Diversity Within the *O*-linked Protein Glycosylation Systems of *Acinetobacter* Species*□

**Nichollas E. Scott‡‡‡, Rachel L. Kinsella§‡‡, Alistair V. G. Edwards¶,
Martin R. Larsen¶, Sucharita Dutta∥, Julian Saba∥, Leonard J. Foster‡,
and Mario F. Feldman§****

The opportunistic human pathogen *Acinetobacter baumannii* is a concern to health care systems worldwide because of its persistence in clinical settings and the growing frequency of multiple drug resistant infections. To combat this threat, it is necessary to understand factors associated with disease and environmental persistence of *A. baumannii*. Recently, it was shown that a single biosynthetic pathway was responsible for the generation of capsule polysaccharide and O-linked protein glycosylation. Because of the requirement of these carbohydrates for virulence and the non-template driven nature of glycan biogenesis we investigated the composition, diversity, and properties of the *Acinetobacter* glycoproteome. Utilizing global and targeted mass spectrometry methods, we examined 15 strains and found extensive glycan diversity in the *O*-linked glycoproteome of *Acinetobacter*. Comparison of the 26 glycoproteins identified revealed that different *A. baumannii* strains target similar protein substrates, both in characteristics of the sites of *O*-glycosylation and protein identity. Surprisingly, glycan micro-heterogeneity was also observed within nearly all isolates examined demonstrating glycan heterogeneity is a widespread phenomena in *Acinetobacter* O-linked glycosylation. By comparing the 11 main glycoforms and over 20 alternative glycoforms characterized within the 15 strains, trends within the glycan utilized for *O*-linked glycosylation could be observed. These trends reveal *Acinetobacter* *O*-linked glycosylation favors short (three to five residue) glycans with limited branching containing negatively charged sugars such as GlcNAc3NAcA4OAc or legionaminic/pseudaminic acid derivatives. These observations suggest that although highly diverse, the capsule/*O*-linked glycan biosynthetic pathways generate glycans with similar characteristics across all *A. baumannii*. *Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.038315, 2354–2370, 2014.*

Acinetobacter baumannii is an emerging opportunistic pathogen of increasing significance to health care institutions worldwide (1–3). The growing number of identified multiple drug resistant (MDR)[1] strains (2–4), the ability of isolates to rapidly acquire resistance (3, 4), and the propensity of this agent to survive harsh environmental conditions (5) account for the increasing number of outbreaks in intensive care, burn, or high dependence health care units since the 1970s (2–5). The burden on the global health care system of MDR *A. baumannii* is further exacerbated by standard infection control measures often being insufficient to quell the spread of *A. baumannii* to high risk individuals and generally failing to remove *A. baumannii* from health care institutions (5). Because of these concerns, there is an urgent need to identify strategies to control *A. baumannii* as well as understand the mechanisms that enable its persistence in health care environments.

Surface glycans have been identified as key virulence factors related to persistence and virulence within the clinical setting (6–8). *Acinetobacter* surface carbohydrates were first identified and studied in *A. venetianus* strain RAG-1, leading to the identification of a gene locus required for synthesis and export of the surface carbohydrates (9, 10). These carbohydrate synthesis loci are variable yet ubiquitous in *A. baumannii* (11, 12). Comparison of 12 known capsule structures from *A. baumannii* with the sequences of their carbohydrate synthesis

[1] The abbreviations used are: MDR, multiple drug resistant; CID, collision induced dissociation; HCD, higher energy collisional dissociation; ETD, electron transfer dissociation, LC, liquid chromatography; MS, mass spectrometry; ZIC-HILIC, zwitterionic hydrophilic interaction liquid chromatography.

loci has provided strong evidence that these loci are responsible for capsule synthesis with as many as 77 distinct serotypes identified by molecular serotyping (11). Because of the non-template driven nature of glycan synthesis, the identification and characterization of the glycans themselves are required to confirm the true diversity. This diversity has widespread implications for *Acinetobacter* biology as the resulting carbohydrate structures are not solely used for capsule biosynthesis but can be incorporated and utilized by other ubiquitous systems, such as *O*-linked protein glycosylation (13, 14).

Although originally thought to be restricted to species such as *Campylobacter jejuni* (15, 16) and *Neisseria meningitidis* (17), bacterial protein glycosylation is now recognized as a common phenomenon within numerous pathogens and commensal bacteria (18, 19). Unlike eukaryotic glycosylation where robust and high-throughput technologies now exist to enrich (20–22) and characterize both the glycan and peptide component of glycopeptides (23–25), the diversity (glycan composition and linkage) within bacterial glycosylation systems makes few technologies broadly applicable to all bacterial glycoproteins. Because of this challenge a deeper understanding of the glycan diversity and substrates of glycosylation has been largely unachievable for the majority of known bacterial glycosylation systems. The recent implementation of selective glycopeptide enrichment methods (26, 27) and the use of multiple fragmentation approaches (28, 29) has facilitated identification of an increasing number of glycosylation substrates independent of prior knowledge of the glycan structure (30–33). These developments have facilitated the undertaking of comparative glycosylation studies, revealing glycosylation is widespread in diverse genera and far more diverse then initially thought. For example, Nothaft et al. were able to show *N*-linked glycosylation was widespread in the *Campylobacter* genus and that two broad groupings of the *N*-glycans existed (34).

During the initial characterization of *A. baumannii* O-linked glycosylation the use of selective enrichment of glycopeptides followed by mass spectrometry analysis with multiple fragmentation technologies was found to be an effective means to identify multiple glycosylated substrates in the strain ATCC 17978 (14). Interestingly in this strain, the glycan utilized for protein modification was identical to a single subunit of the capsule (13) and the loss of either protein glycosylation or glycan synthesis lead to decreases in biofilm formation and virulence (13, 14). Because of the diversity in the capsule carbohydrate synthesis loci and the ubiquitous distribution of the PglL *O*-oligosaccharyltransferase required for protein glycosylation, we hypothesized that the glycan variability might be also extended to *O*-linked glycosylation. This diversity, although common in surface carbohydrates such as the lipopolysaccharide of numerous Gram-negative pathogens (35), has only recently been observed within bacterial proteins

glycosylation system that are typically conserved within species (36) and loosely across genus (34, 37).

In this study, we explored the diversity within the *O*-linked protein glycosylation systems of *Acinetobacter* species. Our analysis complements the recent *in silico* studies of *A. baumannii* showing extensive glycan diversity exists in the carbohydrate synthesis loci (11, 12). Employing global strategies for the analysis of glycosylation, we experimentally demonstrate that the variation in *O*-glycan structure extends beyond the genetic diversity predicted by the carbohydrate loci alone and targets proteins of similar properties and identity. Using this knowledge, we developed a targeted approach for the detection of protein glycosylation, enabling streamlined analysis of glycosylation within a range of genetic backgrounds. We determined that; *O*-linked glycosylation is widespread in clinically relevant *Acinetobacter* species; inter- and intra-strain heterogeneity exist within glycan structures; glycan diversity, although extensive results in the generation of glycans with similar properties and that the utilization of a single glycan for capsule and *O*-linked glycosylation is a general feature of *A. baumannii* but may not be a general characteristic of all *Acinetobacter* species such as *A. baylyi*.

MATERIALS AND METHODS

Bacterial Strains—*Acinetobacter* strains are provided in Table I. All *Acinetobacter* strains were grown in Luria Bertani (LB) (10g Tryptone, 5g yeast extract, and 10g of NaCl per 1L of dH$_2$O supplemented with 15g of agar per liter of broth when needed) broth/agar at 37 °C with shaking at 200rpm. For protein purification (supplementary Methods) an additional 50 μg/ml of Kanamycin was added with 0.2% (w/v) L-arabinose also added for induction when required. For the generation of material for protein purification studies and ZIC-HILIC analysis 1L cultures were grown overnight as described above. Cells were harvested, washed twice in phosphate buffered saline and either used instantly for protein purification or freeze dried for ZIC-HILIC analysis preparation.

Glycopeptide Identification from Purified A1S_1193—Purified proteins (Supplementary Methods) were resolved using 10% SDS-PAGE and stained with Coomassie blue to assess purity. In-gel trypsin digestion of the protein band corresponding to glycosylated A1S_1193 was performed according to Shevchenko et al. (2006) (38). Briefly, bands were washed with water and dehydrated with 100% acetonitrile (ACN), followed by repeated rehydration with 1:1 (v/v) water and ACN and dehydration with 100% ACN. Next disulfide bonds were reduced through treatment with 10 mM DTT in 50 mM NH$_4$HCO$_3$ for 60 min at 37 °C. Cysteine thiol groups were then alkylated with 50 mM iodoacetamide in 50 mM NH$_4$HCO$_3$ in the dark for 60 min at room temperature. Gel pieces were then washed with 50 mM NH$_4$HCO$_3$, dehydrated with 100% ACN and dried. A1S_1193 was then digested with 0.02 mg/ml trypsin in 50 mM NH$_4$HCO$_3$ (Promega, Madison, WI) at 37 °C for 16 h. Peptides were eluted from the gel through addition of 100% ACN and water, and lyophilized for mass spectrometry analysis. Peptides were resuspended in 0.1% trifluoroacetic acid and loaded onto a ZipTipC18 (Millipore, USA) column for desalting. Peptides were eluted with 60% ACN, dried down in a Speedvac and resuspended in 0.1% Formic acid (FA). The peptides were analyzed using a Q-TOF Premier (Waters, Manchester, UK) coupled to a nanoACQUITY (Waters) ultra-performance liquid chromatography system as briefly described (39) with MassLynx, v. 4.1 (Waters) employed for data analysis.

