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Bacterial cell wall modification with a glycolipid substrate

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Abstract

Despite the ubiquity and importance of glycans in biology, methods to probe their structures in cells are limited. Mammalian glycans can be modulated using metabolic incorporation, a process in which non-natural sugars are taken up by cells, converted to nucleotide-sugar intermediates, and incorporated into glycans via biosynthetic pathways. These studies have revealed examples in which glycan intermediates can be shunted through multiple pathways, and this complexity can be heightened in bacteria, as they can catabolize diverse glycans. We sought to develop a strategy that probes structures recalcitrant to metabolic incorporation and that complements approaches focused on nucleotide sugars. We reasoned lipid-linked glycans, which are intermediates directly used in glycan biosynthesis, would offer an alternative. We generated synthetic arabinofuranosyl phospholipids to test this strategy in *Corynebacterium glutamicum* and *Mycobacterium smegmatis*, organisms that serve as models of *Mycobacterium tuberculosis*. Using a *C. glutamicum* mutant that lacks arabinan, we identified synthetic glycosyl donors whose addition restores cell wall arabinan, demonstrating that non-natural glycolipids can serve as biosynthetic intermediates and function in chemical complementation. The addition of an isotopically labeled glycan substrate facilitated cell wall characterization by NMR. Structural analysis revealed that all the five known arabinofuranosyl transferases could process the exogenous lipid-linked sugar donor allowing for the full recovery of the cell envelope. The lipid-based probe could also rescue wild type cells treated with an inhibitor of cell wall biosynthesis. Our data indicate that surrogates of natural lipid-linked glycans can intervene in the cell's traditional workflow, indicating biosynthetic incorporation is a powerful strategy to probe glycan structure and function.

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ASSOCIATED CONTENT

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website. Procedures for 2D NMR experiments, supplementary NMR spectra, chemical shift data, protocols for microbiological assays, Additional detailed synthetic protocols, analytical data for all compounds (pdf).

Graphical Abstract

INTRODUCTION

Bacterial glycans play vital roles in pathogen survival, virulence, invasion, and subversion of the host immune response.^{1,2} Elucidating the functional roles of bacterial glycans is typically challenging. Small molecule probes enable perturbation of glycans *in vitro* and *in vivo*^{3–7}, making them powerful tools to interrogate glycan biology. Indeed, integrating non-natural carbohydrate substrates^{8,9} into mammalian cell surface glycans via metabolic incorporation has facilitated the introduction of reporter groups.¹⁰ Although metabolic incorporation for probing mammalian glycans is well established, the use of non-natural carbohydrate substrates in bacteria is less studied.^{11–24}

Applications of metabolic incorporation in bacteria are complicated by dissimilarities in mammalian and bacterial glycobiology; bacteria employ a more diverse set of building blocks,^{25,26} and use distinctly different pathways to acquire, metabolize, and biosynthesize monosaccharide intermediates²⁷. The metabolic incorporation of non-natural carbohydrates in mammalian cells often relies on cellular esterases to unmask membrane-permeable probes,²⁸ but many bacterial species lack such esterases. An alternative involves the direct import of monosaccharides through glycan salvage pathways,¹¹ but often there is no available uptake mechanism for a monosaccharide in a bacterial species of interest. Even in mammalian cells, probes can experience unintended fates that lead to undesired labeling.^{28,29} These issues can be compounded in bacteria, which utilize a wide range of monosaccharides as energy sources; as a result, there are multiple opportunities for metabolic glycan precursors to undergo degradation and off-target labeling.¹³

An early example of bacterial cell wall labeling involved using a cell wall precursor, a fluorophore-tagged Park nucleotide, with EDTA-treated *Escherichia coli*.¹⁸ EDTA was required for uptake of this functionalized UDP-sugar derivative. The Grimes group has devised a general strategy to circumvent problems associated with bacterial metabolic incorporation by engineering bacteria to possess specific salvage pathways. They generated bacterial strains that import saccharide building blocks, including non-natural versions, that can be used to label the peptidoglycan.¹⁵ As with other metabolic labeling strategies, their strains convert saccharides into nucleotide-sugar donors. To complement this approach, we sought strategies that do not require genetic engineering and avoid intracellular processing.

Our interest in bacterial polysaccharides led us to examine whether a non-natural glycolipid donor could be assimilated into a biosynthetic pathway (Figure 1). We postulated that such a donor could bypass metabolic enzymes and be used by glycosyltransferases directly. Intervening with a sugar donor rather than a precursor, could decrease probe catabolism. Thus, glycans could be probed using the strategy termed *biosynthetic incorporation*.

