A promising bioconjugate vaccine against hypervirulent *Klebsiella pneumoniae*

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Hypervirulent *Klebsiella pneumoniae* (hv*Kp*) is globally disseminating as a community-acquired pathogen causing life-threatening infections in healthy individuals. The fact that a dose as little as 50 bacteria is lethal to mice illustrates the dramatic increase of virulence associated with hv*Kp* strains compared with classical *K. pneumoniae* (c*Kp*) strains, which require lethal doses greater than 10^7 bacteria. Until recently, these virulent strains were mostly antibiotic-susceptible. However, multidrug-resistant (MDR) hv*Kp* strains have been emerging, spawning a new generation of hypervirulent "superbugs." The mechanisms of hypervirulence are not fully defined, but overproduction of capsular polysaccharide significantly impedes host clearance, resulting in increased pathogenicity of hv*Kp* strains. While there are more than 80 serotypes of *K. pneumoniae*, the K1 and K2 serotypes cause the vast majority of hypervirulent infections. Therefore, a glycoconjugate vaccine targeting these 2 serotypes could significantly reduce hv*Kp* infection. Conventionally, glycoconjugate vaccines are manufactured using intricate chemical methodologies to covalently attach purified polysaccharides to carrier proteins, which is widely considered to be technically challenging. Here we report on the recombinant production and analytical characterization of bioconjugate vaccines, enzymatically produced in glycoengineered *Escherichia coli* cells, against the 2 predominant hypervirulent *K. pneumoniae* serotypes, K1 and K2. The *K. pneumoniae* bioconjugates are immunogenic and efficacious, protecting mice against lethal infection from 2 hv*Kp* strains, NTUH K-2044 and ATCC 43816. This preclinical study constitutes a key step toward preventing further global dissemination of hypervirulent MDR hv*Kp* strains.

glycoconjugate | bioconjugation | vaccine | hypervirulent *Klebsiella pneumoniae*

Klebsiella pneumoniae is an encapsulated, Gram-negative bacterium of the Enterobacteriaceae family recognized as an opportunistic pathogen causing nosocomial infections (1). *K. pneumoniae* is notorious mostly due to the emergence of carbapenem-resistant strains (2); however, the rise and global dissemination of a hypervirulent form of *K. pneumoniae* is alarming (3). While the majority of *K. pneumoniae* infections manifest in the hospital setting or in immunocompromised individuals (termed classical *K. pneumoniae* [c*Kp*] infection), a subset of highly invasive, community-acquired *K. pneumoniae* infections, termed hypervirulent *K. pneumoniae* (hv*Kp*) infections, are steadily increasing in frequency (3).

First observed in the 1980s in Taiwan, hv*Kp* infections are pyogenic and mainly present as hepatic abscesses that can be complicated by endophthalmitis, meningitis, osteomyelitis, and necrotizing fasciitis (4–7). One of the most notable bacterial phenotypes associated with hv*Kp* is the overproduction of the capsular polysaccharide (CPS) (8), which results in a hypermucoviscous phenotype. This phenotype can be demonstrated by a positive string test: a greater than 5 mm "string" between an inoculating loop and a plated bacterial colony (9). Overproduction of the CPS has been directly linked with increased resistance to host clearance via impaired complement-mediated bacterial killing (10) and phagocytosis by neutrophils and macrophages (11).

More than 80 *K. pneumoniae* CPS serotypes have been identified (12); however, only 2 serotypes, the K1 and K2 serotypes, are responsible for the vast majority of hv*Kp* infections. In fact, K1 and K2 serotypes have been associated with ∼70% of all hv*Kp* infections across many clinical institutions worldwide (8, 13–15). Additionally, while these infections have historically been susceptible to most antibiotic classes, there are now increasing reports emerging of hv*Kp* strains acquiring multiple antibiotic-resistance determinants, rendering them refractory to most therapeutic regimens (16, 17). Given the severity of disease associated with hv*Kp* infections; their propensity for young, healthy hosts; the increasing rise of drug resistance in hv*Kp* strains; and the observation that the majority of hv*Kp* infections are caused by 2 serotypes, a bivalent glycoconjugate vaccine against the K1 and K2 serotypes would be an optimal prophylactic option.

Glycoconjugate vaccines, composed of a bacterial polysaccharide covalently attached to a carrier protein, are lifesaving prophylactic agents used to prevent colonization and disease by certain bacterial pathogens. Moreover, glycoconjugate vaccines elicit immunological

Significance

***Klebsiella pneumoniae* is considered a nosocomial pathogen, usually infecting immunocompromised patients. However, a pathotype of *K. pneumoniae*, termed hypervirulent *K. pneumoniae* (hv*Kp*), has emerged and is spreading throughout the community, causing severe, often fatal, disease in healthy individuals. Moreover, reports on multidrug-resistant hv*Kp* isolates are increasing in frequency. It is imperative that strategies to combat hv*Kp* begin immediately to prevent further dissemination of this new class of "superbugs." Here, we show that bioconjugate vaccines targeting the capsule of hv*Kp* can provide immunity and protection against extremely lethal hv*Kp* strains. Further, we demonstrate that bioconjugation is a promising technology for rapid development of efficacious vaccines against emerging bacterial threats.**

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memory in all age groups, including infants and children, which is not the case for purely polysaccharide vaccines (18). Traditionally, glycoconjugate vaccines have been manufactured via chemical conjugation (19); however, this process requires the use of complex/multiple-step chemical protocols, making them labor-intensive, ultimately hindering the timely development of next-generation conjugate vaccines against emerging bacterial threats like hv*Kp* (20). As an alternative, we and others have been developing methods to generate glycoconjugate vaccines by exploiting prokaryotic glycosylation systems in a process termed bioconjugation (21).

