The Capsule Polymerase CslB of Neisseria meningitidis Serogroup L Catalyzes the Synthesis of a Complex Trimeric Repeating Unit Comprising Glycosidic and Phosphodiester Linkages*

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Background: The trimeric repeating unit forming the capsular polysaccharide of Neisseria meningitidis serogroup L comprises glycosidic and phosphodiester bonds.

Results: We identified the two-domain capsule polymerase CslB of N. meningitidis serogroup L to assemble this complex trimer with UDP-GlcNAc as substrate.

Conclusion: CslB represents a unique capsule polymerase in group 2 capsule expressing bacteria.

Significance: Understanding capsule biosynthesis is mandatory for combating encapsulated bacterial pathogens.

Neisseria meningitidis is a human pathogen causing bacterial meningitis and sepsis. The capsular polysaccharide surrounding N. meningitidis is a major virulence factor. The capsular polysaccharide is polyhexosamine phosphates in N. meningitidis serogroups A and X. The capsule polymerases (CPs) of these serogroups are members of the Stealth protein family comprising d-hexose-1-phosphate transferases from bacterial and protozoan pathogens. CslA, one of two putative CPs of the pathophysiologically less relevant N. meningitidis serogroup L, is one of the smallest known Stealth proteins and caught our attention for structure-function analyses. Because the N. meningitidis serogroup L capsule polymer consists of a trimeric repeating unit ([1→3]-β-d-GlcNAc-[1→3]-β-d-GlcNAc-[1→3]-α-d-GlcNAc-[1→OPO3]2)n, we speculated that the two predicted CPs (CslA and CslB) work together in polymer production. Consequently, both enzymes were cloned, overexpressed, and purified as recombinant proteins. Contrary to our expectation, enzymatic testing identified CslB to be sufficient to catalyze the synthesis of the complex trimeric N. meningitidis serogroup L capsule polymer repeating unit. No polymerase activity was detected for CslA, although the enzyme facilitated the hydrolysis of UDP-GlcNAc. Bioinformatics analyses identified two glycosyltransferase (GT) domains in CslB. The N-terminal domain modeled with 100% confidence onto a number of GT-A folded proteins, whereas the C-terminal domain modeled with 100% confidence onto TagF, a GT-B folded teichoic acid polymerase from Staphylococcus epidermidis. Amino acid positions known to have critical catalytic functions in the template proteins were conserved in CslB, and their point mutation abolished enzyme activity. CslB represents an enzyme of so far unique complexity regarding both the catalyzed reaction and enzyme architecture.

The human-specific pathogen Neisseria meningitidis (or meningococcus) is one of the leading causes of epidemic meningitis and fatal sepsis (1). In the developed world, meningococcal disease appears sporadically with <1–3 infections/100,000 people/year (2), but outbreaks can occur in situations of crowding (3) and have been recorded in the sub-Saharan meningitis belt in intervals of 8–10 years over the past century (1). These epidemics reached incidence rates up to 1% and mortality rates up to 75% in children and adolescents without any treatment (2). Survivors of invasive meningococcal disease often suffer lifelong from sequelae like deafness, limb deformation, and psychological symptoms (4, 5). Thus, invasive meningococcal disease remains a major burden at the individual and socio-economic level.

Major virulence factors of pathogenic N. meningitidis are the extracellular capsular polysaccharides (CPSs) (6). Twelve N. meningitidis serogroups have been defined so far according to their CPS composition, of which six (A, B, C, W, X, and Y) are important pathogens (7). Based on their chemical characteristics and export machinery, capsules are divided into four groups (8). Group 2 capsules, as present in all N. meningitidis serogroups, exhibit a high charge density and are exported by

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2 The abbreviations used are: CPS, capsular polysaccharide; CPSL, CPSB, CPS, CPSW, and CPSY, capsular polysaccharide of N. meningitidis serogroup L, B, C, W, and Y, respectively; CPSL, in vitro synthesized CPS; CPSL, CPSL isolated from a natural source; oligoCPSL, oligoCPS, oligosaccharides derived from CPSL; AEC, anion exchange chromatography; CIP, calf intestinal alkaline phosphatase; CP, capsule polymerase; GlcNAc, N-acetylglucosamine; GT, glycosyltransferase; MBP, maltose-binding protein.
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ATP-binding cassette transporter-dependent systems (6). In the CPSs of serogroups B, C, W, and Y (CPSB, -C, -W, and -Y), negative charge results from the acidic nonasugar sialic acid, which is the only building block in CPSB and CPSC or one of two building blocks in CPSW and CPSY (9, 10). Very different from the sialic acid-containing CPSs, in which glycosidic bonds link the monomers, phosphodiester link the sugar monomers (N-acetylmannosamine and GlcNAc, respectively) in CPSA and CPSX and also introduce the negative charge (11–13).

