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1 **A low-cost recombinant glycoconjugate vaccine confers immunogenicity and**
2 **protection against enterotoxigenic *Escherichia coli* infections in mice**
3
4 Asher J. Williams[1], Katherine F. Warfel[2,3,4], Primit Desai[5], Jie Li[6], Jen-Jie Lee[6], Derek A.
5 Wong[2,3,4], Sarah E. Sobol[2,3,4], Michael C. Jewett[2,3,4], Yung-Fu Chang[6] and Matthew P.
6 DeLisa[1,5,7]*
7
8 [1]Robert F. Smith School of Chemical and Biomolecular Engineering, Cornell University,
9 Ithaca, NY 14853 USA
10 [2]Department of Chemical and Biological Engineering, Northwestern University, 2145
11 Sheridan Road, Technological Institute E136, Evanston, IL 60208, USA
12 [3]Chemistry of Life Processes Institute, Northwestern University, 2170 Campus Drive,
13 Evanston, IL 60208, USA
14 [4]Center for Synthetic Biology, Northwestern University, 2145 Sheridan Road,
15 Technological Institute E136, Evanston, IL 60208, USA
16 [5]Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853
17 [6]Department of Population Medicine and Diagnostic Sciences, College of Veterinary
18 Medicine, Cornell University, Ithaca, NY, 14853 USA
19 [7]Cornell Institute of Biotechnology, Cornell University, Ithaca, NY 14853 USA
20
21 *Address correspondence to: Matthew P. DeLisa, Robert Frederick Smith School of
22 Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853 USA. Tel:
23 607-254-8560; Email: md255@cornell.edu
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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the primary etiologic agent of traveler's diarrhea and a major cause of diarrheal disease and death worldwide, especially in infants and young children. Despite significant efforts over the past several decades, an affordable vaccine that significantly reduces mortality and morbidity associated with moderate to severe diarrhea among children under the age of 5 years remains an unmet aspirational goal. Here, we describe robust, cost-effective biosynthetic routes that leverage glycoengineered strains of non-pathogenic *Escherichia coli* or their cell-free extracts for producing conjugate vaccine candidates against two of the most prevalent O serogroups of ETEC, O148 and O78. Specifically, we demonstrate site-specific installation of O-antigen polysaccharides (O-PS) corresponding to these serogroups onto licensed carrier proteins using the oligosaccharyltransferase PglB from *Campylobacter jejuni.* The resulting conjugates stimulate strong O-PS-specific humoral responses in mice and elicit IgG antibodies that possess bactericidal activity against the cognate pathogens. We also show that one of the prototype conjugates decorated with serogroup O148 O-PS confers protection against ETEC infection in mice. We anticipate that our bacterial cell-based and cell-free platforms will enable creation of multivalent formulations with the potential for broad ETEC serogroup protection and increased access through low-cost biomanufacturing.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of diarrheal disease worldwide and the leading cause of traveler's diarrhea, especially in locations where clean water and sanitation remain limited [1, 2]. In addition to acute diarrhea-associated morbidity, ETEC is also one of the leading causes of mortality, disproportionately affecting children under the age of 5 years who lack immunity from prior exposure [3, 4]. Deaths from infectious diarrhea have been on the decline for a few decades, due in part to the introduction of oral rehydration therapy. However, ETEC continues to wreak havoc in terms of acute morbidity and associated sequelae that compound the impact of infection in young children, including growth stunting, malnutrition, and impaired cognitive impairment [5, 6].

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1 A vaccine that prevents both acute illness as well as the sequelae associated with
2 ETEC infection has long been a priority of the World Health Organization (WHO) [7]. At
3 present, ETEC vaccine development efforts are primarily centered on inducing immune
4 responses against a subset of important virulence factors including colonization factor/cell
5 surface (CF/CS) antigens and enterotoxins, namely heat-stable toxin (ST) and heat-labile
6 toxin (LT) [8-11]. These factors comprise a classical model for ETEC molecular
7 pathogenesis in which ETEC colonizes the small intestine using plasmid-encoded CF/CS
8 antigens followed by production of one or more enterotoxin (*e.g.*, LT, ST) that drive fluid
9 export and diarrhea [12]. Despite significant efforts over several decades, human trials
10 involving vaccine candidates based on this classical paradigm have met limited success
11 [11, 13-15]. Hence, further improvements are needed to increase the levels of protection
12 against more severe ETEC diarrhea and to expand protection to the breadth of ETEC
13 serotypes. Consequently, there are still no licensed vaccines for ETEC.

14 To expand the repertoire of antigens that could be targeted in future vaccine
15 designs, several studies have assessed adaptive immune responses in humans following
16 either experimental challenge with ETEC or oral administration of an inactivated ETEC
17 vaccine [16-18]. A number of non-canonical antigens beyond the classic vaccine targets
18 were identified, including secreted proteins (*e.g.*, EatA, EtpA, and YghJ), cell surface-
19 expressed proteins (*e.g.*, Ag43, OmpW), and lipopolysaccharide (LPS), among others.
20 Unlike the secreted and cell-surface protein antigens, LPS are glycolipids that include an
21 outermost O-antigen polysaccharide (O-PS) component that is composed of repeating
22 subunits that extend from the surface of the bacteria [19]. Interestingly, the inactivated
23 ETEC vaccine strain, ETVAX, elicited response frequencies against serogroup O78 LPS
24 (which is expressed on the strain) that were comparable to or higher than those against
25 the vaccine CFs in infants. This suggests that LPS is a potent antigen that may contribute
26 to vaccine-induced protection [17]. Other studies have indicated that O-PS is common
27 among ETEC strains associated with diarrheal illness [20, 21], with more than 78 O
28 serogroups identified in ~1,000 ETEC isolates from widespread locations [20]. While this
29 number is impractically high for developing a broadly protective, multivalent vaccine, 10
30 of these serogroups (O6, O8, O9, O25, O27, O78, O128, O148, O153 and O159) account
31 for >75% of the isolates, suggesting that a 10-valent polysaccharide vaccine could afford

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broad protection with the fewest components possible. At present, however, virtually no attention has been paid to ETEC LPS/O-PS as a subunit vaccine antigen.

One barrier to the development of an LPS/O-PS-based vaccines in general is the fact that purified polysaccharides, while partially immunogenic in adults, are often completely incapable of eliciting an antibody response in infants and children, the population for whom an ETEC vaccine is most needed. This problem results from an inability of polysaccharides to interact with the receptors on T cells, but can be solved by covalently coupling the LPS or O-PS structure to a $CD4^+$ T cell-dependent antigen such as an immunogenic protein carrier [22]. The resulting conjugates invoke a T-cell response that results in strong polysaccharide-specific antibody responses, immunological memory, and high immunogenicity in young children [23]. Indeed, conjugates are a highly efficacious and safe strategy for protecting against virulent pathogens, with successful vaccines licensed worldwide against *Haemophilus influenzae*, *Neisseria meningitidis* serogroups (tetravalent), *Streptococcus pneumoniae* (up to 20-valent), and *Salmonella typhi*, and many others in clinical development [23].

Despite their effectiveness, traditional conjugate vaccines are not without their drawbacks. Most notable among them is the complex, multistep process required for the purification, isolation, and conjugation of bacterial polysaccharides, which is expensive, time consuming, and low yielding [24]. A greatly simplified and cost-effective alternative involves metabolic engineering of bacteria to generate recombinant strains that serve as mini factories for one-step production of an unlimited and renewable supply of pure conjugates [25]. This bioconjugation approach is based on engineered protein glycosylation in non-pathogenic *E. coli* strains, wherein an O-PS molecule is conjugated to a co-expressed carrier protein by the *Campylobacter jejuni* oligosaccharyltransferase (OST) PglB (*Cj*PglB) [26]. To date, several unique conjugates have been produced by this method, with a few currently under clinical investigation [25]. Building on these cell-based efforts, we recently described a method called iVAX (*in vitro* conjugate vaccine expression) that enables conjugate vaccine biosynthesis using cell-free extracts derived from glycosylation-competent *E. coli* strains [27]. The iVAX platform promises to accelerate vaccine development and enable decentralized, cold chain–independent biomanufacturing by using cell lysates, rather than living cells, to make conjugates *in vitro*.

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In the current study, we describe two biosynthetic routes – one cell-based and the other cell-free – for low-cost production of conjugate vaccine candidates against two of the most prevalent O serogroups of ETEC, O148 and O78 [20]. Both routes enabled site-specific installation of ETEC O-PS onto carrier proteins used in licensed vaccines, namely the nonacylated form of protein D (PD) from *Haemophilus influenzae* and cross-reactive material 197 (CRM_{197}), a genetically detoxified variant of the *Corynebacterium diphtheriae* toxin (DT). The resulting conjugates stimulated strong O-PS-specific IgG antibody titers in mice, with the resulting antibodies possessing bactericidal activity against the cognate pathogens. For one of the prototype conjugates decorated with serogroup O148 O-PS, we further demonstrated protective efficacy against ETEC infection in mice. Overall, our work expands the inventory of antigens for ETEC vaccine design and provides an important first step towards the creation of a custom, multivalent vaccine with potential for broad ETEC coverage and increased access through adoption of simplified, low-cost biomanufacturing platforms.