TABLE I
Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. baumannii		
ATCC 17978	Reference strain	[74]
ATCC 17978 Δ*pglL*	O-linked glycosylation negative strain	[14]
SDF	Body lice isolate	[75]
AYE	Human isolate	[75]
ATCC 19606	Reference strain	[76]
1224	Clinical isolate, thigh	[77]
1225	Clinical isolate, coccyx	[77]
1441 C1	MDR Clinical isolate	This study
1441 C3	MDR Clinical isolate	This study
Arg1	MDR Clinical isolate	This study
Arg2	MDR Clinical isolate	This study
A. calcoaceticus 1217	Clinical isolate, urine	[77]
A. calcoaceticus 1218	Clinical isolate, leg	[77]
A. pittii 1219	Clinical isolate, urine	[77]
A. baylyi ADP1	Soil isolate	[78]
A. nosocomialis 1222	Clinical isolate, iscial ulcer	[77]
E. coli		
DH5α	General cloning and plasmid propagation	Invitrogen
Plasmids		
pEXT20–A1S_1193His10X	Cloning and expression vector, IPTG inducible, Amp^r, with A1S_1193 inserted at *Bam*H1 and *Xba*I	[28]
pEC-A1S_1193His10X	Arabinose inducible, $K_m{}^r$, plasmid used to express A1S_1193 (Inserted at *Hinc*II and *Sal*I) in *Acinetobacter* for glycan fishing	[28]
pBAVMCS	$K_m{}^r$ pBAV1K-T5-gfp derivative with gfp ORF removed, Constitutive *E. coli*/*Acinetobacter* shuttle vector	[10]
pBAVMCS-A1S_1193His10X	Constitutive plasmid, $K_m{}^r$, used to express A1S_1193 (inserted at *Bam*HI and *Sal*I) in *Acinetobacter*	This study

Membrane Preparation for Glycopeptide Enrichment—Lipid free membranes were prepared according to Pessione et al. (40). Briefly, cells suspended in ice-cold 40 mM Tris (pH7.4) and lysed using three rounds of disruption at 30 kpsi using a cell disruptor (Constant System ltd, Kennesaw, GA). Lysates were then centrifuged at 100,000 $\times$ g for 70 min and the resulting pellet resuspended in 1 ml of 50 mM ammonium bicarbonate. Membrane pellets were delipidated using 4 ml of 2:1 v/v trifluoroethanol/chloroform that was allowed to incubate at 4 °C for 1 h and were mixed every 10 min. The delipidated samples were spun down at 10,000 $\times$ g at 4 °C for 10 min and the upper phase collected and dried before use.

Protease Digestion for Glycopeptide Enrichment—Dried membrane proteins were resuspended in 6 M urea, 2 M thiourea, and 40 mM NH$_4$HCO$_3$ and reduced/alkylated prior to digestion with Lys-C (1/200 w/w) and then trypsin (1/50 w/w) as previously described (30). All peptide digests were dialyzed against ultra-pure water overnight using a Mini Dialysis Kit with a molecular mass cut off of 1000 Da (Amersham Biosciences, Buckinghamshire, UK) and on completion were collected and lyophilized.

Identification of Glycopeptides using ZIC-HILIC Enrichment and Reversed Phase LC-MS/MS—ZIC-HILIC enrichment was performed according to (30) with minor modifications. Micro-columns composed of 10 μm ZIC-HILIC resin (Sequant, Umeå, Sweden) packed into p10 tips containing a 1 mm^2 excised C$_8$ Empore™ disc (Sigma) were packed to a bed length of 0.5 cm. Prior to use, the columns were washed with ultra-pure water, followed by 95% ACN and then equilibrated with 80% ACN and 5% FA. Samples were resuspended in 80% ACN, 5% FA and insoluble material removed by centrifugation at 20,000 $\times$ g for 5 min at 4 °C. Samples were adjusted to a concentration of 2 μg/μl and 100 μg of peptide material loaded onto a column and washed with 10 load volumes of 80% ACN, 5% FA. Peptides were eluted with three load volumes of ultra-pure water into low-bind tubes and concentrated using vacuum centrifugation. ZIC-HILIC fractions were resuspended in 0.5% formic acid and separated using EASY-nLC system (Thermo Scientific, San Jose, CA, USA) coupled to either an LTQ-Orbitrap XL with ETD, an LTQ-Orbitrap velos or an Orbitrap Elite (Thermo Scientific). Samples were eluted using a gradient from 100% buffer A (0.5% acetic acid) to 40% buffer B (0.5% acetic acid, 80% ACN) over 148 mins at a constant flow of 300 nL/min. The instrument was operated using Xcalibur v2.2 (Thermo Scientific) in a data-dependent mode automatically switching between MS and HCD/CID on the Orbitrap Elite and Velos whereas CID/ETD was used on the Orbitrap XL. ETD and CID scan events were analyzed with ITMS whereas HCD scans were analyzed using FTMS. On all instruments the 5 most abundant precursor ions were selected and dynamic exclusion of 30 s enabled. MS resolution was set to 60,000 with an ACG target of 1 $\times$ 10^6, maximum fill time of 500 ms and a mass window of 600 to 2000 *m/z*. HCD fragmentation (normalized collision energy 40) was carried out with an ACG of 2 $\times$ 10^5, maximum fill time of 250 ms, resolution set to 7500 and mass window 200 to 2000 *m/z*. CID (normalized collision energy 35), while CID fragmentation was carried out with an ACG target of 2 $\times$ 10^4 and maximum fill time of 100 ms. ETD fragmentation was carried out with

an ACG target of 2 × 10⁵, ETD reaction time of 100ms. Duplicate enrichments were generated for each glycopeptide analysis.

Glycopeptide Data Processing—The raw files were then processed within Proteome Discover version 1.3 (Thermo Scientific) to generate mgf files and searched using Sequest against a composite FASTA database of strain SDF, ATCC17978, and AYE (NCBI accession: NC_010400.1, NC_009085.1 and NC_010410.1 respectively), obtained from NCBI on 10/07/2012). Scan events that did not result in peptide identification from Sequest searches were exported to Excel (Microsoft, Redmond, WA, USA). To identify possible glycopeptides within this list, the "mgf graph" MS-MS module of GPMAW 8.2 was utilized to identified all scan events within the generated mgfs containing the diagnostic oxonium 301.104 or 204.086 *m/z* ion. Using Excel, all scan events that were not matched by Sequest and contained a predicted marker of glycosylation were identified. These events were manually inspected and identified as possible glycopeptides based on the presence of the glycan fragment within the CID scan. To facilitate glycopeptide assignments from HCD scans, the ions below the mass of the predicted deglycosylated peptides were extracted with Xcalibur v2.2 using the Spectrum list function. Ions with a deconvoluted mass above that of the deglycosylated peptide and ions corresponding to known carbohydrate oxoniums were removed in a similar approach to post-spectral processing of ETD data (41, 42). MASCOT v2.2 searches were using the Walter and Eliza Hall Institute Mascot server (https://sysbio-mascot.wehi.edu.au/mascot/home.html) of the proteobacteria taxonomy of the LugwigNR database. Searches were carried out with a parent ion mass accuracy of 20 ppm and a product ion accuracy of 0.02 Da with no protease specificity as well as the fixed modification carbamidomethyl (C) and variable modifications, oxidation (M), deamidation (N), and N-terminal formylation. The instrument setting of MALDI-QIT-TOF was chosen because of previous studies showing quadrupole-like fragmentation within HCD spectra (43) (generating a, b, and y ions) and our observation of internal cleavage products that are all included in this setting. All spectra were searched with the decoy option enabled and no matches to this database were detected (FDR 0%). To further validate glycopeptide matches, all spectra HCD spectra were annotated using the Expert Annotation tool (44) (http://www.biochem.mpg.de/mann/tools/) with a mass accuracy of 10ppm, whereas ETD data was annotated manually with a mass accuracy of 0.6 Da to ensure all major peaks were match providing further confidence of identity and localization. All annotated spectra are providing within supplemental Tables S1, S3, S4, S6*A*, and S7–S10. Isotopic distribution analysis was accomplished with the aid of the MS-Isotope module of Protein Prospector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msisotope).

<div align="center">RESULTS</div>

A. baumannii Strains Produced Diverse O-glycan Structures—Protein glycosylation and the carbohydrate synthesis loci thought to be responsible for the production of the *O*-linked glycan are ubiquitous in *Acinetobacter* (13, 14). As glycans are produced in a non-template driven manner we began exploring the potential *O*-glycan diversity by undertaking glycopeptide enrichment of commonly used sequenced *A. baumannii* strains as well as clinical isolates. These strains included; ATCC 19606, predicted to produce the same O-glycan as the previously characterized ATCC 17978 glycan (supplemental Fig. S1) (14), two strains thought to produce divergent glycans; AYE and SDF (supplemental Fig. S1); as well as clinical isolates of unknown glycan composition; Arg1, Arg2,

1441 C1, and 1441 C3. ZIC-HILIC enriched preparations of ATCC 19606 resulted in the identification of 50 unique glycopeptides (supplemental Table S1) corresponding to 13 unique glycoproteins. Of these glycoproteins, eight were previously unknown (supplemental Table S2). In agreement with their matching carbohydrate synthesis loci, the glycan moieties in ATCC 19606 and ATCC 17978 were identical, consisting of pentasaccharide β-GlcNAc3NAcA4OAc-4-(β-GlcNAc-6-)-α-Gal-6-β-Glc-3-β-GalNAc- (Fig. 1*A*).

