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### Recommended Citation

Clark, Emily L.; Emmadi, Madhu; Krupp, Katharine L.; Podilapu, Ananda R.; Helble, Jennifer D.; Kulkarni, Suvarn S.; and Dube, Danielle H., "Development of Rare Bacterial Monosaccharide Analogs for Metabolic Glycan Labeling in Pathogenic Bacteria" (2016). *Chemistry Faculty Publications*. 5.  
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# Development of Rare Bacterial Monosaccharide Analogs for Metabolic Glycan Labeling in Pathogenic Bacteria

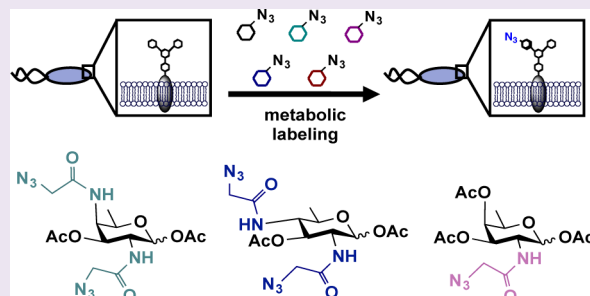
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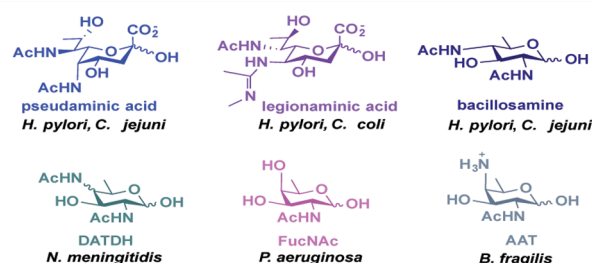
## S Supporting Information

**ABSTRACT:** Bacterial glycans contain rare, exclusively bacterial monosaccharides that are frequently linked to pathogenesis and essentially absent from human cells. Therefore, bacterial glycans are intriguing molecular targets. However, systematic discovery of bacterial glycoproteins is hampered by the presence of rare deoxy amino sugars, which are refractory to traditional glycan-binding reagents. Thus, the development of chemical tools that label bacterial glycans is a crucial step toward discovering and targeting these biomolecules. Here, we explore the extent to which metabolic glycan labeling facilitates the studying and targeting of glycoproteins in a range of pathogenic and symbiotic bacterial strains. We began with an azide-containing analog of the naturally abundant monosaccharide *N*-acetylglucosamine and discovered that it is not broadly incorporated into bacterial glycans, thus revealing a need for additional azidosugar substrates to broaden the utility of metabolic glycan labeling in bacteria. Therefore, we designed and synthesized analogs of the rare deoxy amino D-sugars *N*-acetylfucosamine, bacillosamine, and 2,4-diacetamido-2,4,6-trideoxygalactose and established that these analogs are differentially incorporated into glycan-containing structures in a range of pathogenic and symbiotic bacterial species. Further application of these analogs will refine our knowledge of the glycan repertoire in diverse bacteria and may find utility in treating a variety of infectious diseases with selectivity.



One widespread challenge in the healthcare industry is the increasing ineffectiveness of existing antibiotics due to the evolution of microbial resistance. Even when existing antibiotics are effective at treating bacterial disease, these treatments are often broad-spectrum and suffer from deleterious effects on our beneficial bacteria.<sup>1</sup> Such disruptions to the normal gut microbiome can precipitate long-term health problems<sup>2</sup> including obesity, autoimmune disorders, allergies, malnutrition, and expansion of enteric pathogens in the gut.<sup>3,4</sup> Thus, there is an urgent need to develop novel antibiotics that target specific bacterial populations while leaving the rest of the microbiome intact.

Bacterial glycoproteins serve as attractive targets of therapeutic intervention because they are often linked to pathogenesis, their structures differ markedly from those found in eukaryotes, and they contain distinctive monosaccharide building blocks that are exclusively present on a small number of bacterial pathogens.<sup>5,6</sup> For example, the gastric pathogen *Helicobacter pylori* synthesizes glycoproteins that contain the rare amino- and deoxy-monosaccharides pseudaminic acid, legionaminic acid, and bacillosamine (Figure 1).<sup>7,8</sup> These sugars have limited expression on pathogenic bacteria, and there are no reports of these three monosaccharides in nonpathogenic bacteria that dominate the human gut microbiome<sup>3</sup> (e.g., *Bacteroides* spp., *Prevotella* spp.). More broadly, *Neisseria*



**Figure 1.** Structures of rare, exclusively bacterial deoxy amino monosaccharides and examples of bacteria that install them in their glycans.

*meningitidis* utilizes 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH).<sup>9</sup> *Pseudomonas aeruginosa* incorporates FucNAc residues,<sup>10</sup> and the symbiotic *Bacteroides fragilis* appends 2-acetamido-4-amino-2,4,6-trideoxygalactose (AAT)<sup>11</sup> into its cell surface polysaccharides (Figure 1). These distinctive building blocks appear to be exclusively present on a small number of bacterial species.<sup>6,12</sup>

**Received:** September 11, 2016

**Accepted:** October 20, 2016

**Published:** October 21, 2016



Despite the potential of distinct monosaccharides within bacterial glycans to serve as molecular targets, the systematic discovery of bacterial glycoproteins is hampered by the presence of rare deoxy amino sugars, which are refractory to traditional glycan-binding reagents.<sup>13,14</sup> In particular, lectins and glycosidases employed to detect and remove eukaryotic glycans, respectively, often cannot be utilized to study bacterial glycans due to their restricted binding specificity.<sup>15–17</sup> The development of chemical tools that label bacterial glycans is a crucial step toward discovering and targeting these biomolecules.

