The molecular mechanism of N-acetylglucosamine side-chain attachment to the Lancefield group A Carbohydrate in *Streptococcus pyogenes*.

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Abstract

Streptococcus pyogenes or Group A *Streptococcus* (GAS) causes numerous diseases in humans, ranging from minor skin and throat infections, to life-threatening systemic conditions such as bacteremia, necrotizing fasciitis, and toxic-shock syndrome. GAS synthesizes a key antigenic surface polymer — the Lancefield group A Carbohydrate (GAC). GAC is attached to peptidoglycan and consists of a polyrhamnose polymer, with N-acetylglucosamine (GlcNAc) side chains, that is an essential virulence determinant. The molecular details of the mechanism of polyrhamnose modification with GlcNAc are currently unknown. In this report we demonstrate that GAC biosynthesis requires two distinct undecaprenol-linked GlcNAc-lipid intermediates: GlcNAc-pyrophosphoryl-undecaprenol (GlcNAc-P-P-Und) produced by GacO and GlcNAc-phosphate-undecaprenol (GlcNAc-P-Und) produced by GacI. The GAC polyrhamnose backbone is assembled on GlcNAc-P-P-Und. Furthermore, our data suggests that GlcNAc-P-Und is used by the membrane glycosyltransferase GacL to transfer GlcNAc from GlcNAc-P-Und to the polyrhamnose polysaccharide. In addition, GacJ, a small membrane protein, forms a complex with GacI and significantly stimulates its catalytic activity. We show that GlcNAc modification of polyrhamnose protects GAS from amidase-induced lysis. Thus, our study significantly expands our understanding of the biosynthesis of GAS cell wall polysaccharide and points to the functional importance of polysaccharide modifications in protection of peptidoglycan from lytic enzymes.

IMPORTANCE

In many species of Lactobacillales peptidoglycan is decorated by polyrhamnose polysaccharides that are critical for cell envelope integrity and cell shape and represent key antigenic determinants. Despite the biological importance of these polysaccharides, their biosynthetic pathways have received limited study. The important human pathogen, *Streptococcus pyogenes*, synthesizes a cell wall-associated polyrhamnose polysaccharide with N-acetylglucosamine (GlcNAc) side chains. The GlcNAc side chain is an important virulence determinant used by bacteria to evade the host innate immune defense. Here we identify the molecular mechanism of polyrhamnose modification with GlcNAc in *S. pyogenes*. We show that GacI synthesizes GlcNAc-phosphate-undecaprenol (GlcNAc-P-Und) aided by GacJ. GlcNAc-P-Und is apparently used by

46 GacL to transfer GlcNAc to polyrhamnose. We demonstrate that GacI homologs
47 perform a similar function in *Streptococcus agalactiae* and *Enterococcus faecalis*. Thus,
48 the elucidation of polysaccharide biosynthesis in *S. pyogenes* enhances our
49 understanding of how other Gram-positive bacteria produce essential components of
50 their cell wall.
51

52 **INTRODUCTION**

53 The cytoplasmic membrane of Gram-positive bacteria is surrounded by a thick cell
54 wall consisting of multiple peptidoglycan layers decorated with proteins and a variety of
55 carbohydrate-based polymers. Rhamnose (Rha) is the main component of the
56 carbohydrate structures in many species of the Lactobacillales order (1). These
57 polymers play essential roles in maintaining and protecting bacterial cell envelopes and
58 in pathogenesis of infections caused by *Streptococcus pyogenes* and *Enterococcus*
59 *faecalis* (1). It is of paramount importance to understand the molecular mechanisms of
60 Rha-containing cell wall polysaccharide biosynthesis for developing novel therapeutics
61 against these bacterial pathogens.

62 *S. pyogenes* or Group A *Streptococcus* (GAS) is associated with numerous
63 diseases in humans ranging from minor skin and throat infections such as impetigo and
64 pharyngitis to life-threatening invasive infections such as streptococcal toxic syndrome
65 and necrotizing fasciitis (2). The main component of GAS cell wall is the Lancefield
66 group A Carbohydrate (GAC) that comprises about 40-60% of the total cell wall mass
67 (3). Serological grouping of beta-hemolytic streptococci (A, B, C, E, F, and G groups),
68 introduced by Rebecca Lancefield in 1933, is based on the detection of carbohydrate
69 antigens present on the cell wall (4). GAC is presumably covalently linked to N-
70 acetylmuramic acid (MurNAc) of peptidoglycan, although the molecular structure of the
71 linkage unit has not been established (5). All serotypes of GAS produce GAC consisting
72 of a polyrhamnose backbone with N-acetylglucosamine (GlcNAc) side chains (6, 7). The
73 average molecular mass of GAC has been reported to be 8.9±1.0 kDa, corresponding
74 to an average of 18 repeating units of [→3)α-Rha(1→2)[β-GlcNAc(1→3)]α-Rha(1→] (8).
75 Since Rha is absent in mammalian cells, GAC is an attractive candidate for a universal
76 GAS vaccine (8, 9). Moreover, the GlcNAc side chains are an important virulence

77 determinant in GAS (5). GAS mutants lacking GlcNAc are susceptible to innate immune
78 clearance by neutrophils and antimicrobial agents, and are significantly attenuated in
79 animal models of GAS infection (5).
80 Similar to the biosynthesis of other cell-envelope polymers — peptidoglycan,
81 lipopolysaccharide, wall teichoic acid (WTA), and capsular polysaccharide — the
82 synthesis of Rha-containing carbohydrate structures is likely initiated on the inside of
83 the cytoplasmic membrane and proceeds through several steps including the
84 attachment of the first sugar residue to a lipid carrier, undecaprenyl phosphate (Und-P),
85 followed by elongation of the polysaccharide through stepwise addition of activated
86 sugar residues to the lipid carrier (1). After translocation of the polysaccharide across
87 the membrane and further modifications, it is presumably attached to peptidoglycan via
88 a phosphate ester linkage (1). The first membrane step of O-antigen biosynthesis in
89 enterobacteria and WTA biosynthesis in *Bacillus subtilis* and *Staphylococcus aureus*
90 (10) is catalyzed by the UDP-GlcNAc:Und-P GlcNAc-1-phosphate transferase encoded
91 by WecA homologs (11, 12). WecA transfers GlcNAc-phosphate from UDP-GlcNAc to
92 Und-P, forming GlcNAc-pyrophosphoryl-undecaprenol (GlcNAc-P-P-Und). Although
93 GAS strains do not produce WTA, all GAS genomes contain a *wecA* homolog, *gacO*
94 (5). Significantly, GAC biosynthesis is sensitive to tunicamycin, a known inhibitor of
95 WecA (5) and the *Streptococcus mutans* GacO homolog, RgpG, has been shown to
96 complement WecA activity in *E. coli* (13). These observations support an essential role
97 for GacO in the biosynthesis of GlcNAc-P-P-Und and suggest GlcNAc-P-P-Und may
98 function as a lipid-anchor in the initiation of GAC biosynthesis.
99 Other genes required for GAC biosynthesis and transport are found in a separate
100 location on the GAS chromosome and comprise a 12-gene locus (*gacA*-*gacL*) (Fig.1A).
101 The first three genes, *gacA*-*gacC* together with *gacG,* are conserved in many species of
102 the Lactobacillales order (1). *GacA* encodes a dTDP-4-dehydrorhamnose reductase,
103 the enzyme responsible for dTDP-rhamnose biosynthesis (14). *GacB*, *gacC*, and *gacG*
104 encode putative cytoplasmic rhamnosyltransferases. In *S. mutans* homologous genes
105 *rgpA*, *rgpB* and *rgpF* are involved in rhamnan backbone biosynthesis (15). In GAS,
106 *gacA*-*gacC* are essential for viability and cannot be deleted (5). However, deletion
107 mutants have been obtained in other genes of the GAC gene cluster (5). It has been

108 shown that *gacI*, *gacJ* and *gacK* are non-essential for viability, but are required for
109 GlcNAc side-chain addition to the polyrhamnose (5). They encode a putative
110 cytoplasmic glycosyltransferase, a small membrane protein and a protein with homology
111 to the Wzx family of membrane proteins involved in the export of O-antigen and teichoic
112 acids, respectively. In contrast, inactivation of *gacD*, *gacE*, *gacF*, *gacG*, *gacH* or *gacL*
113 has no effect on GAS viability and the GAC produced by these mutants is reported to
114 display a wild type (WT) antigenic profile, indicating the presence of the
115 immunodominant GlcNAc side chains (5). *GacD* and *gacE* encode the components of
116 an ABC transport system. In *S. mutans* the *gacD* and *gacE* homologs are responsible
117 for rhamnan polysaccharide transport (15). *GacF*, *gacH* and *gacL* encode a cytosolic
118 glycosyltransferase, a putative membrane-associated glycerol phosphate transferase
119 and a membrane-associated glycosyltransferase, respectively.
120 In this study we address the molecular mechanism of GlcNAc attachment to
121 polyrhamnose. We demonstrate that GacI catalyzes formation of GlcNAc-P-Und, and
122 GacJ stimulates the catalytic activity of GacI. Subsequently GacL transfers GlcNAc from
123 GlcNAc-P-Und to the polyrhamnose backbone of GAS polysaccharide. Moreover, we
124 confirm GacO function in GlcNAc-P-P-Und formation and demonstrate the role of this
125 GlcNAc-lipid in initiation of polyrhamnose biosynthesis.
126
127 **RESULTS**
128 **GacL is required for GlcNAc attachment to polyrhamnose.** GacL, a polytopic (12–
129 13 transmembrane segments) membrane protein, is reported to be dispensable for
130 GlcNAc attachment to polyrhamnose (5). To investigate the function of GacL in GAC
131 biogenesis, we disrupted *gacL* in the hyperinvasive *S. pyogenes* M1T1 serotype strain,
132 MGAS5005 (16), creating the 5005Δ*gacL* mutant. In agreement with published data (5)
133 5005Δ*gacL* did not display any detectable growth phenotype in comparison to the WT
134 strain. Surprisingly, we found that 5005Δ*gacL* cells failed to bind GlcNAc-specific anti-
135 GAC antibodies, suggesting a loss of the GlcNAc antigenic epitope (Fig. 1B). The
136 5005Δ*gacL* phenotype was restored by expressing the WT copy of *gacL* on the mutant
137 chromosome (Fig. 1B). To confirm that 5005Δ*gacL* cells are deficient in GlcNAc addition
138 to polysaccharide, we measured the binding of fluorescently labeled succinylated wheat

139 germ agglutinin (sWGA), a lectin that specifically binds non-reducing terminal β-GlcNAc
140 residues, to WT and 5005Δ*gacL* cells. Deletion of *gacL* led to a significant decrease in
141 binding of WGA to the 5005Δ*gacL* strain as compared with the WT (Fig. 1C), indicating
142 that the mutant has substantially less GlcNAc-containing saccharides on the cell
143 surface. Furthermore, direct compositional analysis of cell wall purified from WT and
144 5005Δ*gacL* cells confirmed a significant decrease of GlcNAc content in the 5005Δ*gacL*
145 cell wall sample (Fig.1D, E and F). These data strongly support a role for GacL in
146 GlcNAc side-chain attachment to polyrhamnose.

147 **A *gacL* deletion mutant accumulates GlcNAc-P-Und.** To investigate further
148 the effect of GacL deletion in *S. pyogenes*, we isolated the phospholipid fractions from
149 the membranes of WT and 5005Δ*gacL* cells. TLC analysis revealed accumulation of a
150 previously unidentified phosphoglycolipid in 5005Δ*gacL* (Fig. 2A). The novel lipid was
151 found to be stable to mild alkaline methanolysis, but sensitive to mild acid (0.1 N HCl,
152 50 ºC, 50% isopropanol) and reacted with orcinol spray (17), Dittmer-Lester
153 phospholipid spray reagent (18) and with anisaldehyde, an isoprenol-specific reagent
154 (19), consistent with its tentative identification as a glycophosphoprenol (data not
155 shown). High-resolution negative ion ESI-MS analysis of the purified lipid identified a
156 molecular ion $[M-H]^{-1}$ of M/z = 1048.73621 (Fig. 2B). In addition, prominent high-
157 resolution fragment ions characteristic of PO_3, H_2PO_4, N-acetyl hexosamine-phosphate
158 and Und-P were also found (Table 1). These data are consistent with a glycolipid
159 comprised of N-acetyl hexosamine-phosphate-undecaprenol. In experiments described
160 in detail below, we found that when membrane fractions from GAS are incubated with
161 UDP-[^{3}H]GlcNAc, a [^{3}H]GlcNAc-lipid which co-chromatographs on TLC with this novel
162 lipid is rapidly formed. Importantly, the product ion spectra shown in Figure 2B does not
163 contain a fragment ion containing $[Und\text{-}PO_4\text{-}C_2H_2NHCOCH_3]^-$ (M/z=929.7), arising from
164 a cross-ring fragmentation reaction, suggesting that the hydroxyl of the anomeric carbon
165 and the nitrogen at the 2-position of the glycosyl ring are *trans* to the plane of the ring
166 (20, 21). This observation strongly suggests that the anomeric hydroxyl is present in the
167 β configuration. Furthermore, isolated [^{3}H]GlcNAc-P-Und phospholipid (described in
168 detail below) is extremely sensitive to incubation with 50% phenol at 68 °C — a property
169 that is consistent with β-GlcNAc-P-Und (22) (Table S4). Taken together, these data

170 identify the novel glycolipid accumulating in the 5005Δ*gacL* strain as β-GlcNAc-P-Und
171 and suggest that GacL is a GlcNAc transferase using β-GlcNAc-P-Und as GlcNAc
172 donor for the addition of the GlcNAc side-chains to GAC.

173 **GAS synthesizes two GlcNAc-lipids *in vitro*.** To investigate the biosynthetic
174 origin of the novel glycolipid accumulating in 5005Δ*gacL* cells, membrane fractions from
175 MGAS5005 were incubated with UDP-[^{3}H]GlcNAc and analyzed for lipid products as
176 described in Material and Methods. Experiments showed that [^{3}H]GlcNAc was efficiently
177 transferred from UDP-[^{3}H]GlcNAc into two detectable GlcNAc-lipids (Fig. 3A, Table 2).
178 The major lipid product co-migrates on TLC with the novel lipid accumulating in the
179 5005Δ*gacL* strain and is identified as β-[^{3}H]GlcNAc-P-Und, as described above. The
180 minor product is assumed to be [^{3}H]GlcNAc-P-P-Und, since it co-migrates with authentic
181 GlcNAc-P-P-Und synthesized in *B. cereus* membranes (Fig. 3B) (23) and its formation
182 is potently inhibited by tunicamycin (Fig. S1).

