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Transcriptome-based antigen identification for *Neisseria meningitidis*

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Abstract

The identification of suitable antigens is crucial to successful vaccine development based on subunit approaches. While many methods exist for the identification of vaccine candidates which are surface-exposed or secreted, immunogenic and conserved, contain B and T cell epitopes, most of these have a major drawback: they do not yield any information on whether the antigen is indeed expressed by the pathogen during infection. However, DNA microarrays offer a novel tool for the investigation of the transcriptional activity of all genes of a pathogenic microorganism under in vivo conditions. Employing whole genome DNA microarrays, we have analyzed the transcriptome of *Neisseria meningitidis* serogroup B bacteria during different stages of infection, i.e. exposure to human serum and the interaction with human epithelial and endothelial cells. Combined with data derived from genome-based approaches (such as reverse vaccinology) and immunogenicity studies, this novel transcriptome-based antigen identification should reveal ideal vaccine candidates against serogroup B meningococcal infection.
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1. Introduction

Neisseria meningitidis is a leading cause of purulent meningitis and septicemia in the world with estimated 500,000–1,000,000 cases per year and affects predominantly infants between 6 months and 2 years of age as well as teenagers [1]. Based on the chemical composition and the immunological characteristics of their capsular polysaccharides, meningococci are divided into 12 serogroups. Five of these—serogroups A, B, C, W_{135} and Y—account for virtually all cases of meningococcal disease. While serogroups B and C cause the majority of cases in industrialized countries, group A meningococci are the main pathogens involved in periodic epidemics in China, Middle East, South America, and particularly sub-Saharan Africa [1]. Vaccination against *N. meningitidis* may provide the only measure to significantly reduce the burden of meningococcal disease. During the 20th century, a wide range of vaccine candidates has been evaluated for prevention of meningococcal disease with varying success [2]. Based on the capsular polysaccharides, vaccines specific for *N. meningitidis* serogroups A, C, Y and W_{135} have been developed. However, these vaccines are not very effective in young children [3], i.e. the age group that most importantly requires these vaccines. In addition, since the immune responses against polysaccharides do not involve T cells, mostly low-affinity IgM antibodies are elicited, an immunological memory is not established and booster effects against polysaccharide antigens are principally lacking. These limitations in the use of meningococcal polysaccharide antigens for vaccines can be overcome by linkage of the T cell-independent polysaccharide antigen to a T cell-dependent protein carrier molecule. This results in an enhanced immune response against the conjugated polysaccharides which occurs in infants below the age of 18 months, thus eliciting high titer and boostable protective antibody titers even in this age group. Conjugate vaccines against group C meningococci have been licensed and conjugates with the group A, W_{135} and Y capsular antigens are currently under development or clinical evaluation. A nation-wide immunization program in UK with serogroup C conjugate vaccines resulted in an excellent efficacy and a dramatic decline in the incidence of serogroup C disease [4]. These data provide evidence for a possible prevention of non-serogroup B meningococcal disease. However, polysaccharide conjugate approaches

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are not suitable against serogroup B, which is responsible for most meningococcal disease in USA and Europe [5]. The capsular polysaccharide of serogroup B meningococci is non-immunogenic and bears the risk of inducing autoimmunity as it is indistinguishable from the carbohydrate modification of the eukaryotic neural cell adhesion molecule (N-CAM) [6].

Alternative approaches are based on subcapsularly located antigens like the lipooligosaccharide (LOS) or membrane proteins like PorA and PorB. However, most of the surface-exposed meningococcal antigens exhibit antigenic variability. Hence, antibodies induced against these structures are bactericidal, but type-specific, while immune responses directed against conserved structures often do not confer protection [7]. A broader range of outer membrane antigens is contained in vaccines based on meningococcal outer membrane vesicles (OMV) which were recently developed in Cuba, Norway and The Netherlands. These vaccines have been shown to elicit functional serum bactericidal antibodies and to protect against meningococcal disease in clinical trials. However, the bactericidal titers were mostly serosubtype-specific and the immune responses weakest in younger children and infants [8–10]. The recent availability of the whole genome sequences of *N. meningitidis* serogroup A and B strains [11,12] has dramatically changed antigen screening and built the framework for a novel approach, called "reverse vaccinology", to identify meningococcal subunit vaccine candidates. The genomic information allowed the in silico screening of the meningococcal genome for surface-exposed proteins and secreted antigens. Three hundred and fifty of these antigens were expressed in *Escherichia coli*, analyzed for their immunogenicity and 25 were indeed found to induce bactericidal antibodies against homologous bacteria. Due to their conserved sequence in the meningococcal population they are even expected to have broad protective capacity [5,13].

