Molecular Cloning and Functional Characterization of Components of the Capsule Biosynthesis Complex of Neisseria meningitidis Serogroup A

TOWARD IN VITRO VACCINE PRODUCTION

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Background: The isolation of capsular polysaccharides from pathogenic bacteria for vaccine production is cost-intensive. Results: We describe the cloning, recombinant expression, and functional characterization of three enzymes from Neisseria meningitidis serogroup A that facilitate in vitro synthesis of the capsule polymer. Conclusion: The study presents a novel basis for efficient vaccine production. Significance: Economic vaccine production is prerequisite to combat meningococcal diseases.

The human pathogen Neisseria meningitidis (Nm) is a leading cause of bacterial meningitis and sepsis globally. A major virulence factor of Nm is the capsular polysaccharide (CPS), which in Nm serogroup A consists of N-acetyl-mannosamine-1-phosphate units linked together by phosphodiester linkages (–6)-α-D-ManNAc-(1→OPO₃⁻)ₙ. Acetylation in O-3 (to a minor extent in O-4) position results in immunologically active polymer. In the capsule gene cluster (cps) of Nm, region A contains the genetic information for CPSA biosynthesis. Thereby the open reading frames csaa, -b, and -c are thought to encode the UDP-N-acetyl-d-glucosamine-2-epimerase, poly-ManNAc-1-phosphate-transferase, and O-acetyltansferase, respectively. With the aim to use a minimal number of recombinant enzymes to produce immunologically active CPSA, we cloned the genes csaa, csab, and csac and functionally characterized the purified recombinant proteins. If recombinant CsaA and CsaB were combined in one reaction tube, priming CPSA-oligosaccharides were efficiently elongated with UDP-GlcNAc as the donor substrate, confirming that CsaA is the functional UDP-N-acetyl-d-glucosamine-2-epimerase and CsaB the functional poly-ManNAc-1-phosphate-transferase. Subsequently, CsaB was shown to transfer ManNAc-1P onto O-6 of the non-reducing end sugar of priming oligosaccharides, to prefer non-O-acetylated over O-acetylated primers, and to efficiently elongate the dimer of ManNAc-1-phosphate. The in vitro synthesized CPSA was purified, O-acetylated with recombinant CsaC, and proven to be identical to the natural CPSA by 1H NMR, 31P NMR, and immunoblotting. If all three enzymes and their substrates were combined in a one-pot reaction, nature identical CPSA was obtained. These data provide the basis for the development of novel vaccine production protocols.

NmA is the major cause of meningococcal disease in the African meningitis belt. Besides seasonal epidemics that occur with almost annual frequency, NmA has been the cause of severe pandemics in the last century (1, 2). A major virulence factor of NmA is the negatively charged capsular polysaccharide (CPS). CPSA consists of N-acetyl-mannosamine-1-phosphate units linked by phosphodiester linkages to give the polymer (–6)-α-D-ManNAc-(1→OPO₃⁻)ₙ (3). Of note, the six most virulent NmA serogroups (NmA, -B, -C, -W, -Y, and -X) bear negative CPSs. Negative charge in CPSA and CPSX is due to the phosophodiester group, whereas negative charge in CPSB, -C, -W, and -Y results from the incorporation of sialic acid (2). These NmA CPSs are immunogenic (except CPSB, which is identical to polysialic acid in the human host) and cause the production of antibodies that are bactericidal in the presence of complement (1, 4). In fact, this early observation has made the use of polysaccharide-protein conjugates the gold standard in the development of vaccines against NmA strains. A number of mono- and tetravalent (the latter comprising serogroups A, C, W, and Y) conjugate vaccines against NmA have been licensed (5).

The abbreviations used are: NmA, Neisseria meningitidis serogroup A; AEC, anionic exchange chromatography; DP, degree of polymerization; avDP, averaged DP; CP, capsule polymerase; CPS, capsular polysaccharide; CPSA, CPS of NmA; CPS₅₋1₀, hydrolyzed CPSA; deP, dephosphorylated; CsaA, UDP-GlcNAc-2-epimerase; CsaB, poly-ManNAc-1-phosphate transferase; CsaC, O-acetyltransferase; 1P, 1-phosphate.
Crucial to the success of vaccination programs in the sub-Saharan meningitis belt is the provision of safe high quality vaccines. MenAfriVac/H23041, a conjugate vaccine with CPSA coupled to tetanus toxoid as carrier protein, has been specifically designed to address these needs (6). With a cost of under 50 cents per dose (7), mass vaccination campaigns were possible in Burkina Faso, Mali, and Niger and installed herd immunity (8–10) protecting not only vaccinated but also non-vaccinated individuals and young children (11).

Recent progress made with the cloning and functional expression of capsule polymerases (CPs) (12–15) and pioneering studies that demonstrate the suitability of recombinant enzymes for the in vitro production of CPs (16, 17) have opened new perspectives for the economic and safe production of conjugate vaccines. The goal of our study, therefore, was to isolate the minimal number of enzymes needed for in vitro synthesis of immunologically active CPSA and to pioneer protocols for the use of recombinant enzymes in CPSA production chains. As the sugar building block UDP-ManNAc is commercially not available, it was clear from the start that establishing a successful production chain requires the in situ synthesis of UDP-ManNAc from cheap UDP-GlcNAc. Moreover, as the immunogenicity of CPSA depends on O-acetylation (18), an O-acetyltransferase capable to perform this modification was necessary.