183 **GacI encodes a UDP-GlcNAc:Und-P GlcNAc transferase activity.** The GAS
184 polysaccharide gene cluster contains a gene*, gacI*, reported to be required for GlcNAc
185 addition to cell wall polysaccharide (5) and annotated as a GT-A type
186 glycosyltransferase. The following studies were conducted to determine if GAS GacI
187 might be responsible for the synthesis of GlcNAc-P-Und. To determine if GacI function
188 was essential for formation of GlcNAc-P-Und, we generated a deletion of *gacI* in the
189 MGAS5005. In agreement with published data (5) 5005Δ*gacI* lost reactivity with anti-
190 GAC antibodies, indicating a loss of GlcNAc modification in polyrhamnose (Fig. 1B).
191 When 5005Δ*gacI* membrane fractions were incubated with [^{3}H]UDP-GlcNAc no
192 incorporation into [^{3}H]GlcNAc-P-Und was observed and only the minor lipid,
193 [^{3}H]GlcNAc-P-P-Und was found (Fig. 3C, Table 2), strongly supporting an essential role
194 for GacI in GlcNAc-P-Und synthesis. The observation that the 5005Δ*gacL* strain
195 synthesizes normal levels of [^{3}H]GlcNAc-P-Und *in vitro* (Fig. 3D) indicates that
196 5005Δ*gacL* does not fail to add GlcNAc side-chains to polyrhamnose due to a lack of
197 GlcNA-P-Und and supports the conclusion that GacL may be the GlcNAc-P-
198 Und:polyRhamnan GlcNAc transferase.

199 To confirm the role of GacI in GlcNAc-P-Und biosynthesis we expressed GAS GacI
200 in *E. coli*. When membrane fractions from *E. coli* carrying an empty vector were

201 incubated with UDP-[^{3}H]GlcNAc and analyzed by silica gel TLC, a small peak of
202 [^{3}H]GlcNAc-P-P-Und was found (Table 2, Fig. S4A). In contrast, the membranes of
203 recombinant *E. coli* expressing GacI accumulated two products corresponding to a
204 small amount of GlcNAc-P-P-Und and a very large amount of GlcNAc-P-Und (Table 2,
205 Fig. S4B). Thus, our data strongly indicate that GacI is the GlcNAc-P-Und synthase.
206 **GacI homologs in GBS and *E. faecalis* function in the biosynthesis of GlcNAc-**
207 **P-Und.** The presence of GlcNAc-P-P-Und and GlcNAc-P-Und have been previously
208 reported in *B. cereus* and *Bacillus megaterium* (12, 23). Our *in vitro* studies show that
209 the MGAS5005 strain synthesizes two [^{3}H]GlcNAc-lipids that exactly co-migrate on TLC
210 with the previously reported [^{3}H]GlcNAc-lipids from *B. cereus* (Fig. 3A and 3B).
211 Moreover, an analysis of bacterial genomes using GacI in a BLAST search identified a
212 GacI homolog (60% identity) in *B. cereus* and *B. megaterium*, suggesting its role in the
213 GlcNAc-P-Und biosynthesis in these bacteria. Furthermore, *gacI* homologs were
214 identified in the polysaccharide biosynthesis gene clusters of the important human
215 pathogens *E. faecalis* (*epaI* with 48% sequence identity) (24) and *S. agalactiae* (Group
216 B Streptococcus or GBS) (SAN_1536 gene with 46% sequence identity) (Fig. S2). To
217 investigate whether the GBS homolog of GacI also catalyzes the formation of GlcNAc-
218 P-Und, we engineered a knock out of the GacI homolog in GBS COH1. Although GBS
219 COH1 membranes synthesize both [^{3}H]GlcNAc-P-P-Und and [^{3}H]GlcNAc-P-Und (Fig.
220 4B and Fig. S3A), GBS COH1Δ*gacI* membranes no longer synthesize [^{3}H]GlcNAc-P-
221 Und (Table 2 and Fig. S3B).
222 To confirm that the GacI homolog detected in *E. faecalis*, EpaI (24), also possesses
223 GlcNAc-P-Und synthase activity, EpaI was expressed exogenously in *E. coli* and found
224 to actively catalyze the formation of [^{3}H]GlcNAc-P-Und (Table 2, Fig. S4C). Altogether,
225 our results are consistent with the function of GacI homologs from GAS, GBS and *E.*
226 *faecalis* in the transfer of GlcNAc from UDP-GlcNAc to Und-P forming GlcNAc-P-Und.
227 **ATP stimulates GlcNAc-P-Und biosynthesis.** Preliminary enzymatic properties
228 for the synthesis of [^{3}H]GlcNAc-P-Und established that Mg^{2+} was the preferred divalent
229 cation and that the formation of GlcNAc-P-Und was substantially stimulated by
230 exogenously added Und-P (as a dispersion in CHAPS detergent). In early studies of
231 GAC biosynthesis in GAS (25), it was shown that the addition of ATP dramatically

232 stimulated incorporation of radioactive UDP-[^{3}H]GlcNAc into [^{3}H]GlcNAc membrane
233 lipids and [^{3}H]GlcNAc-polysaccharide. We confirmed that inclusion of 1 mM ATP
234 significantly stimulated the incorporation of [^{3}H]GlcNAc into [^{3}H]GlcNAc-P-Und (Fig. 5
235 and Fig. S5), as well as [^{3}H]GlcNAc-P-P-Und (Fig. S5). However, the inclusion of ATP
236 did not stimulate GacI activity in *in vitro* assays of CHAPS-soluble, affinity purified GacI
237 solely dependent on exogenously added Und-P as acceptor. These data suggest that
238 the effect of ATP addition is most likely due to formation of Und-P, *in situ,* by
239 phosphorylation of endogenous undecaprenol by undecaprenol kinase (26).

240 **GacJ forms a complex with GacI and enhances its catalytic efficiency.** *GacJ* is
241 located immediately downstream of *gacI* in the GAS GAC biosynthesis gene cluster and
242 encodes a small 113 aa membrane protein (Fig. 1A). We hypothesized that GacI might
243 form an obligate complex with GacJ. This hypothesis is based on the observation that
244 *gacJ* and *gacI* are frequently located adjacent to each other on bacterial chromosomes
245 and are sometimes fused to form a single polypeptide (accession numbers: GAM11018,
246 ADH85075, ALC15489 and ADU67183). To test this hypothesis, we solubilized
247 membranes from *E. coli* co-expressing GacJ and amino-terminal His-tagged GacI with
248 the zwitterionic detergent CHAPS, and isolated GacI complexes using Ni-NTA
249 chromatography (Fig. 6A). SDS-PAGE of the affinity-purified sample revealed two
250 bands corresponding to the anticipated molecular sizes of GacI and GacJ (Fig. 6B).
251 Proteomics analysis of the excised protein bands confirmed the identities of the
252 recovered proteins. This result indicates that GacI and GacJ form a stable, CHAPS-
253 soluble complex and co-purify during affinity chromatography.

254 To investigate if GacJ performs any catalytic function in association with GacI, we
255 tested the proteins for GlcNAc-P-Und synthase activity *in vitro*, individually and in
256 combination. When GacI was expressed singly in *E. coli*, enzymatically active protein
257 was found in the membrane fraction (Table 2, Fig. S4B), indicating that GacI does not
258 require GacJ for activity or membrane association. GacJ was found to be catalytically
259 inactive when expressed by itself in *E. coli*, consistent with the observation that
260 5005Δ*gacI* membranes show no residual GlcNAc-P-Und synthase activity. Kinetic
261 analysis of GlcNAc-P-Und synthase activity for Und-P in membrane fractions of *E. coli*
262 expressing GacI revealed an apparent Km of 18.7 μM (Table 3, Fig. S6A). However, co-

263 expression of GacJ dramatically lowered the apparent Km of GacI for Und-P to 1.1 μM,
264 suggesting a significant change in affinity for the lipid acceptor. Moreover, the V_{max} of
265 GacI activity increased from 54.2 pmol/min/mg to 18.5 nmol/min/mg in the presence of
266 GacJ (Table 3, Fig. S6B). A kinetic analysis of GlcNAc-P-Und synthase for Und-P in
267 MGAS5005 strain gave an apparent Km of 6.4 μM and a V_{max} of 333 pmol/min/mg
268 (Table 3, Fig. S6C), similar to the enzymatic parameters of GacI co-expressed with
269 GacJ in *E. coli*. These enzymatic parameters are in marked contrast to those of the
270 GlcNAc-P transferase that synthesizes GlcNAc-P-P-Und. When a similar kinetic
271 analysis was performed in 5005Δ*gacI*, so that only GlcNAc-P-P-Und synthesis could be
272 scored, an apparent Km for Und-P of 19.3 μM was obtained and the V_{max} was only 2.75
273 pmol/min/mg (Table 3, Fig. S6D). Clearly, GacI displays a much greater catalytic
274 efficiency for the synthesis of GlcNAc-P-Und than the enzyme synthesizing GlcNAc-P-
275 P-Und, which is reflected in the dramatic difference in the rates of synthesis of the two
276 glycolipids. In summary, our data indicate that GacJ forms a stable association with
277 GacI and stimulates the catalytic activity of GacI.

278 **GlcNAc-P-P-Und is required for initiation of polyrhamnose polysaccharide**
279 **biosynthesis.** The GAS genome contains a close relative, *gacO*, of *E. coli wecA*, the
280 tumicamycin-sensitive GlcNAc-phosphate transferase responsible for the synthesis of
281 GlcNAc-P-P-Und in many bacteria (11, 12). Significantly, rhamnopolysaccharide
282 synthesis in GAS is inhibited by tunicamycin (5) and a close homolog of GacO, *S.*
283 *mutans* RpgP, has been shown to be required for rhamnopolysaccharide synthesis and
284 can complement WecA deficiency in *E. coli* (13). Our analysis of UDP-[^{3}H]GlcNAc
285 incorporation in GAS and GBS membrane lipids, revealing the presence of two GlcNAc-
286 lipids, GlcNAc-P-Und and GlcNAc-P-P-Und, prompted us to investigate the role of
287 GlcNAc-P-P-Und in the initiation of GAC biosynthesis. First, we investigated the role of
288 GacO in the synthesis of GlcNAc-P-P-Und. When membrane fractions from the WecA
289 deficient *E. coli* strain (CLM37) expressing GacO, CLM37:GacO, were incubated with
290 UDP-[^{3}H]GlcNAc and Und-P (added as a dispersion in 1 % CHAPS), [^{3}H]GlcNAc-P-P-
291 Und was formed at an enzymatic rate that is similar to that found in the WecA over-
292 expressor strain, PR4019 (Fig. 7A). CLM37 carrying an empty vector synthesizes no
293 detectable GlcNAc-P-P-Und under these conditions (Fig. 7A).

294 To test whether GlcNAc-P-P-Und might function as a membrane anchor for the
295 synthesis of the polyrhamnose chain of the rhamnopolysaccharide, membrane fractions
296 from MGAS5005 or the 5005Δ*gacI* mutant were pre-incubated with UDP-[^{3}H]GlcNAc
297 and ATP, to form [^{3}H]GlcNAc-P-P-Und *in situ*, and chased with non-radioactive dTDP-
298 rhamnose. The formation of the resultant [^{3}H]GlcNAc-lipids in 5005Δ*gacI* was analyzed
299 by TLC on silica gel G after 30 min incubation as shown in (Fig. 7B and C). In the
300 absence of TDP-rhamnose, 5005Δ*gacI* membranes produced only [^{3}H]GlcNAc-P-P-Und
301 (Fig. 7B), whereas in the presence of TDP-rhamnose two radioactive products,
302 [^{3}H]GlcNAc-P-P-Und and an additional product with slower mobility on TLC was
303 observed (Fig 7C). Figure S7 shows the time course of accumulation of the new
304 GlcNAc-lipid product. Significantly, parallel incubations with MGAS5005 showed that
305 GlcNAc-P-Und was not glycosylated further. These results strongly support the
306 possibility that GlcNAc-P-P-Und is an acceptor for rhamnosyl units in GAS and may
307 function as the lipid anchor for polyrhamnosyl polysaccharide synthesis.
308 **The GacL mutant displays increased sensitivity to peptidoglycan amidases.**
309 The Gram-positive cell wall protects interior structures, plasma membrane and
310 peptidoglycan, from host defense peptides and hydrolytic and antimicrobial enzymes. It
311 has been shown that the GacI mutant which is GlcNAc-deficient is more sensitive to LL-
312 37-induced killing (5). To test the hypothesis that the loss of GlcNAc decorations in GAC
313 alters cell wall permeability, we investigated the sensitivity of 5005Δ*gacL* to
314 peptidoglycan amidases: PlyC (27), PlyPy (28) and CbpD (29). When 5005Δ*gacL* cells
315 were grown in increasing concentrations of CbpD (Fig. 8A), PlyPy (Fig. 8B) or PlyC (Fig.
316 8C) cellular growth was dramatically inhibited compared to MGAS5005, indicating
317 increased sensitivity to the presence of amidases.
318
319 **DISCUSSION**
320 In almost all Gram-positive bacteria, cell wall-attached glycopolymers are critical for cell
321 envelope integrity and their depletion is lethal (30). Most streptococcal species including
322 two human pathogens GAS and GBS do not synthesize WTA and instead produce Rha-
323 containing glycopolymers as functional homologs of WTA (1). GAC is the major cell wall
324 component of GAS and plays important roles in bacterial physiology and pathogenesis.

