Biochemical Characterization of Bifunctional 3-Deoxy-β-o-manno-oct-2-ulosonic Acid (β-Kdo) Transferase KpsC from Escherichia coli Involved in Capsule Biosynthesis*

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3-Deoxy-β-o-manno-oct-2-ulosonic acid (Kdo) is an essential component of bacterial lipopolysaccharides, where it provides the linkage between lipid and carbohydrate moieties. In all known LPS structures, Kdo residues possess α-anomeric configurations, and the corresponding inverting α-Kdo transferases are well characterized. Recently, it has been shown that a large group of capsular polysaccharides from Gram-negative bacteria, produced by ATP-binding cassette transporter-dependent pathways, are also attached to a lipid anchor through a conserved Kdo oligosaccharide. In the study reported here, the structure of this Kdo linker was determined by NMR spectroscopy, revealing alternating β-(2→4)- and β-(2→7)-linked Kdo residues. KpsC contains two retaining β-Kdo glycosyltransferase domains belonging to family GT99 that are responsible for polymerizing the β-Kdo linker on its glycolipid acceptor. Full-length Escherichia coli KpsC was expressed and purified, together with the isolated N-terminal domain and a mutant protein (KpsC D160A) containing a catalytically inactivated N-terminal domain. The Kdo transferase activities of these proteins were determined in vitro using synthetic acceptors, and the reaction products were characterized using TLC, mass spectrometry, and NMR spectroscopy. The N- and C-terminal domains were found to catalyze formation of β-(2→4) and β-(2→7) linkages, respectively. Based on phylogenetic analyses, we propose the linkage specificities of the glycosyltransferase domains are conserved in KpsC homologs from other bacterial species.

Pathogenic bacteria are often covered by a protective layer of polysaccharide known as the capsule. Capsules are important virulence determinants because, depending on the pathogen, they can protect bacteria against phagocytosis and complement-mediated killing, as well as helping in adherence to host cells (1). In Gram-negative bacteria, capsular polysaccharides (CPS) are synthesized and exported onto the surface of the outer membrane via one of two widely disseminated and fundamentally different assembly systems (1, 2). The ATP-binding cassette (ABC) transporter-dependent system is the focus of this study. In the nomenclature of Escherichia coli, these systems generate “group 2” capsules (1, 2). In group 2 CPS assembly, biosynthesis is initiated by KpsS, which transfers a single 3-deoxy-β-o-manno-oct-2-ulosonic acid (Kdo) residue from its precursor (CMP-β-Kdo) to (lyso)phosphatidylglycerol, in a reaction located at the cytoplasm-membrane interface (1). The KpsS product is then extended by KpsC to form the β-Kdo linker, which typically consists of five to nine Kdo residues (3). The serotype-specific glycosyltransferases (GTs) extend this linker to complete polymerization of CPS in the cytoplasm before it is exported by the pathway-defining ABC transporter and then translocated to the cell surface (1, 2).

The native lipid-linked β-Kdo-oligosaccharides were isolated from E. coli K1 and K5 and Neisseria meningitidis group B by enzymatic depolymerization of high molecular mass CPS, followed by isolation of the hydrophobic glycolipid components (3). GC-MS analysis of partially methylated 3-deoxyoctitoll acetates derived from the methylated β-Kdo-oligosaccharide showed the presence of (→4)- and (→7)-substituted Kdop in roughly equal amounts. This finding suggested a linear structure with alternating (2→4) and (2→7) linkages, although other glycosylation patterns (e.g. a tetrasaccharide repeat unit) were not excluded. Structural analysis of E. coli K1 and K5 β-Kdo-oligosaccharides by NMR spectroscopy identified the β-anomeric configuration of Kdo residues, but the inherent heterogeneity of the samples, and significant signal overlap, precluded the complete assignment of NMR spectra.

Polymerizing KpsC β-Kdo transferases from E. coli and N. meningitidis are functionally exchangeable in vivo. Both are composed of two GT domains, which may correlate with the predicted β-(2→4)- and β-(2→7)-linkages in the oligosaccharide linker. The domains share 35–36% sequence identity and

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§ The abbreviations used are: CPS, capsular polysaccharide; Kdo, 3-deoxy-β-o-manno-oct-2-ulosonic acid; ABC, ATP-binding cassette; GT, glycosyltransferase; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating-frame Overhauser enhancement spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; aa, amino acid.

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possess similar predicted secondary structures. Functional activity was observed in vivo when the domains were expressed as separate polypeptides, suggesting they fold independently, but neither domain alone was sufficient to reconstitute CPS synthesis in vivo (4). The oligosaccharides synthesized in vitro by N. meningitidis KpsS and KpsC on a lipid acceptor have been characterized by LC-MS, but due to a small amount of obtained products, their precise structure has never been elucidated (4).

Recently, we reported the crystal structure of a prototype β-Kdo GT domain from a multidomain protein WbbB from Raoultella terrigena (5). The structure possesses dual Rossmann-fold motifs typically found in GTs with the widespread GT-B fold (6), including the α-Kdo transferase, WaaA (7). However, the β-Kdo GT structure showed extensive insertions, deletions, and structural rearrangements, compared with the canonical fold, which led to WbbB becoming the first example of a new GT99 family in the CAZy database (8). Bioinformatic analyses identified several other putative β-Kdo GTs genes in bacterial strains that produce β-Kdo-containing CPS and/or lipopolysaccharide O-antigens (5). Despite extremely low sequence similarity shared with the WbbB prototype, multiple sequence alignment revealed two motifs within the enzyme active site, which are invariant across all identified β-Kdo transferases, including KpsC. The HP motif is located within the donor substrate binding pocket and involved in coordinating the cytosine ring of CMP-Kdo, similar to the coordination of CMP-sialic acid by some bacterial sialyltransferases. The QXXXD motif is unique to β-Kdo GTs and is located in a Kdo binding pocket. The Asp residue of this motif is essential for WbbB activity and is considered as a potential catalytic nucleophile.