Beginning in the 1990s, efficient conjugate vaccines against N. meningitidis serogroups A, C, W, and Y have been developed, which all used purified CPSs (before or after mild acidic hydrolysis) coupled to adjuvant carrier proteins (14). After the successful introduction of these glycoconjugate vaccines in developed countries (15), mass vaccination campaigns with N. meningitidis serogroup A were carried out in countries of the African meningitis belt to induce herd immunity (16–18), providing protection not only to the vaccinated individuals but also to unvaccinated individuals and, most importantly, to young children (19).

Despite the undeniable benefits of anti-meningococcal vaccines, recent reports discuss the emergence of previously less prominent serogroups in reply to vaccination campaigns against the prevalent serogroups (20–25). One of the mechanisms underlying serogroup replacement is called capsule switching, a genetic event in which the genes necessary for capsule biosynthesis are exchanged between serogroups (21, 26, 27). Nevertheless, the sporadic occurrence of invasive meningococcal disease due to serogroups with currently low pathogenic potential (28, 29) demonstrates that eradication of invasive meningococcal disease will profit from vaccines against rare serogroups.

In this context, we focused our work on capsule polymerases (CPs) of both pathogenic and rarely occurring meningococcal serogroups and thereby follow two research lines: (i) exploitation of recombinant CPs for in vitro production of glycoconjugate vaccines (30, 31) and (ii) delineation of structure-function relationships as a basis for drug target evaluation (32–39).

To generate the plasmids pΔN29-cslB-His₆ (tac) and pΔN37-cslB-His₆ (tac) for the expression of ΔN29-CslB-His₆ and ΔN37-CslB-His₆ without N-terminal MBP tag under tac promoter control, the truncated cslB sequences were amplified using primers CL5/TF38 and CL6/TF38, respectively, and cloned via NdeI/XhoI into p-MBP-cslA-His₆ (tac) and p-MBP-cslB-His₆ (tac), cslA and cslB are expressed under tac promoter control as fusion proteins with an N-terminal maltose-binding protein (MBP) fused by a protease-resistant S₆N₆ linker (44) and a C-terminal His₆ tag. New constructs were verified by sequencing.

Mutations at positions Asp¹³⁸ and Asp¹⁴⁰ in CslB were introduced in p-MBP-cslB-His₆ (tac), and mutations at positions H595, H733 in CslB were introduced in p-MBP-cslB-His₆ (tac) using the Q5® site-directed mutagenesis kit (New England Biolabs) and the respective primers shown in Table 1 according to the manufacturer’s guidelines.

Expression and Purification of Recombinant CslA and CslB—For expression of recombinant proteins, Escherichia coli M15[pREP4] were transformed with expression plasmids (Table 1) and grown overnight in LB medium at 37 °C and 200 rpm in the presence of 200 μg/ml carbenicillin. The preculture was used to inoculate 500 ml (or smaller volumes for test expressions) of PowerBroth® (ATHENAES), and cells were grown at 37 °C and 200 rpm up to an A₆₀₀ of 0.6–1.0. Subsequently, growth was decelerated at 4 °C for 20 min, and protein expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (Merck) to a final concentration of 1 mM and further incubation at 15 °C and 200 rpm for 21 h. Finally, the culture was centrifuged, and the pellet was resuspended in 10–20 ml of lysis buffer and incubated on ice for 10–20 min. The lysate was sonified (Branson Digital Sonifier, 50% amplitude, 8 × 30 s, interrupted by cooling on ice), and soluble and insoluble fractions were separated by centrifugation (20,000 × g, 4 °C, 40 min). Soluble fractions were filtered (0.8-μm pore size; Millex), and recombinant His₆-tagged proteins were enriched by immobilized metal ion affinity chromatography.
TABLE 1
Plasmids, recombinant proteins, and primers used in this study