RESULTS

Expression of ETEC serogroup O148 O-PS antigen in non-pathogenic *E. coli* cells.

Biosynthesis of the O-PS antigen from ETEC serogroup O148 involved plasmid pMW07-O148 [28, 29], which encodes the 10.2 kb O-PS gene cluster from ETEC strain B7A (serotype O148:H28) [30] (**Fig. 1a**). To confirm O-PS expression, we took advantage of the fact that O-PS antigens assembled in the cytoplasmic membrane of *E. coli* cells are transferred onto lipid A-core by the O-antigen ligase, WaaL. The lipid A-core-linked O-PS molecules are then shuttled to the outer membrane, becoming displayed on the cell surface where they are readily detectable with antibodies or lectins having specificity for the O-PS structure. As expected, non-pathogenic *E. coli* W3110 cells, which carry a copy of the *waaL* gene, were observed to express the ETEC O-PS antigen on their surface as evidenced by cross reactivity of nitrocellulose-spotted cells with an anti-ETEC O148 antibody (**Supplementary Fig. 1a**). The binding observed for these cells was on par with that measured for ETEC strain B7A [31], which natively expresses the O148 O-PS antigen. In contrast, both plasmid-free W3110 cells and W3110 cells carrying empty pMW07 plasmid showed little to no cross reactivity. A similar lack of cross reactivity was observed

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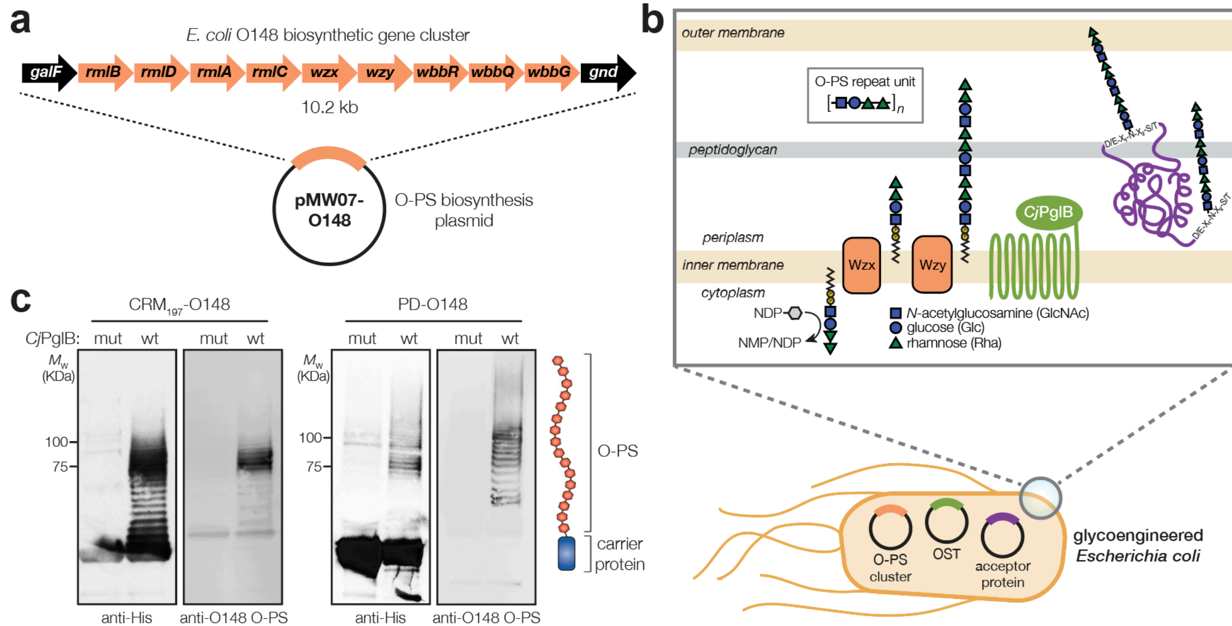

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2 **Figure 1. Biosynthesis of glycoconjugate vaccine candidates against ETEC bacteria.** (a) Biosynthesis
3 of ETEC O148 O-PS from plasmid pMW07-O148, which encodes the entire O-PS locus from ETEC strain
4 B7A (serotype O148:H28) between *galF* and *gnd*. (b) Glycoconjugate biosynthesis is enabled by
5 combining engineered O-antigen biosynthesis with *N*-linked protein glycosylation by *C. jejuni* PglB
6 (*Cj*PglB). Several plasmid-encoded glycosyltransferases sequentially add the repeat-unit sugars to the
7 lipid carrier undecaprenol pyrophosphate in the cytoplasmic membrane. The lipid-linked
8 oligosaccharide is flipped by Wzx and polymerized by the Wzy polymerase, and subsequently
9 transferred to 'DQNAT' acceptor sites in the carrier protein by PglB. Deletion of *waaL* in the host strain
10 is used to eliminate transfer of the O-PS to lipid A-core and ensure efficient transfer to the acceptor
11 protein, while deletion of *lpxM* results in pentaacylated lipid A structure having significantly reduced
12 toxicity. (c) Immunoblot analysis of purified carrier proteins derived from *E. coli* CLM24 cells carrying a
13 plasmid encoding either $CRM_{197}^{4xDQNAT}$ or $PD^{4xDQNAT}$ along with plasmid pMW07-O148 encoding the ETEC
14 O148 O-PS biosynthetic pathway and plasmid pMAF10 encoding wild-type *Cj*PglB (wt) or an inactive
15 mutant of *Cj*PglB (mut) as indicated. Blots were probed with anti-His antibody to detect acceptor proteins
16 and anti-ETEC O148 antibody to detect O-PS. Images depict aglycosylated and multiply glycosylated forms
17 of $CRM_{197}^{4xDQNAT}$ or $PD^{4xDQNAT}$. Molecular weight (M_W) markers are indicated on the left. Results are
18 representative of three biological replicates.

19

20 for CLM24 cells, which have a deletion in the *waaL* gene, confirming that the recombinant
21 O-PS antigen was assembled via the canonical lipid A-core pathway.

22 **Glycosylation of licensed vaccine carrier proteins with ETEC O148 O-PS.** To
23 generate a strong IgG response and lasting immunity, it is desirable to employ a highly
24 immunogenic protein as a carrier for the polysaccharide antigen, in this case ETEC
25 serogroup O148 O-PS. To this end, we sought to engineer non-pathogenic *E. coli* with
26 the ability to glycosylate a set of carrier proteins, namely PD and CRM_{197}, that are
27 currently used in licensed conjugate vaccines (**Fig. 1b**). To enable conjugation of O-PS
28 antigens to these carrier proteins, both were modified at their C termini with four tandem

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1 repeats of an optimized bacterial glycosylation motif, DQNAT [32], followed by a 6x-His tag
2 to enable detection via Western blot analysis and purification by Ni-NTA chromatography.
3 A signal peptide sequence derived from the *E. coli* DsbA protein was fused to the N-
4 terminus to localize CRM_{197} and PD to the periplasm in a manner that is compatible with
5 *N*-linked glycosylation [33]. Each of the resulting plasmids, pTrc99A-$CRM_{197}^{4xDQNAT}$ and
6 pTrc99A-$PD^{4xDQNAT}$, were used to transform *E. coli* strain CLM24 carrying plasmid
7 pMW07-O148 that encoded the O-PS biosynthetic enzymes and plasmid pMAF10 that
8 encoded *Cj*PglB [26]. CLM24 cells were used because they have a deletion of the gene
9 encoding the WaaL O-antigen ligase that makes undecaprenol pyrophosphate (UndPP)-
10 linked glycans including O-PS structures exclusively available to *Cj*PglB by preventing
11 their unwanted transfer to lipid A-core [26].

