INTRODUCTION

Bacteria control the length of their polysaccharides, which can control cell viability, physiology, virulence, and immune evasion. Polysaccharide chain length affects immunomodulation, but its impact on bacterial physiology and antibiotic susceptibility was unclear. We probed the consequences of truncating the mycobacterial galactan, an essential linear polysaccharide of about 30 residues. Galactan covalently bridges cell envelope layers, with the outermost cell wall linkage point occurring at residue 12. Reducing galactan chain length by approximately half compromises fitness, alters cell morphology, and increases the potency of hydrophobic antibiotics. Systematic variation of the galactan chain length revealed that it determines periplasm size. Thus, glycans chain length can directly affect cellular physiology and antibiotic activity, and mycobacterial glycans, not proteins, regulate periplasm size.

RESULTS

To generate mutants with shorter galactan, we characterized M. smeg Gift2 (gene ID: Msme_6403). Chain elongation experiments with recombinant WT M. smeg Gift2 afforded polysaccharides of similar length as those generated by M. tuberculosis Gift2 (Fig. 1, B and C). We generated D267E and D267A variants of M. smeg Gift2, as these variants have substitutions at the metal ion binding motif; therefore, they should bind UDP-Galf less well and afford shorter galactan chains (Fig. S1) (14). In a chain elongation assay, D267A Gift2 was only able to transfer a few Gal residues to acceptor, but D267E Gift2 afforded products about half the length of those obtained using WT Gift2 (Fig. 1C). We therefore introduced gift2 point mutants to an M. smeg strain lacking endogenous gift2. Using a modified approach to mycobacterial recombineering (15), we deleted gift2 at its endogenous site while providing complementation through exogenous expression of Flag-gift2 from an integration proficient plasmid (16). Expression of D267A Flag-gift2 did not complement, but the D267E Flag-gift2 construct did (Fig. S2).

To determine whether the galactan length had been altered in cells, we analyzed the monosaccharide composition of WT, Flag-gift2 complemented (WTcomp), and D267E Flag-gift2 complemented (D267Ecomp) cells (Fig. S3) (17). Because each galactan chain contains one rhamnose (Rha) residue, the ratio of galactose to rhamnose (Gal:Rha) is a surrogate for average galactan chain length. Applying this analysis revealed that D267Ecomp cells generate galactan chains approximately twofold shorter than WT cells (Table 1 and fig. S3).
The arabinosylation of the D267E comp strain was within 5% of the WT comp strain, implying that the galactan chain in the mutant undergoes arabanin formation. Next, we determined whether any other saccharide units or lipids in the D267E comp cell envelope had changed. No change in the identity of covalently associated lipids or the relative amount of galactan appended to the cell wall was detected. Any changes in the composition of free lipids and lipoarabinomannan (LAM) were minimal (fig. S3). Thus, the major perturbation of D267E comp cells is a truncated galactan.

A growth defect of D267E comp cells was readily apparent, as their doubling time was approximately 1.5-fold slower than that of WT (Fig. 2A). Extracellular glycans can influence a cell’s ability to withstand osmotic pressure (18, 19); therefore, we tested whether hyperosmotic stress would further exacerbate this defect. We evaluated osmolyte tolerance for WT, WT comp, and D267E comp cells and found that the mutant strain was much less resistant to hyperosmotic stress (fig. S4). When exposed to high salt concentration (~500 mM sodium chloride), the growth rate was severely restricted.

We profiled cells cultured in hyperosmotic medium containing 250 mM sodium chloride (about 15-fold higher than standard conditions) using RNA sequencing (RNA-seq). Data from the surviving population indicate that the genes associated with nutrient acquisition (Fig. 2B and table S1) and proteostasis are up-regulated. Increasing the levels of proteins that mediate proteostasis can mitigate osmotic stress (20). The mutant cells strongly up-regulate these pathways, thereby underscoring their high sensitivity.