Bioconjugation relies on a conjugating enzyme, known as an oligosaccharyltransferase (OTase), to transfer polysaccharides from lipid-linked precursors to carrier proteins, all within the periplasm of Gram-negative bacterial expression systems such as *Escherichia coli*. Three conjugating enzymes have been utilized for bioconjugate vaccine development: PglB, PglL, and PglS (21–23). Each OTase has unique properties enabling the transfer of different polysaccharide substrates to different carrier proteins. At least 2 bioconjugate vaccines are being tested in human clinical trials: Flexyn2a (24) and ExPEc4V (25), which target *Shigella flexneri* and extraintestinal *E. coli*, respectively. Many more bioconjugate vaccines are in different stages of preclinical development (26, 27). The overwhelming majority of these are being developed using PglB and, to a limited extent, PglL. However, both PglB and PglL are unable to conjugate polysaccharides containing glucose at the reducing end (the first sugar in the growing polysaccharide chain), such as most of the *Streptococcus pneumoniae* (28) and the K1 and K2 *Klebsiella* capsules (12). Recently, we identified a new class of conjugating enzyme, termed PglS, that is capable of transferring a diverse array of polysaccharides, including those that contain glucose as the reducing end sugar (23, 29). Importantly, more than 50% of all *K. pneumoniae* capsular serotypes are composed of polysaccharides with glucose at the reducing end, including both the K1 and K2 serotypes (12). Thus, PglB and PglL cannot be used to generate a bioconjugate vaccine against hv*Kp*.

Here we sought to develop a first-of-its-kind bioconjugate vaccine against hv*Kp* infection. We have glycoengineered strains of *E. coli* for the recombinant production of a bivalent K1/K2 *K. pneumoniae* bioconjugate vaccine. We present data on our glycoengineering approach and the analytical characterization of the resultant K1/K2 bioconjugate vaccines, and demonstrate the efficacy of the hv*Kp* biconjugate vaccines in a murine model of infection.

Results

Glycoengineered Strains of *E. coli* Require RmpA for Heterologous K1 and K2 CPS Expression. Prokaryotic glycoengineering exploits conserved polysaccharide synthesis and export pathways for the reprogrammable assembly and transfer of designer glycans in *E. coli* (*SI Appendix*, Fig. S1). The first step in glycoengineering bioconjugate vaccines against hv*Kp* required building strains of *E. coli* for the heterologous expression of the K1 and K2 *K. pneumoniae* CPSs. As such, we cloned the K1 and K2 CPS loci from *K. pneumoniae* NTUH K-2044 (30) and *K. pneumoniae* 52.145 (31), respectively (Fig. 1 *A* and *B*). The cloned K1 CPS locus contained the genes from *wzx* to *ugd*. The cloned K2 CPS locus contained the genes from *wcuF* to *ugd*. The CPS regulatory genes *wza*, *wzb*, and *wzc* and export gene *wzi* are not required for heterologous CPS expression in *E. coli* (32) and were not included in the constructs. The *galF and orf2* genes were also excluded because *E. coli* carries its own copy of *galF* and the role of *orf2* in *K. pneumoniae* CPS production is unclear. The plasmids containing the biosynthesis machinery for the K1 or K2 CPS were then introduced into *E. coli* CLM37, a reporter strain for heterologous polysaccharide expression and assembly (33). CLM37 cannot produce its natural O16 antigen due to a mutation in the WecA initiating glycosyltransferase. However, CLM37 expresses the WaaL O antigen ligase that transfers lipid-linked polysaccharides, like the K1 and K2 polysaccharides, to the outer core saccharide of LPS (34). After IPTG induction and overnight growth, LPS was extracted, separated by SDS/PAGE, and silver-stained from CLM37 cells expressing either the K1 or K2 CPS plasmids. As seen in Fig. 1 *C* and *D*, no appreciable O antigen polysaccharide was observed. We detected only the core saccharide, indicating that the K1 and K2 glycans were either not expressed or not transferred by the

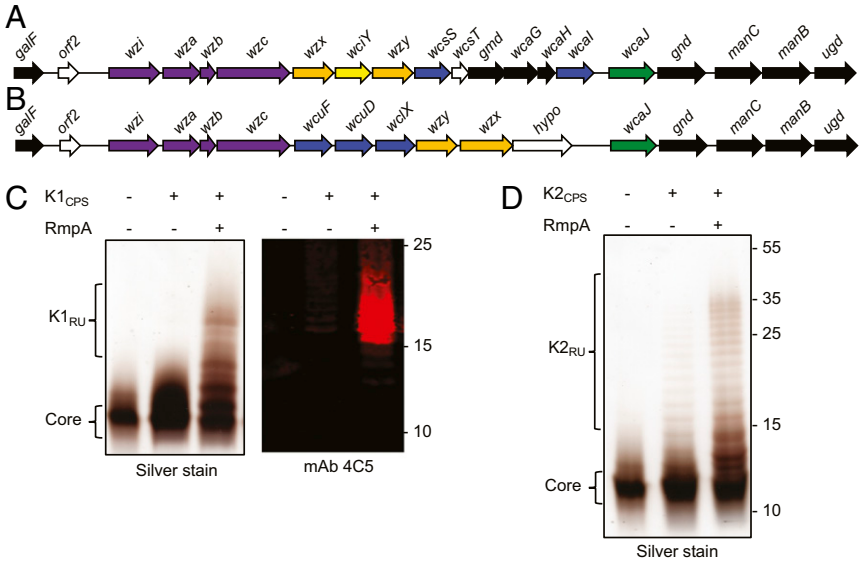


Fig. 1. RmpA enhances expression of the K1 and K2 glycans in *E. coli* CLM37. (*A*) The *K. pneumoniae* K1 CPS gene map and (*B*) the *K. pneumoniae* K2 CPS gene map. Black arrows indicate UDP-sugar biosynthesis genes, purple arrows indicate CPS regulatory/surface export genes, orange arrows indicate transport/polymerase genes, blue arrows indicate glycosyltransferase genes, and green arrows indicate initiating glycosyltransferase genes. (*C*) Silver staining and Western blot analysis of LPS extracted from CLM37, CLM37 expressing the K1 locus, or CLM37 coexpressing the K1 locus and RmpA. (*D*) Silver staining of LPS extracted from CLM37, CLM37 expressing the K2 locus, or CLM37 coexpressing the K locus and RmpA.

