Interrupting biosynthesis of O-antigen or the lipopolysaccharide core produces morphological defects in *Escherichia coli* by sequestering undecaprenyl phosphate

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Running title: O-antigen and LPS mutants sequester Und-P

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Abstract

Undecaprenyl phosphate (Und-P) is a member of the family of essential polyprenyl phosphate lipid carriers and, in the Gram-negative bacterium *Escherichia coli*, is required for synthesizing the peptidoglycan (PG) cell wall, enterobacterial common antigen (ECA), O-antigen and colanic acid. Previously, we found that interrupting ECA biosynthesis indirectly alters PG synthesis by sequestering Und-P via dead-end intermediates, causing morphological defects. To determine if competition for Und-P was a more general phenomenon, we determined if O-antigen intermediates caused similar effects. Indeed, disrupting the synthesis of O-antigen or LPS core oligosaccharide induced cell shape deformities, which were suppressed by preventing the initiation of O-antigen biosynthesis or by manipulating Und-P metabolism. We conclude that accumulating O-antigen intermediates alters PG synthesis by sequestering Und-P. Importantly, many previous experiments addressed the physiological functions of various oligosaccharides and glyconjugates, but these studies employed mutants that accumulate deleterious intermediates. Thus, conclusions based on these experiments must be re-evaluated to account for possible indirect effects of Und-P sequestration.

38 **Importance**

39

40 Bacteria use long chain isoprenoids like undecaprenyl phosphate (Und-P) as lipid
41 carriers to assemble numerous glycan polymers that comprise the cell envelope. In any
42 one bacterium, multiple oligosaccharide biosynthetic pathways compete for a common
43 pool of Und-P, which means that disruptions in one pathway may produce secondary
44 consequences that affect the others. Using the Gram-negative bacterium *Escherichia*
45 *coli* as a model, we demonstrate that interrupting the biogenesis of O-antigen, a major
46 outer membrane component, indirectly impairs peptidoglycan synthesis by sequestering
47 Und-P into dead-end intermediates. These results strongly argue that the functions of
48 many Und-P-utilizing pathways must be re-evaluated, because much of our current
49 understanding is based on experiments that did not control for these unintended
50 secondary effects.

Introduction

Long-chain isoprenoids are widely conserved lipid carriers that translocate glycan intermediates across biological membranes, where they are assembled onto other polymers or released (reviewed in 1). In bacteria, the primary lipid carrier is undecaprenyl phosphate (Und-P), a 55-carbon isoprenoid (C_{55}-P) also referred to as bactoprenol, which is the dephosphorylated product of undecaprenyl pyrophosphate (Und-PP) as synthesized by UppS (2, reviewed in 3). Homologues with shorter chains serve the same function in species of *Mycobacteria* and *Corynebacteria* (C_{50}-P) (4, 5) and *Paracoccus denitrificans* (C_{45}-P) (6). Und-P is required for synthesizing numerous bacterial glycans, including cell wall peptidoglycan (PG) (7, 8), wall teichoic acids (WTA) (9), enterobacterial common antigen (ECA) (10), O-antigen (11), capsular polysaccharides (12), several commercially important exopolysaccharides (reviewed in 13) and a variety of other carbohydrates (14-17). These glycan polymers are synthesized on the cytoplasmic side of the plasma membrane, where individual sugar residues are transferred sequentially from sugar-nucleotide precursors onto Und-P (18-22). The resulting Und-P-linked oligosaccharides are translocated ("flipped") across the plasma membrane and transferred from the lipid carrier onto individual glycan chains (23). The leftover Und-PP carrier is then recycled into the cytoplasm, either before or after being dephosphorylated, where it rejoins the pool of free Und-P (24).

Mutations that prevent Und-P recycling lead to the accumulation of Und-PP-linked intermediates, resulting in a variety of deleterious or toxic effects (24-28). Because any

one organism normally uses Und-P to synthesize multiple products, these intermediates could either be toxic in and of themselves or they might exert their effects indirectly by sequestering Und-P so that it becomes unavailable for use in other pathways. Although there is a long history of ascribing these effects to Und-P sequestration, only a few experiments have addressed this question directly. For example, some *Corynebacterium glutamicum* mutants accumulate lethal amounts of arabinogalactan and lipoarabinomannan intermediates, but the cells survive if the pool of decaprenylphosphate is increased (5). Similarly, several *Staphylococcus aureus* mutants produce lethal WTA intermediates, but increasing the pool of Und-P restores cell growth (29). And lastly, increasing the amount of Und-P reverses the morphological and membrane defects caused by dead-end Und-PP-linked ECA intermediates in *Escherichia coli* (30). In all these cases the intermediates were not overtly toxic. That is, they did not directly inhibit a particular reaction. Instead, the results strongly argue that these dead-end intermediates sequester so much lipid carrier that they impede other, essential biosynthetic pathways (e.g., PG synthesis). Thus, cell death and many other physiological deficiencies were simply secondary effects caused by the inability to synthesize multiple products.

Apart from these examples, little is known about how different biosynthetic pathways compete for Und-P or how mutants in each pathway affect the others, including those which disrupt O-antigen biosynthesis. Thus, we sought to determine if the PG and O-antigen biosynthetic pathways from *E. coli* compete for Und-P and, if so, to describe the physiological effects of this competition. By using a genetic approach, we demonstrate

that disrupting O-antigen biogenesis indirectly alters cell wall synthesis by interfering with Und-P metabolism, and we conclude that the O-antigen and PG biosynthetic pathways compete for a common pool of Und-P. More broadly, we extend this conclusion to interactions among all Und-P-utilizing pathways. An important implication of these observations is that much work regarding the physiological functions of various glycans should be reevaluated, because many of these experiments employed mutants that accumulate similar dead-end Und-PP-linked intermediates. This means that these previous experiments may not reflect the true biological roles of individual glycan products. Instead, the phenotypes observed in such mutants may be contaminated by unintended secondary effects caused by Und-P sequestration.

Materials and Methods

Bacterial strains, plasmids and media. Bacterial strains, plasmids and primers are listed in Table S1, S2 and S3, respectively. Bacteria were grown in LB broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl). When appropriate, kanamycin was used at 50 μg/mL.