The chromosomal locus cps (for capsular polysaccharide synthesis) contains the genetic information for CPSA synthesis, modification, and surface transport. The locus is sub-structured into six regions: A–D, D’, and E (Fig. 1A). The sequences encoded in region A are serogroup-specific and encode among other things the polymerases responsible for CPS synthesis (19). Regions B and C are highly conserved and encode the proteins necessary for export and assembly of the polysaccharide on the cell surface. In NmA, region A comprises four open reading frames (ORF) csaA, -B, -C, and -D (previously designated sacA-D or mynA-D). Using insertion-mutagenesis Swartley et al. (20) demonstrated that each of these genes is involved in the production of the NmA capsule. In a later study the gene product encoded in csaC was shown to be an acetyltransferase with specificity for the O-3 and O-4 positions in ManNAc (21) (Fig. 1B). Based on their nucleotide and predicted amino acid sequence, csaA was presumed to encode a UDP-N-acetyl-D-glucosamine-2-epimerase and csaB to encode a capsule polymerase (20) (Fig. 1B). Additional evidence that the product of csaB is in fact the NmA-specific capsule polymerase arose from the demonstration that the protein is part of the stealth family comprising exclusively D-hexose-1-phosphate transferases (22).

Here we describe the molecular cloning of the genes csaA, csaB, and csaC from NmA, the production of recombinant proteins, and the characterization of their functional properties. Testing a series of synthetic primer compounds, a ManNAc dimer linked together by phosphodiester linkages and carrying a phosphodiester at the reducing end was found to be the minimal acceptor structure. This artificial primer as well as oligosaccharide primers isolated from natural sources was preferen-

FIGURE 1. The capsule biosynthesis gene cluster. A, schematic representation of the chromosomal locus (cps) of NmA. Products of genes forming region A are involved in the synthesis of the capsule polysaccharide and are serogroup-specific. For more information, see the Introduction (adapted from Harrison et al. (19)). B, reactions catalyzed by the gene products of csaA, csaB, and csaC (1). The putative UDP-N-acetyl-D-glucosamine-2-epimerase CsaA catalyzes the epimerization of UDP-GlcNAc to UDP-ManNAc (2). The putative capsule polymerase CsaB transfers ManNAc-1P from the resulting UDP-N-acetyl-mannosamine onto the non-reducing end of the growing CPSA (3). CsaC O-acetylates CPSA at O-3 or O-4 in the presence of the acetyl-donor acetyl-CoA.
The Capsule Biosynthesis Machinery of NmA

**TABLE 1**

| Primer pair | Resulting construct |
|-------------|---------------------|
| GGAGATCT| Strepl-I-CsaA-His6 |
| CGGCTGTG| Strepl-I-CsaB-His6 |
| GACGTCAC| Strepl-I-D69-CsaB-His6 |
| GCGGCCGC| Strepl-I-CsaC-His6 |
| GCTCGAG| Strepl-I-CsaD-His6 |

**EXPERIMENTAL PROCEDURES**

**General Cloning**—The genomic DNA isolated from NmA strain Z2491 was a kind gift from Dr. Heike Claus (Institute for Hygiene and Microbiology, University of Würzburg). The csaB sequence was codon-optimized for use in *Escherichia coli* BL21(DE3) using the Gene Designer software package (DNA 2.0) (23) and the codon frequency tables published by Welch et al. (24). The mean codon frequency for each amino acid was calculated from the codon frequency tables FreqA and FreqB (24), and the resulting codon frequency table was used as the template for the *in silico* generation of csaBco, csaBco flanked 5′ by a BamHI site and 3′ by a XhoI site was synthesized from Eurofins MWG Operon. All other csaC sequences described herein were amplified by polymerase chain reaction (PCR) using the primers shown in Table 1 and genomic DNA from NmA strain Z2491 or csaBco as template. PCR products were cloned via the restriction sites shown in Table 1 into the corresponding sites of the vector pET22b-Strep (25) driving the expression of recombinant proteins under the control of the T7 promoter. PCR products digested with BglII were cloned into the BamHI site of pET22b-Strep.

**Expression and Purification of Recombinant CsaA, CsaB, and CsaC**—Freshly transformed *E. coli* BL21(DE3) were grown at 15 °C in PowerBroth medium for 18 h. At an optical density of \( O_d_{600} = 1 \), protein expression was induced by the addition of 0.1 mM isopropyl-\( \beta \)-D-1-thiogalactopyranoside and allowed to proceed for a period of 20 h. In test expressions, 0.2 ml of culture-volume were pelleted with 16,000 \( \times \) g for 1 min. Cell pellets were lysed with 0.1 ml of lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1 mg/ml lysozyme). The lysis was intensified by 3 cycles of sonication (Branson sonifier 450, 100% amplitude) interrupted by 3 min of cooling on ice. Soluble and insoluble fractions were separated by centrifugation (16,000 \( \times \) g, 30 min, 4 °C), and the supernatant was mixed (1:1) with Laemmli buffer and used for PAGE as described below.