325 The polyrhamnose core of GAC is modified with GlcNAc in an approximately 2:1 ratio
326 (3, 7) of rhamnose to GlcNAc. Collectively, the results of our study suggest a molecular
327 mechanism of GAC biosynthesis in which rhamnan polymer is assembled at the
328 cytoplasmic face of the plasma membrane, translocated to the cell surface and modified
329 by GlcNAc on the outer side of the membrane as illustrated in Figure 9. We report that a
330 lipid carrier, GlcNAc-P-P-Und, synthesized by GAS GacO, is a potential acceptor for
331 initiation of rhamnan backbone biosynthesis. We speculate that the next step of
332 rhamnan biosynthesis involves the action of the GacB, GacC, GacG and GacF
333 glycosyltransferases. The lipid-anchored polyrhamnose is then translocated across the
334 membrane by the ABC transporter encoded by *gacD* and *gacE*. This hypothesis is
335 supported by studies of rhamnan biosynthesis in *S. mutans* (13, 31, 32).
336 Biosynthesis of the rhamnan-backbone of GAC is likely essential for GAS viability
337 because the GacA enzyme involved in dTDP-rhamnose biosynthesis (14), and GacB
338 and GacC glycosyltranferases are indispensable in GAS (5). In contrast, deletion of
339 genes required for GlcNAc attachment to the rhamnan backbone does not affect GAS
340 viability (5). This observation supports our hypothesis that biosynthesis of polyrhamnose
341 and its translocation to the cell surface occur separately from the pathway involved in
342 polyrhamnose modification with GlcNAc. In this study, we found that GlcNAc
343 modification of rhamnan requires GlcNAc-P-Und synthesis. Previously, GlcNAc-P-Und
344 was isolated from various *Bacilli* membranes (12, 23). However the enzyme required for
345 GlcNAc-P-Und biosynthesis was not identified and the biological function of this lipid
346 remained unknown. Our *in vitro* analysis of UDP-GlcNAc incorporation into GlcNAc-
347 lipids by GAS membranes showed that GacI was required for the biosynthesis of
348 GlcNAc-P-Und. Moreover CHAPS-soluble, affinity-purified GacI protein catalyzed the
349 transfer of GlcNAc from UDP-GlcNAc to Und-P yielding GlcNac-P-Und in an *in vitro*
350 reaction system.
351 Interestingly, we found that, in contrast to *B. cereus*, GAS and GBS membranes
352 incubated with UDP-[^{3}H]GlcNAc synthesized primarily [^{3}H]GlcNAc-P-Und. Since GacI
353 and GacO utilize a common pool of Und-P, this phenomenon is presumably due to a
354 much higher apparent affinity of GacI for Und-P compared to GacO. Significantly, we
355 observed a measurable increase in [^{3}H]GlcNAc incorporation into [^{3}H]GlcNAc-P-P-Und

356 in the GacI deletion mutant. This observation further confirmed that the relative
357 synthetic rates of GlcNAc-P-Und and GlcNAc-P-P-Und biosynthesis are determined
358 largely by Und-P availability. Furthermore, we found that the formation of GlcNAc-P-Und
359 and GlcNAc-P-P-Und in GAS membranes was significantly stimulated by ATP. Since
360 GacI activity is not stimulated directly by ATP, it is likely that the effect of ATP is due to
361 increased formation of Und-P, *in situ*, via undecaprenol kinase activity. In *S. mutans,*
362 membrane-associated undecaprenol kinase catalyzes the ATP-dependent
363 phosphorylation of undecaprenol to Und-P (26). It is likely that the homolog of this
364 enzyme encoded by M5005_Spy_0389 is responsible for Und-P biosynthesis in GAS.
365 Wzx flippases translocate Und-P-P-linked oligosaccharides from the cytoplasmic
366 side to the periplasmic side of membranes in bacteria (33). We suggest that GacK may
367 function to transport GlcNAc-P-Und to the extracellular space for utilization as GlcNAc
368 donor in the GlcNAc modification of polyrhamnose. This hypothesis is based on the
369 GlcNAc-deficient phenotype of the GacK mutant (5) and the high domain homology
370 between GacK and proteins of the Wzx family of flippases. However, further research
371 will be required to confirm this hypothesis. The phenotypes of the GacL mutant:
372 absence of the GlcNAc side-chains in GAC and accumulation of GlcNAc-P-Und in the
373 membrane is consistent with GacL functioning in the transfer of GlcNAc from GlcNAc-P-
374 Und to polyrhamnose yielding GlcNAc-modified polysaccharide. Bioinformatics
375 searches with the HHpred program against available protein structures identified the
376 GT-C superfamily of glycosyltrasferases as the closest structural homologs of GacL.
377 The members of the GT-C superfamily consist of proteins characterized with multiple
378 transmembrane domains and a large periplasmic loop close to the N-terminus that
379 contains a metal-binding motif (DxD, ExD, DDx or DEx) (34, 35). The GT-C enzymes
380 act on the outside of the plasma membrane by transferring sugars from the lipid carrier,
381 Und-P, onto either glycolipid or protein. GacL contains 12 transmembrane segments
382 and the highly conserved DEX motif in the first extracellular loop. Thus, the predicted
383 structural homology of GacL is in agreement with the proposed GacL function in GlcNAc
384 transfer from GlcNAc-P-Und to polyrhamnose.
385 Thus, we suggest that the proposed mechanism of GlcNAc attachment to
386 polyrhamnose in GAS is similar to the mechanism of O-antigen modification in *Shigella*

387 *flexneri* in which the GtrB glycosyltransferase, together with GtrA flippase and GtrV
388 membrane protein, are required for addition of a glycosyl group to rhamnose of the O-
389 antigen backbone polysaccharide, which is a part of lipopolysaccharide (36). The
390 modification with glycosyl residues takes place after O-unit assembly and before
391 transfer of the mature O-polysaccharide to the lipid A-core region of the
392 lipopolysaccharide. Moreover, in *Mycobacterium tuberculosis* the modification of cell
393 wall arabinogalactan with galactosamine occurs through similar mechanism involving N-
394 acetyl galactosaminyl-phosphate-undecaprenol synthase, PpgS, and a membrane
395 associated GT-C enzyme, Rv3779, that transfers a galactosamine residue from
396 galactosaminyl-phosphate-undecaprenol to arabinogalactan (37).
397 The last step of GAC biosynthesis is probably similar to the last step of WTA
398 biosynthesis: it involves attachment of GAC to certain MurNAc residues in
399 peptidoglycan via a phosphate ester linkage (38). It is likely catalyzed by members of
400 LytR-CpsA-Psr (LCP) phosphotransferase family encoded by M5005_Spy_1099 and
401 M5005_Spy_1474.
402 GacI is predicted by the HHpred server to have structural homology to
403 polyisoprenyl-glycosyltransferase GtrB from *Synechocystis* which is a homolog of *S.*
404 *flexneri* GtrB (39). GtrB homologs in bacteria and eukaryotes belong to GT-A
405 superfamily of glycosyltransferases and they are responsible for the addition of UDP-
406 glucose to the bactoprenol carrier in the cytoplasm yielding the Und-P-glucose
407 precursor in bacteria and dolichol phosphate-glucose precursor in eukaryotes (34, 35).
408 Our BLAST search using the GacI sequence as query found homologs of this gene in
409 many bacterial species, with the broadest diversity observed in the phylum Firmicutes
410 (Supplemental File). To determine evolutionary relationships between the GacI
411 homologs, these sequences were analyzed using CLANS (CLuster ANalysis of
412 Sequences) (40) (Fig. 10). It is noteworthy that the *B. cereus* GacI has two homologs
413 clustered in two different groups. Our bioinformatics analysis shows that these *gacI*
414 homologs are located in different gene clusters encoding proteins involved in
415 polysaccharide biosynthesis and transport. Moreover, the GAS GacI subgrouped with
416 one *B. cereus* GacI. The GBS GacI and *E. faecalis* GacI homologs are located in the
417 cluster well-separated from the GAS GacI subgroup. Thus, this observation points to

418 possible horizontal gene transfer between GAS and *Bacillus*. Analysis of UDP-GlcNAc
419 incorporation into GlcNAc-lipids in a GBS *gacI* knock-out strain confirmed the function of
420 this gene in the biosynthesis of GlcNAc-P-Und in GBS. Additionally, we demonstrated
421 that the *E. faecalis* GacI homolog (EpaI) functions in the biosynthesis of GlcNAc-P-Und.
422 Significantly, the presence of GacI homologs in GBS and *E. faecalis* matches the
423 reported occurrence of GlcNAc residues in the cell wall polysaccharides of these
424 bacteria (41-44).
425 GacJ, a small membrane protein with three transmembrane α-helices, is required
426 for GlcNAc side-chain attachment to polyrhamnose (5). GacJ belongs to DUF2304
427 family of proteins according to the Pfam protein family database (45). In *Geobacter* sp.,
428 *Desulfuromonas* sp., *Desulfurivibrio alkaliphilus, Desulfuromonas soudanensis* and
429 *Desulfurispirillum indicum*, DUF2304 homologous domains are fused with *gacI*
430 homologs (Fig. 10 and supplemental file). In *M. tuberculosis* a GacJ homolog, Rv3632,
431 is co-transcribed with the gene encoding N-acetyl galactosaminyl-phosphate-
432 undecaprenol synthase, PpgS, and is found to stimulate PpgS activity (37). Consistent
433 with this finding we showed that GacI and GacJ form a complex and co-expression of
434 GacI with GacJ significantly enhanced GacI catalytic activity. Further work is under way
435 to characterize the mechanism of GacJ action on GacI activity.
436 The identification of the mechanism of GlcNAc attachment to polyrhamnose raises
437 the question of how this modification functions biologically in bacteria. It has been
438 previously found that the Δ*gacI* mutant is hypersusceptible to human antimicrobial
439 peptide LL-37 and the antimicrobial action of factors released by thrombin-activated
440 platelets, suggesting a role of GlcNAc modification in protecting the plasma membrane
441 from antimicrobial agents (5). Our study identified the importance of GlcNAc for
442 protection of GAS peptidoglycan from amidase-induced lysis. In *E. faecalis* the GacI
443 homolog, EpaI, is involved in biosynthesis of a cell wall-attached polysaccharide (24),
444 however the polysaccharide structure of the mutant has not been elucidated. The Δ*epaI*
445 mutant was defective in conjugative transfer of a plasmid and resistance of bacteria to
446 detergent and bile salts (24).
447 In conclusion, our study provides a platform for elucidation of novel pathways of
448 glycopolymer biosynthesis in other bacterial pathogens including important drug-

449 resistant bacteria such as *E. faecalis* and *Clostridium sordellii* that possess GacI and
450 GacJ homologs. Since the enzymes involved in the biosynthesis of cell wall attached
451 glycopolymers are promising targets for novel antimicrobials and the glycopolymers
452 represent important features for diagnostics and vaccine targets, our data may provide
453 opportunities for developing novel therapeutics against antibiotic-resistant bacterial
454 pathogens.

455 **MATERIALS AND METHODS**

456 **Bacterial strains and growth conditions.** All plasmids, strains and primers used in
457 this study are listed in Tables S1 and S2 in the supplemental material. The strains used
458 in this study were GAS M1-serotype strain MGAS5005 (16), *Bacillus cereus* (ATCC
459 14579), *Streptococcus agalactiae* COH1 (Group B *Streptococcus* or GBS), *E. coli*
460 CLM37 (46), *E. coli* PR4019 (12), *E. coli* DH5α and *E. coli* Rosetta (DE3). GAS and
461 GBS cultures were grown in Todd-Hewitt broth (BD) supplemented with 0.2% yeast
462 extract (THY), or on THY agar plates at 37 °C. *E. coli* and *B. cereus* strains were grown
463 in Luria-Bertani (LB) medium or on LB agar plates at 37 °C. When required, antibiotics
464 were included at the following concentrations: ampicillin at 100 μg ml^{-1} for *E. coli*;
465 streptomycin at 100 μg ml^{-1} for *E. coli*; erythromycin at 500 μg ml^{-1} for *E. coli* and 1 μg
466 ml^{-1} for GAS and GBS; chloramphenicol at 10 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for GAS;
467 spectinomycin at 200 μg ml^{-1} for *E. coli* and 100 μg ml^{-1} for GAS and GBS.

468 **DNA techniques.** Plasmid DNA was isolated from *E. coli* by commercial kits
469 (Qiagen) according to the manufacturer's instructions and used to transform *E. coli*,
470 GAS and GBS strains. Plasmids were transformed into GAS and GBS by
471 electroporation as described previously (47). Chromosomal DNA was purified from GAS
472 and GBS as described in (48). To construct single-base substitutions or deletion
473 mutations, we used the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene)
474 according to the manufacturer's protocol. Constructs containing mutations were
475 identified by sequence analysis. Primers for site-directed mutagenesis are listed in
476 Table S3. All constructs were confirmed by sequencing analysis (Eurofins MWG
477 Operon).

478 **Construction of the *gacI* deletion mutant in GAS.** For construction of strain
479 5005Δ*gacI*, MGAS5005 chromosomal DNA was used as a template for amplification of

480 two DNA fragments using two primers pairs: GacIm-BamHI-f/GacIdel-r and GacIdel-
481 f/GacIm-XhoI-r (Table S2). Primer GacIdel-f is complementary to primer GacIdel-r. The
482 two gel-purified PCR products containing complementary ends were mixed and
483 amplified using a PCR overlap method (49) with primer pair GacIm-BamHI-f/GacIm-
484 XhoI-r to create the deletion of *gacI*. The PCR product was digested with BamHI and
485 XhoI and ligated into BamHI/SalI-digested temperature-sensitive shuttle vector
486 pJRS233 (50). The plasmid was designated pJRS233Δ*gacI* (Table S1). The resulting
487 plasmid was transformed into MGAS5005, and erythromycin resistant colonies were
488 selected on THY agar plates at 30 °C. Integration was performed by growth of
489 transformants at 37 °C with erythromycin selection. Excision of the integrated plasmid
490 was performed by serial passages in THY media at 30 °C and parallel screening for
491 erythromycin-sensitive colonies. Mutants were verified by PCR sequencing of the loci.
492 **Construction of the *gacL* deletion mutant in GAS.** To create BglII and XhoI
493 cloning sites in the polylinker region of pUC19, site-directed mutagenesis of the
494 plasmid, using the primer pair listed in Table S3 was carried out. The plasmid was
495 designated pUC19BX. The nonpolar *aadA* spectinomycin resistance cassette was
496 amplified from pLR16T (Table S2) using primers Spec-SalI-f/Spec-BamH-r (Table S2),
497 digested with SalI/BamHI, and ligated into SalI/BamHI-digested pUC19BX to yield
498 pUC19BXspec. MGAS5005 chromosomal DNA was used as a template for
499 amplification of two DNA fragments using two primers pairs: gacLup-BglII-f/gacLup-SalI-
500 r and gacLdown-BamHI-f/gacLdown-XhoI-r (Table S2). The first PCR product was
501 digested with BglII/SalI and ligated into BglII/SalI-digested pUC19BXspec. The resultant
502 plasmid, pUC19BXspecL1, was digested with BamHI/XhoI and used for ligation with the
503 second PCR product that was digested with BamHI/XhoI. The resultant plasmid,
504 pUC19BXspecL2, was digested with BglII and XhoI to obtain a DNA fragment
505 containing *aadA* flanked with the *gacL* upstream and downstream regions. The DNA
506 fragment was ligated into pHY304 vector (51) and digested with BamHI/XhoI to yield
507 pHY304Δ*gacL*. The resulting plasmid was transformed into MGAS5005, and
508 erythromycin resistant colonies were selected on THY agar plates at 30 °C. The
509 mutants were isolated as describe above. 5005Δ*gacL* mutants were screened for
510 sensitivity to spectinomycin and verified by PCR sequencing of the loci.