Strain construction. The parent strain for this study was MG1655 *wbbL*$^+$ (31). Restoration of O-antigen production in MG1655 *wbbL*$^+$ obscures the LPS core oligosaccharide, preventing P1-mediated generalized transduction. Consequently, all gene deletions were generated by using lambda Red recombination (32). Kanamycin

119 resistance markers were evicted via the FLP recombinase produced from pCP20 (33).
120 All gene deletions were verified by colony PCR.
121
122 **Plasmid construction.** Plasmids for rescue of Δ*waaL* and Δ*waaC* cells were
123 constructed in pDSW361, which is a kanamycin-resistant derivative of pDSW204 (34).
124 pMAJ41 (P_{204}::*waaL*) was constructed by amplifying *waaL* from MG1655 DNA with
125 primers P229 and P251. The 1275-bp product was cut with EcoRI and PstI and ligated
126 to the same sites of pDSW361. Plasmids pMAJ42 (P_{204}::*waaC*), pMAJ44 (P_{204}::*wecG*)
127 and pMAJ45 (P_{204}::*uppS*) were constructed similarly using the following primer pairs:
128 P231/P252 (pMAJ42), P233/P253 (pMAJ44) and P21/P254 (pMAJ45). pMAJ43 was
129 constructed by digesting pMAJ19 (30) with EcoRI and PstI and ligating the *murA* insert
130 to the same sites of pDSW361. All reference sequences were obtained from the
131 EcoGene 3.0 database (35). All cloning was verified by sequencing at the UAMS DNA
132 Sequencing Core Facility.
133
134 **Suppression of Δ*waaL* and Δ*waaC* shape defects.** Overnight cultures were diluted
135 1:2000 into LB medium containing kanamycin and 100 μM IPTG (unless otherwise
136 noted) and grown at 37°C to an OD_{600} = 0.5-0.6 (approximately 10 doublings). Cells
137 were fixed with 4% paraformaldehyde, spotted onto 1% agarose pads and visualized by
138 phase-contrast microscopy. Our microscope and camera have been described
139 previously (36).
140

Labeling of O16 antigen by concanavalin A-AF488. Overnight cultures were diluted 1:2000 into LB medium and grown at 37°C to an OD_{600} = 0.5-0.6. Cells were pelleted by centrifugation, washed twice in PBS (137 mM NaCl, 3 mM KCl, 9 mM NaH_2PO_4 and 2 mM KH_2PO_4, pH 7.4) and incubated with 2 μM concanavalin A conjugated to Alexa Fluor 488 (Thermo Fisher Scientific) to label the O16 antigen. After 30 minutes, cells were pelleted, washed twice in PBS and visualized by phase-contrast and fluorescence microscopy.

Flow cytometry analysis. Live cells were prepared for flow cytometry as described previously (30). Cell size was assayed by using the forward scatter detector mode of a BD LSRFortessa flow cytometer, housed in the UAMS Flow Cytometry Core Facility. All flow data was analyzed using FlowJo version 10.1.

Results

Mutations that interrupt O-antigen biosynthesis induce morphological defects. The accumulation of unprocessed Und-PP-linked ECA intermediates disrupts cell shape by restricting the availability of Und-P for PG synthesis (30). To determine if interrupting other Und-P-utilizing pathways would provoke similar effects, we constructed a series of mutations in the O-antigen biosynthesis pathway of *E. coli* MG1655 and assayed their effects on cell shape. One complicating factor was that commonly used derivatives of *E. coli* K-12 (e.g. MG1655) normally do not produce O-antigen because the rhamnosyl transferase gene *wbbL* is disrupted by an insertion element (*wbbL*::IS*5*) (37).

164 Rendueles *et al*. recently constructed a derivative of *E*. *coli* MG1655 in which the wild
165 type *wbbL* allele was reintroduced (MG1655 *wbbL*$^{+}$) (31). We obtained this strain and
166 confirmed that it produced the O16 antigen (Figs. 1A and 1B). We then made stepwise
167 deletions in the O-antigen biosynthesis pathway (with the exception of *wbbL*) and
168 evaluated the mutants for changes in cell shape by microscopy and flow cytometry.
169
170 Of the mutants that were predicted to accumulate Und-PP-linked O-antigen
171 intermediates, those lacking either the O-antigen flippase, WzxB (also known as
172 Wzx[O16]) or the WaaL ligase produced the most severe morphological alterations
173 (Figs. 2B and S1A). Cells lacking *wzxB* were swollen, filamentous or chained (Figs. 2B
174 and S1A). When examined by flow cytometry, a population of Δ*wzxB* cells exhibited a
175 3.8-fold greater distribution in forward scatter area (FSC-A) (Fig. 2C), confirming that
176 the cells were enlarged. In addition, Δ*wzxB* cells lysed frequently (e.g., see the empty
177 cells in Fig. 2B). The morphological defects associated with Δ*wzxB* cells were unstable,
178 and the mutant readily developed suppressor mutations that reversed these shape
179 abnormalities (Figs. S2A and S2B). Among these suppressors were IS insertions into
180 *wbbL* and *rmlC* (Fig. S2C), which prevent formation of O-antigen intermediates (Fig.
181 2A). These results parallel the genetic instability associated with mutations in the
182 *Pseudomonas aeruginosa wzx* gene, a homologue of *E*. *coli wzxB* (38). The *P.*
183 *aeruginosa wzx* mutant also acquires second-site mutations in *wbpL*, which eliminates
184 the initial step of O-antigen synthesis and prevents the accumulation of Und-PP-linked
185 O-antigen intermediates. Finally, *E. coli* cells lacking the WaaL ligase also induced
186 morphological alterations in *E. coli*, causing cells to grow on average 40% wider and

62% longer than wild type cells (n=254) (Figs. 2B and 2C, and not shown). Δ*waaL* cells also lysed, but less frequently than did the Δ*wzxB* mutant (not shown).