The chemical technique known as metabolic oligosaccharide engineering (MOE), which was pioneered by Bertozzi *et al.*,<sup>18,19</sup> Reutter *et al.*,<sup>20</sup> and others for the study of mammalian glycoproteins, offers a powerful approach to label and detect bacterial glycoproteins.<sup>21</sup> In MOE, cells are supplemented with an unnatural sugar that is taken up, processed by endogenous carbohydrate biosynthetic pathways, and installed into glycans in place of natural monosaccharides. Using this approach, bio-orthogonal chemical reporters, such as azides,<sup>22</sup> can be incorporated into cellular glycans (Figure 2A). Once

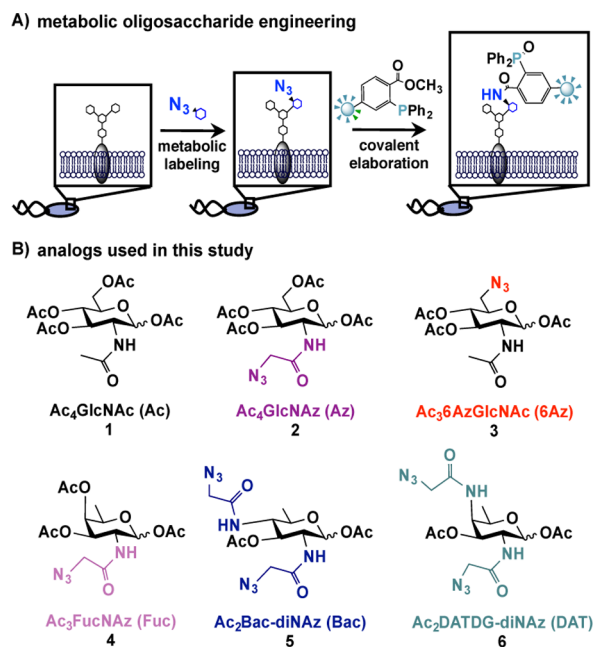
MOE has been utilized to incorporate an azide-bearing analog of the common monosaccharide *N*-acetylglucosamine (GlcNAc) into glycoproteins on the gastric pathogen *Helicobacter pylori*,<sup>21</sup> In particular, the GlcNAc analog peracetylated-*N*-azidoacetylglucosamine (Ac<sub>4</sub>GlcNAz (2), Figure 2B) was robustly incorporated into *H. pylori*'s glycoproteins and facilitated the identification of 125 candidate *H. pylori* glycoproteins.<sup>27</sup> In addition to enabling bacterial glycoprotein discovery, Ac<sub>4</sub>GlcNAz could form the basis of a bacterial glycan targeting strategy as it is minimally presented onto the surfaces of mammalian cells,<sup>28</sup> despite its robust incorporation into mammalian O-GlcNAc modified nuclear and cytosolic proteins.<sup>29</sup> Indeed, our work led to the development of immune-stimulant bearing phosphines that catalyzed the destruction of azide-covered *H. pylori* in an immune-cell dependent manner.<sup>30</sup> Thus, Ac<sub>4</sub>GlcNAz is a reasonable choice from the perspective of discovering and targeting *H. pylori* glycoproteins and may have broad utility for probing glycoproteins or other glycan-containing structures in diverse bacteria.

Here, we explored the extent to which MOE facilitates the study of glycoproteins in a range of pathogenic and symbiotic bacterial strains. Our results demonstrate that relatively few bacterial strains are azide-labeled upon treatment with Ac<sub>4</sub>GlcNAz and thus suggest that differential labeling of bacterial populations is possible. However, these results reveal a need for additional azidosugar substrates to broaden the utility of MOE in bacteria. To meet this need, we synthesized azide-containing analogs of rare bacterial monosaccharides (Figure 2B; compounds 4, 5, 6) and established that pathogenic bacteria effectively process these distinctive sugars. This work sets the stage for facilitating the study of glycoproteins in diverse bacterial species and providing a means to inactivate select bacteria based on their distinctive glycan coating.

## RESULTS AND DISCUSSION

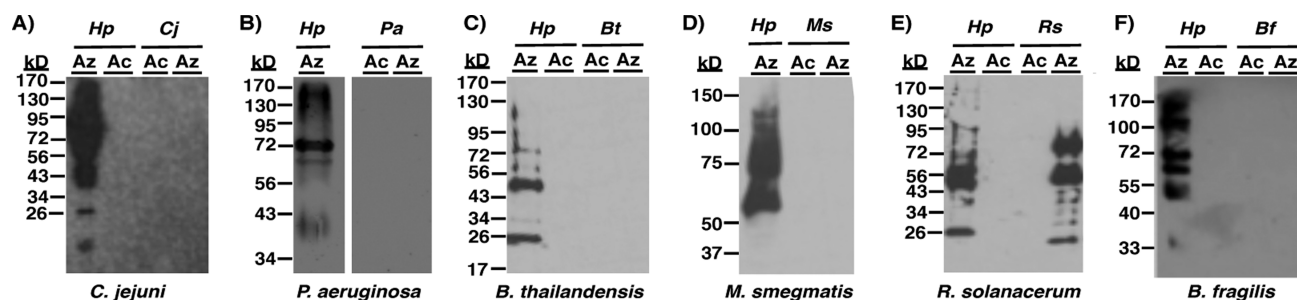
To facilitate the studying and targeting of glycans in a range of pathogenic bacteria, we set out to assess the metabolic incorporation of a range of azide-bearing monosaccharides in diverse bacteria. Since incorporation of azide-containing sugars into a wide range of bacterial glycoproteins is beneficial from the perspective of glycoprotein discovery but detrimental for creating narrow-spectrum glycan-targeted therapeutics,<sup>6</sup> we explored the incorporation of azide-bearing analogs of both common and rare monosaccharide substrates. We reasoned that rare monosaccharides with restricted metabolic fates (e.g., conversion to a single product) would be processed into glycans on fewer bacterial strains than common monosaccharides with broad metabolic fates.

**Differential Labeling of Bacterial Glycoproteins with Ac<sub>4</sub>GlcNAz.** To build upon the established utility of Ac<sub>4</sub>GlcNAz (2),<sup>29</sup> an azide-containing analog of the common monosaccharide GlcNAc, for discovering<sup>27</sup> and targeting<sup>30</sup> *Helicobacter pylori*'s glycoproteins, we first sought to investigate the utility of this substrate to study glycoproteins in bacteria beyond *H. pylori*. We began by assessing the levels of incorporation of Ac<sub>4</sub>GlcNAz into bacterial glycans in a range of species including pathogenic and symbiotic bacteria. In particular, we focused on incorporation into the Gram-negative pathogens *Campylobacter jejuni* and *Pseudomonas aeruginosa*, close relatives of pathogens *Burkholderia thailandensis* and *Mycobacterium smegmatis*, the plant pathogen *Ralstonia*