511 **Complementation of the 5005Δ*gacL* mutant with *gacL* (5005Δ*gacL gacL*$^{+}$).**
512 To construct the plasmid for complementation of the 5005Δ*gacL* mutant, MGAS5005
513 chromosomal DNA was used as a template for amplification of a wild-type copy of *gacL*
514 using the primer pair GacL-XhoI-f and GacL-BglII-r (Table S2). The PCR products were
515 digested with XhoI and BglII and cloned in pBBL740 (Table S1) previously digested with
516 the respective enzymes. The integrational plasmid pBBL740 does not have a replication
517 origin that is functional in GAS, so the plasmid can be maintained only by integrating
518 into the GAS chromosome through homologous recombination. The resultant plasmid
519 pGacL was transformed into 5005Δ*gacL* by electroporation and transformants were
520 selected on agar plates containing chloramphenicol. Several chloramphenicol resistant
521 colonies were selected and *gacL* integration into the chromosome was confirmed by
522 sequencing a PCR fragment.
523 **Construction of the *gacI* (**SAN_1536) **deletion mutant in GBS.** GBS COH1
524 chromosomal DNA was used as a template for amplification of two DNA fragments
525 using two primers pairs: gbsIup-BglII-f/gbsIup-SalI-r and gbsId-BamHI-f/gbsId-XhoI-r
526 (Table S2). The plasmid for *gacI* (SAN_1536) knock-out was constructed using the
527 same strategy described for *gacL* deletion (see above). The resultant plasmid,
528 pHY304GBSΔI, was transformed into GBS COH1, and erythromycin resistant colonies
529 were selected on THY agar plates at 30 °C. The mutants were isolated as described
530 above. GBS COH1 Δ*gacI* mutants were screened for sensitivity to spectinomycin and
531 verified by PCR sequencing of the loci.
532 **Construction of the plasmids for *E. coli* expression of GacI, GacJ and GacO.**
533 To create a vector for expression of GacI from GAS, the gene was amplified from
534 MGAS5005 chromosomal DNA using the primer pair GacI-NcoI-f and GacI-XhoI-r
535 (Table S2). The PCR product was digested with NcoI and XhoI, and ligated into
536 NcoII/XhoI-digested pRSF-NT vector (Table S1). The resultant plasmid, pGacI,
537 contained *gacI* fused at the N-terminus with a His-tag followed by a TEV protease
538 recognition site. To create a vector for expression of GacI and GacJ, the bicistronic
539 DNA fragment was amplified from MGAS5005 chromosomal DNA using the primer pair
540 GacI-NcoI-f and GacJ-XhoI-r (Table S2). The vector was constructed as described
541 above. The plasmid was designated pGacIJ. pET21_NESG plasmid for expression of

542 the GacI homolog, EpaI, from *Enterococcus faecalis* was obtained from the DNASU
543 repository (52). The construct was confirmed by sequencing analysis. The plasmids
544 were transferred into competent *E. coli* Rosetta (DE3) (Novagen) using the
545 manufacturer's protocol.
546 To create a vector for expression of GacO, the gene was amplified from MGAS5005
547 chromosomal DNA using the primer pair GacO-Xba-f and GacO-HindIII-r (Table S2).
548 The PCR product was digested with XbaI and HindIII, and ligated into XbaI/HindIII-
549 digested pBAD33 vector. The resultant plasmid, pGacO, was transferred into competent
550 *E. coli* CLM37 strain that has a deletion of the *wecA* gene.
551 **Construction of the plasmids for *E. coli* expression of PlyPy, CbpD and PlyC.**
552 To create a vector for expression of CbpD amidase (29), the gene was amplified from
553 MGAS5005 chromosomal DNA using the primer pair 28-NcoI-f and 28-stop-r (Table
554 S2). The PCR product was digested with NcoI and XhoI, and ligated into NcoI/XhoI-
555 digested pRSF-NT vector. The resultant plasmid, pCbpD, contained *cbpD* fused at the
556 N-terminus with a His-tag followed by a TEV protease recognition site. To create a
557 vector for expression of PlyPy amidase (28), the gene was amplified from MGAS5005
558 chromosomal DNA using the primer pair PlyPy-NcoI-f and PlyPy-XhoI-r. The PCR
559 product was digested with NcoI and XhoI, and ligated into NcoI/XhoI-digested pET-21d
560 vector. The resultant plasmid, pPlyPy contained the gene fused at the C terminus with a
561 His-tag.
562 To create a vector for expression of PlyC amidase (53), a DNA fragment spanning
563 bicistronic operon which encodes *plyCA* and *plyCB* (27) was synthesized by
564 ThermoFisher Scientific. The plasmid was digested with NcoI and XhoI, and ligated into
565 NcoI/XhoI-digested pET21d vector. The resultant plasmid, pPlyC contained *plyCA*
566 followed by *plyCB* fused at the C terminus with a His-tag.
567 **Expression and purification of GacI, EpaI and GacI/GacJ complex.** For
568 expression of GacI, EpaI and GacI/GacJ complex, *E. coli* Rosetta (DE3) cells carrying
569 the respective plasmid were grown to an OD_{600} of 0.4-0.6 and induced with 1 mM IPTG
570 at 18 °C for approximately 16 hours. The cells were lysed in 20 mM Tris-HCl pH 7.5,
571 300 mM NaCl with two passes through a EmulsiFlex-C5 microfluidizer cell disrupter
572 (Avestin, Inc., Ottawa Ontario,CA). The lysate was centrifuged at 7000 x g for 30

573 minutes, 4°C. The supernatant was centrifuged at 30,000 x g for 60 minutes, 4°C to
574 isolate the membrane fraction.

575 To isolate GacI/GacJ complexes the membrane proteins were solubilized in 2.5%
576 CHAPS, 20 mM Tris-HCl pH 7.5, 300 mM NaCl for 60 minutes, rotating at room
577 temperature. Insoluble material was removed by centrifugation at 30,000 x g for 60
578 minutes, 4°C. Solubilized GacI/GacJ were purified by Ni-NTA chromatography with
579 washes of 2.5% CHAPS, 20 mM Tris-HCl pH 7.5, 300 mM NaCl and 2.5% CHAPS, 20
580 mM Tris-HCl pH7.5, 300 mM NaCl, 10 mM imidazole, and elution with 2.5% CHAPS, 20
581 mM Tris-HCl pH 7.5, 300 mM NaCl, 250 mM imidazole. The GacI/GacJ complex was
582 further purified by size exclusion chromatography on a Superdex 200 (GE Biosciences)
583 column in 2.5% CHAPS, 20 mM HEPES pH 7.5, 100 mM NaCl, with monitoring for
584 protein elution at 280 nm.

585 **Expression of GacO.** For expression of GacO, *E. coli* CLM37 cells carrying the
586 pertinent plasmid were grown to an OD_{600} of 0.8 and induced with 13 mM L-arabinose
587 at 25 °C for approximately 3 hours. The cells were lysed in 20 mM Tris-HCl pH 7.5, 300
588 mM NaCl with two passes through a microfluidizer cell disrupter. The lysate was
589 centrifuged at 1000 x g for 15 minutes, 4 °C. The supernatant was centrifuged at 40,000
590 x g for 60 minutes, 4 °C to isolate the membrane fraction.

591 **Expression and purification of PlyPy, CbpD and PlyC.** For expression and
592 purification of PlyPy, CbpD and PlyC, *E. coli* Rosetta (DE3) cells carrying the respective
593 plasmids were grown to an OD_{600} of 0.4-0.6 and induced at 18 °C with 1 mM IPTG for
594 approximately 16 hours. The cells were lysed in 20 mM Tris-HCl pH 7.5, 300 mM NaCl
595 with two passes through a microfluidizer cell disrupter. The soluble fraction was purified
596 by Ni-NTA chromatography with washes of 20 mM Tris-HCl pH 7.5, 300 mM NaCl and
597 20 mM Tris-HCl pH7.5, 300 mM NaCl, 10mM imidazole, and elution with 20 mM Tris-
598 HCl pH 7.5, 300 mM NaCl, 250 mM imidazole. The PlyPy and CbpD eluate was further
599 purified by size exclusion chromatography on a Superdex 200 column in 20 mM HEPES
600 pH 7.5, 100 mM NaCl for PlyPy, or 20 mM MOPS pH 6.5, 100 mM NaCl for CbpD. PlyC
601 was further purified by anion exchange chromatography on a MonoQ 5/50 GL (GE
602 Biosciences) column in 10 mM sodium phosphate pH 6.0, and a 20 column volume
603 elution gradient of 0-500 mM NaCl.

604 **Isolation of GAS and GBS membranes.** Bacteria were grown at 37 °C to an OD_{600}
605 of 0.8. To obtain GAS cell membranes, cell pellet was re-suspended in phosphate-
606 buffered saline (PBS) and incubated 1 h with PlyC lysin as described in (54). To obtain
607 GBS membranes, the cell pellet was re-suspended in acetate buffer (50 mM
608 CH_3COONa, 10 mM CaCl, 50 mM NaCl, pH 5) and incubated with mutanolysin (200
609 U/ml, Sigma–Aldrich) for 2 h at 37 °C. After hydrolytic enzyme treatment the bacterial
610 suspension was sonicated using a Fisher Scientific™ Model 505 Sonic Dismembrator,
611 15 times ×15 s. After centrifugation at 8,000 *g* for 10 min at 4 °C, the supernatant was
612 collected and centrifuged at 40,000 *g* for 60 min. The pellet was collected as the
613 membrane fraction.

614 **Mass-spectrometry analysis of GacI and GacJ.** LC-MS/MS for proteomic
615 analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher
616 Scientific) coupled with an Eksigent Nanoflex cHiPLC system (Eksigent) through a
617 nanoelectrospray ionization source. The LC-MS/MS data were subjected to database
618 searches for protein identification using Proteome Discoverer software V. 1.3 (Thermo
619 Fisher Scientific) with a local MASCOT search engine.

620 **Dot-blot analysis.** Bacterial cells (1 ml) from exponential phase cultures
621 (OD_{600}=0.8) was centrifuged, washed with PBS, resuspended in 100 µl of PBS and
622 incubated with 1 µl PlyC (1.5 mg/ml) for 1 h at 37°C. After centrifugation at 16,000 *g* for
623 2 min, 5 µl of the supernatant was spotted on a nitrocellulose membrane. The
624 membrane was blocked 1 h with 7% skim milk in PBS with 0.1% Tween 20, and
625 incubated overnight with an anti-GAC antibody diluted 1:5,000 (ab9191; Abcam). Bound
626 antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin
627 G antibody, and the Amersham ECL (enhanced chemiluminescence) Western blotting
628 system.

629 **Binding of succinylated wheat germ agglutinin (sWGA) to bacteria.** MGAS5005
630 and 5005Δ*gacL* cells were collected during mid-exponential phase (OD_{600}=0.6), washed
631 three times with BSA-saline solution (0.5% BSA, 0.15 M NaCl), resuspended in BSA-
632 saline solution and incubated for 30 minute at 37 °C with mixing. GlcNAc specific
633 fluorescein-sWGA was added to final concentrations of 0, 12.5, 25, 50, 100 µg/ml. After
634 1 h of incubation at 37 °C with mixing, the cells were centrifuged, washed twice, and

635 resuspended in BSA-saline. Bound fluorescein-sWGA was quantified in a fluorimeter
636 SpectraMax M5 (Molecular Devices) using an excitation of 544 nm and emission of 590
637 nm.

638 **Isolation of cell wall from GAS.** MGAS5005 and 5005Δ*gacL* cell wall was
639 prepared from exponential phase cultures (OD_{600}=0.8) by the SDS-boiling procedure as
640 described for *Streptococcus pneumoniae* (55). Purified cell wall samples were
641 lyophilized and used for composition analysis.

642 **Carbohydrate composition analysis.** Carbohydrate composition analysis was
643 performed at the Complex Carbohydrate Research Center (Athens, GA) by combined
644 gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS)
645 derivatives of the monosaccharide methyl glycosides produced from the sample by
646 acidic methanolysis as described previously by (56).

647 **Assay of lytic activity of PlyPy, CbpD and PlyC.** Aliquots of MGAS5005 and
648 5005Δ*gacL* frozen stocks [prepared as described in (57)] were inoculated into THY
649 medium 1:20. To a 96 well plate, 200 µL aliquots of culture were dispensed in duplicate,
650 per condition. The plates were grown at 37 °C without aeration for 2 hours. An assay
651 plate was set up containing PlyPy (8 µg mL^{-1}, 16 µg mL^{-1}, 32 µg mL^{-1}), CbpD (0.31 µg
652 mL^{-1}, 0.63 µg mL^{-1}, 1.26 µg mL^{-1}) or PlyC (1.6 ng mL^{-1}, 3.1 ng mL^{-1}, 6.2 ng mL^{-1}) in a
653 volume of 100 µL. To the assay plate, 100 µL of culture was added to each well giving
654 final concentrations of: PlyPy (4 µg mL^{-1}, 8 µg mL^{-1}, 16 µg mL^{-1}), CbpD (0.16 µg mL^{-1},
655 0.31 µg mL^{-1}, 0.63 µg mL^{-1}), or PlyC (0.8 ng mL^{-1}, 1.6 ng mL^{-1}, 3.1 ng mL^{-1}). After
656 mixing, the absorbance at 600 nm was measured for each well (t_0) and the plate was
657 incubated at 37 °C for 1 h. The absorbance at 600 nm was then measured again (t_1).
658 The percentage difference (t_1-t_0) in growth relative to 0 mM of enzyme/antimicrobial, for
659 each concentration was calculated (i.e. 0 mM PlyPy = 100%).