Lipid-linked glycans are a class of sugar donors whose use in glycan perturbation is unexplored. These building blocks can either be acceptors or donors. In the case of the latter, they are employed by membrane-localized glycosyltransferases to generate cell surface glycans and cell wall polysaccharides. Glycan probes that take advantage of cell wall-associated enzymes would avoid import and processing constraints that affect nucleotide sugar-based strategies. We also postulated that lipid-linked sugar donors could be incorporated efficiently because they localize to the cell membrane in the vicinity of the relevant glycosyltransferases. Finally, a synthetic glycosyl donor used directly by a cellular glycosyltransferase allows specific glycan structures to be modified based on sugar donor probe identity, side-stepping off-target incorporation.

We examined the proposed biosynthetic incorporation strategy using lipid-linked monosaccharide donors. Our test case involved bacteria from the suborder *Corynebacterianae*, which contains several human pathogens, including *Mycobacterium leprae*, *Corynebacterium diphtheriae*, and *Mycobacterium tuberculosis (Mtb)*. These bacteria can be recalcitrant to antibiotic intervention, partially due to their shared cell envelope architecture (Figure 2A). Common cell wall features include the peptidoglycan, arabinogalactan, and mycolic acid layers collectively referred to as the mycolyl-arabinogalactan peptidoglycan (mAGP) complex.

The mAGP complex requires arabinofuranose, a monosaccharide absent in humans and other mammals. The tuberculosis drug ethambutol blocks arabinan biosynthesis,³⁰ highlighting the essential role of this polysaccharide in *Mtb*. Not only is this polysaccharide essential, but it also modulates host immune responses.³¹ Despite its importance, little is known about the arabinan at the molecular level;³² therefore, we sought probes of arabinan biosynthesis to address this knowledge gap. A series of membrane-associated arabinofuranosyl transferases (AraTs) assemble the arabinan using the endogenous sugar donor decaprenyl phosphoryl arabinofuranose (DPA).^{33–39} We postulated that surrogate glycosyl donors could be used for arabinan biosynthesis. As a test of this strategy, arabinan biosynthesis is ideal because the process does not directly use arabinose nor arabinose monosaccharide derivatives; DPA's biosynthetic precursor is phosphoribosyl pyrophosphate (Figure 2B). A previous attempt to exploit the arabinose salvage pathway^{27,40} did not lead to detectable probe incorporation in arabinose-containing glycolipids.¹³ Thus, we initially sought to evaluate this biosynthetic approach using a DPA-deficient strain of *Corynebacterium glutamicum (Cg ubiA)*.³³ Rescue of cell wall arabinan in this mutant by a glycolipid analog would indicate biosynthetic processing by the enzymes that mediate arabinan construction.

Herein we report the successful generation of lipid-linked sugar donors⁴¹ and their use in biosynthetic incorporation. We synthesized potential arabinofuranose donors and identified

compounds that the cell can process to generate cell wall arabinan. We used these findings to introduce specific carbon-13 labels into the arabinan, facilitating NMR characterization of the cell wall polysaccharide, to assess whether each of the AraTs can process the probe. Electron microscopy revealed that arabinan-deficient cells treated with our glycolipid sugar donors regenerate structurally complete cell walls. Wild-type cells can take up and process our designed probe: The glycolipid probe could rescue *C. glutamicum* and *M. smegmatis* treated with an antibiotic that blocks DPA biosynthesis. These findings highlight that glycolipid surrogates can precisely target biosynthetic pathways to modify cell surface glycans.

RESULTS AND DISCUSSION

Synthetic DPA analogs recover cell wall arabinan

We used arabinan-deficient *Cg ubiA* to assess selective glycan labeling by non-natural lipid-linked sugar donors. These mutant cells fail to generate the endogenous sugar donor; consequently, incorporation of an exogenous donor should be readily detected (Figure 2C). In this mutant, cell wall arabinan was restored by the addition of DPA,⁴² but whether substrate analogs could be used was unclear.

