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Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element

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Cell surface-located sialic acids of the capsule and the lipooligosaccharide (LOS) are both pivotal virulence factors in *Neisseria meningitidis*, promoting survival and dissemination of this pathogen which can cause both sepsis and meningitis. With the aid of a unique set of isogenic meningococcal mutants defective in the expression of cell surface-located sialic acids, we have demonstrated that encapsulation hinders the primary event in the development of the disease, but the spontaneous switching of encapsulated wild-type bacteria to a capsule-negative phenotype promotes meningococcal adherence and invasion into mucosal epithelial cells. Genetic analysis of the capsule-negative, invasive bacteria revealed a unique mechanism for modulation of capsule expression based on the reversible inactivation of an essential sialic acid biosynthesis gene, *siaA*, by insertion/excision of a naturally occurring insertion sequence element, IS*1301*. Inactivation of *siaA* regulates both capsule expression and endogenous LOS sialylation. This is the first example of an insertion sequence element-based genetic switch mechanism in the pathogenic bacterium and is an important step in the understanding of bacterial virulence.

Keywords: capsule/insertion sequence element/invasion/ *Neisseria meningitidis*

Introduction

Neisseria meningitidis, a Gram-negative diplococcus, is an invasive human pathogen causing sepsis and meningitis. Dissemination occurs from the nasopharynx, which is colonized by *N.meningitidis* in up to 20% of the human population. Despite this high frequency of colonization, meningococcal disease is a relatively rare event with a morbidity of 3 per 100 000 in developed countries (Schlech *et al.*, 1985; Berger *et al.*, 1988). The mechanism by which the bacteria penetrate the mucosal barrier and get access to the bloodstream is only poorly understood. Since *Neisseria* species are exclusively human pathogens, no appropriate animal models exist for studies on bacteria–host cell interactions. However, during recent years, some insights into mucosal cell invasion were obtained by cell and organ culture systems (Stephens, 1989). After entry into the epithelial cell by a phagocytosis-like mechanism, *N.meningitidis* survives within the phagosomes. However, it is unknown at present how the bacteria reach the subepithelial space and get access to the bloodstream. An alternative way to enter the subepithelial space may be by cytotoxic factors which disrupt the integrity of the epithelial cell layer (Stephens and Farley, 1991).

In recent years, several bacterial cell wall constituents that promote the adherence and entry of *N.meningitidis* into epithelial cells have been identified. Initial adherence of the encapsulated bacteria to human nasopharyngeal tissue requires pili, which are long hair-like protrusions extending from the bacterial surface (Stephens and Farley, 1991). In a later stage of the infection, the outer membrane proteins Opa and Opc are thought to strengthen this adherence and to promote entry of the bacteria into the host cell (Virji *et al.*, 1992, 1993). Initial experiments using capsule-deficient meningococci indicated that both Opa- and Opc-mediated invasion of host cells are inhibited in the presence of the thick polysaccharide capsules expressed by *N.meningitidis*, suggesting that capsule formation may need to be down-regulated during the first steps of an infection (Stephens *et al.*, 1993; Virji *et al.*, 1993).

The capsular polysaccharides of the meningococcal serogroups B and C, which predominate in the Northern hemisphere, are homopolymers of sialic acids with α-2,8 and α-2,9 linkages, respectively (Jennings *et al.*, 1977). Interestingly, sialic acid is also found as a modification of the meningococcal lipooligosaccharide (LOS). Substitution of the terminal galactose residue of the LOS with sialic acid depends on the availability of endogenously synthesized CMP-NeuNAc in serogroups with a sialic acid-containing capsule and/or on the exogenous presence of CMP-NeuNAc in culture medium or body fluids (Mandrell *et al.*, 1990). The large amount of sialic acid expressed on the surface of these invasive bacteria is associated with their virulence, mediating resistance to both phagocytosis and to complement-mediated killing via alternative pathway activation (Hammerschmidt *et al.*, 1994; Jarvis, 1995). Thus, meningococcal penetration of the mucosal barrier may require a down-regulation or off-switching of capsule expression, enabling passage through the mucosal epithelium, followed by a re-expression of the cell surface sialic acids to resist the host's immune defences. In this report, we describe a novel mechanism of genetic variation in meningococci that allows a reversible concurrent phase (on- and off-) switching of capsule synthesis and endogenous LOS sialylation.

Results

Influence of capsule and LOS-derived sialic acids on adhesion and invasion of epithelial cells

To analyse the role of cell surface-located sialic acids in adhesion and entry into mucosal cells, we used a set of