WaaL ligase. Given that the WaaL has highly relaxed substrate specificity, we hypothesized that the absence of K1 and K2 polysaccharides was due to their poor expression. Previously, Arakawa et al. demonstrated that the K2 CPS could be detected on the surface of *E. coli* JM109 when the complete K2 locus and the transcriptional activator RmpA were coexpressed (35). Therefore, we cloned *rmpA* from *K. pneumoniae* NTUH K-2044 (NCBI accession no. BAH65944) into pACT3, a low-copy IPTG-inducible vector, and introduced this plasmid into CLM37 strains containing the K1 or K2 CPS-expressing plasmids. When RmpA was coexpressed with either the K1 or K2 CPS expressing plasmids in CLM37, purified LPS contained observable O antigen polysaccharides (Fig. 1 *C* and *D*). Further, Western blot analysis using the monoclonal antibody 4C5, specific to the K1 CPS of *K. pneumoniae* (36), reacted with LPS purified from CLM37 coexpressing the K1 CPS locus and RmpA, indicating that the polysaccharide produced by this glycoengineered *E. coli* strain has a K1 structure. There are no commercially or publicly available antibodies to the K2 polysaccharide.

A

[→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-α-L-Fuc2/3OAc*p*-(1→]

D

[→3)-β-*D*-Glc*p*-(1→4)]-β-D-Man*p*-(1→4)-α-D-Glc*p*-(1→]

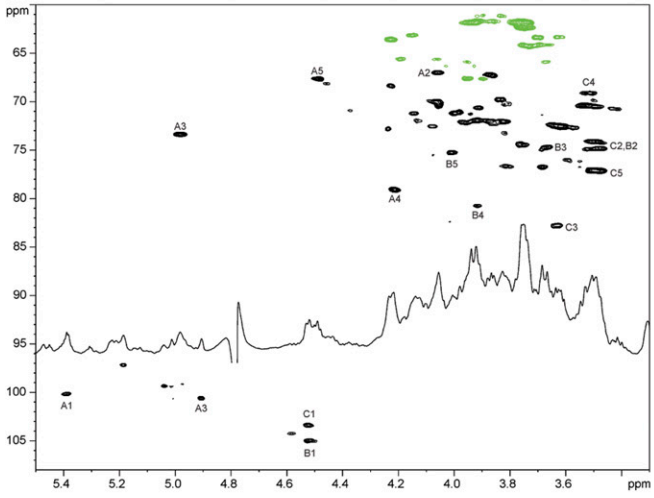
3

↑

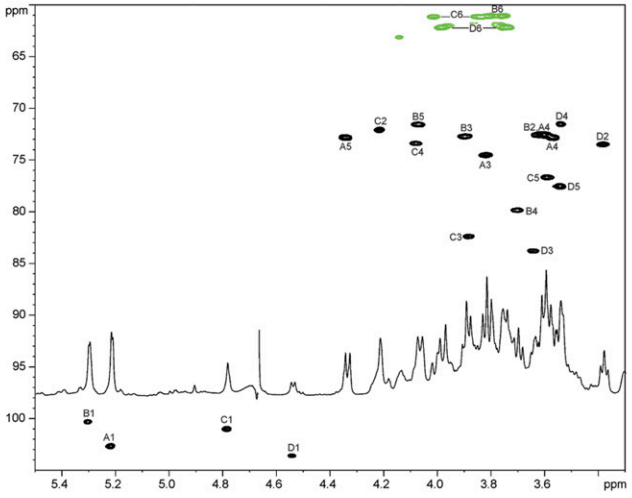
1

α-D-Glc*p*A

B



E



C

Unit		H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6a;b
α-Fuc A	H	5.39	4.06	4.98	4.21	4.48	1.28
	C	100.3	67.0	73.4	79.0	67.6	16.0
β-GlcA B	H	4.52	3.48	3.67	3.92	4.01	
	C	105.0	74.8	74.7	80.7	75.3	
β-Glc C	H	4.52	3.49	3.63	3.51	3.49	3.76; 3.93
	C	103.4	74.0	82.7	69.0	77.0	61.8
pyruvate	H			1.53			
	C	175.1	100.2	26.1			

F

Unit		H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6a;b
α-GlcA A	H	5.22	3.57	3.82	3.60	4.34	
	C	102.7	72.8	74.5	72.5	72.8	180.3
β-Glc D	H	4.54	3.38	3.64	3.54	3.54	3.74; 3.98
	C	103.6	73.5	83.8	71.5	77.5	62.2
β-Man C	H	4.78	4.21	3.88	4.08	3.59	3.84; 4.01
	C	101.0	72.1	82.4	73.4	76.7	61.1
α-Glc E	H	5.30	3.38	3.89	3.70	4.07	3.75; 3.80
	C	100.3	73.5	72.7	79.9	71.5	61.0

Fig. 2. Two-dimensional NMR analysis of K1- and K2-containing LPS extracted from CLM37. (*A*) The core structure of the K1 repeat unit. (*B*) The ^{1}H-^{13}C HSQC spectrum of *K. pneumoniae* K1 polysaccharide produced in *E. coli*. Signals of the 2 variable *O*-acetylated fucoses are not shown because of low signal intensity. (*C*) NMR data for the *K. pneumoniae* K1 polysaccharide and the *E. coli* core (D_2O, 25 °C, 600 MHz). Data for the main structure with *O*-acetylation (OAc) at Fucose A O-3. OAc 2.08/21.6 ppm. (*D*) The core structure of the K2 repeat unit. (*E*) The ^{1}H-^{13}C HSQC spectrum of *K. pneumoniae* K2 polysaccharide produced in *E. coli* (D_2O, 35 °C, 600 MHz). (*F*) NMR data for the *K. pneumoniae* K2 polysaccharide and the *E. coli* core (D_2O, 35 °C, 600 MHz).