| Plasmid name | encoded protein | molecular mass (kDa) |
|--------------|-----------------|---------------------|
| pMBP-cslAhis6-loc | MBP-CslA-His6 | 86 |
| pMBP-cslB-His6-loc | ΔN-CslB-His6 | 100-101 |
| pN37-cslH-His6-loc | ΔN29-CslB-His6 | 101 |
| pN37-cslB-His6-loc | ΔN-CslB-His6 | 100 |
| pN37-cslH-His6-loc | ΔN-CslB-His6-loc | 100-101 |
| pN37-cslH-His6-loc | ΔN-CslB-His6-loc | 100 |

Primers used for generating cslA and cslB wild type constructs. Restriction sites are underlined.

**TF33**  5'-CGGAGCTCCCGATATTTCAATCCGTACGGC-3'
**TF34**  5'-CGGAGCTCCCGATATTTCAATCCGTACGGC-3'
**TF37**  5'-CGGAGCTCCCGATATTTCAATCCGTACGGC-3'
**TF38**  5'-CGGAGCTCCCGATATTTCAATCCGTACGGC-3'
**CL5**  5'-CCATATGATGAAATACAAAAATTTTACG-3'
**CL6**  5'-CCATATGATGAAATACAAAAATTTTACG-3'

Primer pairs used for generating cslB mutants

ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'
ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'
ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'
ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'
ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'
ΔN-CslB<sup>His6</sup>-loc | ΔC3 | 4-ATGGCCCTGGGACCCCAATGAC-3'

was supplemented with different divalent cations to determine optimal reaction conditions for CslB. CslB activity could be observed in the presence of Ca<sup>2+</sup> and Mg<sup>2+</sup>, but the highest activities were obtained in the presence of 5–10 mM Mn<sup>2+</sup> (data not shown). No activity was observed in the presence of EDTA, Co<sup>2+</sup>, or Zn<sup>2+</sup> (data not shown). Reactions were started by the addition of 2 mM UDP-GlcNac (Calbiochem) containing 0.05 μCi of UDP-[<sup>14</sup>C]GlcNac (American Radiolabeled Chemicals). The assay was performed in the presence and absence of 2 μl of heat-inactivated *N. meningitidis* serogroup L lysate. Reaction samples were incubated at 37 °C. After defined time points ranging from 0 to 60 min, 5-μl aliquots were spotted onto Whatman 3MM CHR paper, and free radioactivity was eluted by descending paper chromatography using a mixture of 30% 1 M ammonium acetate, pH 7.5, and 70% ethanol (v/v). The chromatographically immobile <sup>14</sup>C-labeled material was quantified by scintillation counting.

**HPLC and NMR Analysis**—HPLC-based anion exchange chromatography (HPLC-AEC) was performed on a Prominence UFLC-XR (Shimadzu) equipped with a CarboPac PA-100 column (2 × 250 mm; Dionex). Samples were separated as described (46) with the minor adjustment that H<sub>2</sub>O and 1 M NaCl were used as mobile phases M1 and M2, respectively. 5 μl of the samples were loaded for the detection of nucleotides at 280 nm and 50 μl for the detection of *in vitro* synthesized CPSL (CPSL<sub>iv</sub>) at 214 nm. Products were separated using an elution gradient consisting of a 2-μl curved gradient from 0 to 30% M2 over 4 min followed by a linear gradient from 30 to 84% M2 over 33 min. Hydrolytic activity of MBP-CslA-His<sub>6</sub> was analyzed in the presence of both 10 mM MnCl<sub>2</sub> (Fig. 4B, 37 °C, overnight incubation) and 10 mM MgCl<sub>2</sub> (Fig. 4, C–E, 37 °C, 45-min incubation) because Mg<sup>2+</sup> is known to promote Stealth activity (30, 31, 47) and was found to stabilize UDP-Glc and UDP-Gal. In general, all enzymes as well as all other reactants were used in the same concentrations as in the radioactive incorporation assay described above.

