Biochemical characterization of a *Neisseria meningitidis* polysialyltransferase reveals novel functional motifs in bacterial sialyltransferases

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Summary

The extracellular polysaccharide capsule is an essential virulence factor of *Neisseria meningitidis*, a leading cause of severe bacterial meningitis and sepsis. Serogroup B strains, the primary disease causing isolates in Europe and America, are encapsulated in α-2,8 polysialic acid (polySia). The capsular polymer is synthesized from activated sialic acid by action of a membrane-associated polysialyltransferase (*NmB*-polyST). Here we present a comprehensive characterization of *NmB*-polyST. Different from earlier studies, we show that membrane association is not essential for enzyme functionality. Recombinant *NmB*-polyST was expressed, purified and shown to synthesize long polySia chains in a non-processive manner *in vitro*. Subsequent structure–function analyses of *NmB*-polyST based on refined sequence alignments allowed the identification of two functional motifs in bacterial sialyltransferases. Both (D/E-D/E-G and HP motif) are highly conserved among different sialyltransferase families with otherwise little or no sequence identity. Their functional importance for enzyme catalysis and CMP-Neu5Ac binding was demonstrated by mutational analysis of *NmB*-polyST and is emphasized by structural data available for the *Pasteurella multocida* sialyltransferase *Pm*ST1. Together our data are the first description of conserved functional elements in the highly diverse families of bacterial (poly)sialyltransferases and thus provide an advanced basis for understanding structure–function relations and for phylogenetic sorting of these important enzymes.

Introduction

*Neisseria meningitidis* (*Nm*) is a leading cause of bacterial meningitis and sepsis in children and adolescents. Sporadic cases as well as outbreaks and epidemic waves are observed. Despite the availability of potent antimicrobial agents, case-fatality rates are high and survivors frequently suffer from sequelae such as limb loss and deafness (van Deuren *et al*., 2000). Essential virulence factors of disease causing meningococci are their extracellular polysaccharide capsules. Serogroup B strains (*NmB*), the primary disease-causing isolates in Europe and America, are encapsulated in α-2,8 polysialic acid (polySia). The *NmB* capsule was shown to mediate resistance to phagocytosis and complement-mediated bacteriolysis and it is chemically and immunologically identical to polySia found in the human host (Mackinnon *et al*., 1993; Hammer-schmidt *et al*., 1994; Vogel *et al*., 1997; Vogel and Frosch, 1999). This mimicry also prevents the generation of effective polysaccharide-based vaccines against *NmB* strains. Bypass of host defence mechanisms by polySia capsules has not only been described for *NmB*, but is also an important virulence determinant of other disease-causing pathogens such as *Escherichia coli* K1 and K92, *Moraxella nonliquefaciens*, *Pasteurella haemolytica* and *N. meningitidis* serogroup C (Troy, 1992). The polySia capsules of *NmC* and *E. coli* K92 are connected by α-2,9 (Bhattacharjee *et al*., 1975) or alternating α-2,8/α-2,9 (Egan *et al*., 1977) glycosidically linked sialic acids, respectively. Because of their critical function in bacterial pathogenesis enzymes involved in polySia biosynthesis are interesting targets for therapeutic intervention. Biosynthesis of polySia is catalysed by membrane-associated polysialyltransferases (polySTs) at the cytoplasmic side of the inner
membrane of Gram-negative bacteria (Masson and Holbein, 1983; Troy, 1992). The polymerization reaction proceeds by transfer of sialic acid from the donor substrate CMP-Neu5Ac to the non-reducing end of a growing polySia chain and was proposed to be processive, as no reaction intermediates could be detected (Steenberg and Vimr, 2003). Polysialyltransferases have been cloned from E. coli K1 and K92 as well as from N. meningitidis serogroup B and C (Weisgerber et al., 1991; Vimr et al., 1992; Edwards et al., 1994; Claus et al., 1997). Identity is highest between the pair of E. coli (82% identity) and meningococcal enzymes (65% identity) and is lower between the two genera (33% identity). Interestingly, the neisserial enzymes are elongated by a C-terminal domain not present in the E. coli polySTs.

While only few studies included the neisserial polySTs (Masson and Holbein, 1983; Swartley et al., 1997; Steenberg and Vimr, 2003), the E. coli enzymes have been studied more extensively (Whittfield, 2006; Troy, 1992). They are unable to start de novo biosynthesis of polySia, but elongate exogenously added acceptors including oligo- and polysialic acids, siaelygangliosides and synthetic acceptors (Steenberg and Vimr, 1990; Cho and Troy, 1994; McGowen et al., 2001). The nature of the priming endogenous acceptor is still unknown, but recently gene products NeuE and KpsC were shown to be required for de novo synthesis of polySia in E. coli (Andresishcheva and Vann, 2006) and the functional complex of the E. coli K92 polysialyltransferase was found to be larger than a monomer (Vionnet et al., 2006). Construction of chimeric proteins between the closely related E. coli K1 and K92 enzymes, synthesizing α-2,8- and alternating α-2,8/α-2,9-linked polySia, respectively, mapped the region responsible for linkage specificity to primary sequence elements located between amino acids 53 and 85 in both enzymes (Steenberg and Vimr, 2003). However, so far no data on isolated proteins are available. Attempts to purify or solubilize polySTs failed and resulted in inactivation of the enzymes and it was therefore proposed that membrane association is required for polyST activity (Steenberg and Vimr, 2003; Vionnet et al., 2006). The lack of purified native or recombinant protein furthermore prevented detailed structure function analyses of these important enzymes.

No conserved motifs have been described for bacterial sialyltransferases. This is different from the eukaryotic sialyltransferases, where four conserved motifs have been shown to be involved in binding of donor and acceptor substrates and in enzyme catalysis (Drickamer, 1993; Livingston and Paulson, 1993; Geremia et al., 1997; Jeannneau et al., 2004). Moreover, based on primary sequence similarities all eukaryotic sialyl- and polysialyltransferases can be grouped into a single family GT-29 of the CAZy database (http://www.cazy.org) while the bacterial enzymes are distributed into four families (Coutinho et al., 2003). Bacterial polysialyltransferases are found in CAZy family GT-38, while families GT-42, GT-52 and GT-80 contain sialyltransferases that sialylate bacterial lipooligosaccharide (LOS). Structural information is available for the Campylobacter jejuni sialyltransferase cst-II (member of CAZy family GT-42) (Chiu et al., 2004) and the sialyltransferase PmST1 of Pasteurella multocida (member of CAZy family GT-80) (Ni et al., 2006). While cst-II belongs to the glycosyltransferase-A-like (GT-A) structural group, PmST1 has a GT-B-like fold.