*M. smeg* mutants with defects in cell wall biosynthesis are hypersensitive to select antibiotics (7, 21). We, therefore, assessed the viability of the D267E comp strain after antibiotic exposure (Fig. 2C). We reasoned that loss of proper glycan chain length could result in either an alteration of cell envelope permeability to facilitate antibiotic uptake or an increase in the susceptibility of the relevant biosynthetic pathway to inhibition. Consequently, we tested a panel of antibiotics with diverse hydrophobicities and mechanisms of action. D267E comp cells were more susceptible than WT to numerous antibiotics (fig. S5); however, those antibiotics did not share a related mechanism of action. For example, no potency increases were observed for antibiotics targeting the cell envelope. In addition, hydrophilic antibiotics, such as streptomycin or meropenem, exhibited no change in activity. These findings align with results from our lipid analysis experiments indicating that the mutant has an intact mycolic acid–rich outer membrane, as this layer is likely the major barrier for hydrophilic antibiotics. In contrast, hydrophobic antibiotics were more effective on the mutant. Specifically, hydrophobic agents with unrelated mechanisms of action, such as rifampicin and novobiocin, were more potent (fig. S5).

Deletion of nonessential genes involved in cell wall polysaccharide biosynthesis can alter bacterial cell shape and cell envelope architecture (19, 22). Consequently, we evaluated cell shape of D267E comp cells using microscopy. Using a membrane stain to illuminate the cell outline, we found that relative cell length had decreased (fig. S6). To better observe any defects in cell growth and division, we next conducted time-lapse microscopy in a microfluidics chamber (movies S1 to S6). *M. smeg* cells are normally long, thin rods; however, defects in cell envelope biosynthesis can result in “blebbing,” a phenotype characterized by the appearance of circular protrusions (Fig. 3A). Analysis of time-lapse microscopy revealed that D267E comp cells undergo blebbing, and numerous instances of cell rupture were detected during division. These phenotypes also have been observed with mutants of PG biosynthesis, in which atomic force microscopy indicated a substantial decrease in cell wall rigidity (23). These data underscore the underappreciated contributions the galactan makes to cell structure and rigidity.

The effect of the galactan on cell length and rigidity prompted us to examine the architecture of the mutant cell envelope. We used transmission electron microscopy (TEM) to visualize WT, WT comp, and D267E comp cells. The morphology of multiple D267E comp cells was indicative of blebbing, consistent with our previous observations (fig. S6). In the mutant, the periplasm, the region where the galactan resides, had contracted (Fig. 3B and fig. S6). On average, the thickness of the periplasm of D267E comp cells was decreased by about 30% (Fig. 3C). To better understand the relationship between galactan chain length and cell shape, we generated a set of strains whose galactan length varied systematically.

### Table 1. Monosaccharide composition analysis of cell envelope isolated from WT, WT comp, and D267E comp strains. Ratios are the average of three biological replicates

|        | Ara | Gal | Rha |
|--------|-----|-----|-----|
| WT     | 64  | 34  | 1   |
| WT comp| 59  | 33  | 1   |
| D267E comp| 56 | 16  | 1   |
Orthologs of GlfT2 have been identified across the Corynebacterineae suborder, and previous investigations indicate that orthologous GlfT2s have distinct chain length control (24). We introduced the glfT2 genes from either Nocardioides brasiliensis (Nbras) or Corynebacterium diphtheriae (Cdiph) into our glfT2-deficient M. smeg strain. These orthologs complemented growth, and in both CdiphComp and NbrasComp strains, the galactan chain length was shorter than that of WT (Fig. 3D and fig. S7). The frequency of cell bleeding increases as galactan size decreases (fig. S6). We therefore imaged these cells using TEM. The results showed that periplasm thickness and galactan chain length were correlated (Fig. 3, E and F).

To validate the relationship between galactan chain length and periplasm size, we examined these strains using cryo-TEM. Because chemical fixation, dehydration, or embedding of samples was not required, the cells can be imaged in a near-native state. We visualized WT, WTComp, CdiphComp, NbrasComp, and D267EComp strains using cryo-TEM and measured average periplasm size for each strain (Fig. 4A). The data from this analysis recapitulated the trend seen using conventional TEM (Fig. 4, B and C). Among these strains, galactan chain length correlated directly with periplasm size, with an average contribution of 3.2 Å per Glf residue.