All of the <sup>1</sup>H and <sup>31</sup>P NMR experiments were recorded as described previously (31). To detect the <sup>13</sup>C chemical shifts (Table 3), a <sup>1</sup>H, <sup>13</sup>C heteronuclear multiple quantum coherence experiment was recorded on a BrukerAvance III 400-MHz spectrometer equipped with a 5-mm broadband probe (Bruker). <sup>13</sup>C signals were referenced to the free acetate peak at 22.37 ppm.

**Upscaling the *in Vitro* Synthesis of CPSL and Purification of Synthesized Polymer**—For synthesis of CPSL<sub>iv</sub>, 3 nmol of ΔN37-CslB-His<sub>6</sub> were incubated overnight at 37 °C in reaction buffer (20 mM Tris, pH 8.0, 8 mM MnCl<sub>2</sub>) with 15 mM UDP-GlcNac in a total volume of 2 ml. CPSL<sub>iv</sub> was purified by AEC using a MonoQ HR5/5 column (GE Healthcare) and a linear NaCl gradient starting from 0–1 M NaCl at a flow rate of 1 ml/min. CPSL<sub>iv</sub>-containing fractions eluting at 460 mM NaCl were pooled, dialyzed against water (ZelluTrans, Roth, 1,000 molecular weight cut-off), and freeze-dried before further analysis.

**Acid Hydrolysis and Dephosphorylation of CPSL<sub>iv</sub>**—To obtain oligosaccharides derived from CPSL<sub>iv</sub> (oligoCPSL<sub>iv</sub>) for priming the CslB reaction, 7 mg of CPSL<sub>iv</sub> were hydrolyzed in 50 mM acetic buffer (pH 4.2) in a total volume of 1 ml for up to 6 h at 80 °C. The hydrolysis reaction was stopped by neutraliza-
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FIGURE 1. Schematic representation of the capsule structure and capsule gene cluster of N. meningitidis serogroup L. A, chemical structure of the trimeric CPSL repeating unit consisting of two GlcNAc residues in β1,3-glycosidic linkage followed by a phosphodiester-linked GlcNAc in α-configuration. B, the capsule gene cluster cps of N. meningitidis serogroup L is subdivided into six regions designated regions A–D, D′, and E (adapted from Ref. 7). Genes in regions B and C are conserved among group 2 capsule-expressing bacteria and encode proteins necessary for the assembly and export of the polysaccharide capsule to the bacterial surface. Proteins encoded in region A participate in capsular polysaccharide biosynthesis and are serogroup-specific. The three open reading frames identified in region A of N. meningitidis serogroup L were predicted to encode a δ-hexose-1-phosphate transferase (cslA), a bifunctional glycosyltransferase (cslB), and an acetyltransferase (cslC).

FIGURE 2. Production of recombinant enzymes. A, immunoblot analysis of CslB and CslA constructs using an antibody directed against the C-terminal His6 tag. Full-length MBP-CslA-His6 (86 kDa) could be highly enriched but was accompanied by a 37-kDa N-terminal degradation product. Full-length MBP-CslB-His6 (146 kDa) could only be detected in the insoluble fraction. However, the soluble degradation product ∆N-CslB-His6 α (indicated by white arrows) could be purified and was identified as a mixture of ∆N29 and ∆N37 truncations with theoretical molecular masses of 101 and 100 kDa, respectively. The corresponding N-terminal truncations (∆N29- and ∆N37-CslB-His6) were generated and analyzed together with purified MBP-CslA-His6, on a Coomassie-stained SDS-polyacrylamide gel (B).

Results

Cloning, Expression, and Purification of CslA and CslB with Different Epitope Tags—In N. meningitidis, the genetic information for proteins responsible for CPS synthesis, modification, and transport to the surface is encoded in the highly conserved chromosomal locus cps (capsular polysaccharide synthesis). The cps is subdivided into six regions designated A–D, D′, and E (Fig. 1B). Genes clustered in regions B and C are highly conserved in sequence and architecture even among different species of capsule group 2-expressing bacteria and encode enzymes involved in CPS chain initiation and transport (6, 7, 48). In contrast, genes forming the operon called region A are responsible for the serogroup-specific capsule biosynthesis and therefore unique for the respective serogroup. The three ORFs identified in region A of N. meningitidis serogroup L (Fig. 1B) were predicted to encode a δ-hexose-1-phosphate transferase (cslA), a bifunctional glycosyltransferase (cslB), and an acetyltransferase (cslC) (7).

