Biosynthesis of the Escherichia coli K1 group 2 polysialic acid capsule occurs within a protected cytoplasmic compartment

Susan M. Steenbergen and Eric R. Vimr*
Laboratory of Sialobiology and Comparative Metabolomics, Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802, USA.

Summary
Capsular polysaccharides are important virulence determinants in a wide range of invasive infectious diseases. Although capsule synthesis has been extensively investigated, understanding polysaccharide export from the cytoplasm to the external environment has been more difficult. Here we present the results of a novel protection assay indicating that synthesis and export of the Escherichia coli K1 group 2 capsular polysialic acid (K1 antigen) occur within a protected subcellular compartment designated the sialisome. In addition to the polymerase encoded by neuS, localization and complementation analyses indicated that the sialisome includes the accessory membrane protein NeuE. The requirement for NeuE was suppressed by overproducing NeuS, suggesting that NeuE functions by stabilizing the polymerase or facilitating its assembly in the sialisome. Although an interaction between NeuE and NeuS could not be demonstrated with a bacterial two-hybrid system that reconstitutes an intracellular cell-signalling pathway, interactions between NeuS and KpsC as well as other sialisome components were detected. The combined results provide direct evidence for specific protein–protein interactions in the synthesis and export of group 2 capsular polysaccharides under in vivo conditions. The approaches developed here will facilitate further dissection of the sialisome, suggesting similar methodology for understanding the biosynthesis of other group 2 capsules.

Introduction
Microbial exopolysaccharides, defined here as cell-adherent capsules or excreted, unattached or loosely attached slime, are used as additives in a wide range of food and cosmetic products, medical replacements in ocular and joint surgeries, and are essential components of microbial biofilms (Sutherland, 1998; 2001). Some exopolysaccharides are also important virulence determinants in bacterial or fungal invasive disease where, as capsules surrounding the microbial cell surfaces, they function as ligands for cell adhesion or modulators in the avoidance or inhibition of innate and acquired immunity (Vimr et al., 2004). Capsular polysaccharides conjugated to protein carriers conferring T-cell dependency are among the most effective anti-capsule vaccines in current medical use: Streptococcus spp., group b Haemophilus influenzae, and all groups except group B Neisseria meningitidis, with more anti-capsule vaccines in development (Robbins and Schneerson, 2004). Supporting these applied uses of exopolysaccharides are basic studies to understand the molecular details of capsule biosynthesis, which we define here as the processes of intracellular polysaccharide synthesis and export (secretion or translocation) to the external environment. In many cases, the linkage of polysaccharides to the outer membrane of Gram-negative bacteria or cytoplasmic membrane of Gram-positive organisms is unknown (Whitfield, 2006).

Escherichia coli is a model organism for investigating bacterial exopolysaccharide function and biosynthesis. Different types of E. coli capsules have been subdivided into one of four biosynthetic groups (Whitfield, 2006). After synthesis of the required nucleotide sugar(s) unique to a given polymeric structure, functionally diverse glycosyltransferases catalyse synthesis of homo- or heteropolysaccharides that are exported to the cell surface by ATP-binding cassette-like (ABC) transporters or Traffic ATPases, in the case of groups 2 and 3 capsules, or by Wzy-dependent machineries required for groups 1 and 4 export. In the case of groups 2 and (probably) 3 polysaccharides, some sugar chains are anchored to the cell membrane by esterification to phospholipid, further distinguishing them from the lipid A-linked somatic or O
antigens of lipopolysaccharide. Mechanistic distinctions are also based on the genetic regulation and map positions of the capsule gene clusters. We suggest a fifth group of polysaccharides in which the glycosyltransferase (polymerase) simultaneously functions as the translocase. This system, where the polymerase is also the cytoplasmic membrane exporter, has been inferred for the biosynthesis of Gram-positive and -negative polyglucosamines and hyaluronans (Wang et al., 2004; Fluckiger et al., 2005; Tlapak-Simmons et al., 2005). Despite our understanding of groups 1 and 4 Wzy-dependent export systems, transport of group 2 capsular polysaccharides has been a more difficult process to study (Whitfield, 2006). This difficulty stems in part from problems in reconstituting the translocation process after subcellular fractionation (Vimr and Steenbergen, 1993).

Attempts to reconstitute polysialic acid (PSA) synthesis and export using inverted (inside-out) membrane vesicles and an in vitro protection assay indicated that only a relatively small and temporally constant fraction of the total PSA synthesized by this system was resistant to endo-N digestion, implying a failure of the system to export polysialic acid (Vimr and Steenbergen, 1993). Endo-N is a K1-specific bacteriophage-derived depolymerase that cleaves α2,8-linked sialic acid residues of PSA chains \( \geq 7 \) monomers in length (Petter and Vimr, 1993). Our in vitro results suggested that some factor or physical property of the system was lost during subcellular fractionation which prevented efficient export of PSA despite continual polymer synthesis in the presence of exogenously added sugar nucleotide donor cytidine 5′-monophospho N-acetylmuraminic acid (CMP-Neu5Ac) and ATP, and thus continual sensitivity to endo-N, a homotrimer composed of 76 kDa subunits. Although the ‘flippase’ activity of Wzx found in groups 1 and 4 systems has been demonstrated in vitro (Rick et al., 2003), we are unaware of any successful reconstitution of group 2 polysaccharide export. We concluded from our in vitro protection studies that PSA biosynthesis involves essential protein–protein, protein–polysaccharide or protein–lipid interactions that are disrupted during fractionation. In the current study, we describe an in vivo investigation of PSA biosynthesis that provides the first direct evidence for a novel subcellular compartment, which we propose to designate the sialosome. Similarly privileged cytoplasmic compartments may exist in other group 2 polysaccharide biosynthetic systems.

Results and discussion

Mechanisms and models of capsular polysaccharide biosynthesis

Groups 2 and 3 capsule biosynthetic gene clusters include loci unique to the structural requirements of a given polysaccharide (region 2) and sets of shared regions 1 and 3 genes encoding the ABC transporters and several proteins with unclear functions which are nonetheless required for polymer export (Fig. 1). We suggest three possible biosynthetic mechanisms to explain the synthesis and export of group 2 capsules. In model 1 (Fig. 2), synthesis and export are obligatorily coupled processes. In such a coupled system, loss of export function would result in the concomitant loss of capsule synthesis, as observed for the \( E. coli \) K30 polysaccharide that fails to accumulate in a mutant lacking the outer membrane protein Wza (Whitfield and Paiment, 2003). By contrast, group 2 export-defective mutants accumulate full-length intracellular polysaccharides in all regions 1 and 3 or neuE mutants so far investigated (reviewed in Vimr et al., 2004).