12 Following overnight expression of *Cj*PglB along with either $CRM_{197}^{4xDQNAT}$ or
13 $PD^{4xDQNAT}$ in the presence of the ETEC serogroup O148 biosynthetic enzymes, cells were
14 lysed, and His-tagged carrier proteins were purified by Ni-NTA chromatography. Elution
15 fractions from each sample were separated by SDS-PAGE and subjected to
16 immunoblotting using an anti-His antibody to detect the carrier proteins or antiserum
17 specific for ETEC O148 LPS to detect the O-PS antigen. This analysis revealed that both
18 $CRM_{197}^{4xDQNAT}$ and $PD^{4xDQNAT}$ were readily glycosylated with ETEC O148 O-PS glycans
19 (**Fig. 1c**). Importantly, we observed a ladder-like banding pattern for both O148 O-PS-
20 linked carrier proteins (hereafter CRM_{197}-O148 and PD-O148), which is characteristic of
21 *Cj*PglB-mediated O-PS transfer [26] and results from variability in the chain length of O-PS
22 antigens generated by the Wzy polymerase [19]. The most intense laddering signal was
23 observed above 75 kDa, suggesting that the carriers were heavily decorated with ETEC
24 O148 O-PS structures comprised of >10 repeating units (RUs). Control reactions with
25 CLM24 cells that lacked the O-PS biosynthetic plasmid or expressed a catalytically
26 inactive *Cj*PglB enzyme, generated by introducing D54N and E316Q substitutions [34],
27 confirmed that O-PS conjugation to the carrier proteins depended on both the O-PS
28 biosynthetic enzymes and *Cj*PglB (**Fig. 1c**; shown for mutant *Cj*PglB).

29 **Immunogenicity of PD-O148 glycoconjugate in mice.** To investigate conjugate
30 immunogenicity, BALB/c mice were immunized subcutaneously (s.c.) with glycosylated
31 PD bearing the ETEC O148 O-PS antigen and serum from these animals was analyzed

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by enzyme-linked immunosorbent assay (ELISA) to determine antibody titers. BALB/c mice were immunized with 50 µg doses of protein, either PD alone or PD-O148 conjugate, adjuvanted with aluminium phosphate, and subsequently injected with identical booster doses at 21 and 42 days after the initial injection (**Fig. 2a**). Upon analyzing sera collected on day 56, we found that BALB/c mice receiving the PD-O148 conjugate produced high titers of IgG antibodies that specifically recognized LPS derived from ETEC strain B7A (**Fig. 2b**). These serum IgG levels were significantly elevated (~2 orders of magnitude) compared to the titers measured in sera of control mice receiving buffer or aglycosylated PD. The ability of the PD-O148 conjugate to elicit strong IgG titers against ETEC B7A LPS provides further validation for using non-pathogenic *E. coli* strains engineered with protein glycosylation machinery as hosts for conjugate vaccine production.

Functionality of ETEC O-PS-specific serum antibodies. Next, PD-O148 conjugate vaccine-induced serum antibodies were evaluated for the ability to promote complement-mediated killing of ETEC strain B7A by serum bactericidal assay (SBA). SBA is an established method by which the activity of IgGs against bacterial pathogens can be measured. It often correlates with protection for serotypes of a pathogen and is a key *in vitro* method for measuring the functional activity of antibodies [35]. Of relevance here, several groups have developed bactericidal assays for evaluating whether serum IgG antibodies can potentiate the killing of different ETEC strains [36, 37]. Using a similar methodology, we investigated the idea that O-PS-specific serum IgGs elicited by the PD-O148 glycoconjugate would bind to the corresponding LPS on the surface of ETEC strain B7A and, in the presence of components of the human complement system, would mediate bacteriolysis of the enteric pathogen. For the sera derived from mice vaccinated with the PD-O148 conjugate, ~50% survival of ETEC B7A cells (corresponding to ~50% killing activity) was observed at dilutions as high as 10-fold (**Fig. 2c**). In contrast, virtually no killing was observed for sera derived from mice treated with PBS or the aglycosylated PD carrier protein as evidenced by pathogen survival that was close to 100%. Additionally, near complete killing was observed for the undiluted sera of vaccinated mice, whereas no killing was observed for the PBS and aglycosylated PD groups at the same serum dilution. These results confirm the immunological functionality of PD-O148 conjugate vaccine-induced IgGs present in the sera of immunized mice and predict the

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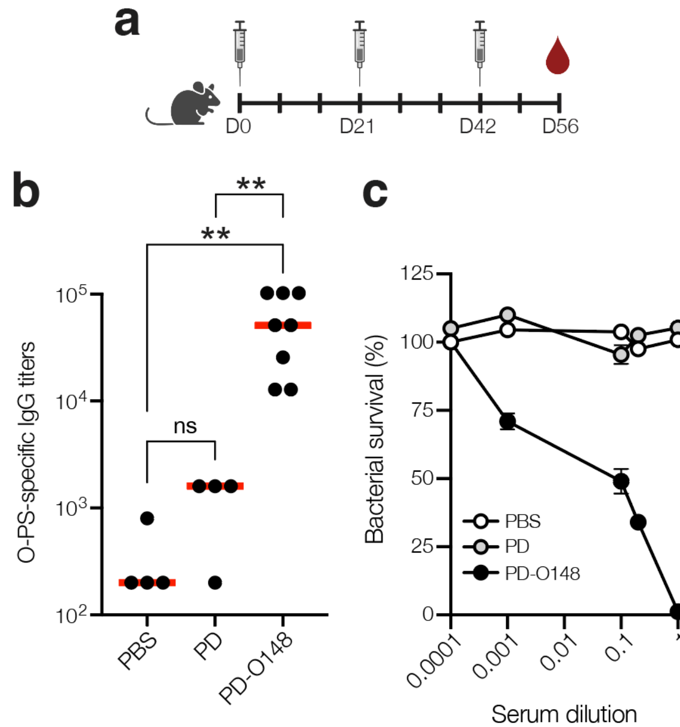

1
2 **Figure 2. Glycoconjugates are immunogenic.** (a) Schematic of the prime-boost immunization schedule.
3 Mice received an initial injection on day 0 and identically formulated booster injections on days 21 and 42.
4 Blood was drawn on days 0, 35, 49, and at study termination on day 56. (b) O-PS-specific IgG titers in day
5 56 serum of individual mice (black circles) and mean titers of each group (red lines) as determined by ELISA
6 with LPS derived from ETEC strain B7A (serotype O148:H28) as immobilized antigen. Groups of three
7 BALB/c mice were immunized s.c. with 100 µL PBS alone or PBS containing 50 µg of either aglycosylated
8 PD carrier protein or glycosylated ETEC O148 conjugate (PD-O148) adjuvanted with aluminium
9 phosphate adjuvant. Mice were boosted on days 21 and 42 with identical doses of each immunogen.
10 Statistical significance was determined by unpaired *t* test with Welch's correction (*$p < 0.05$; **$p < 0.01$;
11 ns, not significant). (c) Bactericidal killing activity of antibodies in the serum of mice immunized with
12 PBS, unmodified PD carrier protein, or ETEC O148 conjugate (PD-O148). Survival data were derived
13 from a standard SBA where dilutions of serum from immunized mice were tested against ETEC strain
14 B7A (serotype O148:H28) in the presence of human complement. SBA curves were generated with
15 pooled sera from each group. Data are the mean of three biological replicates ± SEM.

16 efficacy and protectiveness of our glycoengineered vaccine candidate.

17 **Protective efficacy of PD-O148 glycoconjugate in mice.** Encouraged by the SBA
18 results, we tested the ability of the PD-O148 conjugate to protect mice in a murine model
19 of an orally administered ETEC B7A infection. A major hurdle in developing enteric
20 vaccines is the lack of a suitable small animal model to study the efficacy and
21 immunogenicity of potential ETEC vaccines prior to testing in larger animals or humans.
22 Nevertheless, ETEC infection is commonly induced by the pathogen via oral
23 administration, with mice becoming colonized with ETEC following oral challenge using
24 inocula as small as 10^3 colony-forming units (CFUs) [38]. Oral gavage was selected as an
25 infection model as it closely reflects the route of infection and potentially recapitulates

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1 relevant outcomes of ETEC infection seen in humans [39]. In this study, mice were
2 pretreated with the antibiotic streptomycin, allowing them to more closely mimic the
3 disease symptoms that are often seen in humans [39, 40]. Following immunization according
4 to an identical schedule as above, mice were infected by oral gavage with ~1x10^4 CFUs
5 of ETEC strain B7A (**Fig. 3a**). After challenge infection, mice were monitored for signs
6 and symptoms of enteric illness (*e.g.*, water diarrhea). Diarrhea induced by ETEC
7 infection was greatly reduced in the PD-O148-vaccinated group overall three days post-
8 infection, which was in stark contrast to buffer and aglycosylated PD control groups that
9 developed watery diarrhea. We also collected stool samples to detect shedding of the
10 challenge strain. Diarrheal illness, when it occurs, is associated with higher fecal shedding
11 levels of ETEC in animals and humans [39, 41]. Post-challenge shedding levels of the
12 challenge strain ETEC B7A was detected using quantitative PCR (qPCR) to examine
13 fecal pellet DNA extracts for the presence of LT encoded by the *eltA* gene. Consistent
14 with the observed protection against diarrhea, only mice receiving the PD-O148 conjugate
15 exhibited a significant reduction in ETEC stool shedding detected at day 3 post-infection
16 (~2-log reduction compared to the buffer and aglycosylated PD carrier protein control
17 groups) (**Fig. 3b**). Taken together, these results clearly demonstrated the ability of the
18 PD-O148 conjugate vaccine candidate to confer protection against ETEC infection in
19 mice, consistent with the conjugate's ability to stimulate high titers of IgG antibodies that
20 possess potent bactericidal activity.