Interestingly, aside from the morphological changes observed for the Δ*wzxB* and Δ*waaL* mutants (Fig. 2B), the other O-antigen biosynthesis mutants exhibited little to no change in cell shape (Figs. S1A and S1B). This was surprising because the accumulation of Und-PP-linked ECA intermediates, particularly later stage intermediates, induces cell shape defects (30). However, in the *E. coli* MG1655 strain in which the O-antigen pathway was reconstituted, the O-antigen and ECA biosynthesis pathways compete for a common starting substrate, Und-PP-*N*-acetylglucosamine (GlcNAc). It was therefore possible that Und-PP-GlcNAc was being redirected into the ECA pathway and that this side reaction was reducing the accumulation of Und-PP-linked O-antigen intermediates. To test this possibility, we deleted O-antigen pathway genes (*wbbK* and *wbbJ*) in an ECA-negative Δ*wecB* mutant. WecB helps convert UDP-GlcNAc to UDP-*N*-acetylmannosaminuronic acid (Fig. 3A) (39, 40), so that in the absence of WecB Und-PP-GlcNAc cannot be utilized in ECA biosynthesis (Fig. 3A). Deleting *wecB*, *wbbK* or *wbbJ* individually had little effect on cell shape (Figs. 3B and 3C). However, cells of double mutants lacking *wecB wbbK* or *wecB wbbJ* were greatly enlarged and occasionally branched (Fig. 3B), and the mean cell sizes increased substantially (Fig. 3C). Like the Δ*wzxB* mutant, Δ*wecB* Δ*wbbJ* cells were also genetically unstable, readily accumulating suppressor mutations (not shown). In short, the presence of a functional ECA pathway suppressed the negative effects caused by disrupting the early stages of O-antigen biosynthesis.

Taken together, these results strongly indicate that interrupting the O-antigen biosynthesis pathway induces morphological defects by producing dead-end intermediates.

Disrupting O-antigen assembly limits Und-P availability. Most studies characterizing cell shape in *E. coli* have been conducted in strains lacking O-antigen. However, restoring O-antigen synthesis had no impact on cell shape (Figs. S1A and S1B, compare WT cells to the O-antigen negative *wbbL*::IS*5* mutant). Because cells lacking WbbL do not make O-antigen (Fig. 2A), it was clear that O-antigen itself was not required to maintain normal morphology. Thus, the morphological defects provoked by deleting other genes in the pathway (Figs. 2B, 3B and S1A) cannot be explained by the absence of O-antigen but are instead best explained by the accumulation of Und-PP-linked intermediates. If so, then deleting *wecA*, which initiates O-antigen biosynthesis by transferring GlcNAc-1-phosphate onto the Und-P carrier (Fig. 2A) (41), should reverse the shape defects by eliminating these intermediates. Indeed, removing *wecA* reversed the shape defects observed in Δ*wzxB* and Δ*waaL* cells (Figs. 2B and 2C), confirming that the morphological defects were not caused by the absence of O-antigen but by the presence of deleterious Und-PP-linked intermediates. [We note, though, that cells lacking WecA were slightly larger than wild type (Figs. 2B and 2C) (30), being, on average, 7% longer and 9% wider (n=219). What causes this small difference is unknown.]

233 At this point, we considered it likely that the morphological defects caused by
234 accumulating dead-end O-antigen intermediates were due to impaired PG synthesis
235 caused by Und-P sequestration. If so, then artificially increasing the pool of Und-P
236 should suppress shape defects in O-antigen mutants. To test this possibility, we
237 overproduced the undecaprenyl pyrophosphate synthase UppS (also known as IspU) to
238 increase the pool of Und-P in a Δ*waaL* mutant, which cannot ligate O-antigen to the lipid
239 A-core and therefore accumulates several pathway intermediates. As predicted, the
240 presence of additional UppS returned Δ*waaL* cells to wild type shape and size (Figs. 4A
241 and 4B, compare the p*uppS*-containing strain to cells containing p*waaL* and those
242 containing vector only). Thus, cell shape defects that accompany interruptions of the O-
243 antigen biosynthetic pathway are most likely caused by diminished levels of Und-P.

244

245 To further confirm that a lack of Und-P was altering PG synthesis and causing shape
246 defects, we skewed the balance of shared metabolites in favor of the PG synthesis
247 pathway as another way of alleviating Und-P sequestration. For many *E. coli* strains,
248 including MG1655 (42), GlcNAc is incorporated at the reducing end of the O-antigen
249 (Fig. 1A) (reviewed in 43). This is important because both the PG and O-antigen
250 biosynthetic pathways compete for the nucleotide precursor of this sugar, UDP-GlcNAc.
251 Since the first committed step for PG synthesis is the transfer of enolpyruvate to UDP-
252 GlcNAc by MurA (44), we reasoned that overexpressing *murA* would increase the flux of
253 UDP-GlcNAc into the PG synthesis pathway at the expense of O-antigen production,
254 thereby alleviating the shape defect of Δ*waaL* cells. Indeed, overexpressing *murA*
255 reversed the morphological defects of Δ*waaL* cells (Figs. 4A and 4B), though the

256 reversal was not quite as effective as *uppS* overexpression (discussed above). MurA
257 activity is feedback inhibited by UDP-*N*-acetylmuramic acid, the product of the second
258 step in PG synthesis (45). This regulatory circuit probably limited the amount of UDP-
259 GlcNAc that could be diverted into the PG pathway, and thus the extent of shape
260 recovery. (Feedback inhibition of MurA presumably ensures that sufficient levels of
261 UDP-GlcNAc are available for synthesizing other products, like O-antigen.)
262
263 Finally, we employed a third strategy for increasing the amount of Und-P available for
264 PG synthesis in Δ*waaL* cells. The glycosyltransferases WecG and WbbL compete for
265 Und-PP-GlcNAc to elongate ECA and O-antigen, respectively (18, 42) (Fig. 3A). We
266 reasoned that overexpressing *wecG* would bolster the available pool of Und-P in the
267 Δ*waaL* mutant by diverting more Und-P into the ECA pathway, thereby increasing Und-
268 P turnover and reducing the accumulation of dead-end O-antigen intermediates. As
269 predicted, additional WecG reversed the shape defects of Δ*waaL* cells (Figs. 4A and
270 4B), consistent with the idea that Und-P was recycled and made available for PG
271 synthesis. Interestingly, leaky expression of *wecG* from the plasmid was sufficient to
272 suppress the shape defects in Δ*waaL* cells (Figs. 4A and 4B), but further increases in
273 *wecG* expression caused these cells to grow as longer and longer filaments (e.g., Figs.
274 S3A and S3B, compare 0 to 100 μM IPTG). This profound filamentation strongly
275 suggests that diverting excessive amounts of Und-PP-GlcNAc into the ECA pathway
276 also sequestered the Und-P pool, thereby inhibiting PG synthesis and cell division
277 (although we cannot completely rule out other indirect effects). We note that
278 overexpressing *wecG* caused only moderate elongation of wild type cells (Figs. S4A