660 **Extraction and characterization of bacterial glycolipids.** Two liters of bacterial
661 cells from exponential phase cultures (OD_{600}=0.8) were recovered by sedimentation at
662 10,000 x g for 30 min, and washed with ice-cold PBS 2x. Cells were resuspended in 50
663 ml PBS, sensitized by incubation with 10 ng mL^{-1} PlyC for 1 h at 37 °C and stirred
664 vigorously with two volumes of CH_3OH and one volume of $CHCl_3$ for 30 min at room
665 temperature. The mixture was divided into 5 mL aliquots in 12 mL glass centrifuge

666 tubes. Insoluble material was removed by centrifugation at 200 *g* and the organic
667 extract was transferred to a separatory funnel. The insoluble residue was further
668 extracted with 1 mL of $CHCl_3/CH_3OH$ (2:1) per tube, two times, and the extracts were
669 combined with the previous organic phase. The organic extract was supplemented with
670 $CHCl_3$ and 0.9% NaCl to give a final composition of $CHCl_3/CH_3OH$/0.9% NaCl (3:2:1),
671 mixed vigorously, and allowed to stand in the cold until phase separation was achieved.
672 The organic phase was drained off into a second separatory funnel and the organic
673 layer was washed with 1/3 volume of $CHCl_3/CH_3OH$/0.9% saline (3:48:47), two times.
674 The aqueous layers were discarded. The organic extract was dried on a vacuum rotary
675 evaporator, dissolved in a small volume of $CHCl_3/CH_3OH$ (2:1) and transferred to a 12.5
676 x 100 mm screw cap glass tube (with Teflon lined cap). The organic extract was dried
677 under a stream of nitrogen gas and the glycerolipids were destroyed by deacylation in
678 0.1 M KOH in toluene/CH_3OH (1:3) at 0 °C, 60 min. Following deacylation, the reactions
679 were neutralized with acetic acid, diluted with two volumes of $CHCl_3$, 1 volume of
680 $CHCl_3/CH_3OH$ (2:1) and 1/5 volume of 0.9% NaCl/10 mM EDTA. The two-phase mixture
681 was mixed vigorously and centrifuged to separate the phases. The organic phase was
682 dried under nitrogen, spotted on a 20 x 20 cm sheet of Silica Gel G and developed in
683 $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:4:1). Bacterial lipids were visualized by staining with
684 iodine vapors and pertinent spots were scraped from the thin layer plate, eluted from the
685 silica gel with $CHCl_3/CH_3OH$ (2:1) and reserved for further analysis.
686 **Mass spectrometry analysis of a phospholipid isolated from 5005Δ*gacL*.** A
687 phospholipid accumulated by 5005Δ*gacL* was purified as described above using
688 preparative TLC in silica gel. The compound was analyzed by LC-MS using a Q-
689 exactive mass spectrometer and an Ultimate 3000 ultra high performance liquid
690 chromatography system (Thermo Fisher Scientific, San Jose, CA) on a Kinetex C18
691 reversed-phase column (2.6 mm × 100 mm, 2.1 µm, Phenomenex, USA). Two solvents
692 were used for gradient elution: (A) acetonitrile/water (2:3, v/v), (B)
693 isopropanol/acetonitrile (9:1, v/v). Both A and B contained 10 mM ammonium formate
694 and 0.1% formic acid. The column temperature was maintained at 40 °C, and the flow
695 rate was set to 0.25 ml/min. Mass spectrometric detection was performed by
696 electrospray ionization in negative ionization mode with source voltage maintained at

697 4.0 kV. The capillary temperature, sheath gas flow and auxiliary gas flow were set at
698 330 °C, 35 arb and 12 arb, respectively. Full-scan MS spectra (mass range m/z 400 to
699 1500) were acquired with resolution R = 70,000 and AGC target 5e5. MS/MS
700 fragmentation was performed using high-energy C-trap dissociation with resolution R =
701 35,000 and AGC target 1e6. The normalized collision energy was set at 30.

702 **Assay for incorporation of GlcNAc into polyisoprenol-linked glycolipid**
703 **intermediates.** Reaction mixtures for measuring the incorporation of [^{3}H]GlcNAc into
704 lipids contained 50 mM Tris, pH 7.4, 0.25 M sucrose, 20 mM $MgCl_2$, 0.5 mM β-
705 mercaptoethanol, 5 μM UDP-[6-^{3}H]GlcNAc [(100-2000 cpm/pmol) American
706 Radiolabelled Chemicals] and bacterial membrane suspension (50-250 μg bacterial
707 membrane protein) in a total volume of 10 to 100 μL. In some experiments, 1 mM ATP
708 was included, as indicated. Following incubation at 30 °C, the enzymatic reactions were
709 terminated by the addition of 40 volumes of $CHCl_3/CH_3OH$ (2:1), thoroughly mixed and
710 incubated for 5 min at room temperature. Insoluble material was sedimented at 200 *g*
711 and the organic extract was transferred to a 12x100 mm glass tube. The residue was
712 re-extracted with 1 ml $CHCl_3/CH_3OH$ (2:1), two times, and the organic extracts were
713 combined. The pooled organic extracts were freed of unincorporated radioactivity by
714 sequential partitioning with 1/5 volume of 0.9% saline/10 mM EDTA and then with 1/3
715 volume of $CHCl_3/CH_3OH$/0.9% saline (3:48:47) 3 times, discarding the aqueous phase
716 each time. The washed organic phases were dried under nitrogen and re-dissolved in
717 $CHCl_3/CH_3OH$ (2:1). A carefully measured aliquot was removed and analyzed for
718 radioactivity by liquid scintillation spectrometry after drying. The remainder of the
719 sample was analyzed by thin layer chromatography on Silica Gel G, developed in
720 $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:4:1), using a BioScan AR2000
721 radiochromatoscanner. Incorporation of [^{3}H]GlcNAc into individual [^{3}H]GlcNAc-lipids
722 was calculated using the peak integration values obtained from the BioScan.

723 **Degradation of GlcNAc-lipids by mild acid and mild alkaline treatment.**
724 Bacterial lipids were subjected to mild acid hydrolysis (50% isopropanol, 0.1 M HCl, 50
725 °C, 1 h) and mild alkaline methanolysis (0.1 M KOH in toluene/CH_3OH (1:3), 0 °C, 1 h).
726 Following incubation, the reactions were neutralized with either 1 M Tris or concentrated
727 acetic acid, diluted with $CHCl_3$, CH_3OH and 0.9% NaCl to give a final composition of

728 3:2:1, respectively and partitioned as described above. The organic phases were dried
729 under nitrogen and either quantified for radioactivity or analyzed by thin layer
730 chromatography and detected by iodine staining.

731 **Phenolysis of GlcNAc-P-Und.** Purified [^{3}H]GlcNAc-P-Und was dried under
732 nitrogen in a 12x100 mm conical screw-cap tube and heated to 68 °C in 0.2 ml 50%
733 aqueous phenol as described by Murazumi et al. (22). Following phenolysis, 0.1 ml
734 water was added and the samples were thoroughly mixed. The aqueous and phenolic
735 layers were separated, dried and analyzed for radioactivity by scintillation spectrometry.

736 **Analytical methods.** Membrane protein concentrations were determined using the
737 BCA protein assay kit (Pierce Chemical Co.) employing the method of Ruiz et al. (33).
738 Radioactivity was quantified by liquid scintillation spectrometry on a Packard Tri-Carb
739 Liquid Scintillation Spectrometer using Econosafe Complete Counting Cocktail
740 (Research Products International, Inc., Elk Grove IL).

741 **Phylogenetic analysis of GacI homologs.** Sequences of GacI homologs were
742 retrieved from the non-redundant database using BLAST (58). One thousand
743 sequences were downloaded and were manually curated to remove sequences with
744 shorter that 90% sequence length overlap. The redundancy was reduced using CD-HIT
745 with 0.98 cut off (59). Sequences were manually curated to correct misannotated
746 translation start codons. The pair-wise similarities were analyzed and visualized using
747 CLANS with an E-value cut off 1 e^{-80} (40, 60, 61).

748 **Bioinformatics tools.** The TOPCONS (http://topcons.net/) (62) web server was
749 employed to predict trans-membrane regions of GacI, GacJ and GacL. Homology
750 detection and structure prediction were performed by the HHpred server
751 (https://toolkit.tuebingen.mpg.de/#/tools/hhpred) (63).

752 **Statistical Analysis.** Unless otherwise indicated, statistical analysis was carried out
753 from at least three independent experiments. Quantitative data was analyzed using the
754 paired Student's t-test. A P value equal to or less that 0.05 is considered statistically
755 significant.

756

757 **Supplemental Material**

758 Additional Supplemental Material may be found in the online version of this article at the
759 publisher's web-site.
760 Table S1
761 Table S2
762 Table S3
763 Table S4
764 Figure S1
765 Figure S2
766 Figure S3
767 Figure S4
768 Figure S5
769 Figure S6
770 Figure S7
771 Supplemental File 1
772
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1001

1002

1003 Tables

1004 Table 1. The major fragments of the precursor ion at *m/z* 1048

m/z	Formula	Mass error (ppm)
78.95731	PO_3	-8.19
96.96791	H_2PO_4	-6.31
300.04883	$C_8H_{15}O_9NP$	3.12
845.65894	$C_{58}H_{88}ONP$	-1.02

1005 Fragment ions originating from the precursor molecular ion of 1048 were subjected to
1006 MS/MS fragmentation as described in materials and methods. The proposed molecular
1007 formulae of the product ions are supported by the observed isotope patterns for the
1008 relevant ions.

1009

1010

1011 Table 2. GlcNAc-lipid synthesis in membrane fractions from various bacterial strains.[a]

1012

Enzyme Source	GlcNAc-P-Und, pmol/mg	GlcNAc-P-P-Und, pmol/mg
MGAS5005	259.4±8.2	7.8±1.8
5005Δ*gacI*	Not Detected	15.9±2.5
GBS COH1	62±0.4	5.6±0.8
GBSΔ*gacI*	Not Detected	12±2
E. coli Rosetta (DE3)	Not Detected	19.5±0.3
E. coli:GacI	102.8±3.3	5.4±0.05
E. coli:EpaI	189±3.9	7±1.5

1013

1014 [a]Reaction mixtures contained 50 mM Tris-HCl, pH 7.4, 5 mM 2-mercaptoethanol, 20
1015 mM $MgCl_2$, 1 mM ATP, 5 μM UDP-[^{3}H]GlcNAc (486 cpm/pmol) and bacterial membrane
1016 suspension (50-100 μg membrane protein) in a total volume of 0.01 ml. Following a 10
1017 minute pre-incubation at 30°C, GlcNAc-lipid synthesis was initiated by the addition of
1018 UDP-[^{3}H]GlcNAc. After 5 min, reactions were processed for GlcNAc-lipid synthesis as
1019 described in Materials and Methods. The results are representative of at least three
1020 separate experiments.

1021

1022 Table 3. Kinetic parameters of GlcNAc-lipid synthases in membrane fractions from
1023 various bacterial strains.[a]
1024

Enzyme source	GlcNAc-P-Und		GlcNAc-P-P-Und	
	Km, μM	Vmax, pmol/min/mg	Km, μM	Vmax, pmol/min/mg
MGAS5005	6.4	333	ND	ND
5005Δ*gacI*	ND	ND	19.3	2.75
E. coli:GacI	18.7	54.2	ND	ND
E. coli:GacI/J	1.1	18,500	ND	ND

1025 [a]Membrane fractions from the indicated bacterial strains were assayed for the formation
1026 of [^{3}H]GlcNAc-lipids in the presence of increasing amounts of Und-P, added as a
1027 dispersion in 1% CHAPS. Apparent kinetic parameters for Und-P were calculated from
1028 Michaelis-Menton plots (Fig. S5) using the linear regression algorithm in Sigma Plot ver.
1029 12 (Systat Software, Inc. San Jose, CA). R squared values were 0.9953 for GAS GacI
1030 expressed in *E. coli*; 0.983 for GAS GacI co-expressed with GacJ in *E. coli*, 0.9976 for
1031 GacI in MGAS5005 and 0.931 for GacO in MGAS5005. The results are representative
1032 of at least two separate experiments. ND = Not Detected.
1033

1034

1035 **Figure legends:**

1036 **Figure 1.** Map of *S. pyogenes* genes involved in Group A Carbohydrate (GAC)
1037 biosynthesis and analysis of 5005Δ*gacI* and 5005 Δ*gacL* deletion mutants. (A) GAC
1038 biosynthesis gene cluster. Numbers below represent MGAS5005 gene designations (B)
1039 Representative immunoblot analysis of cell-wall fractions isolated from MGAS5005,
1040 5005Δ*gacI*, 5005Δ*gacL* and 5005Δ*gacL gacL*$^{+}$. Data are representative of biological
1041 triplicates. (C) Binding of N-acetyl glucosamine specific fluorescein-succinylated-WGA
1042 to whole MGAS5005 and 5005Δ*gacL* was measured. Data are the average of three
1043 replicates ± standard deviation. (D) Rhamnose and GlcNAc mole percentage of total
1044 carbohydrate was determined by GC-MS for cell wall material isolated from MGAS5005
1045 and 5005Δ*gacL* following methanolysis as described in Materials and Methods. Data
1046 are the average of four replicates ± standard deviation. The asterisk indicate statistically
1047 different values (* $p < 0.05$, ** $p < 0.01$) as determined by the Student's t-test. (E and F)
1048 GC-MS chromatograms for glycosyl composition analysis of cell wall isolated
1049 MGAS5005 and 5005Δ*gacL*. The deduced schematic structure of the repeating unit of
1050 GAC is shown for each strain. The chromatograms are representative of four separate
1051 analyses performed on two different cell wall preparations.

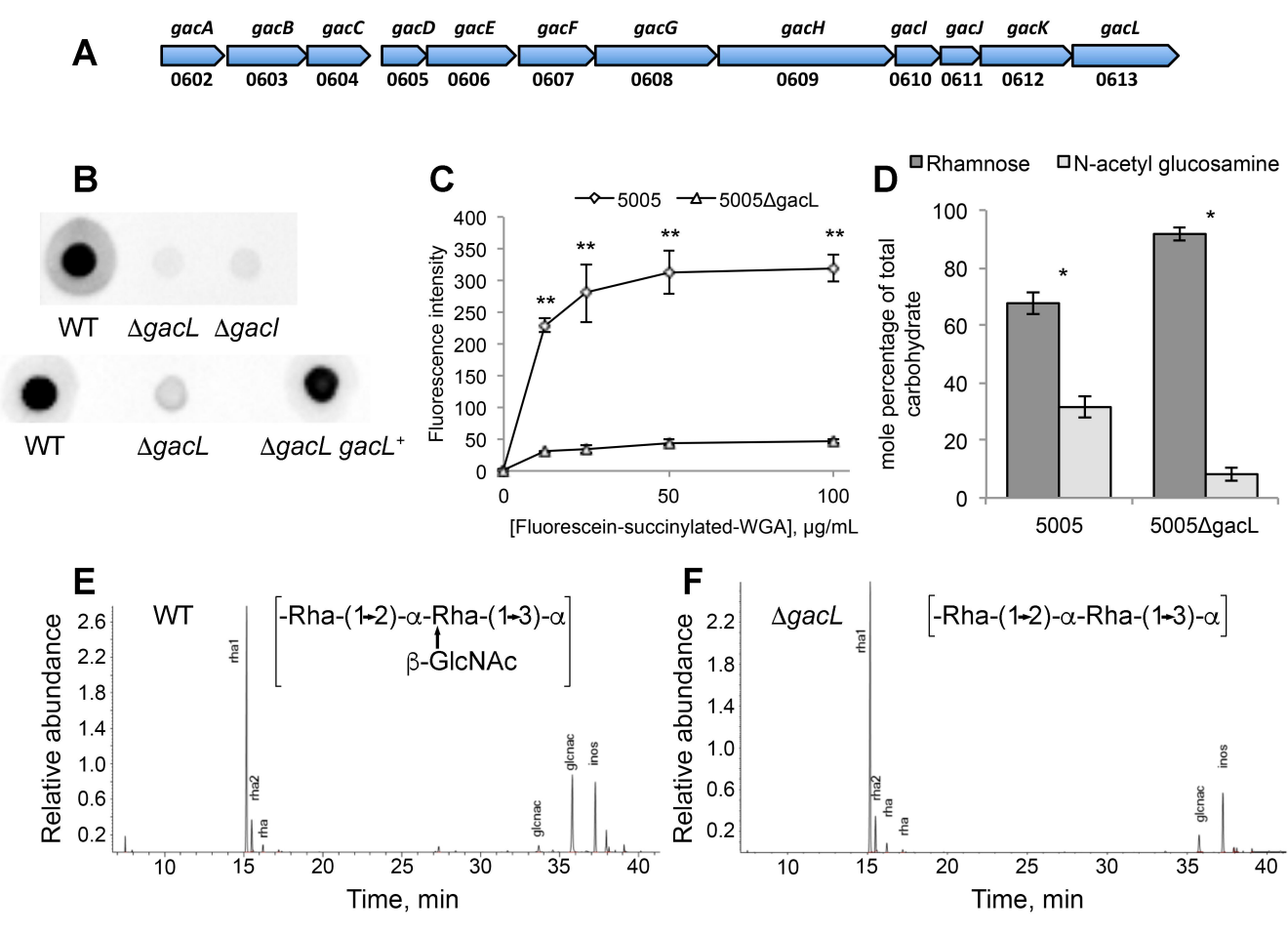
1052 **Figure 2.** Purification and identification of GlcNAc-phosphate-undecaprenol in
1053 5005Δ*gacL.* (A) Thin layer chromatography (TLC) analysis of phospholipids isolated
1054 from MGAS5005 and 5005Δ*gacL*. Phospholipids extracted from bacterial strains were
1055 separated by TLC on Silica Gel G in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:4:1). Position of
1056 the novel, alkaline-resistant and acid-labile, phospholipid accumulating in 5005Δ*gacL* is
1057 indicated by the arrow. The results are representative of three separate experiments.