Model 2 indicates a two-step process in which synthesis occurs independently of export, and where phospholipid modification of the reducing terminus may trigger activation of or signal PSA entry into the export apparatus. This hypothesis is consistent with the accumulation of full-length polysaccharides in group 2 mutants with region 1 or 3 export defects, potentially explaining how a conserved export apparatus transports structurally distinct polysaccharides by recognizing a common chemical modification or secondary structural feature (reviewed in Vimr et al., 2004). Because the products of the region 1 \( kpsC \) and \( kpsS \) genes are homologous with certain enzymes of lipid metabolism, and because mutations in these genes were reported to cause accumulation of...
intracellular group 2 polysaccharides lacking the reducing terminal phospholipid modification (Bronner et al., 1993; Frosch and Muller, 1993), model 2 seemed to be supported. However, the experimental observations supporting model 2 are inconsistent with the phenotypes of _E. coli_ K1 kpsC or kpsS mutants, which accumulate lipidated PSA, suggesting that lipidation is insufficient for group 2 capsule export (Cieslewicz and Vimr, 1996). The explanation for the experimental discrepancies is unclear, but suggests that neither KpsS nor KpsC is required for lipid addition, calling model 2 into question. This conclusion is supported by a recent report that group B meningococcal kpsS (lipB or ctrC) and kpsC (lipA or ctrD) mutants also accumulate lipidated, intracellular PSA (Tzeng et al., 2005). The reason why previous studies did not detect polysaccharide lipidation in _E. coli_ K1 kpsC or kpsS mutants in the _E. coli_ K5 or meningococcal systems may have been failure to control for the heterologous or non-stoichiometric expression systems used in these studies, or relatively low sensitivity of the detection methods. In contrast, studies detecting lipidation of PSA in _E. coli_ K1 and group B meningococcal kpsS and kpsC mutants were carried out with strains containing single-copy capsule gene clusters and enzymatic or structural methods that unambiguously detected phospholipid attachment.

Model 3 is a hybrid of models 1 and 2 and predicts that while polysaccharide synthesis occurs in the absence of transport, only those polymers synthesized in the presence of an intact biosynthetic complex are exported. In the extreme case, assembly of the functional translocase would occur only when triggered by nascent group 2 polysaccharide. Model 3 suggests intimate contact between synthetic and export components of group 2 biosynthetic complexes, consistent with the reduced polymerase activities observed for all regions 1, 3 and neuE mutants (reviewed in Vimr et al., 2004). This phenotype indicates that polymerase catalytic efficiency is affected by the operation or structure of the export apparatus, which is qualitatively similar to the feedback mechanism inferred for group 1 exopolysaccharides.

**Polysialic acid synthesis and export occur within a protected subcellular compartment operationally defined as the sialisome**

To distinguish between models 2 and 3 (Fig. 2), we transformed the wild-type K1 strain EV36 with plasmid expressing endo-N under control of a lac promoter and assayed for PSA expression by sensitivity to phage K1F. Endo-N is a PSA-specific depolymerase that is also the tailspike protein for K1-specific bacteriophage, where it functions as a necessary phage organelle for infection (Vimr et al., 1984; Petter and Vimr, 1993). As shown in Fig. 3, the transformed strain was as susceptible to phage
infection as the EV36 (wild type) control. As productive lytic infection requires an extracellular PSA receptor, the results suggested that polymer synthesis and all of the steps necessary for or leading to translocation occur within an endo-N-inaccessible compartment, operationally defined here as the sialisome, and providing direct experimental support for model 3 (Fig. 2). Similar results were obtained whether IPTG was present in the plates or not, or whether the recipient was co-transformed with pRep4, which carries the lacI repressor, indicating that endo-N gene expression was not limiting.

As shown in Fig. 4B, EV36 had the same efficiency of K1F plating as its uninduced (Fig. 4C) or induced (Fig. 4D) derivatives harbouring pRep4 and pEndo-N. As expected, acapsular mutant EV93 (kpsC) did not support phage infection (Fig. 4A) because it lacks surface capsular polysaccharide, although it accumulates intracellular, unexported PSA in centrally located compartments termed lacunae to indicate their electron transparency (Cieslewicz and Vimr, 1996). Although plaque size differed under the three infection conditions (Fig. 4B–D), the results indicated that capsule was produced independently of the amount of intracellular endo-N, from none (Fig. 4B) to approximately 2% of the intracellular protein concentration of induced cells harbouring pRep4 and pEndo-N (Fig. 4D). We suggest that recombinant endo-N released by phage lysis accounts for the morphological plaque differences by altering cell-surface capsule or PSA released by disrupted cells. However, note that plaque size in the induced strain (Fig. 4D) was at least twice that of wild type (Fig. 4B) despite a reduced host growth rate caused by enzyme overproduction, indicating that even a massive accumulation of intracellular endo-N did not

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**Fig. 3.** PSA biosynthesis is protected from endo-N in vivo. Approximately 75 µl of K1-specific phage K1F (1 × 10^10 pfu ml^-1 phage stock solution) was streaked (arrow) down the middle of the plate with a sterile wooden stick, and bacteria were dragged across the phage from left to right. The plate was photographed after overnight incubation at 37°C. A, E. coli K-12 strain MC4100; B, acapsular mutant EV93; C, EV36 harbouring pEndo-N; D, EV36.

**Fig. 4.** Effects of recombinant endo-N overproduction on K1-specific phage sensitivity. Bacteria were grown to early stationary phase with appropriate drugs and IPTG added where indicated to a final concentration of 1 mM to induce endo-N production when cells reached an A_600 of 0.3. Bacteria (0.15 ml) were plated with approximately 85 K1F plaque forming units in top agar on LB and incubated at 37°C overnight prior to photography. A, Acapsular mutant EV93. B, EV36 wild type. C, EV36 harbouring pRep4 and pEndo-N, uninduced. D, EV36 harbouring pRep4 and pEndo-N, induced and plated to LB containing 1 mM IPTG.