In contrast, *A. baumannii* strains SDF and AYE lack the genes required for the generation of GlcNAc3NAcA4OAc (supplemental S. S1), and consequently, no potential glycopeptides could be identified using the GlcNAc3NAcA4OAc oxonium ion. To enable the detection of divergent glycan attachments, we assessed the presence of the oxonium ion 204.086 *m/z,* generated by the presence of HexNAc moieties within ZIC-HILIC enriched samples of *A. baumannii*. Despite multiple attempts, glycosylation could not be identified within strain AYE on either the peptide or protein level, using Periodic Acid Schiff's (PAS) staining. Interestingly, PAS staining also failed to identify the present of capsular polysaccharide (supplemental Fig. S2) suggesting the absence of products from the AYE carbohydrate locus. As the capsular polysaccharide is required for complement resistant (13) we assess the resistance of AYE to complement-mediated killing. Consistent with the lack of the capsular polysaccharide AYE was highly sensitive to complement-mediated killing, compared with ATCC 17978 and all recent clinical isolates examined (supplemental Fig. S3). The levels of sensitivity were also consistent with that of the known capsule mutant ATCC 17978 ΔpglC (supplemental Fig. S3) (13), supporting the absence of carbohydrate locus products within AYE.

Within SDF the use of ZIC_HILIC enrichment lead to the identification of 13 unique glycopeptides containing a tetrasaccharide glycan composed of HexNAc₂-Hex-NulO (mass 884.34 Da) (Fig. 1*B*), where NulO corresponds to a 316.13 Da nonulosonic acid sugar. The identification of this unique glycan attached to five unique proteins (Table II and supplemental Table S3) both confirmed SDF has an active general *O*-linked glycosylation system and produces a glycan different to the pentasaccharide characterized in ATCC 17978 and 19606. Analysis of glycan related fragments within identified glycopeptides of SDF revealed the mass and isotopic distribution of the 317.13 *m/z* oxonium ion (the MH+ of the 316.13 residue, supplemental Fig. S4*A*), was consistent with legionaminic acid (elemental composition $C_{13}H_{21}O_7N_2$) (45). To further assess the identity of the 316 Da moiety, analysis of the low mass region of the HCD spectra was undertaken (supplemental Fig. S4*B*) confirming fragmentation consistent with that of the legionaminic or pseudaminic acid oxonium ion (45). These observations are in line with the genetic analysis of the glycan locus of SDF, which contains homologs for legionaminic acid biosynthesis as noted previously (12) (supplemental Fig. S1 and S4*C*).
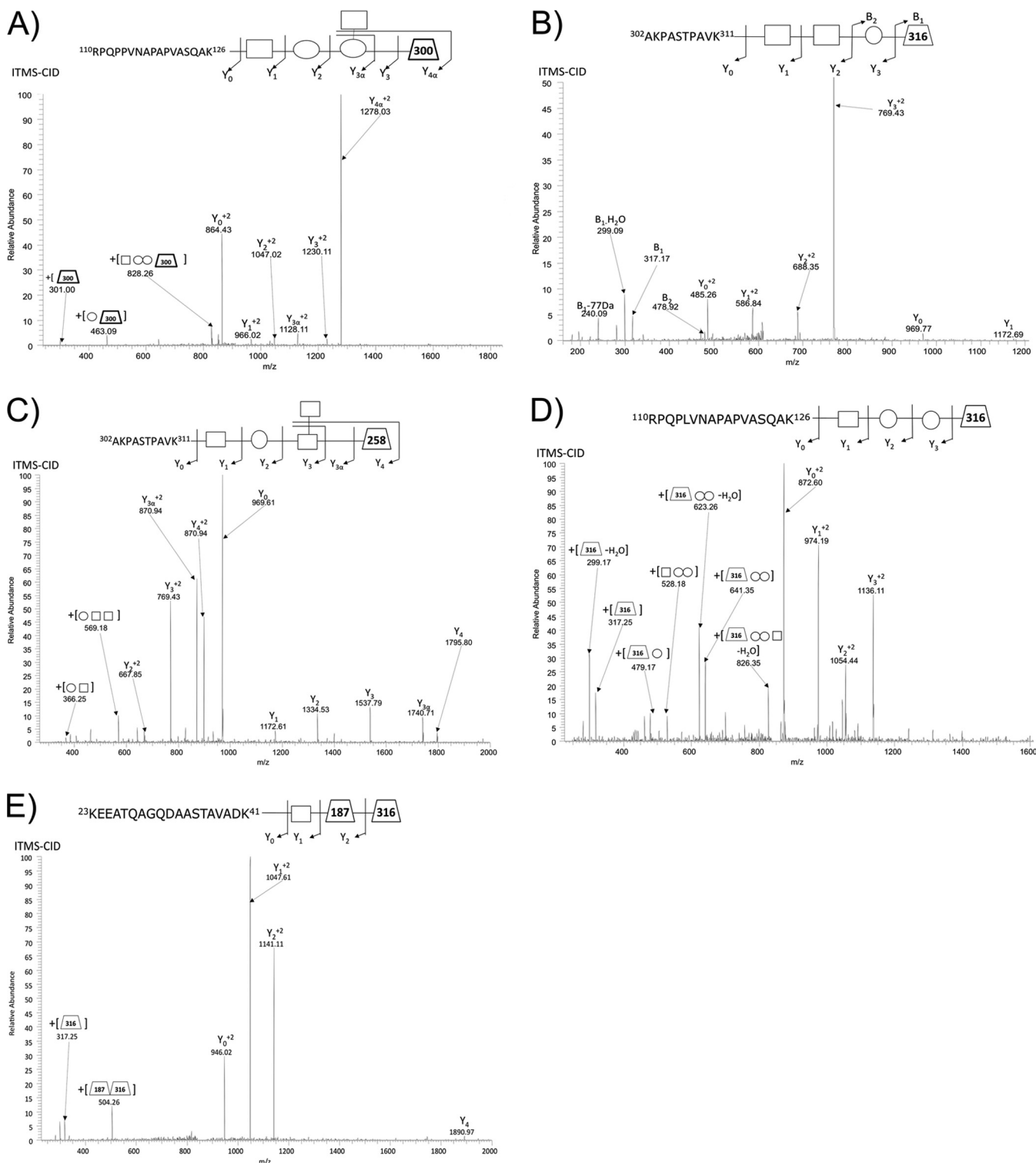
FIG. 1. **Major *O*-glycan structure identified using ZIC-HILIC enrichment.** ITMS-CID fragmentation results in near exclusive glycan fragmentation of *A. baumannii* glycopeptides leading to the identification of *A*, the previously pentamer glycan (14) within strain ATCC 19606 ([110]RPQPPVNAPAPVASQAK[126] of D0CDA9_ACIBA), *B*, a 4-mer glycan containing a 316 Da residue in strain SDF ([302]AKPASTPAVK[311] of B0VKN6_ACIBS), *C*, a novel pentamer glycan within strain Arg1 containing a 258 residue ([302]AKPASTPAVK[311] of A3M265_ACIBT), *D*, a novel 4-mer glycan within strain Arg2 ([110]RPQPLVNAPAPVASQAK[126] of J5IPS4_ACIBA) containing a similar 316 Da residue to SDF, and *E*, the novel trisaccharide identified in strains 1441 C1 and C3 ([23]KEEATQAGQDAASTAVADK[41] of A7FB63_ACIBT) containing the 316 Da residue of SDF and Arg 1.

TABLE II

Glycans Identified within Acinetobacter species. The 15 strains investigated within this study are denoted with the total number of glycans identified, number of shared glycans and the number of unique glycan also indicated for each strain. Annotated examples of each unique glycoform are provided within Fig. 2 to 5, 7 to 9 and supplementary Figs. Major glycans (define by the most observed glycan species) are denoted with*

Strain name	Glycans observed	Shared Glycans	Unique Glycans	Glycan 1	Glycan 2	Glycan 3	Glycan 4	Glycan 5	Glycan 6	Glycan 7	Glycan 8	Glycan 9	Glycan 10
A. baumannii ATCC 17978	8	6	2	HexNAc-Hex2-HexNAc-300*	HexNAc-Hex2-HexNAc-258	HexNAc-Hex2-258	HexNAc-Hex2-HexNAc-314	386-Hex2-HexNAc-300	HexNAc-Hex2-300	Double glycan; HexNAc-Hex2-HexNAc-300 and HexNAc-Hex2-300	HexNAc-Hex-HexNAc2-300		
A. baumannii ATCC 19606	7	6	1	HexNAc-Hex2-HexNAc-300*	HexNAc-Hex2-HexNAc-258	HexNAc-Hex2-258	HexNAc-Hex2-HexNAc-314	Double glycan; HexNAc-Hex2-HexNAc-300 and HexNAc-Hex2-300	Double glycan; 2*(HexNAc-Hex2-HexNAc-300)	HexNAc-Hex2-300			
A. baumannii SDF	3	0	3	HexNAc2-Hex-316*	HexNAc2	HexNAc2-Hex-358							
A. baumannii Arg1	9	2	7	HexNAc-Hex2-HexNAc-258*	HexNAc-Hex2-258	244-Hex2-HexNAc-258	HexNAc-Hex-HexNAc2-258	Double glycan; HexNAc-Hex2-HexNAc-258	HexNAc-154-Hex2-HexNAc-258	HexNAc-Hex	HexNAc-Hex2-HexNAc	Double glycan; HexNAc-Hex2-HexNAc-258-HexNAc-Hex2-258	
A. baumannii Arg2	8	1	7	HexNAc-Hex2-316*	HexNAc2-Hex2-316	Double glycan; HexNAc-Hex2-316	HexNAc	HexNAc-Hex-316	HexNAc-Hex-HexNAc-316	HexNAc-Hex2	HexNAc2-316		
A. baumannii 1441 C1	10	6	4	HexNAc-187-316*	Double glycan; HexNAc-187-316	Triple glycan; HexNAc-187-316	HexNAc-187	HexNAc-187-316-HexNAc-187-331	HexNAc	HexNAc-187-316-HexNAc-187	HexNAc-145-316	HexNAc-187-331	HexNAc-172-316
A. baumannii 1441 C3	5	5	0	HexNAc-187-316*	Double glycan; HexNAc-187-316	Triple glycan; HexNAc-187-316	HexNAc-187	HexNAc-187-316-HexNAc-187					
A. baumannii AYE	0	0	0										
A. calcoaceticus 1217	1		1	HexNAc-176-HexNAc2Hex*									
A. calcoaceticus 1218	1	0	1	HexNAc-HexNAcA-HexNAc2*									
A. pittii 1219	1		1	HexNAc-176-HexNAc2-Hex*									
A. nosocomialis 1222	1	0	1	Triple glycan; HexNAc-Hex2*									
A. baumannii 1224	1	0	1	HexNAc-Hex-HexNAc2-158-Hex*									
A. baumannii 1225	2	0	2	272-258-HexNAc-258*	diNAcBac-258-HexNAc-258								
A. baylyi ADP1	2	0	2	285-HexNAcA-HexNAcOAc2-HexNAc*	285-HexNAcA-HexNAcOAc-HexNAc2								

