Unique import of host-derived sugar *N*-acetylglucosamine-1-P by Rickettsiae for biosynthesis of cell envelope glycoconjugates

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***We hypothesize** rickettsiae uniquely import host-derived NAG-1-P for biosynthesis of cell envelope components and that metabolite thievery from the host amino sugar biosynthesis pathway is essential for rickettsial intracellular replication and survival.*

Rickettsial growth is dependent on host NAG-1-P metabolism

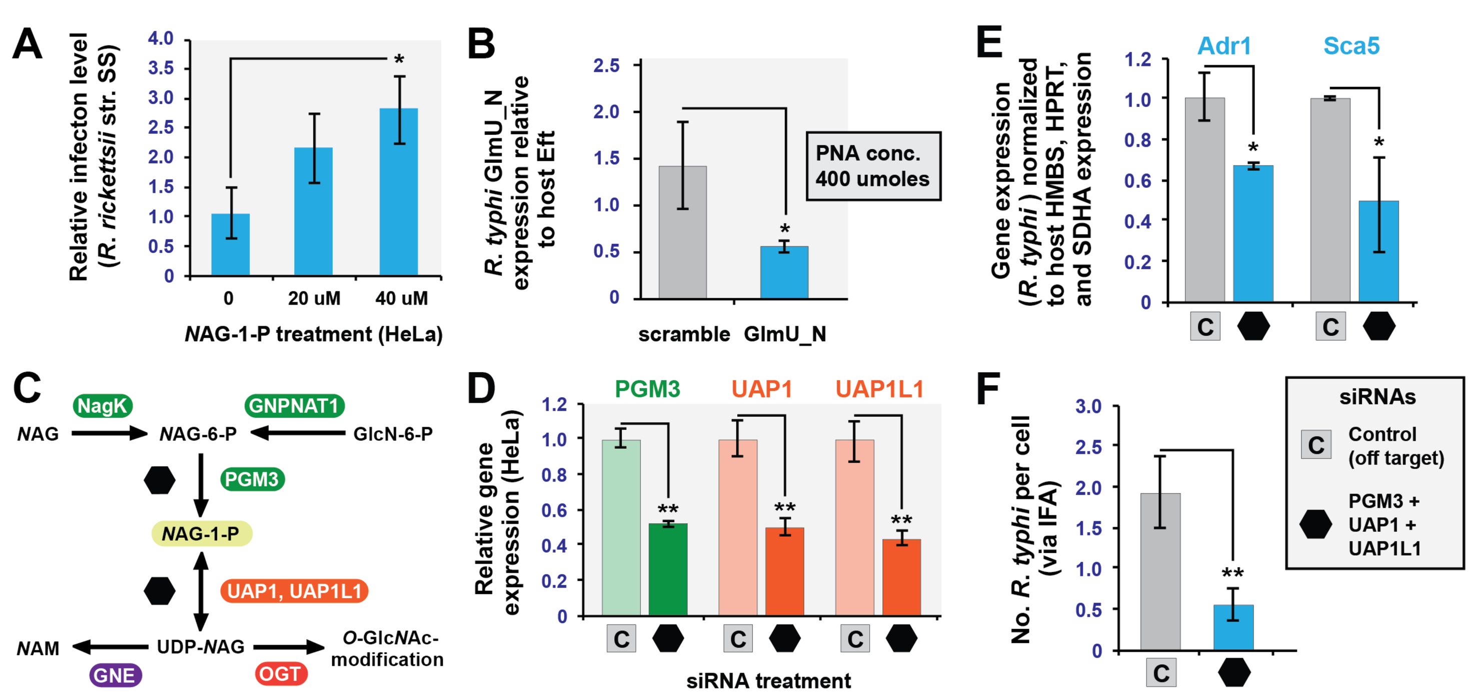

FIG 2. Rickettsiae need host *N*AG-1-P for growth. *(A)* *N*AG-1-P aggression assay. HeLa cells were trypsinized, resuspended in DMEM-10, and transferred to a 24-well plate. *N*AG-1-P was added for overnight incubation at 20°C. Cells were infected with *R. rickettsii* (MOI1) for 8 hr., followed by total RNA extraction. cDNA was synthesized using the iScript kit (Bio-Rad). Gene expression was measured with qPCR, with *Rickettsia* 16S rDNA normalized to host Porphobilinogen deaminase (HMBS), Hypoxanthineguanine phosphoribo-syltransferase (HPRT) and succinate dehydrogenase (SDHA) genes. Error bars represent mean ±SD (* ≤0.05 by Student's two-sided t-test). ***(B)*** PNA-based knockdown of GlmU_N expression in *R. typhi*. Scramble and *glmU_N* PNAs were electroporated into purified rickettsiae prior to Vero cell infection. After 24 hr, bacteria were purified