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To demonstrate that both basal and induced endo-N productions by EV36 transformed with pRep4 and pEndo-N were sufficient for PSA degradation, we carried out radial immunodiffusion against anti-PSA antibody recognizing PSAs ≥ 8–10 sialic acid residues. If endo-N is present, PSA is degraded to oligomers < 8 residues in length that do not react with antibody. As shown in Fig. 5, extracts of both wild type (well 1) and mutant strain EV93 (well 2) contained PSA, as evident by the precipitin halos surrounding the respective wells. Note that strain EV93 PSA is derived from unexported, intracellular PSA whereas the wild-type antigen comes from capsular polysaccharide released during cell disruption (Cieslewicz and Vimr, 1996). In contrast, PSA from EV36 was degraded in the presence of basal or induced amounts of endo-N, resulting in the absence of precipitin halos (Fig. 5, wells 3 and 4 respectively). To directly demonstrate the susceptibility of intracellular PSA to endo-N, extracts of EV93 were mixed 1:1 with those of uninduced (Fig. 5, well 5) or induced (Fig. 5, well 6) extracts from EV36 harbouring pRep4 and pEndo-N. The absence of PSA antigen under either condition indicates that the expression of even a basal amount of endo-N is sufficient to depolymerize intracellular PSA.

An alternative argument for the results shown in Figs 3 and 4 is that endo-N is inactive when expressed intracellularly. To show that endo-N is active in vivo, we investigated PSA stability by transmission electron microscopy (TEM) of intact and thin-sectioned cells. We had previously shown by TEM that E. coli K1 export mutants accumulate lipidated PSA in intracellular lacunae unbounded by a membrane and displaying morphologies unique to a particular export defect (Cieslewicz and Vimr, 1996; 1997), indicating that E. coli normally lacks depolymerase(s) for turnover of the PSA that accumulates in these export mutants. In the case of strain EV93, the unexported PSA tends to accumulate in centrally located lacunae evident in thin sections (Cieslewicz and Vimr, 1996), a result that was confirmed independently for this study (Fig. 6D). The PSA in these accretion bodies bear terminal phospholipids that we assume result from the intracellular micelles formed by otherwise highly soluble and electrostatically charged polysaccharides. Lacunae thus represent intracellular organelles resulting from synthesis of full-length, lipidated PSA that either does not enter the translocation pathway or is aborted during biosynthesis. When whole mounts of negatively stained EV93 were examined by TEM, lacunae were apparent as bubble-like organelles in the intact cells (Fig. 6A and B). In contrast, lacunae were absent in EV93 transformed with pEndo-N with or without IPTG induction (Fig. 6C), indicating that basal endo-N expression was sufficient to degrade intracellular PSA. Similarly, lacunae were not detected when EV93 harbouring pEndo-N was examined by TEM of thin-sectioned samples (Fig. 6E). Taken together, the results of the intracellular endo-N protection assay demonstrate in vivo depolymerase activity against intracellular PSA, supporting the conclusion that wild-type PSA synthesis and export occur within an environment inaccessible to endo-N. Note that this operational definition of the siasosome does not preclude the concept of a kinetic compartment, in which the synthesis of PSA is so rapid that endo-N, even at induced concentrations, does not have time to degrade nascent PSA. However, because even one intra-chain clip would be expected to disrupt biosynthesis, we think it is more likely that the siasosome represents the functional biosynthetic complex that has been previously shown to involve multiple protein–protein interactions (McNulty et al., 2006).
important, our results tend to exclude model 2 by favouring the directed coupling of PSA biosynthesis to its export shown in model 3 (Fig. 2).

Complementation and suppression of the neuE translocation defect by neuS overexpression

We previously showed that inactivation of neuE results in reduced NeuS activity and accumulation of intracellular PSA, a translocation-defective phenotype similar in all regions 1 and 3 mutants so far investigated, suggesting that NeuE is not obligatory for PSA synthesis but is required for export (Vimr and Steenbergen, 1993; Vimr et al., 2004). However, a recent study indicated that NeuE was part of a NeuS complex, suggesting that NeuE might function directly in synthesis (Andreishcheva and Vann, 2006). Because our previous studies could not distinguish between a polar effect of the neuE mutation in EV725 on neuS expression and a direct effect of the mutation on translocation, we carried out a series of complementation experiments to distinguish between these possibilities. As shown in Fig. 7, complementation in trans with neuE+ carried by pSX94 restored the unencapsulated recipient to an encapsulated phenotype, as detected by sensitivity of the transformant to K1-specific lytic phage infection. Surprisingly, plasmid pSX92, which carries a truncated copy of neuE but has neuS+ oriented in the same direction as the lacZ promoter, complemented EV725 (Fig. 7), suggesting that neuS overexpression suppresses the requirement for neuE. This conclusion was supported by maxi-cell analysis and the complementation pattern for plasmids pSX90 and pSX91 with oppositely oriented neuS (Figs 7 and 8A). As expected, plasmid pSR426, which carries all of regions 2 and 3, complemented EV725 (Fig. 7). In agreement with the translational start of neuE overlapping the upstream neuC gene (Andreishcheva and Vann, 2006), plasmid pSX93 did not complement strain EV725 (Fig. 7).

NeuE has been shown to include a putative C-terminal polyprenoid recognition signal (PIRS) that could play a role in polyprenol binding during PSA synthesis or translocation (Fig. 8B) (Steenbergen et al., 1992; Zhou & Troy, 2003, 2005). However, NeuS activity is not eliminated in a neuE null mutant, indicating that PIRS is not obligatory for polymerization (Vimr et al., 2004), a conclusion supported by the analyses shown in Figs 7 and 8A. To determine if the NeuE PIRS domain functions as a membrane anchor, and thus a possible structural role consistent with the export defect of a neuE mutant (Vimr et al., 2004), we...
carried out localization experiments with maltose binding protein (MBP) fused to the domain.