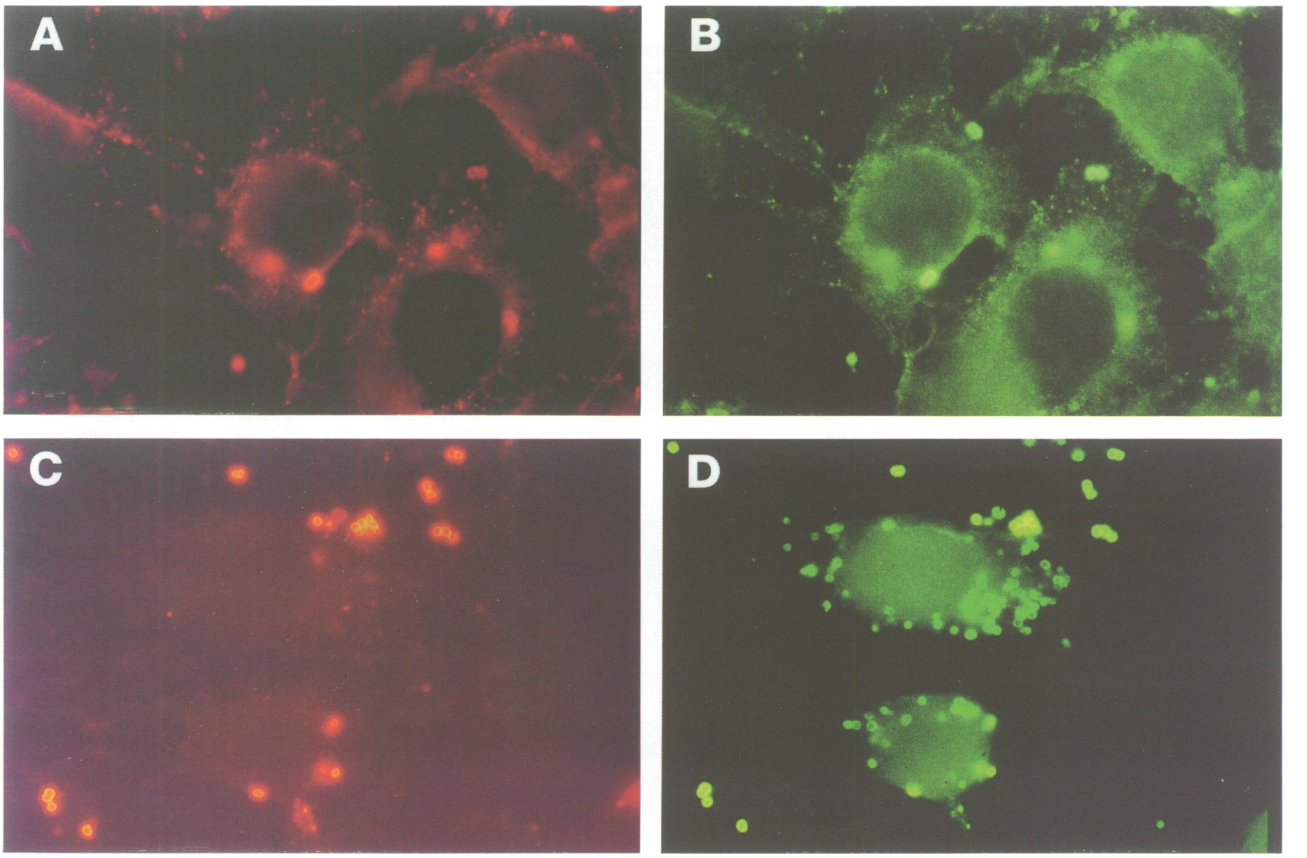

Fig. 1. Immunofluorescence microscopy of adherent and invasive meningococci in Chang epithelial cell cultures. Wild-type cells of strain B1940 are shown in (**A**) and (**B**), the capsule-deficient mutant B1940*siaD*$^-$ is shown in (**C**) and (**D**). All cells were labelled with a mouse antiserum raised by immunization with whole meningococci. Extracellular bacteria were stained with TRITC-labelled anti-mouse Ig (A and C). After permeabilization of the cells and re-incubation with the mouse serum, the bacteria were stained with FITC-labelled anti-mouse Ig for detection of intra- and extracellular bacteria (B and D). (A and B) and (C and D), respectively, represent identical microscopic areas.

isogenic mutants of the invasive serogroup B isolate B1940 defective in capsule (*siaD*$^-$), LOS sialylation (*galE*$^-$) or both (*cps*$^-$) (Hammerschmidt *et al.*, 1994). Chang conjunctiva epithelial and Hep-2 larynx carcinoma cell lines were infected with these bacteria and the numbers of adherent and intracellular bacteria were determined. After 2–16 h of infection, the capsule-deficient mutants B1940*cps*$^-$ and B1940*siaD*$^-$ adhered much more to both types of cells than the parental strain B1940 (Figures 1 and 2; Table I). The *cps*$^-$ mutant with the additional defect in LOS biosynthesis adhered slightly better (~2-fold) than its full-sized LOS counterpart (Table I). The increase in adhesion of unencapsulated bacteria may reflect an additional unmasking of meningococcal cell surface adhesins. Similarly, the number of the unencapsulated mutants entering the epithelial cells exceeded that of the parent strain 50- to 100-fold, irrespective of whether bacterial entry was scored microscopically or by gentamicin selection (Table I). This difference in invasion was particularly evident during the first 6 h of infection when the number of intracellular unencapsulated mutant bacteria increased steadily and the encapsulated parent bacteria remained extracellularly (Figure 2). Due to the absence of any other detectable differences in surface components between these strains (no differences in the amount or molecuar weight of Opa, Opc and pilin were observed), these data indicate that the state of encapsulation is a critical determinant in the interaction of meningococci with the epithelial cells. This was further confirmed when we analysed the intracellularly located bacteria of the wild-type strain for capsule production. Using the serogroup B capsule-specific monoclonal antibody mAb 735 (Frosch *et al.*, 1985) in the immunofluorescence microscopy-based invasion assay, we found that none of the relatively few bacteria located intracellularly expressed the capsular polysaccharide, whereas all extracellularly adherent meningococci were encapsulated. The unencapsulated phenotype of the intracellular bacteria remained after recovery and growth of the intracellular bacteria on agar plates, indicating that the loss of capsule was not due to a change in microenvironment. The low level of intracellular parent bacteria during the infection may represent unencapsulated bacteria already present in the bacterial population of the inoculum and increasing during infection. In accordance with this, colony blotting of the inoculum with mAb 735 revealed capsule-negative variants at a frequency of 1×10^{-4}. The LOS immunotype (L3,7,9) and its potential to exogenously sialylate the terminal lacto-*N*-neotetraose was unaffected in isolated invasive bacteria (data not shown) as determined by enzyme-linked immunosorbent assay (ELISA) using LOS-specific monoclonal antibodies and measurements of the incorporation of exogenous radiolabelled CMP-NeuNAc into the LOS.