**DISCUSSION**

Many polysaccharides, including the galactan, are assembled in the cytoplasm and then transported into the extracellular space; therefore, export and chain length control are tightly coupled (25, 26). Our ability to isolate a mycobacterial strain with a truncated galac-
For mycobacteria, decreasing periplasm size appears to disrupt normal nutrient uptake, as D267Ecomp cells subjected to hyperosmotic challenge strongly up-regulate nutrient acquisition machinery. Last, in *Escherichia coli*, the Rcs system detects periplasm stress, in part by the protein RcsF (34). In *M. smeg*, there are two proteins with sequence similarity to RcsF (table S1 and fig. S8). The transcript for one (Msme_0699) is among the most highly up-regulated in D267Ecomp cells during normal growth and hyperosmotic challenge. This finding suggests that periplasm size is a cellular attribute that mycobacteria maintain and monitor.

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**Fig. 3. Galactan chain length affects cell shape and periplasm size.** (A) Time-lapse microscopy images of *M. smegmatis* glfT2 mutants. Galactan truncation led to blebbing morphology (arrows) and, in multiple instances, cell rupture during division. (B) TEM of *M. smegmatis* glfT2 mutants highlighting the periplasm (arrows). (C) Galactan truncation decreases periplasm size in *M. smegmatis* (*P* < 0.0001). (D) Introduction of glfT2 orthologs affords access to strains with altered galactan chain length. (E) TEM images were used to quantify periplasm thickness across ortholog complemented strains. (F) Periplasm size and galactan chain length correlate linearly.

**Fig. 4. Native-state imaging of glfT2 engineered *M. smeg*.** (A) Cryo-TEM micrographs of WT, WTcomp, and D267Ecomp strains. Boundaries of periplasm are highlighted with red lines (left). (B) Quantification of periplasm size. (C) Cryo-TEM data analysis correlates with galactan chain length and periplasm size.
Polysaccharides decorate the surface of all cells. The length of the glycan chains is controlled, but how chain length affects bacterial physiology has been unclear. Before this study, the only known role of chain length had been to influence immunomodulatory properties (1). We found that polysaccharide chain length affects bacterial physiology and numerous cellular attributes, including cell permeability, morphology, mechanical integrity, resistance to environmental stress, periplasm size, and antibiotic susceptibility. Our findings highlight how bacteria can modulate polysaccharide length to respond to environmental stresses. The observation that strains of bacteria can modulate galactan chain length (7) highlights the flexibility endowed by the template-independent synthesis used to create glycans. This flexibility could be an advantage in responding to dynamic changes in the environment. Last, our data suggest that agents that block galactan biosynthesis could enhance susceptibility to known hydrophobic antibiotics.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

*M. smegmatis* mc^155 and derived mutants were grown with shaking at 37°C in Middlebrook 7H9 broth (BD, Franklin Lake, NJ) supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol, 0.5% (w/v) bovine serum albumin (United States Biological, Salem, MA), catalase (4 mg/liter) (Sigma-Aldrich), 15 mM sodium chloride, and 0.05% (v/v) Tween 80. Cells were maintained on agar plates using 7H10 agar (BD, Franklin Lake, NJ) supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol, 0.5% (w/v) bovine serum albumin (United States Biological, Salem, MA), catalase (4 mg/liter) (Sigma Aldrich), and 15 mM sodium chloride. When necessary, antibiotic selections were performed using kanamycin (20 μg/ml), hygromycin B (50 μg/ml), and gentamicin (2.5 μg/ml).

**Molecular biology methods and cloning**

All cloning was performed using DH5α (NEB) grown in LB supplemented with kanamycin (50 μg/ml) or ampicillin (100 μg/ml) when appropriate. Plasmids and oligonucleotide primers used in this study are described in table S2. For protein expression and purification, *M. smegmatis* glfT2 was cloned into pET28a. Site-directed mutation constructs were amplified by polymerase chain reaction (PCR), phosphorylated by T4 Polynucleotide Kinase (NEB), ligated by cloning vectors plasmids were used to develop point variants of integration-proficient vectors harboring mutations were created by next-generation sequencing.