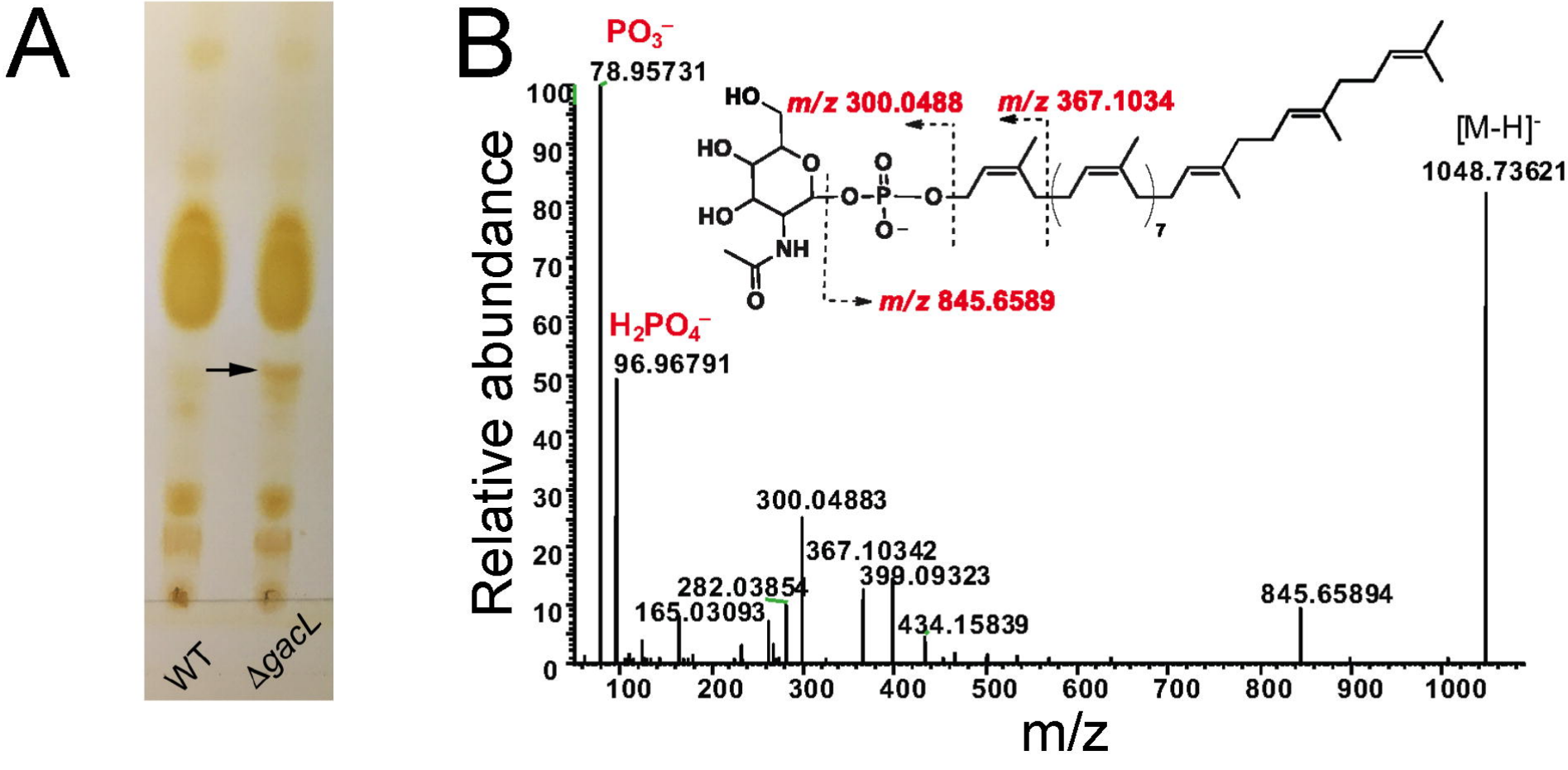
1058 (B) ESI-MS/MS analysis of the novel phospholipid isolated from 5005Δ*gacL*. The
1059 spectrum is assigned to GlcNAc-phosphate-undecaprenol.
1060 **Figure 3.** Thin layer chromatography of [^{3}H]GlcNAc-lipids from *in vitro* incubations of
1061 GAS mutants and *Bacillus cereus* membranes with UDP-[^{3}H]GlcNAc. Membrane
1062 fractions from MGAS5005 (panel A), *B. cereus* (Panel B), 5005Δ*gacI* (Panel C), or
1063 5005Δ*gacL* (Panel D) were incubated with UDP-[^{3}H]GlcNAc and analyzed for
1064 [^{3}H]GlcNAc lipid synthesis by thin layer chromatography. Reaction mixtures contained
1065 50 mM Tris-Cl, pH 7.4, 5 mM 2-mercaptoethanol, 20 mM $MgCl_2$, 1 mM ATP, 5 μM UDP-
1066 [^{3}H]GlcNAc (486 cpm/pmol) and bacterial membrane suspension (100-200 μg
1067 membrane protein) in a total volume of 0.02 ml. Following a 10 minute pre-incubation at
1068 30°C, GlcNAc-lipid synthesis was initiated by the addition of UDP-[^{3}H]GlcNAc. After 10
1069 min, reactions were processed for GlcNAc-lipid synthesis as described in Materials and
1070 Methods. The organic layers were dried, dissolved in a small volume of $CHCl_3/CH_3OH$
1071 (2:1) and a portion was removed and assayed for radioactivity by liquid scintillation
1072 spectrometry. The remainder was spotted on 10 x 20 cm plate of silica gel G and
1073 developed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:1:4). [^{3}H]GlcNAc-lipids were detected
1074 by scanning with an AR2000 Bioscan Radiochromatoscanner. The results are
1075 representative of three separate experiments.
1076 **Figure 4.** Thin layer chromatography of [^{3}H]GlcNAc-lipids from *in vitro* incubations of
1077 GAS and GBS membranes with UDP-[^{3}H]GlcNAc. Membrane fractions from WT
1078 MGAS5005 (panel A) or GBS COH1 (Panel B) were incubated with UDP-[^{3}H]GlcNAc
1079 and analyzed for [^{3}H]GlcNAc-lipid synthesis by thin layer chromatography. Reaction
1080 mixtures were exactly as described in the legend to Figure 3. After 5 min incubation with

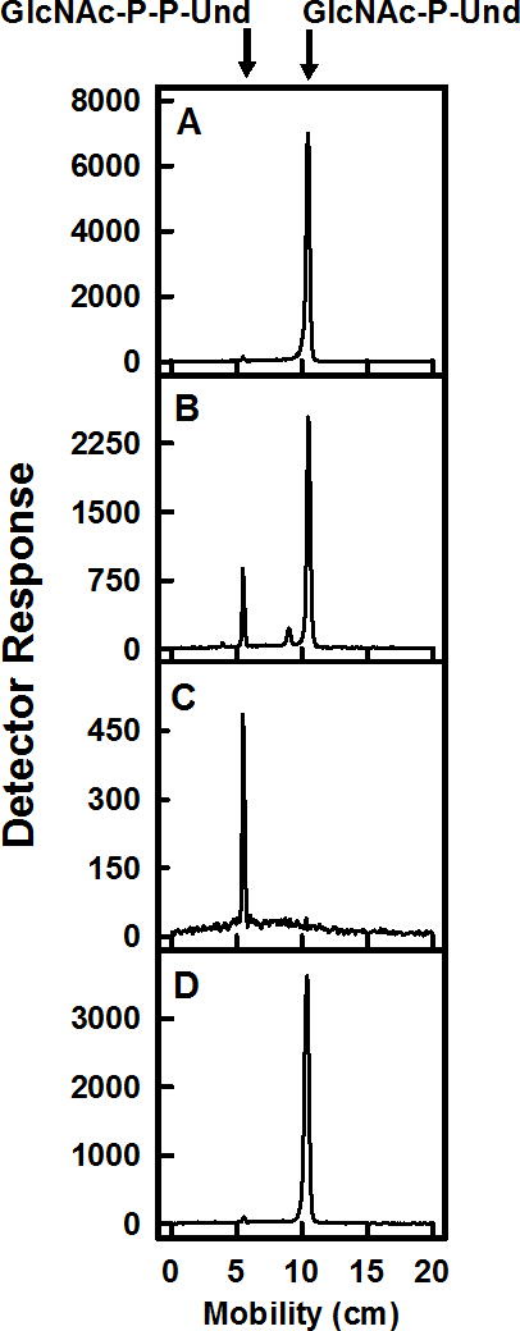
1081 UDP-[^{3}H]GlcNAc, reactions were processed for GlcNAc-lipid synthesis as described in
1082 Materials and Methods. The organic layers were dried, dissolved in a small volume of
1083 $CHCl_3/CH_3OH$ (2:1) and a portion was removed and assayed for radioactivity by liquid
1084 scintillation spectrometry. The remainder was spotted on 10 x 20 cm plate of silica gel G
1085 and developed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:1:4). [^{3}H]GlcNAc-lipids were
1086 detected by scanning with an AR2000 Bioscan Radiochromatoscanner. The results are
1087 representative of three separate experiments.
1088 **Figure 5**. Effect of ATP on GlcNAc-P-Und synthesis *in vitro* in MGAS5005 membrane
1089 fractions. GlcNAc-P-Und synthesis in MGAS5005 membranes was assayed after the
1090 indicated time at 30 °C in the presence (○) or absence (●) of 1 mM ATP. Reaction
1091 mixtures were identical to those described in Figure 3 except for the presence of ATP.
1092 Following incubation, incorporation into [^{3}H]GlcNAc-P-Und was determined as
1093 described in Materials and Methods. The results are representative of three separate
1094 **Figure 6.** GacI and GacJ exist as a detergent-stable complex in the membrane. GacJ
1095 and His-tagged GacI were co-expressed in *E. coli* Rosetta DE3 cells and extracted from
1096 the membrane fraction in 2.5% CHAPS. The proteins were purified using Ni-NTA
1097 agarose in the presence of 2.5% CHAPS. (A) Fractions collected during Ni-NTA
1098 purification were analyzed by immunoblot using anti-His antibodies. (B) The eluted
1099 proteins were analyzed by SDS-PAGE. The results are representative of three separate
1100 experiments.
1101 **Figure 7.** Analysis of GacO function in MGAS5005. (A) GacO catalyzes the synthesis of
1102 GlcNAc-P-P-Und in *E. coli* membranes**.** Reaction mixtures contained 50 mM Tris-HCl,
1103 pH 7.4, 5 mM 2-mercaptoethanol, 20 mM $MgCl_2$, 0.5 % CHAPS, 20 μM Und-P

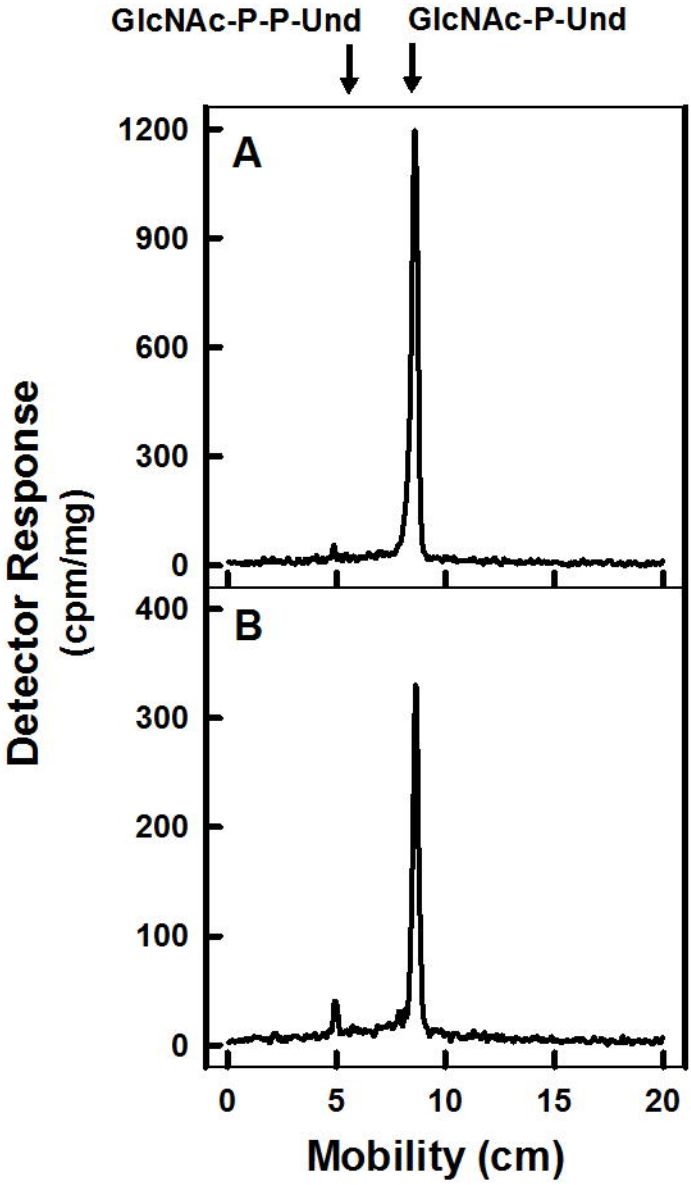
1104 (dispersed by ultrasonication in 1 % CHAPS), 5 μM UDP-GlcNAc (452 cpm/pmol) and
1105 *E. coli* membrane fraction from either PR4019, CLM37 or CLM37:GacO strains.
1106 Following incubation for 10 min at 30 ºC, incorporation of [^{3}H]GlcNAc into [^{3}H]GlcNAc-P-
1107 P-Und was determined as described in Materials and Methods. Data are the average of
1108 three replicates ± standard deviation.
1109 (B and C) GlcNAc-P-P-Und can function as an acceptor substrate for rhamnosylation to
1110 form Rhamnosyl-GlcNAc-P-P-Und in 5005Δ*gacI* membranes**.** Incubation conditions
1111 were as described in the legend to Figure 3. (B) After 35 minutes incubation with UDP-
1112 [^{3}H]GlcNAc, the reactions were analyzed for formation of [^{3}H]GlcNAc lipids as described
1113 in Figure 3. (C) After 5 minutes incubation with UDP-[^{3}H]GlcNAc, the reactions were
1114 incubated with 20 μM TDP-Rhamnose for an additional 30 min and analyzed for
1115 formation of [^{3}H]GlcNAc lipids as described in Figure 3. The results are representative
1116 of three separate experiments.
1117 **Figure 8.** The absence of GacL increases sensitivity to cell wall amidases. Mid-
1118 exponential phase MGAS5005 and 5005Δ*gacL* were grown in the indicated
1119 concentrations of (A) CbpD, (B) PlyPy, and (C) PlyC. The change in growth is
1120 represented as a percentage of growth where no amidase was present. Data are the
1121 average of three replicates ± standard deviation. The asterisk indicate statistically
1122 different values (** $p < 0.01$) as determined by the Student's t-test.
1123 **Figure 9.** Schematic diagram of GAC biosynthesis. GAC is anchored to peptidoglycan
1124 presumably via phosphodiester bond. GAC biosynthesis is initiated on the inner leaflet
1125 of the plasma membrane where GacO produces GlcNAc-P-P-Und which serves as a
1126 membrane-anchored acceptor for polyrhamnose synthesis catalyzed by the GacB,