21 **Biosynthesis and immunogenicity of an ETEC serogroup O78-directed conjugate.**

22 As a first step towards such a multivalent formulation, we sought to create a
23 glycoconjugate vaccine candidate against ETEC serogroup O78 by implementing an
24 identical strategy as outlined above for serogroup O148. We chose serogroup O78
25 because it is one of the most widely distributed and most frequently occurring ETEC
26 serogroups that, together with ETEC serogroups O6, O8, O27, O148, O153 and O169,
27 accounts for nearly 25% of the nearly 1,000 isolated identified worldwide [20]. Introduction
28 of plasmid pMW07-O78 [27, 29, 42] (**Supplementary Fig. 2a**) into non-pathogenic *E. coli*
29 strain W3110 enabled expression of cell-surface O78 O-PS molecules that were readily
30 detected by dot blot analysis (**Supplementary Fig. 1b**). By combining plasmid pMW07-
31 O78 together with the plasmids for expressing *Cj*PglB and the CRM_{197} carrier protein in

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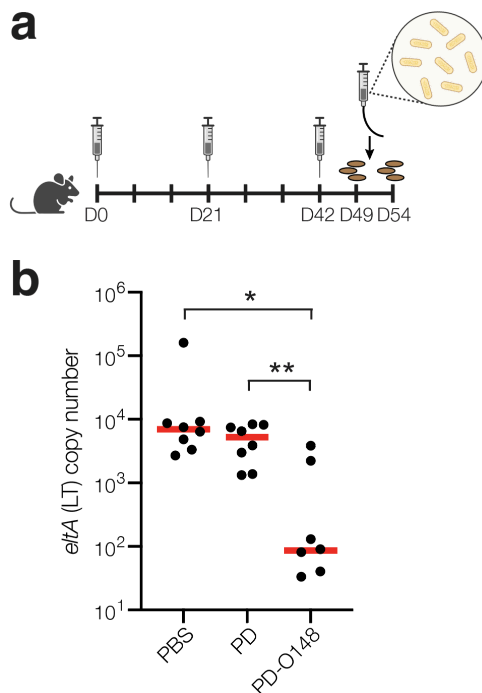

1
2 **Figure 3. Glycoconjugates are functional and protective.** (a) Schematic of the immunization and
3 challenge schedule. Mice received an initial immunogen injection on day 0 and identically formulated
4 booster injections on days 21 and 42. On day 49, mice received antibiotics in the drinking water to eradicate
5 normal flora and fecal pellets were collected. On day 51, mice were subsequently infected by gavage and
6 checked daily for 3 days. On day 54, fecal pellets were collected, and mice were sacrificed. (b) Vaccine
7 effects on stool shedding of ETEC B7A in infected mice 3 days post-infection. Groups of 8 BALB/c
8 mice receiving PBS, unmodified PD carrier protein, or PD-O148 were infected with ~1 x 10^4 CFUs of
9 ETEC B7A. Fecal samples were collected for DNA extraction and analyzed by qPCR to detect
10 pathogen. ETEC burden in fecal pellets was measured by qPCR of the *eltA* gene, which encodes heat-
11 labile endotoxin (LT). Data are the mean (red bars) of individual mice (black circles). Statistical
12 significance was determined by unpaired *t* test with Welch's correction (*$p < 0.05$; **$p < 0.01$).
13
14 CLM24 Δ*lpxM* cells, we were able to produce glycosylated CRM_{197} bearing the O78 O-
15 PS antigen (**Supplementary Fig. 2b**). The glycosylated CRM_{197}-O78 conjugate was used
16 to immunize BALB/c mice and was observed to induce high titers of IgG antibodies that
17 specifically recognized LPS derived from ETEC strain H10407 (serotype O78:H11) [43]
18 (**Supplementary Fig. 2c**). Collectively, these results highlight the modularity of the
19 biosynthetic approach, enabling facile production of an additional serogroup-specific
20 conjugate simply by swapping out the O-PS biosynthesis plasmid. Such
21 interchangeability will be key to generating a multivalent conjugate formulation that
22 confers broad ETEC serogroup protection, with the results here representing an important
23 first step in that direction.

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1 **Cell-free biosynthesis of ETEC O78 O-PS conjugate.** In parallel to using living cells,
2 we also explored whether a cell-free protein synthesis (CFPS) approach could be used
3 to produce glycoengineered conjugate vaccine candidates against ETEC. To this end, we
4 leveraged a modular technology for *in vitro* conjugate vaccine expression (iVAX) in
5 portable, freeze-dried lysates from detoxified, non-pathogenic *E. coli* [27] (**Fig. 4a**). Previous
6 studies demonstrated that iVAX reactions are capable of synthesizing clinically relevant
7 doses of protective conjugate vaccines comprised of pathogen-specific O-PS antigens
8 linked to licensed carrier proteins. Here, we produced an iVAX lysate from *E. coli* CLM24
9 Δ*lpxM* cells expressing the ETEC O78 O-PS biosynthetic pathway and *Cj*PglB. This
10 lysate, which contained lipid-linked ETEC O78 O-PS and active *Cj*PglB, was used to
11 catalyze iVAX reactions primed with plasmid DNA encoding the $PD^{4xDQNAT}$ carrier protein.
12 The products of these reactions were immunoblotted with anti-His antibody or a
13 commercial anti-ETEC O78 antibody specific to the ETEC O78 O-PS. Similar to cell-
14 based expression, cell-free iVAX reactions produced $PD^{4xDQNAT}$ that was clearly
15 glycosylated with the O78 O-PS antigen and exhibited the characteristic ladderlike
16 banding pattern associated with O-PS chain-length variability (**Fig. 4b**). Control reactions
17 with lysates from cells lacking the ETEC O78 O-PS were devoid of any detectable
18 glycosylation. Following immunization of BALB/c mice with the iVAX-derived conjugate,
19 we observed strong induction of IgG antibodies that specifically recognized LPS derived
20 from ETEC strain H10407 (serotype O78:H11) (**Fig. 4c**), with serum IgG titers comparing
21 favorably to those observed following immunization with the CRM_{197}-O78 conjugate that
22 was made in living cells. Finally, PD-O78 conjugate vaccine-induced serum antibodies
23 were evaluated for the ability to promote complement-mediated killing of ETEC strain
24 H10407 by SBA. Greater than ~50% killing activity of ETEC H10407 cells was observed
25 for the sera derived from PD-O78-vaccinated-mice at dilutions as high as 10-fold,
26 whereas no measurable killing was observed for sera derived from mice treated with PBS
27 or aglycosylated PD (**Fig. 4d**).

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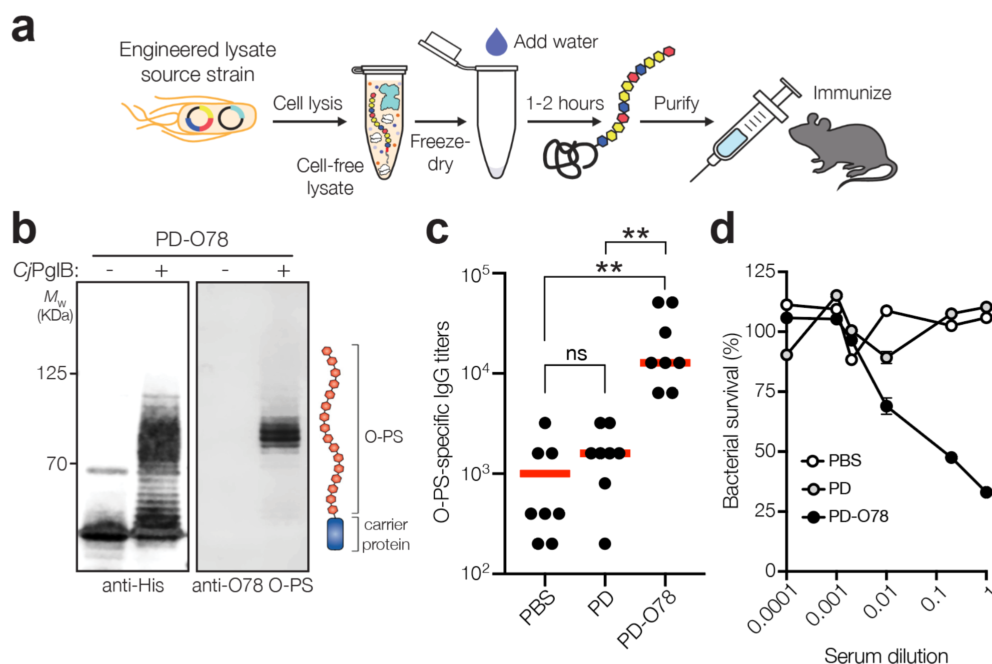

