted with lysine, a change that inhibits secretion through the Tat (39, 40) pathway, cells remained fluorescent and exhibited the same localization patterns as observed with cells producing wild-type proteins. These experiments indicate that our *M. chelorycally mycolyltransferases are not secreted through the Tat system but rather likely through the Sec translocon. Cells harboring plasmids encoding mycolyltransferases lacking signal sequences displayed broad cytoplasmic fluorescence but showed no localized fluorescence at the poles or septa ([SI Appendix, Fig. S18]). When the strain producing Msmeg_6398-mCherry was exposed to QTF, colocalized fluorescence emission was asymmetric and distributed at the poles (Fig. 4D). Overall, the findings support our conclusion that QTF is a reporter of mycolyltransferase activity, and that extracellular mycolyltransferase activity is coincident with sites of peptidoglycan biosynthesis.

**Discussion**

Small molecule probes of cell envelope biogenesis typically report on peptidoglycan assembly, an essential process in virtually all bacterial species (37–41). Probes of distinct classes of bacteria would enable selective visualization and diagnosis. Mycobacteria are distinguished from other prokaryotes by the unique composition of their cell envelope, notably their mycolic acid-rich outer membrane. The mycolic acid layer is constructed from secreted, extracellular mycolyltransferases, we generated probes of distinct classes of bacteria to serve as real-time reporters of mycolyltransferase activity. These labeling strategies require either multiple steps or use fluorophore-substituted trehalose substrates whose fluorescence signal is indistinguishable from their metabolic products. We envisioned complementing the available tools with a mycolyltransferase-triggered fluorogenic probe. To this end, we synthesized QTF, a selective fluorogenic probe that obviates the need for washing and reports on mycobacterial cell wall assembly in live bacteria in real time.

Fluorescence derived from QTF arises through mycolyltransferase-promoted cleavage. Our data indicate this process occurs through the formation and subsequent hydrolysis of an acyl-enzyme intermediate (23). The fluorescent lipid product produced remains at the site of its generation because of the low diffusion rates of the mycobacterial membrane (13). Mycobacteria treated with QTF exhibit fluorescence at the cell poles and cell septum, concentrations (i.e., 250 nM) afforded a robust signal with high contrast and low background. The fluorescence was localized to the septa and cell poles, with enhanced signal at the older poles (i.e., the poles inherited from the previous cell cycle) (Fig. 4A and B, for videos see SI Appendix and Movie S1). To examine whether the fluorescence arising from mycolyltransferase activity is correlated with peptidoglycan biosynthesis, we incubated *M. smegmatis* with QTF and 7-hydroxycoumarin-3-carboxylic acid-3-amino-2-aminoc acid (HADA), a fluorescent analog of d-alanine that is incorporated into nascent peptidoglycan (37, 38). Mycolyltransferase activity (i.e., QTF-derived fluorescence) was coincident with HADA-based fluorescence. Both signals colocalized at the faster growing, older pole (Fig. 4C).

 franchesci, E. coli, M. smegmatis, C. glutamicum, and B. subtilis were treated with 0.1% Triton X-100 for 10 minutes, washed twice, and incubated with 2 μg/mL of QTF in PBS for 10 minutes. Cells were then fixed with 3% paraformaldehyde in PBS for 10 minutes and washed three times with PBS.

**Fig. 3.** QTF processing by mycolyltransferases in live bacteria. (A) Flow cytometry analysis of *M. smegmatis*, C. glutamicum, E. coli, and B. subtilis with QTF. Cells were treated with QTF (1 μM) for two to three doubling times and then fluorescence was compared with that of untreated bacteria. Data are representative of two independent experiments (see SI Appendix, Fig. S8 for scatterplots and controls). (B) Confocal fluorescence microscopy images of different bacterial species exposed to QTF (1 μM) before flow cytometry analysis. Scale bar: 5 μm. (C) Confocal fluorescence microscopy overlay of all channels of a mixed culture of *C. glutamicum* (red), *E. coli* (green), and *B. subtilis* (blue). (D) Fluorescence emission from incubation of QTF (1 μM) with *M. smegmatis* culture filtrate protein (CFP) (200 ng) at 37 °C for 12 h. Probe turnover in the presence of bacteria (10 μM) was monitored. SE from three replicates is depicted. (E) *M. smegmatis* CFP (9 μg) analyzed by native-PAGE ([Left] Coomassie stain; [Right] Immersion in QTF solution [2.5 μM]). Fluorescent bands were excised and analyzed by protein digest mass spectrometry. Identified mycolyltransferases are listed (see also SI Appendix, Fig. S10 and Tables S2 and S3 for protein list). (F) High-resolution mass spectrometry (HRMS) analysis following exposure of QTF (50 μM) to *M. smegmatis* CFP (1 μg). Signals corresponding to QTF and predicted cleavage standards Q-Tre and lipid-FL are labeled (see also Fig. 1 and SI Appendix, Fig. S11).
indicating mycolyltransferase activity is localized to these sites. Thus, despite the abundant secretion of mycolyltransferases, they selectively associate with the cell envelope at the poles and septa.