For protein purification, pellets from 125 ml of expression culture were pelleted by centrifugation (6000 \( \times \) g, 10 min, 4 °C). After a washing step with PBS, cells were resuspended in 7.5 ml of binding buffer (50 mM Tris, pH 8.0, 300 mM NaCl) supplemented with 40 \( \mu \)g/ml bestatin (Sigma), 1 \( \mu \)g/ml pepstatin (AppliChem), 100 \( \mu \)M PMSF (Stratagene) and sonicated (Branson Digital Sonifier, 50% amplitude, 8 \( \times \) 30 s, interrupted by cooling on ice). After centrifugation at 27,000 \( \times \) g for 30 min, the soluble fractions were directly loaded onto HisTrap columns (GE Healthcare) to enrich the recombinant proteins by immobilized metal ion affinity chromatography. Columns were washed with binding buffer (50 mM Tris, pH 8.0, 300 mM NaCl), and proteins were eluted in step gradients using 10, 30, 50, and 100% elution buffer (binding buffer containing 500 mM imidazole). Fractions containing recombinant protein were pooled, and the buffer was changed to storage buffer (50 mM Tris, pH 8.0, 50 mM NaCl for CsaA/CsaB; 50 mM Hepes, pH 7.05, 100 mM NaCl, 5 mM MgCl₂, and 1 mM EDTA for CsaC) using the HiPrep 26/10 desalting column (GE Healthcare). Isolated proteins were concentrated using Amicon Ultra centrifugal devices (Millipore 30 MWCO). After separation into aliquots, samples were snap-frozen in liquid nitrogen and stored at −80 °C.

**SDS-PAGE and Immunoblotting**—SDS-PAGE was performed under reducing conditions using 2.5% (v/v) \( \beta \)-mercaptoethanol and 1.5% (w/v) SDS. Proteins were stained using Roti-Blue (Carl Roth GmbH) according to the manufacturer's guidelines. For Western blot analysis samples and standard proteins were blotted onto PVDF membranes (Millipore). Histo-tagged proteins were detected with 0.5 \( \mu \)g/ml anti-penta-His antibody (Qiagen) and goat anti-mouse IR800 antibody (LI-COR) as second antibody. Second antibodies were used in a 1:20,000 dilution.

**Preparation of CPSA Oligosaccharides**—CPSA oligosaccharide samples with an averaged degree of polymerization (avDP) of 6 and 15, respectively, were generated by acidic hydrolysis of long CPSA chains isolated from bacterial cultures (CPSA\(_n\)). Solutions containing 2.5 mg/ml CPSA in sodium acetate buffer (50 mM sodium acetate, pH 4.8) were incubated at 73 °C for 6 h, and 2 pool fractions (avDP 6) and 15, respectively) were purified by anionic exchange chromatography (Q-Sepharose column, GE Healthcare) using a sodium chloride gradient. The avDP and the dispersion of saccharide chains was determined by \( ^{31} \)P NMR and high performance anionic exchange chromatography-pulsed amperometric detection (HPAEC-PAD) analysis following an established protocol (26). If used in enzymatic reactions, hydrolyzed CPSA (CPSA\(_{hyd}\)) was dephosphorylated.
Chemical Synthesis of CsaB Acceptors—A short summary of the synthesis of ManNAc and ManNAc derivatives as well as of ManNAc disaccharide units linked together by phosphodiester linkages is provided in the supplemental schemes S1 and S2. A manuscript describing the detailed chemical synthesis and the characterization of these compounds is under preparation.5

Activity Testing of CsaA/CsaB by Use of a Radioactive Assay System—CsaA/CsaB activity was analyzed using an adaptation of a radioactive incorporation assay previously described for the N-acetylglucosamine-1-phosphate transferase from NmX (17). Briefly, assays were carried out with 5 μl of the soluble fractions of bacterial lysates expressing either recombinant CsaB or CsaA (see Fig. 2C) or with purified and epitope-tagged proteins (112 pmol of StrepII-CsaA-His6; 88 pmol of CsaBco-His6) in a total volume of 25 μl of assay buffer (50 mM Tris pH 8.0 or various pH for determination of the pH optimum). Divalent cations were added from stock solutions. The reaction was primed with 5 ng of avDP15 and started by the addition of 0.05 μmol of UDP-GlcNAc (Calbiochem) containing 0.05 μCi of UDP-[14C]GlcNAc (American Radiolabeled Chemicals). Samples were incubated at 37 °C, and 5-μl aliquots were spotted onto Whatman 3MM Chr paper after 0, 5, 10, and 30 min. After descending paper chromatography, the chromatographically immobile 14C-labeled CPSA was quantified by scintillation counting.