1
2 **Figure 4. Glycoconjugates are functional and protective.** (a) The iVAX platform provides a rapid
3 means to develop and distribute conjugate vaccines against bacterial pathogens. (b) Immunoblot analysis
4 of $PD^{4xDQNAT}$ generated using iVAX lysate. Specifically, 5-mL reactions with glyco-enriched S12 extract
5 derived from CLM24 Δ*lpxM* cells carrying pMW07-O78 and pSF-*Cj*PglB-LpxE were primed with plasmid
6 pJL1-$PD^{4xDQNAT}$. Blots were probed with anti-His antibody to detect acceptor proteins and anti-ETEC
7 O78 antibody to detect O-PS antigens. Images depict aglycosylated and multiply glycosylated forms
8 of $PD^{4xDQNAT}$. Molecular weight (M_W) markers are indicated on the left. Results are representative of
9 three biological replicates. (c) O-PS-specific IgG titers in day 56 serum of individual mice (black circles)
10 and mean titers of each group (red lines) as determined by ELISA with LPS derived from ETEC strain
11 H10407 (serotype O78:H11) as immobilized antigen. Groups of three BALB/c mice were immunized s.c.
12 with 100 μL PBS alone or PBS containing 24 μg of either aglycosylated PD carrier protein or
13 glycosylated ETEC O148 conjugate adjuvanted with aluminium phosphate adjuvant. Mice were
14 boosted on days 21 and 42 with identical doses of each immunogen. Statistical significance was
15 determined by unpaired *t* test with Welch's correction (*$p < 0.05$; **$p < 0.01$; ns, not significant). (d)
16 Bactericidal killing activity of serum antibodies from mice immunized with same immunogens as in (c).
17 Survival data were derived from a standard SBA where serum dilutions were tested against ETEC
18 strain H10407 in the presence of human complement. SBA curves were generated with pooled sera
19 from each group. Data are the mean of three biological replicates ± SEM.
20

21 **DISCUSSION**

22 In the present study, we describe robust cell-based and cell-free bioconjugation strategies
23 for producing conjugate vaccine candidates against two widespread O serogroups of
24 ETEC, O148 and O78 [20]. These strategies leveraged glycoengineered strains of non-
25 pathogenic *E. coli* and their cell-free extracts for site-specific installation of two different
26 ETEC O-PS structures onto the PD and CRM_{197} carrier proteins that are used in licensed
27 vaccines. The resulting PD- and CRM_{197}-based conjugates were strongly immunogenic
28 in mice, eliciting high titers of O-PS-specific IgG antibodies that also exhibited potent

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1 bactericidal activity against ETEC strains B7A (serotype O148:H28) and H10407
2 (serotype O78:H11). In proof-of-concept challenge studies, mice vaccinated with the PD-
3 O148 conjugate were protected against ETEC infection as evidenced by the lack of overt
4 water diarrhea and significantly decreased ETEC stool shedding. Overall, our findings
5 expand the repertoire of available ETEC antigens to include molecules that have largely
6 been overlooked for subunit vaccine engineering. Moreover, the cell-based and cell-free
7 platforms described here lay the foundation for future creation of a custom, multivalent
8 vaccine with the potential for broad ETEC coverage and increased access through
9 adoption of simplified, low-cost biomanufacturing platforms.

10 The immunogenicity and protective efficacy of our conjugates substantiate the use
11 of ETEC O-PS as a subunit vaccine antigen. This antigenic expansion is significant for
12 several reasons. First, even though glycoconjugate vaccines are one of the safest and
13 most effective methods for preventing bacterial infections, there are surprisingly few that
14 have been fully licensed. That said, these numbers are poised to increase in the coming
15 years as cell-based and cell-free technologies for glycoengineering recombinant
16 vaccines, such as those described here and elsewhere, [25] reach full maturity. As part of
17 this maturation, it is imperative to continue growing the pipeline with as many promising
18 anti-bacterial vaccine candidates as possible, especially as diarrheal and other vaccine-
19 preventable diseases continue to be unmet challenges and as drug-resistant bacteria are
20 predicted to threaten up to 10 million lives per year by 2050 [44]. Second, all ETEC vaccines
21 to enter clinical testing thus far are focused on the classical paradigm of ETEC
22 pathogenesis and attempt to induce immune responses to select colonization factor
23 (CF/CS) antigens and LT [8-11]. However, the risk of focusing on the classical paradigm is
24 that it constrains ETEC vaccinology to a subset of canonical antigens, which could be
25 problematic if our current view of ETEC pathogenesis is incomplete, as has been
26 suggested recently [10]. For this reason, we decided to investigate O-PS from ETEC
27 serogroups O148 and O78 with the goal of validating these non-canonical targets and
28 supplementing the existing inventory of ETEC vaccine antigens. One potential drawback
29 of O-PS as an ETEC vaccine target is the fact there are more than 78 O serogroups.
30 Fortuitously, however, as few as 10 serogroups (O6, O8, O9, O25, O27, O78, O128,
31 O148, O153 and O159) account for >75% of known ETEC isolates [20], suggesting that a

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1 10-valent conjugate vaccine could afford broad protection. This number is much more
2 feasible for multivalent ETEC vaccine development, especially considering that some of
3 the most effective licensed conjugates achieve a valency of >10 by attachment of distinct
4 polysaccharides from the most important serogroups (*e.g.*, Prevnar13, Prevnar20). It is
5 also worth noting that multivalency is not a unique challenge for an O-PS-based vaccine.
6 There are 25 distinct CF/CS antigens identified to date that would need to be combined
7 in some manner in order to achieve ~75% coverage of all isolates expressing the most
8 common colonization factors [10]. Hence, both O-PS and protein antigen-based vaccine
9 candidates face complicated paths to a broadly protective, multivalent vaccine.

10 The ETEC O148 and O78 structures are now part of an ever-expanding list of
11 polysaccharides that can be transferred to acceptor proteins by the *Cj*PglB biocatalyst.
12 *Cj*PglB is well known for its remarkably relaxed oligosaccharide substrate specificity that
13 allows transfer of diverse Und-PP-linked glycans including numerous different O-PS
14 structures [26, 45]. The ability of *Cj*PglB to site-specifically modify diverse acceptor proteins
15 is aided by the introduction of a genetically encoded *N*-linked glycosylation tag that can
16 be appended N- or C-terminally in single or multiple copies, or can be inserted at internal
17 locations in the acceptor protein [33]. Here, the introduction of four tandemly repeated
18 DQNAT motifs at the C-termini of CRM_{197} and PD facilitated their use as acceptor protein
19 substrates for *Cj*PglB and significantly expanded the set of carrier proteins available for
20 bioconjugation, which historically has focused on a narrow set of carriers that are not
21 currently used in any licensed vaccines – most notably *Pseudomonas aeruginosa*
22 exotoxin A (ExoA) [25]. Importantly, the in-built flexibility of bacterial OSTs like *Cj*PglB
23 together with programmable glycosylation motifs makes it straightforward to produce an
24 array of custom vaccines comprised of different polysaccharide/protein combinations.