and GlmU_N expression was assessed by Western blot using an anti-GlmU_N antibody. Densitometry was used to compare GlmU_N expression to host Eft. Error bars reflect three biological replicates. ***(C)*** Black hexagons, siRNA targets. ***(D)*** Targeted silencing of *N*AG-1-P metabolism. HeLa cells were transfected with off target or target (PGM3+UAP1+UAP1L1) siRNA for 24 hr. followed by qPCR. The normalized target gene expression (PGM3 or UAP1 or UAP1L1) with respect to GAPDH from three independent assays are plotted. Protein expression was also observed by immunoblotting with Abs against PGM3, UAP1 and UAP1L1 (data not shown). ***(E)*** Rickettsial growth (gauged by Adr1 or Sca5 expression) determined by qPCR. *adr1* or *sca5* expression was normalized to HMBS, HPRT and SDHA for off target or target gene siRNA treatment. Rickettsial growth determined by IFA. HeLa cells transfected with control or target siRNA for 24 hr., followed by *R. typhi* infection (MOI5) for 2 hr. *R. typhi* was detected with anti-*R. typhi* serum and Alexa Fluor 488 secondary antibody. The number of *R. typhi* per cell was counted for 100 individual cells in three independent experiments. All experiments (C-F) were done in triplicate, with error bars representing mean ±SD (**, P < 0.01; *, P < 0.05 by Student's two-sided t-test).

Rickettsia cell envelope glycoconjugate synthesis is powered by host cell metabolites