Depending on the programme used, neuE is predicted to encode a polypeptide with one to five membrane-spanning regions (Table 1). Each of the nine programmes identified the putative C-terminal PIRS domain while four of them uniquely identified PIRS as the only membrane-spanning region (Table 1). To confirm that PIRS functions as membrane anchor, we fused the BglII-BamHI fragment from pSX92 to the MBP polylinker in plasmid pMal-c2, generating plasmid pSX101 expressing an N-terminal MBP lacking its leader peptide sequence fused to the PIRS domain and the last 25 amino acid residues of NeuE. As shown in Fig. 8C (lanes 4 and 5), an overproduced polypeptide of the expected molecular weight was localized to the membrane fraction after differential centrifugation and denaturing polyacrylamide gel electrophoresis, indicating that PIRS anchored a soluble polypeptide to what we presume is the inner membrane. No overproduced polypeptide was detected in the soluble fraction (Fig. 8C, lanes 6–9), indicating efficient localization of the putative fusion to the membrane. However, the PIRS anchor is unlikely to span the membrane because nine of the 25 amino acid residues following it to the C-terminus are positively charged and therefore unlikely to cross into the periplasm. On the basis of secondary structural predictions (MacVector 9.0.2), we further suggest that the PIRS domain is unlikely to be an alpha helix, and more likely to be an amphipathic beta sheet reminiscent of the proposal for a similar anchoring domain in hyaluronan synthase (Heldermon et al., 2001). Thus, while the exact function of NeuE is still unclear, our results

Table 1. Computer-assisted analysis of NeuE TM regions.

| Program       | No. of NeuE TM regions | NeuE orientation N-terminus | C-terminus |
|---------------|------------------------|-----------------------------|------------|
| DAS           | 3                      | ND                          | ND         |
| HMMTOP 2.0    | 4                      | i                           | i          |
| PRED-TMR 2    | 1                      | ND                          | ND         |
| SPLIT 3.5     | 1                      | ND                          | ND         |
| TMAP          | 3                      | ND                          | ND         |
| TM-Finder     | 3                      | ND                          | ND         |
| TMHMM 2.0     | 1                      | i                           | i          |
| TMpred        | 5                      | i                           | o          |
| TopPred II    | 1                      | i                           | o          |

ND, not determined; o, outside (periplasmic); i, inside (cytoplasmic).
indicate that it is not obligatory for PSA synthesis, although necessary for export. We have not yet tested whether deletion of this domain affects the export function.

Controls with anti-MBP confirmed that the overproduced fusion polypeptide included the expected MBP epitope(s). Thus, the pMal-c2 vector alone synthesized the expected 53 kDa polypeptide (MBP-β-gal-α), whereas fusion of an almost full-length copy of neuE (pSX104) synthesized the expected 65 kDa polypeptide resulting from truncation of the lacZ α peptide of the vector by cloning the EcoRI-HindIII fragment from pSX90 into the pMal-c2 polylinker (Fig. 8D, lanes 3 and 1 respectively). The polyclonal anti-MBP antibody also detects lower-molecular-weight bands probably arising from proteolysis, including prominent bands near the 43 kDa size of native MBP, and a band common to strains independent of IPTG induction (Fig. 8D, lanes 1–5), representing an E. coli antigen recognized by the polyclonal antibody preparation. Induction of the strain expressing the malE-BglII-BamHI fragment from pSX90 (pSX101), which codes for the PIRS domain and last 25 amino acid residues of NeuE, stably produced the expected 45 kDa fusion polypeptide (Fig. 8D, lane 2) that was observed in the membrane fraction of induced cells (Fig. 8C, lanes 4 and 5). The results of this Western blot analysis confirm the identity of the overproduced membrane polypeptide as MBP fused to the NeuE PIRS domain. We conclude that this domain functions as NeuE membrane anchor, as predicted by the results in Table 1.

Predictions of the sialisome concept

Involvement of the siaisome in PSA synthesis and translocation predicts interactions between NeuS and the export apparatus. Silver and colleagues demonstrated that KpsM and KpsT function as the inner membrane exporter and ATPase components of the group 2 ABC transporter respectively (Pigeon and Silver, 1994; Bliss et al., 1996). KpsS is thought to include a large periplasmic domain that might connect the ABC transporter to the outer membrane, possibly involving KpsD as chaperone or outer membrane pore (Arrecubieta et al., 2001; McNulty et al., 2006). KpsC and KpsS share homology with enzymes of lipid metabolism and appear from Western blot analyses to interact with the inner membrane (Rigg et al., 1998). The requirement or function in PSA biosynthesis of KpsF (isomerase) and KpsU (CMP-ketodeoxyoctonate, KDO, synthetase) is uncertain, although in the K5 system KDO is thought to link the capsular polysaccharide chain to phospholipid (Whitfield, 2006 and references cited therein). Except for the ABC transporter, the exact functions of other kps gene products are unclear, although mutation of most region 1 or region 3 and neuE genes results in an acapsular phenotype and diminution of in vitro NeuS activity (reviewed in Vimr et al., 2004).

To develop an in vivo approach for investigating PSA synthesis and export, we used a two-hybrid bacterial system for the detection of both homo- and heterotypic protein–protein interactions. Daines and Silver (2000) previously used a Lex-based system to identify an interaction between NeuD and NeuB, implicating NeuD as a stabilizer or activator of the synthase (Fig. 2), a function recently supported by protein engineering of the homologous group B streptococcal NeuD (Lewis et al., 2006). This conclusion is consistent with the function of NeuD as a monomeric sialic acid transacetylase (Steenbergen et al., 2006), in which case direct interaction with NeuB might ensure efficient acetylation during the synthase-catalysed condensation of ManNAc with PEP to produce sialic acid. As an alternative to the Lex system, which requires the use of special strains to prevent possibly conflicting homodimeric interactions, we tested the applicability of the Bordetella pertussis adenylate cyclase system (Karimova et al., 1998; 2000). This system depends on reconstitution of an active adenylate cyclase and subsequent transcriptional amplification of cAMP-dependent promoters, such as lac or mal expressed in an E. coli cya host. Because the signalling cascade is intracellular, we did not expect to observe interactions between extracellular components of the PSA biosynthetic machinery, as supported by the lack of interactions between KpsD and all prey tested (Table S1). In contrast, both homo- and heterotypic interactions were detected between a variety of other kps and soluble or membrane-bound neu gene products.

Detection of interacting pairs

The two-hybrid system tests genes or gene fragments fused at the C-terminus of T25 (pKT25 bait derivatives), and prey or prey constructs fused to the N- or C-terminus of T18 in pUT18 and pUT18C respectively. Plasmids co-transformed into a cya background are examined for their Mal phenotypes (white, no interaction; red, strong interaction; pink, weak interaction) on MacConkey-maltose agar plates, and then assayed for beta-galactosidase using the standard reaction with o-nitrophenyl β-D-galactoside substrate (Miller, 1972). Backcrossing plasmids into fresh backgrounds confirms that the phenotypes are dependent on the expressed fusions. Positive controls detect interaction between leucine-zipper (Zip) fragments cloned into pKT25 and pUT18C (Karimova et al., 1998). We found that apparent instability of the BTH101 recipient strain sometimes resulted in mixed phenotypes, presumably caused by spontaneous mutation of the reporter gene to cAMP inde-
Two-hybrid analysis of selected pair constructs in EV727 and EV729, capsule-negative and capsule-positive, respectively, 