To further understand the *O*-linked glycosylation diversity and glycan characteristics within *A. baumanii* we analyzed four recent MDR clinical isolates (Arg1, Arg2, 1441 C1, and 1441 C3). As with SDF, the analysis of these strains revealed a diverse array of glycans with the major glycopeptides identified containing the glycans HexNAc-Hex$_2$-HexNAc-258 (mass 988.34 Da), HexNAc-Hex$_2$-NulO (mass 843.31 Da), and HexNAc-dHexNAc-NulO (mass 706.30 Da) within Arg1, Arg2, and 1441 C1 and 1441 C3 respectively (Fig. 1*C*–1*E* supplemental Tables S4–S7, and Table II). These glycopeptides corresponded to both previously identified O-glycosylation substrates and a multitude of novel protein acceptors (supplemental Tables S2 and S4–S7). Inspection of the mass and isotopic distribution of oxonium ions generated by these moieties (corresponding to the 258 and 316 Da residues) supported the assignments of these residues as a deaceylated form of GlcNAc3NAcA4OAc, a carbohydrate residue previously identified within the glycan of *Acinetobacter lwoffii* F78 (46), and legionaminic/pseudaminic acid (supplemental Fig. S5*A*, S5*B*). From these results, we confirm that diverse glycans are utilized in a range of strains, and that similar substrates are targeted for glycosylation across *A. baumannii* strains.

A. baumannii Strains Display Glycan Micro-heterogeneity Producing Multiple Related Glycan Structures—In addition to the major glycoform described above, at least three alternative glycoforms were unexpectedly identified within each *A. baumannii* strain, including SDF, Arg1, Arg2, 1441 C1, and 1441 C3 (Fig. 2*A*-*J*, Table II, supplemental Tables S3–S7). Glycan diversity appeared to be largely the result of chemical exchange and/or addition of functional groups such as acetyl and methyl groups to the bacterial specific residue observed within each strain, although examples of truncated glycan were also observed (Fig. 2*A*–2*C*). Inspection of the mass and isotopic distribution of oxonium ions generated by the modified bacterial specific residues (corresponding to the 331 and 358 Da residues) supported the assignments of these residues as methylated or acetylated forms of the nonulosonic sugar found in the major glycoform (supplemental Fig. S6). These modified forms although common and found on multiple peptide substrates appear less abundant, based on frequency of identification and ion intensity relative to the major glycoform within each strain (data not shown).

As glycan heterogeneity was not considered during the initial analysis *A. baumannii* ATCC 17978 and ATCC 19606, re-inspection of ZIC-HILIC enrichment datasets were carried out leading to the confirmation of glycan heterogeneity within both strains (Fig. 2*D*, 2*E*; supplemental Tables S1, S2, S8, and Table II). The dominant glycan structure produced by ATCC 17978 and ATCC 19606 is HexNAc-Hex$_2$-HexNAc-GlcNAc3NAcA4OAc, however, alternative glycans composed of HexNAc-Hex$_2$-HexNAc-258 and HexNAc-Hex$_2$-HexNAc-314 were also identified (Fig. 2*D*, 2*E*) in both strains (supplemental Table S1 and S8). Examination of the low mass region

of the HCD spectrum within these alterative glycans revealed the presence of two novel ions corresponding to 259 and 315 *m/z* (consistent with MH+ of the 258.09 and 314.12 Da residues respectively, supplemental Fig. S7). The observed masses and isotopic distributions of these oxonium ions were consistent with a methylated form and a deacetylated form of GlcNAc3NAcA4OAc, of 315.11733 Da and 259.09112 Da, respectively (supplemental Fig. S7). Within ATCC 17978 we also noted the existence of two unique glycoforms that differed in the internal carbohydrate of the glycan, one corresponding to the exchange of a HexNAc for a Hex residue (Fig. 2*F*) and the other to the exchange of the linking carbohydrate for a moiety of 386 Da (Fig. 2*G*). The analysis of the low *m/z* region of the HCD spectra confirmed the presence of a 387.11937 Da MH+ ion and provided insight into the identity of this moiety. By comparing spectra generated from modified and non-modified glycans (supplemental Fig. S8*A,* S8*B*) a putative characterization was possible suggesting this moiety corresponds to a composition of $C_{13}H_{24}N_1O_{10}S_1$ that is consistent with the mass and isotopic pattern of this residue. (supplemental Fig. S8*C*). A molecule with this formula has not been previously reported within a bacterial glycopeptide. This predicted composition does not match any known carbohydrate and appears to represent a minor *O*-linked glycoform of ATCC 17978.

It should be noted that in addition to heterogeneity because of the exchange and/or addition of functional groups within the glycan, polymerized forms of the O-linked glycan, glycan oligomers, were also observed within all strains (Table II). These glycan oligomers were present on numerous peptides (supplemental Tables S1, S3–S7, Table II, and supplemental Figs. S9–S23) with dimeric glycans (Fig. 2*I*) identified in all strains. In addition to glycan dimers, trimers (Fig. 2*J*) were also readily detectible within strains 1441 C1 and 1441 C3, which produces the smallest *A. baumannii* O-glycan characterized to date, a linear trisaccharide of 706.39 Da.

Acinetobacter baumannii O-glycosylates Multiple Conserved Protein Substrates via Serine Residues in Low-complexity Regions—Examination of multiple *A. baumannii* strains expanded the repertoire of known glycosylation substrates adding 19 novel glycoproteins to the seven previously identified substrates (supplemental Table S2). Interestingly few glycoproteins identified were unique to a single strain; for example within the laboratory strains examined glycopeptides corresponding to the same eight glycoproteins were observed in SDF, ATCC 17978, and ATCC 19606 (Fig. 3*A*), whereas seven were unique to a single strain. This trend was seen across all *A. baumannii* strains examined where ~70% of all glycoproteins were identified within at least two strains (Fig. 3*B*). These observations in combination with genetic analyses of multiple genome-sequenced strains of *A. baumannii* confirm that the identified glycoproteins are both conserved and targeted for glycosylation across divergent strains (supplemental Table S9). An example of this is the conserved, puta-
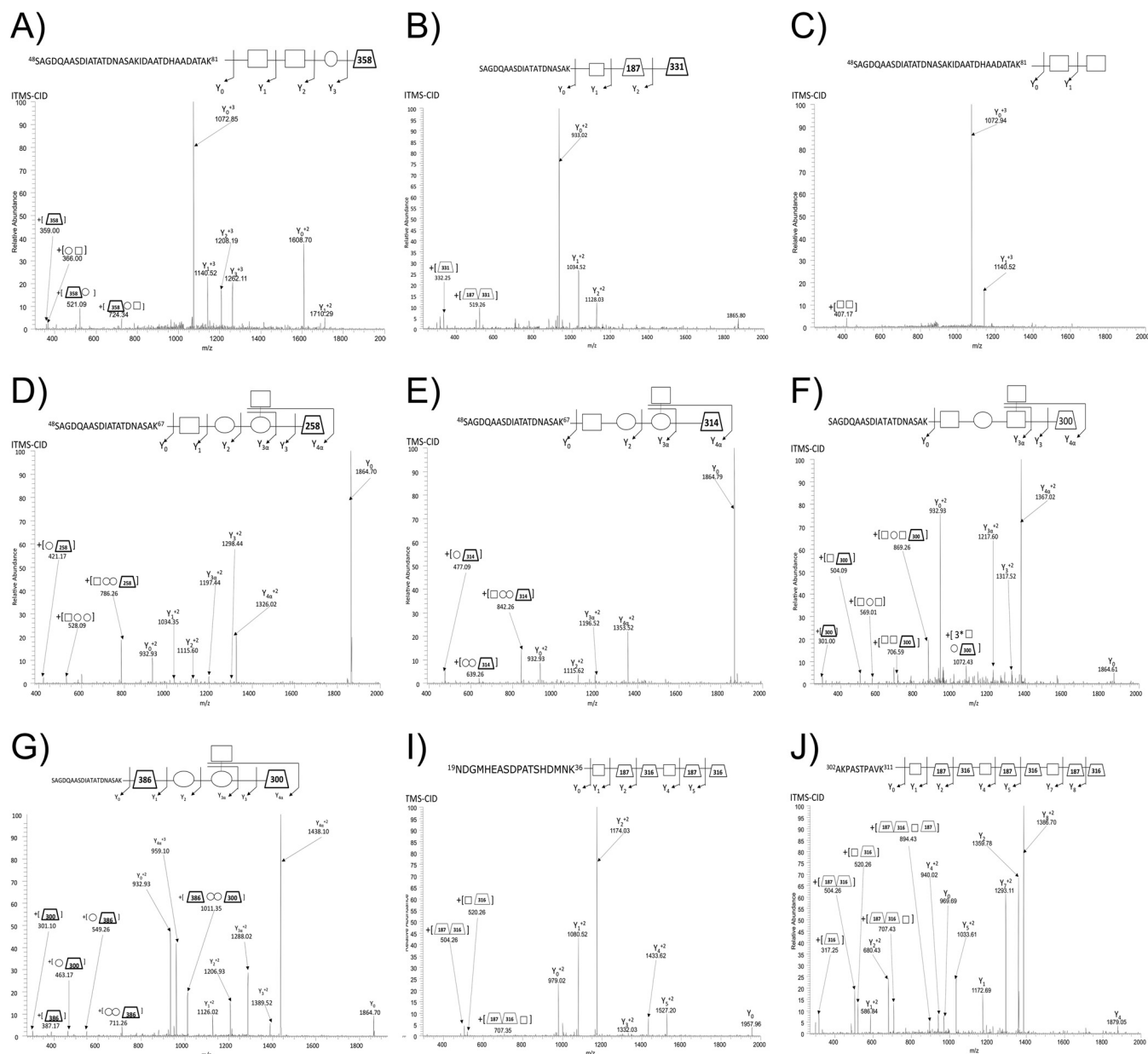
FIG. 2. **Alterative *O*-glycan structure identified using ZIC-HILIC enrichment with *A. baumannii* strains.** ITMS-CID fragmentation enabled the identification of *A. baumannii* glycopeptides containing alterative glycoforms within all strains examined including: *A*, A tetramer glycan composed of HexNAc$_2$-Hex-NulOAc within *A. baumannii* SDF ([48]SAGDQAASDIATATDNASAKIDAATDHAADATAK[81] of B0VLI0_ACIBS), *B*, A trisaccharide composed of HexNAc-dHexNAc-NulOCH3 within *A. baumannii* 1441 C1 ([48]SAGDQAASDIATATDNASAK[67] of A7FB63_ACIBT), *C*, A disaccharide composed of HexNAc$_2$ within *A. baumannii* SDF ([48]SAGDQAASDIATATDNASAK[67] of B0VLI0_ACIBS), *D*, A pentamer glycan within *A. baumannii* 19606 composed of HexNAc-Hex$_2$-HexNAc-GlcNAc3NAcA ([48]SAGDQAASDIATATDNASAK[67] of D0CEI7_ACIBA), *E*, A pentamer glycan within *A. baumannii* 19606 and 17978 composed of HexNAc-Hex$_2$-HexNAc-GlcNAc3NAcA4OAcCH3 ([48]SAGDQAASDIATATDNASAK[67] of A7FB63_ACIBT), *F*, The known pentamer glycan of *A. baumannii* ATCC 17978 where a Hex has been exchanged for a HexNAc residue compared with the previously reported glycan (14) ([48]SAGDQAASDIATATDNASAK[67] of A7FB63_ACIBT), *G*, The known pentamer glycan of *A. baumannii* ATCC 17978 where the linking HexNAc residue has been exchanged for the unique 386.11 Da residues compared with the previously reported glycan (14) ([48]SAGDQAASDIATATDNASAK[67] of A7FB63_ACIBT), *I*, A dimer of the trisaccharide *O*-glycan of *A. baumannii* 1441 C1 ([19]NDGMHEASDPATSHDMNK[36] of A7FB95_ACIBT), and *J*, A trimer of the trisaccharide *O*-glycan of *A. baumannii* 1441 C1 ([302]AKPASTPAVK[311] of A3M265_ACIBT).