NMR Analysis of Glycoengineered K1 and K2 LPS and the *K. pneumoniae* NTUH K-2044 CPS. Next, we characterized the K1- and K2-containing LPSs purified from the glycoengineered strains of CLM37. The published structure of the *K. pneumoniae* NTUH K-2044 K1 CPS repeat unit has been reported as (→3)-β-D-Glc-(1,4)-[2,3-(*S*)-pyruvate]-β-D-GlcA-(1,4)-α-L-Fuc-(1→) with varying degrees of acetylation at the C_2-OH and C_3-OH of the fucose residue (37, 38). The NMR spectra and chemical shifts for the K1-containing LPS (Fig. 2 *A*–*C*) contained many signals from the core saccharide due to the short K1 polysaccharide chains, which can also be observed as low molecular weight polysaccharides on the silver-stained K1-containing LPS (Fig. 1*C*). Analysis of the 2D spectra (gCOSY, TOCSY, NOESY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) allowed for the identification of the K1 repeating units containing the structure -3-β-Glc-4-β-GlcA-4-α-L-Fuc2/3OAc-, where fucose was present in 3 variants: nonacetylated or acetylated at positions 2 or 3. The published structure of the K1 CPS includes the same repeating unit as well as pyruvylation at the O-2, O-3 positions of the glucuronic acid (GlcA) residue. Although the spectra contained small signals attributable to pyruvate, there was no indication that it was linked to the GlcA residue or any other monosaccharide of the repeating unit. Given the varying degrees of acetylation and pyruvylation between the previously published K1 structures (37, 38), we purified the capsular polysaccharide from *K. pneumoniae* NTUH K-2044 and reexamined it by NMR analysis. The NMR analysis (*SI Appendix*, Fig. S2) showed an identical structure to the K1-containing LPS without pyruvylation. Acetylation of the fucose was evident with *O*-acetylation at the O-2 (~20%, A″) and the O-3 (~40%, A′) of fucose, while the rest of fucose (A) was not acetylated. Small signals of terminal fucose A* were also visible. Last, signals of B4 and B5 were shifted between the native and deacylated K1 polysaccharide, likely due to different pH or salt formations by glucuronic acid. The data obtained here indicate that the polysaccharide structures produced by the K1 glycoengineered strain of *E. coli* and that of the *K. pneumoniae* K1 are nearly identical.

The K2-containing LPS was also extracted from glycoengineered *E. coli* CLM37 cells and analyzed by NMR to determine structure and linkage. As shown in Fig. 2 *D*–*F*, the K2 polysaccharide matched perfectly with the published structure of the native K2 polysaccharide (39).

Glycoengineering a K1 and K2 Bioconjugate Vaccine in *E. coli*. Previously, we established a bioconjugation system for transferring polysaccharides containing glucose at the reducing end to carrier proteins (23). The system employs the use of the oligosaccharyltransferase PglS from *Acinetobacter baylyi* ADP1 to transfer polysaccharides from a lipid-liked precursor to a genetically deactivated variant of exotoxin A protein from *Pseudomonas aeruginosa* (EPA) fused to a fragment of ComP, the natural acceptor protein of PglS. As such, we introduced either the K1 or the K2 CPS-expressing plasmid along with the transcriptional activator RmpA into a glycocompetent strain of *E. coli*, strain SDB1, expressing the PglS bioconjugation platform. SDB1 is Δ*waaL* derivative of CLM37, which prevents the use of the capsule for LPS synthesis. After induction and overnight growth, K1-EPA and K2-EPA glycoproteins were purified using affinity, anionic exchange, and size-exclusion chromatography and examined by Coomassie staining and Western blotting. As seen in Fig. 3*A*, the unglycosylated EPA fusion protein migrates between the 75-kDa and 95-kDa markers (the theoretical molecular weight is 75,526.15 Da). The K1-EPA glycoprotein existed almost entirely as a semihomogenous glycoform with a smeared electrophoretic mobility slightly larger than the 95-kDa marker. A similar electrophoretic mobility was also observed for the K1-containing LPS; however, more resolution can be seen due to the fact that the K1-containing LPS was separated by using a 15% polyacrylamide gel whereas the K1-EPA glycoprotein was resolved on an 8% polyacrylamide gel. While this presentation for a bioconjugate vaccine is unusual, the smear-like banding is commonly observed for glycoconjugate vaccines prepared with chemical methodologies and is likely a product of nonstoichiometric levels of acetylation and pyruvylation of the K1 repeat unit. Western blotting using the 4C5 monoclonal antibody specific to the K1 capsule confirmed that the K1-EPA bioconjugate was indeed carrying K1 glycans (Fig. 3 *B*–*D*). The K2-EPA glycoprotein ran more similarly to conventional bioconjugate vaccines, exhibiting a modal, ladder distribution with each band corresponding to a glycoform with an additional K2 subunit.

Intact Mass Spectrometry of Bioconjugate Vaccines. Due to the lack of polyclonal or monoclonal antibodies specific to the K2 glycan, we next performed intact mass spectrometry analysis on the K2-EPA bioconjugate. This technique provides analytical data on the relative glycosylation ratios for bioconjugates existing in multiple different glycoforms as well as relative masses associated with each glycoform. As seen in Fig. 4*A*, the intact mass spectrum of the K2-EPA bioconjugate showed a modal, ladder distribution of K2-EPA bioconjugates, each separated by ~662 Da, the mass of the K2 repeat unit (39). As many as 12 K2 repeat units were observed to be covalently attached to the EPA carrier protein by mass spectrometry; however, analysis of the Coomassie-stained purified glycoprotein preparation indicates that as many as 16 repeat units were transferred (Fig. 4*A*). Relative quantification of the different

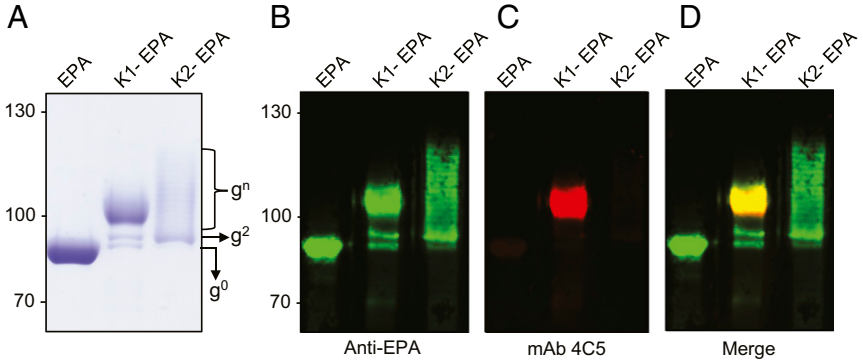


Fig. 3. K1-EPA and K2-EPA bioconjugate vaccines. (*A*) Coomassie-stained image of purified EPA, K1-EPA, and K2-EPA. Each lane was loaded with ~5 μg of glycoconjugate based on total protein. The unglycosylated EPA exists a single band. The K1-EPA exists as multiple glycoforms migrating in smear-like pattern. The K2-EPA bioconjugate migrates with a modal, ladder distribution. Each lane was loaded with ~0.5 μg of glycoconjugate based on total protein. (*B*–*D*) Western blot analysis probing for EPA and the K1 glycan. (*B*) Anti-EPA Western blot, (*C*) anti-K1 Western blot, and (*D*) merge image.