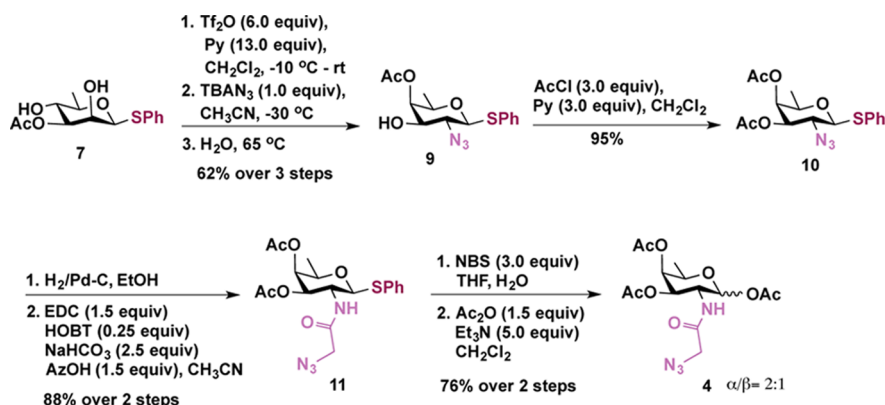
**Figure 2.** Schematic overview of metabolic oligosaccharide engineering (MOE) experiments performed and structures of monosaccharide analogs used in this study. (A) In MOE experiments, cells are first metabolically labeled with an unnatural azide-containing sugar. Azide-bearing glycans then undergo Staudinger ligation with phosphine probes to enable their detection on cells or in cell lysates. (B) Metabolic incorporation of azide-containing analogs of the naturally abundant monosaccharide *N*-acetylglucosamine (GlcNAc) as well as of the rare bacterial monosaccharides FucNAc (4), bacillosamine (5), and DATDG (6) were explored in this work.

incorporated into cellular glycoproteins, the azide acts as a chemical handle that undergoes covalent elaboration with exquisitely selective reaction partners, including triarylphosphines<sup>23</sup> and cyclooctynes,<sup>24</sup> for the attachment of biological probes<sup>25</sup> (Figure 2A). Incorporation of azide-containing sugars into bacterial glycans thus facilitates detection, enrichment, and identification of bacterial glycoproteins and enables targeting with covalent therapeutics to incite cell damage.<sup>26</sup>



**Figure 3.** Western blot analyses indicate that Ac<sub>4</sub>GlcNAz (2) supplementation leads to installation of azides into glycoproteins in select bacterial pathogens. (A) *Helicobacter pylori* (Hp), *Campylobacter jejuni* (Cj), (B) *Pseudomonas aeruginosa* (Pa), (C) *Burkholderia thailandensis* (Bt), (D) *Mycobacterium smegmatis* (Ms), and (E) *Ralstonia solanacerum* (Rs) were grown in media supplemented with 1 mM Ac<sub>4</sub>GlcNAz (2, Az) or the azide-free control sugar Ac<sub>4</sub>GlcNAc (1, Ac) for 5 days, then harvested by lysis. (F) Due to its faster doubling time, *Bacteroides fragilis* (Bf) was grown in media supplemented with 1 mM Ac<sub>4</sub>GlcNAz (2, Az) or the azide-free control sugar Ac<sub>4</sub>GlcNAc (1, Ac) for 2 days, then harvested by lysis. The presence of azides in cellular glycoproteins was detected by reacting lysates with 250  $\mu$ M Phos-FLAG for 6–12 h at 37  $^{\circ}$ C and then analyzing samples via Western blot with anti-FLAG antibody. The data shown are representative of replicates ( $n \geq 3$ ).

#### Scheme 1. Synthesis of Ac<sub>3</sub>FucNAz (Fuc) 4

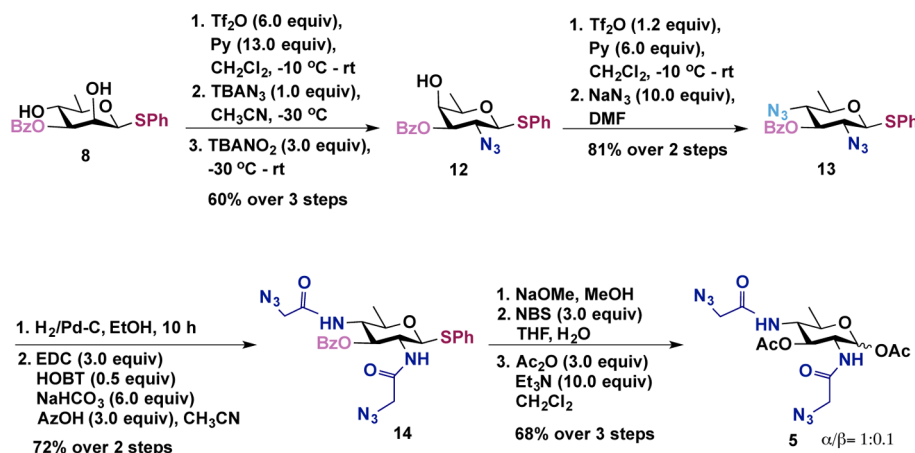
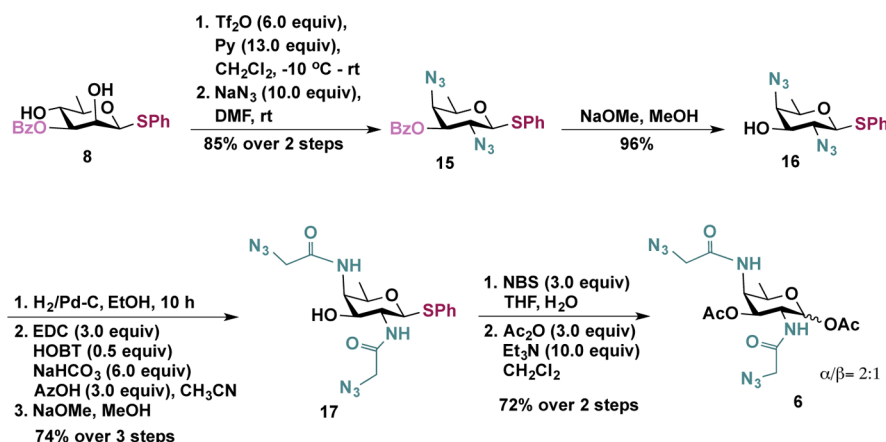