Table I. Invasion and adherence of *N.meningitidis* serogroup B strain B1940 and derived mutants defective in capsule and/or LOS biosynthesis

Strain/mutant	No. of adherent bacteria		No. of invasive bacteria	
	Chang cells	Hep-2 cells	Chang cells	Hep-2 cells
B1940	1.9×10^5	2.2×10^5	3.5×10^4 (2.7×10^1)	2.5×10^4 (3.8×10^1)
B1940*siaD*$^-$	2.4×10^6	5.6×10^6	7.5×10^5 (1.3×10^3)	2.2×10^6 (1.1×10^3)
B1940*cps*$^-$	5.1×10^6	1.5×10^7	1.0×10^6 (3.4×10^3)	2.4×10^6 (4.5×10^3)
B1940*galE*$^-$	1.0×10^6	2.3×10^6	6.7×10^4 (3.3×10^2)	2.9×10^5 (4.8×10^2)
B1940*siaA*::IS*1301*	8.4×10^6	3.3×10^6	9.4×10^5 (1.3×10^3)	5.6×10^5 (8.6×10^2)

Adherence and invasion were measured by microscopy and gentamicin selection assay (in parentheses). The numbers given represent the number of microorganisms per 2×10^5 cells. The intracellular survivors of the gentamicin assay represented only a small moiety of the cells which were seen microscopically. This is a known phenomenon (van Putten, 1993) and due to the sudden metabolic change from intracellular growth to artificial growth conditions on nutrient agar, release of lysosomal enzymes during host cell lysis and damage of bacteria by saponin treatment. However, the intracellular capsule-negative variants isolated from the infection assay with the wild-type strain are representive and there is no artifical intracellular selection for capsule-negative variants, since we analysed and varied only one or two phenotypic markers, i.e. capsule and LOS expression, and observed for all strains and variants an identical percentage of gentamicin survivors. Mutant B1940*siaD*$^-$ (capsule–, LOS sialylation+) was derived from the wild-type strain B1940 by insertional inactivation of the polysialyltransferase gene (Frosch *et al.*, 1991). B1940*galE*$^-$ (capsule+, LOS sialylation–) was established by replacing the *galE* and *rfb* genes with a chloramphenicol resistance cassette, resulting in expression of a truncated LOS (Hammerschmidt *et al.*, 1994). B1940*cps*$^-$ (capsule–, LOS sialylation–) was obtained by deletion of the complete *cps* locus, including *galE* (Frosch *et al.*, 1990).

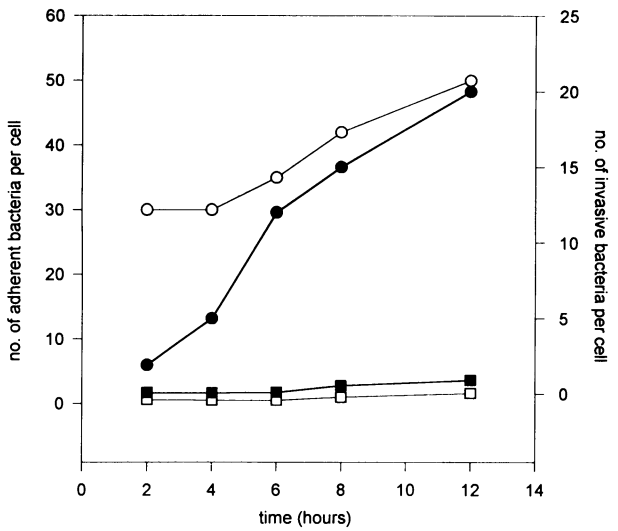

Fig. 2. Adhesion (open symbols refer to the left *y*-axis) and invasion (closed symbols refer to the right *y*-axis) of *N.meningitidis* strain B1940 (squares) and the capsule-deficient mutant B1940*siaD*$^-$ (circles) during a time course of 12 h. Note the dramatic difference in adherence and invasiveness between the unencapsulated mutant and encapsulated parental strain.