In a test tube, arabinofuranose derivative **1** can serve as a donor for the branching arabinofuranosyltransferase AftC, suggesting simplified DPA analogs might be substrates in cells.⁷ Still, an *in vitro* assay of AraT activity indicated that indicated that AraF derivatives with shorter diterpene lipids were not arabinose donors.⁴³ We, therefore, produced a collection of DPA analogs in which the structure of the lipid was varied (Figure 3).⁴¹ The lipids tested were chosen based on their commercial availability, aqueous solubility, length, and structural similarity to the endogenous decaprenyl lipid.⁴¹ We also factored in analog accessibility by chemical synthesis. These lipids fall into three structural classes: terpenoid, alkyl, and cyclic. FPA (**1**) was included because it is a known substrate for AftC.⁷ The diterpenes Z-neryl (**2**) and (R)-citronellyl (**3**) were also evaluated as controls, as previous findings suggested that they would be poor arabinose donors.⁴³ Octyl (**4**), phenyloctyl (**5**), and dodecyl (**6**) analogs served as inexpensive, commercially available lipids that could shed light on the scope of the lipid group required for function. Finally, a naphthyl analog (**7**) was assessed to provide a structural outlier. We synthesized each of the compounds and evaluated their ability to function as glycosyl donors in cells.

To determine whether any of these analogs would afford arabinan production, we treated cultures of *C. glutamicum ubiA* with each DPA analog (**1–7**) for one doubling time, isolated the cell wall, and carried out carbohydrate composition analysis. The appearance of arabinose in the cell walls of treated bacteria indicated that several of the synthetic analogs led to arabinose incorporation (Figure 3 and Figure S1). We observed variations in the extent of recovery based on the identity of the lipid tail. Most derivatives gave little to no arabinose incorporation. However, consistent with previous work, FPA (**1**) led to significant arabinose incorporation.⁷ Because our experiment was limited to a single doubling time, extant galactan within probe-treated *Cg ubiA* was expected to distort the arabinose/galactose ratio, as we observed. We determined that the arabinose production was highest with FPA (**1**), so we used this derivative in all subsequent experiments.

Characterization of the recovered arabinogalactan from 1-¹³C-FPA treated cells

C. glutamicum uses at least five AraTs to synthesize the arabinan (Figure 4 and Table 1). AftA primes the galactan with an α -1,5-Araf residue at the 8th, 10th, and 12th galactose of the galactan.^{33,34} Emb produces linear α -1,5-Araf.³³ AftC primes the α -1,3-Araf branches.^{35,38} These branches are extended with α -1,5-Araf by AftD.³⁸ Finally, AftB caps the polymer with α -1,2-Araf residues.³⁶ Each of these glycosyltransferases must use the DPA mimic for full arabinan production. We characterized the resultant arabinan to determine which transferases processed FPA as a substrate. We used NMR spectroscopy because it is not destructive, requires no sample derivatization, and has been used to characterize the arabinogalactan of *M. bovis* BCG and *M. smegmatis*.⁴⁴⁻⁴⁶ We obtained ¹H-¹³C HSQC spectra of isolated, soluble arabinogalactan from *C. glutamicum* ATCC 13032 and galactan from untreated *C. glutamicum ubiA* for comparison. The data indicate that the chemical shifts of the relevant resonances are consistent between species and strains (Figure S2, Table S1).⁴⁶ This outcome is compatible with the shared architecture of the core polysaccharide of *C. glutamicum*, *M. smegmatis*, and *M. bovis* BCG.

With the chemical shifts of the *C. glutamicum* arabinan established, we generated a 1-¹³C-labeled version of FPA (**8**) (Scheme S1). The anomeric label provides the means to identify chemical shifts using ¹H-¹³C 2D-NMR experiments, the standard experiments used for carbohydrate characterization. The advantages of using 1-¹³C-FPA (**8**) include selective labeling of saccharides produced from DPA, and a 100-fold increase in signal intensity and, therefore, sensitivity. We treated cells with 1-¹³C-FPA (**8**) and isolated the mAGP complex. The complex was hydrolyzed with base thereby liberating succinyl and mycolyl esters, and cleaving the polysaccharide from the peptidoglycan to yield soluble arabinogalactan.⁴⁷ The labeled arabinogalactan was characterized using ¹H-¹³C HSQC, ¹H-¹³C HMBC, and ¹H-¹³C HSQC-TOCSY. When we compared the ¹H-¹³C HSQC spectrum of labeled arabinogalactan to that of arabinogalactan from wild-type cells (Figure 5), all of the expected resonances (Table S1) were present. The assignment of the anomeric peaks was confirmed using HSQC-TOCSY and HMBC, which elucidated the spin system of the saccharide residue and the connectivity of glycosidic bond, respectively. In the HSQC-TOCSY spectrum (Figure 6) five cross peaks were observed, corresponding to the putative anomeric resonance of the terminal t-Araf residue. The chemical shifts of the observed TOCSY peaks in the F2 dimension, δ 4.05, 3.95, 3.81, 3.69, and 3.59 ppm, matched the resonances of the t-Araf residue of the arabinogalactan. The cross peaks in the HSQC-TOCSY spectrum also matched the chemical shifts obtained in previous characterizations of the arabinogalactan. In the HMBC experiments, the ¹³C- satellites that correspond to H1/C1 of the t-Araf are visible. The cross peak at 3.80/100.63 ppm matches a resonance seen in the HSQC-TOCSY, indicating it is a correlation to H4 of the terminal residue. The other cross peak at 4.09/100.63 ppm is not within the spin system: therefore, this peak is the correlation across the glycosidic bond of the terminal residue.