*solanacerum*, and the symbiotic bacterium *Bacteroides fragilis*. These bacteria were selected as their ability to produce glycosylated proteins has been previously established.<sup>31–37</sup> To gauge metabolic incorporation, bacteria were treated with 1 mM Ac<sub>4</sub>GlcNAz (2) or the azide-free control sugar peracetylated *N*-acetylglucosamine (Ac<sub>4</sub>GlcNAc (1), Figure 2B), then their proteins were harvested by cell lysis and reacted with a phosphine probe comprising a FLAG peptide<sup>38</sup> (Phos-FLAG). In all Western blot analyses, lysates from *H. pylori* treated with Ac<sub>4</sub>GlcNAz followed by Phos-FLAG were included as a positive control.<sup>21</sup> Analysis of proteins from treated *H. pylori* and *R. solanacerum* yielded an array of azide-labeled glycan-containing structures that were detected by Western blot analysis with anti-FLAG antibodies (Figure 3). In contrast, similar treatment of the other bacterial strains with Ac<sub>4</sub>GlcNAz led to no detectable azide-labeled glycoproteins (Figure 3). Control samples from cells treated with the azide-free compound peracetylated GlcNAc (Ac<sub>4</sub>GlcNAc, 1) yielded no azide-dependent signal, as expected. Zinc or Coomassie staining of electrophoresed samples confirmed that all samples contain proteins (Supporting Information Figure 1), indicating that the lack of detectable azides in some samples was not due to low protein levels. Counter to our expectation, these data reveal that metabolic incorporation of azides into bacterial glycans upon treatment with Ac<sub>4</sub>GlcNAz is not a widespread phenomenon but rather appears limited to select bacterial strains.

**Synthesis of Azide-Containing Variants of Rare Bacterial Monosaccharides.** Due to the observed narrow incorporation of Ac<sub>4</sub>GlcNAz into bacterial glycans, we wondered whether analogs of rare bacterial monosaccharides might facilitate glycoprotein discovery while setting the stage for novel antibiotic strategies that interfere with select pathogenic strains but leave the microbiome largely intact. Therefore, we designed a panel of azide-containing variants of the rare bacterial sugars FucNAc, bacillosamine, and DATDG (Figure 2B, 4–6). Given that a variety of carbohydrate biosynthetic pathways are permissive of *N*-azidoacetyl substituents in place of the natural *N*-acetyl moieties,<sup>18,26</sup> we reasoned that the requisite bacterial enzymes would likely process analogs bearing *N*-azidoacetyl groups. Further, we chose to temporarily mask the hydrophilic hydroxyl groups of these analogs with hydrophobic acetyl groups<sup>39</sup> to maximize efficiency of uptake by bacterial strains.<sup>21</sup>

To access these compounds, we built upon a strategy developed by Kulkarni and co-workers to synthesize rare bacterial amino and deoxy monosaccharide building blocks including FucNAc, bacillosamine, and DATDG.<sup>40,41</sup> In this strategy, changing the sequence of addition of nucleophiles in a series of one-pot double serial and double parallel displacement reactions using azide, phthalimide and nitrite ions as nucleophiles and mannose-derived 2,4-bis-trifluoromethanesulfonates (triflates) as electrophiles enabled rapid access to various orthogonally protected rare deoxy amino sugar building blocks.<sup>40,41</sup> Accordingly, the 2,4-diol substrates (Scheme 1,



Scheme 2. Synthesis of Ac<sub>2</sub>Bac-diNAz (Bac) 5Scheme 3. Synthesis of Ac<sub>2</sub>DATDG-diNAz (DAT) 6

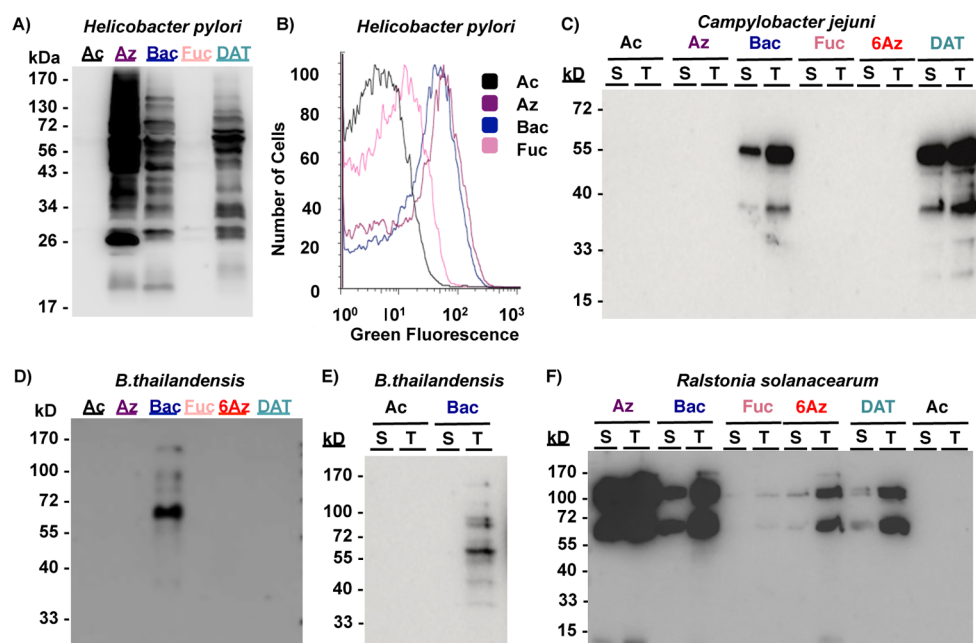
compound 7 and Scheme 2, compound 8) for the one-pot double displacement reaction were first prepared starting from cheaply available D-mannose in six steps.<sup>41</sup>

Transformation of D-rhamnosyl 2,4-diol 7 into D-fucosamine derivative 4 is shown in Scheme 1. Following our earlier established procedure, compound 7 was converted to the corresponding 2,4-bistriflate derivative, which upon a brief workup was subjected to a one-pot, regioselective, nucleophilic displacement reaction. Accordingly, a sequential C2-OTf displacement of the crude bistriflate with a stoichiometric amount of tetrabutylammonium azide ( $\text{TBAN}_3$ ) at  $-30\text{ }^\circ\text{C}$  followed by water mediated intramolecular displacement of the C4-OTf by C3-OAc, via the ring opening of the corresponding orthoester intermediate, resulted in the formation of the C4-OAc derivative of D-fucosamine 9 in 62% yields over three steps.<sup>41</sup> The remaining 3-OH group was capped as an acetate to form 10 (95%), which upon azide reduction under hydrogenation conditions, followed by EDC mediated coupling of the so-formed amine with azidoacetic acid,<sup>19</sup> afforded the azido derivative 11 in 88% yield over two steps. Finally, hydrolysis of the thioglycoside by using NBS and water generated the corresponding hemiacetal, which upon subsequent treatment with acetic anhydride in the presence of triethylamine afforded triacetate compound 4 ( $\alpha/\beta = 2:1$ ;  $\text{Ac}_3\text{FucNAz}$ , Fuc) in 76% yield over two steps.