ABC transporter-dependent pathways involving synthesis of a β-Kdo linker are found in prominent human pathogens, including E. coli, N. meningitidis, and Haemophilus influenzae (1, 5). In E. coli, they are found in extra-intestinal isolates, including the globally disseminated multidrug-resistant ST131 group responsible for the majority of extra-intestinal infections by this species (9). The same assembly pathway is also used by opportunistic human pathogens (e.g. Moraxella nonliquefaciens and Kingella kingae), zoonotic pathogens (Pasteurella multocida) and pathogens of livestock (e.g. Actinobacillus pleuropneumoniae, Mannheimia hemolytica and P. multocida) (1, 5, 10). The distribution in human pathogens, and the importance of the capsule layers in the virulence of those bacteria, makes the KpsS and KpsC β-Kdo GTs attractive targets for small molecule inhibitors in anti-virulence strategies, where unmasked bacteria would be susceptible to host defenses. In this work, we extend our current understanding of capsule assembly pathways and β-Kdo GT function by characterizing the precise catalytic activities of the dual E. coli K1 KpsC domains and determining the unequivocal structure of its β-Kdo oligosaccharide product.

Results

In Vitro Analysis of the Activity of KpsC in Whole Cell Lysates—E. coli K1 KpsC (GenBank™ accession number KIE82100) is a protein containing 675 amino acids with two domains corresponding to residues 38–299 and 379–623, respectively, based on Conserved Domain Database search (Fig. 1A). We aimed to examine the function of each KpsC domain by either expressing them as separate polypeptides or, in a complementary approach, by generating constructs with one of the domains catalytically inactivated. Regions corresponding to amino acids 2–675, 2–352, 303–675, and 353–675 of the enzyme contained conserved HP and QxxxD motifs that are invariant across all identified β-Kdo GTs.

FIGURE 1. Activity of KpsC constructs in in vitro whole cell lysate assays. (A) Sequence alignment of conserved domains in KpsC. Mutation sites in each domain and the position of the break points used to separate the domains are indicated. B, structure of acceptor 1. C, TLC analysis of the in vitro Kdo transferase activity of KpsC toward acceptor 1. The reaction mixtures contained 10 μg of total protein from E. coli BL21 expressing either empty vector pET28a(+) or different KpsC constructs. Lane 1, pET28a(+); lane 2, KpsC(2–675); lane 3, KpsC(2–675) H194A/P195A/H522A/P523A; lane 4, KpsC(2–675) D160A/D488A; lane 5, KpsC(2–675) H194A/P195A; lane 6, KpsC(2–675) D160A; lane 7, KpsC(353–675); lane 8, KpsC(303–675); lane 9, KpsC(2–675) H522A/P523A; lane 10, KpsC(2–675) D488A; lane 11, KpsC(2–352); lane 12, KpsC(2–675) H194A/P195A and KpsC(2–675) H522A/P523A; lane 13, KpsC(2–675) D160A and KpsC(2–675) D488A; lane 14, KpsC(2–352) and KpsC(2–675) D160A. The reactions were incubated at 30 °C for 30 min and visualized under UV light.

D motifs that are invariant across all identified β-Kdo GTs. The distribution in human pathogens, and the importance of the capsule layers in the virulence of those bacteria, makes the KpsS and KpsC β-Kdo GTs attractive targets for small molecule inhibitors in anti-virulence strategies, where unmasked bacteria would be susceptible to host defenses. In this work, we extend our current understanding of capsule assembly pathways and β-Kdo GT function by characterizing the precise catalytic activities of the dual E. coli K1 KpsC domains and determining the unequivocal structure of its β-Kdo oligosaccharide product.
each domain of KpsC(2–675) were replaced with two alanine residues, and an additional construct was made with both domains mutated in this way. Independently, the predicted catalytic nucleophilic Asp was changed to Ala in both domains, separately and in tandem. The generated plasmids (Table 1) were overexpressed in E. coli BL21 (DE3) to examine the solubility and enzymatic activity of KpsC constructs. Full-length KpsC and its N-terminal domain were expressed in amounts of soluble protein sufficient for purification and analysis. In contrast, the KpsC(353–675) protein and all of the site-directed mutants containing one inactivated domain were mostly found in the membrane fraction.

The activities of the constructs were initially examined in vitro in a coupled assay using whole cell lysates and a fluorescently labeled β-(2→4)-linked Kdo disaccharide acceptor 1 (Fig. 1B). The activated sugar donor, CMP-β-Kdo, is inherently unstable (11) and was generated in situ using CTP, Kdo, and purified CMP-Kdo synthetase (KdsB). Reaction products were analyzed by thin layer chromatography (TLC) (Fig. 1C). As expected, lysates containing KpsC(2–675) converted the acceptor to a product that was retained at the origin, consistent with addition of multiple Kdo residues. As negative controls, lysates from cells transformed with the empty vector, or lysates containing mutant proteins with both β-Kdo GT domains inactivated (KpsC(2–675) H194A/P195A/H522A/P523A, and KpsC(2–675) D160A/D488A), were all completely inactive in reactions with acceptor 1. Mutations affecting only the C-terminal domain (KpsC(2–675) H522A/P523A, KpsC(2–675) D488A) also resulted in inactivity, whereas mutations to only the N-terminal domain (KpsC(2–675) H194A/P195A and KpsC(2–675) D160A) resulted in enzymes capable of full conversion of acceptor 1 to a product that migrates slower on TLC.