We overlaid the HMBC spectrum of the arabinogalactan derived from chemical complementation and the HSQC spectrum of the arabinogalactan from wild-type cells (Figure 7). The overlay indicates that the putative terminal Araf residue is attached to the 2-position of the penultimate residue of the arabinogalactan. Similar methods of

characterization confirmed the presence of other expected anomeric peaks (Figure S4, Table S1). These data provide a high level of confidence that all of the expected arabinose residues are present when the mutant is exposed to FPA (**1**).

The detection of the terminal residue has several implications for biosynthetic incorporation. First, with labeling, 2D-heteronuclear correlations can be used to characterize the structure of arabinogalactan from cells treated with the glycolipid analog. Second, the presence of all arabinose residues by NMR indicates that cells can construct full-length arabinogalactan from the exogenous DPA analog FPA (**1**). Thus, FPA is processed by each of the AraTs that build the arabinan.

Recovery of the *Corynebacterianeae* cell wall by chemical complementation

FPA treatment led to the production of cell wall arabinan, but whether FPA treated cells could generate a full cell wall, including the mycolic acid layer was unclear. Accordingly, we treated *Cg ubiA* cells with 500 μ M FPA (**1**) and visualized the cells using electron microscopy (Figure 8). The appearance of a second opaque region around the periphery of the cell is indicative of the mycolic acid bilayer.^{48,49} Therefore, the arabinan synthesized from FPA can support the attachment of the mycolic acids. These data highlight that chemical complementation with FPA can regenerate the critical arabinan and mycomembrane components of the cell wall in *Cg ubiA* cells.

FPA rescues *M. smegmatis* from BTZ043 induced death

Given that *C. glutamicum ubiA* cells could utilize FPA, we wanted to examine FPA use in wild-type cells. Therefore, we determined whether it could rescue cells treated with an inhibitor of DPA biosynthesis. We employed *M. smegmatis mc²155*, a widely used, fast-growing, model for pathogenic mycobacteria. We postulated that processing of FPA by wild-type cells should compensate for the inhibition of DPA biosynthesis and rescue cell viability. Peptidoglycan has been labeled using a related strategy,¹⁵ and inhibitors of DPA biosynthesis are known. One class, benzothiazinones, block the enzyme DprE1, which catalyzes an epimerization reaction in the last step of DPA biosynthesis. The compound BTZ043, a commercially available benzothiazinone, is a potent antibiotic against *M. tuberculosis* (MIC = 1–4 ng/mL), *C. glutamicum* (MIC=20 μ g/mL), and *M. smegmatis* (MIC= 0.1–80 ng/mL).^{50,51} BTZ043 has been suggested to lead to cell death in *C. glutamicum ATCC 13032* by preventing decaprenyl phosphate recycling by sequestering the lipid in the form of decaprenyl phosphoryl ribose.^{51,52} We hypothesized that FPA processing might facilitate lipid recycling and thereby rescue cells from a lethal dose of BTZ043.⁵³