and S4B, compare p*wecG* to the vector only), an effect not nearly as profound as that seen in the Δ*waaL* mutant (Figs. S3A and S3B). Also, with the exception of *wecG*, overexpressing other genes had no effect on the morphology of wild type cells (Figs. S4A and S4B).

In sum, the preceding results reinforce the hypothesis that the general accumulation of Und-PP-linked intermediates indirectly impairs PG synthesis, most likely by limiting the availability of Und-P.

***Disruptions in LPS core biosynthesis sequester Und-P*.** During LPS assembly, the WaaL ligase connects O-antigen subunits to the core oligosaccharide (Fig. 5A) (reviewed in 46). Thus, in cells producing O-antigen, any mutation that produces an incomplete core oligosaccharide should also accumulate dead-end Und-PP-linked O-antigen intermediates (Fig. 5A). Because the PG and O-antigen biosynthetic pathways compete for Und-P, sequestering Und-P in this way should impair PG synthesis and produce cell shape defects in mutants that lack a complete core oligosaccharide. To test this idea, we deleted *waaC* and *waaF* from MG1655 *wbbL*$^+$. WaaC and WaaF add the first and second heptose residues to the Kdo-2 moiety of the LPS inner core (Fig. 5B) (47, 48). Loss of WaaC or WaaF caused cells to swell, usually beginning at one end, so that the cells adopted a shape resembling a bowling pin or tadpole (Figs. 5C and S5). These defects were distinct from those observed in Δ*wzxB* or Δ*waaL* cells (Figs. 2B and S1A). Three lines of evidence indicated that these morphological changes were probably due to Und-P sequestration. First and foremost, preventing the

synthesis of O-antigen by deleting *wecA* suppressed the shape defects in Δ*waaC* (Figs. 5C and 5D) and Δ*waaF* cells (Fig. S5). Second, increasing the pool of Und-P by overexpressing *uppS* reversed the shape defects of Δ*waaC* cells (Figs. 6A and 6B). And third, restricting the biosynthesis of O-antigen, either by increasing the flux of UDP-GlcNAc into the PG synthesis pathway (by overexpressing *murA*) or by redirecting Und-PP-GlcNAc to the ECA pathway (by overexpressing *wecG*) returned Δ*waaC* cells to near-normal wild type morphology (Figs. 6A and 6B). The results of these three approaches were identical to those that suppressed the morphological effects exhibited by mutants in the O-antigen biosynthetic pathway, as discussed above. Similarly, suppression of shape defects in the Δ*waaC* mutant paralleled the suppression of defects observed in the Δ*waaL* mutant. In particular, overexpressing *murA* suppressed but did not fully restore wild type morphology to Δ*waaC* cells (Figs. 6A and 6B), and expressing *wecG* too highly induced cell filamentation (Figs. S3C and S3D). We note, though, that we do not understand why mutants lacking Wzx, WaaL or WaaC differ from one another in morphology. Presumably, these mutants have comparable effects on the pool of Und-P and should therefore have similar phenotypes. The fact that they differ suggests that additional factors are at play. Collectively, the data demonstrate that shape defects associated with disruptions of the LPS core are mostly likely caused not by the loss of LPS *per se* but are instead indirect effects caused by sequestering Und-P and interfering with PG synthesis.

Discussion

Und-P and its variants are universal lipid carriers employed by all bacteria for synthesizing numerous glycan polymers (reviewed in 1). Because it frequently serves as an intermediate in multiple synthetic pathways, Und-P unites what are otherwise biochemically and functionally diverse oligosaccharides. Consequently, disruptions of one Und-P-dependent pathway may have indirect effects on the others. Here, we demonstrate that disrupting the biogenesis of *E. coli* O-antigen compromises PG synthesis by sequestering Und-P. We conclude that the O-antigen and PG synthetic pathways compete for a common pool of Und-P (Fig. 7). We further posit that because of this competition, the phenotypes exhibited by many previous O-antigen and LPS mutants may not accurately reflect the physiological functions of these cellular components. Instead, because such mutants would sequester Und-P and might therefore impair cell wall synthesis or the production of other compounds, the behaviors of such mutants would represent an unknown combination of contributing factors. Of course, these considerations would not apply to most mutations in *E. coli* K-12, which does not synthesize O-antigen.

Mutations that disrupt LPS biosynthesis sequester Und-P. To our knowledge, the present study describes the first controlled experiments to examine the secondary effects of Und-P sequestration as it relates to LPS biosynthesis. However, several lines of independent evidence corroborate the results reported here. One of the best examples centers on the study of the O-antigen flippase, WzxB. Loss of WzxB causes the accumulation of Und-PP-linked O-antigen intermediates (49), which in turn limits the availability of Und-P for PG synthesis. Because of this, *wzxB* mutants are notoriously