Attempts to create active constructs containing only the C-terminal domain met with mixed success; KpsC(353–675) was inactive, but a slightly larger construct (KpsC(303–675)) showed partial conversion of substrate to a product co-migrating with those obtained with mutationally inactivated N-terminal domains. When both KpsC(2–675) H194A/P195A and KpsC(2–675) H522A/P523A (or KpsC(2–675) D160A and KpsC(2–675) D488A) were added together in a single reaction mixture, a new product was observed that mostly remains at the origin of TLC plate. This finding showed that constructs with inactive C-terminal domains retain their ability to contribute to polymerization despite their inability to use the acceptor 1 when examined in isolation. The same was true for N-terminal domain KpsC(2–352), which, on its own, was inactive in reaction with 1 but contributed to polymerization when KpsC(2–675) D160A was also present in the reaction.

Based on the solubility and the observed activity in these whole cell lysate assays, the WT KpsC(2–675), its N-terminal domain KpsC(2–352), and a mutant with catalytically inactivated N-terminal domain KpsC(2–675) D160A (hereafter referred as KpsC, KpsC-N, and KpsC-C, Fig. 2A) were chosen for protein purification and detailed structural characterization of their reaction products.

### In Vitro Activity Assay Using Purified KpsC Constructs with Acceptor 2—To up-scale the enzymatic reactions and to produce sufficient amounts of products for NMR analysis, we moved away from disaccharide acceptor 1 to a more readily obtainable acceptor 2, comprising one Kdo residue linked, through an eight-carbon chain, to a p-methoxybenzamide tag (Fig. 2B). The change of tag overcame (photo)degradation problems we routinely experience with fluorescein-tagged acceptors. The activities of purified KpsC-N and KpsC toward substrate 2 were confirmed in vitro using a coupled assay, as described above. Switching to an acceptor with a single Kdo residue potentially imposes more structural constraints for catalysis with respect to proximity of the acceptor tag and the alkyl linker to the active site(s), as these differ from the native acceptor structure (the KpsS product). As anticipated, significantly lower conversion was observed for both KpsC-N and KpsC in reaction with acceptor 2, following incubation times that were sufficient for complete conversion of 1 by KpsC under the same conditions. A similar decrease in activity was observed when a single Kdo attached to fluorescein tag was used (data not shown), which excluded the possibility that the lower reactivity arises from the p-methoxybenzamide tag itself. Instead, it appears that the enzyme prefers to have acceptors containing at least two Kdo residues. Increasing incubation times (longer than 1 h) did not significantly improve the conversion. This was likely because of instability of both KpsC constructs, which precipitated over time in the reaction mixture, and/or the instability of in situ generated CMP-β-Kdo, which undergoes rapid hydrolysis to CMP and Kdo (11). To overcome the low conversion issues, an excess of KpsC-N was included in the reaction, and this facilitated complete conversion of 2 to product 3 within 1 h. The product 3 was purified and fully characterized via NMR spectroscopy and ESI MS, as described below, and then used as an acceptor to analyze activities of KpsC and KpsC-C (Fig. 2C).
**KpsC β-Kdo Glycosyltransferase Activity**

**A**

$\text{KpsC}$

$\begin{align*}
2 & \rightarrow 6 \\
\text{His}_5 & \rightarrow 675
\end{align*}$

$\text{KpsC-N}$

$\begin{align*}
2 & \rightarrow 352 \\
\text{His}_5 & \rightarrow 675
\end{align*}$

$\text{KpsC-C}$

$\begin{align*}
2 & \rightarrow 3 \\
\text{His}_5 & \rightarrow 675
\end{align*}$

$\text{D160A}$

$\begin{align*}
2 & \rightarrow 3 \\
\text{His}_5 & \rightarrow 675
\end{align*}$

**B**

$\text{HO}_2\text{C} - \text{O} - \text{CH}_2\text{OH}$

$\text{HO}_2\text{C} - \text{O} - \text{CH}_2\text{OH}$

$\text{OCH}_3$

**C**

$\begin{align*}
\beta & \rightarrow 2,4 \\
\text{KpsC-N} & \rightarrow 3 \\
\beta & \rightarrow 2,7 \\
\beta & \rightarrow 2,4 \\
\text{KpsC-C} & \rightarrow 3 \\
\beta & \rightarrow 2,7 \\
\beta & \rightarrow 2,4 \\
\text{KpsC-C} & \rightarrow 6 \\
\beta & \rightarrow 2,4 \\
\beta & \rightarrow 2,7 \\
\beta & \rightarrow 2,4
\end{align*}$

![FIGURE 2. Biochemical characterization of KpsC activity. A, KpsC constructs purified for detailed characterization. B, structure of acceptor. C, schematic summary of the in vitro reactions and products used to assign activities to KpsC constructs. The products 3–6 from scaled-up reactions were purified and characterized by NMR spectroscopy and mass spectrometry.](image)