A drawback of these approaches is that they do not yield any information on the expression level of antigens during meningococcal infection. Meningococci are well known to rapidly alter the expression level of a wide range of proteins by excessive exchange and mutation of their DNA and phase variation due to slipped-strand mispairing [14–16], which ensures the existence of a highly diverse bacterial population and immune evasion by alteration of surface structures. Hence, a serum antibody response may well be bactericidal against meningococci grown in the laboratory in vitro, but lack any protective efficacy under in vivo conditions of infection. Combined with the lack of an animal model of meningococcal sepsis and meningitis which is suitable for vaccine testing, this fact hampers the development of meningococcal vaccines based on antigens of proteinaceous nature. On the other hand, screening methods based on the in vitro growth of *N. meningitidis* may well miss protective antigens which are only expressed at detectable levels during in vivo infection [13]. Recently, Tang and co-workers [17] employed signature-tagged mutagenesis to search for proteins which are essential for meningococcal replication in the infant rat model of meningococcal bacteremic infection, leading to the identification of about 70 candidates.

In humans, meningococcal infection starts with colonization of the nasopharyngeal and tonsillar mucosa. Following adherence, adaptation and proliferation, meningococci can initiate a parasite directed endocytosis by non-ciliated epithelial cells and gain access to the circulation [18]. After a limited phase of bacteremia, meningococci are able to bind to and cross the blood brain barrier and subsequently elicit meningococcal meningitis [19]. DNA microarrays offer an ideal tool for the simultaneous transcriptional analysis of all genes present in a bacterial genome. In the present work we utilized oligonucleotide-based DNA microarrays as a technology platform to analyze transcriptional changes in *N. meningitidis* serogroup B in model systems of these three key steps of meningococcal infection: RNA was isolated from meningococci incubated in human serum as well as adherent to human epithelial cells and endothelial cells. Subsequent hybridization to whole genome DNA microarrays enabled us to characterize the meningococcal transcriptome in these model systems of human infection. With this novel approach we found a wide range of meningococcal surface proteins which are induced under in vivo conditions. These antigens are now available for the rational design of protein-based vaccines for the prevention of meningococcal disease.

2. Materials and methods

2.1. Bacterial strains

The meningococcal strain used in this study is the piliated *N. meningitidis* serogroup B strain MC58 that was isolated 1985 from a clinical case in the UK [20].

2.2. Isolation of meningococcal RNA

For isolation of RNA from serum-treated bacteria, MC58 WT was grown in supplemented protease peptone medium (PPM+) to mid-logarithmic growth phase. Cultures were washed once in PBS, split and one half was incubated in native human serum for 30 min, whilst the other half was incubated in PBS. Subsequently RNA isolation was performed as previously described [21,22]. Quality of RNAs was assessed by formaldehyde–agarose gel electrophoresis. Potential traces of DNA were removed by treatment with RNAse-free DNAse I (Roche Diagnostics GmbH, Mannheim, Germany). Absence of neisserial DNA was confirmed by PCR with primers specific for ORFs NMB0829 (*hsdM*) and NMB1972 (*groEL*), absence of nucleic acids from human cells by RT-PCR using primers specific for human ORFs XM_004814 (*ACTB*) and XM_009352 (*GAPDS*). Infection of human epithelial and endothelial cells and RNA isolation from cell-adherent bacteria were done as previously described [22].