tive uncharacterized protein D0C6C0_ACIBA (Fig. 3*C*), which was identified in five out of the seven strains examined (supplemental Table S2). Interestingly, it was also noted that the observed glycoproteins are unique to *Acinetobacter*, with no homologs outside of this genus, and are highly conserved with >97% identity between strains (supplemental Table S9).
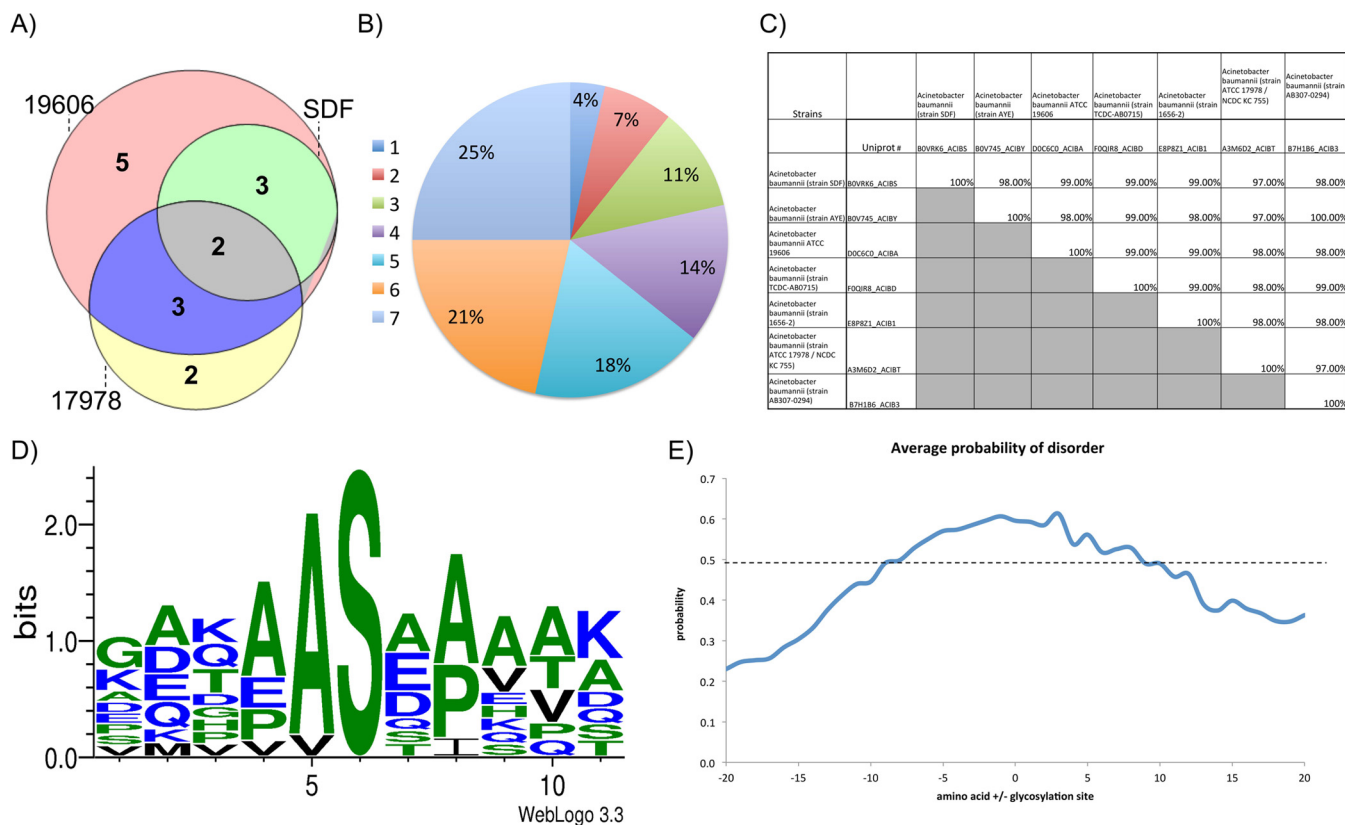
A)

B)

C)

Strains	Uniprot #	Acinetobacter baumannii (strain SDF) B0VRK6_ACIB5	Acinetobacter baumannii (strain AYE) B0V745_ACIBY	Acinetobacter baumannii ATCC 19606 D0C6C0_ACIBA	Acinetobacter baumannii (strain TCDC-AB0715) F0QIR8_ACIBD	Acinetobacter baumannii (strain 1656-2) E8P8Z1_ACIB1	Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755) A3M6D2_ACIBT	Acinetobacter baumannii (strain AB307-0294) B7H1B6_ACIB3
Acinetobacter baumannii (strain SDF)	B0VRK6_ACIB5	100%	98.00%	99.00%	99.00%	99.00%	97.00%	98.00%
Acinetobacter baumannii (strain AYE)	B0V745_ACIBY		100%	98.00%	99.00%	98.00%	97.00%	100.00%
Acinetobacter baumannii ATCC 19606	D0C6C0_ACIBA			100%	99.00%	99.00%	98.00%	98.00%
Acinetobacter baumannii (strain TCDC-AB0715)	F0QIR8_ACIBD				100%	99.00%	98.00%	99.00%
Acinetobacter baumannii (strain 1656-2)	E8P8Z1_ACIB1					100%	98.00%	98.00%
Acinetobacter baumannii (strain ATCC 17978 / NCDC KC 755)	A3M6D2_ACIBT						100%	97.00%
Acinetobacter baumannii (strain AB307-0294)	B7H1B6_ACIB3							100%

D)

E)

FIG. 3. *A. baumannii* prominently glycosylates Serine residues with Alanine residues in the −1 position. Analysis of glycoproteins and glycosylation sites identified of *A. baumannii* strains. *A*, Comparison of glycoproteins identified within each strain. Showing the high level of overlap between strains. *B*, Analysis of the overlap between strains glycoproteins identified in all seven *A. baumannii* strains. *C*, Comparison of the sequence identity of proteins between strains of *A. baumannii* demonstrating the high level of sequence identity between strains of the identified glycoproteins. *D*, Motif analysis of identified glycosylation sites showing a strong preference for the sequence AS. *E*, Comparison of the region of disorder around the identified sites of glycosylation, a disordered prediction >0.5 is consisted to be disordered according to PreDisorder, http://casp.rnet.missouri.edu/predisorder.html.