In the current study we focused on the characterization of the polyST from N. meningitidis serogroup B and throughout succeeded with soluble expression and purification of recombinant Nmb-polyST. We thereby demonstrate that membrane association is not a prerequisite for the formation of functional enzyme. We also show that removal of the C-terminal extension present in Nmb but not in the homologous E. coli enzymes, completely abolished enzymatic activity, proving it as an essential functional domain. Using site-directed mutagenesis and refined protein alignment strategies, we identified two functionally important motifs, which are highly conserved in a number of bacterial (poly)sialyltransferases of otherwise unrelated sequences. With these data we provide the first evidence for the conservation of catalytic features among bacterial sialyl- and polysialyltransferases and thereby improve the basis for design of sialyltransferase-specific drugs.

Results

Recombinant expression of Nmb-polyST

Detailed structure--function analyses of bacterial polysialyltransferases were hitherto prevented by insufficient supply of the enzymes. Expression levels were described to be low and polyST activity was found associated with bacterial membranes (Shen et al., 1999; Steenberg and Vimr, 2003; Vionnet et al., 2006). As attempts to solubilize polyST were either unsuccessful or accompanied by inactivation of the enzyme, all analyses have been performed with crude membrane fractions as enzyme source. With the aim of obtaining purified enzyme in yields sufficient to perform structure--function studies, we began the current work with a systematic search for conditions that would allow production of recombinant Nmb-polyST. To test the influence of N-terminal fusion partners on Nmb-polyST expression and activity, constructs were generated either with short N-terminal epitope tags (T7, Strep II) or with additional large fusion parts like NusA and maltose-binding protein (MBP) (Fig. 1A). The constructs were expressed in E. coli BL21 (DE3) and soluble and insoluble fractions of the bacterial lysates were analysed.

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for expression and activity of NmB-polyST. Although soluble protein could be detected for all constructs (Fig. 1B), the addition of large N-terminal fusions considerably increased the amount of active protein in the soluble fractions (Fig. 1C). Compared with polySTs carrying only short N-terminal epitope tags, additional fusion of NusA or MBP increased the soluble activity of NusA– and MBP–polyST two- and threefold, respectively. Also, the specific activity of both fusion proteins was increased twofold compared with enzymes with short tags (Fig. 1D). Subsequent experiments were therefore carried out with either NusA or MBP fusion proteins.

**Definition of the minimal active domain of NmB-polyST**

It has been reported for other bacterial sialyltransferases that N- or C-terminal truncations significantly increase protein solubility by eliminating membrane interaction domains (Chiu *et al.*, 2004; Ni *et al.*, 2006). Consequently, the second series of experiments was designed to define the minimal catalytic domain of NmB-polyST. N- and C-terminal truncations of the enzyme were generated as NusA fusion proteins carrying a C-terminal His-tag for detection. Full-length and truncated proteins were expressed in *E. coli* and soluble and insoluble cell fractions were tested for expression and enzymatic activity of NmB-polyST. As depicted in Fig. 2, approximately 50% of the wild-type polyST was soluble (Fig. 2B) and enzymatically active (Fig. 2D), whereby the detected activity was threefold higher in the soluble than in the insoluble fraction. Removal of 23 (Δ23NmB-polyST) and 33 (Δ33NmB-polyST) amino acids from the N-terminus had only slight effects on solubility and activity of NmB-polyST. However, deletion of the first 64 amino acids (Δ64NmB-polyST) shifted the majority of the expressed protein to the insoluble fraction and no enzymatic activity was detected in soluble or insoluble fractions.

Primary sequence analysis revealed that NmB-polyST carries a C-terminal extension of 95 amino acids that is not present in the polySTs of *E. coli* K1 and *E. coli* K92 (Fig. 2A). To investigate the role of this additional
protein part, we generated a set of truncated NmB-polySTs lacking the C-terminal domain either partially (NmB-polySTΔ22, NmB-polySTΔ60) or completely (NmB-polySTΔ94, NmB-polySTΔ95, NmB-polySTΔ97). As displayed in Fig. 2C, all variants could be expressed as soluble proteins at similar or, in the case of constructs with entirely deleted C-terminal domain, even higher levels than the full-length enzyme. However, each C-terminal truncation completely abolished enzymatic activity (Fig. 2D), indicating that the C-terminal domain is indispensable for NmB-polyST activity.

The fusion protein MBP–NmB-polyST produces capsular polySia in vivo

Our efforts to express recombinant NmB-polyST in the E. coli expression strain BL21 (DE3) clearly revealed beneficial effects of large N-terminal fusion parts on the expression of active, soluble enzyme. However, to analyse if polyST fusion proteins maintain enzymatic activity also in vivo, wild-type and MBP–NmB-polyST were subcloned into a neisserial expression vector and transformed into the polyST-deficient neisserial strain.
2517. Parental strain and transformants were analysed for capsular polySia in a quantitative whole-cell ELISA. Equal loading of the microtitre plates with bacteria was confirmed in a parallel ELISA directed against the meningococcal major outer membrane protein PorA. Interestingly, no difference in capsule expression was observed between strains complemented with wild-type or the MBP fusion construct (Fig. 3A). Moreover, Western blot analysis of the neisserial lysates revealed a similar polySia staining of wild-type and fusion protein that was not detectable in samples treated with endoN (Fig. 3B), which is a bacteriophage-derived enzyme that degrades polySia with high substrate specificity (Mühlenhoff et al., 2003; Stummeyer et al., 2005). This clearly demonstrates that MBP–NmB-polyST is enzymatically active in vivo.

Purification of recombinant NmB-polyST

As MBP–NmB-polyST was shown to be active in vitro and in vivo and because MBP could be directly utilized for affinity chromatography, purification was optimized for the MBP–NmB-polyST construct schematically depicted in Fig. 1A. The fusion construct additionally carries two short epitope tags (N-terminal Strep II-, C-terminal His-tag), which were used for detection of the protein throughout the purification. After overexpression in E. coli BL21(DE3) at 15°C, the recombinant MBP–NmB-polyST was purified in two consecutive steps by MBP affinity and size exclusion chromatography. Trials to also utilize the C-terminal His-tag for purification failed, indicating that the epitope is not accessible in the native enzyme. As shown in Fig. 4A, the applied purification procedure yielded a highly enriched MBP–NmB-polyST (protein of 100 kDa). Major bands of smaller molecular weight still visible in the Coomassie-stained SDS-PAGE of the purified pool were also detected in a Western blot directed against the Strepl-tag, but not in a blot stained with an anti-His-tag antibody. This indicates that these bands represent breakdown products of MBP–NmB-polyST caused by C-terminal degradation.

Throughout the purification, polyST activity was monitored by the radiochemical polyST assay using colominic acid as the acceptor. Enzyme activity was detected in all MBP–NmB-polyST-containing fractions and specific activity increased about 20-fold from cell lysate to the elute obtained after gel filtration (see Fig. 4A, bottom). As depicted in Fig. 4B, the fusion protein eluted in a single peak at the upper limit of the applied Superdex 200 gel filtration column, which suggests association of MBP–NmB-polyST as hexameric or higher-order oligomers. However, the purified protein was soluble and remained completely in the supernatant after high-speed centrifugation (100 000 g, Fig. 4C). One litre of expression culture yielded 1.3 mg of the highly enriched MBP–NmB-polyST. The protein was concentrated to 2 mg ml$^{-1}$ and stored at 4°C for 3–4 weeks without detectable loss of activity or further degradation.