2.3. Construction of oligonucleotide-based whole genome DNA microarrays

For each of the 2158 ORFs present in the published genome of *N. meningitidis* serogroup B strain MC58 [12], oligonucleotide-based microarrays were manufactured as previously described [21,22]. Three oligonucleotides (40-mers) per gene comprising gene-specific internal fragments (covering 5′, central and 3′ parts) were designed. All oligonucleotides (manufactured by MWG-Biotech AG, Ebersberg, Germany) carried a C6 amino linker modification at the 5′-end for covalent attachment to the slide surface. The oligonucleotides were spotted using the Affymetrix 417™ Arrayer (MWG-Biotech AG) on Super Aldehyde Slides (TeleChem International, Sunnyvale, CA, USA) and the slides processed according to the manufacturers' instructions.

2.4. Preparation of labeled cDNA, microarray hybridization and data analysis

Equal amounts of the RNAs to be compared were labeled differentially with Cy3-dCTP and Cy5-dCTP (Amersham Pharmacia, Freiburg, Germany), respectively, during a first-strand reverse transcription (RT) reaction using Superscript II RNase H^- reverse transcriptase (Life Technologies, Karlsruhe, Germany) and a balanced mixture (20 pmol each) of C-terminal primers specific for all genes present on the microarrays. The two differentially labeled cDNA samples were combined, brought to 3× SSC/0.1% (w/v) SDS and hybridized to the array at 50 °C for 16 h. Arrays were washed and scanned using the Affymetrix 418™ Scanner (MWG-Biotech AG). Average signal intensity and local background measurements were obtained for each spot using ImaGene 4.0 software (Biodiscovery Inc., Los Angeles, CA, USA). The two channels were normalized with respect to the mean values of all *N. meningitidis* DNA spots and the Cy3/Cy5 fluorescence ratios were calculated from the normalized values. The transcript ratios of all ORFs were calculated from the average signal ratios of all oligonucleotides per gene. Biological experiments and subsequent hybridizations were repeated at least three times and data of the independent experiments were combined. ORFs exhibiting a minimal 1.6-fold deregulation on average and in more than 50% of the independent experiments were defined as differentially transcribed.

3. Results and discussion

3.1. Transcriptional changes after confrontation with human serum

Oligonucleotide DNA microarrays were employed which previously exhibited a high sensitivity, specificity and reproducibility, allowing a 1.6-fold deregulation to be detected at a level of confidence of 99.7% [21]. Cultures of MC58 WT bacteria were incubated for 30 min in native human serum before RNA preparation. RNA was isolated from serum-treated and control bacteria incubated in PBS. The RNAs of serum-treated and control meningococci were labeled differentially and hybridized to whole genome DNA microarrays. This approach led to the identification of 279 differentially regulated genes. Of these, 134 were induced and 145 repressed (Table 1). The degree of deregulation ranged from 11.9-fold upregulation to 5.3-fold downregulation. The number of differentially regulated genes accounts for 12.9% of the meningococcal genome. The ORFs can be grouped in the following functional categories: metabolism, transcription, translation, DNA synthesis and modification, membrane proteins and transporters, as well as a wide range of hypothetical ORFs. Thirty-four of the differentially transcribed ORFs were previously shown to be virulence associated or essential under in vivo conditions, suggesting that they are necessary for meningococcal survival and dissemination in the bloodstream.

An ideal vaccine candidate should be exposed on the surface of intact bacteria in order to be highly accessible to the

Table 1
N. meningitidis serogroup B ORFs differentially regulated in serum-treated meningococci

Functional category	Differentially regulated genes	Minimum to maximum	Induced	Repressed	Virulence genes	Present in serogroup A	Phase variable
Capsule	3	−1.9 to +2.3	1	2	3	2	0
Cell cycle	2	−2.9 to +4.2	1	1	1	2	0
DNA	12	−2.4 to +2.8	5	7	1	12	0
Membrane	35	−2.4 to +3.6	14	21	16	32	3
Transport	20	−3.9 to +3.1	9	11	4	20	0
Metabolism	63	−5.2 to +5.7	14	49	8	62	3
Phage related	1	−1.9	0	1	0	1	0
Protein fate	5	−3.1 to +2.4	2	3	0	5	0
Transcription	10	−3.0 to +2.6	3	7	1	10	0
Translation	40	−2.8 to +7.6	27	13	0	40	0
Hypothetical	90	−3.6 to +11.9	60	30	0	76	0
Total	279	−5.2 to +11.9	134	145	34	259	6