**N-terminal Domain of KpsC Catalyzes Addition of a Single β-(2→4)-Kdo Residue—Product 3 from a scaled-up reaction was purified using a Sep-Pak C$_{18}$ cartridge followed by gel permeation chromatography on Sephadex G-25 and analyzed by NMR spectroscopy. The complete assignment of $^1$H and $^{13}$C NMR signals of 3 was performed using a combination of $^1$H NMR, proton-coupled $^{13}$C NMR, two-dimensional $^1$H,$^1$H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating-frame Overhauser enhancement spectroscopy (ROESY), $^1$H,$^{13}$C heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments. The HSQC spectrum of product 3 (Fig. 3) showed two pairs of methylene signals at $\delta_{	ext{C}}$ 35.0 and 36.4, characteristic of Kdo C-3 (12), indicating the presence of two Kdo residues (labeled A and B). Correlations from H-3 to H-5 were traced in the COSY and TOCSY spectra, and the signals for corresponding carbons were assigned by HSQC. A small $^{3}J_{\text{C}-\text{H}}$ coupling constant impeded coherence transfer after H-5, and the remaining assignment was completed using ROESY and HMBC data. The NMR chemical shifts for the carbohydrate moiety of acceptor 2 and product 3 are given in Table 2. As judged by $^{3}J_{\text{C}-\text{H},-3ax}$ coupling constants (13) of $\sim$4 Hz, as well as relatively large difference between chemical shifts of methylene protons (12), both Kdo residues are $\beta$-linked. The $^{13}$C NMR chemical shifts of residue B were indicative of its terminal position, whereas residue A C-4 signal at $\delta$ 73.3 was shifted downfield compared with its position at $\delta$ 68.7 in acceptor 2, because of the $\alpha$-glycosylation effect. Based on $^{13}$C NMR data and the correlation with $^1$H signals in HMBC spectrum, i.e. C-2/O-CH$_2$-C at $\delta$ 102.3/3.39 and 102.3/3.66 (Fig. 3), residue A was recognized as the Kdo moiety attached to the alky linker. Finally, the (2→4)-linkage between Kdo residues was unambiguously confirmed by the correlation in the HMBC spectrum between the anomeric carbon and proton at the substitution position B C-2/A H-4 at $\delta$ 103.4/3.83.

The structure of product 3 was further confirmed by electrospray ionization mass spectrometry (ESI MS). The ESI mass spectrum of 3 in negative ion mode contained the major peak [M – H]$^-$ at $m/z$ 718.30, which showed a gain of 220 atomic mass units compared with acceptor 2, corresponding to one added Kdo residue (Fig. 4A). Thus, the reaction product of N-terminal KpsC domain has structure 3 shown in Fig. 2C.

**C-terminal Domain of KpsC Catalyzes Addition of a Single β-(2→7)-Kdo Residue—**The fully characterized product 3 was used as a substrate in a scaled-up reaction with KpsC-C. The reaction product 4 was purified and analyzed essentially as described for 3. The $^1$H and HSQC spectra showed the presence of three Kdop residues (labeled A, B, and C) (Fig. 3), and the signals within each spin system were assigned using homonuclear and heteronuclear NMR experiments, as described above (Table 2). The $\beta$-anomeric configuration was inferred based on the difference between chemical shifts of H-3ax and H-3eq of $\Delta$ 0.51 ppm (A) and 0.59 ppm (B and C), by comparing published data (12) for the methyl esters of methyl $\alpha$- and $\beta$-glycosides of Kdop (i.e. $\Delta\delta$ 0.27 and 0.64 ppm, respectively). The signals for residue A were close to those for the (2→4)-substituted Kdo residue A in product 3, whereas the signals for residue C are indicative of its terminal position. The substitution of residue B in 4 at position O-7 was inferred from a positive $\alpha$-glycosylation effect on C-7 and the negative $\beta$-glycosylation effect on neighboring carbons in residue B (C-6 and C-8) compared with $^{13}$C NMR signals of the terminal Kdo residue in 3. The HMBC spectrum showed the correlation between residue C C-2 and residue B H-7 at $\delta$ 101.9/4.40, which, taking in consideration the substitution positions, determined the sequence of Kdo residue as follows: C(2→7)B(2→4)A. Thus, the C-terminal domain of KpsC catalyzed the addition of a single Kdo residue at position O-7 of (2→4)-linked Kdo residue.

The ESI MS spectrum of 4 showed a predominant ion peak [M – H]$^-$ at $m/z$ 938.36, which corresponded to a trisaccharide (Fig. 4B). The spectrum also contained a peak at $m/z$ 718.30, which could be explained by traces of contaminating unreacted substrate 3, slow degradation of 4 during sample handling, and/or cleavage of (2→7) linkage because of in-source fragmentation of parent ion. Similar in-source fragmentation resulted in loss of terminal Kdo residue was reported earlier for a Kdo-(2→3)-Rha-(1→3)-GlcNAc trisaccharide derivative (5). TLC analysis of product 4 left over after ESI MS analysis confirmed that the amount of contaminating 3 (if present) is very low (Fig. 4C).
**TABLE 2**

1H and 13C NMR data for the carbohydrate moiety of acceptor 2 and products 3–5 (δ, ppm)

| Sugar residue | C-1 | C-2 | H-3αx | H-3eq | H-4 | H-5 | H-6 | H-7 | H-8α | H-8β |
|---------------|-----|-----|-------|-------|-----|-----|-----|-----|-------|-------|
| **Acceptor 2** |     |     |       |       |     |     |     |     |       |       |
| β-Kdo-(2→)    | 1.78| 2.40| 3.75  | 3.94  | 3.60| 3.90| 3.74| 3.85|       |       |
| β-Kdo-(2→)    |     |     | 3.06  |       |     |     |     |     |       |       |
| **Product 3** |     |     |       |       |     |     |     |     |       |       |
| →4)-β-Kdo-(2→ |     |     |       |       |     |     |     |     |       |       |
| A             | 1.81| 2.35| 3.83  | 4.18  | 3.60| 3.88| 3.73| 3.84|       |       |
| B             | 175.0| 102.3| 35.0 |       |     |     |     |     |       |       |
| **Product 4** |     |     |       |       |     |     |     |     |       |       |
| →4)-β-Kdo-(2→ |     |     |       |       |     |     |     |     |       |       |
| A             | 1.81| 2.32| 3.82  | 4.16  | 3.57| 3.87| 3.73| 3.84|       |       |
| B             | 175.1| 102.4| 34.9 |       |     |     |     |     |       |       |
| **Product 5** |     |     |       |       |     |     |     |     |       |       |
| →4)-β-Kdo-(2→ |     |     |       |       |     |     |     |     |       |       |
| A             | 1.94| 2.31| 3.88  | 4.15  | 3.42| 3.90| 3.79| 3.86|       |       |
| B             | 175.0| 101.8| 34.0 |       |     |     |     |     |       |       |