Activity Testing of CsaA/CsaB by Use of a Multienzyme Spectrophotometric Assay—1.2 μM CsaA and 1 μM CsaB were assayed in the presence of 0.25 mM UDP-GlcNAc (Calbiochem), 20 mM MgCl2, and 50 mM Tris, pH 8.0, in a total volume of 100 μl. The consumption of UDP-GlcNAc was coupled to nicotinamide adenine dinucleotide (NADH) consumption using the following enzymes/substrates: 0.25 mM adenosine triphosphate (ATP, Roche Applied Science), 1 mM phosphoenolpyruvate (ABCR), 0.3 mM NADH (Roche Applied Science), 9–15 units/ml pyruvate kinase, 13.5–21 units/ml lactic dehydrogenase (PK/LDH mix Sigma), and 0.05 mg/ml nucleoside monophosphate kinase (Roche Applied Science). Absorption was measured at 340 nm every 10 s for 30 min using a Biotek EL 808 96-well plate reader.

Physicochemical Analysis of CPSAiv—To produce sufficient CPSA (CPSAiv) for PAGE and NMR analyses, 0.84 nmol (1.2 μM final) of StrepII-CsaA-His6, 88 pmol of Δ69CsaBco-His6 in a total volume of 25 μl of assay buffer (50 mM Tris pH 8.0 or various pH for determination of the pH optimum). Divalent cations were added from stock solutions. The reaction was primed with 5 ng of avDP15 and started by the addition of 0.05 μmol of UDP-GlcNAc (Calbiochem) containing 0.05 μCi of UDP-[14C]GlcNAc (American Radiolabeled Chemicals). Samples were incubated at 37 °C, and 5-μl aliquots were spotted onto Whatman 3MM Chr paper after 0, 5, 10, and 30 min. After descending paper chromatography, the chromatographically immobile 14C-labeled CPSA was quantified by scintillation counting.

5 D. V. Yashunsky, A. J. Black, and A. V. Nikolaev, manuscript in preparation.
The Capsule Biosynthesis Machinery of \( \text{NmA} \)

aration on high percentage (25%) PAGE and visualized by a combined Alcian blue/silver staining procedure (27).

The residual sample was freeze-dried, solubilized in 0.75 ml of deuterium oxide (\( \text{D}_2\text{O} \), 99.9% atom \( \text{D} \); Aldrich) to give a concentration of 0.5–1 mg/ml saccharide and used for product characterization by NMR. All the \( ^1\text{H} \) and \( ^{31}\text{P} \) NMR experiments were recorded as previously described (17).

HPLC-anion exchange chromatography (AEC) was performed on a Prominence UFLC-XR (Shimadzu) equipped with a CarboPac PA-100 column (\( 2 \times 250 \) mm, Dionex). Samples were separated as described by Keys et al. (28) with the minor adjustment that \( \text{H}_2\text{O} \) and 1 M NaCl were used as mobile phases M1 and M2, respectively. 5 \( \mu \)l of the samples were loaded for the detection of nucleotides at 280 nm and 50 \( \mu \)l for the detection of CPSA at 214 nm. Products were separated using an elution gradient consisting of a 2-curved gradient from 0 to 30% M2 over 4 min followed by a linear gradient from 30 to 84% M2 over 33 min. Enzyme concentrations were used as indicated in Fig. 6. All other reactants were used in the amounts described above.

\textbf{Analysis of 2-Acetamidoglycol—Assignments of \( ^1\text{H} \) NMR spectrum were in agreement with those reported in literature (29). \( 1\text{H} \) NMR (\( \text{D}_2\text{O} \), 400 MHz): \( \delta \) = 6.68 (d, 1 H, \( J_{1,2} \) 1.0, H-1), 4.25 (dd, H 1, \( J_{3,4} \) 6.5 Hz, H-3), 3.99 (dt, 1 H, \( J_{4,5} \) 8.4, \( J_{5,6a} \) = \( J_{5,6b} \) 4.2 Hz, 6.5 Hz, H-5), 3.86 (d, 2 H, H-6), 3.77 (dd, 1 H, H-4), 2.05 (s, 3 H, CH\textsubscript{3}CO). Significant signals from \( ^{13}\text{C} \) NMR (\( \text{D}_2\text{O} \), 100 MHz): \( \delta \) = 141.47 (C-1), 78.70 (C-5), 68.70 (C-3), 68.36 (C-4), 59.84 (C-6), 21.84 (2 \( \times \) CH\textsubscript{3}CO).