immune system. Therefore, membrane proteins are likely to be evaluated as antigens. The meningococcal cell surface contains a variety of specific proteins and structures, which enable *N. meningitidis* to interact with host cells, serve as transporters, control the intracellular environment and shield the bacteria from immune responses. Together with the capsular polysaccharide, outer membrane proteins represent the principal antigens of *N. meningitidis*. Of the 55 transporters and membrane proteins differentially transcribed in human serum, 23 were induced and 32 repressed (Table 2). Of these proteins, 20 represent virulence genes.

Of the differentially regulated virulence factors, type IV pili are essential for initial cell-to-cell contact, DNA uptake and motility. Interestingly we found *pilC1*/*C2*, which are crucial for cell interaction as being induced, whereas *pilT* (necessary for pilus retraction), *pilG* and the *pilM–pilQ* operon were repressed. Further virulence associated genes that were upregulated are the *frpC* operon, the putative adhesin NMB0586 and *tonB*: *frpC* codes for a secreted potential toxin of unknown function that is present in invasive strains and likely to be expressed under invasive conditions [23]. The protein encoded by *tonB* is crucially involved in iron uptake and intracellular survival [24,23].

Among the downregulated virulence genes were *mtrC*, *mtrD*, encoding a multiple transferable resistance system, *mtrE*, coding for a multi-drug efflux pump channel protein, the putative hemolysin NMB1646, the class IV outer membrane protein NMB0382, NMB0543 (coding for a putative L-lactate permease) and *fetB* (encoding part of an iron(III) ABC transporter).

The 23 meningococcal membrane and surface proteins induced in human serum may constitute attractive vaccine candidates, and several are already under evaluation. However, some of these—like the general membrane proteins NspA, TspA and class IV—are downregulated in serum. This may point towards an immune evasion by transcriptional repression and should have important implications for vaccine development. Interestingly, the highly variable membrane protein PorA that does not induce cross-protective immune responses is induced in human serum.

3.2. Analysis of the transcriptome of meningococci associated to the surface of HEp-2 cells

We had previously analyzed the transcriptome of meningococci adherent to human epithelial cells (HEp-2) and human brain microvascular endothelial cells (HBMEC) [22]. HEp-2 cells were recently found to be derived from the HeLa epithelial cell line. Infection of HEp-2 with *N. meningitidis* strain MC58 *siaD* (Fig. 1) revealed the differential regulation of 72 genes upon adherence of meningococci to HEp-2 cells (equivalent to 3.3% of the meningococcal genome). Sixty-seven of these were upregulated, five were downregulated.

