Biochemical Analysis and Structure Determination of Bacterial Acetyltransferases Responsible for the Biosynthesis of UDP-\(N,N'\)-Diacetylbacillosamine

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Received for publication, August 22, 2013, and in revised form, September 11, 2013 Published, JBC Papers in Press, September 24, 2013, DOI 10.1074/jbc.M113.510560

Background: A connection between glycoproteins containing \(N,N'\)-diacetylbacillosamine and pathogenicity has previously been shown in Campylobacter jejuni.

Results: Structural and kinetic studies of two bacterial acetyltransferases show the diversity within the binding pockets responsible for UDP-\(N,N'\)-diacetylbacillosamine production.

Conclusion: Carbohydrate acetyltransferases from \(O\)-linked glycosylation pathways exhibit significant divergence from their \(N\)-linked counterparts.

Significance: Acetyltransferase characterization increases our understanding of the diverse nature of bacterial glycosylation.

UDP-\(N,N'\)-diacetylbacillosamine (UDP-diNAcBac) is a unique carbohydrate produced by a number of bacterial species and has been implicated in pathogenesis. The terminal step in the formation of this important bacterial sugar is catalyzed by an acetyl-CoA (AcCoA)-dependent acetyltransferase in both \(N\)- and \(O\)-linked protein glycosylation pathways. This bacterial acetyltransferase is a member of the left-handed \(\beta\)-helix family and forms a homotrimer as the functional unit. Whereas previous endeavors have focused on the Campylobacter jejuni acetyltransferase (PglD) from the \(N\)-linked glycosylation pathway, structural characterization of the homologous enzymes in the \(O\)-linked glycosylation pathways is lacking. Herein, we present the apo-水晶 structures of the crystallographic domain (ATD) from the bifunctional enzyme PglB (Neisseria gonorrhoeae) and the full-length acetyltransferase WeeI (Acinetobacter baumannii). Additionally, a PglB-ATD structure was solved in complex with AcCoA. Surprisingly, this structure reveals a contrasting binding mechanism for this substrate when compared with the AcCoA-bound PglD structure. A comparison between these findings and the previously solved PglD crystal structures illustrates a dichotomy among \(N\)- and \(O\)-linked glycosylation pathway enzymes. Based upon these structures, key residues in the UDP-4-amino and AcCoA binding pockets were mutagenized to determine their effect on binding and catalysis in PglD, PglB-ATD, and WeeI. Last, a phylogenetic analysis of the aforementioned acetyltransferases was employed to illuminate the diversity among \(N\)- and \(O\)-linked glycosylation pathway enzymes.

The unique, bacterial sugar \(N,N'\)-diacetylbacillosamine (diNAcBac)\(^2\) has recently attracted attention due to its role in bacterial pathogenesis in Campylobacter jejuni (1–3). Importantly, the enzymes responsible for the biosynthesis of this sugar have also been found in other human pathogens, including selected strains of Acinetobacter baumannii (4) and Neisseria gonorrhoeae (5). In all three bacteria, UDP-diNAcBac is biosynthesized from UDP-\(N\)-acetylglucosamine (UDP-GlcNAc) by a series of three enzymes. The first two enzymes, an NAD\(^+\)-dependent dehydratase and a pyridoxal-5’-phosphate-dependent aminotransferase, form the UDP-4-amino sugar that acts as a substrate for the final step in diNAcBac biosynthesis. Acetylation of the C4 amine on this sugar is accomplished by an acetyl coenzyme A (AcCoA)-dependent acetyltransferase to generate UDP-diNAcBac (Fig. 1). This reaction is catalyzed by an active site histidine that acts as a general base to abstract a proton from the C4 amine, resulting in nucleophilic attack on the thioester of AcCoA. The biosynthetic machinery necessary for UDP-diNAcBac production has been found in both asparagine (\(N\)-linked) and serine/threonine (\(O\)-linked) protein glycosylation pathways, with this sugar acting as the anchor point for further carbohydrate elaboration. Both glycosylation pathways rely on the sequential build-up of sugars on a polyprenyl diphosphate-linked isoprene lipid carrier and transfer of the oligosaccharide \textit{en bloc} onto an acceptor protein. For the \(N\)-linked glycosylation pathway in C. jejuni, the ultimate glycan is a heptasaccharide consisting of GalNAc-\(\alpha\)-1,4-GalNAc-\(\alpha\)-1,4-(Glc-\(\beta\)-1,3)-GalNAc-\(\alpha\)-1,4-GalNAc-\(\alpha\)-1,4-GalNAc-\(\alpha\)-1,3-diNAcBac (6, 7). Conversely, the N. gonorrhoeae \(O\)-linked glycosylation pathway utilizes the Gal-\(\beta\)-1,4-Gal-\(\alpha\)-1,3-diNAcBac trisaccharide (5, 8). The final oligosaccharide for the \(O\)-linked pathway in the AYE strain of A. baumannii is still unknown; however, the glycan in a less

\(^*\) This work was supported, in whole or in part, by National Institutes of Health Grants GM097241 (to B. I.) and T32-GM08334 (Biotechnology Training Program) (to M. J. M).

The atomic coordinates and structure factors (codes 4M98, 4M99, and 4M9C) have been deposited in the Protein Data Bank (http://wwpdb.org).

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\(^2\) The abbreviations used are: diNAcBac, \(N,N'\)-diacetylbacillosamine or 2,4-diacetamido-2,4,6-trideoxy-\(\alpha\)-d-glucose; AcCoA, acetyl-coenzyme A; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; ATD, acetyltransferase domain; UDP-4-amino, UDP-2-acetamido-4-amino-2,4,6-trideoxy-\(\alpha\)-d-glucose.
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C. jejuni-dNAcBac Pathway

N. gonorhoeae-dNAcBac Pathway

FIGURE 1. The C. jejuni (top) and N. gonorhoeae (bottom) glycosylation pathways that utilize dNAcBac as the reducing end sugar.

pathogenic strain (ATCC 17978) of A. baumannii was recently characterized (9) and found not to include dNAcBac.