*Data were from the HSQC experiment.*

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β-Kdo Oligosaccharide Consists of Alternating β-(2→4)- and β-(2→7)-linked Kdo Residues—The acceptor substrate specificity of the N- and C-terminal domains and the structural data for the products 3 and 4 suggested that they together generate an alternating linkage pattern in the native linker oligosaccharide. To confirm unequivocally this proposal and to elucidate...
the structure of Kdo linker, the products 3 and 4 were used as acceptors in reactions with purified KpsC and a 20-fold molar excess of donor substrate. The obtained products 5 and 6 were purified and characterized by NMR spectroscopy.

The 1H NMR spectra of 5 and 6 were essentially identical (Fig. 5); therefore, the 2D NMR experiments were performed only on product 5. The 13C HSQC spectrum of 5 (Fig. 6) demonstrated the presence of a disaccharide repeat unit. Downfield displacement of the signals for residue A C-4 and residue B C-7 because of the α-glycosylation effect indicated the positions of substitution. The HMBC spectrum showed the correlation between residue A C-2 and residue B H-7 at δ 101.8/4.37 that confirmed the A(2→7)B linkage. The B(2→4)A linkage cannot be concluded unequivocally from the HMBC spectrum because of the absence of the corresponding correlation. However, this linkage could be inferred taking into account the disaccharide repeat unit, the substitution of residue A at position 4, and the structural data obtained for 3 and 4.

In addition to the major series of signals corresponding to internal repeat units, the NMR spectra contained a minor series resulting from the residues in terminal repeat units (labeled A4, A7, and B3) (Fig. 5). Correlations from the minor residue A4 H-3ax signal were traced in the TOCSY spectrum, and the A4 H-4 and H-5 signals mostly overlap with those from residue A in internal repeat units. The position of residue A4 C-6/H-6 in the HSQC spectrum inferred from the ROESY correlation residue A4 H-5/H-6 was close to that of residue A in product 4 (Fig. 3). Hence, residue A4 is the first Kdo attached to the alkyl linker. Well resolved minor H-5 resonances were used as starting points to assign the residue A7 and B7 spin systems. The residue B7 C-7 signal (overlapped with residue A C-7) at δ 70.3 and C-6 signal at δ 75.0 indicated that B7 occupies the terminal non-reducing position of the Kdo oligosaccharide (compare with residue B C-7 and C-6 in 3, Fig. 3). Residue A7 was substituted at position O-4 and was tentatively assigned to the terminal non-reducing B7(2→4)A7 repeat unit. No minor signals that would correspond to non-substituted A residues were found in NMR spectra. From these results, it is apparent that all of the chains terminate with β-(2→4)-linkages.

**Discussion**

The kpsS and kpsC genes found in many capsule synthesis gene clusters encode β-Kdo GTs belonging to family GT99 (4, 5). In this work, we biochemically characterized the activity and specificity of individual KpsC domains, and we unambiguously established the structure of the β-Kdo linker. Site-directed mutagenesis studies confirmed the essential role of the conserved HP and QXXXD motifs in KpsC for β-Kdo transferase activity predicted based on multiple sequence alignment with the WbbB prototype, indicating mechanistic similarities with the GT99 family enzymes. In the prototype GT99 protein, WbbBGTV99 substitution of either His or Pro to Ala reduced catalytic activity, without affecting protein solubility. Double mutations of both His and Pro residues in the KpsC domains resulted in complete loss of activity of the targeted domain, based on TLC. However, the mutated KpsC and isolated C-terminal KpsC domain were significantly less soluble than wild-type KpsC.

We recently determined the structure of WbbBGTV99 a KpsC homolog (5); however, the sequence similarity between these proteins is weak, and individual KpsC domains are significantly shorter than WbbBGTV99 (~330 aa versus 400 aa). The C-terminal α/β domain, where most of the catalytically essential residues are located, shows by far the strongest similarity, with all core secondary structural motifs conserved (Fig. 7). KpsC also shows two substantive and conserved insertions in this domain.
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**FIGURE 5. Comparison of ¹H NMR spectra of enzymatic products.** The spectra of 5 and 6 were recorded after adjusting pH to 11 with NH₄OH for a more accurate comparison. All other 1D and 2D spectra shown in the paper were recorded without pH adjustment (at pH ~ 6). Asterisks indicate signals from low molecular weight impurities. Dashed lines highlight the position of the minor signals from the residues in terminal repeat units in products 5 and 6 compared with those in products 3 and 4.

(as well as a longer one in the first portion of the α-helical domain), which may contribute the active site. KpsC also has a predicted C-terminal helical region similar in length to α-helical domain in WbbB GT99, but poor sequence similarity makes detailed comparisons difficult. More interesting is the observation that KpsC has no apparent sequence similarity N-terminal to the WbbB GT99 α4 helix (from the penultimate α/β repeat of the N-terminal α/β domain). This is not simply a matter of sequence divergence, as all KpsC domains have a large deletion in this region relative to WbbB GT99 (~90 aa in E. coli KpsC-N). Although only or two more α/β motifs may theoretically be present, most of this domain, already reduced in WbbB GT99, is necessarily absent. Possibly the insertions in the C-terminal α/β domain allow this domain to take on functions (such as mediating acceptor binding) originally served by the N-terminal α/β domain. Whereas a definitive understanding of this issue will ultimately require an experimental KpsC structure, this analysis suggests that KpsC is likely a GT-B glycosyltransferase with a single Rossmann-fold domain, which, paradoxically, is the feature central to the definition of the GT-A fold.