To characterize the enzymes that synthesize the CPSL repeating unit consisting of (→3)-β-D-GlcNAc-(1→3)-β-D-GlcNAc-(1→3)-α-D-GlcNAc-(1→OPO3)α, cslA and cslB were amplified via PCR and subcloned into a vector that drives the expression of N-terminally MBP-tagged and C-terminally His6-tagged proteins (MBP-CslA-His6 and MBP-CslB-His6) and was successfully used to express other CPS (31, 32, 38). From 500-ml transformed E. coli M15[pREP4] expression cultures, 15 mg of MBP-CslA-His6 and 0.6 mg of MBP-CslB-His6 were obtained after immobilized metal ion affinity chromatography and size exclusion chromatography. The Western blot carried out with an anti-His6 tag antibody demonstrated that full-length MBP-CslA-His6 (86 kDa) was enriched together with a 37-kDa N-terminal degradation product (Fig. 2A). Protein purification from expression cultures transformed with plasmid pMBP-cslB-His6 (tac) did not contain full-length MBP-
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we used an HPLC-AEC assay that allows the recording of nucleotide sugar consumption and product formation (30). In a total volume of 75 μl, 0.15 nmol of ΔN37-CslB-His₆ were incubated with 4 mM UDP-GlcNAc. Products and educts were monitored immediately after the start of the reaction (time point 0) and after 75 min and 210 min (Fig. 4A). HPLC-AEC separation coupled to UV absorbance detection at 280 nm demonstrated the consumption of the donor sugar UDP-GlcNAc and the simultaneous production of UMP and UDP (Fig. 4A, left). In the profiles recorded at 214 nm, the appearance of a negatively charged polymer strongly suggested that ΔN37-CslB-His₆ was sufficient to synthesize CPSLᵣ. Moreover, the enzyme was capable of initiating the polymerization reaction de novo (Fig. 4A). Testing of MBP-CsLA-His₆ under the same experimental conditions did not produce any product peak in the 214-nm channel even after an overnight reaction (Fig. 4B). However, the profile obtained in the 280-nm channel demonstrated the formation of UMP at the expense of UDP-GlcNAc (Fig. 4B), whereas UDP-GlcNAc was stable if incubated in the absence of MBP-CsLA-His₆. Because enzyme-facilitated hydrolysis of donor substrates has been described for many glycosyltransferases that were tested without or with poor acceptors (49), the production of UMP indicated that MBP-CsLA-His₆ is an active enzyme and that UDP-GlcNAc is the correct donor substrate. To confirm this assumption, the experiment was repeated by including UDP-Gal and UDP-Glc as controls (Fig. 4, C–E). Besides naturally occurring hydrolysis of UDP-Gal and UDP-Glc, which was also observable in the absence of MBP-CsLA-His₆ (Fig. 4, C and D), enzyme-facilitated hydrolysis was only detected when MBP-CsLA-His₆ was incubated with UDP-GlcNAc, emphasizing that this sugar nucleotide is the MBP-CsLA-His₆ substrate.

CPSLᵣ Synthesized by ΔN37-CslB-His₆ Is Identical to CPSL Isolated from Natural Source (CPSLᵣ)—We used NMR spectroscopy to confirm that the polymer synthesized by ΔN37-CslB-His₆ in vitro (CPSLᵣ) was identical to CPSL from a natural source (CPSLᵣ) as described by Jennings et al. (42). The production of CPSLᵣ was up-scaled, and synthesized polymer was purified by preparative AEC (Fig. 5A). Samples were treated with CIP before loading onto the column to reduce the size of the nucleotide peaks (UDP and UMP) so that CPSLᵣ could be well separated from other reaction products using an NaCl gradient (Fig. 5A). Still interrogating whether the presence of CslA might influence the quality of the product synthesized by CslB, a second reaction was carried out in the presence of both MBP-CsLA-His₆ and ΔN37-CslB-His₆. NMR spectra obtained with the two polymer samples demonstrated identical signals in both ¹H NMR (Fig. 5D; the two spectra are shown) and ³¹P NMR (Fig. 5C; only the product of ΔN37-CslB-His₆ is shown) profiles and confirm that, under in vitro reaction conditions, MBP-CsLA-His₆ has no influence on the function of ΔN37-CslB-His₆. In detail, the ¹H NMR shows a doublet of doublets at δ 5.40 ppm with coupling constants consistent with an anomeric phosphate linkage in the α-d-configuration (Fig. 5, B and C, and Table 2). Two doublets at δ 4.64 and 4.58 ppm with comparably large coupling constants (Fig. 5C and Table 2) indicated the presence of two GlcNAc residues in β-configuration. The bidimensional ¹H,³¹P heteronuclear multiple bond corre-
lation NMR spectrum confirmed the presence of a 1→3 phosphodiester linkage between the constituents A and C (Fig. 5B). 1H, 13C heteronuclear multiple quantum coherence allowed us to assign the 13C spectrum of the polymer (Table 3). All of the 1H, 31P, and 13C NMR profiles, even if collected at different temperatures and frequencies, are in full congruence with the data reported by Jennings et al. (42) for CPSL