348 difficult to isolate (38, 49, 50) and are deleterious in the presence of a fully functional O-
349 antigen-producing machinery (51, 52). We recreated both of these circumstances in the
350 present study, and in both instances the accumulation of Und-PP-linked O-antigen
351 intermediates induces morphological defects that are reversed by eliminating the
352 production of O-antigen. Thus, it is not surprising that *wzxB* flippase mutants acquire
353 suppressing mutations that prevent the formation of O-antigen intermediates (38).
354 Indeed, investigators who study WzxB function in *Salmonella enterica* do so in a genetic
355 background where expression of O-antigen is controlled by a *galE* mutation, which
356 removes the first committed step in the biosynthetic pathway so that no Und-PP-linked
357 O-antigen intermediates can accumulate until galactose is supplied (49, 51, 52). The
358 deleterious effects of Und-P sequestration have also been noted for other transport
359 mutants (53, 54), as well as mutants that are unable to ligate O-antigen intermediates to
360 the lipid A core (24) or that truncate the core oligosaccharide (55). *In toto*, then, our
361 LPS and O-antigen findings are consistent with the conditional phenotypes that Und-P
362 sequestration produces in mutants that disrupt the synthesis of wall teichoic acids (56,
363 57), capsular polysaccharides (58-60), exopolysaccharide (61) and ECA (30, 62).
364
365 The present results, especially when combined with previous observations (29, 30),
366 strongly suggest that the accumulation of any Und-PP-linked intermediate will interfere
367 with PG synthesis. This possibility, which we consider highly probable, has significant
368 implications for past and future experiments that explore the physiological relevance of
369 any glycan polymer whose synthesis requires a Und-P intermediate. Here, we point out
370 that this consideration is of special concern with regard to understanding the

physiological roles played by LPS and O-antigen. Many experiments have investigated the biological functions of these compounds by studying mutants which we now predict were also accumulating dead-end O-antigen intermediates. It is very likely that the phenotypes of such mutants represent a combination of behaviors: those caused by the loss of LPS or O-antigen, plus those caused by secondary defects due to Und-P sequestration. A further complication is that one of these secondary effects, impaired cell wall synthesis, itself induces widespread cell envelope stress responses (63-65). Thus, phenotypes exhibited by such mutants will also be contaminated by stress behaviors. In short, mutants that accumulate Und-PP-linked intermediates will probably exhibit phenotypes with a complicated mixture of causes, many of which will be unrelated to the direct functions of LPS or O-antigen. More broadly, we wish to highlight the danger of drawing conclusions about the physiological function of any compound or cellular component based on the behavior of mutants that accumulate similar Und-PP-linked intermediates (e.g., 30, 66).

Finally, to be clear, we note that Und-P sequestration does not explain all previous phenotypes associated with disruptions in the biosynthesis of LPS or O-antigen. For example, the absence of LpxL (formerly HtrB), a lauroyl acyltransferase required for the maturation of Kdo-2-lipid-A (67), induces cell shape defects in *E. coli* even though that strain does not produce O-antigen (68). Similarly, depleting LPS transport proteins induces cell shape defects in the absence of O-antigen (69). Und-PP-linked O-antigen intermediates are present in neither of these cases, so no Und-P should be sequestered. These results, along with many others, clearly indicate that LPS plays a

394 distinct physiological role. What we are urging here is that the mutants to be studied
395 must be carefully selected to avoid unnecessary confusion in interpreting the results of
396 similar experiments.
397
398 **Evidence for relaxed substrate specificity or reversible reactions.** As mentioned
399 earlier, loss of the O-antigen flippase WzxB is extremely deleterious, including gross
400 morphological changes and lysis (Figs. 2B and S1A). These effects are much greater
401 than those in mutants that disrupt earlier steps in the O-antigen biosynthetic pathway
402 (Figs. 3B and S1A). Interestingly, WzxB can translocate different repeat units (70, 71),
403 and under certain conditions mutants lacking WzxB express LPS linked to O-units (51,
404 62), suggesting that other Wzx translocases exhibit substrate cross-reactivity. This
405 seemingly relaxed substrate specificity may mean that more than one Und-PP-linked
406 intermediate accumulates in a *wzxB* mutant. If so, then PG synthesis may be
407 compromised to a greater degree than if WzxB flipped O-antigen intermediates
408 exclusively, which may explain why a *wzxB* mutant is so deleterious. Alternately, the
409 reaction catalyzed by WbbI may be the first irreversible reaction during elongation of O-
410 antigen intermediates. Thus, any mutation before or at the WbbI step (Fig. 2) would
411 accumulate fewer Und-PP-linked intermediates than if the reactions were not reversible.
412 That O-antigen glycosyltransferase reactions might be reversible would not be too
413 surprising, given that initiation of O-antigen synthesis by WbaP (a WecA homolog) is
414 reversible in *Salmonella typhimurium* (72). In summary, the absence of reaction
415 reversibility could explain why disruptions in WzxB and later steps are so deleterious.
416

Is the level of Und-P defined? Competition for Und-P among a variety of biochemical pathways raises the interesting question of whether the steady-state levels of Und-P can be changed. In fact, increasing the amount of cellular Und-P would seem to be the simplest way to bypass the deleterious consequences of any dead-end intermediates, as we show in principle by producing more UppS, which reverses the effects of Und-P sequestration. The answer was partially uncovered by Barreteau *et al*. who determined that the intracellular levels of Und-PP and Und-P are quite similar between *E. coli* and *S. aureus* (73). It is remarkable that the basal levels of these C_{55}-isoprenoid pools have remained relatively unchanged between the two organisms, given that the two have such different amounts of PG (reviewed in 74) and that the two diverged some 2 billion years ago (75). Why have the relative amounts of this lipid carrier remained unchanged in these very different organisms? One possibility is that an overabundance of membrane-associated polyisoprenols may disrupt the architecture of the typical phospholipid bilayer (reviewed in 76). If so, then the conserved levels of bacterial Und-P may represent an evolutionary compromise that balances glycan biosynthesis with biophysical realities. This pressure to maintain a constant pool of Und-P might play an important role in determining the number of Und-P-dependent pathways that can be accommodated in any one organism. Because PG is essential, this structure may be the foundation around which all other Und-P-consuming pathways must be organized and delimited.