Most of the KpsC homologs from other bacteria are 640–730-amino acid proteins with similar dual domain structure (5). Phylogenetic analysis of separated KpsC domains from a number of human pathogens, including *N. meningitidis* and *H. influenzae*, and *Campylobacter jejuni* (5), showed the C- and N-terminal domain groups in separate subclades, suggesting that there are conserved β-(2→4)- and β-(2→7)-linkage specificities in the corresponding domains of all KpsC proteins. The separated domains are able to reconstitute capsule assembly *in vivo* (4) and the biosynthesis of the β-Kdo oligosaccharide *in vitro* (this study). KpsC domains naturally encoded by separate open reading frames are rare but do exist. A thermophilic sulfur-disproportionating bacterium *Thermosulfurimonas dismutans* provides one example, where 318- and 348-aa proteins (GenBank™ accession numbers OAQ19866 and OAQ20765) are single domain homologs of KpsC domains that fall into N- and C-terminal domain phylogenetic subclades, respectively. The biological role of these proteins has not been investigated.

It remains to be established which KpsC domain acts first on its natural substrate, (lyso)phosphatidylglycerol-β-Kdo produced by KpsS. The specificity of the KpsC domains toward the KpsS product defines the linkage configuration between the first and second Kdo residues at the reducing end of the β-Kdo linker. The lipid-linked β-Kdo oligosaccharides isolated from *E. coli* and *N. meningitidis* CPS were found to contain either an odd or an even number of Kdo residues, depending on serotype (3). This may be explained by the acceptor specificity of the GT that transfers the first residue of the CPS repeat-unit domain onto the poly-Kdo linker (1). However, in this scenario, the consistency in odd or even numbers of β-Kdo residues for a particular serotype demands that the entire population of β-Kdo-linkers has the same defined linkage type between first and second Kdo residues, to retain the same register. NMR spectroscopic analysis showed that all (within the limits of detection) *in vitro* KpsC products possess a β-(2→4) linkage at the non-reducing end. This could be explained by the lower activity of the C-terminal KpsC domain compared with the N-terminal domain, so the limiting step is the formation of the β-(2→7) linkage. However, the non-physiological conditions used in the *in vitro* reactions may affect the range of products.
FIGURE 6. Part of $^1$H,$^13$C HSQC spectrum of product 5. The numbers refer to H/C pairs in Kdo residues, which are designated by letters. The corresponding $^1$H NMR spectrum is shown along the axis. O-$\text{CH}_2$-C, C-$\text{CH}_2$-N, and C-$\text{CH}_2$-C signals belong to 8-carbon methylene linker in the acceptor.

FIGURE 7. Topology of WbbB$_{GT99}$/H$_{9252}$-$\beta$-Kdo transferase and the inferred topological features of KpsC domains. KpsC differs from WbbB by the deletion of most of the N-terminal $\alpha/\beta$ domain. The positions of conserved KpsC insertions in the C-terminal $\alpha/\beta$ domain are marked with red triangles; possibly present elements of the N-terminal $\alpha/\beta$ domain are denoted with dashed lines.
and not fully reflect the natural situation in the cell. The isolated C-terminal domain demonstrated only weak activity in whole cell lysate assay, but this must be interpreted with caution because the purified KpsC-C construct was significantly less stable than either KpsC and KpsC-N. In addition, we found that the β-(2→7) linkage is more labile to hydrolysis than β-(2→4) linkage (data not shown), and this could also influence the range of products detected. The β-(2→7)-linkages in products 5 and 6 might be partially breaking down during purification steps, which would result in an increase of the portion of oligosaccharides terminated with β(2→4)-linkages.

In summary, we have defined the activity of KpsC, an enzyme with dual β-Kdo GT domains critical for the production of group 2 capsules in E. coli and other prominent Gram-negative pathogens. With these capsules providing vital roles in pathogenesis, the conservation and novel activities of KpsC homologs provide potential therapeutic targets active against a range of encapsulated bacteria.

### Experimental Procedures

**General DNA Methods**—Genomic DNA from E. coli serogroup K1 (strain EV36 (14)) was prepared using the PureLink Genomic DNA mini kit (Invitrogen). Plasmid DNA was purified using the PureLink plasmid miniprep kit (Invitrogen), and DNA fragments from PCRs and restriction digestions were purified using the PureLink PCR purification kit (Invitrogen). Restriction endonucleases and T4 DNA ligase (New England Biolabs) were used according to the manufacturer’s instructions. Recombinant plasmid constructs used in this study are listed in Table 1. Single amino acid substitutions were introduced by PCR using Kod Hot Start DNA polymerase (Novagen) and pWQ879 (encoding KpsC(2–675)) as a template. The template strand was digested with DpnI, and the digestion mixtures were used to transform competent E. coli DH5α (φ80d deor lacZΔM15 endA1 recA1 hisdR17 (rpsL::mam-1) supE4 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169F−) cells to generate the plasmids pWQ883–pWQ888. All constructs were confirmed via DNA sequencing performed at the Advanced Analysis Centre at the University of Guelph. Custom oligonucleotide primers (Sigma) are listed in Table 3.