**Protein expression and purification**

A homology model for *M. smegmatis GlfT2* was generated using SWISS-MODEL (35). Protein was expressed in DE3 Tuner cells. Starter cultures were used to inoculate 1-liter cultures of LB supplemented with kanamycin to 50 μg/ml. These cultures were grown to mid-log at 37°C, and once reaching OD₆₀₀ (~0.6), they were chilled on ice. Protein production was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.1 mM. Cultures were then incubated overnight at 16°C. Cells were then harvested by spinning at 4000 relative centrifugal force (RCF) for 10 min. Cell pellets were frozen at ~80°C until purification.

Cell pellets were thawed on ice and resuspended in buffer A [50 mM sodium phosphate, 400 mM NaCl, 20 mM imidazole, 15% glycerol (pH 7.6)] supplemented with lysozyme (1 mg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, and 1250 U benzonase (Millipore Sigma). Cell lysis was completed by mechanical disruption at 22 kpsi (Constant Systems Cell Disruptor). The lysate was clarified by centrifugation at 20,000 RCF, and the supernatant was filtered using a 0.22-μm PES EasyFlow syringe-drive filter (Millipore Sigma). GlfT2 constructs with N-terminal hexahistidine tags were purified using IMAC (immobilized metal affinity chromatography). The filtered supernatant was loaded onto an Akta Fast protein liquid chromatography system and applied to a 1-ml HisTrap HP column (GE Healthcare). A linear, 5%/min gradient of buffer B [50 mM sodium phosphate, 400 mM NaCl, 400 mM
imidazole, 15% glycerol (pH 7.6)] was used to elute GlfT2 constructs. Fractions containing GlfT2 constructs were identified by SDS-PAGE, pooled, vitrified, and stored at −80°C.

**GlfT2 in vitro assays**

GlfT2 acceptor elongation experiments were set up similarly to previous reports (12). Kinetics parameters were determined using UDP-Glo (Promega). Enzyme acceptor binding was saturated by addition of acceptor to 200 μM, and donor sugar (UDP-Galf) concentration varied from 15 to 1500 μM with UDP-Galf. Kinetic reactions were initiated by the addition of GlfT1 to 50 nM and quenched with UDP-Glo reagent after 40 min at room temperature. Reaction volumes were 10 μl and were performed in triplicate. This time point was chosen to avoid any artifacts owing to quenching the reaction during kinetic lag phase (36).

**Cell length determination**

Cells were grown in supplemented 7H9 medium and incubated with FM-464 (5 μg/ml) to stain the cell membrane. Cells were placed on a 1.0% agarose pad. Images were collected using a Nikon Eclipse Ti-E epifluorescence microscope using a 100× objective. Length determination was determined using NIS-Elements software. Cell boundaries were determined using thresholding for bright objects, and of the cells selected, only single cells were measured. Statistical significance was determined by unpaired t test (P < 0.0001).

**Transmission electron microscopy**

Cells were grown to mid-log in supplemented 7H9 medium. Fixation, dehydration, embedding, sectioning, and microscopy were performed similar to as previously described (37). Periplasm size was determined using ImageJ. Distance between the inner and outer membranes was measured in at least 10 locations per cell, and the average of those individual measurements was taken as the periplasm size for that cell. For initial imaging using WT, WTcomp, and D267Ecq strains, 100 cells were analyzed for each strain. Statistical significance was determined by unpaired t test (P < 0.0001). Subsequent imaging using WT, Cdihcomp, Nbrascomp, and D267Ecq strains analyzed 30, 30, 25, and 23 cells, respectively.

**Cryo-TEM**

Cells were cultured to mid-log in complete 7H9 and concentrated 50-fold by pelleting by centrifugation and resuspending in a 50-fold smaller volume. Concentrated cells were plunged-frozen in liquid ethane using an automated Leica EM GP system (Leica Microsystems) using R2/2 200 mesh grids (Quantifoil) and imaged using a Talos 120 kV cryo-transmission electron microscope. For quantification, the distance between the inner and outer membranes was measured an average of 10 individual locations per cell. The mean of those individual measurements was taken as the periplasm size for that cell. Twenty-five cells were analyzed for each strain.