To test this hypothesis, we first treated *M. smegmatis* exposed to a lethal dose of BTZ043 (6 ng/mL) and FPA. FPA addition yielded a concentration-dependent rescue of cell viability (Figure S5A). At 100 μ M FPA, inhibitor-treated cells grew at similar rates to untreated cells. We then tested the expected product of FPA processing, (Z,Z)-farnesyl phosphate. When a cell uses the natural substrate DPA, the arabinan is produced and decaprenyl phosphate is generated. The mitigating effects of FPA raised the possibility that its activity was due to the production of (Z,Z)-farnesyl phosphate. We, therefore, tested this possibility. No concentration of (Z,Z)-farnesyl phosphate used (150 pM to 100 μ M) in *M. smegmatis*

afforded any rescue (Figure S5B). We obtained similar results with *C. glutamicum*, as FPA rescued the effects of BTZ0243, but (Z,Z)-farnesyl phosphate had no effect. Our cumulative data indicate that when cells are treated with an inhibitor of DPA, the glycolipid FPA can rescue cells. These findings are consistent with our other data showing that the AraTs process FPA to generate the cell wall arabinan. The inability of the (Z,Z)-farnesyl phosphate to compensate for the effects of BTZ0243 suggest that FPA's role as a sugar donor is essential to the mechanism of rescue. Moreover, our findings suggest there are multiple pathways of lipid recycling in *Corynebacteriaceae*, highlighting the value of these glycan probes.

CONCLUSION

Using mimics of glycolipids, we found that biosynthetic incorporation can be used to manipulate bacterial glycans.

Specifically, we synthesized lipid-linked arabinofuranose donors that install arabinose directly into the mycobacterial cell wall. An isotopically labeled derivative of the most effective donor, FPA (**1**), facilitated characterization of the resultant cell wall polysaccharide. The data show that FPA was a substrate for the cellular enzymes and its presence resulted in the generation of an intact arabinogalactan. Electron microscopy of arabinan-deficient cells exposed to the synthetic glycolipid revealed the mycolic acid membrane was restored. These data indicate that FPA is a substrate for each of the arabinofuranosyltransferases that mediate cell wall construction; thus, FPA is a competent DPA surrogate. The identification of an accessible DPA surrogate enables new experiments to examine the structure of the arabinan by NMR and mass spectrometry. Our rescue experiments highlight the potential utility of these probes to uncover new features of glycan biosynthesis. Also, we anticipate that cells can incorporate other arabinofuranose derivatives bearing modifications for structural and functional studies.

Though our studies focused on the arabinan of *Corynebacteriaceae* species, biosynthetic incorporation using glycolipids should apply to other bacterial glycans. For example, an immunomodulatory glycolipid in mycobacteria known as the lipoarabinomannan requires both lipid-linked mannose and lipid-linked arabinose as the sugar donors for its synthesis.^{57,58} We envision probing the function of this glycan using biosynthetic incorporation. In addition, Gram-negative bacteria, including some pathogenic strains employ oligosaccharyltransferases and lipid-linked sugar donors for glycan and glycoconjugate biosynthesis.⁵⁹ We envision glycolipid probes could act as both a donor and acceptor. Glycans would be built on the probe and then transferred to its target. Thus, biosynthetic incorporation could be applied to study how oligosaccharide structure affects the virulence of many important human pathogens including *Neisseria meningitidis*, *Campylobacter jejuni*, and the gut commensal *Bacteroides fragilis*.^{60–64}

Glycolipids are used in multiple biosynthetic pathways in bacteria, and these pathways can be exploited. Trehalose-based glycolipid analogs can be processed by enzymes to modify the mycobacterial cell envelope and image the bacteria.^{65,66} We found FPA can rescue antibiotic-treated cells, but the product of FPA utilization, (Z,Z)-farnesyl phosphate, cannot.

These findings highlight the need to better understand how different lipids are recycled. We envision our biosynthetic strategy could be adapted to track the fate of glycosyl donor polyprenyl groups in cells. Thus, we anticipate glycolipid probes provide a new means to monitor, label, and visualize critical pathways in bacteria.

MATERIALS AND METHODS

Materials and Instrumentation

All chemicals were purchased from Sigma Aldrich unless otherwise stated. BTZ043 was purchased from Ark Pharm. Unlabeled DPA analogs (**1-7**) were prepared using methods as previously reported (See the Supporting Information for procedures and characterization data).⁴¹ 1-¹³C-D-arabinose was purchased from Cambridge Isotope Labs (CLM-715). 1-¹³C-(*Z,Z*)-farnesyl phosphoryl-β-D-arabinofuranose (**8**, Scheme S1) was prepared following a previously published route.⁴¹ (*Z,Z*)-farnesyl phosphate was purchased from Isoprenoids, LLC (2z6zFP003). Nuclear magnetic resonance spectra were recorded on a 300 MHz spectrometer (acquired at 300 MHz for ¹H and 75 MHz for ¹³C), 400 MHz spectrometer (acquired at 400 MHz for ¹H and 100 MHz for ¹³C), or a 500 MHz spectrometer (acquired at 500 MHz for ¹H and 125 MHz for ¹³C). Chemical shifts are reported relative to tetramethylsilane or residual solvent peaks in parts per million (CHCl₃: ¹H, 7.27, ¹³C, 77.23; MeOH: ¹H, 3.31, ¹³C, 49.15). High-resolution mass spectra (HRMS) were obtained on an electrospray ionization-time of flight (ESI-TOF) mass spectrometer.