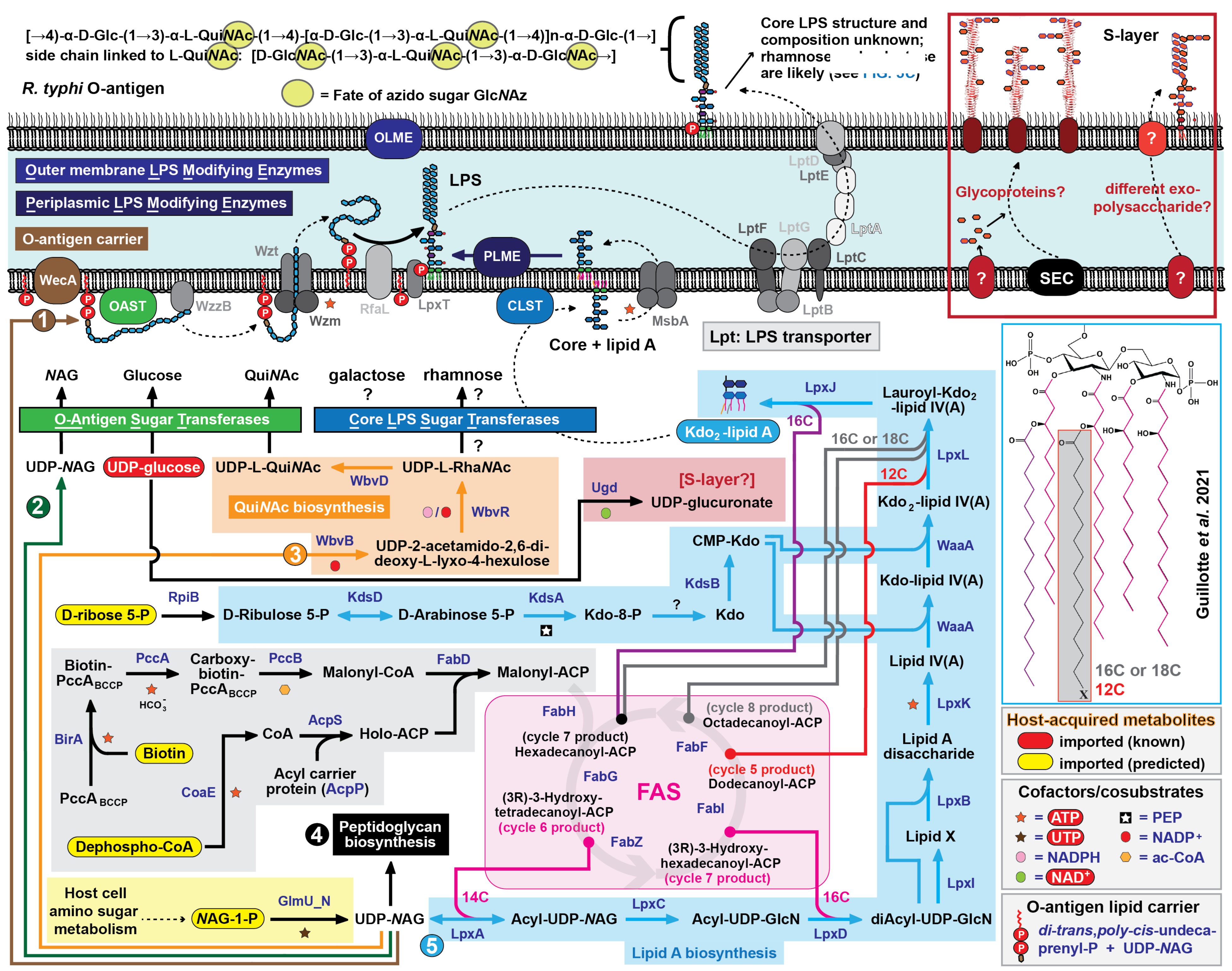

FIG 1. The crucial pilfering of host *N*AG-1-P by rickettsiae (highlighted yellow, **bottom left corner**) is evinced by five separate offshoots of UDP-*N*AG (synthesized by GlmU_N): **1)** synthesis of the O-antigen lipid carrier (**brown**), **2)** direct incorporation into O-antigen (**dark green**), **3)** conversion to Qui*N*ac (**orange**), **4)** initiation of PGN synthesis (**black**), and 5) initiation of lipid A synthesis (**light blue**). **Inset at center-right** shows the results of our recently published work (Guillotte *et al.* 2021; PMID: 33952661), which generated four *Rickettsia* lipid A structures: three (*R. akari*, *R. typhi*, and *R. montanensis*) that have longer (C16/C18) 2' acyl chains (**gray**) and the smaller structure for *R. rickettsii* (strs. Sheila Smith and Iowa) with a shorter (C12) 2' acyl chain (**red**). Question marks indicate uncertain synthesis (UDP-glucose via Ugd) or manner of incorporation (rhamnose and galactose) into LPS. The proposed O-antigen of *R. typhi* is shown at top; highlighting shows the fates of azido sugars incorporated into rickettsial glycoconjugates. **Box at top right** shows predicted incorporation of sugars in S-layer, with glucoronate a main sugar of *Rickettsia* S-layer. Modified from: *Wholly Rickettsia! Reconstructed Metabolic Profile of the Quintessential Bacterial Parasite of Eukaryotic Cells* (PMID: 28951473).