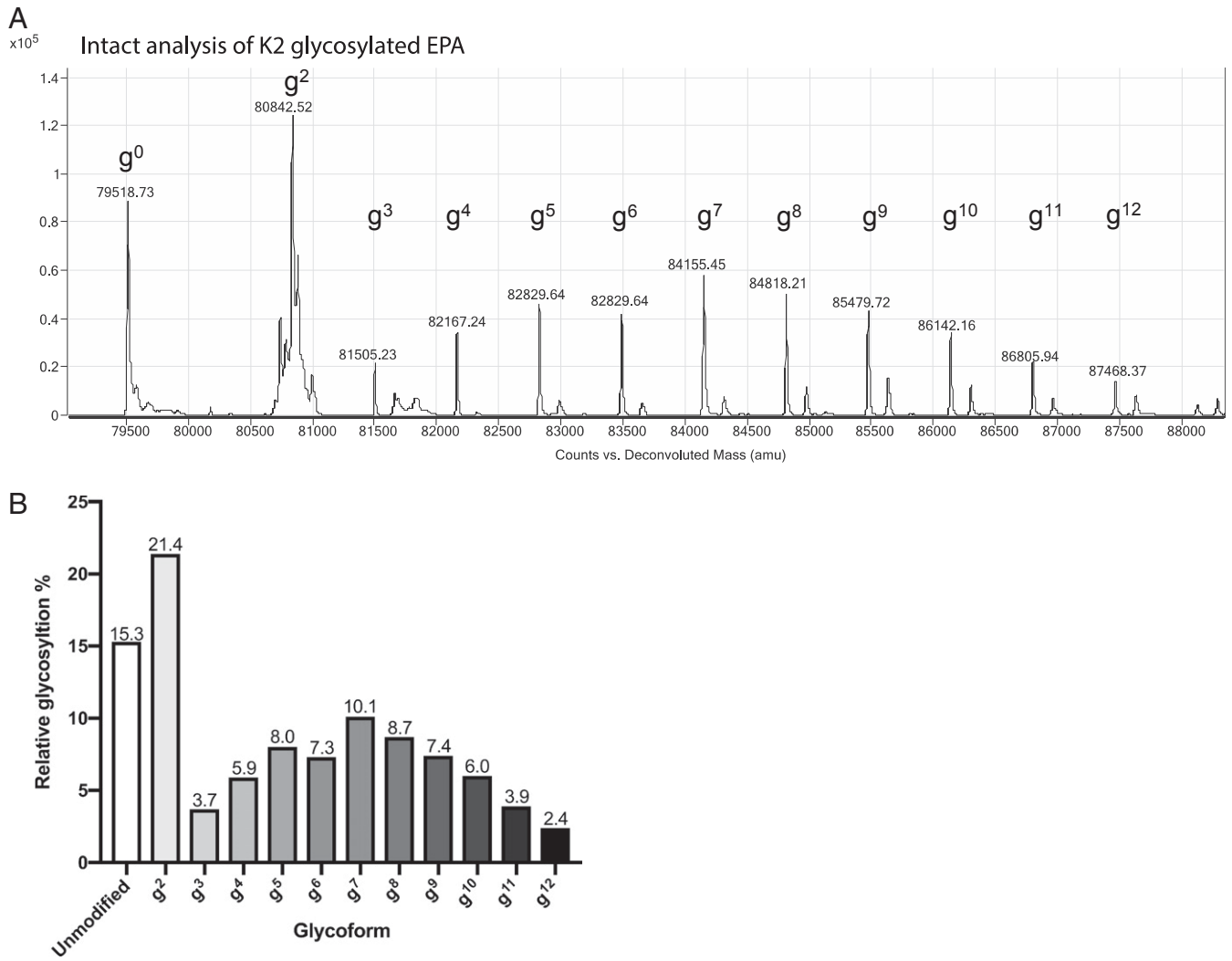


Fig. 4. Mass spectrometry analysis of K2-EPA. (*A*) Intact protein mass spectrometry analysis showing the MS1 mass spectra for K2-EPA. The EPA fusion protein has a theoretical mass of 79,526.15 Da and can be observed as the peak at 79,518.73 Da. The EPA fusion protein was also observed in multiple states of increasing mass corresponding to the K2 repeat unit, which has a mass of 662 Da. Varying glycoforms of K2-EPA were observed and are denoted by "$g^{numeric}$", where "g" stands for glycoform and the "numeric" corresponds to the number of repeating CPS8 subunits. (*B*) Quantification of the relative abundance of each K2 glycoform.

glycoforms showed that the most abundant glycoform contained 2 repeat units, with the next most abundant glycoform containing 7 K2 repeat units (Fig. 4*B*). Interestingly, when examined collectively, the K2-EPA glycoprotein seemed to be glycosylated with the K2 glycan in a semi–bell-curve distribution, indicating that PglS may prefer certain-sized lipid-linked polysaccharides as substrates. We also performed intact mass spectrometry on the K1-EPA bioconjugate. The EPA fusion protein was observed as a series of peaks compatible with different glycoforms containing the inherently heterogenous K1 repeat units, which is nonstoichiometrically modified with acetylation at the fucose residue and/or pyruvylated at the glucuronic acid residue (*SI Appendix*, Fig. S3).