For the synthesis of Ac<sub>2</sub>Bac-diNAz (Bac) 5, we started from 3-OBz derivative 8 (Scheme 2). Diol 8 upon similar triflation

followed by sequential, one pot  $\text{S}_\text{N}2$  reactions with  $\text{TBAN}_3$  and with tetrabutyl ammonium nitrite ( $\text{TBANO}_2$ ), at C2 and C4, respectively, afforded D-fucosamine derivative 12 in 60% overall yield.<sup>41</sup> Concomitant triflation of the axial 4-OH group and its displacement with  $\text{NaN}_3$  furnished bacillosamine 13.<sup>41</sup> Reduction of 2,4-azido groups in 13 followed by EDC coupling with azidoacetic acid afforded 14 in 72% yield over two steps. Debenzoylation, conversion of anomeric thioglycoside to the corresponding hemiacetal with NBS/water, and acetylation afforded the target molecule Ac<sub>2</sub>Bac-diNAz (Bac) 5 ( $\alpha/\beta = 1:0.1$ ) in 68% yield over three steps.

The synthesis of an analog of the rare sugar DATDG is outlined in Scheme 3. A double displacement of the 2,4-bistriflate, obtained from 8, with excess  $\text{NaN}_3$  cleanly afforded diazido derivative 15 in excellent overall yield.<sup>41</sup> The 3-O-benzoate group was cleaved using NaOMe to obtain 16 (96%). This step was deemed necessary in order to avoid the possible migration of benzoate (C3  $\rightarrow$  C4) during the ensuing reduction of azides to corresponding amines. The azides were then reduced under a hydrogen atmosphere, and so formed amines were coupled with the azidoacetic acid using EDC as the coupling reagent. In this reaction, along with the desired di-N-acylated product, we also obtained fully acylated product. Treatment of the mixture of acylated products with NaOMe in MeOH gave the desired product 17 in 74% yield over three steps. Thioglycoside was treated with NBS/water, and the so formed hemiacetal was acetylated to give the desired derivative



**Figure 4.** Metabolic incorporation of azide-containing rare bacterial monosaccharide analogs onto bacterial pathogens. *H. pylori*, *C. jejuni*, *B. thailandensis*, and *R. solanacearum* were grown in media supplemented with 1 mM  $\text{Ac}_4\text{GlcNAz}$  (2, Az),  $\text{Ac}_2\text{Bac-diNAz}$  (5, Bac),  $\text{Ac}_3\text{FucNAz}$  (4, Fuc),  $\text{Ac}_3\text{6AzGlcNAc}$  (3, 6Az),  $\text{Ac}_2\text{DATDG-diNAz}$  (6, DAT), or the azide-free control sugar  $\text{Ac}_4\text{GlcNAc}$  (1, Ac) for 5 days, then probed for the presence of azide-labeled glycans on cells or within lysates by reaction with 250  $\mu\text{M}$  Phos-FLAG and subsequent detection with anti-FLAG antibody. (A) The presence of azides in *H. pylori*'s lysates was detected via Western blot analysis with anti-FLAG-HRP. (B) Presentation of azides on *H. pylori* cell surfaces was assessed via flow cytometry with anti-FLAG-FITC. (C) Incorporation of azides into *C. jejuni*'s surface-accessible ("S") versus total ("T") glycoproteins was probed by reacting intact cells or cell lysates, respectively, with Phos-FLAG, then analyzing samples via Western blot analysis. (D, E) Display of azides in *B. thailandensis* lysates (D) or in surface-accessible versus total glycoproteins (E) was analyzed via Western blot. (F) Incorporation of azides into *R. solanacearum* surface-accessible versus total glycoproteins was assessed via Western blot analysis. All lanes contain equivalent protein levels. The data shown are representative of replicate experiments ( $n \geq 3$ ).

$\text{Ac}_2\text{DATDG-diNAz}$  (DAT) 6 ( $\alpha/\beta = 2:1$ ) in 72% yield over 2 steps.

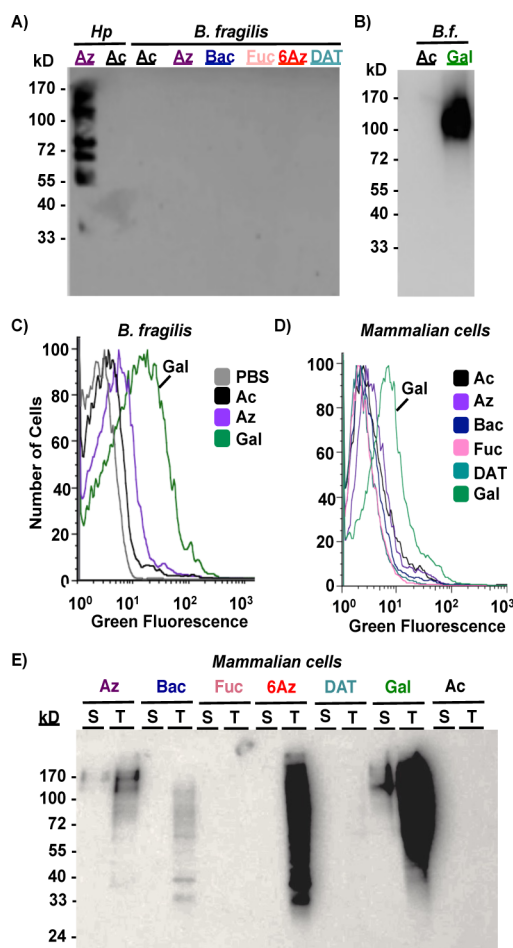
**Rare Bacterial Monosaccharide Analogs Are Incorporated into Diverse Bacterial Pathogens.** With access to these rare bacterial monosaccharide analogs, we performed metabolic labeling experiments to assess incorporation into diverse bacterial species. We first evaluated metabolic incorporation into bacterial pathogens or close relatives of pathogens. For these experiments, bacteria were grown in media supplemented with one of each of the panels of rare bacterial monosaccharide analogs ( $\text{Ac}_3\text{FucNAz}$  (4),  $\text{Ac}_2\text{Bac-diNAz}$  (5), or  $\text{Ac}_2\text{DATDG-diNAz}$  (6)), or with one of three GlcNAc analogs ( $\text{Ac}_4\text{GlcNAz}$  (2),<sup>28</sup> the peracetylated 6-azido GlcNAc analog  $\text{Ac}_3\text{6AzGlcNAc}$  (3),<sup>42</sup> or the azide-free control  $\text{Ac}_4\text{GlcNAc}$  (1); Figure 2B) that are not appreciably incorporated onto mammalian cell surfaces.<sup>28,42</sup> After metabolic labeling, proteins were harvested by cell lysis and reacted with Phos-FLAG for Western blot analysis with anti-FLAG antibody to yield a snapshot of all ("total") azide-labeled glycans from the bacteria. Alternatively, intact cells were rinsed and reacted with the cell impermeable compound Phos-FLAG to probe for the presence of surface accessible ("surface") azides on the bacteria;<sup>30</sup> these surface accessible azide-labeled species were then detected by flow cytometry or Western blot analysis with an anti-FLAG antibody.