Previous structural characterization of the dNAcBac biosynthetic pathway has focused on the acetyltransferase PglD, an N-linked glycosylation pathway enzyme from C. jejuni (10, 11). Additionally, genetic studies have shown that deletion of the pglD gene in C. jejuni results in the loss of the final heptasaccharide and dramatic reduction of colonization in a chick animal model; however, a low level of glycosylation was still detected by lectin blotting and mass spectrometry (12). PglD is a member of the left-handed β-helix family and consists of two separate domains. The N-terminal domain contains a β-α-β-α-β-α Rossman fold motif to accommodate UDP-4-amino sugar binding. A hexapeptide repeat motif defines the C-terminal domain that is responsible for the left-handed β-helix and AccoA binding. The oligomeric state of PglD consists of a homotrimer that utilizes the left-handed β-helix motif of two protomers to form the cleft for AccoA binding. Structures of other bacterial N-acetyltransferases have recently been reported (13–15), although they are distant homologues of PglD based upon their divergent sugar substrates. However, the sugar acetyltransferases maintain the same overall protein fold by forming a trimer as the biological unit. In addition, they utilize the same left-handed β-helix motif from adjacent protomers to form the AccoA binding pocket. Structures of mammalian acetyltransferases, such as HAT1 (16), belonging to the GNAT (GCN5-related N-acetyltransferase) superfamily, bear no resemblance to their bacterial counterparts. This is most likely due to the considerable difference between their respective acyl acceptor substrates, histone H4 (HAT1) and UDP-4-aminococca (PglD). Interestingly, AccoA has been shown to adopt one of two distinct conformations, either bent or curved, depending upon the specific acetyltransferase in question (17). Similar to citrate synthase (18), AccoA bound to PglD adopts a compact conformation with a bend at the pyrophosphate moiety.

To further our understanding of acetyltransferases from the different UDP-diNAcBac biosynthetic pathways and to gain insight into the divergent nature of N- and O-linked protein glycosylation in prokaryotes, acetyltransferases from N. gonorhoeae (PglB-ATD) and A. baumannii (WeeI) were investigated. To this effect, these enzymes were purified and crystallized, and the structures were solved to high resolution. In addition, a co-crystal structure of PglB-ATD bound to AccoA was determined. In this context, a comparison between these structures and the previously solved C. jejuni acetyltransferase (PglD) crystal structures (10) was explored. Interestingly, the assumption that these bacterial acetyltransferases should closely resemble each other because they catalyze the identical reaction is not founded. Surprisingly, the substrate binding pockets for each of these enzymes vary considerably. Based upon this structural comparison, a series of active site mutations was carried out on all three acetyltransferases, and the enzymes were characterized kinetically for both AccoA and UDP-4-aminococca substrates to gain insight into the catalytic mechanism. These studies suggest that although each enzyme catalyzes the acetyltransferase reaction with identical substrates, key residues within the binding pockets lead to a diverse set of catalytic efficiencies. Last, a phylogenetic analysis of acetyltransferases that catalyze the conversion to UDP-diNAcBac in N- and O-linked glycosylation pathways is examined. The three acetyltransferases presented exhibit a high level of evolutionary diversity despite their ability to generate the identical final UDP-diNAcBac sugar. Unexpectedly, PglB-ATD from the O-linked glycosylation pathway shares a more common ancestral lineage with the PglD (N-linked) when compared with WeeI (O-linked).

EXPERIMENTAL PROCEDURES

Common Materials—All chemicals were purchased from Sigma-Aldrich unless otherwise stated. The UDP-4-aminococca sugar was biosynthesized as described previously from the C. jejuni enzymes PglF and PglE (19).

Molecular Biology—The acetyltransferase domain (ATD) of the pglB gene from N. gonorhoeae FA1090 was identified through a Clustal Omega alignment (20) with the C. jejuni acetyltransferase (PglD). The gene encoding this domain was amplified via the polymerase chain reaction (PCR) with the forward primer

C. jejuni-dNAcBac Pathway

N. gonorhoeae-dNAcBac Pathway

UDP-4-amino

UDP-dNAcBac

UDP-4-amino

UDP-dNAcBac
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5′-CGCGGATCCATGGCCGGAATTCAAAACCTCG-3′ and the reverse primer 5′-GCAACCGGGAACAGCCCTTTAGCTCGAGCCG-3′ from the N. gonorrhoeae FA1090 strain (8). The weel gene was amplified via PCR from the genomic DNA from the A. baumannii AYE strain (ATCC BAA-1710) (21). BamHI and XhoI restriction sites were engineered to facilitate cloning of each construct into a modified pET30b(+) vector (Novagen) containing an N-terminal His8 tag followed by a tobacco etch virus protease site prior to the BamHI site. Amplifications were accomplished with the PfuTurbo DNA polymerase (Stratagene) as described by the manufacturer. Amplicons were purified and double-digested with BamHI and XhoI restriction enzymes (New England Biolabs). Digested inserts and linearized vectors were fractionated by agarose gel electrophoresis and purified with the Wizard SV Gel and PCR Cleanup Kit (Promega). Ligations were conducted with the T4 DNA ligase kit (Promega) using a 15-min incubation at room temperature. Sequencing by Genewiz (Cambridge, MA) confirmed the presence of all gene products. Site-directed mutagenesis was accomplished utilizing the QuikChange protocol (Stratagene) with pglD-pET24a (+), pglB-ATD-pET24a (+), and weel-pET24a (+) (from C. jejuni, N. gonorrhoeae, and A. baumannii, respectively) as the template plasmids from previous studies (4, 5, 19).

Protein Expression—The modified pET30b(+) plasmid containing each gene was used to transform Escherichia coli BL21(DE3)pLysS RIL competent cells (Stratagene). One liter of LB medium containing 50 μg/ml kanamycin and 30 μg/ml chloramphenicol was inoculated with 8 ml of an overnight culture of cells. The cells were then allowed to grow at 37 °C while shaking until an optical density of ~0.8 (λ = 600 nm) was reached. The culture was cooled to 16 °C and induced with 0.5 mM iso-β-d-thiogalactosylpyranoside. After incubating for 18 h with shaking at 16 °C, the cells were harvested by centrifugation (2600 × g, 30 min) and stored at −80 °C until needed.