Taken together these data demonstrating identity between CPSL

provide unequivocal evidence that /H9004N37-CslB-His6 is sufficient to synthesize the complex trimeric repeating unit found in CPSL

Oligosaccharides Derived from CPSL

Prime the /H9004N37-CslB-His6 Reaction by Being Elongated at the Non-reducing End—Chain elongation catalyzed by group 2 CPs proceeds at the non-reducing end and usually needs an acceptor to be initiated (6). However, self-priming, as demonstrated for CslB in the current study, has been observed for various other CPs and is considered a coincidental event in which a donor sugar is elongated as acceptor (30, 31, 50, 51). Both the hexose-1-phosphate transferases from N. meningitidis (30, 31) and the glycosaminoglycan CPS synthases from Pasteurella multocida (50) have been reported to assemble longer chains in the absence than in the presence of acceptors. Priming of enzymes with acceptors allows more elongation reactions to take place at the same time, eventually leading to larger populations of shorter chains. To test whether the product length synthesized by ΔN37-CslB-His6 can be influenced by the addition of oligosaccharide primers, the CPSL

fraction characterized by NMR (see Fig. 5) was subjected to mild acid hydrolysis to obtain oligosaccharide primers (see “Experimental Procedures”). As shown in Fig. 6A, CPSL

chains were progressively converted into smaller split products. The oligosaccharide pool (oligoCPSL

obtained after 360 min was utilized for further experiments. Because mild acid hydrolysis is known to selectively cleave the phosphoester bond at the anomeric carbon (52), it was likely that the obtained oligoCPSL

were capped with a stable phosphomonoester at the non-reducing end. Therefore, one half of the oligoCPSL

fraction was treated with CIP to free the non-reducing end (CIP-oligoCPSL

Corresponding to the experiment displayed in Fig. 4, a ΔN37-CslB-His6 reaction was performed in the absence of any acceptor and in the presence of either oligoCPSL

chains were capped with a stable phosphomonoester at the non-reducing end. Therefore, one half of the oligoCPSL

fraction was treated with CIP to free the non-reducing end (CIP-oligoCPSL

After overnight incubation, reaction products were separated by HPLC-AEC and detected in the 214-nm channel. The product profiles obtained in the absence of acceptor (self-priming) and the reaction primed with oligoCPSL

showed reduced retention times corresponding to reduced polymer lengths. Together, these data demonstrate that extension of priming oligomers by ΔN37-CslB-His6 proceeds via free non-reducing ends.

-Fold Recognition Tools Predict Two Glycosyltransferase Domains in CslB—Using the structure prediction tool PHYRE2 (53), two separate glycosyltransferase (GT) domains were pre-
dicted in the CslB primary sequence: an N-terminal GT-A fold and a C-terminal GT-B fold. The GT-A fold was modeled with 100% confidence onto 13 different glycosyltransferases. We chose the homology model that was based on the glucuronic acid transferase domain of the CP K4CP from *E. coli* K4, an enzyme that synthesizes a chondroitin-like CPS, for further analyses (54). The C-terminal domain of CslB was modeled with 100% confidence (18% sequence identity with amino acid residues 460–877 of CslB) onto TagF, a teichoic acid polymerase from *Staphylococcus epidermidis* that uses CMP-glycerolphosphate as a donor substrate (55).