K1 and K2 Bioconjugates Elicit Serotype-Specific IgG Responses. The K1-EPA and K2-EPA bioconjugates were then tested for their abilities to induce serotype-specific IgG responses. Four immunization groups, each containing 5 mice, were vaccinated with either a placebo (the unglycosylated EPA fusion protein), the K1-EPA bioconjugate, the K2-EPA bioconjugate, or a bivalent mixture of the K1- and K2-EPA bioconjugates. All vaccines were coformulated with an equal mixture of Imject Alum as an adjuvant (50 μL vaccine to 50 μL alum). The EPA-alone group, K1-EPA group (222 ng of K1 glycan), and K2-EPA group (195 ng of K2 glycan) all received 5 μg of vaccine based on total protein quantity. The total polysaccharide content was measured using a modified anthrone-sulfuric assay (40). The bivalent vaccine was formulated by combining the K1-EPA and K2-EPA vaccine doses; thus, bivalent groups received a total of 10 μg of EPA, 222 ng of K1 glycan, and 195 ng of K2 glycan. Mice were vaccinated 3 times s.c. on days 0, 14, and 28 and killed on day 42. Serum was collected throughout the course of the trial and used to characterize the IgG responses via ELISA with plates coated with either *K. pneumoniae* NTUH K-2044 (K1) or *K. pneumoniae* ATCC 43816 (K2) whole cells.

As seen in Fig. 5, mice vaccinated with K1-EPA bioconjugates had increased K1-specific IgG titers compared with mock-vaccinated mice. Mice receiving the bivalent K1-/K2-EPA vaccine also had similar increases in K1-specific IgG titers compared with mock-vaccinated mice. The response was slightly lower than that of the single K1-EPA vaccinated group, but no statistically significant difference was observed. No K2-specific cross-reactivity was observed for K1-EPA vaccinated mice. In addition, mice

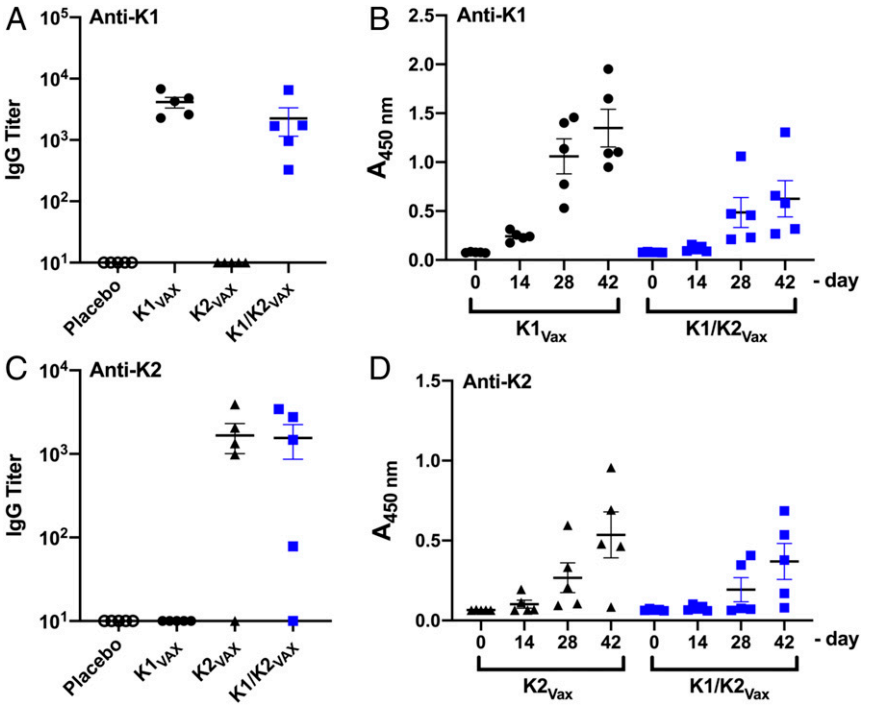


Fig. 5. IgG responses to K1 and K2 bioconjugate vaccines. (*A*) Titers of K1-specific IgG antibodies in mice immunized with EPA, K1-EPA, K2-EPA, or a bivalent K-/K2-EPA. (*B*) K1-specific IgG kinetics over the course of the immunization as measured by ELISA and quantified by absorbance at 450 nm. (*C*) Titers of K2-specific IgG antibodies in mice immunized with EPA, K1-EPA, K2-EPA, or a bivalent K-/K2-EPA. (*D*) K2-specific IgG kinetics over the course of the immunization as measured by ELISA and quantified by absorbance at 450 nm.

vaccinated with the K2-EPA biconjugate had increased K2-specific IgG titers compared with mock-vaccinated mice; however, 1 mouse receiving the K2-EPA vaccine did not show increases in K2-specific IgG titers. Mice receiving the bivalent K1-/K2-EPA vaccine also had similar increases in K2-specific IgG titers compared with mock-vaccinated mice. As was the case for the K2-EPA group, 1 mouse did not show an increase in K2-specific IgG titers. No K1-specific cross-reactivity was observed for mice vaccinated with the K2-EPA bioconjugate.

Mice sera were further examined for serotype-specific IgG kinetics over the course of the vaccination. As seen in Fig. 5, the kinetics of K1-specific total IgG responses increased over time, with K1-EPA vaccinated mice showing a more robust response compared with bivalent K1-EPA/K2-EPA vaccinated mice. The kinetics of total IgG responses specific to K2 were also measured, and a similar trend was observed, with mice vaccinated with the K2-EPA bioconjugate showing the most robust response compared with bivalent vaccinated mice.

Next, mouse sera were pooled and examined by whole-cell ELISA for IgG subclass distributions specific to the K1 or K2 antigen. As seen in *SI Appendix*, Fig. S4, pooled K1-EPA vaccinated sera and pooled K1-/K2-EPA vaccinated sera displayed an exclusively IgG1-specific response to the K1 antigen. The same trend was also observed for the pooled K2-EPA and pooled K1-/K2-EPA vaccinated sera when probing for specificity to the K2 antigen.