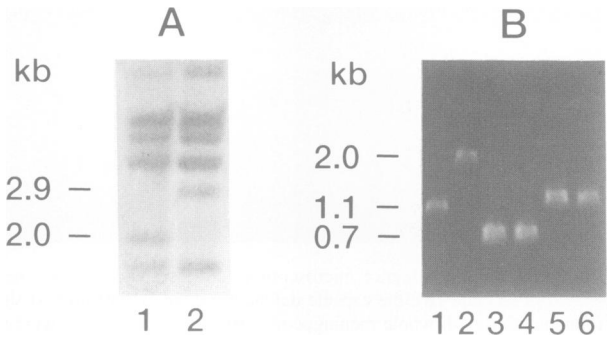

Fig. 3. (**A**) Southern blot analysis of *Eco*RI-digested chromosomal DNA of wild-type strain B1940 (lane 1) and a capsule-negative invasive derivative (lane 2). The DNA was hybridized with the digoxigenin-labelled plasmid pMF32.35 harbouring the complete *cps* locus of strain B1940 (Frosch *et al.*, 1989). (**B**) PCR products obtained when *siaA*-specific (lanes 1 and 2), *siaB*-specific (lanes 3 and 4) and *siaC*-specific (lanes 5 and 6) oligonucleotides were used for amplification. Lanes 1, 3 and 5: template DNA was from strain B1940; lanes 2, 4 and 6: template DNA was from the capsule-negative invasive derivative.

Characterization of the mechanism for capsule phase variation

The mechanisms behind the loss of encapsulation accompanying invasion of the parental strain B1940 were explored by searching for mutations within the genes required for biosynthesis of the α-2,8-linked polysialic acid in the wild-type-derived capsule-deficient invasive derivatives. For this purpose, extracellular bacteria from five independent invasion experiments with strain B1940 were killed and the intracellularly located bacteria were released by saponin treatment of the Chang epithelial cells. The bacteria were plated on nutrient agar and monitored for capsule production by colony blotting using mAb 735. Among 388 gentamicin survivors recovered from the five experiments, 322 (83% with a range of 60–100% in the five experiments) were capsule negative. The recovered encapsulated bacteria probably result from the reported incomplete penetration of the antibiotic in clumps of strongly adherent bacteria (de Vries *et al.*, 1994), although a high frequency reversion of a small subpopulation of unencapsulated intracellular bacteria once plated on nutrient agar cannot formally be excluded at this point.

A total of 30 capsule-negative colonies randomly selected from these experiments were investigated further by Southern blot analysis. *Eco*RI-restricted chromosomal DNA of the capsule-negative variants was hybridized with the cloned *cps* gene cluster (Frosch *et al.*, 1989). Two of these variants exhibited a molecular weight shift from 2 to 2.9 kb in one *Eco*RI fragment of region A of *cps* (Figure 3A; Frosch *et al.*, 1989; Edwards *et al.*, 1994). The other investigated clones showed no altered *cps* restriction pattern. The reason for the loss of capsule expression in this second class of capsule-negative variants is unclear and currently under investigation. The capsule-negative derivatives with the enlarged *Eco*RI fragment exhibited the same high adherence and invasion potential as the capsule-deficient mutants, when applied to the

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                                                                    M  A  R  T  A  I  T  D  N  I   10
ttcgtcattctatta<u>GGGCGTGTCCCTAATTTGA</u>ATAAGCCCAAAAGAGCCCTATTTTACCCTATATTCCAACGAAATATAGGCAAACACAAACAATGGCGAGAACCGCAATAACTGACAACATA 110

 W  E  Q  L  Q  T  T  M  K  A  H  G  C  H  Q  W  K  N  D  R  T  V  M  E  A  I  L  W  K  L  R  T  G  A  P  W  R  D  I  P  I  E  52
TGGGAACAATTACAAACAACCATGAAAGCGCACGGCTGTCATCAATGGAAGAACGACCGTACCGTGATGGAAGCCATACTATGGAAGCTAAGAACAGGTGCACCATGGCGAGACATACCTATTGA 235

 L  G  S  W  K  T  A  Y  N  R  F  N  R  W  S  K  K  G  L  W  Q  N  F  F  L  I  Y  E  K  K  L  T  K  N  G  Y  S  S  T  E  V   93
GCTAGGGTCATGGAAAACCGCTTATAACCGCTTTAACCGATGGTCTAAAAAAGGCTTGTGGCAGAATTTTTTTTTGATCTACGAAAAGAAATTGACAAAGAATGGGTATTCATCGACGGAAGTTA 360
                                                    V  A  E  F  F  F  D  L  R  K  E  I  D  K  E  W  V  F  I  D  G  S  Y  23

 M  Y  G  V  I  N  M  Q  V  E  L  G  V  V  S  I  E  Q  L  D  K  A  V  A  E  T  R  Q  K  Y  T  Y  V  W  T  R  M  E  I  R  S  I  135
TGTACGGTGTCATCAACATGCAAGTGGAGCTCGGCGTGGTTTCGATAGAGCAATTGGACAAAGCCGTGGCGGAAACACGACAAAAATACACCTATGTGTGGACTCGCATGGAAATCCGCTCGATT 485
  V  R  C  H  Q  H  A  S  G  A  R  R  G  F  D  R  A  I  G  Q  S  R  G  G  N  T  T  K  I  H  L  C  V  D  S  H  G  N  P  L  D   64

 L  K  S  L  G  V  T  C  T  T  V  K  L  Q  T  T                                                                                151
TTAAAGTCACTGGGGGTAACGTGCACGACGGTCAAGTTGCAAACGACTTGATAGAAGTCATACAAGAAGCCCAGTATTTTATCGCTGACAAAGGGTATGACTCGCAAGAAATCAGAGATAAAGCG 610
F  K  V  T  G  G  N  V  H  D  G  Q  V  A  N  D  L  I  E  V  I  Q  E  A  Q  Y  F  I  A  D  K  G  Y  D  S  Q  E  I  R  D  K  A  106