As depicted in Figure 4, the gastric pathogen *H. pylori* robustly incorporates  $\text{Ac}_4\text{GlcNAz}$  (2),  $\text{Ac}_2\text{Bac-diNAz}$  (5), and  $\text{Ac}_2\text{DATDG-diNAz}$  (6) into surface glycans, as revealed by Western blot (Figure 4A) and flow cytometry (Figure 4B; Supporting Information Figure S2B) analysis. In contrast,

$\text{Ac}_3\text{FucNAz}$  (4) is minimally incorporated into *H. pylori*'s glycans and *H. pylori* treated with  $\text{Ac}_4\text{GlcNAc}$  (1) remain azide-free (Figure 4A,B). The gut pathogen *C. jejuni* displays a different metabolic incorporation phenotype; Western blot analysis reveals robust processing of only  $\text{Ac}_2\text{Bac-diNAz}$  (5) and  $\text{Ac}_2\text{DATDG-diNAz}$  (6) into select primarily surface-accessible glycans in *C. jejuni* (Figure 4C). The opportunistic pathogen *B. thailandensis* incorporates  $\text{Ac}_2\text{Bac-diNAz}$  (5) but no other azidosugars into its glycan-containing structures (Figure 4D); follow up analysis of the  $\text{Ac}_2\text{Bac-diNAz}$  (5) labeled species from *B. thailandensis* indicates that they are not surface accessible (Figure 4E). Finally, the plant pathogen *R. solanacearum* exhibits the broadest incorporation profile, with treatment with  $\text{Ac}_4\text{GlcNAz}$  (2),  $\text{Ac}_2\text{Bac-diNAz}$  (5),  $\text{Ac}_2\text{DATDG-diNAz}$  (6),  $\text{Ac}_3\text{6AzGlcNAc}$  (3), and, to a lesser extent,  $\text{Ac}_3\text{FucNAz}$  (4) yielding surface accessible azides detectable by Western blot (Figure 4F) and flow cytometry (Supporting Information Figure 3). In all cases, Coomassie staining of electrophoresed samples confirmed equivalent protein levels (Supporting Information Figure 2), indicating that the lack of detectable azides in some samples was not due to low protein content. Further, the negative control samples from cells treated with  $\text{Ac}_4\text{GlcNAc}$  (1) consistently lacked an azide-dependent signal. Taken together, these data suggest that the extent of incorporation into bacterial glycans is dependent upon the structure of the metabolic input and upon the bacteria under study. Relative to  $\text{Ac}_4\text{GlcNAz}$ , analogs of rare bacterial monosaccharides appear better suited to study bacterial glycans.

**Rare Bacterial Monosaccharide Analogs Are Not Incorporated onto Symbiotic or Mammalian Cells.**

With compelling evidence that this panel of azidosugars could be used to study or target glycans in a variety of bacterial pathogens, we next explored whether these azidosugars are incorporated into the beneficial bacteria *Bacteroides fragilis*. Such information is critical to assess potential undesired impacts on innocent bystanders of a rare bacterial monosaccharide-based antibiotic strategy. *B. fragilis* supplemented with the panel of azide-containing sugars did not incorporate azides into glycoproteins at a detectable level by Western blot analysis (Figure 5A) despite high protein content in these samples (Supporting Information Figure 4A). In contrast,



**Figure 5.** Metabolic incorporation of azide-containing rare bacterial monosaccharide analogs onto symbiotic *Bacteroides fragilis* and mammalian Madin Darby Canine Kidney (MDCK) cells. (A–C) *B. fragilis* were grown in media supplemented with 1 mM  $\text{Ac}_4\text{GlcNAz}$  (2, Az),  $\text{Ac}_2\text{Bac-diNAz}$  (5, Bac),  $\text{Ac}_3\text{FucNAz}$  (4, Fuc),  $\text{Ac}_3\text{6AzGlcNAc}$  (3, 6Az),  $\text{Ac}_2\text{DATDG-diNAz}$  (6, DAT), or the azide-free control sugar  $\text{Ac}_4\text{GlcNAc}$  (1, Ac) for 2 days, then probed for the presence of azide-labeled glycans on cells or within lysates by reaction with 250  $\mu\text{M}$  Phos-FLAG and subsequent detection with anti-FLAG antibody via Western blot (A, B) or by reaction with DIBO-488 (20  $\mu\text{M}$ , 1 h) followed by flow cytometry (C). (D, E) MDCK cells were cultured with 0.25 mM azidosugar-supplemented media for 3 days, then probed for the presence of azide-labeled glycans using Phos-FLAG and anti-FLAG antibody. (D) Analysis of intact MDCK was performed by flow cytometry with anti-FLAG-FITC. (E) Incorporation of azides into MDCK surface-accessible (“S”) versus total (“T”) glycoproteins was probed by reacting intact cells or cell lysates, respectively, with Phos-FLAG, then analyzing samples via Western blot analysis. Data are representative of replicate experiments ( $n \geq 3$ ).

Western blot and flow cytometry analyses revealed that the azidosugar peracetylated *N*-azidoacetylgalactosamine ( $\text{Ac}_4\text{GalNAz}$ ),<sup>43</sup> an analog of *N*-acetylgalactosamine, was robustly displayed in *B. fragilis* surface glycans (Figure 5B,C; Supporting Information Figure 4B); this result is consistent with a recent demonstration by Kasper and co-workers that  $\text{Ac}_4\text{GalNAz}$  is processed into polysaccharide A in *B. fragilis*.<sup>44</sup> These data indicate that, though it is possible to incorporate azides into *B. fragilis* surface glycans, such incorporation is negligible with the rare bacterial monosaccharide analogs reported here. Thus, narrow range antibiotics based on differential incorporation of azides onto the surface of pathogenic but not symbiotic bacteria may be possible.