**Synthesis of Acceptor 1**—The synthesis of 1 started with the previously reported thioglycoside 7 (Fig. 8) (15). Glycosylation of 8-azidooctanol with 7 led to the formation, in 80% yield, of 8, which was then deacetylated to give 9 (96% yield). Protection of O-7 and O-8 as an isopropylidene acetal was achieved by reaction of 9 with dimethoxypropane and p-toluenesulfonic acid giving 10 in 80% yield. The diol moiety in 10 was acetylated by formation of the orthoester and acidic hydrolysis providing 11 in 80% yield over the two steps. Glycosylation of 11 with 7 resulted in the formation, in 70% yield, of disaccharide 12. The acetates in 12 were cleaved by methanolysis affording 13 in 95% yield. Disaccharide 13 was then completely deprotected and converted to amine 15 in three steps and 60% overall yield. Reaction of 15 with FITC gave a 74% yield of 1. In all cases, the anomeric configuration of the product formed in the glycosylation reactions was established by measurement of the $\beta$-1,4H3ax (13). All experimental and characterization details can be found in the supplemental material.

**Synthesis of Acceptor 2**—As illustrated in Fig. 8, acceptor 2 was synthesized from 9, which was produced as an intermediate in the preparation of 1. Cleavage of the methyl ester in 9 was achieved by treatment with sodium hydroxide to give 16, which was then reduced to amine 17 in 80% yield over the two steps. Amidation of 17 with p-anisoyl chloride gave, in 75% yield, 2. Experimental and characterization details can be found in the supplemental material.

**In Vitro Kdo transferase Assay Using Cell Lysates and Acceptor 1**—KpsC constructs were expressed in E. coli BL21 (DE3) (F−ompT gal dcm hsdS6 (rB− mB−) λ (DE3)). Overnight cultures were used to inoculate 5 ml of LB medium at a 1:100 dilution. Cultures were grown at 37 °C until an A600 of ~0.6 was achieved and expression of the recombinant protein was then induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.3 mM. The cultures were grown for a further 2.5 h at 37 °C. Cells were harvested by centrifugation at 5,000 × g for 5 min. The cell pellet was resuspended in 400 μl of buffer (50 mM Tris-HCl, 250 mM NaCl, pH 7.4) supplemented with Complete mini EDTA-free protease inhibitors (Roche Applied Science). The cells were lysed by ultrasonication for a total of 1 min with 10% amplitude in pulses (10 s on/15 s off). The total protein concentration was measured using DC protein assay (Bio-Rad). In vitro reactions were performed in PCR tubes at 30 °C in total volume of 20 μl. Acceptor 1 concentration was estimated by measuring the absorbance at 490 nm in 0.01 M NaOH, using an extinction coefficient of 88,000 M$^{-1}$ cm$^{-1}$ (16). The coupled assay contents were 50 mM HEPES, pH 8.0, 10 mM MgCl$_2$, 2 mM Kdo, 5 mM CTP, 0.2 mM synthetic

### Table 3

**Primer sequences**
The sequence complementary to the template is shown in uppercase. The introduced NcoI and XhoI restriction sites are underlined.

| Primer | Sequence (5′→3′) | Plasmid |
|--------|------------------|---------|
| OL1034 | gatoccatgggcATGCGATTTACGCGCTGCG | pWQ879, pWQ880 |
| OL1035 | gatoccatgggcGCGAATCCTGACGTTACGAA | pWQ879, pWQ882, pWQ881 |
| OL1036 | gatoccatgggcGCGAATCCTGACGTTACGAA | pWQ880 |
| OL1037 | gatoccatgggcACCGCCTGATTTTTCGAC | pWQ882 |
| OL1126 | gatoccatgggcAACCGACGAGAAATGCGG | pWQ881 |
| OL1089 | CTTCCTCCATGATACGCTGCGgACAGCTCTCACCTGACGTT | pWQ883, pWQ885 |
| OL1090 | CCGAATTTGCTGAGTTGCGggccgCAAGTACTGGTGGGGAAT | pWQ883, pWQ885 |
| OL1091 | ATTCGCCACAGTAGACGCTGCGgACAGCTCTCACCTGACGTT | pWQ883, pWQ885 |
| OL1099 | CCTACATTGTTTATAAACCGgccgCGGATGTACTGGTGGGGAAT | pWQ884, pWQ885 |
| OL1100 | GCTCAGTACATTTAATTGAtgCGACGTTACGACG | pWQ886, pWQ888 |
| OL1101 | CTGTTTTAATAGAGGCGgGTCGACTCACCTGACGTT | pWQ887, pWQ888 |
| OL1102 | GGGTCAGCGGAGCGGTCGACTCACCTGACGTT | pWQ887, pWQ888 |
Kpsβ-Kdo Glycosyltransferase Activity

acceptor 1, 1 µg of KdsB (expressed and purified essentially as described (17)), and 10 µg total protein of each KpsC mutant lysate. Cell lysate prepared from E. coli BL21 cells transformed with the empty vector pET28a(+) served as a negative control. The reaction was initiated by addition of KdsB and was incubated for 30 min. The reaction was stopped by spotting 1 µL onto a TLC plate (Silica Gel 60 F254). The TLC plate was developed in freshly prepared chloroform/methanol/water/acetic acid mixture (25:15:4:2, v/v) and the products were visualized using a hand-held UV lamp.

Overexpression and Purification of KpsC and KpsC-N—KpsC(2–675)-His6 and KpsC(2–352)-His6 were expressed in E. coli BL21 (DE3). Overnight cultures of transformants containing pWQ879 and pWQ880 were used to inoculate 1 liter of LB medium at a 1:100 dilution, and protein expression was carried out as described above. Cells were harvested by centrifugation at 5,000 × g for 10 min and stored at −80 °C until needed. The cell pellet was resuspended in 25 ml of buffer A (50 mM Tris-HCl, 250 mM NaCl, pH 7.4) containing 10 mM imidazole, and cells were lysed by ultrasonication for a total of 3.5 min with 30% amplitude in pulses (10 s on/15 s off). The suspension was cleared via successive centrifugation steps at 4,000 × g for 10 min, 12,000 × g for 20 min, and 100,000 × g for 1 h at 4 °C. The proteins were purified from the residual supernatant using 2 ml of nickel-nitrilotriacetic acid-agarose resin (Qiagen). The column was conditioned initially with 20 ml of buffer A containing 10 mM imidazole before sample was applied. The column was then washed with 24 ml of buffer A.