Protein Purification—Each protein purification step was carried out at 4 °C. For crystallization experiments, the cell pellet (~3 g) was resuspended in 40 ml of 50 mM HEPES, pH 7.4, 100 mM NaCl, 30 mM imidazole (Buffer A) and then lysed by sonication. The lysate was then cleared by centrifugation (145,000 × g, 60 min) and added to 2 ml of nickel-nitrilotriacetic acid resin (Qiagen). The slurry was allowed to tumble for 3 h and then packed into a fritted PolyPrep column (Promega). Ligations were conducted with the T4 DNA ligase kit (Promega) using a 15-min incubation at room temperature. Sequencing by Genewiz (Cambridge, MA) confirmed the presence of all gene products. Site-directed mutagenesis was accomplished utilizing the QuikChange protocol (Stratagene) with pglD-pET24a (+), pglB-ATD-pET24a (+), and weel-pET24a (+) (from C. jejuni, N. gonorrhoeae, and A. baumannii, respectively) as the template plasmids from previous studies (4, 5, 19).

Data Collection—All crystals were grown as hanging drops by combining 1.5 μl of a 10 mg/ml protein solution in SEC buffer with 1.5 μl of reservoir solution at 25 °C. Each well contained a final volume of 500 μl of reservoir solution. For the co-crystallization of PglB-ATD with AcCoA, the substrate was added to the protein so that the final concentration was 10 mM and incubated for 45 min at 25 °C. The reservoir solution for apo-PglB-ATD contained 0.1 M sodium acetate, pH 4.6, 0.02 M calcium chloride, and 30% 2-methyl-2,4-pentanediol. The AcCoA-bound PglB-ATD reservoir solution contained 0.1 M BisTris, pH 5.5, 3.0 M NaCl. For apo-Weel, the well solution contained 0.1 M sodium acetate trihydrate, pH 4.5, 3.0 M NaCl, 0.7% 1-butanol. After the crystals were fully grown (~24 h), they were cryoprotected in reservoir solution containing 20% glycerol. For AcCoA-bound PglB-ATD, this solution was also supplemented with 10 mM substrate. Diffraction data were collected on beamline X25 (National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY) at 100 K using a Pilatus 6M detector. Data sets were processed using HKL2000 (22), MOSFLM (23), TRUNCATE (24, 25), and SCALA (24). Parameters from the data collection are listed in Table 1.
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TABLE 1

Data collection and refinement statistics

|          | PgB-ATD | PgB-ATD(AcCoA) | WeeI |
|----------|---------|----------------|------|
| **Data collection** |         |                |      |
| Space group | P2,3    | P4,2,2         | P3,21|
| Unit cell dimension (a, b, c) (Å) | 86.22, 86.22, 86.22 | 97.70, 97.70, 173.95 | 148.29, 148.29, 182.41 |
| Resolution (Å) | 43.2-1.7 | 43.2-1.7       | 87.0-2.6 |
| No. of observed reflections | 25,105 | 26,704          | 180,938 |
| Rexp (%)<sup>a</sup> | 7.8 (53.7) | 10.5 (50.8) | 7.5 (47.5) |
| completeness (%) | 23.2 (4.2) | 19.1 (5.2) | 22.4 (4.4) |
| Redundancy | 100 | 100 | 99.6 |

| **Refinement** |         |                |      |
| Resolution | 43.2-1.7 | 64.2-2.6 | 48.5-2.1 |
| Rwork/Rfree (%)<sup>b</sup> | 16.7/18.8 | 19.8/23.7 | 19.1/22.6 |
| Total no. of atoms | 1543 | 4619 | 10,138 |
| Protein | 1434 | 4326 | 9498 |
| Water | 109 | 139 | 640 |
| Ligands | 0 | 154 | 0 |
| B factors (Å<sup>2</sup>) | 23.2 (4.2) | 19.1 (5.2) | 22.4 (4.4) |
| Overall | 22.4 | 48.3 | 34.6 |
| Protein | 21.8 | 47.6 | 34.4 |
| Water | 29.8 | 44.9 | 37.7 |
| Ligand | 71.5 | 71.5 | 71.5 |
| Ramachandran plot (%)<sup>c</sup> | 98.5/1.5/0 | 96.1/3.6/0.3 | 96.3/3.2/0.5 |
| r.m.s. deviation | 0.006 | 0.006 | 0.007 |
| Bond lengths (Å) | 1.13 | 1.16 | 1.10 |
| Bond angles (degrees) | 4M98 | 4M99 | 4M99 |
| Protein Data Bank code |         |                |      |

<sup>a</sup> Statistics for the highest resolution bin are in parentheses.

<sup>b</sup> R<sub>work</sub> = Σ||F<sub>obs</sub>|| - |F<sub>calc</sub>||/Σ|F<sub>obs</sub>|, where |F<sub>obs</sub>| is the intensity of a reflection and |F<sub>calc</sub>| is the mean intensity of a group of equivalent reflections.

<sup>c</sup> Ramachandran plot statistics are given as core/allowed/generously allowed and are for all chains.

Structure Determination and Refinement—Preliminary electron density maps for the PgB-ATD and Weel structures were generated in PHASER (26), utilizing the previously solved PglD structure (Protein Data Bank code 3BSW) (10) as the molecular replacement search model. Refinement and model building of each structure were accomplished with COOT (27) and PHENIX (28). Water molecules were added using COOT, and the AcCoA ligand was modeled into PgB-ATD after the R<sub>free</sub> value was <30%. Refined structures were validated using MolProbity (29). Composite omit maps for the AcCoA-bound PgB-ATD structure were generated with PHENIX. The final refinement statistics are listed in Table 1.

Acetyltransferase Activity Assay—Enzyme mutants were analyzed for activity utilizing a 5,5'-dithiobis-(2-nitrobenzoic acid) spectrophotometric assay as described previously (4). Briefly, each assay was carried out at 50 mM HEPES, pH 7.4, 2 mM MgCl<sub>2</sub>, 0.05% BSA, 0.001% Triton X-100, and 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid). The substrate concentrations of the reaction curve over a 5-min time period at 25 °C.