Lipid A structural divergence in Spotted Fever Group pathogens

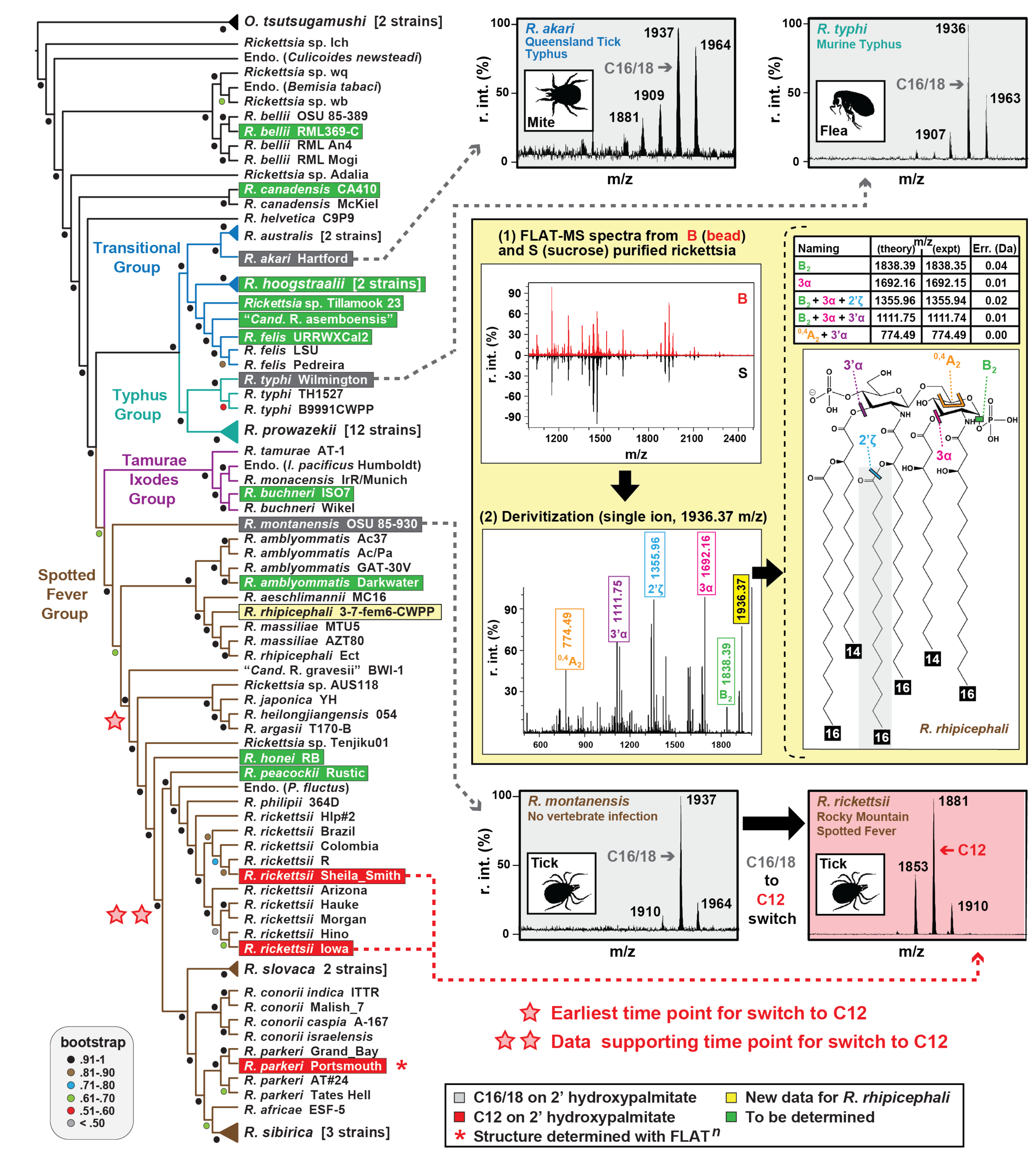

FIG. 6. Five lipid A structures using the "Caroff" lipid A microextraction-purification-chemical derivatization method (Guillotte *et al.* 2021), as well as our unpublished results using FLAT^n to solve the structure of *R. rhipicephali* lipid A. The arthropod hosts for these species are shown, though all structures were obtained from rickettsia-infected Vero76 (African Green Monkey) cells. The yellow enclosed area depicts the FLAT^n procedure used to generate lipid A structure with minimal input sample. **(1)** FLAT-MS spectra from *R. rhipicephali* purified from Vero76 cells using either a bead (B) or sucrose gradient (S) purification strategy. **(2)** Derivatization of a single ion (1936.37 