| Bait/prey plasmids | Gene pairs (bait/prey) tested | Phenotypes on MacConkeya | [β-Galactosidase activityb] | Phenotypes on MacConkey | [β-Galactosidase activityb] |
|-------------------|-------------------------------|--------------------------|-----------------------------|---------------------------|-----------------------------|
|                   |                               | Early | Late | Early | Late | Early | Late |
| pKT25/pUT18C      | None                          | –     | 24 ± 9 | 23 ± 1 | –     | 31 ± 2 | 26 ± 2 |
| pKT25-zip/pUT18C-zip | zips                          | ++   | 830 ± 90 | 5130 ± 140 | ++   | 360 ± 120 | 4820 ± 450 |
| pSX750/pSX753     | kpsC/kpsE                     | +     | 220 ± 60 | 540 ± 60 | +     | 200 ± 80 | 760 ± 180 |
| pSX750/pSX758     | kpsC/kpsE                     | +     | 65 ± 13 | 120 ± 20 | +     | 230 ± 4 | 1170 ± 250 |
| pSX750/pSX762     | kpsC/neuS                    | –     | 18 ± 1 | ND     | –     | 15 ± 1 | ND     |
| pSX751/pSX753     | neuS/kpsC                    | ++   | 560 ± 73 | 4700 ± 70 | ++   | 420 ± 80 | 5540 ± 280 |
| pSX751/pSX758     | neuS/kpsE                    | –     | 58 ± 3 | 500 ± 75 | +     | 140 ± 19 | 1050 ± 120 |
| pSX571/pSX767     | neuS/neuE                    | –     | 17 ± 1 | ND     | –     | 16 ± 1 | ND     |

a. Negative (white); ++, strong positive (deep red); +, weak positive (red). Colonies were scored for Mal phenotypes on MacConkey-maltose agar plates after 2 days of incubation at 30°C.

b. [β-Galactosidase activity was measured when cells were between A600 values of 0.2–0.5 (early) or after overnight incubation (late). Data are given as the mean ± SEM for triplicate experiments.

ND, not determined.

dependence or reversion of the cya defect. Expressing plasmids in the recA strain DHM1 did not solve this inconsistency of the strain background, as expected by the lack of kps and neu genes in the E. coli K-12 genome. Therefore, we constructed E. coli K-12 (BW30270) and K1 (EV36) cya::kan strains EV726 and EV728, respectively, by P1 transduction from strain MERR4. After curing the kan cassettes with pCP20 (Datsenko and Wanner, 2000), the EV727 and EV729 recipients were used for co-transformation of bait and prey plasmids in the event that wild-type kps or neu gene products present in the EV36-derived background might influence some interactions. In general, results comparing the two host genetic backgrounds were qualitatively identical, although in some cases, as discussed in the following section, the genetic background had a quantitative effect on the interaction.

Figure S1 shows a schematic diagram for one representative experiment testing interactions between a KpsC bait and a variety of prey plasmids expressed in BTH101, with some prey fusions tested in either of two orientations relative to the B. pertussis cya fusion partners. Note that both homo- and heterotypic interactions were detected, suggesting that KpsC has multiple binding domains.

Table 2 shows the data for the positively and selected negatively interacting pairs in the EV727 and EV729 backgrounds. Note that NeuS and KpsC interacted strongly, but only when NeuS was the bait (Table 2). This orientation dependency is likely to reflect steric constraints imposed by the B. pertussis Cya domains, T25 or T18 (Karimova et al., 1998; 2000). Note that all plasmid fusions were sequenced to verify that translation would be in frame. Although we have not confirmed the positive interactions with antibody pull-down experiments, an immunological study in the E. coli K5 system indicated that KpsC-K5 was required for membrane localization of the heparosan (K5 antigen) synthase (Rigg et al., 1998). However, previous results established that NeuS does not require any other kps or neu gene product for membrane association (Steenbergen et al., 1992), suggesting an alternative function for KpsC in group 2 capsule biosynthesis. Surprisingly, we could not detect an interaction between NeuS and NeuE (Table 2) despite the report that such an interaction might be necessary for polymerization (Andreishcheva and Vann, 2006). However, other interactions between KpsC and itself or KpsE were detected (Table 2), suggesting that KpsC plays a role in connecting PSA export to the polymerase, NeuS, adding support to model 3 (Fig. 2).

Vann and colleagues previously detected an in vitro requirement for KpsC in NeuS-catalysed polymerization (Andreishcheva and Vann, 2006). Furthermore, the estimated size of the radiation-sensitive polymerase complex was 78 kDa larger than the predicted molecular weight of the NeuS monomer (approximately 48 kDa), supporting interaction(s) between NeuS and one or more molecular species totalling less than 48 kDa (Vionnet et al., 2006). Note that NeuS did not interact with itself (Table S1), suggesting that the polymerase might function as a monomer as part of a larger complex. Also note that assaying the polymerase after irradiation only detects the size of the complex necessary for polymerization in vitro, indicating that the functional size of the biosynthetic complex (synthesis and export) could be much larger than suggested by enzyme assay. When taken together, the results of our two-hybrid analysis strongly support a direct interaction between KpsC and NeuS. Many other potentially interacting pairs of kps or neu gene products tested...
in the two-hybrid system yielded negative results (Table S1), suggesting no or only weak interactions that may have been below the limits of detection. We reasoned that potentially weak interactions might be distinguishable from true negatives if a more sensitive reporter assay was employed.