Because amino acid residues with critical catalytic functions have been described for both template proteins, we next used sequence analysis tools to identify correlating positions in CslB. The GT-A-specific DXD motif (56) could be allocated to the amino acid residues Asp138-Pro139-Asp140 in CslB, and two conserved histidine residues that characterize the GT-B folded TagF protein-like family (57) coincide with His595 and His733 in the CslB sequence (see Fig. 7, A–C). The conserved positions were mutated to alanine to give the double mutant CslBD138A/D140A and the single mutants CslBH595A and CslBH733A. Mutant constructs were expressed in *E. coli*, and recombinant proteins purified at levels comparable with wild type (0.6–0.8 mg/500 ml of culture). However, when activity was controlled in the HPLC-based system, all mutant proteins were found to be inactive (Fig. 7D). Supposing that each mutant should still contain one functional domain, we next performed trans-complemen-
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TABLE 3

| Constituent | C1  | C2  | C3  | C4  | C5  | C6  | CH3 (NHOOCCH3) |
|-------------|-----|-----|-----|-----|-----|-----|----------------|
| A           | 93.7| 52.9| 78.0| 68.4| 72.5| 60.4| 22.2           |
| B           | 100.6| 54.4| 78.1| 68.4| 75.0| 60.4| 22.4           |
| C           | 100.9| 54.4| 78.0| 68.7| 75.2| 60.4| 22.4           |

Discussion

To reconstruct the synthesis of the complex N. meningitidis serogroup L capsular polysaccharide in vitro, we cloned and expressed the two putative CPs, CslA and CslB, encoded in region A of the capsule biosynthesis locus of N. meningitidis serogroup L. Functional testing and the detailed product analysis by one- and two-dimensional NMR provided unequivocal evidence that CslB is sufficient to synthesize nature-identical CPSL with only UDP-GlcNAc as donor substrate. This ability to start the polymerization reaction de novo is not uncommon among CPs and has previously been described for GAG CPS synthases and the CPs of N. meningitidis serogroups A and X (30, 31, 50, 51). Although facilitated hydrolysis of UDP-GlcNAc, indicated by the release of UMP, was observed in the presence of the Stealth family member CslA and argued for a hexose-1-phosphate transferase activity, no explicit function could be attributed to CslA in the course of this study. Bioinformatics analyses applied to CslB identified an enzyme with two different GT-folds, an N-terminal GT-A and a C-terminal GT-B domain, that are separated by a long amino acid stretch (residues 270–459). CPs with two GT domains have been described previously (6), but CslB is, to the best of our knowledge, unique among the CPs of group 2 capsule-expressing bacteria in that two different GT domains are combined in one polypeptide chain, and a trisaccharide repeating unit is formed that comprises two β-glycosidic linkages and one α-phosphodiester bond.

Other two-domain CPs consist of two equally folded domains, like the GT-A folded GAG CPS synthases isolated from P. multocida (58, 59) and E. coli K4 (54) or the GT-B folded CPs from N. meningitidis serogroups W and Y (39). All of these earlier described enzymes assemble CPSs consisting of dimeric repeating units that only contain glycosidic linkages, and they use two different nucleotide sugars as donors. Nevertheless, a GT domain organization similar to the one identified in CslB has been predicted for members of the TagF polymerase family, including TagF from S. epidermidis (55), the template on which the C-terminal domain of CslB was modeled. In contrast to CslB, where attempts to separately express the two GT domains were yet unsuccessful (data not shown), the removal of the N-terminal TagF GT-A domain (that is hypothesized to be involved in teichoic acid decoration) released an active teichoic acid polymerase (55).
Superimposing the predicted CslB GT domains with the template structures revealed that amino acid residues known to have catalytic function in the template enzymes were also present in CslB. The amino acid stretch Asp^{138}-Pro^{139}-Asp^{140} in the N-terminal GT-A domain modeled perfectly onto the DXD motif of the glucoronic acid transferase domain of K4CP (54). The well defined and highly conserved DXD motif in GT-A folded glycosyltransferases interacts with the phosphate groups of the nucleotide donor through the coordination of a divalent cation (usually Mn^{2+}) (54, 56). Single point mutations of the two aspartate residues to alanine (D138A and D140A; data not shown) as well as simultaneous mutation of both residues abolished enzymatic activity. The assumption that Asp^{138}-Pro^{139}-Asp^{140} represents the DXD motif in CslB is corroborated by the finding that CslB requires Mn^{2+} for activity. Two histidine residues were shown to be conserved in the TagF family (57). In TagF from S. epidermidis, His^{444} acts as an active base deprotonating the hydroxyl group of the glycerol acceptor at C-1, whereas His^{584} is involved in coordinating the pyrophosphate of the donor substrate (55). Both positions superimpose with histidine residues of the CslB homology model (His^{595} and His^{733}), and mutation of either histidine to alanine abolished CslB activity, thus suggesting equivalent functions.