mz) illustrating five major fragmentation products. These products are named in the table with theoretical and experimental sizes shown, with error calculation illustrating robust interpretation. Genome-phylogeny estimated with RAxML (see PMID: 28951473).

Rickettsiae has a unique strategy to overcome no glycolytic enzymes

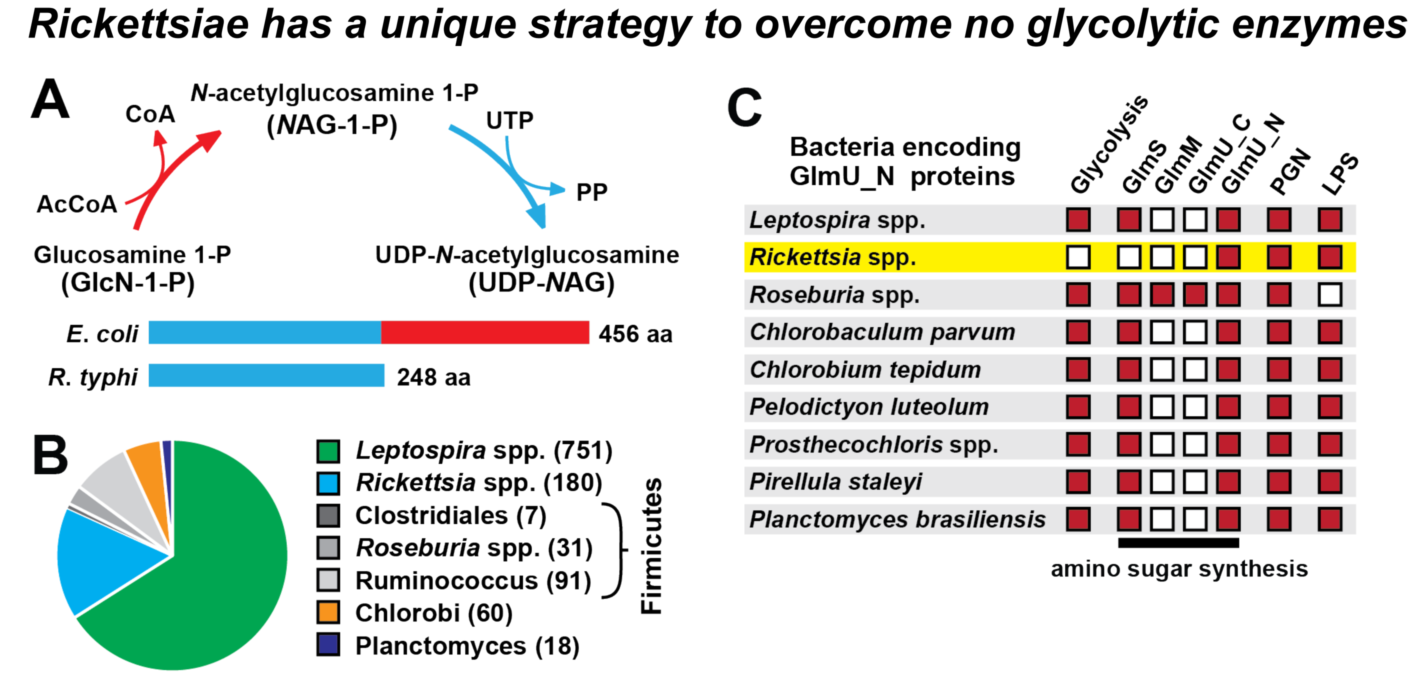

FIG 3. The GlmU_N protein. *(A)* Rickettsiae differ from most other bacteria that synthesize LPS and/or PGN by lacking the ability to synthesize *N*AG-1-P from endogenous GlcN-1-P. ***(B)*** Breakdown of 1149 GlmU_N proteins belonging to PATRIC Global Family PGF_00023782 (11 proteins belong to metagenome datasets and were discarded). ***(C)*** Metabolic profiles for exemplar GlmU_N-containing taxa (pathways analyzed using KEGG).