K1/K2 Bioconjugate Vaccines Protect Mice from hv*Kp* Infection. The correlates of immunity that confer protection from classical or hypervirulent *K. pneumoniae* infection are not known. Therefore, we evaluated the protectiveness of the bivalent K1-/K2-EPA vaccine using a murine acute pulmonary infection model with 2 separate hypervirulent *K. pneumoniae* strains: NTUH K-2044 (K1) and ATCC 43816 (K2). Both strains are hyper-capsule producers (hypermucoviscous) and extremely virulent in mice. In fact, the LD_{50} for both strains has been shown to be less than 100 colony forming units (CFUs) in murine respiratory infection models (41). Two groups of mice (20 female BALB/c mice per group) were vaccinated with the carrier protein alone or with the bivalent K1-/K2-EPA bioconjugate vaccine. Briefly, mice were vaccinated on days 0, 14, and 28 using the same dosing and adjuvant formulation as described above. After the vaccination regimen, the 2 groups (EPA placebo or bivalent bioconjugate vaccinated) were then challenged with either the K1 or K2 strain at doses close to the published LD_{50} values. Specifically, 10 mice from each vaccination group were inoculated by aspiration with the hypervirulent K1 strain (NTUH K-2044) and 10 were inoculated with the hypervirulent K2 strain (ATCC 43816) and subsequently monitored for survival and changes in weight for a 2-wk period (Fig. 6 *A* and *B*). As seen in Fig. 6*A*, 80% of placebo-vaccinated mice challenged with only 50 CFUs of the hypervirulent K1 strain (*K. pneumoniae* NTUH K-2044) succumbed to infection, whereas only 20% of bioconjugate-vaccinated mice died ($P = 0.0057$ by log-rank test). While *K. pneumoniae* ATCC 43816 was not as virulent in this mouse model as *K. pneumoniae* NTUH K-2044, a dose of only 250 CFUs was able to kill 30% of the placebo-vaccinated mice (Fig. 6*B*), whereas no mice from the bioconjugate-vaccinated group challenged with ATCC 43816 died ($P = 0.0669$ by log-rank test). Additionally, placebo-vaccinated mice that did survive low-dose challenge had lower body weights than bioconjugate-vaccinated mice (*SI Appendix*, Fig. S5).

Given the success of the bioconjugate vaccine in preventing death from infection of hv*Kp* at doses close to the LD_{50} values, we further challenged a separate group of placebo- and bioconjugate-vaccinated mice with hv*Kp* strains at 100× the published LD_{50} titers. For *K. pneumoniae* NTUH K-2044 (K1), all of the placebo-vaccinated mice rapidly died (Fig. 6*C*). The majority of bioconjugate-vaccinated mice also succumbed to this high-dose infection; however, the bioconjugate-vaccinated mouse

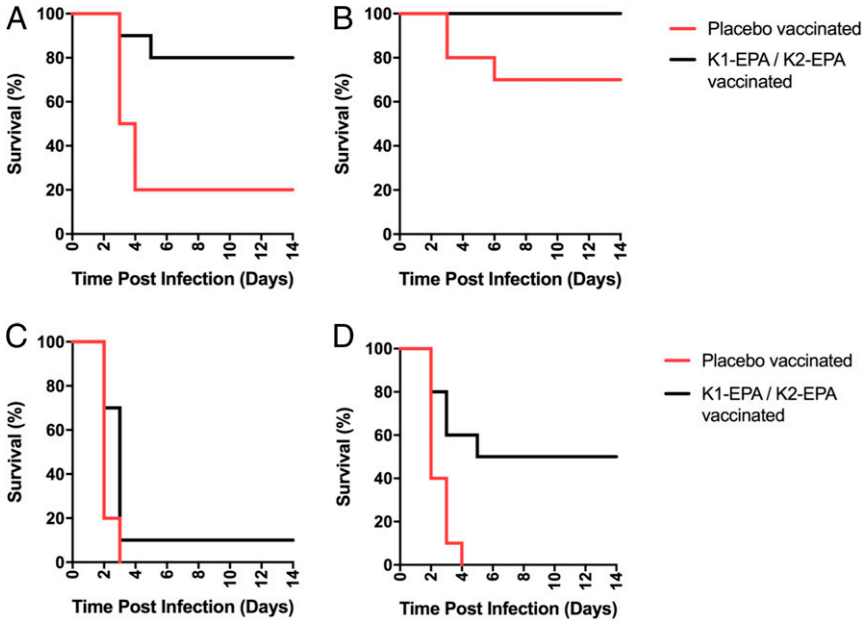


Fig. 6. Survival of placebo- and bivalent bioconjugate-vaccinated mice after lethal challenge with hv*Kp*. Groups of mice were vaccinated with either the placebo or the bivalent K1-/K2-EPA bioconjugate on days 0, 14, and 28. Anesthetized mice were aspirated with either the *K. pneumoniae* NTUH K-2044 or ATCC 43816 and monitored for survival for 14 d. For low-dose challenge studies, mice were infected with (*A*) 50 CFU of NTUH K-2044 or (*B*) 250 CFU of ATCC 43816. For high-dose challenge studies, mice were infected with (*C*) 4,700 CFU of NTUH K-2044 or (*D*) 4,300 CFU of ATCC 43816. Each graph represents data from a single experiment with $n = 10$ mice per group. Statistical analysis was performed via log-rank (Mantel–Cox) tests.

group had a statistically significant increase in survival compared with the placebo-vaccinated group ($P = 0.0250$ by log-rank test). When mice were challenged with close to 100× the published LD_{50} value of ATCC 43816 (K2), all placebo-vaccinated mice died by day 4, whereas 5 of the 10 mice survived the infection ($P = 0.0038$ by log-rank test; Fig. 6*D*). These data suggest that the biconjugate vaccine is efficacious in protecting some mice from K1 or K2 hv*Kp* challenge, even at very high inocula.

Discussion

cKp infections are traditionally associated with nosocomial infections among hospitalized or immunocompromised patients, while hv*Kp* can target healthy, immunocompetent hosts. Until recently, hv*Kp* strains have been susceptible to common antibiotic agents; however, many cases of carbapenem-resistance and even colistin-resistance phenotypes in hv*Kp* isolates have been recently reported (42–45). Furthermore, hv*Kp* strains generated via the acquisition of hypervirulence plasmids by MDR c*Kp* are also emerging (16, 17). The extreme virulence of these strains coupled with a lack of antimicrobial treatment options is worrisome. As such, vaccines to prevent possible outbreaks of these hypervirulent, antimicrobial-resistant infections are urgently needed (46), and possibly prevent the spread of the hypervirulent plasmid. Here we report on the recombinant production and analytical characterization of rapidly produced bioconjugate vaccines against the 2 most common hypervirulent serotypes of *K. pneumoniae* (K1 and K2), which account for more than 70% of the hv*Kp* cases (15). Using a bioconjugation approach in glycoengineered *E. coli*, we show that K1 and K2 bioconjugates are immunogenic and efficacious, protecting mice from lethal infection from 2 different hypervirulent strains of *K. pneumonia*.