Soluble recombinant MBP–NmB-polyST produces polySia chains in a non-processive manner

Capsular polysaccharide purified from serogroup B N. meningitidis was shown to exhibit a high degree of polymerization (DP) (Gotschlich et al., 1969; Frosch and Müller, 1993). This implies that NmB-polyST is able to produce large polymers when the enzyme is part of the
native capsule biosynthesis complex associated with the inner bacterial membrane. To analyse products synthesized by purified NmB-polyST in more detail, pentameric (DP5) α-2,8-linked oligosialic acid was used as the primer for the recombinant enzyme and the polymerization reaction was started by addition of radiolabelled sugar donor substrate CMP-[14C]-Neu5Ac. Products were separated by high percentage polyacrylamide gel electrophoresis and visualized by autoradiography. Because polySia can be specifically degraded with bacteriophage endosialidases (endoN) (Mühlenhoff et al., 2003; Stummeyer et al., 2005), the nature of the synthesized products was controlled by treating one of two parallel samples with endoN prior to electrophoresis. As shown on the gel in Fig. 5A, which separates polymerization products from DP 10 to >100, a large fraction of the synthesized polySia was longer than 100 residues per chain. All reaction products were specifically degraded by endoN. This demonstrates that the recombinant MBP–NmB-polyST is able to synthesize long polySia chains starting with short oligomeric acceptors in vitro. However, in addition to the high-molecular-weight polySia, also shorter oligosialic acid reaction products were detectable (Fig. 5A). To validate the presence of short reaction products and analyse the mode of chain elongation in more detail, we studied the influence of acceptor concentrations on chain length distribution. Therefore, DP5 concentrations were altered over three orders of magnitude starting with 1 μM, while the concentration of enzyme and donor substrate CMP-Neu5Ac was kept constant. As shown in Fig. 5B, increasing acceptor concentrations augment the concentration of short- and medium-sized reaction products until – at the point of equimolar concentrations of DP5 and CMP-Neu5Ac (lanes 4 and 5) – virtually all radiolabelled products remain below DP11 (Fig. 5B). An identical correlation of chain length distribution and acceptor concentration was obtained for trimeric (DP3) α-2,8-linked oligosialic acid (Fig. S1). These data argue against processivity of NmB-PolyST in vitro, as in case of a highly processive mechanism synthesis of few long chains should be favoured over synthesis of many short chains. To evaluate these data in a second assay system, we used our recently developed fluorescence-based polyST assay (Vionnet and Vann, 2007) that utilizes the trisialylganglioside analogue GT3-FCHASE as artificial acceptor substrate. In a first experiment GT3-FCHASE was incubated with purified MBP–NmB-polyST and increasing concentrations of the donor substrate CMP-Neu5Ac (0, 5, 50, 500 μM). After an incubation time of 30 min the synthesized reaction products were analysed by ion exchange HPLC. As shown in Fig. 6, the chain length of synthesized polySia increased with increasing concentra-
tions of donor substrate and resulted in a complete conversion of the acceptor substrate to high-molecular-weight polymer at a CMP-Neu5Ac concentration of 500 μM. This clearly demonstrates synthesis of long polySia chains. To verify the suggested non-processive elongation mode of NmB-polyST (Fig. 5B and Fig. S1), we performed a time-course experiment under reaction conditions that, as shown above, result in synthesis of long polySia chains (Fig. 7). If the reaction was stopped after 2 min predominantly short oligosialic acid-containing products were found (Fig. 7A) that were further elongated as the polymerization progressed (Fig. 7B and C) to finally yield high-molecular-weight polymer (Fig. 7D). This is reflected by a shift of the detected product peaks to longer retention times (Fig. 7). The occurrence of intermediates in the polymerization reaction argues, in agreement with the data obtained from the radioactive assay system, for non-processive chain elongation by MBP–NmB-polyST and indicates that the enzyme dissociates from the product after each addition of sialic acid.

Fig. 5. PolySia biosynthesis of purified NmB-polyST.
A. Purified NmB-polyST was assayed for 30 min in the presence of 0.1 mM pentameric α2,8-linked sialic acid (DP5) and 1 mM CMP-[^14C]-Neu5Ac. Subsequently, half of the sample was digested with polySia-specific endoN. Radiolabelled reaction products were separated by acrylamide gel electrophoresis (10%) and detected by autoradiography. The following dyes were used as standards and correspond to polySia chain length given in brackets: trypan blue (DP100), xylene cyanol (DP52), bromophenol blue (DP19), brom cresol purple (DP11).

B. Dependence of chain length distribution on acceptor concentration. The assay was performed as described in (A) but DP5 concentrations were varied as indicated. Samples were separated on a 25% polyacrylamide gel. To display single oligomeric-reaction products at the highest acceptor concentration more clearly, less sample volume was applied in lane 4.

Fig. 6. Dependence of GT3-FCHASE extension by purified NmB-polyST on CMP-Neu5Ac concentration. Purified NmB-polyST was incubated with GT3-FCHASE and CMP-Neu5Ac for 5 min as described in Experimental procedures. The respective CMP-Neu5Ac concentrations are indicated. The reaction mixtures were then adjusted to 25% ethanol. The supernatants were applied to a DNA Pac PA-100 column and chromatographed with a gradient of NaNO3 according to Inoue et al. (2001) and Inoue and Inoue (2003).
Identification of two conserved motifs in bacterial sialyl- and polysialyltransferases

To identify amino acid residues critical for (poly)sialyltransferase activity, we searched for common sequence motifs in the available bacterial sialyltransferase sequences. By iterative steps of pairwise and multiple alignments combined with visual inspection of the sequences, we identified two short motifs (D/E-D/E-G and HP). Both motifs are present in a range of enzymes with otherwise little identity (Fig. 8) that, based on functional and sequence properties, had been allocated to different CAZy families (GT-38 and GT-52) and to pfam family 05855. While GT-38 contains bacterial polysialyltransferases, GT-52 includes bacterial LOS sialyltransferases and pfam 05855 groups sialyltransferases similar to the sialyltransferase Lst of Haemophilus ducreyi (Bozue et al., 1999). The D/E-D/E-G and the HP motif identified in this study are conserved in all members of the three families and, interestingly, are also found in the bacterial LOS sialyltransferases grouped in GT-80. However, the relation to GT-80 family members is not easily seen in an alignment, as the stretch of sequence between the D/E-D/E-G and HP motif is approximately 50 amino acids longer than in the other families.