To explore whether these rare bacterial monosaccharide analogs may have the potential to underpin a covalent antibiotic strategy aimed at gastrointestinal pathogens, we set out to evaluate the incorporation of these analogs in mammalian cells. As a model system, we turned to the well-studied Madin Darby Canine Kidney (MDCK) epithelial cell line, as this cell line is an established model for epithelial cells that line the gastrointestinal tract. In experiments analogous to those described above, MDCK cells were treated with each of a panel of azidosugars, and metabolic incorporation of azides into cellular glycans and onto cell surfaces was assessed using Phos-FLAG and anti-FLAG antibody.<sup>19</sup> Western blot and flow cytometry analyses revealed that only the positive control sugar  $\text{Ac}_4\text{GalNAz}$ , which is robustly incorporated into mucin-type O-linked glycoproteins,<sup>43</sup> led to significant delivery of azides onto the surface of MDCK cells (Figure 5D,E). In contrast, none of the other azidosugars resulted in an appreciable display of surface-accessible azides (Figure 5E, “surface” lanes); Coomassie staining confirmed that the differential azide labeling was not due to appreciable differences in protein loading across samples (Supporting Information Figure 4C). As expected based on previous literature reports,  $\text{Ac}_4\text{GlcNAz}$  (2) and  $\text{Ac}_3\text{6AzGlcNAc}$  (3) were incorporated into internal MDCK glycoproteins, consistent with the metabolic incorporation of these sugars into nuclear and cytosolic O-GlcNAc residues (Figure 5E, “total” lanes).<sup>42</sup> Unexpectedly, bacillosamine analog 5 was processed by MDCK cells and led to installation of azides into some internal MDCK structures (Figure 5E, “total lane”). This result is surprising due to the assumed lack of metabolic enzymes to process this exclusively bacterial sugar analog. Regardless, the finding that none of the rare bacterial monosaccharide analogs led to appreciable presentation of azides on the surface of MDCK cells bodes well for the potential of these sugars to form the basis for selective covalent antimicrobial applications.

**Discussion.** Here, we explore and expand the scope of metabolic glycan labeling across diverse bacterial species. Presumably due to the distinctive monosaccharide building blocks employed by bacteria, the analog  $\text{Ac}_4\text{GlcNAz}$  (2) appears to have some but limited utility for metabolic labeling of bacterial glycoproteins. To address this limitation, we produced a series of novel bacteria-specific azidosugar substrates that are robustly incorporated into glycans in a variety of pathogenic bacteria. Our data indicate that the choice of azidosugar substrate dictates which bacterial species process azides into their glycans.

The ability of bacterial strains to process monosaccharide analogs into their glycans could reflect underlying differences in glycosylation machinery present within each species. For example, *H. pylori* and *C. jejuni* have biosynthetic pathways



that convert bacillosamine to pseudaminic acid. A likely metabolic fate of Ac<sub>2</sub>Bac-diNAz (5) in these bacteria, therefore, is conversion to azido-pseudaminic acid and installation onto flagellin glycoproteins. In contrast, *B. fragilis* does not have the pseudaminic acid biosynthetic pathway enzymes, nor does it process Ac<sub>2</sub>Bac-diNAz into its glycans. Thus, metabolic incorporation of azidosugars into bacterial glycans is linked to which carbohydrate active enzymes are present within each species. The differences in glycan labeling across bacterial species observed in this work could also be a reflection of the labeling conditions used. Due to the dependence of glycan expression on a variety of factors (e.g., cell cycle, the environment), bacterial strains might exhibit a different metabolic incorporation phenotype at different growth stages or in different environments. For example, under different environmental conditions, bacterial strains might incorporate monosaccharide analogs that were not displayed in their glycans under the conditions used in this study. Moreover, exploring a range of sugar analog concentrations (e.g., higher and lower than 1 mM) has the potential to broaden or narrow metabolic incorporation into glycans. Even within the scope of this work, where fixed concentrations of sugars were utilized for fixed periods of time, subtle differences in glycoprotein fingerprints were observed across samples; Ac<sub>4</sub>GlcNAz-treated *H. pylori* displayed altered labeling profiles (Figure 3), likely due to phase variation causing changes in protein and glycan expression levels.

Metabolic incorporation sets the stage to use the azide as a chemical handle to enrich these labeled species, ultimately enabling glycoproteomic analyses.<sup>19</sup> Indeed, Woo *et al.* recently reported an azido-glycoproteomic method that could be applied to identify which bacterial proteins are glycosylated and to elucidate the structures of the modifying glycans.<sup>45</sup> Moreover, metabolic labeling of bacterial glycoproteins provides an approach to monitor bacterial glycan dynamics; time- and environment-dependent changes in glycan expression have the potential to yield a dynamic picture of bacterial glycosylation, ultimately providing insight into the functions of these critical biomolecules. Expanding the scope of metabolic glycan labeling to a range of bacterial species is therefore an important step to facilitate the study of bacterial glycoproteins.

In addition to easing bacterial glycoprotein discovery, this work sets the stage for covalent targeting of distinctive bacterial glycans with molecules that incite damage or permit visualization.<sup>6</sup> Our data indicate that the choice of azidosugar substrate dictates which bacterial species become azide-covered. Differential incorporation of azides across bacterial cells could form the basis for narrow-spectrum antibiotics that induce killing of pathogenic bacteria yet leave symbiotic bacteria unharmed. For example, azide-coated bacteria could be targeted with immune stimulant-bearing phosphines via Staudinger ligation<sup>30</sup> or with copper-free click-chemistry<sup>46</sup> delivered payloads. Analogously, azide-reactive molecules bearing imaging agents<sup>47,48</sup> could be employed to track bacterial infection in an animal model. Taken together, the work presented here provides expanded tools for metabolic labeling of bacterial glycans, which might be harnessed for glycoprotein discovery, targeted cell killing, or molecular imaging.