**Time-lapse microscopy**

Cells were grown to mid-log and diluted to OD₆₀₀ of 0.1. Cells were loaded into a CellASIC (BO4A) plate with constant fully supplemented 7H9 flow in a 37°C chamber. Images were taken every 15 min on an inverted Nikon TI-E microscope using a 60× objective. Cells were imaged using phase contrast. For analyzing frequency of blebbing, cells were loaded into the BO4A plate, and images from the initial time point were used to score for blebbing. Undistorted rod-shaped cells were counted as phenotypically normal, and cells with circular protrusions were counted as undergoing blebbing. Scoring was performed in technical quadruplicate.

**Minimum inhibitory concentration determination**

Antibiotic sensitivity was determined using an alamarBlue assay similar to previous reports (38). Briefly, cells were cultured to late-log phase using supplemented 7H9 medium and diluted to OD₆₀₀ = 0.05. An antibiotic of interest was then added and diluted serially twofold in a black 96-well plate (Corning). The final volume of cultures was 100 μl. Cells were incubated with shaking for 24 hours at 37°C. Cell viability was determined by adding 6 μl of alamarBlue (Thermo Fisher Scientific) in each well. The plate was then incubated at 37°C for 1 hour, and fluorescence (emission, 590 nm) was measured.

**SDS sensitivity**

Minimum inhibitory concentration (MIC) for SDS sensitivity was conducted similar to antibiotics. Viability of cells following exposure to SDS was determined by culturing strains to mid-log, supplementing with SDS to 0.05% (w/v), and incubating for 4 hours at room temperature. CFU were then determined for each strain, with and without SDS added. Concentration of SDS was chosen based on previous hypersusceptibility phenotypes.

**RNA sequencing**

Cells were grown to mid-log in either supplemented 7H9 or supplemented 7H9 with 250 mM NaCl. Cells were harvested by centrifugation at 3000 RCF for 10 min, and cell pellets were resuspended in TRIzol. Cell suspensions were frozen at −80°C. RNA extraction, library preparation, and sequencing were performed as previously described (39). Raw data analysis was performed similar to previous work (40). Statistical significance was determined by unpaired t test.

**Mycolyl-arabinogalactan-peptidoglycan isolation**

Cell envelope material was extracted similar to previous protocols. Isolation of mycolyl-arabinogalactan-peptidoglycan (mAGP) was conducted similar to previous reports (8, 41). Briefly, cell pellets were thawed on ice and resuspended in 50 ml of phosphate-buffered saline (PBS) supplemented with 2% Triton X-100. Cells were lysed at 22 kpsi with a benchtop cell disruptor from Constant Systems (Kennesaw, GA), and lysate was clarified by centrifugation (20,000 RCF for 1 hour). The supernatant was discarded, and insoluble pellet was extracted three times with 2% SDS in PBS for 1 hour at 95°C. Following the third extraction, mAGP material was washed with milli-Q water, 80% acetone in water, and 100% acetone. Residual acetone was allowed to evaporate overnight.

**Cell envelope composition analysis**

**Galactan length determination**

Composition analysis was performed using 300 to 400 μg of sample. A 10-μg aliquot of inositol was added to the samples, which were then dried. Samples were hydrolyzed in 2 M trifluoroacetic acid (TFA) for 2 hours in a sealed tube at 121°C, reduced with NaBD₄, and acetylated using acetic anhydride/TFA. Resulting alditol acetates were analyzed on an Agilent 7890A gas chromatograph (GC) interfaced to a 5975C MSD, electron impact ionization mode. Separation was performed on a 30-m Supelco SP-2331 bonded phase fused silica capillary column. Alongside the samples, a standard containing 1 μg of rhamnose, 15 μg of arabinose, 10 μg of inositol, and 10 μg of...
galactose was run. The reported Ara:Gal:Rha ratios were an average of either three biological replicates (WT and D267Fcomp) or two biological replicates (WTcomp, Cdíphcomp, and Nbrascomp).