FIGURE 8. Synthesis of 1 and 2. a, HO(CH₂)₆CH₂N₃, IBr, AgOTf, 2:3 CH₂Cl₂–CH₃CN, −70 °C, 1 h, 80%. b, NaOCH₃, CH₃OH, room temperature, 30 min (96% for 9, 95% for 13). c, 2,2-dimethoxypropane, p-TsOH, dimethylformamide, room temperature, 2 h, 80%. d, (i) CH₃C(OCH₃)₃, CSA, CH₃CN, room temperature, 30 min, and (ii) 1 N HCl, room temperature, 10 min, 80% over two steps. e, 7, NIS, TFOH, 2:3 CH₂Cl₂–CH₃CN, −70 °C, 1 h, 70% (β-anomer). f, 80% AcOH, 60 °C, 1 h. g, 1 N NaOH, room temperature, 30 min. h, H₂, Pd(OH)₂/C, H₂O, room temperature, 5 h, (60% over three steps for 15, 80% over two steps for 17). i, FITC, NaHCO₃, H₂O, DMF, 0 °C, 2 h, 74%. j, p-Anisoyl chloride, K₂CO₃, H₂O, room temperature, 2 h, 75%.

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A containing 25 mM imidazole, before elution with 15 ml of buffer A containing 350 mM imidazole; eluent was collected in 1-ml fractions. Protein was concentrated and exchanged into imidazole-free buffer A using a Vivaspin centrifugal concentrator. Protein purity was assessed by SDS-PAGE using 10% resolving gels in Tris-glycine buffer, and the gels were stained with SimplyBlue SafeStain (ThermoFisher). The concentration was estimated from $A_{280}$ values using theoretical extinction coefficients of 106,230 and 63,370 M$^{-1}$ cm$^{-1}$ predicted by Prot-Param for KpsC(2–675)-His$_{6}$ and KpsC(2–352)-His$_{6}$, respectively. Proteins were stored in buffer A containing 10% glycerol at −80 °C and retained their activity for at least a month.

Overexpression and Purification of KpsC-C—KpsC(2–675)-His$_{6}$ D160A was expressed in E. coli BL21 (DE3) in mostly insoluble form. Overnight cultures of transformant containing pWQ886 was used to inoculate 2 liters of LB medium, and the protein expression was induced with isopropyl 1-thio-$\beta$-D-galactopyranoside at a final concentration 0.3 mM. Cells were grown for 2.5 h at 37 °C, harvested by centrifugation at 5,000 × g for 10 min, and stored at −80 °C until needed. Cell lysate were prepared as described above. The suspension was cleared via successive centrifugation steps at 4,000 × g for 10 min, 12,000 × g for 20 min, and 100,000 × g for 1 h at 4 °C. The membranes from the final centrifugation step were resuspended in 32 ml of buffer A containing 10 mM imidazole and 0.1% (w/v) n-dodecyl-$\beta$-D-maltoside, supplemented with protease inhibitors (Roche Applied Science), and incubated overnight at 4 °C with gentle agitation. The extract was cleared by centrifugation at 100,000 × g for 1 h at 4 °C, and the supernatant was loaded onto a 2-ml nickel-nitrilotriacetic acid-agarose resin column. The protein was washed on the column and loaded on to a Sep-Pak C18 column, which was previously washing with 10 ml of acetonitrile and 20 ml of water. The column was washed with 20 ml of water, and the product was eluted in 5 ml of 50% (v/v) acetonitrile/water mixture. The solution was concentrated to 100 μl and further purified using gel chromatography on a column (48 × 0.7 cm) of Sephadex G-25 Superfine, eluted in water with flow rate 0.25 ml/min. Elution was monitored using a refractometer (Knauer) and TLC; the 0.75-ml fractions containing the product were combined and dried using a SpeedVac.

NMR Spectroscopy—NMR studies were performed at the NMR Centre of the Advanced Analysis Centre at the University of Guelph. The products were deuterium-exchanged by dissolving in 99.9% D$_2$O and drying using a SpeedVac. NMR spectra were obtained at 30 °C in 99.96% D$_2$O using a Bruker Avance II 600 MHz spectrometer equipped with a cryoprobe. The chemical shifts are referenced to the internal standard, sodium 3-trimethylsilylpropanoate-2,2,3,3-$d_4$ ($\delta_N = 0$ ppm, $\delta_C = −1.6$ ppm). Two-dimensionalexperimentswereperformedusingstandard Bruker software, and the Bruker TopSpin 2.1 program was used to acquire and process the NMR data. Mixing times in TOCSY were set up to 100 ms. Mixing times in ROESY were set to 300 ms for product 3 and 200 ms for products 4 and 5. The HMBC experiment was optimized for the $J_{4,5}$ coupling constant 8 Hz.

ESI MS—ESI mass spectrometry analyses were performed on Agilent UHD 6530 Q-Tof mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at 250 °C, with a flow rate of 8 liters/min. Nebulizer pressure was 30 p.s.i., and the fragmentor was set to 160. Nitrogen was used as both nebulizing and drying gas. The mass-to-charge ratio was scanned across the $m/z$ range of 50–2000 $m/z$ in 4 GHz (extended dynamic range) negative ion mode. The instrument was externally calibrated with the ESI Tune Mix (Agilent).

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