25 As mentioned above, manufacturing of traditional conjugate vaccines is a complex,
26 multistep process that is expensive, time consuming, and low yielding [24]. The Centers for
27 Disease Control and Prevention (CDC) cost per dose for conjugate vaccines ranges from
28 ~$9.50 for the *H. influenzae* vaccine ActHIB up to ~$75 and ~$118 for the meningococcal
29 vaccine Menactra and pneumococcal vaccine Prevnar 13, respectively [46]. The cell-based
30 and cell-free strategies described here represent greatly simplified alternatives for
31 biomanufacturing conjugate vaccines whereby metabolically engineered *E. coli* or their

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1 cell-free extracts are exploited for one-step production of an unlimited and renewable
2 supply of pure conjugate vaccine product. Because of the dramatic simplification of the
3 process, bioconjugation strategies are anticipated to allow scalable production of large
4 quantities of conjugates at a much more affordable cost, which is especially important for
5 achieving sustained impact on global public health. For large-scale *E. coli*-based
6 production, recent technoeconomic analysis indicates that the manufacturing costs (costs
7 of goods sold, COGS) for a biologic antiviral, Griffithsin (GRFT), are as low as $3.43/g
8 assuming production of >24,000 kg GRFT/yr [47]. These authors concluded that, based on
9 an estimated dose of 12–24 mg, it would be possible to provide ~1 billion doses/yr at a
10 COGS of about $0.08/dose. While economics for a conjugate vaccine would be different
11 due to the smaller dose and need for fewer total doses overall, we anticipate that a large-
12 scale, *E. coli*-based manufacturing process for conjugates would offer a highly
13 competitive cost per dose. Likewise, our own economic analysis of cell-free production
14 revealed that iVAX reactions are inexpensive, costing ~$5/mL for raw materials. This cost
15 can be decreased by approximately four-fold to ~$1.40/mL (~$0.50 per 24 μg dose) by
16 optimizing the cell-free extract formulation to use the low-cost energy substrate
17 maltodextrin in place of the significantly more expensive phosphorylated secondary
18 energy substrate phosphoenolpyruvate (PEP) [42]. Additional advantages of cell-free
19 systems for conjugate production include that they can be: (i) distributed through freeze
20 drying [48] followed by simple rehydration at the point of use [27, 49]; (ii) linearly scaled from 1
21 nL to 100 L [50] for accelerated process development; and (iii) rapidly customized and
22 reconfigured for product switching [49, 51]. Taken together, these features have the potential
23 to advance new paradigms in decentralized manufacturing of conjugate vaccines that we
24 are continuing to explore for ETEC and other high-priority pathogens.
25

26 **MATERIALS AND METHODS**

27 **Bacterial strains and plasmids.** All strains used in this study are provided in
28 **Supplementary Table 1**. Briefly, *E. coli* strain DH5α was used for all molecular biology
29 including plasmid cloning and isolation. *E. coli* W3110 was used for expressing O-PS on
30 lipid A-core and displaying O-PS molecules on the cell surface while *E. coli* CLM24 [26] was
31 used as the host strain for expressing glycoengineered conjugates using intact cells.

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CLM24 is a derivative of W3110 that carries a deletion in the gene encoding the WaaL ligase, thus facilitating the accumulation of preassembled glycans on Und-PP as substrates for *Cj*PglB-mediated protein glycosylation. CLM24 Δ*lpxM* was used as the source strain for expressing glycoengineered conjugates in cell-free reactions. CLM24 Δ*lpxM* lacks the gene encoding the lipid A acyltransferase LpxM, a deletion that yields a pentaacylated lipid A structure with significantly reduced toxicity [52]. The ETEC strains B7A (serotype O148:H28; CS6, LT, STa) [30] and H10407 (serotype O78:H11; CFA/I, LT, STa) [43] were used for SBA and challenge studies as well as a source of LPS. These strains were chosen because both were previously used in human challenge trials [31, 43].

Plasmids used in the study are listed in **Supplementary Table 1**. Briefly, plasmids pTrc99A-ssDsbA-PD^{4xDQNAT} and pTrc99A-ssDsbA-$CRM_{197}^{4xDQNAT}$ [27] were used to express the PD and CRM_{197} acceptor proteins in the *E. coli* periplasm. These plasmids were constructed by PCR amplification of the ssDsbA-PD^{4xDQNAT} and ssDsbA-$CRM_{197}^{4xDQNAT}$ sequences from plasmids pTrc99S-ssDsbA-PD^{4xDQNAT} and pTrc99S-ssDsbA-$CRM_{197}^{4xDQNAT}$, respectively [27], and followed by ligation of the PCR products in pTrc99A. Plasmid sequences were confirmed by Sanger sequencing at the Genomics Facility of the Cornell Biotechnology Resource Center (BRC). Plasmids pMAF10 [26] and pMAF10$^{D54N/E316Q}$ [34] were used for cell-based expression of wild-type *Cj*PglB and an inactive D54N/E316Q mutant of *Cj*PglB, respectively. The bacterial O-PS biosynthetic pathway plasmids were pMW07-O78 [27, 29, 42] for expressing the O-PS of ETEC strain H10407 and pMW07-O148 [28, 29] for expressing the O-PS of ETEC strain B7A. Plasmids used for cell-free expression of conjugate vaccines included pSF-*Cj*PglB-LpxE for co-expressing CjPglB along with *Francisella tularensis* phosphatase LpxE that promotes monophosphorylation of lipid A [27] and pJL1-PD^{4xDQNAT} for expressing PD^{4xDQNAT} [27].

Cell-based glycoconjugate expression and purification. For cell-based glycoconjugate expression, plasmids pTrc99A-ssDsbA-PD^{4xDQNAT} and pTrc99A-ssDsbA-$CRM_{197}^{4xDQNAT}$ encoding conjugate carrier proteins preceded by the DsbA signal peptide for translocation to the periplasm were used to transform CLM24 cells carrying a bacterial O-PS pathway encoded on plasmid pMW07-O148 or pMW07-O78 and *Cj*PglB encoded on plasmid pMAF10 or pMAF10$^{D54N/E316Q}$. Transformed cells were grown in 10 mL LB medium (10 g/L yeast extract, 5 g/L tryptone, 5 g/L NaCl) overnight at 37 °C. The next

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1 day, cells were subcultured into 1 L of LB and allowed to grow at 37 °C until the optical
2 density at 600 nm (OD_{600}) reached 0.6-0.8. The culture was then supplemented with 0.2%
3 arabinose to induce expression of *Cj*PglB and grown at 30 °C for 16 h, after which 0.5
4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the
5 conjugate carrier protein for an additional 8 h at 30 °C. The cells were harvested, and cell
6 pellets were resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 10 mM
7 imidazole; pH 7.5) at 2-5 mL buffer per gram wet weight. Cells were lysed using a
8 EmulsiFlex-C5 homogenizer (Avestin) then centrifuged at 13,000 x g for 30 min. The
9 lysate was filtered through a 0.45-μm syringe filter and loaded onto a gravity flow column
10 containing Ni-NTA resin (Thermo Fisher Scientific) that was washed with 5-10 column
11 volumes of wash buffer (20 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole; pH 7.5). The
12 resin and cell lysate supernatant were allowed to equilibrate for 30 min at 4 °C. After
13 flowthrough of the supernatant, the resin was washed with 5 column volumes of wash
14 buffer. The protein was eluted with 3 column volumes of elution buffer (20 mM Tris-HCl,
15 200 mM NaCl, 300 mM imidazole; pH 7.5). Eluted protein was dialyzed into sterile PBS,
16 concentrated, and quantified measured by Bradford assay.
17 **Periplasmic extract preparation**. Periplasmic extracts containing the expressed
18 glycoproteins were prepared by centrifuging the induced cultures at 13,000 x g and 4 °C
19 for 2 min. The resulting pellets were resuspended in 0.4 M L-arginine (Sigma-Aldrich; 100
20 μL 0.4 M L-arginine per 100 mL culture) and incubated at 4 °C for 1 h with gentle shaking
21 at 10-min intervals. The resuspended pellets were then centrifuged as above to obtain
22 the final periplasmic extracts in the supernatant.
23 **Cell-free extract preparation.** Cell-free extracts were prepared as previously described
24 [42, 53]. Specifically, CLM24 Δ*lpxM* cells were transformed with both pSF-*Cj*PglB-LpxE and
25 pMW07-O78 plasmids for the strain used to generate PD-O78, and only the pSF-*Cj*PglB-
26 LpxE plasmid for the strain used to generate the negative control aglycosylated PD. Cells
27 were grown in a Sartorius Stedim BIOSTAT Cplus bioreactor at the 10-L scale in 2xYTP
28 media supplemented with carbenicillin at 100 μg/mL and chloramphenicol at 34 μg/ml or
29 only carbenicillin at 100 μg/mL in the negative control extract . Cells were inoculated at
30 OD_{600} ≈ 0.08 and induced at OD_{600} ≈ 1 with 0.02% arabinose to induce expression of
31 *Cj*PglB and O-PS enzymes and harvested at OD_{600} ≈ 3. All subsequent steps were