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680 **Figure Titles and Legends**

681

682 **Figure 1. Restoring O-antigen biosynthesis to *E. coli* K-12.** (A) Structure of O16
683 antigen from *E. coli* K-12 (reviewed in 43). Abbreviations: Gal*f*, galactofuranose; Glc,
684 glucose; Rha, rhamnose; GlcNAc, *N*-acetylglucosamine. (B) Detection of O-antigen
685 with concanavalin A-AF488 in cells with the indicated genotypes. Cells were labeled
686 with concanavalin A-AF488, washed and photographed by phase-contrast and
687 fluorescent microscopy (green signal). The white bar represents 3 μm. Concanavalin A
688 binds α-glucose (underlined in panel A) and α-mannose residues. Strains: MAJ1
689 (*wbbL*::IS*5*) and MAJ330 (*wbbL*$^+$).

690

691 **Figure 2. Disrupting O-antigen biosynthesis induces morphological defects in *E.***
692 ***coli*.** (A) O-antigen biosynthesis pathway. Abbreviations: P-Gc, glucose 1-phosphate;
693 dTDP, thymidine diphosphate; Und-P, undecaprenyl phosphate; UDP, uridine
694 diphosphate; G, *N*-acetylglucosamine; Ac-CoA, acetyl coenzyme A; Rh, rhamnose; Gf,
695 galactofuranose. Note that WzxB and WbbH are also known as Wzx(O16) and
696 Wzy(O16), respectively (62). (B) Micrographs of cells with the indicated genotypes.
697 Cells were grown at 37°C in LB for approximately ten doublings until the culture reached
698 an OD_{600} = 0.5-0.6. The cells were then fixed and photographed by phase-contrast
699 microscopy. Additional O-antigen mutants are shown in Figure S1. Micrographs of
700 Δ*wzxB* cells were from overnight cultures because the strain readily develops
701 suppressor mutations that correct the shape defect as shown in Figure S2. The white
702 bar represents 3 μm. (C) Flow cytometry data from live cells in panel B. Histograms of

703 the forward scatter area (FSC-A) from 100,000 events (cells). The mean cell size of the
704 wild type (WT) is represented by the vertical dashed line and is expressed in arbitrary
705 units (AU). Data is representative of two independent experiments. Strains: MAJ330
706 (WT), MAJ339 (Δ*wzxB*), MAJ345 (Δ*waaL*), MAJ343 (Δ*wecA*), MAJ369 (Δ*wecA* Δ*wzxB*)
707 and MAJ370 (Δ*wecA* Δ*waaL*).
708
709 **Figure 3. Synthetically misshapen mutant combinations in the O-antigen and**
710 **ECA pathways.** (A) Biosynthetic pathway illustrating how the ECA and O-antigen
711 synthesis pathways compete for Und-PP-GlcNAc. Abbreviations: UDP, uridine
712 diphosphate; GlcNAc, *N*-acetylglucosamine; ManNAcA, *N*-acetylmannosaminuronic
713 acid; Und-P, undecaprenyl phosphate; Und-PP, undecaprenyl pyrophosphate; ECA,
714 enterobacterial common antigen. (B) Micrographs of cells with the indicated genotypes.
715 Cells were grown and imaged as described in the legend to Figure 2. The white bar
716 represents 3 μm. (C) Flow cytometry from data from live cells in panel B. Histograms
717 of the forward scatter area from 100,000 events (cells). The mean cell size for wild type
718 cells (red graph) is represented by the vertical dashed line and is expressed in arbitrary
719 units (AU). Data is representative of two independent experiments. Strains: MAJ330
720 (WT), MAJ356 (Δ*wecB*), MAJ346 (Δ*wbbJ*), MAJ344 (Δ*wbbK*), MAJ398 (Δ*wecB* Δ*wbbJ*)
721 and MAJ397 (Δ*wecB* Δ*wbbK*).
722
723 **Figure 4. Suppression of Δ*waaL* shape defects.** (A) Micrographs of Δ*waaL* cells
724 containing derivatives of pDSW361 that express the indicated genes. Cells were grown
725 at 37°C in LB containing 100 μM IPTG (0 μM IPTG for p*wecG*) until the culture reached

an OD_{600} = 0.5-0.6. The cells were then fixed and photographed by phase-contrast microscopy. The white bar represents 3 μm. Further characterization of *wecG* overexpression is found in Figure S3. (B) Flow cytometry data from live cells in panel A. Histograms of the forward scatter area from 100,000 events (cells). The mean cell size for Δ*waaL* cells expressing *waaL in trans* (red graph) is represented by the vertical dashed line and is expressed in arbitrary units (AU). Data is representative of two independent experiments. Strains: MAJ434 (p*waaL*), MAJ437 (p*uppS*), MAJ436 (p*wecG*), MAJ435 (p*murA*) and MAJ433 (vector). The effect of expressing the aforementioned derivatives of pDSW361 on wild type cells is shown in Figure S4.

Figure 5. Disrupting LPS core biosynthesis induces morphological defects in *E. coli*. (A) Maturation of Und-PP-linked O-antigen intermediates and their ligation to the Lipid A core. Mutations that truncate the core oligosaccacharide (e.g. Δ*waaC*) prevent attachment of the O-antigen (right side). Abbreviations: IM, inner membrane; OM, outer membrane; A, lipid A. Other abbreviations are listed in the legend to Figure 2. (B) General structure of the *E. coli* K-12 core oligosaccharide (shaded in gray) (reviewed in 77). WaaC and WaaF transfer Hep-I and Hep-II, respectively, onto the growing O-antigen chain. (C) Micrographs of cells with the indicated genotypes. Cells were grown and imaged as described in the legend to Figure 2. The cells were fixed and photographed by phase-contrast microscopy. The white bar represents 3 μm. Similar results were obtained for mutants of *waaF* as shown in Figure S5. (D) Flow cytometry data from live cells in panel C. Histograms of the FSC-A from 100,000 events (cells). The mean cell size of the wild type strain (red graph) is represented by the vertical