No homologs were found in any other bacterium and therefore their function cannot be assigned based on homology.

To accurately assess the local environment of glycosylation, based on the site of attachment, the sites of modification were characterized using ETD fragmentation (supplemental Table S10). Using this approach a total of seven sites of glycosylation could be localized, which in conjunction with four additional sites localized by the presence of only one hydroxyl containing amino acid in the sequence, lead to the localization of eleven glycosylation sites across the examined *A. baumannii* strains (supplemental Table 10). Within this dataset it was noted that only Serine appeared to be glycosylated within the identified glycopeptides, and that the sites of glycosylation seem to have a strong preference for alanine in the −1 position (Fig. 3*D*, supplemental Table S10). Because previous reports have suggested that glycosylation occurs at disordered regions in other bacterial *O*-linked glycosylation systems rather than at a specific sequon (47–49), we also examined the region surrounding the identified glycosylation sites. Similar to other bacterial *O*-linked systems, sites of *A. baumannii O*-linked glycosylation occur in low complexity regions, rich in proline, alanine, and serine (Fig. 3*E*), suggesting that the recognition of substrates by *O*-oligosaccharyl-transferases is conserved in different bacteria.

Targeted Analysis of the Glycan Diversity in Acinetobacter Clinical Isolates—Although the results of the ZIC-HILIC enrichment confirmed our hypothesis of *O*-linked diversity within multiple strains, it also highlighted potential variability in the performance of ZIC-HILIC enrichment. As these methodologies rely on the ability to detect glycosylation based on the presence of diagnostic carbohydrate reporter ions, we reasoned that if strains possessed a glycan lacking known carbohydrate moieties or were ineffectively enriched with ZIC-HILIC, detection and determination of glycosylation would be compromised. In order to refine the analysis of glycan diversity and expand the number of strains analyzed, we developed a targeted approach to assess glycosylation. Based on the observation that the same proteins are glycosylated at sites with similar structural characteristics in multiple strains (Fig. 3*A*, 3*D*, and 3*E*), we reasoned that a His-tagged version of one of these conserved proteins could be expressed in other *Acinetobacter* strains and used as bait in order to isolate
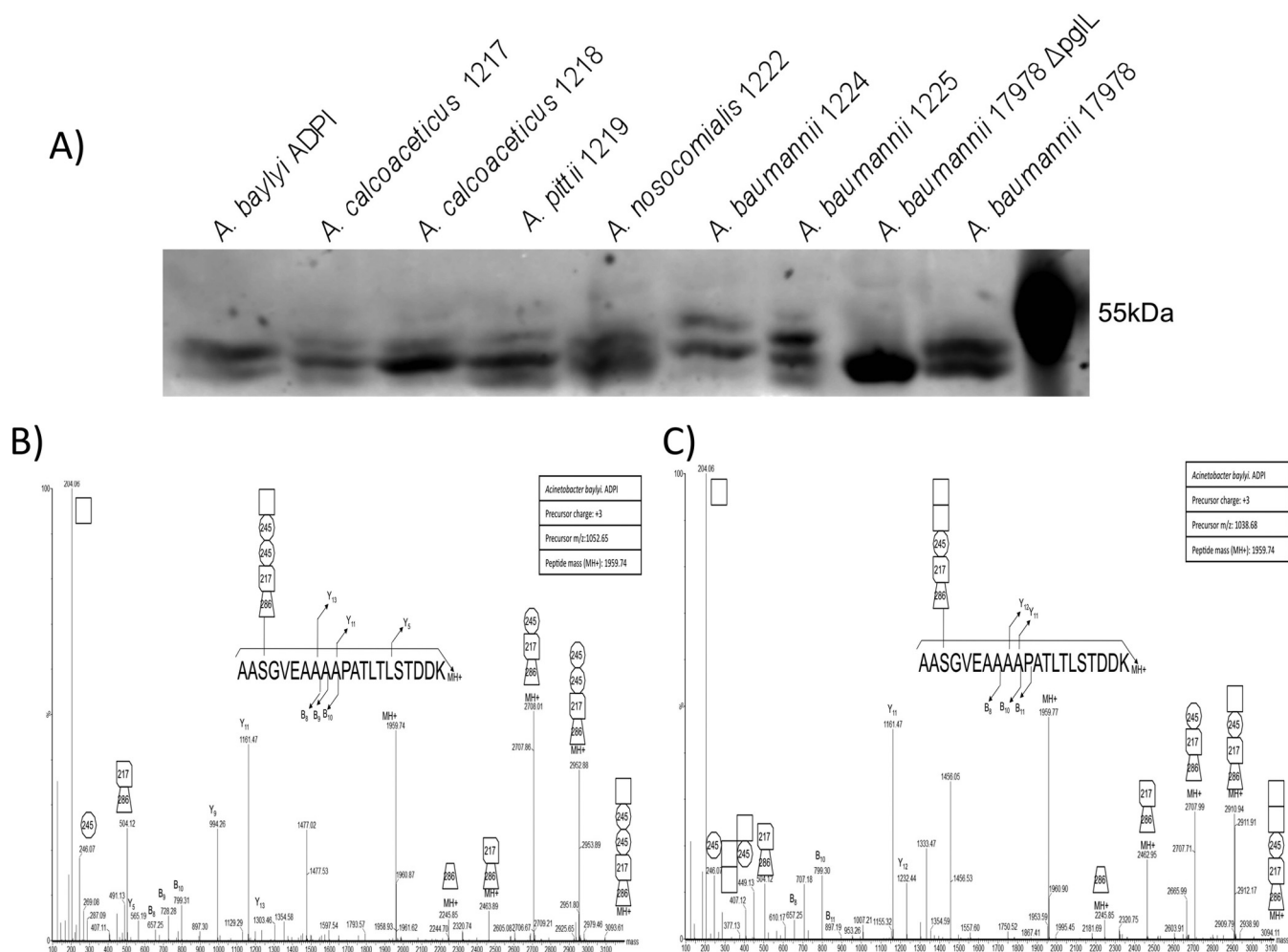
FIG. 4. **Western blot analysis used to resolve the mass difference between glycosylated and unglycosylated A1S_1193.** *A*, Anti-Histidine Western blot analysis of *Acinetobacter* strains recombinantly expressing *Acinetobacter* glycoprotein A1S_1193 with a C-terminal Histidine tag. The slight increase in molecular weight indicates the protein has been post-translationally modified. *B–J*, ESI-QTOF MS/MS Analysis of the fished *A. baumannii* glycoprotein A1S_1193 to elucidate the glycan structure. ESI-QTOF-MS and MS/MS was carried out on purified A1S_1193, expressed in various *Acinetobacter* strains, to characterize the posttranslational modification. *B–C*, ESI-QTOF-MS/MS analysis of tryptic peptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] expressed in *A. baylyi* ADP1 revealed either the pentasaccharide 285-217–245$_2$-HexNAc or 285-217–245-HexNAc$_2$ attached to the glycopeptide. *D*, MS/MS fragmentation of [203]AA**S**GVEAAAAPATLTLSTDDK[223] expressed in *A. calcoaceticus* 1217 displays modification with the pentasaccharide HexNAc-176-HexNAc$_2$-Hex. *E*, *A. calcoaceticus* 1218 glycosylates the tryptic peptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] with the tetrasaccharide HexNAc-217-HexNAc$_2$. *F*, Fragmentation of tryptic peptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] from A1S_1193 expressed in *A. pittii* 1219 reveals glycosylation with a pentasaccharide identical to 1217, HexNAc-176-HexNAc$_2$-Hex. *G*, *A. nosocomialis* 1222 modifies glycopeptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] with the trisaccharide repeat unit HexNAc-Hex$_2$. *H*, *A. baumannii* 1224 uses the hexasaccharide HexNAc-Hex-HexNAc$_2$-158-Hex to glycosylate glycopeptide [203]AA**S**GVEAAAAPATLTLSTDDK[223]. *I–J*, *A. baumannii* 1225 modifies glycopeptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] with one of two tetrasaccharides, 272–258-HexNAc-258 or 228–258-HexNAc-258.

attached glycans. To achieve this goal we expressed the *A. baumannii* glycoprotein A1S_1193 in clinically relevant strains. Previous work from our lab showing the site of glycosylation within A1S_1193 is Serine-205 (28), contained within the tryptic peptide [203]AA**S**GVEAAAAPATLTLSTDDK[223] facilitated characterization of the corresponding glycans (14). Expression of A1S_1193 leads to the decoration of the protein substrate with native glycosylation, enabling the isolation of the protein independent of the chemical properties of the sugars. Importantly, this approach does not require prior

knowledge of the genomic sequence or glycan structure, enabling the assessment of glycosylation in unsequenced clinical strains.