**Luminescent assay for detection of interacting plasmid pairs**

Beta-Glo combines the hydrolysis of a luciferase substrate conjugated to beta-galactoside in a sensitive coupled luminescence assay (Hannah et al., 2003). Relative luminescence units (RLU) are quantified with a luminometer designed for the microtiter-plate format, facilitating data acquisition and replicate analyses. NeuC is an UDP-GlcNAc 2′-epimerase that produces ManNAc as the first committed step in sialic acid biosynthesis. However, the expected homotypic interaction was not statistically significant by standard beta-galactosidase assay (Fig. S2A, pair 9), but was observed in the *E. coli* K-12-derived background, EV727 (Fig. S2B, pair 9). Failure to detect a significant homotypic interaction in the EV36-derived background, EV729, could indicate the competitive effect of the wild-type neuC gene product on the interaction if it competes with the bait or prey hybrid polypeptide (Fig. S2A). In contrast, pairs 5, 7 and 8 that were negative by standard assay (Table 2) remained so in the luminescence assay (Fig. S2). Positive interactions between pairs 3, 4 and 6 (Fig. S2) confirmed the results of the standard assay (Table 2), although the stronger responses relative to the Zip control (Fig. S2, pair 2) when bait and prey plasmids were coexpressed in the EV36-derived background could indicate enhancement by other *kps* or *neu* gene products, which are absent in EV727 (Fig. S2B). Note that all *neuC*, *neuE* and *neuS* bait or prey plasmids tested in Tables 2, S1 and Fig. S2 complemented their cognate isogenic mutants RS2918, EV725 and EV136, respectively, whereas none of the *kps* constructs complemented theirs. The failure of individual *kps* genes to complement cognate mutants has been previously observed (Vimr et al., 1989), presumably representing the requirement for coexpression of single-copy regions 1 and 3 genes to deliver precise amounts of each region’s gene products to the synthetic/export apparatus. DNA sequencing confirmed that the cloning of out-of-frame fusions was not the reason for lack of complementation. Therefore, we carried out deletion analysis of the *kpsC* bait plasmid to define the domains for the homo- and heterotypic interactions of KpsC detected by two-hybrid analysis (Table 2, and Figs S1 and S2).

**Deletion analysis of KpsC bait**

To more precisely define the KpsC binding domains, a series of deletion mutations was prepared by digestion from the 3′ end of *kpsC* in pSX750 with exonuclease III and mung bean exonuclease. The deletions resulted in the removal of 2–633 amino acid residues from the full-length (676 amino acid residues) *kpsC* gene product. As shown in Fig. 9, Δ1775 produced an intermediate phenotype whereas longer deletions in Δ1858 and Δ1892 lost homotypic interactions with the pSX753 *kpsC*-encoded prey. These results strongly implicate an N-terminal region of about 85 amino acid residues for each deletion as determined by DNA sequencing of the bait constructs. All data were for plasmid pairs expressed in EV727; comparable results were obtained in the EV729 background.
domain recognizing NeuS, but this will require separate deletion construction because of the orientation dependence on KpsC–NeuS interactions (Table 2). Although the recent purification of the group B meningococcal NeuS orthologue has been shown to polymerize PSA in the absence of other kps or neu gene products (Freiberger et al., 2007), our current results indicate that capsule biosynthesis requires multiple protein–protein interactions for in vivo operation. It is intriguing to speculate that the multiple heterotypic KpsC interactions are required to guide NeuS to the translocation apparatus, with KpsC linking nascent PSA to the KpsE connector. Whether KpsC also has an enzymatic function remains to be determined.

Roberts and colleagues (Rigg et al., 1998) pointed out the apparent kpsC gene duplication event that gave rise to homologous N- (Domain A, residues 60–270) and C- (Domain B, residues 387–598) terminal domains with 39.2% similarity over 210 amino acid residues and 58% overall nucleotide identity in the E. coli K5 biosynthetic system. Our results indicate that Domain A functions homotypically while Domain B, at least in part, is necessary for heterotypic interaction with KpsE. Interestingly, KpsC-K5 was released by osmotic shock, leading to the speculation that this presumably soluble protein is part of an adhesion complex connecting polysaccharide synthesis and export, potentially involving a KpsE domain (Rigg et al., 1998). Although chemical cross-linking and co-purification with histidine-tagged KpsS-K5 identified apparent interactions between it and KpsM, KpsT, KpsE and KpsD, KpsS-K5 also interacted with other polypeptides whose relevance to capsule biosynthesis is unclear (McNulty et al., 2006), suggesting that chemical cross-linking might produce artefactual results. In contrast, we could not detect interactions between KpsS-K1 and any bait–prey combination tested (Table S1), further suggesting that chemical cross-linking might yield artificial associations or that relevant KpsS interactions are not detectable with the current two-hybrid system. Note that most of our results were obtained with full-length kps and neu gene fusions. However, the orientation effects on NeuS–KpsC interactions, for example, indicate probable steric constraints interfering with some fusions (Table 2). Therefore, we plan to test shorter fusions of negatively interacting pairs as well as those with demonstrated interactions to more precisely define binding domains and, ultimately, identify specific interacting peptides that should define the complete network of homo- and heterotypic interactions in group 2 capsule systems. This approach should lead to a better understanding of capsule biosynthesis while helping to identify new therapeutic targets in a wide range of encapsulated pathogens expressing group 2 or group 2-like capsules.

Conclusions

The most important finding of this study is that PSA biosynthesis (synthesis and export) occurs within an intracellular compartment operationally defined as inaccessible to endo-N, a specific depolymerase previously used to investigate PSA in both bacterial and mammalian systems (Vimr et al., 1984). Our approach provides a novel in vivo protection assay whereby PSA was detected by K1-specific phage infectivity, electron microscopy and immunodiffusion assessing the processes of translocation, intracellular accumulation of unexported PSA and polymerization respectively. Although we do not know the minimum number of PSA chains necessary for productive lytic phage infection, the number must be no less than 5–10% of the normally present 50 000 chains, as below this minimum number only small plaques are produced because of diminished phage binding (Vimr, 1992; Cieslewicz and Vimr, 1997). Because plaque size was not diminished by endo-N overproduction (Fig. 4), we conclude that more than 5000 PSA chains are synthesized and exported to the outer surface of each cell during capsule biosynthesis in the presence of intracellular endo-N. This conclusion was supported by identical precipitin halos surrounding wild type or cells expressing endo-N (data not shown), which is a reflection of the equivalent amounts of capsular polysaccharide being synthesized and exported during cell growth (Steenbergen and Vimr, 1990). Our previous characterization of the export defects in mutants with regions 1 or 3 defects facilitated detection of endo-N activity in vivo. Indeed, our data indicate that there could be two sialosomes: one endo-N-accessible formed by intracellular accumulation of unexported PSA, and the endo-N-inaccessible compartment representing the authentic biosynthetic environment. These results strongly point to model 3 as a dynamic representation of group 2 polysaccharide biosynthesis. (Fig. 2)