D/E-D/E-G and HP motifs are crucial for NmB-polyST activity

To investigate the functional relevance of the identified motifs, point mutations were introduced into NmB-polyST by site-directed mutagenesis. The histidine and proline of the HP motif as well as the glycine and its adjacent glutamate residue of the D/E-D/E-G motif were individually changed to alanine, resulting in the mutants H278A, P279A, E153A and G154A. Mutants were expressed in E. coli BL21(DE3) and lysates were analysed for protein expression and activity by Western blot and the radioactive polyST activity assay, respectively. As shown in Fig. 9A, all mutants were expressed at the level of the wild-type protein and the ratio between soluble and insoluble protein was in all cases similar to wild type. In contrast, enzyme activity was dramatically impaired in all mutants (Fig. 9B). While mutations in the D/E-D/E-G motif (E153A and G154A) fully inactivated the protein, the H278A and P279A mutants of the HP motif maintained
Fig. 8. Conserved motifs in bacterial sialyl- and polysialyltransferases. Sequence alignment of bacterial sialyltransferases identifying two short motifs conserved in CAZy families GT-38 (bacterial sialyltransferases), GT-52 (bacterial LOS-sialyltransferases) and in pfam family 05855 (similar to _H. ducreyi_ sialyltransferase Lst). To improve clarity of the illustration only three representatives of pfam 05855 are shown.

PolySTNmB (AA20478), polyST_Eck92 (AA42415), polyST_Eck1 (CA44305), 2,3ST_cpsK_Sa (EAO06216), 2,3-ST_Hd (AAD28703), SiaA_Lst_Hi (AAL38659), PM0508_Lst_Pm (AAK02592), LsgB_Hi (AAH8755), Cps8K_Sa (AAR29926), 2,3_ST_Nm (AA44544), Ist_Ng (AAY1933), Ist_Ap (AAS66624), Psyc_0663_Pa (AAZ18522). Alignments were made using Mulat1 (Corpet, 1988).
residual activity (below 10% of wild type). However, when both residues of the HP motif were changed to alanine simultaneously (H278A/P279A) enzyme activity was abolished.

To confirm the functional relevance of the D/E-D/E-G and HP motifs in vivo, mutant meningococcal strains were generated by homologous recombination and capsular phenotypes of the mutant strains were analysed by slide agglutination using the polySia-specific antibody 735. In agreement with the in vitro studies, Neisseria strains exhibiting the E153A, G154A or the double mutation H278A/P279A did not synthesize polySia capsules while capsules were still produced in strains carrying the single mutations H278A and P279A (Fig. 9C). However, a whole-cell ELISA used to quantitatively compare polySia synthesis in wild-type bacteria and mutants clearly demonstrated impairment of capsule biosynthesis in mutants with residual in vivo activity. While the PorA control was similar for wild type and mutants, the polySia signal was significantly reduced to 30% and 60% for the mutants carrying the amino acid exchanges H278A and P279A respectively. This demonstrates that mutations within the HP motif not only decrease enzymatic activity in vitro, but also reduce capsule production in vivo.

An enzyme-linked assay system for the functional characterization of purified polysialyltransferases

To compare wild-type and mutant polySTs with partial activity (particularly mutants H278A and P279A) we decided to perform kinetic studies. As a first step towards this goal, the glycosyltransferase assay developed by Gosselin et al. (1994) was adapted to assay polyST activity. This spectrophotometric assay links the release of CMP by polyST during polySia synthesis to NADH oxidation and thus enables continuous monitoring of the enzyme reaction. PolyST activity towards short oligosialic acid acceptors (DP2 to DP5) was measured at constant CMP-Neu5Ac and enzyme concentrations. As shown in Fig. 10A, efficient chain elongation required oligomers of at least DP3. These findings are in agreement with the acceptor dependence obtained when polyST-containing
membrane preparations of *E. coli* K1 or K92 were assayed in radiochemical or HPLC-based test systems (Steenbergen and Vimr, 1990; Ferrero et al., 1991; Chao et al., 1999).

Subsequently, kinetic parameters of MBP–NmB-polyST were determined for the donor substrate CMP-Neu5Ac (Fig. 10B, Table 1). The calculated \( k_m \) value of 0.42 mM is fivefold higher than that obtained for the membrane bound enzyme from *E. coli* K1 polyST (Kundig et al., 1971; Vijay and Troy, 1975). However, because apart from enzyme source and preparation but also assay and buffer conditions differ between earlier and current studies, further interpretation of the observed variations in \( k_m \) appears unreasonable. In summary, the enzyme-linked assay provided a useful tool to carry out kinetic analysis of polySTs and greatly facilitated the acquisition of data which, until now, have required single-point measurements and elaborate detection methods.

The HP motif is involved in CMP-Neu5Ac binding

Because NmB-polySTs carrying the point mutations H278A and P279A retained residual activity in vitro, kinetic properties of these mutants could be determined. Both mutants were expressed as MBP fusion proteins, purified and tested in the enzyme-linked polyST assay. As listed in Table 1, \( V_{max} \) values for CMP-Neu5Ac were decreased by a factor of 4 (P279A) and 6 (H278A) with respect to the wild-type enzyme and, in both proteins, \( k_m \) values for CMP-Neu5Ac were increased three- to fivefold. These data suggest that the HP motif of NmB-polyST is involved in binding of the donor substrate CMP-Neu5Ac.

To further analyse the effect of both mutations on acceptor binding, \( k_m \) values for colominic acid were determined. Interestingly, Michaelis constants were not significantly influenced by either mutation indicating that: (i) the HP motif does not vitally participate in acceptor binding, and (ii) the introduced point mutations did not cause major structural changes as the acceptor binding site appeared to be largely unaffected. Remarkably, the recently solved crystal structure of *P. multocida* sialyltransferase PmST1 (Ni et al., 2006) revealed a similar CMP-Neu5Ac binding function of the HP motif in the GT-80 sialyltransferase family. The enzyme consists of two Rossmann domains that form a deep cleft in which the active site is located and has been crystallized in the presence and absence of donor and acceptor substrates. The active site of PmST1 with bound donor analogue CMP-3F(α)Neu5Ac and acceptor lactose (Ni et al., 2007) is shown in Fig. 11 and illustrates that the histidine residue of the HP motif (H311 Fig. 10.

![Fig. 10. Enzymatic characterization of NmB-polyST.](image)

**A.** To analyse acceptor specificity, oligomeric α2,8-linked sialic acids with a degree of polymerization (DP) ranging from DP2 to DP6 (0.5 mM) were assayed at constant enzyme (4 μM) and CMP-Neu5Ac concentrations (1 mM) using the continuous spectrophotometric assay. PolyST activity is detected as decreasing absorption at 340 nm (NADH oxidation).

**B.** To determine \( k_m \) and \( V_{max} \) of NmB-polyST for the donor substrate CMP-Neu5Ac, measurements were performed at 30°C and 0.28 mg ml⁻¹ colominic acid. The resulting substrate velocity curve and Lineweaver–Burk plot are depicted. Kinetic parameters were obtained by non-linear regression in Prism (GraphPad Software).