ATAGAACACGGTATGAAAGCTATTATACCAAAGCGTAAAAATGCCAAGAAACCTAACCCTGATTTTGATAGCTACCTTTATAAATTACGCCACTTAGTCGAGAATGCATTTGCACGATTAAAGCA 735
 I  E  H  G  M  K  A  I  I  P  K  R  K  N  A  K  K  P  N  P  D  F  D  S  Y  L  Y  K  L  R  H  L  V  E  N  A  F  A  R  L  K  Q  148

GTTTCGTAGTATTGCTACTCGCTATGAGAAATTAGCTCGTAATTTTAAATCGATGCTTTACTTGGCTTGCTCTATTATTCATGCTAAG<u>TTAAATTGAGGACACGCCCTA</u>gtaaactttctcatat 844
  F  R  S  I  A  T  R  Y  E  K  L  A  R  N  F  K  S  M  L  Y  L  A  C  S  I  I  H  A  K  L  N                                179
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Fig. 4. Nucleotide and derived amino acid sequences of IS*1301* (upper case letters). The flanking regions of *siaA* are given in lower case letters. The inverted repeats are underlined. The TA duplication is double underlined. This IS was registered by the Plasmid Reference Centre Prefix Registry, Stanford University, and the name IS*1301* was assigned. For sequence determination of IS*1301*, a PCR fragment of 1197 bp was generated with primers SH38 (5′-GCCATATCGAAGGTGGT-3′) and SH40 (5′-GGTAGATGCTAATGATAT-3′) both complementary to *siaA*. The fragment was ligated into the *Sma*I site of pBluescript plasmid vector and sequenced using the primers SH38, SH40 and additional oligonucleotides derived from the obtained sequence data. Additionally, the PCR fragment was sequenced directly with the same primers to confirm the nucleotide sequence, which was analysed using the PC/GENE software package (Intelligenetics, Montain View, CA).

mucosal cells (Table I), confirming the previous notion that the capsule is a critical determinant of the interaction of meningococci with mucosal cells.

The altered *Eco*RI gene fragment harbours the *siaA* and *siaB* genes and the 5′ end of the *siaC* gene (Edwards *et al.*, 1994), which encode proteins of the biosynthetic machinery required for α-2,8-linked polysialic acid synthesis. Using PCR and primers which flank each of these genes, we could detect an insertion within the *siaA* gene, but not within *siaB* or *siaC* (Figure 3B). The *siaA* gene product is an epimerase, which catalyses the first step in α-2,8-linked polysialic acid synthesis by converting GlcNAc-6-phosphate to ManNAc, a precursor for NeuNAc synthesis (Edwards *et al.*, 1994; Fessner and Frosch, unpublished). The enlarged *siaA* gene was subcloned and sequenced. An insertion at position 587 of the open reading frame (ORF) was detected, which exhibited characteristics of a transposable genetic element (Figure 4). The size of the element was 844 bp. Inverted repeats of 19 bp at both ends with three nucleotide mismatches were found. The GC content of this insertion sequence, termed IS*1301* was 42.2%, which is within the range reported for *Neisseria* species (Vedros, 1984). IS*1301* contained two overlapping ORFs between nucleotides 81 and 533 and between 293 and 829, respectively. In general, insertion sequences have a single ORF encoding a transposase, which extends almost the entire length of the element. It is unclear whether both ORFs of IS*1301* are expressed independently or whether they are fused by a translational frameshift as described for IS*1*, IS*3* and Ty*1* (Mellor *et al.*, 1985; Sekine and Ohtsubo, 1989; Prere *et al.*, 1990). The proteins encoded by both ORFs are basic with pIs of 10.2 and 9.8, which is consistent with their probable DNA binding properties required for acting as a transposase. The insertion site of IS*1301* was sequenced from five other *siaA*::IS*1301* inactivated meningococci. It was found that the insertion sequence element consistently inserted at the same position, and IS*1301* was found in the same orientation in all cases. Insertion of IS*1301* in the *siaA* gene was accompanied by a two nucleotide (TA) target sequence duplication. The sequence of IS*1301* was not similar to other known

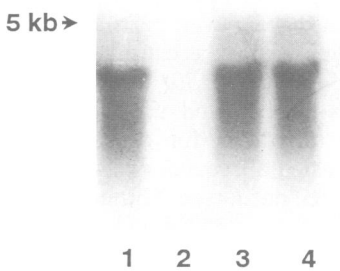

Fig. 5. Northern blot analysis of total mRNA isolated from wild-type strain B1940 (lane 1) and two independently isolated *siaA*::IS*1301* derivatives (lanes 3 and 4). Mutant B1940*cps*$^{-}$, which is characterized by a deletion of the complete *cps* cluster including region A, served as a negative control (lane 2). A ^{32}P-labelled PCR fragment spanning the *siaB*, *siaC* and *siaD* genes was used as a probe.

insertion sequences deposited in the EMBL data library. However, the inverted repeats (IRs) showed a 60% identity to the IR of IS*711* of *Brucella ovis* (Halling *et al.*, 1993).