For efficient bioconjugation of polysaccharides to proteins, usually only 3 components are needed: an oligosaccharyltransferase (also known as a conjugating enzyme), a target protein to be glycosylated, and a polysaccharide to be transferred. However, we found that a fourth factor, the RmpA transcriptional activator, was required to efficiently express K1 and K2 capsules in *E. coli*. Multiple alleles of *rmpA* are associated with hypermucoviscous phenotypes (10, 47). *K. pneumoniae* isolates can carry as many as 3 different *rmpA* alleles. One is commonly found on the chromosome ($rmpA_c$), whereas 2 plasmid-encoded *rmpA* alleles ($_prmpA$ or $_prmpA2$) are located on the virulence plasmid found in hypervirulent isolates of *K. pneumoniae*. In our study, $_prmpA$ functioned as transcriptional activator in glycoengineered *E. coli* cells; however, whether $rmpA_c$ or $_prmpA2$ would have the same or possibly an additive effect on K1 and K2 CPS polysaccharide expression has not been determined.

A few vaccine strategies have been or are in development for *K. pneumoniae*. In fact, a 24-valent, purely capsular polysaccharide vaccine (Klebgen Bema) was developed in the 1980s by the Swiss Serum and Vaccine Institute and tested in human clinical trials (48). While the vaccine was shown to elicit increases in serotype-specific IgG responses in adult cohorts, as frequently happens with polysaccharide-only vaccines, total serotype-specific IgG levels dropped to near baseline levels 18 mo after vaccination for many capsular antigens (48). More recently, a glycoconjugate vaccine composed of 4 *K. pneumoniae* OPS serotypes conjugated to flagellin proteins from *Pseudomonas aeruginosa* was developed (49). The OPS vaccine was immunogenic, and mice passively transferred with the OPS vaccine-induced antibodies were protected against systemic c*Kp* infection. While these preclinical results are promising, OPS-based vaccines more appropriately target c*Kp* strains, as molecular studies have shown that the capsular polysaccharide of hypervirulent isolates can mask the OPS antigens (50). Also, it has not been shown that an OPS-based vaccine would be protective protect against hv*Kp* isolates overproducing capsular polysaccharide. To the best of our knowledge, the K1-/K2-EPA bioconjugate presented here is the first case of a vaccine providing protection from extremely lethal hypervirulent isolates. While our in vivo work suggests efficacy against hv*Kp* in the lung, additional studies need to assess efficacy against hv*Kp* in other niches including the liver, bloodstream, and meninges.

Glycoconjugate vaccines elicit IgM-to-IgG class switching and immunological memory (18). While this is common to all glycoconjugate

vaccines, the distribution of IgG isotypes can be different for each antigenic stimulus, as well as differ based on the age of the vaccinated population. For instance, pneumococcal conjugate vaccines elicit strong IgG1 responses in infants and young children (2–5 y), whereas the same vaccine elicits a predominantly IgG2 response in healthy adults (18–39 y) and geriatric patients (>50 y) (51). Using 6-wk-old BALB/c mice for our immunization model, we observed an exclusively IgG1 response for the both the K1 and K2 antigens. In fact, we were not able to observe any signals for IgG2a, IgG2b, or IgG3 subclasses by ELISA. IgG1 antibodies are known to efficiently activate the classical route of complement (52). Therefore, our data indicate that K1- and K2-specific IgG1 antibodies may be sufficient to provide protection to vaccinated mice challenged with lethal doses of the hv*Kp* isolates. Future experiments will be needed to establish if high levels IgG1 can be employed as an appropriate correlate of immunity and predict protection against hv*Kp*.

While it is currently difficult to define which serotypes should be included in a capsular polysaccharide glycoconjugate vaccine targeting c*Kp* infection or determine which populations are most at risk, the seroepidemiology of hv*Kp* is much clearer. Specifically, hv*Kp* is endemic to certain parts of Asia, and 2 serotypes, K1 and K2, have emerged as the highly predominant disease-causing serotypes (53–55). Moreover, fewer than 10 serotypes have been reported to be associated with hypervirulent infections (K1, K2, K5, K16, K20, K54, K57, and KN1) (3). In addition to the K1 and K2 serotypes, the K5, K16, and K54 serotypes also contain glucose as the reducing end sugar (12), suggesting that they may also be appropriate polysaccharide substrates for the PglS bioconjugation platform. The remaining serotypes (K20, K57, and KN1) contain galactose at their reducing ends, which are also compatible with the PglS bioconjugation platform. Therefore, PglS could be employed to develop a pan-hypervirulent bioconjugate vaccine against *K. pneumoniae*. Importantly, the use of the K1-/K2-EPA bioconjugate would also target K1 and K2 strains of *K. pneumoniae* associated with classical, nosocomial infection. Thus, by expanding the serotype coverage, a broadly protecting glycoconjugate vaccine targeting both classical and hypervirulent pathotypes of *K. pneumoniae* could be developed rapidly by using our bioconjugation platform and employed to significantly reduce the burden of *K. pneumoniae* disease and possibly slow the rates of drug resistance and transmission of the hypervirulence plasmid.

The increasing incidence of community-acquired hv*Kp* MDR strains poses a serious threat to global health. It is imperative that vaccine strategies to combat hv*Kp* begin immediately, as the dissemination of the virulence plasmid into the *cKp* population could have devastating consequences. Our work demonstrates that bioconjugation is a promising approach to rapidly developing efficacious antibacterial vaccines.

Materials and Methods

The bacterial strains, plasmids, and primers used in this study are listed in *SI Appendix, SI Materials and Methods*. A full description of all methods employed for this study is provided in *SI Appendix, SI Materials and Methods*. All data are available in the main text or the *SI Appendix*.

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