**Table 1.** Kinetic parameters of wild-type and mutant NmB-polyST.

| NmB-polyST | \( V_{max} \) (μmol min⁻¹ mg⁻¹) | \( k_m \) (mM) | \( k_m^a \) (mM) |
|-----------|-------------------------------|---------------|----------------|
| Wild type | 25.8 ± 3.6                    | 0.42 ± 0.03   | 0.63 ± 0.10    |
| H278A     | 4.0 ± 1.1                     | 1.37 ± 0.12   | 0.40 ± 0.14    |
| P279A     | 6.0 ± 1.0                     | 2.15 ± 0.51   | 0.51 ± 0.13    |

a. Determined at constant acceptor concentration of 0.28 mg ml⁻¹ colominic acid.

b. Determined at constant donor concentration of 1 mM CMP-Neu5Ac.

c. Kinetic constants were calculated based on an average chain length of 32 residues for colominic acid as estimated using the thiobarbituric acid assay procedure according to Skoza and Mohos (1976).

Kinetic values were obtained using the continuous polysialyltransferase assay and are presented as the means ± SE of three independent determinations.
in PmST1) is directly involved in CMP-Neu5Ac binding. It forms hydrogen bonds with the phosphate group of CMP and with the carboxylate function of the sialic acid moiety. Moreover, this structure shows that the D/E-D/E-G motif is located directly at the active site cleft of PmST1 and is involved in binding of donor and acceptor substrates. The second aspartic acid residue of the DDG sequence forms hydrogen bonds to the acceptor lactose and to the hydroxyl group in position C4 of the sialic acid moiety. In combination, the structural and biochemical data provide strong evidence that the D/E-D/E-G and HP sequences are crucial for CMP-Neu5Ac binding and enzyme catalysis, in bacterial sialyl- and polysialyltransferases that harbour these motifs.

Discussion

In this study, we functionally characterized the polysialyltransferase responsible for capsule biosynthesis in N. meningitidis serogroup B. Although polySTs are inter-
esting therapeutic targets to combat these pathogens, no data on isolated proteins and virtually no information on structure–function relationships, which are crucial for the rational design of inhibitors, have been reported. Attempts to purify or solubilize membrane-associated polySTs have failed or resulted in inactivation of the enzymes, so that membrane association was proposed to be essential for polyST activity (Steenbergen and Vimr, 2003; Vionnet et al., 2006).

With the aim of overcoming the lack in structure–function information, we started this study by screening for production systems that enable expression and purification of active NmB-polyST. Soluble expression of active protein was significantly improved after addition of large N-terminal fusion parts and allowed purification of NmB-polyST fused to MBP. Purified MBP–NmB-polyST was enzymatically active and, as described for membrane bound bacterial polySTs of E. coli K1 and K92 (Steenbergen and Vimr, 1990; Ferrero et al., 1991; Chao et al., 1999), able to synthesize long polySia chains from oligomeric primers of at least DP3. Consequently, our data provide clear evidence that membrane association is not a prerequisite for NmB-polyST activity. Furthermore, we demonstrate that the purified enzyme elongates polySia chains in a non-processive manner. This is in contrast to in vivo studies carried out in E. coli K1 that suggest a processive mode of polySia biosynthesis (Steenbergen and Vimr, 2003). PolySTs were proposed to be part of a large capsule biosynthesis complex, in which biosynthesis and translocation of polySia across the inner and outer bacterial membranes are tightly linked (Steenbergen and Vimr, 2003) and the functional E. coli K92 polysialyltransferase complex was found to be larger than a monomer (Vionnet et al., 2006). Thus, potential interaction partners of polyST could increase the efficiency of polySia biosynthesis and thereby increase processivity. We (W.F.V. and J.V.) recently demonstrated that also membrane-bound E. coli K92 polyST is a non-processive enzyme in vitro (Vionnet and Vann, 2007), but the utilized membranes lacked other gene products of the K92 capsule biosynthesis cluster as well. However, even polySia biosynthesis assayed with intact membrane preparations of E. coli K1, which most likely include all relevant interaction partners of polyST, was found to be less efficient with exogenously added sialyloligomers than with endogenous or lipid bound acceptors (Cho and Troy, 1994; Chao et al., 1999; McGowen et al., 2001). This may argue for a more effective binding of the endogenous acceptor and hence result in increased processivity. In conclusion, the finding that recombinant soluble NmB-polyST is non-processive does not exclude processivity of the enzyme in the living system. The investigation of functional properties of recombinant NmB-polyST in complex with other factors of the capsular biosynthetic machinery is therefore an impor-

Fig. 11. Location of the conserved HP and D/E-D/E-G motifs in the P. multocida sialyltransferase PmST1.
A. Surface representation of PmST1 (PDB entry 2EX1). The motifs are coloured in red (D/E-D/E-G) and blue (HP) while bound CMP is shown in yellow stick representation.
B. Active site view of PmST1. Amino acids that are part of the two motifs are labelled and depicted in green stick representation. Hydrogen bonds are shown as dotted line while the bound donor motifs are labelled and depicted in green stick representation.
tant aim in future studies. Interestingly in this regard, MBP–NmB-polyST expressed in a polyST-deficient Neisseria strain is capable to complement the defect (see Fig. 3). The fusion protein may thus provide an interesting tool for studies aimed at identifying polyST-mediated protein–protein interactions in vivo.

Mapping of the minimal catalytic domain of NmB-polyST has shown that the C-terminal domain, though not conserved in the homologous E. coli enzymes, is essential for catalytic activity. All C-terminally truncated NmB-polySTs were completely inactive. Studies are underway to analyse if potential variations in folding, in the oligomeration status or in substrate binding properties are responsible for inactivation of the truncated polySTs (Fig. 2).

To characterize the catalytic domain of NmB-polyST in more detail, we aimed at identifying key residues for polyST function. So far no functional residues have been described for bacterial polysialyltransferases and no sequence relationships to other bacterial sialyltransferases have been reported. In contrast to the eukaryotic sialyltransferases, which are grouped into a single CAZy family (GT-29) and harbour four highly conserved and well-described sialylmotifs (Drickamer, 1993; Livingston and Paulson, 1993; Geremia et al., 1997; Jeanneau et al., 2004), the less homologous bacterial sialyltransferases are found in four different CAZy families. GT-38 contains bacterial polysialyltransferases, while GT-42, GT-52 and GT-80 include bacterial LOS sialyltransferases. Herefore, no conserved sequence features relating members of different CAZy families have been described. In the current study, we combined sequence alignments and visual inspections and identified two short motifs in bacterial sialyltransferases, the D/E-D/E-G motif and the HP motif. The latter contains bacterial sialyltransferases similar to the Lst family members was provided by Wakarchuk et al. (2001). They showed that the neisserial α2,3-sialyltransferase Lst switches to a bifunctional α2,3/6-sialyltransferase mode upon mutation of the single-residue G168 and predicted this residue to be positioned in an acceptor-binding cavity. Interestingly, G168 is found only two residues upstream of the D/E-D/E-G motif (residues 164–166) in this enzyme. Finally it should be mentioned that structural information is available for a second sialyltransferase the cst-II from C. jejuni (Chiu et al., 2004). However, this enzyme is a
member of CAZy family GT-42, which is the only bacterial sialyltransferase family so far that does not harbour HP- and D/E-D/E-G motifs.