749 dashed line and is expressed in arbitrary units (AU). Data is representative of two
750 independent experiments. Strains: MAJ330 (WT), MAJ374 (Δ*waaC*), MAJ343 (Δ*wecA*)
751 and MAJ384 (Δ*wecA* Δ*waaC*).
752
753 **Figure 6. Suppression of Δ*waaC* shape defects.** (A) Micrographs of Δ*waaC* cells
754 containing derivatives of pDSW361 that express the indicated genes. Cells were grown
755 and imaged as described in the legend to Figure 4 except that IPTG was added to 25
756 μM IPTG for p*wecG*. Overexpression of *murA* does not fully suppress the shape
757 defects of Δ*waaC* cells (e.g., p*murA*, inset). The white bar represents 3 μm. (B) Flow
758 cytometry data from live cells in panel A. Histograms of the forward scatter area from
759 100,000 events (cells). The mean cell size area for Δ*waaC* cells expressing *waaC in*
760 *trans* (red graph) is represented by the vertical dashed line and is expressed in arbitrary
761 units (AU). Data is representative of two independent experiments. Strains: MAJ439
762 (p*waaC*), MAJ442 (p*uppS*), MAJ441 (p*wecG*), MAJ440 (p*murA*) and MAJ438 (vector).
763
764 **Figure 7. Model of competition for Und-P in *E. coli*.** The PG, ECA and O-antigen
765 biosynthesis pathways compete for pools of Und-P (red) and UDP-GlcNAc (blue).
766 Interrupting the biosynthesis of ECA, O-antigen or Lipid A core will sequester part of the
767 pool of Und-P, and will therefore restrict its availability for PG synthesis. Note that the
768 colanic acid biosynthesis pathway, which also utilizes Und-P, is omitted from this
769 illustration because this compound is not produced appreciably under normal growth
770 conditions, though it is expressed highly during stress. Abbreviations: PG,
771 peptidoglycan; UDP-M5, uridine diphosphate *N*-acetylmuramic acid-L-alanine-D-

772 glutamate-mesodiaminopimelic acid-D-alanine-D-alanine; Und-P, undecaprenyl
773 phosphate; GlcNAc, *N*-acetylglucosamine; Und-PP, undecaprenyl pyrophosphate; ECA,
774 enterobacterial common antigen; LPS, lipopolysaccharide.