Strains and growth conditions

C. glutamicum ATCC 13032 and *C. glutamicum ubiA* were grown at 30°C in brain heart infusion sorbitol (BHIS) media. *C. glutamicum pks13*, was grown in brain heart infusion (BHI) media. *C. glutamicum ubiA* and *C. glutamicum pks13* were selected for by supplementing cultures with kanamycin to a final concentration of 25 µg/mL. *Mycobacterium smegmatis mc²155* was grown in Middlebrook 7H9 medium supplemented with Middlebrook ADC and 0.05 % Tween-80.

Chemical Complementation of the Arabinan

Cultures of *C. glutamicum* ATCC 13032 and *C. glutamicum ubiA* were grown to saturation in BHIS media. Refresh cultures were inoculated from the saturated starter cultures (OD₆₀₀=0.02) and grown to mid-log (OD₆₀₀=0.6–0.8). Aliquots (1 mL) were separated into sterile 10 mL baffled flasks, and DPA analogs were added from 100 mM DMSO stocks to the desired final concentration. After growth to saturation, cells were transferred into Eppendorf tubes and pelleted for 15 minutes at 5,000 x g. The supernatant was discarded, and the cell pellets were stored at –80 °C.

Mycolyl-arabinogalactan-peptidoglycan complex isolation

Cell pellets from 1 mL cultures were resuspended in lysis buffer, 2% Triton x-100 in PBS pH 7.2, and disrupted by sonication (6 × 20 sec separated by 2 min off intervals) while on ice. The cell debris was pelleted by centrifugation at 15,000 x g for 15 min. The supernatant was discarded, and the pelleted material was washed three times with a 2% solution of SDS in PBS (500 µL) by heating to 95 °C for 1 h before pelleting as above and discarding the

supernatant. An additional three washes were performed with water, 80% acetone/water, and then acetone (500 μ L each). After discarding the supernatant from the final wash, the pellets were placed under vacuum overnight to remove any remaining acetone before being stored at -20°C .⁴⁵

Alditol acetate preparation for cell wall composition analysis

The previously isolated samples of the mAGP complex were taken up in 2M trifluoroacetic acid (250 μ L) and hydrolyzed at 115°C for 2 h. The suspensions were filtered through syringe filters (Millex ® , PVDF, 0.2 μm pore size) and concentrated under reduced pressure. The monosaccharide mixtures were reduced by stirring with 0.25 M NaBD₄ in 1 M NH₄OH in ethanol (250 μ L) overnight. The remaining deuteride was quenched with 10% acetic acid/methanol (250 μ L) and concentrated under reduced pressure. Residual borate salts were removed by evaporation with 3×250 μ L aliquots of 10% acetic acid/methanol under reduced pressure. The samples were dried overnight under high vacuum. The alditol samples were taken up in a 1:1 mixture of acetic anhydride and pyridine (500 μ L) and stirred at room temperature overnight. The reactions were concentrated under reduced pressure, and residual acetic anhydride and pyridine were removed by co-evaporation with toluene. The alditol acetates were taken up in ethyl acetate (500 μ L), passed through a plug of MgSO₄, and concentrated *in-vacuo*.⁴⁵

Cell wall composition analysis

Alditol acetate samples were dissolved in 1:1 acetonitrile/water (200 μ L) and analyzed by LC-MS. The analysis was performed on a Shimadzu LC-MS-2010A spectrometer fitted with a Supelco Discovery ® BIO wide pore C18 column (150 mm x 2.1 mm, 5 μm pore size) eluting with a gradient from 17–23% acetonitrile in water over 25 minutes with a flow rate of 0.1 mL/min.