To investigate glycan diversity in the clinical setting, six clinical *Acinetobacter* isolates were selected for analysis using this targeted approach. The expression of A1S_1193 within these *Acinetobacter* clinical isolates lead to a detectible mass shift compared with the non-glycosylated control (Fig. 4*A*) suggesting glycosylation of the protein substrates. MS analysis confirmed the addition of glycan structures to
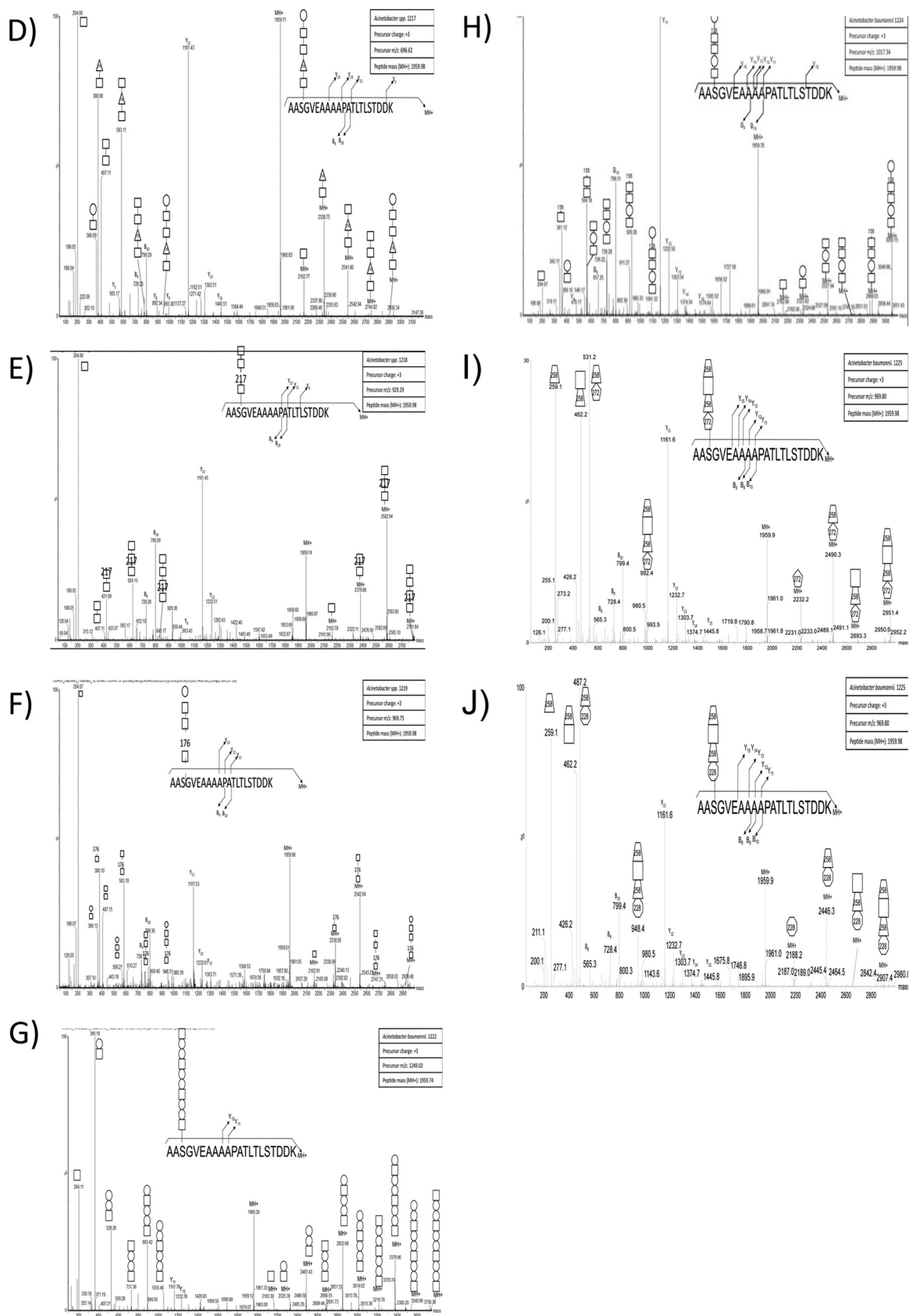
F‌IG. 4—*continued*

A1S_1193 with a total of six novel glycan structures observed across the six strains (Fig. 4*D*–4*J*, Table II). From this analysis it was noted that all glycans were composed of four to six carbohydrates and were largely linear in nature with few branched sugars observed. As with SDF, ATCC 19606, and ATCC 17978, glycan heterogeneity was also observed within clinical isolates; for example within *A. baumannii* 1225 two glycans composed of 272–258-HexNAc-258 and 228–258-HexNAc-258 were identified (Fig. 5*F*, 5*G*). As with previously identified heterogeneity the alteration in *A. baumannii* 1225 occurred on the bacterial specific residues corresponding to the mass of diNAcBac (228 Da; bacillosamine). Interestingly the glycans of *A. baumannii* 1225 appear similar to the previously characterized glycans of *A. lwoffii* F78 and *A. baumannii* AB307–0294 where NMR was utilized to confirm the modification of the capsule with the deacetylated form of GlcNAc3NAcA4OAc (258 Da) or diNAcBac (228 Da) and BacNAc modified with 3-OH-butyrate (272 Da) respectively (46, 50). Furthermore sequencing of *A. baumannii* 1225 isolate has confirmed the presence of the genes necessary for GlcNAc3NAcA4OAc and diNAcBac biosynthesis supporting the assignment of these bacterial specific carbohydrates (supplemental Fig. S1*C*).

Not all O-glycan Structures within Acinetobacter Match the Predicted Carbohydrate Synthesis Locus: The Investigation of A. baylyi ADP1 Glycosylation—With the development of a targeted approach to investigate *Acinetobacter* O-linked glycosylation we also assessed its potential to characterize the *O*-linked glycan of the non-pathogenic model strain *A. baylyi* ADP1. This bacterium is frequently employed as a model *Acinetobacter* strain because of its amenability to genetic manipulation (51). Interestingly *A. baylyi* ADP1 was recently demonstrated to contain a functional glycosylation system (26), although the exact structure has not been elucidated. Using our targeted approach *A. baylyi* ADP1 was found to glycosylate A1S_1193 with a pentasaccharide composed of 285–217-245$_2$-HexNAc or 285–217-245-HexNAc$_2$, which is distinct from the capsule subunit of its parent strain *A. baylyi* BD4 (Fig. *4B*, 4*C*, Table II). The residues of this glycan included two atypical sugars of mass 217 and 245 Da matching 2-acetamido-2-deoxy-d-hexuronic acid and O-acetyl-N-acetylhexosamine moieties respectively, which have also been recently identified within multiple *Campylobacter species* (31). Furthermore within *A. baumannii* AB307–0294 a form of hexuronic acid, galactosaminuronic acid, has been previously noted (50). This observation demonstrates the biosynthetic pathways required to generate the underivatized precursor of the 217 Da do exist in *Acinetobacter*. The identification of a shortened glycan composed of chemically unusual moieties demonstrated the importance of experimental analysis of glycan structure, rather than bioinformatic prediction and the convenience of our targeted based approach to rapidly identify glycosylation in the *Acinetobacter* genus.

DISCUSSION

Protein *O*-glycosylation is a common process in bacterial species, and is required for virulence and biofilm formation in *A. baumannii* ATCC 17978. However, the prevalence, diversity, and specific role of the *O*-glycan modification of *A. baumannii* has yet to be determined (14). Lees-Miller et al. showed that in the strain ATCC 17978 the building blocks employed for capsular polysaccharide and the *O*-linked glycan are identical and employ the same enzymatic machinery for their synthesis, which is encoded in a single glycan locus (13). Genetic comparison of the capsular loci revealed extensive variability in the predicted capsule structure (11). In this work, we analyzed the glycan structures attached to proteins in the strains most commonly used for molecular studies as well as ten clinical isolates and confirmed the presence of extensive *O*-linked glycan diversity. From this analysis a diverse array of glycans were observed across all 15 strains examined with a total of 11 unique main glycoforms identified (Tables II). These observations support the predictions of Hu et al. and the presence of extensive glycan diversity in *A. baumannii*. In addition to the prominent structures, alternative *O*-glycans were also identified demonstrating *A. baumannii* is capable of producing multiple glycoforms within a given strain. We observed that the majority of strains produced an array of unique glycans only found within that strain; exceptions to this are the reference strain ATCC 19606 that produced the identical *O*-linked pentasaccharide containing GlcNAc3NAcA4OAc of ATCC 17978 (Fig. 1*A*, Table II) and the closely related isolates 1441 C1 and 1441 C3 (Fig. 1*E*, Table II). Interestingly, the pentasacccharide of ATCC 17978 and ATCC 19606 has also been identified as the capsule-repeat unit of *A. baumannii* strain SMAL (52). Although the frequency of the observed GlcNAc3NAcA4OAc containing structure would suggest the commonality of this sugar in *A. baumannii*, both the recent bioinformatics analyses (11, 12) and this work suggest that the pentasacccharide of ATCC 17978 (13, 14), ATCC 19606, and SMAL (52) is just one of the multitude of glycans utilized by *A. baumannii*.

Although the majority of glycans were unique to specific strains, multiple glycans contained common bacterial specific sugars such as in the case of the reference strain SDF and clinical isolates Arg2, 1441 C1, and 1441 C3. Within these strains, multiple glycopeptides were identified decorated with residues matching the mass and fragmentation pattern of the negatively charged sugar, legionaminic or its stereoisomer pseudaminic acid (Fig. 1*B*, 1*D*, and 1*E*) (45). Bioinformatic analysis of the carbohydrate clusters of SDF as well as the unpublished clinical isolates *A. baumannii* 1441 C1 and C3 (Weber et al. unpublished data) supports the presence of the legionaminic acid biosynthesis pathway within these strains (supplemental Fig. S1*B* and S4*C*). This finding demonstrates *A. baumannii* is one of the many bacterial species now recognized to utilize legionaminic/pseudaminic acid or their de-

Glycan Mass	Proposed Glycan Identity
300	GlcNAc3NAcA4OAc
258	GlcNAc3NAcA
314	GlcNAc3NAcA4OAcCH$_3$
228	QuiNAc4NAc / diNAcBac
272	QuiNAc4NBu / BacNAc4NBu
316	NulO
331	NulOCH$_3$
358	NulOAc
176	HexA
187	dHexNAc
217	HexNAcA
245	HexNAcOAc
386	C$_{13}$H$_{24}$N$_1$O$_{10}$S$_1$
285, 158, 145 and 172	Unknown moieties[+]

rivatives within protein attached glycans (45, 53, 54). Within the best characterized of these systems, the *O*-linked glycosylation system of *C. jejuni*, these sialic acid analogs are required for ideal protein function where they are essential for autoagglutination, modulation of the hydrophobicity of the flagellin and dampening of the inflammation response by SigLec-10 binding (55–57). Interestingly, the presence of negatively charged sugars appears to be a common feature for most if not all the *A. baumannii* structures identified (Table II). From this trend it is tempting to speculate that these negative sugars may be advantages to *A. baumannii* biology and themselves may be important for virulence as seen for other bacterial species where negative surface carbohydrates can provide resistance to complement killing (58).