Phylogenetic Analysis of UDP-diNACbac Acetyltransferases—Bacterial organisms containing the UDP-diNACbac pathway were identified using the respective oligosaccharyltransferases from C. jejuni (YP_002344519.1), N. gonorrhoeae (YP_207345.1), and A. baumannii (YP_002324267.1). Further selection of the relevant acetyltransferases relied on a >0.35% sequence identity cutoff in BLASTP (30) with PglD (C. jejuni; YP_002344516.1), PgB-ATD (N. gonorrhoeae; YP_207258.1), and Weel (A. baumannii; YP_001715524.1). Acetyltransferase sequences were aligned simultaneously with the software program MUSCLE (31) using a gap-opening penalty of −2.9, a gap extend penalty of 0, and a hydrophobicity multiplier of 1.2. Phylogenetic trees were constructed utilizing the neighbor-joining method (32) and Poisson model (33) with MEGA version 5.2 (34). The confidence level of this process was estimated using a bootstrap analysis with 1000 replicate data sets.

RESULTS

Structure of the N. gonorrhoeae Acetyltransferase PgB-ATD—PgB from N. gonorrhoeae is a bifunctional enzyme containing an N-terminal phosphoglycosyltransferase domain and a C-terminal acetyltransferase domain (ATD) that are homologous to the C. jejuni enzymes PglC and PglD, respectively (5). For crystallographic studies, the membrane-bound phosphoglycosyltransferase domain was removed based upon a Clustal Omega alignment with PglD, thus leaving behind the acetyltransferase domain referred to herein as PgB-ATD. The structure of the apo-form of PgB-ATD was solved by molecular replacement utilizing the previously solved acetyltransferase PglD (sequence identity = 34%) (10). Difficulties in crystallization of this protein were addressed by removing the final 10 amino acid residues from the C-terminal tail based upon a sequence alignment with PglD. The removal of these PgB-ATD residues, which are not present in corresponding PglD sequence, results in a comparable C-terminal tail between the two constructs. PgB-ATD was crystallized in the cubic space group P2<sub>1</sub>3 with a single protomer in the asymmetric unit. Previous work has indicated that bacterial acetyltransferases trimerize in solution (10, 35). Whereas the structure of PgB-ATD shows a single molecule in the asymmetric unit, the homotrimer can be observed through crystallographic symmetry centered on a 3-fold axis (Fig. 3A). This acetyltransferase contains two distinct domains that are responsible for the catalysis of UDP-4-amino to UDP-diNACbac using the AcCoA cosubstrate. The N-terminal section...
(Asn199–Leu285) comprises a binding pocket for the UDP-4-amino sugar substrate through a β-α-β-α-β-α Rossman fold motif. The C terminus (Pro286–Leu403) is composed of a lefthanded β-helix motif that, in conjunction with an adjacent PglB-ATD protomer in the trimeric state, forms an extended cleft that is utilized for AcCoA binding.

Although the *N. gonorrhoeae* acetyltransferase catalyzes the same reaction as PglD from *C. jejuni* and has the same general fold (r.m.s. deviation = 0.79 Å), there are a few notable differences in the structures. The PglB-ATD structure contains a flexible loop (Arg233–Thr246) that is not observed in PglD (Fig. 3B). This loop is tucked in between α-helices 1 and 2 in the N-terminal sugar-binding domain and makes numerous backbone interactions with the second β-sheet (Phe229/Asp231/Asp232). For example, the side chain amide nitrogen of Asn239 has a hydrogen-bonding interaction with the Glu216 acid moiety on helix 1. Similarly, the backbone amide nitrogen of Leu248 and Leu249 on helix 2 interacts with the hydroxyl and carbonyl moiety from the Thr246 loop residue, respectively. In the apo-state, PglD contains a cofactor gate, comprising the final 10 C-terminal residues that interacts with the adjacent, active site protomer (10). To accommodate AcCoA binding, this gate undergoes a conformation change such that an interaction is formed with the cognate protomer in a coiled motif.

Surprisingly, the apo-structure of PglB-ATD reveals that no such cofactor gate is evident (Fig. 3B). Instead, the apo-state structure exists as the coiled motif resembling the AcCoA-bound structure of PglD (3BSY). Additional structures of the apo-form of PglB-ATD were solved under distinct crystallization conditions that further supported the absence of the cofactor gate (data not shown).

Multiple attempts to crystallize PglB-ATD in the presence of UDP-4-amino were unsuccessful. Therefore, a structural alignment of apo-PglB-ATD and UDP-4-amino bound PglD (3BSS) was explored due to the minimal changes within the N-terminal domain upon sugar binding in the PglD structures (r.m.s. deviation = 0.70). PglD residues Asp35 (Asp231 in PglB-ATD), Asp36 (Asp232), and His125 (His333), which accept hydrogen bonds from the ribosyl 3'-hydroxyl group, uridine imide, and pyranose C4-amine, respectively, are strictly conserved between the two structures. Only two notable changes between the structures are observed. The Asn162 amino acid in PglD, which interacts with the carbonyl oxygen of the pyranose C2-acetyl group, is modified to the homologous Gln770 residue in the PglB-ATD
structure. Interestingly, Ser\textsuperscript{13} in the PglD structure, which plays a significant role in the sugar binding pocket by hydrogen bonding to the α-phosphate of UDP-4-amino and N\textsubscript{ε} of Lys\textsuperscript{36}, is replaced by Gly\textsuperscript{208} in PglB-ATD. One cannot rule out the significance of the aforementioned loop in PglB-ATD with respect to sugar binding. Upon UDP-4-amino binding to PglD, an unwinding of helix α2 (Met\textsuperscript{40}–Thr\textsuperscript{45}) to accommodate sugar binding and allow for optimal interactions is apparent in the crystal structure. The PglB-ATD flexible loop is located adjacent to this helix (Fig. 3B) and, upon sugar binding, could elicit a conformational change in this enzyme to mimic missing interactions within this site. Clearly, a PglB-ATD UDP-4-amino-bound structure would be necessary to confirm this hypothesis.