To address further the frequency of *siaA*::IS*1301* inactivated intracellular bacteria among all unencapsulated meningococci recovered from epithelial cells, we hybridized 501 colonies collected from five additional invasion experiments with oligonucleotide SH47, which is complementary to eight nucleotides of the 3′ end of IS*1301* followed by 12 nucleotides complementary to *siaA* downstream of the insertion site. Colonies were replica plated and monitored for capsule expression, which was found to be of the same order of magnitude as described above. Ten to 15% of the capsule-negative isolates from four experiments gave a strong signal after hybridization with oligonucleotide SH47, but 50% of the capsule-negative colonies in one experiment were shown to have an *siaA*::IS*1301* inactivation. The reason for this deviation in one experiment is not known.

Northern blot analysis revealed that the insertion of IS*1301* did not result in the termination of transcription of *siaA* (Figure 5). This is in accordance with the lack of

hairpin structures within the element that could potentially act as transcriptional terminators. This situation contrasts with that found in many other bacterial insertion sequences, which exert a strong polar effect on downstream located genes (Galas and Chandler, 1989). The *siaB*, *siaC* and *siaD* genes, which are located downstream of *siaA* and which constitute a transcriptional unit together with *siaA* (Edwards *et al.*, 1994), were all transcribed, and the respective enzymatic activities were identical to those of the encapsulated parental strain (data not shown). However, since the defect in *siaA* causes substrate deprivation and CMP-NeuNAc was not synthesized in the cell, the capsular polysaccharide could not be synthesized. In addition, this defect also resulted in the inability to sialylate the LOS endogenously, which also requires CMP-NeuNAc as the substrate for sialylation. Thus, IS*1301* regulates both capsule expression and endogenous LOS sialylation by insertion within *siaA*.

The biological significance of inactivation of capsular polysaccharide synthesis and of LOS sialylation by IS*1301* depends on its potential to be reversed, since expression of the capsular polysaccharide and sialylated LOS are required for survival during dissemination (Hammerschmidt *et al.*, 1994; Jarvis, 1995) and unencapsulated bacteria have never been observed as disease isolates. To confirm that the capsular polysaccharide can be re-expressed in *siaA*::IS*1301* inactivated meningococci, single colonies were plated and monitored by colony blotting using mAb 735 for capsule expression. This experiment revealed a frequency of 4×10^{-4} for reversion to the encapsulated phenotype. Thirteen revertant, capsule-positive colonies were isolated and their *siaA* genes were amplified by PCR. Direct nucleotide sequence analysis revealed that IS*1301*, including one copy of the TA duplication, had been deleted and the wild-type *siaA* sequence was restored. This precise excision is reminiscent of the behaviour of Tn*5* and Tn*10* (Berg, 1989; Kleckner, 1989) and the frequency correlates well with the excision ratios of other IS elements (Bartlett *et al.*, 1988; Ou *et al.*, 1988). In most of the transposable genetic elements, excision follows intrastrand pairing of the inverted repeats and subsequent slippage across the directly repeated duplication during DNA replication. The frequency of phase variation from the encapsulated to the unencapsulated state is not known. From 10^5 colonies analysed by colony blotting for capsule expression we could not detect a single capsule-negative variant due to *siaA* inactivation by IS*1301*. We do not know yet whether the frequency changes with the environmental conditions.

Southern blot analysis indicated that insertion of IS*1301* within *siaA* was the result of a replication of one of the 12 copies within the genome, since an additional *Pvu*II gene fragment of 2.7 kb was detected, which is as expected from the size of IS*1301* (which has no internal *Pvu*II restriction site) and the size of the *Pvu*II gene fragment of the *cps* cluster (Figure 6). However, the precise excision restored the original restriction pattern of the wild-type strain, indicating that excision entails loss of the IS*1301* copy from *siaA* and not its transposition to a new site.

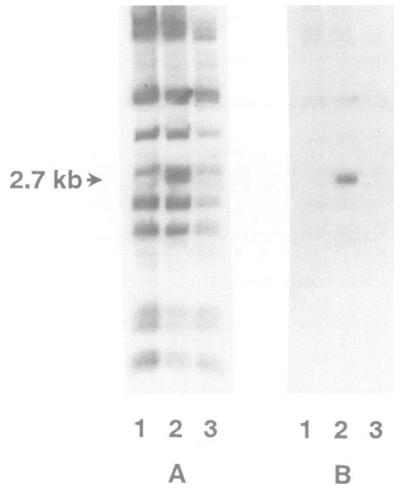

Fig. 6. (**A**) Southern blot analysis of *Pvu*II-digested DNA of wild-type strain B1940 (lane 1), the *siaA*::IS*1301* capsule-inactivated derivative (lane 2) and a capsule-positive revertant isolate (lane 3). For hybridization, a ^{32}P-labelled PCR fragment spanning 443 nt of IS*1301* (between nt 232 and 675) was used. In the *siaA*::IS*1301* capsule-inactivated derivative, a 2.7 kb *Pvu*II fragment hybridized in addition to the 12 copies of IS*1301* present in strain B1940 and the capsule-positive revertant isolate. (**B**) To confirm that the 2.7 kb *Pvu*II fragment corresponds to the insertion within *siaA*, the DNA probe was washed from the filter membrane before it was reprobed with ^{32}P-labelled oligonucleotide SH47, which is complementary to the 3′ joining region of *siaA* and IS*1301* (see also Figure 4).