Lethal point mutations introduced into one domain of CslB did not turn CslB into a single-action transferase, as it was for instance shown for PmiHAS and the two-domain CPs of N. meningitidis serogroups W and Y (39, 60), but completely inactivated the enzyme. Similar to K4CP, in which a mutation...
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in the DxD motif of the C-terminal active site influences binding of UDP-GalNAc to the N-terminal DxD site (61), the destruction of an active site in CslB may influence the functionality of the second, indicating complex interdomain dependencies. Importantly, CslB activity could be recovered when point mutants were tested in trans-complementation experiments. Although the significantly lower activity observed under these conditions indicates suboptimal interactions between domains, the data clearly exclude functional destruction of the second domain due to lethal mutations in the first. The two GT domains in CslB are connected by a linker comprising amino acid residues 270–459. It is most likely that this region, for which no homology could be found using PHYRE2, participates in the catalytic activity. A similar region was identified in the CPs of N. meningitidis serogroups W and Y and shown to contribute to the formation of the functionally active sialyltransferase domain (36, 39). To precisely annotate functions to each domain in CslB, more studies are needed that require the availability of suited, preferably fluorescently labeled acceptors to enable the recording of single transfer reactions.

Based on the reactions catalyzed by the structural templates, it seems reasonable to speculate that the N-terminal GT-A domain of CslB, like the GT-A folded C-terminal domain of K4CP (54), catalyzes the formation of the glycosidic linkages with inverting stereochemistry, whereas the C-terminal GT-B fold, similar to Tagf from S. epidermidis (55), catalyzes the formation of the phosphodiester bond. This allocation of functions first of all highlights that Stealth is not the only enzyme family involved in hexose-1-phosphate transfer reactions in the process of neisserial capsule biosynthesis and, second, suggests that the number of domains correlates with the number of linkage types. In this scenario, the GT-A domain of CslB would catalyze two consecutive GlcNAc transfers before the GT-B fold catalyzes the GlcNAc-1-phosphate transfer. A comparable sequence of enzymatic steps has been described for WbdA<sup>O9a</sup>, the polymerizing mannosyltransferase assembling the E. coli O9a antigen (62). WbdA<sup>O9a</sup> consists of two GT-B domains, and, during the synthesis of the tetrameric repeating unit, each domain is responsible for two consecutive mannose transfers in either α-(1→2)- or α-(1→3)-linkage, respectively (63, 64).

An open question still remains concerning the function of CslA. As discussed before, the recombinant enzyme facilitates the hydrolysis of UDP-GlcNAc to GlcNAc 1-phosphate and UMP. Because this behavior suggests a functional hexose-1-phosphate transferase (31, 49), one may speculate that CslA functions as an initiating glycosyltransferase that transfers the phosphate transferase (31, 49), one may speculate that CslA-like enzymes play an important role in CPS biosynthesis. We will extend our studies to these strains to gain a better understanding of the underlying functional mechanisms.

Taken together, with N. meningitidis serogroup L as a model organism, we have identified a CP (CslB) exhibiting so far unprecedented architecture and function. Moreover, we provide initial evidence that the combination of CslA/CslB and thus the genomic organization of the N. meningitidis serogroup L cps region A is preserved in a larger group of Gram-negative bacteria.

Author Contributions—F. T. and G.-S. R designed the study. L. C. was responsible for cloning, purification, and enzymatic experiments. F. B., M. R. R., and V. P. performed NMR studies and analyzed the data. C. H. and V. U. provided bacterial lysates and reagents and helped with data interpretation. F. T., L. C., and G.-S. R. analyzed the data and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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