NMR sample preparation of arabinogalactan from mycolyl-arabinogalactan-peptidoglycan

The solid mAGP complex was taken up in 1 M sodium hydroxide to a final concentration of 0.1 g of mAGP/mL. The suspension was stirred for 24 h at 70°C . The peptidoglycan was removed by vacuum filtration through a membrane filter (4 μm pore size) to yield soluble arabinogalactan in aqueous base. The solution was desalted by dialyzing into Millipore water for 24 h. The samples were lyophilized to afford fluffy off-white solids. The solids were taken up in D₂O and lyophilized three times before they were stored for later use in a desiccator at -20°C .⁶⁷

2D-NMR Spectroscopy

All 2-dimensional ^1H - ^{13}C spectra were recorded using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm TCI-F or QCI-F Cryoprobe at 298 K in D₂O. All experiments (heteronuclear single quantum coherence, ^1H - ^{13}C HSQC; heteronuclear single quantum coherence-total correlation spectroscopy, ^1H - ^{13}C HSQC-TOCSY; and heteronuclear multiple bond correlation, ^1H - ^{13}C HMBC) were performed using standard pulse sequences provided in the Bruker Software. The mixing time for the TOCSY experiment was 80 or 120 ms.

Electron Microscopy

Pelleted cultures grown with or without FPA were removed from the -80°C freezer, thawed, and fixed with 2.5% glutaraldehyde and 0.05 M lysine in 0.1 M cacodylate buffer (pH 7.4) containing 0.075% Ruthenium Red for 1 h at 4°C (1 mL). Samples were washed five times with 0.5 mL of 0.075% ruthenium red in 0.1 M cacodylate buffer. The washed cells were post-fixed with 1% w/v OsO_4 in 0.1 M cacodylate containing 0.075% Ruthenium Red for 1 h at rt. After rinsing the fixed cells with distilled water, they were resuspended in 1% aqueous uranyl acetate for 1 h and washed five times with water. Suspended cells were dehydrated through a graded ethanol series (50 to 100% ethanol in distilled water) before being embedded in Spurr medium through intermediate 1,2-epoxypropane infiltration. Blocks were conventionally cut, stained, and examined with a Philips CM120 microscope operating under standard conditions.⁵⁵

Rescue of cells treated with BTZ043

Cell viability was quantified in triplicate via the Alamar Blue Assay (Invitrogen).⁵⁶ Starter cultures of *M. smegmatis* were diluted with Luria-Bertani broth to $\text{OD}_{600} = 0.05$. Starter cultures of *C. glutamicum* were diluted with Luria-Bertani broth to $\text{OD}_{600} = 0.001$. Compounds assayed at a constant concentration were added to the master mix. The master mix (100 μL) was added to the second through eleventh wells of the plate leaving the third column empty. To remaining master mix (700 μL) was added the compound being serially diluted. To the empty wells in column three were added 200 μL of master mix doped with a compound. The 100 μL excess in column three was serially diluted by mixing with subsequent columns. Plates containing *M. smegmatis* were incubated at 37°C for 24 h with shaking at 200 rpm. Plates containing *C. glutamicum* were incubated at 30°C for 16 h with shaking at 200 rpm. After the plates had grown, 6 μL of Alamar blue reagent (Invitrogen) was added to each well. The plates were returned to the shaker for 1 h. Plates were read on a Tecan M1000 plate reader. Plates were shaken for 2 seconds (2 mm, linear) immediately before reading well fluorescence ($\lambda_{\text{ex}} = 570 \text{ nm} \pm 5$, $\lambda_{\text{em}} = 585 \pm 5 \text{ nm}$). Each well was read with 50 flashes at 400 Hz from 20 mm above the plate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Monosaccharide probe incorporation strategies.

A) In metabolic incorporation, a monosaccharide probe is converted to a nucleotide sugar by the cell leading to competition with endogenous metabolites and diversion from the desired biosynthetic pathway. B) Biosynthetic incorporation utilizes a lipidated sugar donor eliminating cytosolic processing and decreasing competition.

Figure 2. Schematic depiction of the mycobacterial wall and arabinan biosynthesis.

(A) The essential mycolyl-arabinogalactan-peptidoglycan complex is composed of building blocks not present in mammals. The arabinan (yellow) is appended to the galactan (red) via arabinofuranosyltransferases (AraT) that use the arabinose donor decaprenyl phosphoryl arabinofuranose (DPA). The linkage of the arabinogalactan to the peptidoglycan involves multiples steps (not shown). (B) DPA is biosynthesized from pRpp, complicating the use of arabinose for metabolic incorporation of arabinose. (C) An illustrative arabinosylation reaction catalyzed by the arabinofuranosyltransferase AftA, which appends an arabinofuranose residue to the galactan. (D) The structure of the arabinan depicting the mycobacterial arabinofuranosyltransferases (AftA, Emb, AftD, AftC, and AftB). The relationships between residues in the arabinan and the arabinofuranosyl transferases responsible for their introduction are indicated by dashed lines (AftA, Emb, and AftD) or by colored circles (AftC and AftB).