\textbf{In Vitro Synthesis, Purification, and Immunological Analysis of CPSA\textsubscript{v} and CPSA\textsubscript{v(OAc)}—To generate CPSA\textsubscript{v}, 10 nmol of CsaA and 16 nmol of CsaB were incubated overnight at 37 \( ^\circ \)C in reaction buffer (50 mM Tris, pH 8.0, 20 mM MgCl\textsubscript{2}) with 10 mM UDP-GlcNAc in a total volume of 9 ml. The reaction was primed with 1 \( \mu \)g of CPSA\textsubscript{v(OAc)}-dep of avDP6. Acetylation of 1 mg of CPSA\textsubscript{v} was performed for 4 h at 37 \( ^\circ \)C in the presence of 1.2 nmol of CsaC and 14 mM acetyl-CoA (Sigma) in a total volume of 0.5 ml of acetylation buffer (25 mM Tris, pH 7.5, 50 mM NaCl). Both CPSA\textsubscript{v} and CPSA\textsubscript{v(OAc)} were purified via AEC using a Mono Q HRESS/5 column (GE Healthcare) at a flow rate of 1 ml/min and a linear sodium chloride gradient. CPS containing fractions eluting at 540 mM NaCl were pooled, dialyzed (ZelluTrans, Roth, 1 kDa MWCO) against water, and freeze-dried for further analysis. For dot blot analyses, small aliquots of the purified CPSA\textsubscript{v} and CPSA\textsubscript{v(OAc)} were spotted onto nitrocellulose (Whatman) and incubated with mAb 932 specifically directed against CPSA\textsubscript{v(OAc)} (mAb 935 was generated in the lab of Prof. Dr. D. Bitter-Suermann, Hannover Medical School, Institute for Medical Microbiology, and was kindly provided for this study) in a 1:10,000 dilution. Dot blots were developed with goat anti-mouse IR800 antibody (LI-COR) in a 1:20,000 dilution.

\textbf{RESULTS}

\textbf{Cloning and Expression of csaA, csaB, and csaC; Production of Recombinant Proteins—}Because previous analyses carried out on these genes provided strong evidence that csaA, csaB and csaC encode the UDP-GlcNac-epimerase, the poly-ManNAc-1-phosphate-transferase (20), and the O-acetyltransferase (21), respectively, primers were constructed (see “Experimental Procedures”) to amplify these ORFs. The genomic DNA isolated from \( \text{Nm} \) strain Z2491 was used as a template. Obtained PCR products were cloned into the pET22b-Strep vector (25), allowing the expression of recombinant proteins with N-terminal StrepII- and/or C-terminal His\textsubscript{\textasteriskcentered} tag. After transformation into BL21(DE3) and induction of protein expression (see “Experimental Procedures”), the distribution of recombinant proteins between the soluble (s) and insoluble (i) fraction of bacterial lysates was analyzed by Western blotting against the affinity tags. The recombinant epitope tagged forms of CsaA and CsaC appeared mostly in the soluble fraction and could be purified directly from the bacterial lysates. CsaA was purified by immobilized metal ion affinity chromatography followed by a desalting step and yielded 40 mg of protein/liter expression culture. Although some additional faint bands were visible in Coomassie-stained SDS-PAGE (Fig. 2A), a protein fraction highly enriched in CsaA was obtained. CsaC was purified following the protocol described by Gudlavalleti et al. (21) and yielded 96 mg of homogenously pure protein/liter of culture (Fig. 2A).

Similarly, StrepII-CsaA-His\textsubscript{\textasteriskcentered} encoding the putative poly-ManNAc-1-phosphate-transferase, was well expressed, and the excess of the construct appeared in the soluble fraction. However, the major product revealed with the anti-penta-His antibody in Western blot migrated with an apparent molecular mass of 50 kDa, strongly deviating from the calculated molecular mass of 67 kDa (Fig. 2B, left lanes). Because faint signals with molecular masses of >50 kDa were additionally displayed with the anti-penta-His antibody, we concluded that StrepII-CsaA-His\textsubscript{\textasteriskcentered} is either prone to N-terminal degradation or translated from an alternative start codon. Consequently, we investigated the \( \text{NmA} \) genome with bioinformatics techniques. Indeed, two of the used gene prediction softwares (GenMark and GeMarkS; Refs. 30 and 31) retrieved an additional ATG (starting with position 183528 of the \( \text{NmA} \) genome (NC_003116.1)). In PRODIGAL (32), the prediction for this second start codon was comparable with the published start codon (base no. 183321; Ref. 33).

To investigate if translation from the alternative ATG leads to a stable protein, the corresponding truncation \( \Delta 69\text{CsaB} \) was cloned with (StrepII-\( \Delta 69\text{CsaB}-\text{His} \)) and without (\( \Delta 69\text{CsaB}-\text{His} \)) the N-terminal degradation or translated from an alternative start codon. Consequently, we investigated the \( \text{NmA} \) genome with bioinformatics techniques. Indeed, two of the used gene prediction softwares (GenMark and GeMarkS; Refs. 30 and 31) retrieved an additional ATG (starting with position 183528 of the \( \text{NmA} \) genome (NC_003116.1)). In PRODIGAL (32), the prediction for this second start codon was comparable with the published start codon (base no. 183321; Ref. 33).

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Consecutively, enzymatic activity within the soluble fractions of the bacterial lysates was determined with a radioactive incorporation assay previously developed for the poly-GlcNAc-1-phosphate-transferase from NmX (17). The fractions containing the recombinant CsaB variants were tested in the presence of CsaA and UDP-[14C]GlcNAc. In accordance with the levels of expressed protein (Fig. 2B), StrepII-CsaB-His6 and Δ69-CsaBco-His6 showed identical activity profiles (Fig. 2C). Based on these results the protein variant Δ69-CsaBco-His6 was chosen for further experiments. The protein was purified from the soluble fraction of transformed BL21(DE3) by immobilized metal ion affinity chromatography and size exclusion chromatography, yielding 60 mg of highly pure protein from 1 liter of bacterial culture (Fig. 2D).