A

E. coli O16

β-D-Gal*f*→α-D-Glc→α-L-Rha2Ac→α-D-GlcNAc

B

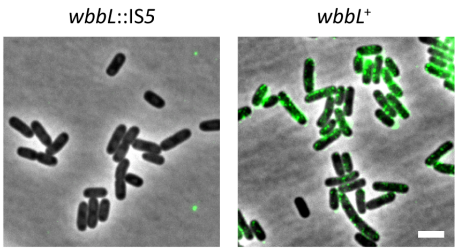

Figure 1

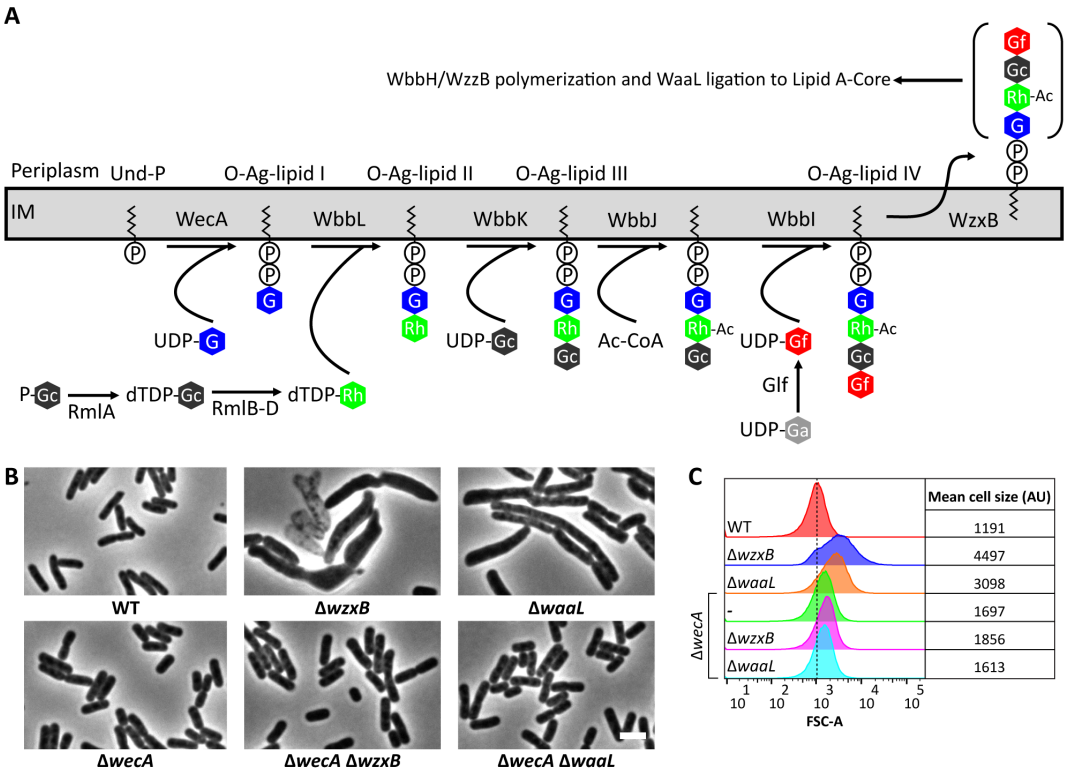

Figure 2

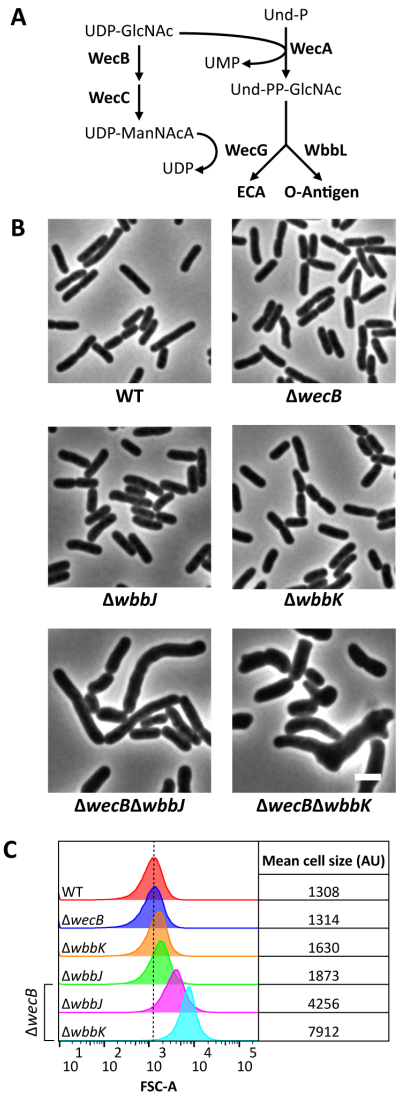

Figure 3

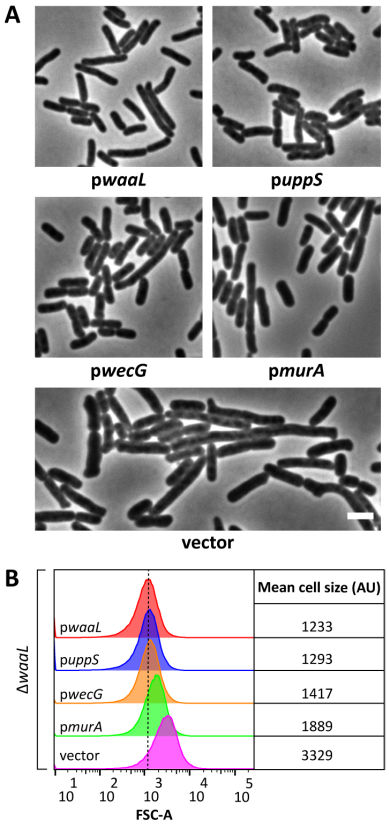

Figure 4

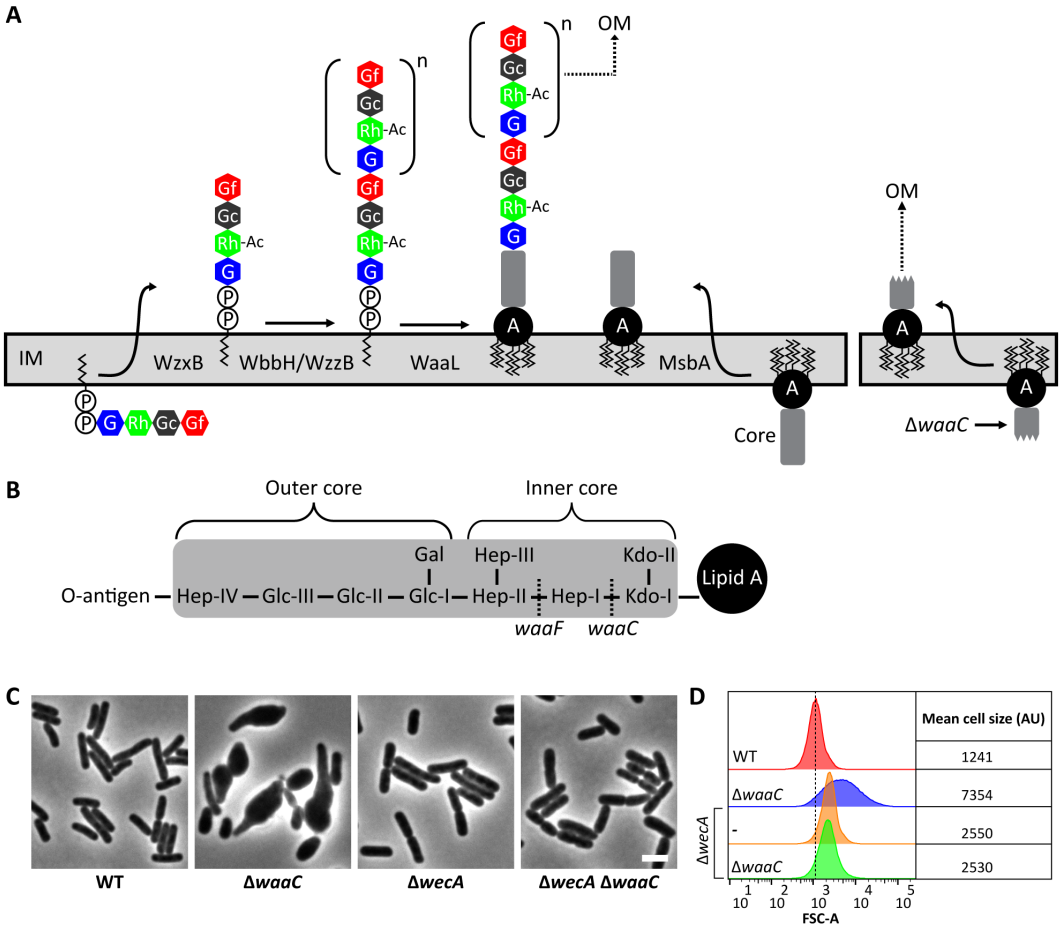

Figure 5

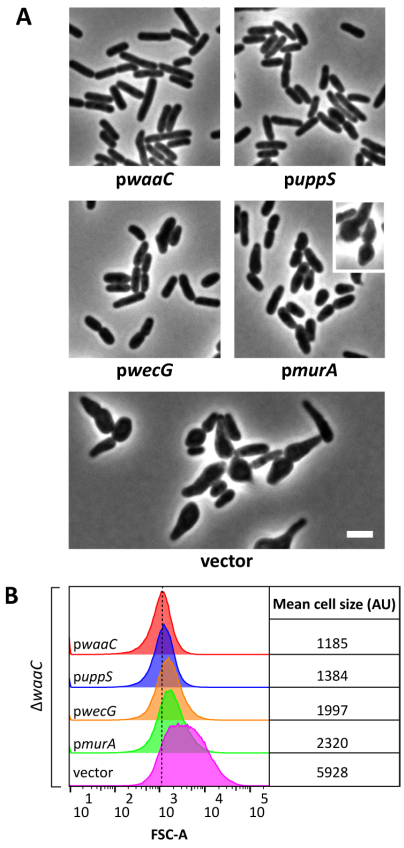

Figure 6

Figure 7