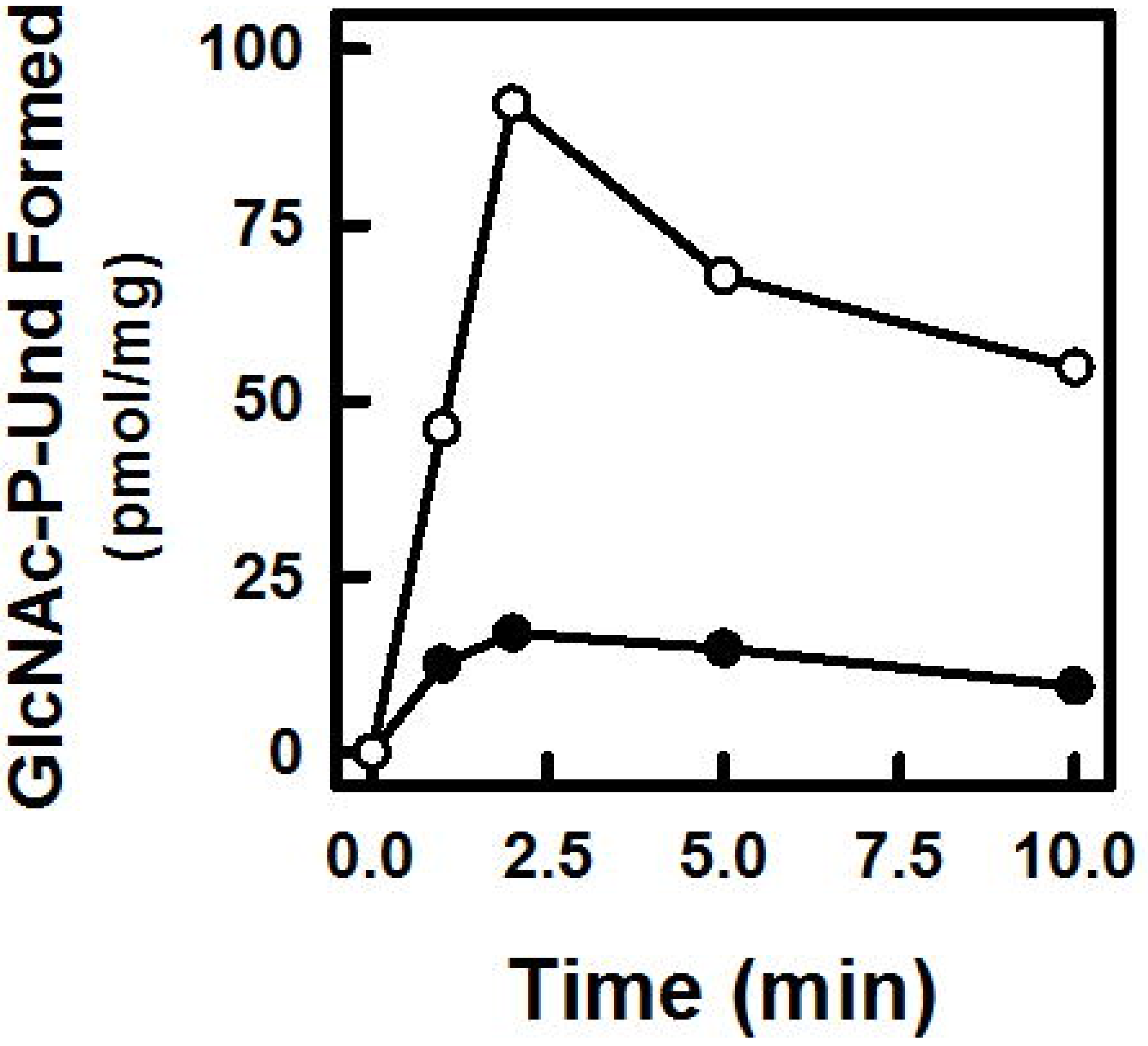
1127 GacC, GacF and GacG rhamnosyltransferases. Following polymerization polyrhamnose
1128 is transferred to the outer leaflet of the membrane presumably by the GacD/ GacE ABC
1129 transporter. Also in the inner leaflet of the membrane, GacI aided by GacJ produces
1130 GlcNAc-P-Und which then diffuses across the plasma membrane to the outer leaflet
1131 aided by GacK. Subsequently, GacL transfers GlcNAc to polyrhamnose using GlcNAc-
1132 P-Und as glycosyl donor. Lastly, protein members of LytR-CpsA-Psr
1133 phosphotransferase family presumably attach GAC to peptidoglycan. Several details of
1134 this biosynthetic scheme are still speculative and further research will be required to
1135 definitively confirm this hypothetical pathway, but the overall organization is consistent
1136 with other isoprenol-mediated capsular polysaccharide pathways.
1137 **Figure 10.** Sequence relationship of GacI family of proteins. Homology between GacI
1138 homologs is graphically displayed using CLANS analysis (40). Dots correspond to
1139 individual protein sequences selected as described in Material and Methods and
1140 provided as Supplementary File. Selected homologs of *S. pyogenes* are highlighted by
1141 colored dots.

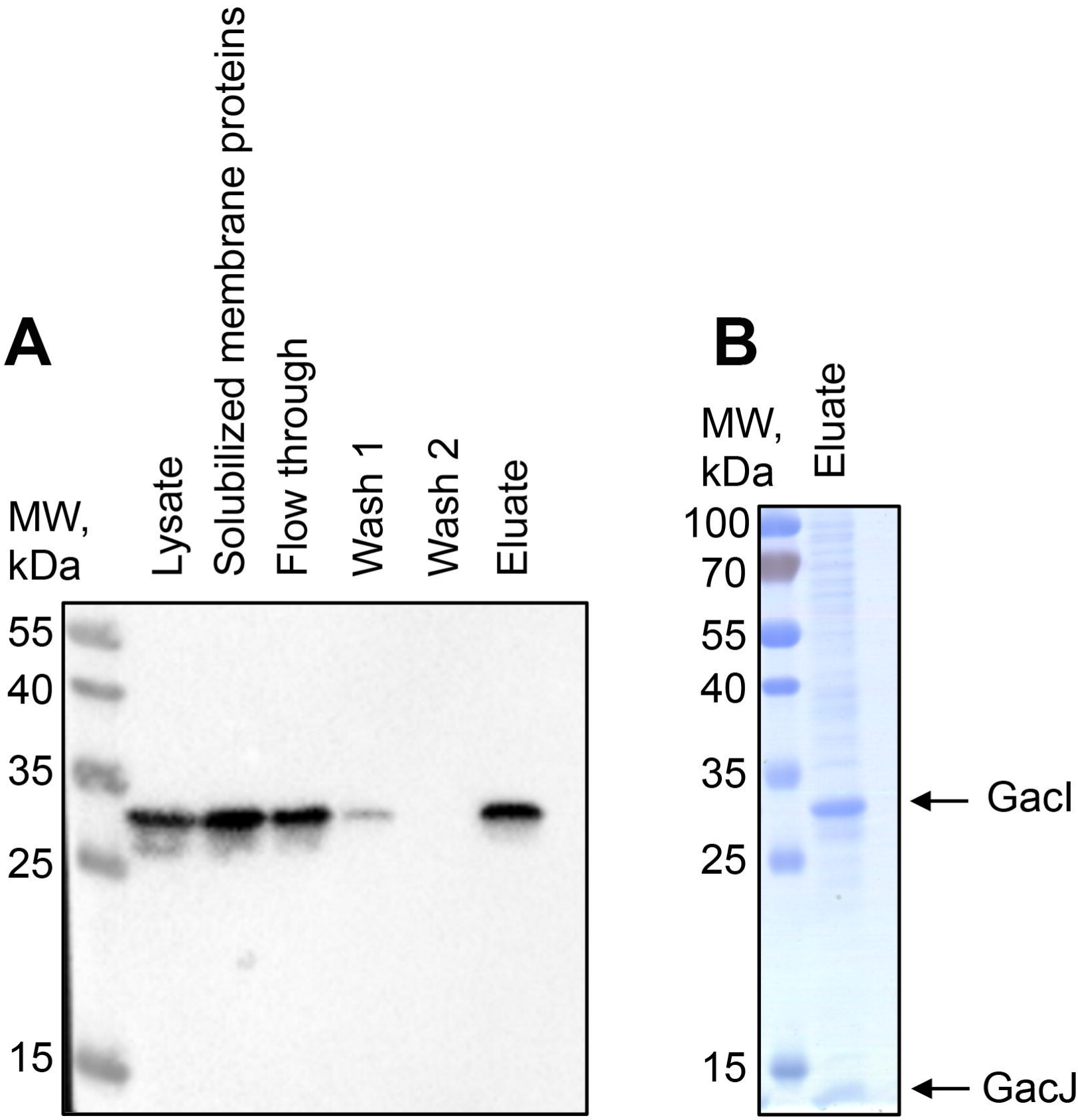

A
gacA gacB gacC gacD gacE gacF gacG gacH gacI gacJ gacK gacL
0602 0603 0604 0605 0606 0607 0608 0609 0610 0611 0612 0613
B
WT ΔgacL ΔgacI
WT ΔgacL ΔgacL gacL+
C
5005 5005ΔgacL
Fluorescence intensity
[Fluorescein-succinylated-WGA], μg/mL
D
Rhamnose N-acetyl glucosamine
mole percentage of total carbohydrate
5005 5005ΔgacL
E
WT
-Rha-(1→2)-α-Rha-(1→3)-α
β-GlcNAc
Relative abundance
Time, min
rha1 rha2 rha glcnac glcnac inos
F
ΔgacL
-Rha-(1→2)-α-Rha-(1→3)-α
Relative abundance
Time, min
rha1 rha2 rha rha glcnac inos

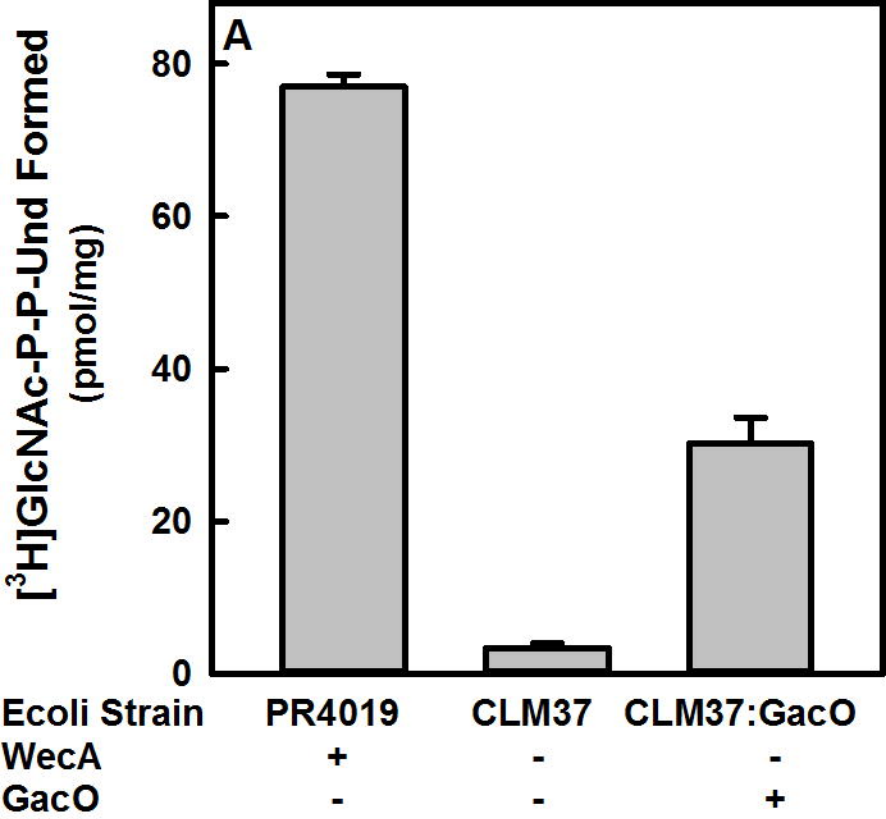

A
WT
ΔgacL
B
PO3−
78.95731
m/z 300.0488
m/z 367.1034
m/z 845.6589
[M-H]-
1048.73621
H2PO4−
96.96791
300.04883
367.10342
399.09323
282.03854
165.03093
434.15839
845.65894
Relative abundance
m/z
HO
NH
O
7