**Conclusions.** Metabolic labeling of bacterial glycoproteins is a relatively new strategy to study and target these biomolecules. Prior to this work, common monosaccharide precursors were employed to metabolically label glycoproteins in *H. pylori*<sup>27</sup> and *Bacteroidales* species,<sup>49</sup> and a bacillosamine

analog was employed as a committed pseudaminic acid precursor to label flagellin glycoproteins in *C. jejuni*.<sup>50</sup> The work reported here adds a new dimension to this field by generating analogs of rare bacterial monosaccharides that are processed by a variety of pathogenic but not symbiotic bacterial strains, thus offering the potential to exploit the incorporated azides for glycoprotein discovery, targeted cell killing, and molecular imaging. Although we focus here on a relatively small panel of pathogenic and symbiotic bacterial strains, this strategy has the potential to be applied to study, target, and track distinctive glycans in complex microbial communities present in an animal infection model. Thus, this strategy opens the door to discovering novel antibiotic targets and treating a wide range of bacterial diseases in a glycosylation-dependent manner.

## METHODS

**Materials and Chemical Synthesis.** Organic chemicals and anti-FLAG antibodies were purchased from Sigma-Aldrich. *H. pylori* strain G27<sup>51</sup> was a gift of Manuel Amieva (Stanford University), and *M. smegmatis* strain Mc<sup>2</sup>155::otsA treS treY<sup>52</sup> was a gift from Peter Woodruff (University of Southern Maine). Bacterial cells (*C. jejuni* ATCC 33560; *B. thailandensis* ATCC 700388; *R. solanacearum* ATCC 33291\* (\*incorrectly cataloged as *C. jejuni*; species confirmed as *R. solanacearum* by 16S rRNA sequencing); *B. fragilis* ATCC 23745; *P. aeruginosa* ATCC 39324) and MDCK cells (ATCC CCL-34) were purchased from ATCC and grown according to the supplier's instructions. Ac<sub>4</sub>GlcNAc (1), Ac<sub>4</sub>GlcNAz (2), Ac<sub>4</sub>GalNAz, and Phos-FLAG were synthesized as previously described.<sup>19,53</sup> Ac<sub>3</sub>6AzGlcNAc (3)<sup>42</sup> was kindly provided by Matthew Pratt (University of Southern California). Ac<sub>3</sub>FucNAz (4), Ac<sub>2</sub>Bac-diNAz (5), and Ac<sub>2</sub>DATDG-diNAz (6) were synthesized using standard organic chemistry procedures and characterized by standard techniques including <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy and mass spectrometry. Ac<sub>3</sub>FucNAz (4), Ac<sub>2</sub>Bac-diNAz (5), and Ac<sub>2</sub>DATDG-diNAz (6) were purified using flash silica gel chromatography.

**Metabolic Labeling.** Bacterial cells were grown in rich liquid media supplemented with 1 mM<sup>21</sup> Ac<sub>4</sub>GlcNAz (2), Ac<sub>3</sub>6AzGlcNAc (3), Ac<sub>3</sub>FucNAz (4), Ac<sub>2</sub>Bac-diNAz (5), Ac<sub>2</sub>DATDG-diNAz (6), Ac<sub>4</sub>GalNAz, or the azide-free control Ac<sub>4</sub>GlcNAc (1) for 2–5 days, depending on the doubling rate of the organism. *H. pylori*, *C. jejuni*, and *R. solanacearum* were metabolically labeled for 5 days under microaerophilic conditions (14% CO<sub>2</sub>, 37 °C). *B. fragilis* was metabolically labeled for 2 days under anaerobic conditions (created by a Becton Dickinson EZ anaerobe Gaspack in an airtight container; 37 °C), and *M. smegmatis*, *B. thailandensis*, and *P. aeruginosa* were grown in 1 mM sugar-containing media for 5 days under aerobic conditions, as appropriate for each bacterial strain. MDCK was grown in Dulbecco's Modified Eagles Medium supplemented with 250 μM of Ac<sub>4</sub>GlcNAc (1), Ac<sub>4</sub>GlcNAz (2), Ac<sub>3</sub>6AzGlcNAc (3), Ac<sub>3</sub>FucNAz (4), Ac<sub>2</sub>Bac-diNAz (5), Ac<sub>2</sub>DATDG-diNAz (6), or Ac<sub>4</sub>GalNAz for 3 days. Cells were then harvested, rinsed with phosphate buffered saline (PBS), and reacted with 250 μM Phos-FLAG for 5 h at 37 °C for detection of cell surface azides; whole cells were then lysed for Western blot, as described below. Alternatively, metabolically labeled cells were harvested, rinsed with PBS, lysed, and then reacted with 250 μM Phos-FLAG overnight at 37 °C. Protein lysates were standardized (BioRad's DC protein concentration assay) and analyzed by Western blot.

**Western Blot.** Reacted lysates from metabolically labeled cells were standardized to a protein concentration of ~3 mg mL<sup>-1</sup>, loaded onto a 4–15% Tris-HCl SDS-PAGE gel, separated by electrophoresis, and transferred to nitrocellulose paper. Anti-FLAG-HRP was employed to visualize FLAG-tagged proteins *via* chemiluminescence.

**Flow cytometry.** Metabolically labeled intact cells were reacted with Phos-FLAG (250 μM) for 1 h, then rinsed and treated with anti-FLAG-FITC to visualize FLAG-covered cells. Alternatively, the presence of azides on cells was probed using Click-IT Alexa Fluor 488 DIBO Alkyne (20 μM) for 5 h in the dark for copper-free click

chemistry detection of azides (ThermoFisher Scientific; ex: 488/em: 519). Cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) instrument, with 20 000 live cells gated for each replicate experiment.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.6b00790.

Detailed procedures for chemical syntheses and biological experiments, scanned NMR data for synthesized compounds, and supporting figures (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank K. Moulton and A. McBride for helpful comments, M. Pratt and K. Chuh for generously providing Ac<sub>3</sub>6AzGlcNAc, and P. Woodruff and M. Amieva for sharing bacterial strains. E.L.C. acknowledges support from a Surdna Foundation Undergraduate Research Fellowship; K.L.K. thanks Maine-INBRE for a summer research fellowship; M.E. and A.R.P. thank CSIR-New Delhi for fellowships; and E.L.C., K.L.K., and J.D.H. received Gruea/O'Connell Research Awards. Research reported in this publication was supported by the National Institutes of Health under grant number R15GM109397, by a Henry Dreyfus Teacher-Scholar award to D.H.D., and by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM0103423, and by Science and Engineering Research Board, Department of Science and Technology, Government of India (Grant No. EMR/2014/000235).

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