In conclusion, the establishment of efficient expression, purification and assay procedures for NmB-polyST allowed us to identify and characterize key groups for polyST function. Alignments with bacterial sialyltransferases revealed two functional motifs, the D/E-D/E-G and the HP motif, that are conserved in otherwise unrelated bacterial sialyltransferases of CAZy families GT-38, GT-52 and GT-80 as well as in pfam 05855. The functional importance of both motifs for enzyme catalysis and/or CMP-Neu5Ac binding was demonstrated by mutational analysis of NmB-polyST and is emphasized by structural and biochemical data available for the P. multocida sialyltransferase PmST1. Our data therefore allow hypothesizing that basic features of substrate binding and enzyme catalysis are conserved in a wide range of bacterial sialyltransferases and improve the basis for design of sialyltransferase-specific drugs.

Experimental procedures

Materials

Colominic acid and CMP-Neu5Ac were purchased from Sigma. Oligomeric sialic acids were from Nacalai Tesque. The pMAL-c Vector was from New England Biolabs, and pET vectors were purchased from Novagen.

Cloning of NmB-polyST expression vectors

NmB-polyST was amplified by PCR using plasmid pUE3 (Frosch et al., 1991) and the primer pair KS23 (5'-G CAT GGA CCC TTA AGA AAA ATA AAA AAA AACT-3') and KS41 (5'-GCA GCC GGC CGC TCT ATC TCT ACC AAT TCT-3') containing BamHI and NotI sites (underlined) respectively. The PCR product was subcloned into the respective sites of pET23a to result in an N-terminally T7- and C-terminally His6-tagged construct (pET23a-NmB-polyST). To generate an expression construct including an N-terminal NusA-/Strep-tag and a C-terminal His6-tag, the N-terminal NusA fusion part followed by a StrepII-tag, adapters Strepa and KS41 (5'-CTA GCC GGC CCC TGG TTC GGC GTG-3') and KS59 (5'-CTA GCC GGC CCC TGG TTC GGC GTG-3') were inserted by adapterligation into the BamHI and Spel restriction sites of pET43a generating pET43a-Strep. The NmB-polyST gene was subcloned from pET23a-NmB-polyST using restriction sites BamHI and NotI for the expression vector pET43a-Strep-NmB-polyST that encodes for the polyST fused with an N-terminal NusA-/Strep-tag and a C-terminal His6-tag. To obtain the vector pMBP-Strep, the MBP was amplified by PCR with primers FF03 (5'-GAT CAT ATG AAA ACT GAA GAA GTA AAA CT-3') and FF04 (5'-GAT CAT ATG AAA ACT GAA GAA GTA AAA CT-3') from the vector pMAL-c and subsequently subcloned into the Ndel and Spel restriction sites of pET43a-Strep. Finally, NmB-polyST was subcloned from pET43a-Strep-NmB-polyST using the Nhel/NotI sites resulting in pMBP-Strep-NmB-polyST that encodes for the polyST fused with an N-terminal MBP/Strep-tag and a C-terminal His6-tag.

Generation of truncated proteins

N-terminally truncated NmB-polySTs were generated by PCR using the forward primers IO07 (5'-G CAT TGG ATC CAC ATC TCC ATT TTA TCT TAC-3') for Δ23 NmB-polyST, IO08 (5'-GCA TGG ATC CAA CAA TTA TTT ATT TGT CAT ATC TA-3') for Δ33 NmB-polyST and IO09 (5'-GCA TGG ATC CTA TAC TTC TTC AAC CTT AAA CTA AAA A-3') for Δ64 NmB-polyST and the reverse primer KS41 (5'-GCA GCC GGC CGC TCT ATC TCT ACC AAT TCT-3'). C-terminally truncated NmB-polySTs were generated by PCR using the reverse primers FF01 (5'-GCA TGG ATC CAC ATC TCC ATT TTA TCT TAC-3') for Δ22, FF02 (5'-GCA TGG ATC CAA CAA TTA TTT ATT TGT CAT ATC TA-3') for Δ33 NmB-polyST and IO09 (5'-GCA TGG ATC CTA TAC TTC TTC AAC CTT AAA CTA AAA A-3') containing BamHI and NotI sites (underlined) respectively. The PCR product was subcloned into the respective sites of pET23a resulting in expression of N-terminally T7- and C-terminally His6-tagged construct (pET23a-NmB-polyST). To generate an expression construct including an N-terminal NusA-/Strep-tag and a C-terminal His6-tag, the N-terminal NusA fusion part followed by a StrepII-tag, adapters Strepa and KS41 (5'-CTA GCC GGC CCC TGG TTC GGC GTG-3') and KS59 (5'-CTA GCC GGC CCC TGG TTC GGC GTG-3') were inserted by adapterligation into the BamHI and Spel restriction sites of pET43a generating pET43a-Strep. The NmB-polyST gene was subcloned from pET23a-NmB-polyST using restriction sites BamHI and NotI for the expression vector pET43a-Strep-NmB-polyST that encodes for the polyST fused with an N-terminal NusA-/Strep-tag and a C-terminal His6-tag. To obtain the vector pMBP-Strep, the MBP was amplified by PCR with primers FF03 (5'-GAT CAT ATG AAA ACT GAA GAA GTA AAA CT-3') and FF04 (5'-GAT CAT ATG AAA ACT GAA GAA GTA AAA CT-3') from the vector pMAL-c and subsequently subcloned into the Ndel and Spel restriction sites of pET43a-Strep. Finally, NmB-polyST was subcloned from pET43a-Strep-NmB-polyST using the Nhel/NotI sites resulting in pMBP-Strep-NmB-polyST that encodes for the polyST fused with an N-terminal MBP/Strep-tag and a C-terminal His6-tag.

Site-directed mutagenesis

Single-point mutations of NmB-polyST were obtained by QuikChange site-directed mutagenesis (Stratagene) following the manufacturer's instructions using the plasmid pET23a-NmB-polyST as template. Mutated polySTs were subsequently subcloned into the BamHI and NotI sites of pET23a resulting in expression of N-terminally T7-tagged proteins. The identity of all constructs was confirmed by sequencing. Mutagenic primers are given below with the mutated base triplets underlined: pET23a-G154A: KS52 (5'-G ACT CAT TTAATT GAT GAA GGC ACT GGA ACA TAT GCT CC-3') and KS53 (5'-GGG AGC ATA TGT TCC AGT CGC TTC ATC AAT AAG ATG AGT C-3'); pET23a-E153A: KS54 (5'-TACG ACT CAT TTAATT GAT GAA GGC ACT GGA ACA TAT GCT CC-3') and KS55 (5'-CGT GAG TAT TCC AGT CGC TTC ATC AAT AAG ATG AGT C-3'); pET23a-H278A: KS56 (5'-ATT AAA GAA GAA ATA TTT ATT AAA CTA CAC GCC CAA AAA GAG ATG GCC AAC AAC-3') and KS57 (5'-GTT TCT GGC CAT CCT TTT TGG GGC TAG TTT AAT AAA GAA GTG CAT CTA AAG AAA ATAA A AACT CTT-3'); pET23a-P279A: KS58 (5'-GGG AAG ATA TTT ATT AAA CTA CAC GCC CAA AAA GAG ATG GCC AAC AAC TA-3') and KS59 (5'-TA GTT GTT GGC CAT CCT TTT TGG GTG TAG TTT AAT AAA CAT TCT TCC-3'). Mutants H278A and P279A were additionally introduced into the pMBP-Strep vector. Therefore, the polyST insert of pET23a-H278A and pET23a-P279A were ligated into pET43a-Strep using restriction sites BamHI and NotI and subsequently subcloned into pMBP-Strep using the Nhel and NotI sites.
Expression of recombinant NmB-PolyST