Discussion

Genetic variation by genomic recombination and gene replacement are common strategies, which are used by pathogenic organisms to adapt to the host during the different stages of an infection and to circumvent the host's defence mechanisms (Robertson and Meyer, 1992). In this study we describe a novel type of genomic recombination in the meningococcus enabling a concurrent phase variation of capsule expression and endogenous sialylation of LOS. This mechanism involves a reversible insertional inactivation of a gene (*siaA*) essentially involved in the synthesis of CMP-NeuNAc, which is required for both capsular polysaccharide synthesis and LOS sialylation. This switching between a non-sialylated and a sialylated bacterial phenotype may provide an adaptive mechanism enabling the bacteria to pass the mucosal barrier and to resist the host's immune defence.

The mechanism of reversible inactivation of the meningococcal *siaA* gene involves the insertion/excision of an insertion sequence element, termed IS*1301*, which is present in multiple copies scattered over the meningococcal chromosome. The exact target sequence in the *siaA* gene is unknown, but may resemble that of the insertion sites of IS*630* found in *Shigella sonnei* (Tenzen and Ohtsubo, 1991) and IS*711* of *B.ovis* (Halling *et al.*, 1993). Like the insertion of IS*1301*, both of these insertion sequence elements are accompanied by a TA duplication. For IS*711*, a consensus target site, YTAR (R, purines; Y, pyrimidines), has been described, which is reminiscent of the target site CTAG of IS*630*. Thus, it is of note that the insertion of IS*1301* in *siaA* is also within the sequence TTAG. A similar mechanism of insertional inactivation of a virulence gene has been found controlling *Citrobacter freundii* Vi antigen expression in *Escherichia coli* (Ou *et al.*, 1988). This system, however, involves the use of

the *E.coli* insertion sequence elements and has not been demonstrated to occur in *Citrobacter*.

The potential biological significance of a mechanism allowing reversible variation of capsule expression and LOS sialylation for meningococcal pathogenesis is illustrated by our findings that the genetically defined capsule-deficient phenotypes showed much higher invasive potential than the encapsulated parental strain and by the apparent selective entry of unencapsulated wild-type bacteria into mucosal cells. Ten to 15% of the re-isolated unencapsulated intracellular bacteria contained the insertion sequence element in *siaA*. The reason for the loss of capsule expression in the other capsule-negative variants is unclear and it is not known yet whether these variants also undergo reversible phase variation of capsule expression and thus that other mechanisms of capsule variation exist or that the loss of capsule is due to irreversible mutations in *cps* genes.

By use of *in vitro* culture systems it is difficult to assess whether the reported novel mechanism of capsule phase variation is also of major biological relevance under *in vivo* conditions during meningococcal infection. However, besides the *in vitro* observations in this study and other reports on the necessity of capsule phase variation as a prerequisite for meningococcal invasion (Stephens *et al.*, 1993; Virji *et al.*, 1993), there is also some clinical evidence for a switching mechanism of capsule expression during the outbreak of meningococcal disease. Meningococci isolated from the nasopharynx of patients suffering from meningococcal disease exhibit a variable degree of encapsulation, and capsule-negative variants are often found (Cartwright, 1995). These variants may be considered as the disease-causing agents, provided that they have the ability to re-express their capsule before they enter the bloodstream. Whether IS*1301* is the dominant modulator of capsule phase variation must be analysed in future studies, e.g. by analysis of unencapsulated nasopharyngeal and encapsulated cerebrospinal fluid isolates from the same infected individual. However, the IS*1301*-induced capsule-negative phenotype described here is reversible and switching-on occurs at a frequency of $\sim 10^{-4}$ (which may be even higher under natural conditions during infection) and therefore fulfills the postulated conditions required for an outbreak of meningococcal disease, although the number of bacteria required to cause disease is unknown. However, in experimental infections in mice, an LD_{50} of <10 meningococci was measured when injected intraperitoneally, bypassing the mucosal barrier (Miller and Castles, 1936; Holbein *et al.*, 1979).

The relevance of IS*1301* for capsule phase variation is further underlined by the fact that this transposable genetic element occurs in a large variety of meningococcal strains, which were isolated from cerebrospinal fluid, blood cultures and sputum. Furthermore, using a serogroup C isolate in the invasion assay, we also observed invasive bacteria with a negative capsular phenotype based on reversible *siaA* inactivation by IS*1301* (S.Hammerschmidt, unpublished). Thus, inactivation of *siaA* by IS*1301* seems to be of general relevance for modulation of meningococcal virulence.

Materials and methods

Bacterial strains

Neisseria meningitidis strain B1940 is a clinical isolate and was obtained from U.Berger, Institute of Hygiene, Heidelberg, Germany. The LOS immunotype of this strain is L3,7,9 and was determined as described below. Mutants derived from this strain exhibit defined defects in capsule and/or LOS biosynthesis (Hammerschmidt *et al.*, 1994). Their characterisics are given in the footnotes to Table I. Strain B1940 and derivatives are all piliated and express Opa and Opc, as shown by electron microscopy and immunoreactivity with the monoclonal antibodies SM1, 4B12 and B306 (kindly provided by M.Virji, John Radcliffe Hospital, Oxford, UK and M.Achtman, Max-Planck-Institut für Molekulare Genetik, Berlin, Germany, respectively). No differences in the amounts or molecular masses of pilin, Opa and Opc were observed by Western blotting. Expression of these determinants was also verified for the microorganisms isolated from the cell culture experiments.