We have carried out the first extensive two-hybrid analysis of capsule biosynthesis in any species. The results confirm and extend previous biochemical approaches involving protein cross-linking and immunological detection (Arrecubieta et al., 2001; McNulty et al., 2006), subcellular fractionation and irradiation (Andreishcheva and Vann, 2006; Vionnet et al., 2006), and complementation analyses (Fig. 7). For example, when the biochemical results are combined with our analysis of KpsC interactions, it is clear that KpsC functions in several homo- and heterotypic interactions important to capsule synthesis and export. The N-terminal region necessary for KpsC homotypic interaction has been identified, while a separate C-terminal KpsC domain was shown to mediate heterotypic interaction with KpsE. In contrast, there was no evidence for NeuS oligomerization.
although this polypeptide was shown to interact with both KpsC and KpsE, supporting the concept of a dynamic biosynthetic apparatus that couples polymerization to export, in further support of model 3 (Fig. 2). This conclusion implies that the signalling event(s) involved in directed coupling of PSA synthesis and KpsC linking the polymerase to the periplasmic connector, KpsE, could mediate export. Experiments are in progress mapping the NeuS domain that interacts with KpsC. Our results suggest that all groups 2 and 3 polymerases will share similar interactive domains. Finally, despite our inability to detect an interaction between the polymerase and NeuE, suppression of a neuE defect by NeuS overproduction supports the conclusion that NeuE is not obligatory for capsule biosynthesis, although it is likely to function in some as yet undefined structural role that is necessary, under the normal single-copy circumstance, for PSA export.

Experimental procedures

Bacterial strains, plasmids, phage and growth conditions

The strains, plasmids and phage used in this study are given in Table 3. Bacteria were routinely grown in Lennox-formulated Luria–Bertani (LB) broth purchased from Fisher (Chicago, IL). Kanamycin, ampicillin and tetracycline were used at 50, 100 and 10 μg ml⁻¹ of final concentrations respectively. Transductions were carried out using P1vir as described previously (Ringenberg et al., 2003). MacConkey agar base with 0.4% maltose was used to screen two-hybrid co-transformants. Plasmid pSX101 was constructed by ligating the BglII-BamHI fragment from plasmid pSX90 into the BamHI site of the pMal-c2 polylinker, which produces an in-frame fusion of the NeuE PIRS domain to MBP. The BamHI site of the pMal-c2 polylinker, which produces an in-frame fusion of the NeuE PIRS domain to MBP. The desired orientation was determined by restriction endonuclease digestion and confirmed by DNA sequencing. Plasmid pSX104 was constructed by ligating the EcoRI-HindIII fragment from plasmid pSX90 into the pMal-c2 polylinker, which produces an in-frame fusion of the NeuE PIRS domain to MBP. The desired orientation was determined by restriction endonuclease digestion and confirmed by DNA sequencing.

Cloning kps and neu genes into two-hybrid plasmids

All bait and prey clones harbour PCR products amplified from boiled EV36. Amplification was carried out using SuperMix High Fidelity (Invitrogen) to eliminate introducing spontaneous mutations into the amplified DNA sequences. In most cases (Table S2), primers were designed to amplify the full-length kps- or neu-coding sequence with restriction sites in the 5’ end of each primer. Restriction sites were chosen as sites not contained in the gene sequence of interest but present in the multiple cloning site of the two-hybrid plasmid. Sites were designed such that digestion of the PCR product and vector with the specific enzymes, followed by ligation, would yield in-frame fusions of the kps or neu gene to the T25- or T18-coding sequences in the vectors. Stop codons were included in some primers when cloning into pKT25 or pUT18C, although these vectors also included their own stop codons. PCR products were purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen) followed by restriction digestion. The digested products were electrophoresed through agarose, the band cut out and the DNA removed from the agarose. This band was then ligated to vector that had been treated with the same restriction enzymes. Insertions were verified by restriction digestion. Fusion junctions described in the text were verified, identified by DNA sequencing as previously described (Steenbergen and Vimr, 2003).

Beta-Glo assay

Bait and prey plasmids were co-transformed into chemically competent EV727 or EV729 by selecting for both ampicillin and kanamycin resistance. Single colonies were inoculated in 2 ml of LB plus the appropriate drugs and grown overnight at 30°C. Three millilitres of fresh media was inoculated with 70 μl of the overnight culture and grown for 2.5–3 h at 30°C. Cultures were then diluted 300-fold and 50 μl portions were added to wells of a white, opaque 96-well microtiter dish (Falcon, number 353296). An equal volume of the Beta-Glo reagent was added to each well and the plate was agitated to mix. The plate was then incubated at room temperature, in the dark, for 30–45 min. Luminescence was quantified on a Wallac Victor2 Multilabel Counter and data expressed as RLU after subtracting background, which was 2000–3000 RLU for medium alone and 3000–6000 RLU for medium plus cells lacking any plasmid. Statistical comparisons were made between cells harbouring vectors without inserts to the Zip control or interacting plasmid pairs after subtraction of the cells alone backgrounds.

Computer-assisted analysis of NeuE transmembrane regions

Nine different transmembrane (TM)-predictive programs were used to analyse NeuE for potential membrane spanning regions. DAS (dense alignment surface method) compares dot plots of NeuE against a collection of non-homologous TM proteins using the RfEm scoring matrix (Cserzó et al., 1994). HMMTOP 2.0 (hidden Markov model for topology prediction) (Tusnády and Simon, 2001) and TMHMM 2.0 (transmembrane helices based on a hidden Markov model) (Krogh et al., 2001) utilize a hidden Markov model distinguishing the inside loop region, inside TM-helix cap, TM helix, outside TM-helix cap and outside loop region, or TM-helix core, caps and loop regions respectively. TMHMM (transmembrane analysis program) incorporates information from multiple alignments of homologous polypeptides to determine the membrane-spanning segments (Persson and Argos, 1994). PRED-TMR2 (prediction of transmembrane regions in proteins) uses the propensities of amino acid residues at the termini of TM helices of proteins compiled by the authors to identify TM segments (Pasquier and Hamodrakas, 1999). TM-Finder uses experimentally derived hydrophobicity and non-polar phase scales
Table 3. Bacterial strains, plasmids and phage used in this study.