To optimize protein production, bacteria were either cultivated at 30°C (only polySTs fused to NusA) or at 15°C. For production at 30°C, freshly transformed *E. coli* BL21(DE3) were grown in PowerBroth (Athena ES) containing 200 µg l⁻¹ carbenicillin at 30°C and 225 r.p.m. At an optical density of OD₆₀₀ = 1.8 expression was induced by adding 1 mM IPTG. Bacteria were harvested 3 h after induction by centrifugation (6000 g for 15 min, 4°C). For 15°C production, bacteria were grown at 30°C to an optical density of OD₆₀₀ = 0.9. Cultures were then rapidly cooled (ice bath) and further grown at 15°C until OD₆₀₀ = 1.8 was reached. Transgene expression was induced by the addition of 1 mM IPTG and cells were harvested 24 h after induction. All pellets were washed once with PBS and stored at −20°C.

Separation of soluble and insoluble fractions of NmB-PolyST

To analyse NmB-PolyST expression and enzymatic activity in bacterial lysates, cells were re-suspended in 50 mM Tris-HCl pH 8.0, 40 mM MgCl₂ and lysed by sonication. Soluble and insoluble fractions were obtained following centrifugation (16 000 g, 15 min, 4°C). The insoluble fraction (pellet) was re-suspended in 50 mM Tris-HCl pH 8.0, 40 mM MgCl₂ in a volume equal to that of the soluble fraction.

Purification of recombinant MBP–NmB-PST fusion protein

Bacterial pellets from 0.5 l of cultures were re-suspended in binding buffer (20 mM Tris; pH 7.4; 1 mM EDTA; 1 mM DTT; 25 mM NaCl) including protease inhibitors (40 mg ml⁻¹ Bestatin, 1 µg ml⁻¹ Pepstatin and 1 mM PMSF) to give a final volume of 20 ml. Cells were disrupted by sonication and samples were centrifuged (30 min; 16 000 g, 4°C) and filtered (Sartorius Minisart 0.8 µm). For affinity absorption of MBP–NmB-PST, pre-swollen amylose resin (New England Biolabs) was added to the cleared supernatant and incubated at 4°C for 1 h. Subsequently, the incubation mixture was transferred to a column and washed with 12 volumes of binding buffer at a flow rate of 0.5 ml min⁻¹. Bound protein was eluted with elution buffer (binding buffer containing 10 mM maltose). Fractions containing the fusion protein were pooled and passed through a desalting column (High Prep 26/10) equilibrated in 50 mM NaH₂PO₄ pH 8.0 and finally concentrated to 2 mg ml⁻¹ using Amicon Ultra centrifugal devices (Millipore). To further enrich the MBP–NmB-PolyST fusion protein, a gel filtration chromatography step was applied. Samples were filtered (Millipore Ultrafree MC 0.2 µm) and loaded on a Superdex 200 10/300 GL column (GE Healthcare). Proteins were eluted at a flow rate of 0.5 ml min⁻¹ with 50 mM NaH₂PO₄ pH 8.0 buffer and fractions of 0.5 ml were collected. Obtained protein samples were stable for more than 3 weeks if stored at 4°C.

SDS-PAGE and immunoblotting

SDS-PAGE was performed under reducing conditions using 2.5% (v/v) β-mercaptoethanol and 1.5% (w/v) SDS. For Western blot analysis, proteins were blotted onto nitrocellulose (Whatman). Proteins containing an N-terminal StreptII-tag were detected by Streptactin-alkaline phosphatase-conjugate (StrepTactin-AP; IBA) according to the manufacturer’s guidelines. His-tagged proteins were detected with 1 µg ml⁻¹ penta-His antibody (Qiagen) followed by goat anti-mouse IgG-AP (Dianova). For quantification by infrared fluorescence detection, samples and standard proteins were blotted onto PVDF membranes (Millipore). His-tagged proteins were detected with 1 µg ml⁻¹ penta-His antibody (Qiagen) followed by 50 ng ml⁻¹ goat anti-mouse IR680 antibody (LI-COR) and quantified according to the recommendations of the Odyssey infrared imaging system (LI-COR).

Radiochemical polyST assays

PolyST activity in bacterial lysates was analysed as described previously (Weisgerber et al., 1991). Briefly, 10 µl of lysate was mixed with 10 µl of TMD buffer (40 mM MgCl₂, 50 mM, Tris/HCl pH 8.0) and 2 mM of colonic acid (100 mg ml⁻¹) as acceptor and reactions were started by adding 2 µl of CMP-[¹⁴C]Neu5Ac (13 mM, 1.55 mCi mmol⁻¹). Samples were incubated at 37°C and 5 µl of aliquots were spotted on Whatman 3MM CHR paper after the respective reaction time. Following descending paper chromatography, the chromatographically immobile [¹⁴C]-labelled polyST reaction products were quantified by scintillation counting. To analyse the products of purified NmB-polyST in more detail, reaction mixtures were analysed in the TBE-buffered (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) electrophoresis system described for analysis of acidic capsular polysaccharides (Pelkonen et al., 1988). Enzyme reactions were carried out in TMD in a total volume of 24 µl as described above, using 10 µg of enzyme and 0.001–1 mM α2,8-linked sialic acid (DP5) as acceptor. Control samples were subsequently treated with 1 µg of polySia-degrading endoN (Stummeyer et al., 2005) for 20 min at 37°C. Equal volumes of sample buffer (2 M sucrose in TBE) were added and samples were electrophoresed at 4°C and 200 V overnight. To visualize [¹⁴C]-labelled reaction products, gels were vacuum-dried immediately after electrophoresis and exposed to an imaging film (BioMax, Kodak).

Fluorescent polyST assays

PolyST activity of purified NmB-polyST was also monitored using the fluorescent acceptor GT3-FCHASE as acceptor (Vionnet and Vann, 2007). Briefly, GT3-FCHASE (0.23 µM), CMP-Neu5Ac (50–500 mM) and purified NmB-polyST (30–180 µg ml⁻¹) were incubated in TMD buffer (40 mM MgCl₂, 50 mM, Tris/HCl pH 8.0) at 37°C. At the indicated time points (2–30 min) reactions were stopped by adding to 25% ethanol and samples were further analysed by HPLC as described (Vionnet and Vann, 2007).