Structure of the N. gonorrhoeae Acetyltransferase PglB-ATD Bound to AccOA—The AccOA-bound PglB-ATD structure was solved by molecular replacement using the apo-PglB-ATD structure. This protein was crystallized in the tetragonal space group P\textsubscript{4}\textsubscript{2}2\textsubscript{1}2\textsubscript{1} with three PglB-ATD protomers in the asymmetric unit. Three AccOA molecules were observed between the clefts formed by adjacent left-handed β-helices in a compact conformation with a bend at the pyrophosphate moiety (Fig. 4). AccOA binds to PglB-ATD in a similar fashion with respect to PglD; however, there are noticeable differences between coenzyme and binding pocket residues. Notably, PglB-ATD utilizes a series of seven residues from both protomers to bind AccOA. In particular, Ser\textsuperscript{356} and the backbone amide nitrogen of Gly\textsuperscript{251} form a hydrogen bond to the carbonyl oxygen of the thioester (Fig. 5A). This is in stark contrast to PglD, where the acetyl group is rotated 180° and forms hydrogen-bonding interactions with His\textsuperscript{134} and Asn\textsuperscript{118} (Fig. 5B). Although the contacts between protomer B in PglD and AccOA are mainly hydrophobic, this protomer plays a much larger role in PglB-ATD. Both hydrogen-bonding interactions of the thioester carbonyl originate from this protomer. Likewise, the water hydrogen-bonding network binding the pyrophosphate moiety and the 3'-phosphate is replaced by Arg\textsuperscript{368} and Lys\textsuperscript{401}, respectively. Only two water molecules are observed binding to AccOA in the PglB-ATD structure, whereas seven water molecules are contributing factors in the PglD structure. In fact, there are no conserved residues responsible for binding to AccOA observed when comparing the two structures. However, backbone interactions between Ala\textsuperscript{381} (PglB-ATD) and Gly\textsuperscript{373} (PglD) serve a similar purpose by hydrogen bonding to a carbonyl oxygen in the pantetheine moiety and the C6 amine on the adenine ring. Further hydrogen-bonding interactions in the PglB-ATD structure can be observed from Thr\textsuperscript{363} (protomer A) and Gln\textsuperscript{369} (protomer B) on the amide adjacent to the thioester in the pantetheine moiety.

Although PglD undergoes a conformational change in the C-terminal tail upon AcCoA binding, no evidence of this change is observed in the PglB-ATD structure. In fact, because the apo-state of PglB-ATD is already in the coiled motif, as observed in the AccOA-PglD structure, no other conformational change is necessary to accommodate AccOA binding. This lack of change between the AccOA-bound state and apo-state of PglB-ATD is reflected in the minor change in r.m.s. deviation between the two structures (0.30 Å). However care must be taken in interpreting these results because a small change in r.m.s. deviation may be biased because the AccOA-bound state was solved by molecular replacement with the apo-PglB-ATD structure. There are only two key conformational changes in the active site cleft necessary for AccOA binding. Most importantly, Arg\textsuperscript{368} in the apo-PglB-ATD structure serves to block access to the channel prior to AccOA binding. Upon binding, Arg\textsuperscript{368} rotates out of the cleft to allow AccOA access to the binding site. This residue is also essential for the binding of AccOA because it has a total of four hydrogen-bonding interactions with the coenzyme (Fig. 5A). Although no such C-terminal cofactor gate exists in PglB-ATD, Arg\textsuperscript{368} may play a similar role to allow for AccOA binding. Glutamine 369 also plays a role in coenzyme binding by rotating 90° to form part of the pantetheine binding pocket and picks up a favorable hydrogen-bonding interaction with AccOA. Analogous residues are not apparent in a structural alignment between PglB-ATD and PglD, adding to the dichotomous nature of these two proteins.

Structure of the A. baumannii Acetyltransferase Weel—The Weel structure was solved by molecular replacement using the previously solved apo-PglB-ATD structure (sequence identity = 26%). This acetyltransferase crystallized in the hexagonal space group P\textsubscript{3}2\textsubscript{1}2\textsubscript{1} and contained six protomers in the asymmetric unit forming a dimer of the biological trimer assembly. Optimization of the crystals was a necessity due to the poor diffraction quality of the original conditions. In particular, the addition of 0.7% 1-butanol to the crystallization buffer improved resolution by 0.8 Å (Hampton additive screen). Similar to PglB-ATD and PglD, Weel is composed of N-terminal (Met\textsuperscript{1}–His\textsuperscript{90}) and C-terminal (Leu\textsuperscript{211}–Leu\textsuperscript{217}) domains that are each responsible for binding to UDP-4-amino and AccOA, respectively (Fig. 6A). A cleft is formed between two adjacent protomers from the C-terminal left-handed β-helix domain that accommodates AccOA binding, as observed previously in the PglB-ATD crystal structure. Unfortunately, multiple screening attempts to solve the Weel structure bound to the UDP-4-amino sugar and AccOA proved unsuccessful.
Similar to the apo-structure of PglB-ATD, WeeI contains a flexible loop between helices α1 and α2 in the N-terminal sugar-binding domain. As previously observed with PglB-ATD, this loop has numerous intramolecular interactions with the protein backbone. Interestingly, the conserved residue Asn46 (Asn239 in PglB-ATD) exhibits a similar hydrogen-bonding interaction with Asn121 on an adjacent protomer. Of note, Pro49 (Pro242 in PglB-ATD) is also conserved in this region and serves to stabilize this loop through hydrogen bonding of the backbone carbonyl to the conserved Phe35 (Phe229 in PglB-ATD) amide nitrogen. The essential sugar binding residues observed in the PglD/UDP-4-amino structure are strictly conserved in WeeI, including Ser13 (Ser11 in PglD), which is conspicuously absent in the PglB-ATD structure. Residues Asp35, Asp36, and His125 in PglD, which contribute hydrogen-bonding interactions with UDP-4-amino, are conserved in WeeI (Asp37, Asp38, and His138). The only exception in this binding pocket is the PglD Asn162 (Gln370 in PglB-ATD) residue. In WeeI, the pyranose moiety of the UDP-4-amino binding pocket is formed by a seven-amino acid loop (Gln174-Pro180) from the adjacent protomer. This loop is not observed in the two other acetyltransferase structures (Fig. 6B) and contains two residues (Gln174 and Thr176) in the vicinity of hydrogen bonding to the carbonyl oxygen of the pyranose C2-acetyl group. Alanine mutagenesis was performed on these two sites to ascertain their relationship to UDP-4-amino binding (see below). In PglD, a conformational change in His15 is observed to accommodate sugar substrate binding. In the apo-structure, this residue occludes the UDP-4-amino pocket. However, upon substrate binding, this residue tucks into the pocket and interacts with the β-phosphate moiety of the sugar. Whereas this residue is conserved in PglB-ATD (His210), the more bulky, hydrophobic phenylalanine residue is found in WeeI. This small change could have a deleterious binding effect on the UDP-4-amino substrate (see below).