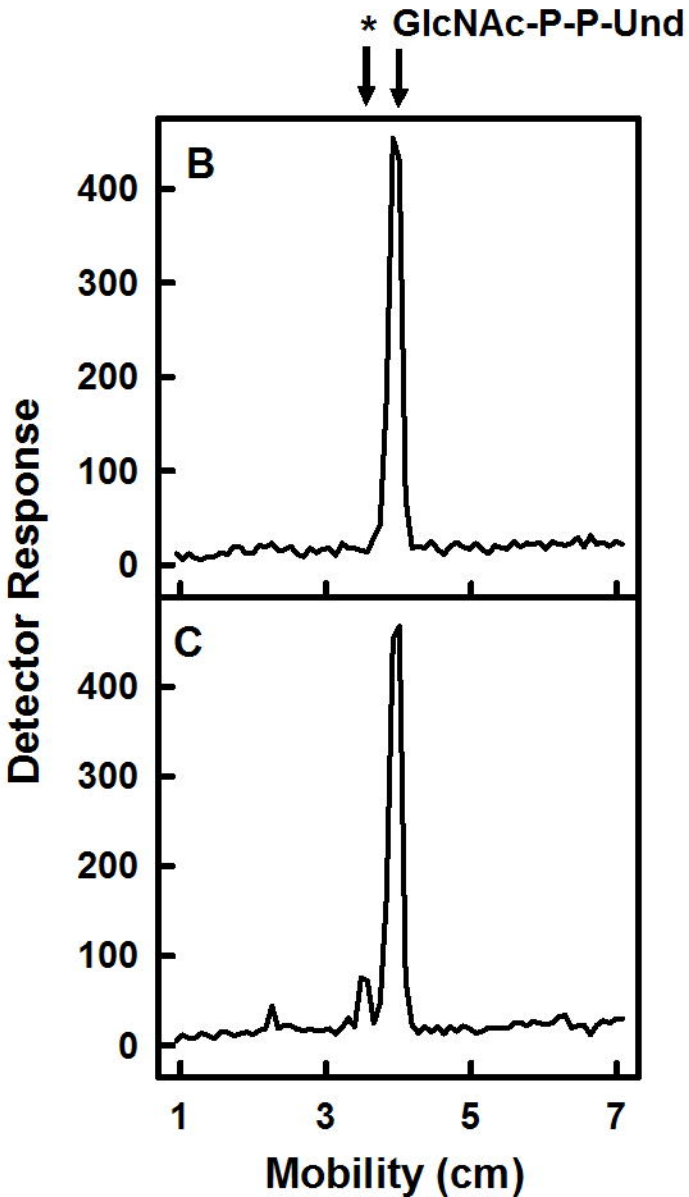

GlcNAc-P-P-Und
GlcNAc-P-Und
A
B
C
D
8000
6000
4000
2000
0
2250
1500
750
0
450
300
150
0
3000
2000
1000
0
Detector Response
0
5
10
15
20
Mobility (cm)

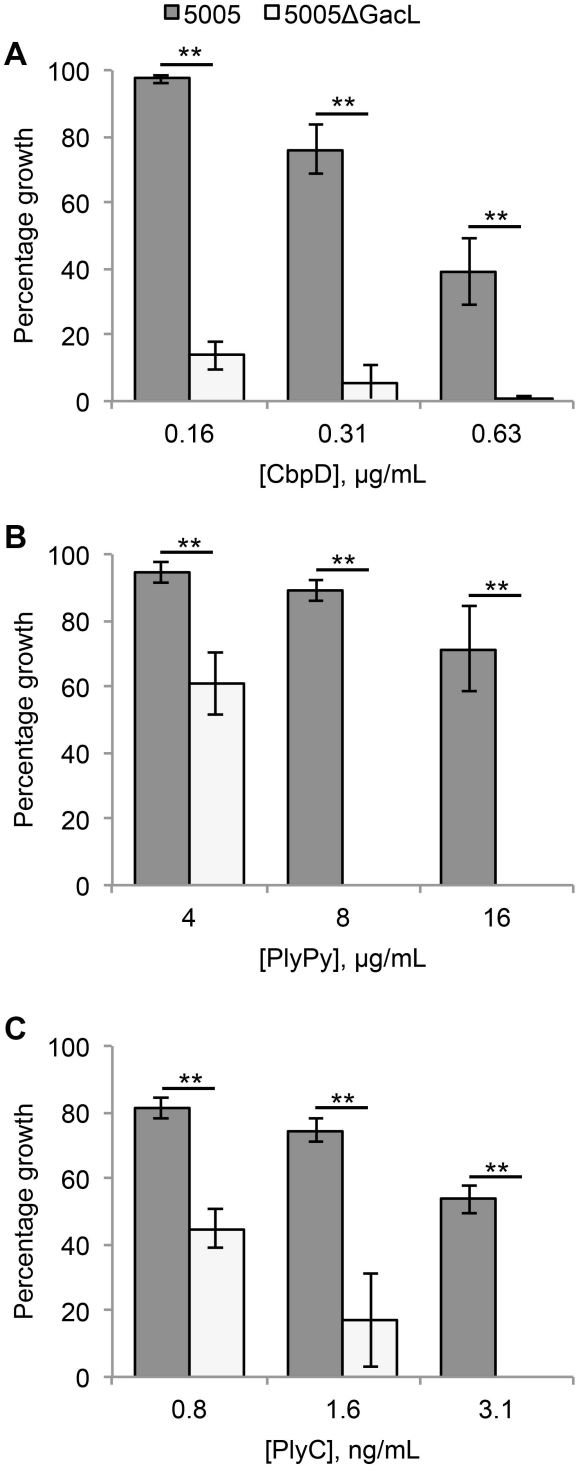

GlcNAc-P-P-Und
GlcNAc-P-Und
A
B
1200
900
600
300
0
400
300
200
100
0
Detector Response
(cpm/mg)
0
5
10
15
20
Mobility (cm)

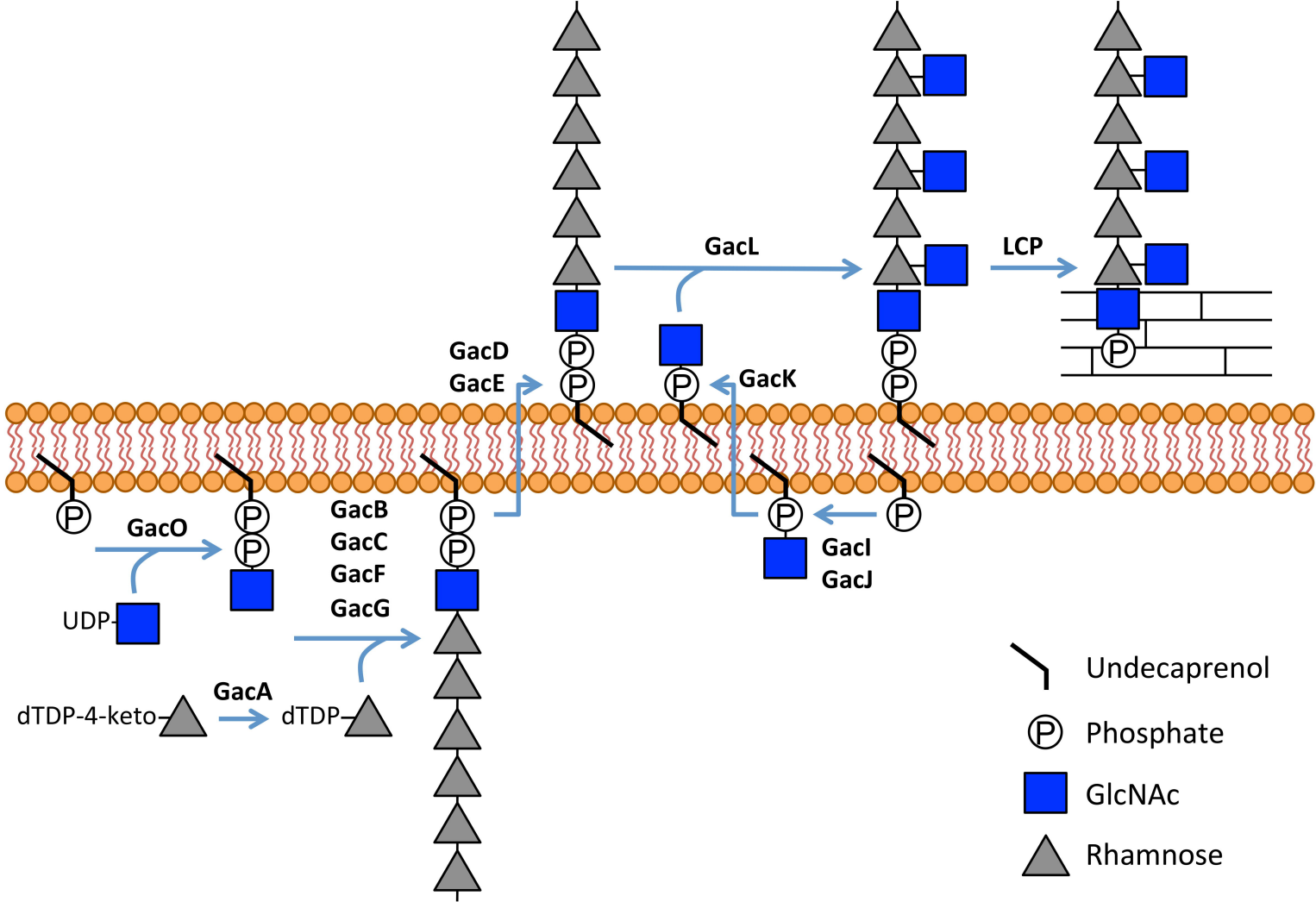

GlcNAc-P-Und Formed
(pmol/mg)
100
75
50
25
0
0.0
2.5
5.0
7.5
10.0
Time (min)

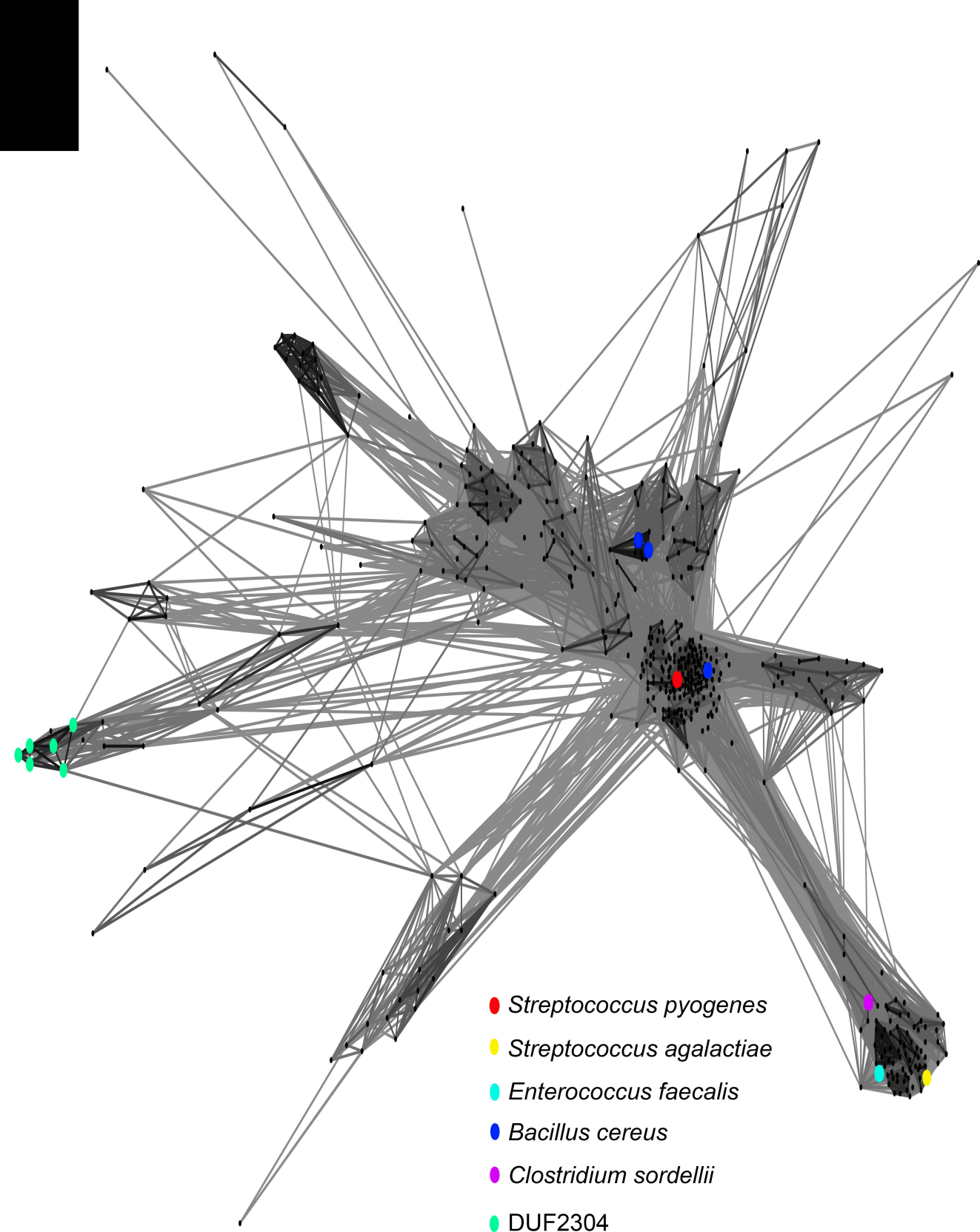

A
MW, kDa
Lysate
Solubilized membrane proteins
Flow through
Wash 1
Wash 2
Eluate
55
40
35
25
15
B
MW, kDa
Eluate
100
70
55
40
35
25
15
GacI
GacJ


A
[3H]GlcNAc-P-P-Und Formed (pmol/mg)
80
60
40
20
0
Ecoli Strain
PR4019
CLM37
CLM37:GacO
WecA
+
-
-
GacO
-
-
+


* GlcNAc-P-P-Und
B
C
Detector Response
400
300
200
100
0
1
3
5
7
Mobility (cm)


5005
5005ΔGacL
A
Percentage growth
100
80
60
40
20
0
**
**
**
0.16
0.31
0.63
[CbpD], µg/mL
B
Percentage growth
100
80
60
40
20
0
**
**
**
4
8
16
[PlyPy], µg/mL
C
Percentage growth
100
80
60
40
20
0
**
**
**
0.8
1.6
3.1
[PlyC], ng/mL


GacL
LCP
GacD
GacE
GacK
GacO
GacB
GacC
GacF
GacG
GacI
GacJ
UDP-
GacA
dTDP-4-keto-
dTDP-
Undecaprenol
Phosphate
GlcNAc
Rhamnose


Streptococcus pyogenes
Streptococcus agalactiae
Enterococcus faecalis
Bacillus cereus
Clostridium sordellii
DUF2304