Optimization of Test Conditions and Characterization of CsaB Substrates—As the donor sugar (UDP-ManNAc) used by CsaB must be produced in situ in the epimerase reaction catalyzed by CsaA, the optimization of test conditions needed the presence of both enzymes. As for CpsPs of other NmA strains, a hydrolysate of CPSA (CPSAhyd) was used to prime the reaction in the presence of CsaA and its substrate UDP-14C]GlcNAc.

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to remove this group. Independent of the size of the primers used to start the reaction, the native acetylated oligomers (CPSA_{hyd(OAc)} of avDP6 and avDP15) were found to be poor acceptors, but activity increased considerably after removal of acetyl groups and even further after release of the capping phosphate residue, making CPSA_{hyd(deOAc)-deP} the most efficient acceptors. The size of the priming polymers was not significant for enzymatic activity (Fig. 4; compare avDP6 and avDP15). The obtained results allowed the conclusion that the chain elongation by CsaB proceeds via the non-reducing end and by transfer of ManNAc-1P onto C6-OH groups. Furthermore, because CPSA_{hyd(deOAc)} was a better acceptor than CPSA_{hyd(OAc)}, it is likely that O-acetylation takes place after polymer synthesis.

Because no information on the minimal length of the priming acceptor for CsaB could be derived from the CPSA_{hyd} fractions, we used well characterized synthetic compounds to interrogate this question. The compounds synthesized are shown in Fig. 5 and varied not only in length but also with respect to O-acetylation (compounds 2, 4, 6 were 3-O-acetylated) and reducing end modifications (37–39). In compounds 1, 2, 5, and 6 the reducing ends were occupied by an n-decyl-phosphate ester, whereas a methyl group (OMe) was present in compounds 3 and 4. The Δ69-CsaB_{co-His6} activity did not go beyond background (no acceptor) with compounds 1-4 but, intriguingly, steeply increased with disaccharides carrying an n-decyl-phosphate ester at the reducing end (compounds 5 and 6) (Fig. 5). With the non-O-acetylated compound 5, activity values similar to those obtained with the optimized acceptor CPSA_{hyd(deOAc)-deP} were measured. In line with the above data (Fig. 4), O-acetylation of compound 5 (resulting in compound 6) reduced the quality of the acceptor. Based on these data, the minimal acceptor recognized by Δ69-CsaB_{co-His6} could be defined as the dimer of ManNAc units linked together by phosphodiester linkages. The presence of a phosphodiester at the reducing end seems obligatory, because compounds ending with OMe groups do not work as acceptor substrates.

FIGURE 5. Determination of the minimal CsaB acceptor. Derivatives of ManNAc ending at the reducing end with a methyl group (compounds 3, 4) or a phospho-n-decyl-ester (compounds 1, 2, 5, 6) were used to prime the Δ69-CsaB_{co-His6} reaction in the continuous spectrophotometric assay. The dimer of ManNAc-1P carrying a phosphodiester at the reducing end was identified as minimal acceptor. Importantly, similar to the natural oligosaccharides the acceptor quality dropped by roughly 20% if the synthetic acceptor was O-acetylated (compare compounds 5 and 6).
**In Vitro Synthesis of CPSA Chains**—To analyze if long CPSA chains can be produced with the recombinant enzymes, test reactions were carried out in the presence of StrepII-CsaA-His6, H9004 69-CsaBco-His6, and CPSAhyd(deOAc)-deP of avDP6 as the priming oligosaccharide. Reactions were started by the addition of UDP-GlcNAc. Control reactions (Fig. 6) showed that long CPSA chains were produced in the presence of all reactants (reaction) and, although in small amounts, also in control 1 where no priming oligosaccharides were added. The production of long CPSA chains in control 1 argues for the capacity of CsaB to start the polymerization de novo.

**FIGURE 6. In vitro synthesis of CPSA.** A, products synthesized in the CsaA/CsaB reaction in the presence of UDP-GlcNAc and CPSA_{hyd(deOAc)} of avDP6 were analyzed by high percentage PAGE and a combined Alcian blue/silver staining. Long chains were produced in the presence of all reactants (reaction) and, although in small amounts, also in control 1 where no priming oligosaccharides were added. The production of long CPSA chains in control 1 argues for the capacity of CsaB to start the polymerization de novo. B, 31P NMR analyses carried out with the reaction, and controls 1 and 4 show signals characteristic for the phosphodiester linkages of CPSA and byproducts of the reaction. C, the 1H NMR analysis of control 4 demonstrates that CsaA catalyzes the UDP-GlcNAc/UDP-ManNAc epimerization via the intermediate 2-acetamidoglucal. D, HPLC analysis of reaction products obtained with variant CsaA:CsaB ratios, as indicated. This experiment clearly showed that UDP formation is prevented if the CsaB concentration is equal to or higher than the concentration of CsaA. UMP, UDP, and UDP-GlcNAc/UDP-ManNAc were detected at 280 nm and CPSA at 214 nm.