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1 performed on ice unless otherwise stated. Cells were harvested by centrifugation at 5,000
2 x g for 15 min and then washed 3 times with S30 buffer (10 mM Tris acetate pH 8.2, 14
3 mM magnesium acetate, and 60 mM potassium acetate). Following washing, cells were
4 pelleted at 7,000 x g for 10 min, then flash frozen and stored at -80 °C. For lysis, CLM24
5 Δ*lpxM* cells were resuspended in 1 mL/g S30 buffer then homogenized using an
6 EmulsiFlex-C3 high-pressure homogenizer (Avestin) with 1 pass at a pressure of ~21,000
7 psig. Following lysis, cells were centrifuged for 12,000 x g for 10 min. Supernatant was
8 then collected and incubated at 37°C for 1 h in a runoff reaction. Cells were then
9 centrifuged once more at 10,000 x g for 10 min and the supernatant was flash frozen and
10 stored at –80 °C as the final extract.
11 **Cell-free protein synthesis reactions.** For cell-free synthesis of PD-O78 and
12 aglycosylated PD, reactions were prepared at the 5-mL scale in 50 mL conical tubes.
13 Reactions producing PD-O78 used extract enriched with both *Cj*PglB and ETEC-O78 O-
14 PS, while reactions productingg aglycosylated PD used extract enriched only with PglB.
15 Each reaction was prepared as described previously [54] to contain 3.33 ng/µL pJL1-PD-
16 4xDQNAT plasmid and 30% (vol./vol.%) extract in addition to: 10 mM magnesium
17 glutamate (Sigma, 49605), 10 mM ammonium glutamate (Biosynth, FG28929), 130 mM
18 potassium glutamate (Sigma, G1501), 1.2 mM adenosine triphosphate (Sigma A2383),
19 0.85 mM guanosine triphosphate (Sigma, G8877), 0.85 mM uridine triphosphate (Sigma
20 U6625), 0.85 mM cytidine triphosphate (Sigma, C1506), 0.034 mg/mL folinic acid,
21 0.171 mg/mL *E. coli* tRNA (Roche 10108294001), 2 mM each of 20 amino acids, 30 mM
22 phosphoenolpyruvate (PEP, Roche 10108294001), 0.4 mM nicotinamide adenine
23 dinucleotide (Sigma N8535-15VL), 0.27 mM coenzyme-A (Sigma C3144), 4 mM oxalic
24 acid (Sigma, PO963), 1 mM putrescine (Sigma, P5780), 1.5 mM spermidine (Sigma,
25 S2626), 57 mM HEPES (Sigma, H3375), and 15-20 µg/mL T7. Reactions were then
26 lyophilized for 16-20 hours using a VirTis Benchtop Pro Lyophilizer (SP scientific). Fully
27 lyophilized reactions were rehydrated with 5 mL nuclease-free water and incubated at
28 30°C for one hour to synthesize the carrier protien (PD). After one hour of protein
29 synthesis, glycosylation was initiated by supplementing 25 mM $MnCl_2$ and 0.1 % wt/vol
30 DDM. Reactions were incubated for one more hour at 30 °C, then were centrifuged at

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1 16,000 x g for 15 minutes. The His-tagged carrier protein was then purified from the
2 soluble cell-free reactions using Ni-NTA affinity resin as previously described [42].
3 **Western blot analysis.** Cell-based glycoconjugate samples were run on NuPAGE 4-12%
4 Bis-Tris gels (Invitrogen). Following electrophoretic separation, proteins were transferred
5 from gels onto 0.45-μm Immobilon-P polyvinylidene difluoride membranes (PVDF) using
6 a mini blot module (Thermo Fisher Scientific) according to the manufacturer's instructions.
7 Membranes were washed twice with TBS buffer (80 g/L NaCl, 20 g/L KCl, and 30 g/L Tris-
8 base) followed by incubation for 1 h in blocking solution (50 g/L non-fat milk in TBS). After
9 blocking, membranes were washed three times in TBS-T (TBS with 0.05% (v/v%) Tween-
10 20) with a 5-min incubation between each wash. For fluorescence-based detection of
11 immunoblots, membranes were probed with both an anti–6x-His tag antibody (R&D
12 Systems, Cat # MAB050; diluted 1:7,500) and anti–ETEC O148 antibody (Abcam, Cat #
13 ab78827; diluted 1:1,000) or anti–ETEC O78 antibody (Abcam, Cat # ab78826; diluted
14 1:1,000) in 1X TBS-T with 5% (w/v) BSA. Probing of membranes was performed overnight
15 at 4 °C with gentle rocking, after which membranes were washed with TBS-T as described
16 above and probed with fluorescently labeled secondary antibodies for 1 h at room
17 temperature in 1X TBS-T with 5% (w/v) nonfat dry milk. The membrane was washed for
18 5 min with TBS-T and then imaged using a ChemiDoc XRS+System (Bio-Rad). For
19 chemiluminescence-based detection of immunoblots, membranes were probed with an
20 anti–6x-His tag antibody (Abcam, Cat # ab9108; diluted 1:7,500) and then with the
21 corresponding anti-mouse HRP-conjugated secondary antibody (Abcam, Cat #
22 ab205718; diluted 1:7,500). Another membrane was separately probed with anti-ETEC
23 O148 antibody (Abcam, Cat # ab78827; diluted 1:1,000) or anti-ETEC O78 antibody
24 (Abcam, Cat # ab78826; diluted 1:1,000) in 1X TBS-T with 5% (w/v) BSA and then with
25 anti-rabbit HRP-conjugated secondary antibody (Abcam, Cat # ab205718; diluted
26 1:7,500). For signal visualization, membranes were briefly incubated at room temperature
27 with Western ECL substrate (Bio-Rad) and imaged using a ChemiDoc XRS+System (Bio-
28 Rad).
29 Cell-free samples were run on 4-12% Bis-Tris gels with SDS-MOPS running buffer
30 supplemented with NuPAGE antioxidant. Samples were then transferred to PVDF 0.45-
31 μm membranes (Millipore, USA) for 55 min at 80 mA per blot using a semi-dry transfer

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1 cell. Membranes were blocked for 1 h at room temperature or overnight at 4 °C in Intercept
2 Blocking Buffer (Licor). Primary antibodies used were anti-His antibody (Abcam, Cat #
3 ab1187; diluted 1:7,500) or anti-ETEC-O78 antibody (Abcam, Cat # ab78826; diluted
4 1:2,500) in Intercept blocking buffer with 0.2% (v/v) Tween 20, and membranes were
5 incubated for 1 h at room temp or overnight at 4 °C. The secondary antibody used was a
6 fluorescent goat anti-rabbit antibody (Licor, Cat # GAR-680RD; diluted 1:10,000) in
7 Intercept blocking buffer, 0.2% (v/v) Tween 20 and 0.1% (w/v) SDS for both anti-His and
8 anti-ETEC-O78 blots. Blots were washed 6 times for 5 min after each of the blocking,
9 primary, and secondary antibody incubations using PBS-T. Blots were imaged with Licor
10 Image studio.

11 **Dot blot analysis.** To detect cell-surface expression of ETEC O148 O-PS, overnight
12 cultures of the following strains were grown: *E. coli* strains W3110 and CLM24 without a
13 plasmid, W3110 carrying empty pMW07, W3110 and CLM24 carrying pMW07-O148, and
14 ETEC strain B7A. A total of 2 µl containing an equivalent amount of each strain, as well
15 as LPS extracted from B7A cells, were spotted onto a nitrocellulose membrane. The
16 membrane was allowed to dry, and then non-specific sites were blocked by soaking in
17 5% (w/v) BSA in TBS-T for 1 h at room temperature, followed by incubation for 30 min
18 with anti–ETEC O148 antibody (Abcam, Cat # ab78827; diluted 1:1,000) in 0.1% (w/v)
19 BSA in TBS-T. The membrane was washed three times with TBS-T, then incubated with
20 secondary antibody conjugated to HRP. After three TBS-T washes, the membrane was
21 incubated with Western ECL substrate (Bio-Rad) and imaged using a ChemiDocTM
22 XRS+System (Bio-Rad). An identical protocol was followed for detection of cell-surface
23 expression of ETEC O78 O-PS.

24 **Immunization.** Groups of four 6-week-old female BALB/c mice (Harlan Sprague Dawley)
25 were immunized s.c. with 50 µL of sterile PBS (pH 7.4, Fisher Scientific) or formulations
26 containing either aglycosylated PD or PD-O148 conjugate. The amount of antigen in each
27 preparation was normalized such that ~25-50 µg of these proteins was administered per
28 injection. The purified protein groups were formulated in sterile PBS and mixed with an
29 equal volume of Adju-Phos aluminium phosphate adjuvant (InvivoGen) before injection.
30 Mice were boosted 21 and 42 days after the initial immunization. For antibody titering,
31 blood was taken on days 0, 35, and 49 via submandibular collection, as well as at the

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1 study termination on day 56 via cardiac puncture. Sera were isolated from the collected
2 blood draws after centrifugation at 5,000 x g for 10 min and stored at −20°C. For bacterial
3 killing assays, final blood serum collections for all the mice within each group were pooled.
4 The protocol number for the animal trial was 2012-0132 and was approved by the
5 Institutional Animal Care and Use Committee (IACUC) at Cornell University.