Figure 3. The extent of arabinose incorporation determined by cell wall composition analysis. Chemical complementation of *Cg ubiA* was performed by growing cells with each potential donor (250 μ M) shown (1-7) for one doubling time. The ratio of arabinose to galactose residues was compared to *Cg* wild-type and the *Cg ubiA* mutant by cell wall composition analysis. (Z,Z)-farnesyl phosphoryl arabinofuranose (1) treatment yielded the highest ratio of arabinose to galactose. Data shown are from a single experiment..

Figure 4. The strategy for labeling the arabinogalactan.

AraTs that process 1- ^{13}C -FPA give rise to products with characteristic NMR chemical shifts.

Roman numeral designations of these spin systems are maintained in subsequent NMR spectra and tabulated resonances.

Figure 5. Stacked ^1H - ^{13}C HSQC spectra of soluble arabinogalactan from *Cg* ATCC 13032 (black) and arabinogalactan from *Cg* *ubiA* complemented with $1\text{-}^{13}\text{C}$ -FPA (8) (red). Spectra were recorded at 600 MHz at 298 K in D_2O . Relevant peaks are tabulated in Table S1.

^1H - ^{13}C HSQC-TOCSY■ *C. glutamicum* $\Delta ubiA$ + 1- ^{13}C -FPA (8) ^1H - ^{13}C HSQC■ *C. glutamicum* ATCC 13032 CH/CH₃■ *C. glutamicum* ATCC 13032 CH₂ **Figure 6.**

Stacked ^1H - ^{13}C HSQC spectra of soluble arabinogalactan from *Cg* ATCC 13032 (black and gray) and ^1H - ^{13}C HSQC-TOCSY of arabinogalactan from *Cg ubiA* complemented with 1- ^{13}C -FPA (8) (red). Spectra were recorded at 600 MHz at 298 K in D_2O . Relevant peaks are tabulated in Table S1.

^1H - ^{13}C HMBC■ *C. glutamicum*Δ*ubiA* + 1- ^{13}C -FPA (8) ^1H - ^{13}C HSQC■ *C. glutamicum* ATCC 13032 CH/CH₃■ *C. glutamicum* ATCC 13032 CH₂ **Figure 7.**

Stacked ^1H - ^{13}C HSQC spectra of soluble arabinogalactan from *Cg* ATCC 13032 (black and gray) and ^1H - ^{13}C HMBC of arabinogalactan from *Cg* *ubiA* complemented with 1- ^{13}C -FPA (8) (red). Spectra were recorded at 600 MHz at 298 K in D₂O. Relevant peaks are tabulated in Table S1.

Figure 8. Transmission electron micrographs and corresponding schematic depictions of mycobacterial walls of *Cg ATCC 13032*, *Cg ubiA* with and without 500 μ M FPA treatment (1), and *Cg pks13*, which lacks mycolic acids⁵⁴.

The opaque outer region represents the surface polysaccharides (PS) and mycolic acid membrane. The middle electron transparent region is indicative of the peptidoglycan (PG), arabinogalactan (AG) and mycolic acid membrane (MAM), and the opaque inner region is the cell membrane (CM).⁵⁵ Scale bars equal to 100 nm.

Figure 9. The effect of FPA (1) on the antibiotic activity of BTZ043 in (A) *M. smegmatis* *mc*²155 and B) *C. glutamicum* ATCC 13032.

Bacterial growth was measured, via the Alamar Blue assay, in the presence of specified concentrations of antibiotic BTZ043 only (black) or BTZ043 and 100 μ M FPA (1) (green). The Y axis depicts the relative fluorescence compared to an untreated control sample.⁵⁶ Experiments were repeated in triplicate. For points lacking error bars the error was too small to be depicted graphically. Error bars represent the standard deviation, $n=3$.

Table 1.

Spin systems produced by specific AraTs.

Enzyme	Linkage	Relevant spin systems
AftA	α 1–5 (Priming)	V
Emb	α 1–5 (Main Chain)	VIII
AftC	α 1–3	I and VII
AftD	α 1–5 (Branches)	V
AftB	β 1–2	I, III, and IV