| Strain, plasmid or phage | Genotype or relevant description | Source (or reference) |
|-------------------------|----------------------------------|-----------------------|
| **Bacteria**            |                                  |                       |
| BTH101                  | F- cya-854, recA1, endA1, gyrA96 (Nal'), thi1, hsdR17, spoT1, rfbD1, glmV44(AS) | Karimova et al. (1998) |
| BW30270                 | rph' derivative of MG1655        | E. coli Genetic Stock Center |
| DH5<sup>e</sup>         | F-, F<o>Φ80lacZ<sub>M15</sub>, recA1, endA1, gyrA96, thi-1, hsdR17 (r<sup>R</sup>, m<sup>±</sup>), supE44, relA1, deoR, Δ(lacZΔ8-argF)U169, phoA | Laboratory stock |
| DHM1                    | F-, cya-99, araD139, galE15, galI6, rpsL1 (Str<sup>R</sup>), hsdR2, mcrA1, mcrB1 | Karimova et al. (1998) |
| EV36                    | K-12/K1 hybrid                  | Vimr and Troy (1985)  |
| EV93                    | EV36 kpsC::tet                   | Vimr et al. (1989)    |
| EV94                    | EV36 kpsS::tet                   | Vimr et al. (1989)    |
| EV136                   | EV36 neuS::tet                   | Steenbergen and Vimr (1990) |
| EV725                   | EV36 neuE::kan                   | Steenbergen and Vimr (1990) |
| EV726                   | BVW30270 cya::kan                | This study            |
| EV727                   | BVW30270 ΔcyA                    | This study            |
| EV728                   | EV36 cya::kan                    | This study            |
| EV729                   | EV36 ΔcyA                        | This study            |
| HB101                   | thi-1, hsdS20 (r<sup>+</sup>, r<sup>−</sup>), supE44m recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 (str<sup>R</sup>), xyl-5, mtl-1 | Laboratory stock |
| MER4                    | F araD139, Δ(argF-lac)U169, rpsL150, relA1, fliB5301, deoC1, ptsF25, rbsR7, cya::kan | Mark D. Gonzalez |
| RS2918                  | EV36 ΔneuC                       | Vann et al. 2004      |
| **Plasmids**            |                                  |                       |
| pUC18                   | High-copy cloning vector         | Laboratory stock      |
| pUC19                   | pUC18 with inverted multiple cloning site | Laboratory stock      |
| pMAL-c2                 | Cytoplasmic expression of malE fusions | New England Biolabs    |
| pGEM-T Easy             | AT cloning vector                | Promega               |
| pSR426                  | 6.7 kb EcoRV-BamHI fragment from the K1 capsule cluster containing neuDBACES in a Bluescript cloning vector | Annunziato et al. (1995) |
| pRep4                   | lacI expression                  | Qiagen                |
| pEndo-N                 | Endo-neuraminidase expression    | Steenbergen et al. (2006) |
| pSX90                   | pUC18 with 2.7 kb EcolI-BamHI fragment from the K1 capsule cluster | Steenbergen and Vimr (1990) |
| pSX91                   | pUC19 with 2.7 kb EcolI-BamHI fragment from the K1 capsule cluster | Steenbergen and Vimr (1990) |
| pSX92                   | pUC18 with 2.3 kb BglII-BamHI fragment from the K1 capsule cluster | Steenbergen and Vimr (1990) |
| pSX93                   | pUC18 with 3.07 kb HindIII-BamHI fragment from the K1 capsule cluster | This study            |
| pSX94                   | PCR product of neuE cloned into pGEM T-Easy | This study            |
| pSX101                  | pMAL-c2 with the 2.3 kb BglII-BamHI fragment from the K1 cluster, fusing the NeuE PIRS domain to MBP | This study            |
| pSX102                  | pSX94 with a mutation at aa381 of neuE | This study            |
| pSX103                  | pSX94 with a mutation at aa385 of neuE | This study            |
| pSX104                  | pMAL-c2 with the 2.7 kb EcoRI-BamHI fragment from the K1 cluster, fusing NeuE to MBP | This study            |
| pKT25                   | Derivative of pSU40 that carries the T25 fragment of B. pertussis adenylate cyclase with a multi-cloning sequence at the 3' end of T25, also carries a kanamycin-resistance gene | Karimova et al. (1998) |
| pUT18                   | Derivative of pUC19 that carries the T18 fragment of B. pertussis adenylate cyclase with a multi-cloning sequence at the 5' end of T18, also carries an ampicillin-resistance gene | Karimova et al. (1998) |
| pUT18C                  | Derivative of pUC19 that carries the T18 fragment of B. pertussis adenylate cyclase with a multi-cloning sequence at the 3' end of T18, also carries an ampicillin-resistance gene | Karimova et al. (1998) |
| pKT25-myc               | Derivative of pKT25 with Myc tag  | M. Gonzalez           |
| pUT18C-flag             | Derivative of pUT18C with Flag tag | M. Gonzalez           |
| pSX750                  | kpsC in pKT25                    | This study            |
| pSX751                  | neuS in pKT25                    | This study            |
| pSX752                  | kpsD in pKT25                    | This study            |
| pSX753                  | kpsE in pUT18                    | This study            |
| pSX754                  | kpsD in pUT18                    | This study            |
| pSX755                  | neuC in pUT18                    | This study            |
| pSX756                  | neuC in pUT18C                   | This study            |
| pSX757                  | kpsD in pUT18C                   | This study            |
| pSX758                  | kpsE in pUT18C                   | This study            |
| pSX759                  | neuS in pKT25-myc                | This study            |
| pSX760                  | neuE in pKT25-myc                | This study            |
| pSX761                  | neuE in pUT18C                   | This study            |
| pSX762                  | neuS in pUT18-FLAG               | This study            |
to detect TM segments (Deber et al., 2001). TMpred (prediction of transmembrane regions and orientation) uses statistical preference matrices in a data set of membrane proteins compiled by experts to determine likely membrane-spanning regions (Hofmann and Stoffel, 1993). TopPred II (topology prediction of membrane proteins) detects hydrophobic segments using a sliding trapezoid window and evaluates topological models using the positive-inside rule (von Heijne, 1992; Claros and von Heijne, 1994). SPLIT 3.5 (University of Split, Croatia) uses integrated scales for amino acids to predict TM regions (Juretic et al., 1993). Analyses were carried out using the derived primary structure of NeuE (Andreishcheva and Vann, 2006) and the default settings for each programme. The neuE sequence chosen for analysis includes the ATG that overlaps 95 bp with the 3’ end of neuC.

Other analytical techniques

The TEM of negatively stained whole mounts or thin sections was carried out as previously described (Cieslewicz and Vimr, 1996; 1997). Maxi-cell analysis and polyacrylamide gel electrophoresis were carried out as described in Cieslewicz et al. (1993). For the standard beta-galactosidase assay (Miller, 1976), cells were grown as described above, except that the final dilution was fivefold or 10-fold prior to assay. Data were expressed in standard Miller Units. Western blot analysis was carried out using anti-MBP prepared against purified MBP purchased from New England Biolabs and used according to the manufacturer’s instructions.

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