The WeeI AcCoA binding pocket exhibits a stronger homology to the PglB-ATD site (56% sequence identity) when compared with PglD (34% sequence identity). Not surprisingly, this
Similar to PglB-ATD, WeeI does not appear to utilize a cofactor gate for AcCoA binding (Fig. 6B). From the apo-structure, the C-terminal tail is in a coiled motif that resembles the AcCoA-bound PglD structure. WeeI also contains a residue analogous to Arg\textsuperscript{368} (PglB-ATD) that may act as a gate to AcCoA binding. Lysine 173 is positioned in a fashion similar to Arg\textsuperscript{368} and obstructs the binding cleft in the apo-state. Although no structure of AcCoA bound to WeeI exists, one can hypothesize that this residue plays an analogous role in coenzyme binding. Key residues that interact with AcCoA in the PglB-ATD crystal structure are mostly conserved in WeeI. PglB-ATD residues Gly\textsuperscript{351} (Gly\textsuperscript{156} in WeeI), Gln\textsuperscript{369} (Gln\textsuperscript{174}), and Thr\textsuperscript{363} (Thr\textsuperscript{168}) are strictly conserved. Substitutions at Lys\textsuperscript{401} (Arg\textsuperscript{211}) and Ser\textsuperscript{350} (Asn\textsuperscript{155}) are complementary in nature, and a similar role can be envisioned at these positions. Of note, the 1\textsuperscript{24}EHE (PglD) and 3\textsuperscript{32}DHD (PglB-ATD) motifs that are critical for catalysis are slightly modified in WeeI (1\textsuperscript{37}AHD). The carboxylate moiety of PglD (Glu\textsuperscript{126}), PglB-ATD (Asp\textsuperscript{334}), and WeeI (Asp\textsuperscript{139}) is hydrogen-bonded to the imidazole ring of histidine, increasing its basicity. This enhancement allows for the N\textsubscript{\textgamma} nitrogen of histidine to act as a general base in catalysis by deprotonating the C4 amine on the UDP-4-amino sugar. Although the carboxylate moiety in PglD (Glu\textsuperscript{124}) and PglB-ATD (Asp\textsuperscript{332}) may serve to recycle histidine back to its precatalytic state by abstracting a proton from N\textsubscript{\textgamma} following substrate turnover (10), this cannot be the case in WeeI due to the alanine moiety at this position.

**Analysis of Acetyltransferase Active Site Mutants**—To better understand the contributions of particular residues in binding and catalysis, a series of mutations was created in the active sites of PglD, PglB-ATD, and WeeI based upon their crystal structures. While holding one substrate at saturating levels for PglD and PglB-ATD, the other was varied to determine kinetic parameters through initial velocity measurements. Ellman’s reagent (5,5’-dithiobis(2-nitrobenzoic acid)) was utilized to monitor AcCoA conversion to CoASH through generation of the TNB\textsuperscript{2–} chromophore (ε\textsubscript{412 nm} = 14,150 M\textsuperscript{–1} cm\textsuperscript{–1}). Due to the poor binding of UDP-4-amino to WeeI, the AcCoA kinetic
parameters were determined at the $K_{m}$ of the UDP-sugar. Typical Michaelis-Menten kinetics were observed for all concentrations of UDP-4-amino and AcCoA. Initial velocity measurements were averaged between two duplicate experiments. UDP-4-amino and AcCoA kinetic parameters for the acetyltransferase mutants are listed in Tables 2 and 3, respectively.

When comparing UDP-4-amino affinity to bacterial acetyltransferases, Weel exhibits poor binding ($K_{m}$ > 10-fold). From a structural alignment standpoint, the phenylalanine at position 13 may contribute to such a poor $K_{m}$ in Weel. This observation is based upon the absence of changes in UDP-4-amino binding pocket residues with respect to PglD and PglB-ATD. The aforementioned His$^{15}$ residue in PglD can be classified as a type of gatekeeper moiety due to its ability to tuck into the pocket to accommodate UDP-4-amino binding and interact directly with this substrate. The histidine is conserved in PglB-ATD, resulting in a similar UDP-4-amino $K_{m}$; however, this site is a phenylalanine in Weel. This change in steric bulk, hydrophobicity, and loss of hydrogen bonding at this key position may result in reduced binding affinity. Therefore, a mutation in PglD (H15F), PglB-ATD (H210F), and Weel (F13A) was explored. This mutation had a deleterious effect on both catalysis and binding on both PglD and PglB-ATD, whereas the F13A Weel mutation mainly affected turnover (Table 2). In order to ascertain if these mutations have any effect on the adjacent AcCoA binding pocket, kinetic parameters were explored for this substrate. Surprisingly, the PglD H15F mutation resulted in a 10-fold increase in binding affinity to AcCoA while decreasing $k_{cat}$ by 20-fold (Table 3). Mutation of H210F (PglB-ATD) and F13A (Weel) resulted in no change in binding affinity; however, $k_{cat}$ decreased considerably. This particular site in the UDP-4-amino binding pocket contributes significantly to binding and catalysis in these acetyltransferases.

The inability to crystallize UDP-4-amino with either PglB-ATD or Weel prompted a series of alanine mutations to determine specific sites within each binding pocket that contribute to binding and catalysis. Based upon an alignment with the UDP-4-amino PglD structure, PglB-ATD (Q369A and Q370A) and Weel (Q174A and T176A) mutants were created. In both cases, these changes are within the vicinity of the PglD residue Asn$^{162}$, which interacts with the carbonyl oxygen of the pyranose C2-acetyl group. Although neither PglB-ATD mutation resulted in a change in UDP-4-amino binding, Q369A had a significant effect on turnover (13-fold decrease). Likewise in the Weel mutations, only T174A resulted in a considerable (270-fold) loss in $k_{cat}$. Due to the proximity of these mutations to the AcCoA binding site, kinetic parameters were also established for this substrate. Mirroring the UDP-4-amino results, a decrease in $k_{cat}$ was only observed for the PglB-ATD Q369A mutant (6-fold) and Weel Q174A (260-fold). It is apparent that the Weel Q174A mutant plays an extremely important role in catalysis of this reaction.