Strategy for tracing host NAG-1-P incorporation into rickettsial glycoconjugates

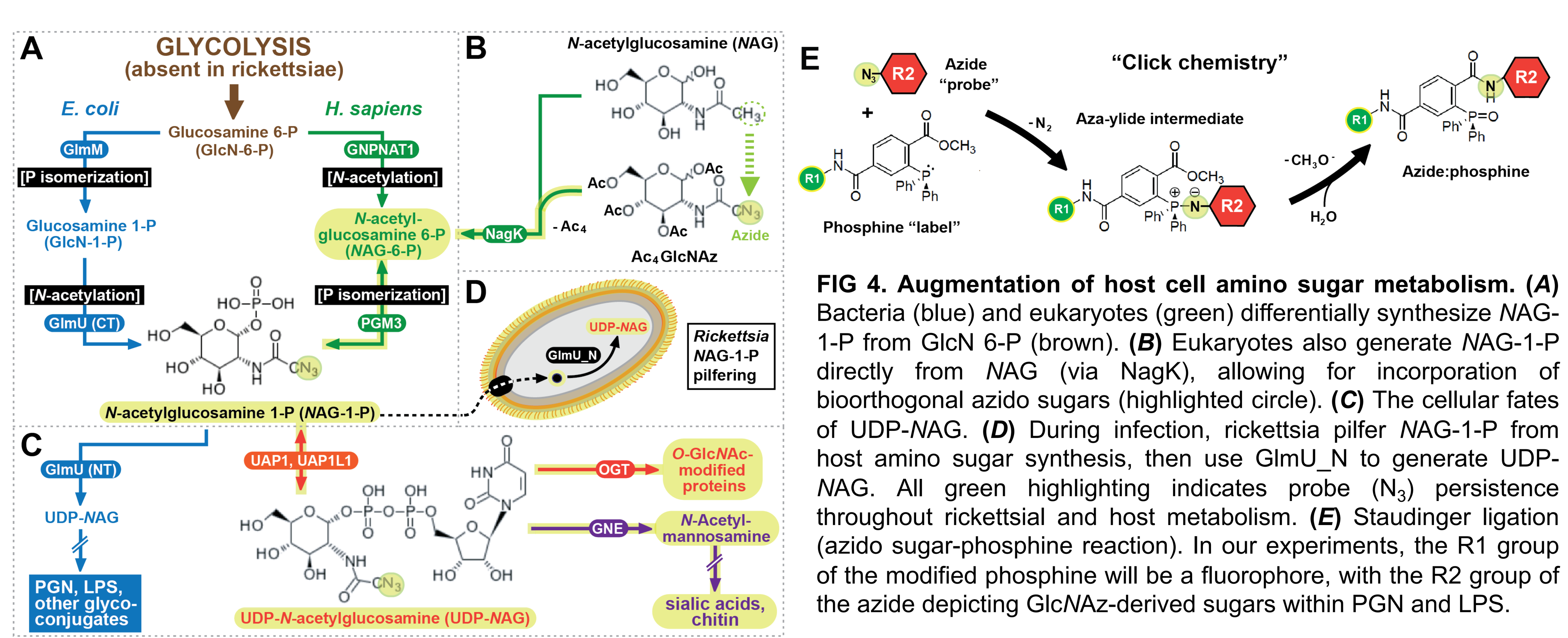

FIG 4. Augmentation of host cell amino sugar metabolism. *(A)* Bacteria (blue) and eukaryotes (green) differentially synthesize *N*AG-1-P from GlcN 6-P (brown). ***(B)*** Eukaryotes also generate *N*AG-1-P directly from *N*AG (via NagK), allowing for incorporation of bioorthogonal azido sugars (highlighted circle). ***(C)*** The cellular fates of UDP-*N*AG. ***(D)*** During infection, rickettsia pilfer *N*AG-1-P from host amino sugar synthesis, then use GlmU_N to generate UDP-*N*AG. All green highlighting indicates probe (N_3) persistence throughout rickettsial and host metabolism. ***(E)*** Staudinger ligation (azido sugar-phosphine reaction). In our experiments, the R1 group of the modified phosphine will be a fluorophore, with the R2 group of the azide depicting Glc*N*Az-derived sugars within PGN and LPS.

Identifying novel sugars in rickettsial exopolysaccharide

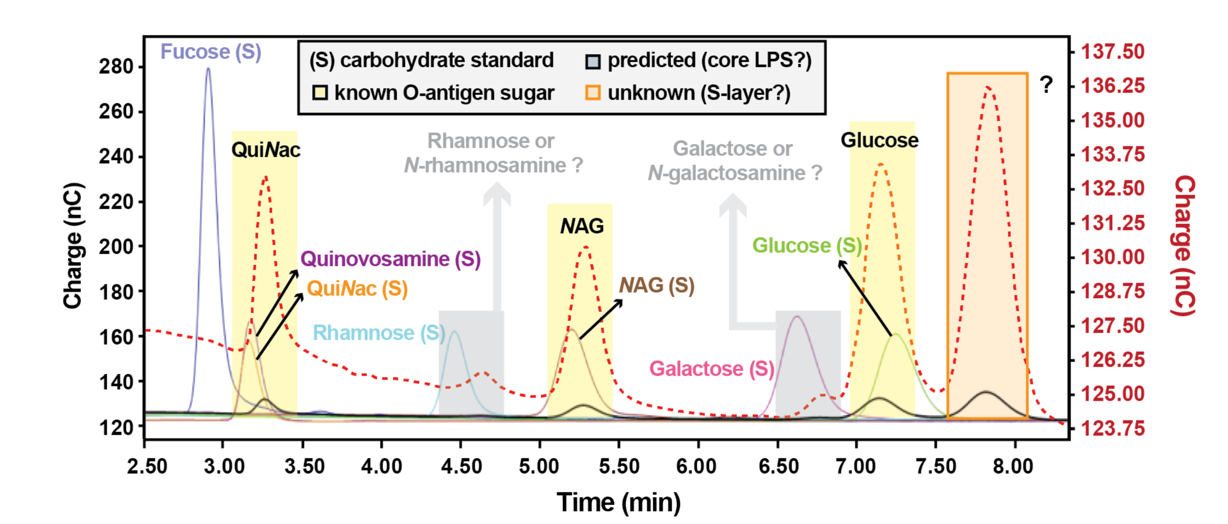

FIG 5. Characterization of rickettsial cell envelope glycoconjugates. Analysis of *R. rhipicephali* exopolysaccharide. Bacteria were purified from ten T75 flasks, and boiled at 100ºC, pH 3.7 for 4 hr. This solution was dialyzed into H_2O, frozen, and lyophilized. The sample was analyzed with **high performance anion exchange chromatography** (**HPAEC**) coupled with **pulsed amperometric detection** (**PAD**) using a Dionex ICS 5000 to obtain monosaccharide content. HPLC-SEC was implemented to obtain general sugar size. Using standards (S) for seven common LPS sugars we show the presence of known *Rickettsia* O-antigen sugars Qui*N*Ac, *N*AG, and glucose (yellow), predicted core LPS sugars rhmanose (or Rha*N*Ac) and galactose (or Gal*N*Ac) (gray), and an unknown sugar likely comprising the S-layer. The *Rickettsia* sample (black) is expanded between net charge 123.75 and 137.5 at right (maroon dashed line), reflecting the low input sample relative to standards set for exopolysaccharide/capsule extracted for free-living bacteria.

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