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mers were purified by AEC using a protocol similar to the one
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CP of CsaA concentrations (50 nM, see “Experimental Procedures”).

reaction
signals were most prominent in control 4 (Fig. 6
StrepII-CsaA-His6 and UDP-GlcNAc present, we speculated
in perfect agreement with published data (29), represented the
NMR analysis carried out with control 4 revealed a signal that,
in perfect agreement with published data (29), represented the
anomeric proton of 2-acetamidoglucal (Fig. 6
13C,1H heteronuclear multiple quantum coherence (see “Experimental
procedures”) NMR analyses allowed us for the first time to assign a
13C spectrum of this intermediate. The complete absence of
signals for the anomeric proton of ManNAc (both α and β)
confirmed that UDP is an intermediate of the CsaA reaction
and not produced by hydrolysis of UDP-ManNAc.

Another interesting observation was the de novo start of
CPSA chains by Δ69-CsaB
, His6 if incubated with equimolar
CsaA concentrations (50 nm, see “Experimental Procedures”).
The in vitro produced CPSA chains (CPSAα) could be doubtless
identified with both PAGE and 31P NMR (Fig. 6 B), with only
StrepII-CsaA-His6 and UDP-GlcNAc present, we speculated
that UDP is a side product of the epimerase reaction. A 1H
NMR analysis carried out with control 4 revealed a signal that,
in perfect agreement with published data (29), represented the
anomeric proton of 2-acetamidoglucal (Fig. 6 C).

To determine on the analytical scale if acetylated, bio-
identical polymer can be produced in a one-step reaction starting
with one substrate and just using the polymerase, we incubated
CsaB in the presence of 3-O-acetylated UDP-ManNAc (UDP-
3OAcManNAc) and analyzed the reaction products by PAGE
and 31P NMR. However, no product signals could be detected
by any of the methods (Fig. 8 A and B) and 31P NMR revealed
that UDP-3OAcManNAc was not used by CsaB.

Finally we explored if O-acetylated CPSA can be produced in
a one-pot reaction. Therefore, the reaction mixture containing
CsaA, CsaB and UDP-GlcNAc was supplemented with recom-
binant CsaC and acetyl-CoA. Moreover, control reactions with
single compounds missing (see Fig. 8 C and D) were carried out in
parallel. After overnight incubation, products were analyzed by
Alcian blue/silver-stained high percentage PAGE (Fig. 8 C)
and immunoblotting with mAb 932 (Fig. 8 D). In the presence of
all components, a product recognized by mAb 932 was pro-
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CsaA and CsaC being UDP-GlcNAc/UDP-ManNAc epimerase
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1, 4, and 6 (Fig. 8 C) indicated that suitable conditions were
established for all enzymes in the one-pot-reaction scheme.

DISCUSSION

Of all pathogenic Nm serogroups, NmA has caused the most
disastrous epidemics in sub-Saharan Africa. The prevalence of
this pathogen provoked an unprecedented endeavor to develop
a highly effective and economic vaccine, MenAfriVac® (7).
With costs of less than 50 cents per dose, MenAfriVac® enabled
mass vaccination campaigns in Burkina Faso, Mali, and Niger
(8–10), which installed herd immunity, leading to protection
not only for vaccinated but also for non-vaccinated individuals
and in particular of young children (11).

All NmA vaccines licensed today are glycoconjugate vaccines
coupled to carrier proteins, with CPSA polysaccharides isolated
from large scale NmA cultures or oligosaccharides having
shorter chain length obtained by acidic hydrolysis (40). To
avoid the significant cost and biohazard in association with
large scale NmA cultures and pyrogen-free production of poly-
saccharides, the enzyme-catalyzed in vitro synthesis of CPSA

1–4), in which components were omitted as indicated, were
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established for all enzymes in the one-pot-reaction scheme.
(CPSA_{iv}) would provide an attractive alternative. Toward this goal, we describe in this study the molecular cloning and functional expression of the three enzymes (UDP-GlcNAc-2-epimerase, CsaA; poly-ManNAc-1-phosphate-transferase, CsaB; O-acetyltransferase, CsaC) that are part of the capsular biosynthesis complex in \( \text{Nm}_{A} \) and represent the minimal number of enzymes needed to produce immunologically active CPSA_{iv}(OAc) in vitro starting from economic precursors. Using the well-characterized BL21(DE3) strain as expression host, C-terminal His\(_6\)-tagged and N-terminal StrepII-tagged versions of CsaA and CsaC could be purified in high quality and remarkable quantity (CsaA and CsaC, 40 and 96 mg/liter bacterial culture, respectively). In CsaB a second start codon was identified encoding methionine 70. In the overexpression system the use of this second start codon (construct \( \Delta69-S\text{CsaB-His}_{6} \)) generated a stable protein that could be purified in remarkable amounts (60 mg/liter bacterial culture) after its DNA sequence had been optimized for codon usage in BL21(DE3). Whether this second start codon is actually used in the natural environment remains an open question.