There is still an ongoing discussion over the acetyltransferase catalytic mechanism and in particular the protonation state of UDP-4-amino substrate (10, 11, 15). Although this study does not address this question specifically, the role that Glu$^{124}$ (PglD) plays in catalysis was explored. This position has been implicated in returning the catalytic histidine (H125) back to its preturnover state by transferring the proton on the imidazole moiety to the thiolate on CoAS$^{-}$ (10). Most homologous acetyltransferases incorporate either a glutamate or aspartate at this position; however, this site is occupied by an alanine in Weel. Interestingly, the catalytic efficiency of Weel is comparable with that of PglD and PglB-ATD. To better understand the catalysis and binding at this site, mutant variants of PglD (E124A) and PglB-ATD (D332A) were prepared. In both cases, $k_{cat}$ was reduced for both substrates (Tables 2 and 3); however, the loss was more significant in PglB-ATD (20-fold). Mutation to alanine in PglD and PglB-ATD then has a detrimental effect on catalysis, yet the wild-type Weel is still a competent enzyme with alanine at this position. Therefore, recycling of the active site must be accomplished in another manner. The closest amino acid site that can act as a general base is Lys$^{13}$; however, that residue is over 6 Å away from the catalytic histidine. The most straightforward solution would rely on the thiolate from CoAS$^{-}$ (following acetylation of the UDP-4-amino sugar) to act as a base to directly remove the proton from the catalytic histidine. This would regenerate the active site to its precatalytic state and explain the absence of a general base adjacent to His$^{138}$ in Weel. In lieu of these results, the glutamate/aspartate moiety in PglD (Glu$^{124}$) and PglB-ATD (Asp$^{332}$) appears to be a non-absolute requirement for catalysis, and its essentiality may have previously been overstated.

Phylogenetic Analysis of Bacterial Acetyltransferases—Although the three acetyltransferases presented here carry out the same reaction and display the same general protein fold, homology within the substrate binding sites is quite divergent. To further our understanding on the evolutionary aspect of acetyl-
transfosses within the diNacBac pathway, a phylogenetic analysis was carried out. Bacteria containing the diNacBac pathway were first identified by having a >35% homology to known oligosaccharyltransferases from C. jejuni, N. gonorrhoeae, and A. baumannii. Comparative assessment of these enzymes allowed for classification of PgdD as an N-linked glycosylation system and PgbB-ATD/WeeI as O-linked systems. Acetyltransferases were further classified similarly to the oligosaccharyltransferase analysis above and sequentially aligned with the software program MUSCLE. Interestingly, the neighbor-joining dendrogram (Fig. 7) is broken up into multiple clades and exhibits evolutionary diversity, which is observed within the acetyltransferase binding pockets. This is somewhat surprising because the acetyltransferases from C. jejuni, N. gonorrhoeae, and A. baumannii carry out the identical reaction to produce the diNacBac sugar. Similar results were observed previously using dehydratase and aminotransferase homologs from Campylobacter and Neisseria (36). Of note, homologous A. baumannii acetyltransferases are evolutionarily more distant with respect to C. jejuni and N. gonorrhoeae.

**DISCUSSION**

Divergence of Bacterial Acetyltransferases in N- and O-Linked Protein Glycosylation Pathways—Glycosylation is a ubiquitous post-translational modification and is known for regulating cellular processes, such as protein folding, stability, and cell signaling (37–38). Significantly, bacteria also utilize protein gly-
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cosylation for purposes of mediating colonization, adhesion, and invasion of eukaryotic cells (1, 12, 39). In fact, recent work on the ATCC 17978 strain of A. baumannii has demonstrated a link between pathogenicity and protein glycosylation (9, 40). To better understand the module responsible for the biosynthesis of UDP-diNAcBac, research has focused on the specific enzymes that lead to the synthesis of this unusual sugar. Bacterial glycosylation can be classified as N-linked (asparagine-linked glycan) and O-linked (serine/threonine-linked glycan). Both modifications, in the context of UDP-diNAcBac biosynthesis, have been studied extensively in C. jejuni (N-linked) (7, 10, 19) and to a lesser extent in N. gonorrhoeae and A. baumannii (O-linked) (4, 5, 8). Although the enzymes responsible for the biosynthesis of this unique, nucleotide sugar are present in these bacteria, they are evolutionarily divergent with regard to their acetyltransferases (Fig. 7). Homologs of these enzymes from their respective organisms are separated into multiple clades within the dendrogram. There are two explanations to account for this observation. First, these enzymes could have convergently evolved by acquiring the biosynthetic enzymes necessary for the production of UDP-diNAcBac. Second, these enzymes could have evolved from a common ancestor and diverged over an extended period of time. This is the simpler explanation and could account for the varying degrees of identity observed within the AccoA and UDP-4-amino binding pockets (4). For instance, the C. jejuni PgdI UDP-4-amino binding pocket shares a higher sequence identity with PgbB-ATD from N. gonorrhoeae. Conversely, WeeI from A. baumannii shares a higher homology with its O-linked counterpart, PgbB-ATD, in the AccoA binding pocket. In either case, it is interesting that the acetyltransferases from two O-linked pathogens (N. gonorrhoeae and A. baumannii) are evolutionarily more divergent with respect to the N-linked C. jejuni enzyme. It is currently unknown whether A. baumannii acquired this enzyme from an N- or O-linked pathway. Although the true significance of UDP-diNAcBac is presently unclear, it is important to recognize its ubiquitous nature in pathogenic bacteria. Why specific bacteria acquired the UDP-diNAcBac biosynthetic pathway remains a mystery. Additionally, questions surrounding the motility of the UDP-diNAcBac module between bacteria in lieu of the entire glycosylation pathway remain unanswered. Further work is warranted to address these questions in the context of bacterial fitness and pathogenicity.