6 **Serum antibody titering.** Serum antibody titers were determined by ELISA using LPS
7 derived from ETEC strains as immobilized antigen. Specifically, O148 and O78 LPS
8 molecules were prepared from ETEC strains B7A and H10407, respectively, by hot
9 phenol water extraction and DNase I (Sigma) and proteinase K (Invitrogen) treatment, as
10 described elsewhere [17]. Briefly, extracted LPS samples were purified using PD-10
11 desalting columns packed with Sephadex G-25 resin (Cytiva), and concentrations were
12 determined using a purpald assay [55]. 96-well plates (MaxiSorp; Nunc Nalgene) were
13 incubated with 0.5 μg/mL of purified LPS diluted in PBS, pH 7.4, 25 μL/well, at 4 °C
14 overnight. Plates were incubated with 50 μL blocking buffer (5% (w/v) nonfat dry milk
15 (Carnation) in PBS) overnight at 4 °C, then washed three times with 200 μL PBS-T (PBS,
16 0.05% (v/v) Tween 20) per well. Serum samples isolated from the collected blood draws
17 of immunized mice were appropriately serially diluted in triplicate in blocking buffer and
18 added to the plates for 2 h at 37 °C. Plates were washed three times with PBS-T (+ 0.03%
19 BSA (w/v)), then incubated for 1 h at 37 °C in the presence of a horseradish peroxidase–
20 conjugated goat anti-mouse IgG antibody (Abcam, Cat # ab97265; diluted 1:25,000).
21 After three PBS-T + 0.3% BSA washes, 50 μL of 3,3′-5,5′-tetramethylbenzidine substrate
22 (1-Step Ultra TMB-ELISA; Thermo Fisher Scientific) was added to each well, and the
23 plates were incubated at room temperature in the dark for 30 min. The reaction was
24 stopped by adding 50 μL of 2 M H_2SO_4, and absorbance was measured at a wavelength
25 of 450 nm using a FilterMax F5 microplate spectrophotometer (Agilent). Serum antibody
26 titers were determined by measuring the lowest dilution that resulted in signals that were
27 3 standard deviations above the background controls of no serum. Statistical significance
28 was determined by unpaired *t* test with Welch's correction using GraphPad Prism 9 for
29 MacOS (Version 9.2.0).

30 **SBA.** A modified version of a previously described SBA method was followed [56]. ETEC
31 B7A cells were grown overnight from a frozen glycerol stock, then seeded 1:20 in LB

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1 medium. Log-phase grown bacteria were harvested, adjusted to an OD_{600} of 0.1, then
2 further diluted 1:5,000 in Hanks' Balanced Salt Solution with 0.5% (w/v) BSA (Sigma
3 Aldrich). Assay mixtures were prepared in 96-well microtiter plates by combining 20 µL of
4 serially diluted heat-inactivated test serum (dilutions ranging from 10^0-10^4), and 10 µL of
5 diluted bacterial suspension. After incubation with shaking for 60 min at 37 °C, 10 µL of
6 active or inactive complement source was added to each well, to a final volume percent
7 of 25% (v/v). Heat-inactivated complement was prepared by thawing an aliquot of active
8 pooled human complement serum (Innovative Research, ICSER1ML), incubating in a 56
9 °C water bath for 30 min, and cooling at room temperature. Assay plates were incubated
10 with shaking at 37 °C for 60–90 min, then 10 µL was plated from each well (diluted to 50
11 µL in LB) on LB agar plates. Serum samples were tested and plated in duplicate, and
12 colonies were counted (Promega Colony Counter) after 16–18 h of incubation at 30 °C.
13 CFUs were counted for each individual serum dilution, and SBA titers were determined
14 by calculating percent survival at various serum dilutions. Data was plotted as percentage
15 survival versus serum dilution.
16 **ETEC infection.** Groups of eight 6-week-old female BALB/c mice (Harlan Sprague
17 Dawley) were immunized s.c. with 50 µL of sterile PBS (pH 7.4, Fisher Scientific) or
18 formulations containing aglycosylated PD or PD-O148 conjugate, according to the 49-day
19 immunization schedule described above. At 7 days post-vaccination (48 h prior to
20 challenge infection), mice received streptomycin (5 g/L) and fructose (6.7% (w/v)) in the
21 drinking water to eradicate normal flora and fecal pellets were collected. Food was
22 withheld 12 h prior to challenge infection and replaced with sterile water without
23 antibiotics. Famotidine (50 mg/kg) (Sigma-Aldrich) was then administered 2 h prior to
24 challenge infection to neutralize gastric acid. At 9 days post-vaccination, mice were
25 subsequently infected with a 200-µL inoculum containing ~1×10^4 CFU of ETEC strain
26 B7A, administered by gavage with a feeding needle directly introduced in the stomach via
27 the esophagus. After challenge infection, mice were checked daily for 3 days, and fecal
28 pellets were collected and stored at -20°C for further analysis. Mice were sacrificed 72 h
29 post-infection. All procedures were carried out in accordance with protocol 2012-0132
30 approved by the Cornell University Institutional Animal Care and Use Committee.

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1 **Quantitative real-time PCR analysis of ETEC burden.** DNA from fecal pellets of
2 individual mice was extracted from thawed stool samples using a QIAamp DNA stool kit
3 (Qiagen) following the manufacturer's instructions. To enhance extraction of pathogen
4 DNA, stool samples were first vigorously homogenized with ~300 mg of 1.0-mm-diameter
5 zirconia beads (Bio Spec) using a Mini-Bead Beater (BioSpec). After extraction, DNA was
6 eluted in elution buffer and stored at -20°C. Stool DNA and tissue were analyzed for the
7 ETEC B7A-specific heat-labile enterotoxin LT encoded by the *eltA* gene to determine the
8 levels of shedding of the organism in stool. Quantification of ETEC was performed by
9 qPCR using Taq DNA polymerase, as described elsewhere [57, 58], using the following
10 conditions: preheating at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 58
11 °C for 30 s, elongation at 72 °C for 1 min. PCR was performed for 55-60 cycles for
12 maximal saturation of signal with final extension at 72 °C for 7 min in a 7500 Fast Real-
13 Time PCR System (Applied Biosystems). The primer sequences used were: *eltA* forward
14 5′- TTCCCACCGGATCACCAA -3′ and *eltA* reverse 5′-CAACCTTGTGGTGCATGATGA
15 - 3′, along with a custom Taqman Probe (Thermo Fisher Scientific) 5'-
16 CTTGGAGAGAAGAACCCT-3' labeled with FAM (6-carboxyfluorescein) at the 5' end and
17 MGB at the 3′ end. Reactions with no DNA template were also included as controls.
18 Reaction components were combined in MicroAmp 96-well reaction plates (Applied
19 Biosystems) and plates were centrifuged at ~500 x g for 1 min before each reaction.
20 **Statistical analysis and reproducibility.** To ensure robust reproducibility of all results,
21 experiments were performed with at least three biological replicates and at least three
22 technical measurements. Sample sizes were not predetermined based on statistical
23 methods but were chosen according to the standards of the field (at least three
24 independent biological replicates for each condition), which gave sufficient statistics for
25 the effect sizes of interest. All data were reported as average values with error bars
26 representing standard error of the mean (SEM). Statistical significance was determined
27 by unpaired *t* test with Welch's correction (*$p < 0.05$, **$p < 0.01$; ns, not significant). All
28 graphs were generated using Prism 9 for MacOS version 9.2.0. No data were excluded
29 from the analyses. The experiments were not randomized. The Investigators were not
30 blinded to allocation during experiments and outcome assessment.
31

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10 **Author Contributions.**
11 Conceptualization: A.J.W., K.F.W., M.C.J., Y.-F.C. and M.P.D.
12 Methodology: A.J.W., P.D., K.F.W., J.L., J.-J.L., D.A.W., S.E.S. and Y.-F.C.
13 Investigation: A.J.W., P.D., K.F.W., J.L., J.-J.L., D.A.W., S.E.S. and Y.-F.C.
14 Visualization: M.P.D.
15 Supervision: M.C.J., Y.-F.C. and M.P.D.
16 Writing - original draft: A.J.W. and M.P.D.
17 Writing - review & editing: A.J.W., P.D., K.F.W., M.C.J., Y.-F.C. and M.P.D.

18

19 **Competing Interests Statement.** M.P.D. and M.C.J. have financial interests in Gauntlet,
20 Inc. and Resilience, Inc. M.P.D. has financial interests in Glycobia, Inc., MacImmune, Inc.,
21 UbiquiTX, Inc., and Versatope Therapeutics, Inc. M.P.D.'s and M.C.J.'s interests are
22 reviewed and managed by Cornell University and Northwestern University, respectively,
23 in accordance with their conflict-of-interest policies. All other authors declare no
24 competing interests.

25

26 **Data Availability.** All data needed to evaluate the conclusions in the paper are present
27 in the paper and/or the Supplementary Information.

28

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