When the CsaA/CsaB reaction was carried out under suboptimal conditions (CsaA concentrations were higher than CsaB concentrations), UDP and 2-acetamidoglucal were formed as side products. A similar finding was made by Sala et al. (41),

**FIGURE 7. Purification and characterization of in vitro synthesized CPSA.**

A. \( \text{in vitro} \) synthesized CPSA (CPSA_{iv}) was separated from all contaminating reaction products using anion exchange chromatography with the indicated sodium chloride gradient. B. purified CPSA_{iv} after O-acetylation (CPSA_{iv}(OAc)) was re-purified under the same conditions resulting in a well separated product peak, which in panel C dot blot analyses were recognized by mAb 932. D. corresponding \( ^{1}H \) NMR analysis of the produced CPSA_{iv} in comparison to CPSA_{n} (from natural source). Slight variations in chemical shifts between CPSA_{n} and CPSA_{iv} are due to pH variations resulting from different purification protocols.
who showed that the *E. coli* UDP-*N*-acetylglucosamine-2-
epimerase, if present at high concentrations, releases the two
intermediates of the epimerization reaction (UDP and 2-acet-
amidoglucal) into solution. Because *E. coli* UDP-*N*-acetylgluco-
samine-2-epimerase and CsaA share significant sequence sim-
ilarity, it is reasonable to believe that CsaA uses the same
catalytic mechanism. This CsaA side reaction could be com-
pletely suppressed if CsaA concentrations were equal or lower
than the CsaB concentrations.

Using a two-step protocol (in test reactions even a one-pot
reaction; see Fig. 8) *O*-acetylated CPSA (CPSA*O*Ac) could be
produced *in vitro* in high purity and at medium scale (1 mg).
The Capsule Biosynthesis Machinery of NmA

Similar to the CPSA isolated from natural source (CPSAₕ) (42), the CPSAₜₐₜ fraction was of high molecular weight, showed ³¹P NMR and ¹H NMR profiles consistent with CPSAₕ and was recognized by mAb 932, a standard reagent in the characterization of immunologically active CPSA. Small differences were seen in the acetylation patterns. While the CPSAₕ reference contained some O-4-acetylation, this modification was not detectable in CPSAₜₐₜ. Although this difference at first sight may suggest the existence of a second enzyme with preference for the 4-O position, this interpretation is highly unlikely since casC-knockouts are completely devoid of O-acetylation (21). Interesting, rather diverse values exist in literature for the relative occurrence of 3-O-Ac, 4-O-Ac, and free hydroxyl groups in CPSAₕ (70:30 (34), 87:8:5 (35), 40:27:33 (21)). To resolve the question of the importance of O-4-acetylation for the immunogenic quality of in vitro produced CPSA, further experimental work is needed.

In the current study we provide clear evidence that the acceptor quality of CPSAₛₜ increases after removal of the O-acetyl-groups. Consequently, also, the synthetic ManNAc-1P dimer carrying 3-O-Ac-groups (compound 6) was a less suited primer than the respective compound 5 without O-acetyl-groups. Asking if UDP-activated and 3-O-acetylated ManNAc (UDP-3OAcManNAc) may be a donor substrate for CsaB, we chemically synthesized this compound using a modification of a literature procedure for the preparation of UDP-GlcNAc (43) (see the supplemental scheme 3). Remarkably, no insertion of the modified compound was seen, strongly arguing for a highly selective recognition of the donor substrate by CsaB and further emphasizing the hypothesis that O-acetylation in NmA takes place on the synthesized polysaccharide. Of relevance in this context are the previous demonstrations that O-acetylation of sialic acid residues in the CPSs of NmC and E. coli K1 takes place after the polymer has reached a certain length and could not be detected on the donor substrate (44, 45).

In 1999 a study by Ravenscroft et al. (36) demonstrated that acidic hydrolysis of CPSA results in fragments that are capped by phosphate at the non-reducing end. We show in the current study that removal of this capping phosphate steeply increases CsaB activity values (Fig. 4) and thus provide clear evidence that chain elongation proceeds by transfer of ManNAc-1P residues to the non-reducing end of the priming oligosaccharide. In addition, the use of synthetic priming compounds demonstrated that the minimal acceptor for CsaB is the ManNAc-phosphate dimer and that the phosphate group at the non-reducing end can be extended with rather large chemical groups (n-decyl ester in the compounds tested in this study). Particularly this latter finding is of biotechnological relevance because it provides the perspective that CPSA chains can be built with priming oligosaccharides that carry functional groups ready for conjugation to carrier proteins (5).

Similar to the capsule polymerase of NmX (17), CsaB is also capable of initiating polymerization in the absence of any acceptor. Remarkably, this de novo activity was not altered in the presence of the artificial compounds 1-4. More experimental work is needed to interpret these findings, but based on these results it is tempting to speculate that the de novo reaction involves two UDP-ManNac residues bound to acceptor and donor site in the enzyme.

In summary, we present data in this study that provide a new basis for the development of efficient and economic protocols (even one-pot-reaction protocols) for the synthesis of highly pure and immunologically active CPSAₜₐₜ. This means a large step forward in the combat of epidemics caused by one of the predominant neisserial serogroups NmA.

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