Mutagenesis of the UDP-4-amino Binding Pocket Reveals Kinetic Diversity—The structures of PgbB-ATD and WeeI add to the growing number of acetyltransferases that are associated with UDP-diNAcBac biosynthesis. Importantly, these structures represent the first O-linked glycosylation pathway enzymes that result in the production of this bacterial sugar. Although the overall architecture of these proteins is similar with respect to PgdI, there are notable differences that contribute to their contrasting kinetic parameters. In particular, WeeI binds to UDP-4-amino with a significantly lower affinity (10-fold) in comparison with PgdI and PgbB-ATD (Table 2). From a structural alignment standpoint, WeeI contains one key residue (Phe134) that may be responsible for this dramatic $K_a$ shift. In PgdI, this position (His155) undergoes a conformational change to accommodate UDP-4-amino binding and interacts with sugar β-phosphate. Site-directed mutagenesis of this position (H155F) resulted in a 10-fold loss in affinity for UDP-4-amino binding with PgdI. However, the same mutation in PgbB-ATD (H210F) produced a more modest loss in binding (3-fold). This position is extremely important for acetyltransferase activity because there is a 100-fold decrease in catalytic efficiency ($k_{cat}/K_m$) when mutating this residue to a phenylalanine in both PgdI and PgbB-ATD. Despite the poor binding affinity of UDP-4-amino, WeeI must contain a compensatory effect because this enzyme retains a similar efficiency with respect to PgdI and PgbB-ATD.

WeeI contains an additional loop (Gln174-Pro180) that forms the UDP-4-amino binding pocket near the pyranose moiety. Residue Gln174 seems to be critical for catalysis because an alanine mutation results in a 270-fold loss in $k_{cat}$ while maintaining its affinity for UDP-4-amino. When aligned to the PgdI UDP-4-amino structure, this position is analogous to Asn162 that interacts with the carbonyl oxygen of the pyranose C2-acetyl group. In the WeeI apo-structure, Gln174 is within 5 Å of the catalytic base (His138) and 3.6 Å of the AccoA thioester when aligned to the PgbB-ATD AccoA structure. Likewise, the Q174A mutation has a detrimental effect on AccoA catalysis with a 260-fold loss in turnover. Clearly, this residue plays a key role in the overall function of WeeI.

Dichotomy among N- and O-Linked Acetyltransferase AccoA Binding Pockets—A general theme of binding and catalysis among homologous proteins is conservation of key amino acids that result in the comparable activity between enzymes. Although the AccoA binding parameters of PgdI and PgbB-ATD are extremely similar (Table 3), the ways in which the enzymes bind the coenzyme are distinct (Fig. 5). AccoA is mainly held into the binding pocket of PgdI by hydrophobic interactions and a network of water molecules. In fact, only two side chains (Asn118 and His134) contribute to the overall affinity of this substrate in PgdI. Surprisingly, these analogous residues in PgbB-ATD play no role in binding to AccoA. Instead, the coenzyme is held in the binding site by a series of hydrogen-bonding interactions from a total of seven residues. Interactions between the phosphate moieties in PgbB-ATD have replaced the water molecules in PgdI with Arg260 and Lys401.

In addition to this major change in binding site functionality, PgbB-ATD does not appear to utilize a C-terminal cofactor gate for AccoA binding. Upon AccoA binding in the PgdI structure, the C-terminal tail undergoes a conformational change to accommodate the coenzyme in the form of a coiled motif. This coiled motif is already apparent in the apo-structure of PgbB-ATD (Fig. 3); however, the removal of the final 10 amino acids from the C-terminal tail for crystallization purposes could have elicited this result. However, this is unlikely because this deletion does not remove the residues responsible for this conformational change, as observed in PgdI. Furthermore, the apo-WeeI structure is of the full-length protein and also does not exhibit a conformational change in this cofactor gate (Fig. 6). However, one cannot rule out the possibility of a crystallographic artifact when discussing these types of small conformational changes between enzymes. Although both O-linked glycosylation acetyltransferases do not contain this cofactor gate, comparisons between the apo- and AccoA-bound structures of
PglB-ATD resulted in the discovery of a residue that may have analogous function. In the apo-structure, Arg^{368} can be observed blocking the AcCoA binding cleft. Upon coenzyme binding, this residue rotates out of the pocket and interacts with the phosphate and pantetheine hydroxyl moieties. When comparing the coenzyme binding pockets in the apo- and AcCoA-bound structures, no other large conformational changes are detected. Similar to this observation, WeeI contains Lys^{173} at this position when aligned with the PglB-ATD AcCoA structure. This residue seems to function in a similar fashion to Arg^{368} in PglB-ATD because the lysine side chain is also observed blocking access to the AcCoA binding channel in the apo-state. These changes are not surprising in the context of homology between N- and O-linked glycosylation pathways because the O-linked acetyltransferases retain a high degree of sequence identity in the AcCoA binding pocket. PglB-ATD and WeeI bear a stronger resemblance in their structural homology when compared with PglD. This observation is also evident in the sequence homology between their respective aminotransferase active sites (4).

In conclusion, the structures of the O-linked glycosylation pathway acetyltransferases PglB-ATD and Weel brings us closer to understanding the intricacies of UDP-diNAcBac biosynthesis. Importantly, these structures establish the divergent nature of the UDP-4-amino and AcCoA binding pockets in contrast to the N-linked acetyltransferase PglD. Although these three enzymes catalyze the same reaction, minor modifications of each binding site can have large ramifications on binding and catalysis. These results provide insight into the surprising structural diversity among bacterial acetyltransferases that catalyze the same reaction with similar efficiencies. C. jejuni, N. gonorrhoeae, and A. baumannii occupy specific and different environments within their host organisms. For instance, C. jejuni is an enteric pathogen and resides in the digestive tract, while A. baumannii colonizes the respiratory tract. The changes outlined in this study may reflect the adaptability of the components in the UDP-diNAcBac pathway to their respective environments. Due to the high catalytic efficiency of these acetyltransferases, pathway flux may be attenuated through these enzymes (4, 19). Depending upon the environment in which the bacterial pathogen resides, virulence factors that rely upon diNAcBac glycosylation may need to be tuned in a positive or negative fashion. Therefore, changes within the acetyltransferase binding pockets may be the result of these circumstances. Additional research is necessary to provide further evidence for this hypothesis. The structural and mutagenesis work presented here strengthens our understanding of bacterial glycosylation in relation to N- and O-linked glycosylation pathways from significant pathogenic bacteria.

Acknowledgments—We are extremely grateful to Professor Robert Sauer, Dr. Robert Grant, and Jeremy Setser for assistance with data refinement and technical advice on crystallography. We thank Dr. Nina Leksa for Weel data collection, Professor Michael Laub for advice on phylogenetic analysis, and Dr. Angelyn Larkin for critical reading of the manuscript. Finally, we thank Austin Travis for PglB-ATD AcCoA data collection and critical reading of the manuscript.

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