In addition to legionaminic acid, other carbohydrates such as the HexNAcA, HexNAcOAc, and diNAcBac residues, whose identity is assigned based on mass comparison to characterized glycans of bacterial glycosylation systems (31), were observed across multiple strains within this study (Table III). Residues with identical masses have been identified as

components of glycans utilized within other bacterial protein glycosylation systems (47, 59–61) suggesting *A. baumannii* utilizes a similar carbohydrate repertoire as other Gram-negative protein glycosylation systems. Although the identities of these residues cannot be confirmed from the mass alone the convergence of similar residues, irrespective of their stereochemistry, is an observation of significant note. A similar concept of the utilization of a conserved carbohydrate repertoire has been noted within *Neisseria* and *Campylobacter* species where both systems utilize diBacNAc yet the enzymes responsible for the generation of diBacNAc represent two phylogenetically distinct clades (62–64). Widespread use of these unique carbohydrates by multiple bacterial glycosylation systems suggests preference for these sugars in protein modification, although the exact advantages of these residues are unknown. If specific carbohydrates are advantageous for virulence or glycosylation functionality this preference may be exploited by potential anti-microbial therapies and the generation of serological reagents, which may aid in the diagnosis and treatment of *A. baumannii* infections and is currently under investigation within our laboratory (65, 66).

With the exception of AYE, at least one glycan structure could be identified within each of the examined strains, with the presence of multiple alternative glycan forms being a common feature of *A. baumannii* strains. Although bacterial glycan heterogeneity is poorly understood it has been suggested that it could contribute to immune evasion (37) Similar to previously observed heterogeneity in the capsule structure of *Acinetobacter* strains (46, 52) glycan diversity appeared to be largely the result of chemical exchange and/or addition of functional groups such as acetyl and methyl groups to the bacterial specific residue observed within each strain, although examples of truncated glycan were also observed (Fig. 2*A*–2*C*). These alterations were not uniformly distributed on all carbohydrate residues but appeared to favor the alteration of bacterial specific carbohydrates related to diNAcBac, GlcNAc3NAcA4OAc, and legionaminic/pseudaminic acid. Within these sugars multiple alterations were associated with augmentation of the levels of acetylation (Table II). This observation is of key interest because of the association of acetylation levels in other capsule systems and resistance to complement mediated killing (58, 67, 68). As the capsule is essential in ATCC 17978 for complement resistance (13) variability in capsule structure may alter the levels of resistance, which may influence virulence. In addition to the exchange and addition of chemical functional groups, variations such as the addition or lack of sugars as well as changes in the order of the sugars (Fig. 2, supplemental Figs. S9–S23) were also observed across strains. These variations suggest that unlike the archetypical glycosylation system of *C. jejuni*, which only utilizes complete correctly formed glycans, *A. baumannii*, is more promiscuous with glycans utilized for protein modification. This promiscuous nature would be in line with other *O*-linked glycosylation systems such as that of *Neisseria*,

which change the *O*-linked glycans because of phase variation of glycosyltransferases involved in the assembly of the glycan to aid in immune evasion (69).

In addition to the diversity resulting from the exchange/alteration of carbohydrates within glycans we also observed heterogeneity in the form of glycan oligomerization (Fig. 2*I* and 2*J*, Table II). This finding is in agreement with the previous work of Lees-Miller et al. (13) showing that polymerized capsule subunits can be attached to protein substrates in ATCC 17978, and support the notion that the shared glycan biosynthesis pathway for capsule and protein glycosylation is a general feature of *A. baumannii*. Interestingly the observed diversity within *Acinetobacter* glycans is consistent with the micro-heterogeneity recently noted in the glycosylation systems of other bacteria genera, such as *Campylobacter, Burkholderia,* and *Francisella* (32–39). These observations suggest that the majority of glycosylation systems utilize a range of related glycans with proteins substrates rarely decoration with a homogenous glycan. Although the physiological significance of the glycan micro-heterogeneity is still unknown, the extent of glycan microdiversity seen within *Acinetobacter* is among the highest reported to date (32–39).

Within this study glycosylation was detected in all strains examined except the multiple drug resistant strain *A. baumannii* AYE. As previously noted (11, 12), this strain carries a glycan cluster containing three glycosyltransferases and the genes required for the synthesis of diBacNAc, which has been demonstrated to lead to the generation of UDP-diBacNAc in heterogeneous expression systems (50). Because of the multiple drug resistant nature of AYE targeted glycosylation could not be undertaken leaving only enrichment of glycopeptides by ZIC-HILIC chromatography, which is a variable means to assess glycosylation. We initially reasoned that our inability to identify glycosylation within AYE using ZIC-HILIC enrichment may be the result of technical caveats including; the oligosaccharides may have failed to alter glycopeptides hydrophilicity sufficiently to enable partitioning to the pseudo-water of the ZIC-HILIC stationary phase (70); or the resulting glycan may have lacked diagnostics ions used to identify glycosylation within other strains. Conversely it is also possible that the glycosylation machinery in this strain is regulated, and only present under certain growth conditions or functionally inactive. However, the absence of PAS reactive capsular polysaccharide and glycoproteins in this strain, as well as its sensitivity to serum killing provides additional evidence that this strain does not produce surface carbohydrates under the conditions tested. The sensitivity of AYE to serum killing is in contrast to the resistance exhibited by recent clinical isolates (supplemental Fig. S3), suggesting without selective pressure *A. baumannii* may regulate or lose surface carbohydrate expression. The loss of surface carbohydrates in the laboratory has been noted in numerous bacterial species. For example, common *E. coli* K12 strains do not produce O antigen because of the mutation in a rhamnosyltransferase (71) Another

example is the human pathogen *Burkholderia cenocepacia* strain J2315 that is extremely sensitive to human serum because of an insertion of IS402 within the glycosyl transferase wbxE (72). The requirement of capsular polysaccharide for serum resistance demonstrates the significant biological role for surface carbohydrates in the virulence and infective lifecycle of *A. baumannii* (13) and the importance for experimental elucidation of both glycans and there phenotypic roles.

Although all strains examined supported our hypothesis of diversity in the *O*-linked glycan, the agreement between the carbohydrate locus and the glycan structure was not always consistent. The large 34 ORF carbohydrate locus of *A. baylyi* ADPI encodes the TDP-rhamnose biosynthetic pathway, which is not necessary for production of the pentasaccharides we detected, that did not contain rhamnose. The locus encodes 10 glycosyltransferases, when only five would be needed for the pentasaccharide (supplemental Fig. S1 and Figs. 4*B*–4*C*). The *O*-linked glycan has an unusual sugar composition consisting of monosaccharides that match residues observed in other recently identified *Campylobacter* glycosylation systems as well as the *A. baumannii* capsule glycan of AB307–0294 (31, 50). As the sugars corresponding to the unusual masses are unknown, one possibility is that this strain generates one or multiple residues from a rhamnose based precursor. However, the capsule of the closely related strain *A. baylyi* BD4, which ADP1 is derived from (73), has been shown to contain L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose (43). Therefore, it is tempting to speculate that in contrast to *A. baumannii*, ADP1 produces a capsule that is unrelated to the *O*-glycan. Consistent with this, ADP1 was the only strain that did not show evidence of higher oligomer glycan by Western blotting of the A1S_1193 bait (Fig. 4*A*). Additionally, the ADPI glycan cluster contains two initiating glycosyltransferase homologs suggesting the potential to produce two unique lipid-linked glycans, one for protein modification and one for capsule (supplemental Fig. S1). Additional work is required to further explore these observations to confirm the segregation of the capsule and *O*-glycan, as it will be interesting to know if and how the two pathways are compartmentalized to avoid possible crosstalk between them.

The ability to assess heterogeneity within this work was because of the use of both targeted and non-targeted technologies for bacterial glycosylation analysis. The ZIC-HILIC approach provides a non-targeted means to assess glycan diversity but requires significant experience in glycopeptide analysis to elucidate sugar structures as well as instrumentation capable of performing multiple fragmentation approaches. To overcome these shortcomings we developed a method to simplify glycosylation analysis that could be achieved on routine MS instrumentation. This method was developed based on the observation of conservation within proteins subjected to glycosylation and the structural properties of these proteins within *Acinetobacter* glycosylation. In this approach, a common tagged-glycosylation acceptor pro-

tein is introduced as "bait" into the strain of interest. The glycosylated protein is then purified via affinity chromatography, digested and subjected to MS analysis. This approach provides a scalable and optimizable means to produce glycosylated proteins without prior knowledge of the glycan structure, allowing analysis of un-sequence clinical strains. As the fragmentation pattern of the "bait" is known, the determination of the glycans attached to it is straightforward. This targeted approach was employed here to characterize the sugar composition of seven different *Acinetobacter* strains. We believe that in the future this strategy could be employed to simplify the characterization of other bacterial glycosylation systems. By using both targeted and non-targeted MS approaches we show extensive diversity exists in the *Acinetobacter* glycoproteome; that at least 26 proteins, most of which are unique to *Acinetobacter*, are subjected to *O*-linked glycosylation; and, further demonstrate that the use of a single glycan for both *O*-linked glycosylation and capsule production appears to be a general feature of *A. baumannii*.

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