LOS serotyping

LOS serotyping was performed by an ELISA with a panel of antibodies generously provided by W.Zollinger, Walter Reed Army Institute, Washington. Briefly, polystyrole microtitre plates (Greiner, Frickenhausen, Germany) were coated with 100 µl poly-D-lysine [5 µg/ml in phosphate-buffered saline (PBS), pH 7.5]. Whole bacterial cells (5×10^6 in 20 µl) were added for 1 h at room temperature and subsequently fixed with 0.05% glutaraldehyde in PBS. After washing three times with PBS, the wells of the microtitre plates were saturated with 150 µl of 5% (w/v) fat-free milk powder in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl. Subsequently, antibodies in appropriate dilutions were added for 2 h at room temperature. After washing three times with PBS, antibody binding was detected by incubation with a peroxidase-labelled anti-mouse immunoglobulin (Dako, Hamburg, Germany) and subsequent addition of a substrate solution of 20 µl of 2.7% 2,2′-azino[di(3-ethylbenzthiazolinesulfonic acid)] (Boehringer Mannheim) Germany, in 10 mM potassium phosphate, pH 6, containing 0.0025% H_2O_2. The peroxidase reaction was measured by the increase in absorbance at 414 nm.

LOS sialyltransferase activity

LOS sialylation was performed as described previously (Mandrell *et al.*, 1993), with the exception that no exogenous LOS was added.

Invasion assay

Confluent Chang epithelial or Hep-2 larynx carcinoma cell lines (2×10^5) maintained in RPMI/5% fetal calf serum (FCS) were inoculated with 10^7 meningococci and incubated at 37°C and 5% CO_2 as described previously (van Putten, 1993). Subsequently, the cells were rinsed several times with PBS and incubated (15 min) with an anti-meningococcal mouse serum or the anti-capsular polysaccharide IgG2a mAb 735 (Frosch *et al.*, 1985), followed by fixation in methanol (–20°C, 30 min). Bound anti-meningococcal antibodies were visualized by immunofluorescence microscopy after incubation with TRITC-labelled anti-mouse Ig. When appropriate, this procedure was followed by staining of intracellularly located meningococci using the anti-meningococcal mouse serum and FITC-labelled anti-mouse Ig. Bacterial adherence and invasion were scored by microscopic counting of extra- and intracellular bacteria in at least 100 epithelial cells. For isolation of the intracellular bacteria, the extracellular meningococci were killed by treatment with gentamicin and the intracellular meningococci were plated on GC agar after saponin-mediated lysis of the epithelial cells (van Putten, 1993). Each experiment described in this communication was performed at least four times, all giving consistent results, although a day-to-day variability of up to 40% was observed.

Colony blot

For detection of encapsulated bacteria by colony blotting, the bacterial colonies were transferred from the nutrient agar to nitrocellulose filters with a pore size of 0.45 µm (Schleicher & Schüll, Dassel, Germany). The filters were placed on Whatman filter paper 3MM soaked with 0.5 M NaOH; 0.5 M Tris–HCl, pH 7.5, 1.5 M NaCl; 2× SSPE; 70% ethanol, stepwise, 2 min each. After drying, the nitrocellulose filters were blocked by incubation in 3% fat-free milk in 10 mM PBS, pH 7.5, for 30 min and ascites fluid of mAb 735 diluted 1:1000 in PBS was added for 1 h. Bound antibody was detected with alkaline phospatase-labelled anti-mouse Ig (Dianova) and a substrate solution containing 0.05 mg of 5-bromo-4-chloro-3-indolylphosphate per ml, 1 mg of nitro blue tetrazolium per ml in 100 mM Tris–HCl, pH 9.6, 4 mM $MgCl_2$.

Recombinant DNA techniques

Bacterial RNA isolation and Northern blot analysis were performed according to standard protocols (Ausubel *et al.*, 1989). Nucleotide sequence determination was performed according to the dideoxy chain

termination method using the T7 sequencing kit from Pharmacia, Freiburg, Germany. The protocol for direct sequencing of PCR fragments was described previously (Lüneberg *et al.*, 1993). Southern blots were performed under stringent conditions using digoxigenin or ^{32}P-labelled probes. For digoxigenin-labelling of DNA fragments, the DIG–DNA labelling and detection kit from Boehringer Mannheim, was used. The multiprime DNA labelling system from Amersham (Braunschweig, Germany) was used for labelling of DNA fragments with [α-^{32}P]dCTP. Oligonucleotides were labelled at the 3′ end with [α-^{32}P]dCTP using calf thymus terminal transferase (Boehringer Mannheim, Germany). Colony hybridization with oligonucleotide SH47 (5′-ACACGCCCTA-GTAAACTTTC-3′) was performed at 54°C in 6× SSC, 5× Denhardt's solution, 0.1% SDS. Amplification of the *siaA* gene by PCR was performed using the oligonucleotides UE43 (5′-ATGAAAAGAATT-CTTTGCATTACAGGTACC-3′) and UE31 (5′-TTAAAGATTCAAAT-CGATAA-3′) and Goldstar *Taq* DNA polymerase (Eurogentec, Seraing, Belgium) under buffer conditions recommended by the manufacturer. Reactions consisted of 36 cycles including 90 s of denaturation at 94°C, 90 s of annealing at 45°C and 120 s of extension at 72°C.

Accession number

Nucleotide sequence data are deposited under EMBL/Genbank accession number Z49092

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