We also observed cell cycle-dependent mycolyltransferase activity: Midcell fluorescence increased only during septal formation, suggesting the localization of Ag85 to the septum is dynamic. Cytokinesis generates incipient poles in the subsequent daughter cells, consistent with the high temporal activity of Ag85 at the septum. Following division, we observed continued mycolyltransferase activity at both poles of the resulting daughter cells; however, the fluorescence at the new pole is markedly lower than that at the older inherited pole. These observations are consistent with the known sites of peptidoglycan biosynthesis detected using fluorescent peptidoglycan probes, and they highlight QTF’s ability to reveal temporal events in the mycobacterial cell cycle.

Our findings that mycolyltransferase activity is localized led us to examine the location of individual enzyme homologs. To this end, we generated C-terminal mCherry fusions of Msmeg_3580, Msmeg_6398, and Msmeg_6399. The resulting fluorescence aligned with that afforded from QTF. Still, there were differences in the details of mycolyltransferase localization. Specifically, Msmeg_6398 was concentrated at the septa, while Msmeg_3580 and Msmeg_6398 exhibited septal and biased polar localization (SI Appendix, Figs. S15–S17). These findings suggest the expression or localization of these enzymes is cell cycle dependent and that these enzymes have distinct roles in building and maintaining the mycolic acid membrane.

Compounds that inhibit proper mycolyltransferase localization could be efficacious, as deletion of a single mycolyltransferase can profoundly affect cell viability (44). Moreover, mycolyltransferases can engage in protein–protein interactions, as Msmeg_6398 has been found to interact with LprG, a lipid transferase (45). Alternatively, since mycolyltransferases are secreted, their mechanism of export could influence their localization. The M. smegmatis mycolyltransferases in this study are likely exported by the Sec translocase system, which can localize proteins at specific subcellular sites, including the cell poles (46, 47).

Inhibitors of Ag85 have been sought as antibiotic leads. In vitro data obtained with QTF confirmed that the Ag85 inhibitor ebselen blocks the M. tuberculosis mycolyltransferases; however, we found ebselen was poorly effective against the tested M. smegmatis orthologs. The M. smegmatis enzymes we tested possess the conserved cysteine residue ebselen targets in M. tuberculosis Ag85 proteins; however, M. smegmatis also encodes an extended linker region directly preceding the helix that the ebselen adduct has been demonstrated to obstruct (34). M. smegmatis mycolyltransferases may be sufficiently flexible to circumvent the inhibitory effect of the ebselen adduct. A major drawback of ebselen is its lack of selectivity; it functions as a general cysteine modification reagent (48). The ineffectiveness of ebselen in M. smegmatis, and the lack of conservation of this cysteine among mycolyltransferases from other species, highlights the need for the continued development of mycolyltransferase inhibitors, especially for those enzymes produced by pathogens.

QTF’s ability to report on Ag85 activity provides unique opportunities. The inherent sensitivity of this fluorogenic probe, together with its requisite aqueous stability and solubility, render QTF an excellent screening tool. The assays we have described could be readily adapted to high-throughput screens for mycolyltransferase inhibitors. Compounds that block Ag85 activity would augment existing antitubercular drug regimens. Moreover, this mycolyltransferase probe could be employed as a diagnostic tool to detect or selectively monitor mycobacteria in mixed bacterial populations. Our findings indicate QTF can be used in mixed populations to sensitively and rapidly detect mycolic acid-containing bacteria with no washing. In this way, the properties of QTF have the potential to enhance current microscopy-based diagnostic methods that examine patient sputum for the presence of mycobacteria. QTF has the potential to reduce the number of processing steps and duration of staining protocols by eliminating the need for washing. Additionally, the probe may afford improved accuracy and sensitivity in mycobacterial cell
identification—traits that are vital for high-burden regions of *M. tuberculosis* with limited resources.

**Materials and Methods**

Synthetic details and characterization of all previously unreported compounds are provided in SI Appendix. This document also contains information regarding the origin or generation of strains, plasmids, proteins, and compounds described herein, as well as protocols for all fluorescence assays, mass spectrometric assays, and microscopic imaging.

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