Continuous spectrophotometric polysialyltransferase assay

For rapid characterization of purified polysialyltransferases, the glycosyltransferase testing system described by Gosselin et al. (1988). Briefly, [³⁵S]CMP-Neu5Ac (50–500 µM) and purified NmB-polyST (30–180 µg ml⁻¹) were incubated in TMD buffer (40 mM MgCl₂, 50 mM, Tris/HCl pH 8.0) at 37°C. At the indicated time points (2–30 min) reactions were stopped by adding to 25% ethanol and samples were further analysed by HPLC as described (Vionnet and Vann, 2007).
et al. (1994) was adapted to polysialyltransferases. All measurements were carried out in 96-half area well plates (Greiner Bio-one) in a total volume of 106.5 μl. In detail, a master solution was prepared containing the linking enzymes pyruvate kinase (16.5 U ml⁻¹, Sigma), lactate dehydrogenase (23.5 U ml⁻¹, Sigma) and nucleotide monophosphate kinase (0.5 U ml⁻¹, Roche) in reaction buffer [1.5 mM ATP (Sigma), 1 mM PEP (Fluka), 0.13 mM NADH (Roche), 14 mM MgSO₄, 56 mM KCl in 100 mM Tris pH 7.5]. As acceptor substrate either colaminic acid or sialyloligomers were added in various concentrations. After addition of the donor substrate CMP-Neu5Ac (62 μM to 2 mM) samples were first monitored at OD 340 nm until a stable baseline was reached (3–5 min). This was essential to metabolize free CMP, which is always present in CMP-Neu5Ac preparations due to hydrolysis of the substrate. Finally, PST was added (10–20 U ml⁻¹) and reactions were followed until the total amount of NADH was metabolized.

Generation and analysis of mutant Neisseria strains

For mutagenesis of N. meningitidis strains, the plasmids pET23a-G154A, pET23a-E153A, pET23a-H278A and pET23a-P279A needed to be modified to allow regular homologous recombination into the meningococcal chromosome and selection for the mutants. The siaD downstream region was amplified from serogroup B strain MC58 with primers GH149 (5′-GGG CGC TTC GAG AAT ACT ATG ACT TCT GC TCT CC-3′) and GH150 (5′-GGG CGC TTC GAG CTA ATG GTA ATT TGA CAA TAG AGC G-3′) and integrated into the meningococcal chromosome downstream of the mutated siaD gene using the respective XhoI sites (underlined). The resulting plasmids were linearized with NotI, blunt ended with T4 DNA polymerase and ligated with the kanamycin resistance cassette excised from pUC4K (GE Healthcare) by HincII. The final plasmids harbouring the kanamycin resistance cassette between the siaD gene and the siaD downstream region were used to transform serogroup B meningococcal strain MC58. Transformants were selected on GC agar supplemented with 100 μg ml⁻¹ kanamycin. Recombination of the mutagenized motifs into the meningococcal siaD gene was verified by sequencing the PCR product obtained with primer pair GH157 (5′-CA GGC CAC TAC TCT TAT C TG-3′)/Kana2 (5′-GAT TTT GAG ACA CAA CGT GG-3′) with either primers GH157 and GH160 (5′-AGG TTC ATT AAT AAC TAC CAG C-3′)/D/E/D/E/G motif) or primers U8ea (5′-AA CGC TAC CCC ATT TCA-3′) and GH160 (HP motif). Furthermore, regular homologous recombination was confirmed by Southern blot hybridizations with the siaD gene and the kanamycin resistance gene used as a probe respectively. The meningococcal capsule phenotype was analysed by whole-cell ELISA as described above (Vogel et al., 2001). Quantitative analysis of capsule expression was performed by whole-cell ELISA as described (Vogel et al., 2001). Briefly, microtitre plates were pre-coated with poly d-lysine (25 μg ml⁻¹ in PBS) for 1 h at room temperature. After three washing steps with PBS, bacterial suspensions (20 μl well⁻¹, OD₆₀₀ = 0.10 in PBS) were applied for 2 h and cross-linked to poly d-lysine by adding glutaraldehyde (0.05% in PBS) for 10 min. Plates were washed three times with PBS and non-specific binding sites were saturated by incubation with 1% BSA in PBS for 1 h. After three washing steps capsular polySia was detected by immunostaining using the polySia-specific mab 735. The amount of bacteria bound to each well was controlled with a parallel set of microplates using mab P1.7 directed against the PorA antigen of the meningococcal strain MC58 for detection. Subsequently, plates were incubated with peroxidase coupled secondary antibody (Dianova) and analysed by colour reaction.

In vivo analysis of MBP–NmB–polyST

Neisserial expression constructs were generated for in vivo comparison of wild type and MBP–NmB–polyST. The MBP–polyST insert of the E. coli expression construct pMBP-Strep-NmB–polyST was amplified with primers HC574 (5′-GGG CGC TTC AGA GAA GGA GAT ATA CAT ATG AAA AC-3′) and HC572 (5′-GGG CGC TTC AGA GAA GGA GAT ATA CAT ATG AAA AC-3′) and integrated between the SpeI and EcoRV sites of the neisserial expression vector pAP1 (Lappann et al., 2006) resulting in pAP1-MBP–NmB–polyST. For comparison also the polyST gene without further tags was amplified with primers HCS73 (5′-GGG CGC TTC AGA GAA GGA GAT ATA CAT ATG GTA ATT AAG AAA ATA AAA AC-3′) and HC572 and integrated into the EcoRV site of pAP1 resulting in pAP1-NmB-polyST. Further in vivo studies were performed using strain 2517, an unencapsulated polyST knockout mutant of the meningococcal serogroup C strain 2120 (Ram et al., 2003) that was transformed with the resulting plasmids. Wild type and transformants were analysed for capsule expression by whole-cell ELISA as described above. Bacterial loading was controlled using anti-PorA antibody P1.2. Additionally, neisserial lysates were analysed by Western blot analysis. Bacteria were pelleted from 2 ml of suspension cultures (OD₆₀₀ = 1.1), re-suspended in 100 μl of Lämmli sample buffer [100 mM Tris pH 6.8, 1.7% SDS, 16.5% glycerol (v/v), 2.5% 2-mercaptoethanol, bromophenol blue] and lysed by sonication. The lysates were centrifuged (16.000× g, 10 min, 4°C) and supernatants were divided in two aliquots. One aliquot was subsequently digested with 1 μg of endoN (Stummeyer et al., 2005) and incubated for 15 min at room temperature followed by a 15 min incubation at 37°C. All samples were incubated for 10 min at 60°C prior to electrophoresis. SDS-PAGE and Western blot were performed as described above.

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**Supplementary material**

The following supplementary material is available for this article:

**Fig. S1.** Elongation of trimeric α-2,8-linked sialic acid (DP3) by purified NmB-polyST.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05862.x

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