**Quantification of MurNAc residues**

Composition analysis was performed by combined GC/mass spectrometry (GC/MS) of heptafluorobutyric acid (HFBA) derivatives of monosaccharide methyl glycosides produced from acidic methanolyis. Briefly, 700 to 900 µg of mAGP were heated with 6 M HCl for 17 hours at 105°C. After heating, the sample was dried thoroughly by a stream of nitrogen and repeated additions of methanol. Methanolysis was carried out by addition of methanolic HCl for 15 min at 80°C. After cooling and removal of the solvent with nitrogen, the sample was treated with 200 µl of acetonitrile and 50 µl of the HFBA reagent. After reacting for 30 min at 105°C, the sample was dried and dissolved in acetonitrile. GC/MS analysis of the HFBA methyl glycosides was performed on an Agilent 7890A GC interfaced to a Mass Selective Detector. To create a comparison of Rha/MurNAc residues, relative abundance of these monosaccharides was calculated as an internal standard. To create a comparison of Rha/MurNAc residues, relative abundance of these monosaccharides was calculated as a percentage of total mAGP mass.

**Biolog determination of osmosensitivity**

Cells were cultured in 7H9 medium to mid-log phase. They were diluted to OD 600 = 0.1, diluted approximately 15-fold further into 7H9 broth supplemented with Dye G (Biolog). This mixture was then added to PM09 plates, with each well containing 100 µl of inoculated culture. Three plates were used per strain as technical replicates. Plates were incubated at 30°C for 72 hours. Data were analyzed subtracting background signal measured from uninoculated wells that acted as negative controls.

**Lipid isolation and analysis**

Cells were cultured (50 ml of complete 7H9) to mid-log and harvested, and cell pellets were frozen at −80°C until further use. Lipid extraction began with resuspension of each cell pellet in 3 ml of 2:1 methanol:chloroform. The initial extraction took place overnight at room temperature with agitation. Delipidated cells were pelleted by centrifugation at 300 RCF, and the supernatant was carefully removed. Delipidation continued with addition of 3 ml of 1:2 methanol:chloroform. Extraction proceeded with agitation at 55°C for 2 hours. Delipidated cells were pelleted by centrifugation at 300 RCF, and the supernatant was carefully removed. This extraction was then repeated a second time, for a total of three extractions. Both the delipidated cells and supernatant were then evaporated overnight under nitrogen. After evaporation, the material from the supernatant fraction was subjected to a Folch wash. These combined fractions were then evaporated under nitrogen. The dried material was dissolved in 2 ml of water and extracted three times with 2 ml of PBS-saturated phenol at 80°C. The samples were then dialyzed, separated via SDS-PAGE, and stained with the Pro-Q Emerald stain (Thermo Fisher Scientific). Assignment of bands was determined using Jankute et al. (43) as a reference.

Last, mycolic acids covalently appended to the mAGP were analyzed by thin-layer chromatography (TLC) using 20:4:0.5 hexanes:ethyl acetate:acetic acid as a reference. These lipids were dissolved in 2:1 chloroform:methanol and were carried forward for LM (lipomannan)/LAM (lipoarabinomannan) analysis, whereas the organic layer contained freely extractable lipids. Permethylation was performed by addition of dichloromethane (DCM) (1.5 ml), milli-Q filtered H2O (1.0 ml), and iodomethane (150 µl). This reaction proceeded for 4 hours at room temperature, yielding mycolic acid methyl esters (MAMEs). After two washes with water (2 ml each), the organic phase was evaporated under nitrogen. Diethyl ether (2.0 ml) was added to each sample to extract MAMEs and after evaporation under nitrogen, MAMEs were resuspended in DCM (100 µl) and analyzed via TLC using 95:5 hexanes:ethyl acetate as the mobile phase. Plates were developed by staining in 10% cupric sulfate and charring. Assignments of lipids were performed using Madacki et al. (42) as a reference.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/38/eaba4015/DC1

View request a protocol for this paper from Bio-protocol.

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