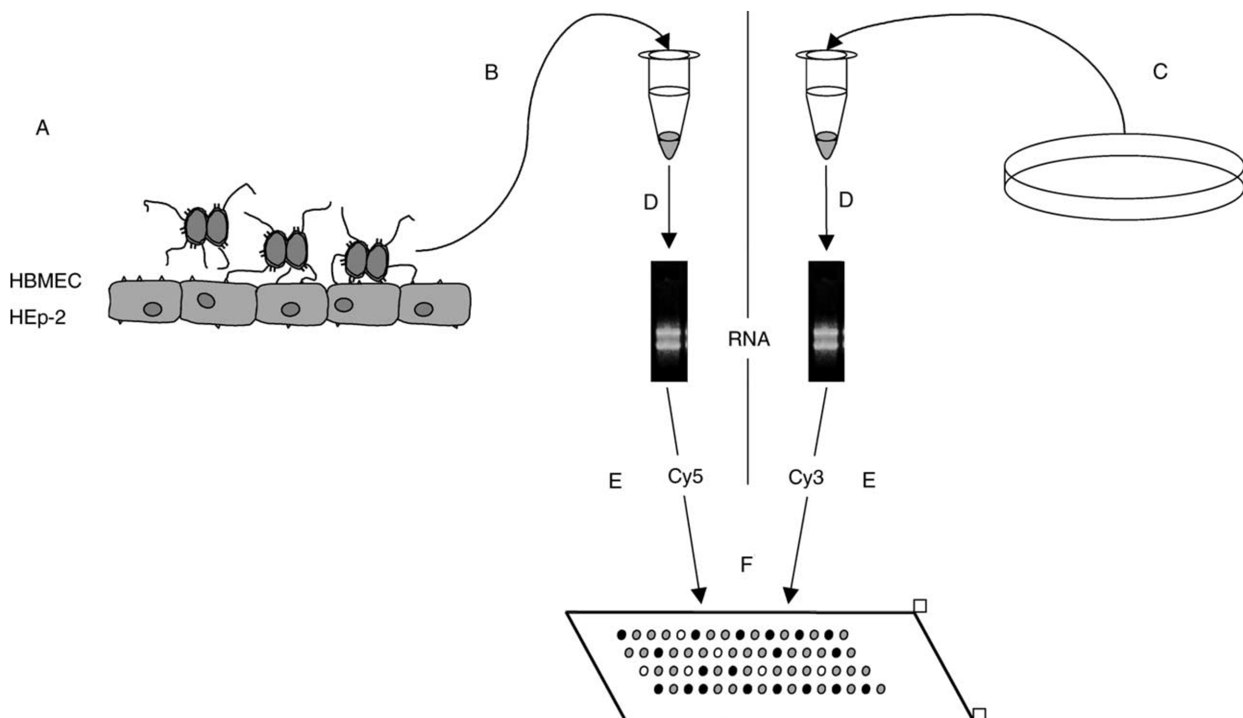

Fig. 1. Analysis of the transcriptome of host cell-adherent meningococci. (A) HEp-2 cells or HBMEC are infected with *N. meningitidis* strain MC58 *siaD*. (B) Six hours post-infection, the cell-adherent bacteria are isolated. (C) Control bacteria are incubated in cell culture medium for 6 h. (D) RNA is extracted from cell-adherent bacteria and control meningococci. (E) RNAs are labeled differentially, (F) combined and hybridized to whole genome oligonucleotide microarrays for detection of differentially transcribed ORFs.

Table 2
Surface and membrane protein encoding *N. meningitidis* serogroup B ORFs differentially regulated in serum-treated meningococci

NMB number[a]	Differential regulation	S.D.	Encoded protein	Virulence gene	NMA number[b]	Phase variable
NMB0034	+2.6	0.3	Conserved hypothetical protein		NMA0280	
NMB0042	−1.8	0.2	Conserved hypothetical protein		NMA0288	
NMB0049	+1.9	0.3	PilC2	+	NMA0293	+
NMB0162	+7.0	4.6	Pre-protein translocase SecY subunit		NMA0109	
NMB0228	−1.7	0.3	Conserved hypothetical protein Rni3		NMA0030	
NMB0260	−1.8	0.1	Hypothetical inner membrane protein		NMA2227	
NMB0288	−1.8	0.3	Hypothetical inner membrane protein		NMA2199	
NMB0333	−1.8	0.2	Pilus assembly protein PilG	+	NMA2155	
NMB0341	−2.1	0.4	*Neisseria*-specific antigen protein TspA		NMA2146	
NMB0364/NMB0584/ NMB1412/NMB1414	+3.6	0.1	FrpC operon protein	+	NMA2124	
NMB0382	−1.7	0.2	Outer membrane protein class IV RmpM	+	NMA2105	
NMB0470	+3.0	0.9	C4-Dicarboxylate transporter		NMA2015	
NMB0490	−1.9	0.1	PspA-related protein		Absent	
NMB0535	−1.9	0.1	Glucose/galactose transporter GluP		NMA0714	
NMB0543	−1.7	0.1	L-Lactate permease, putative	+	NMA0722	
NMB0578	−1.6	0.5	Copper ABC transporter, periplasmic copper-binding protein NosD		NMA0762	
NMB0586	+2.9	0.5	Adhesin, putative	+	NMA0789	
NMB0610	−2.0	0.5	Spermidine/putrescine ABC transporter, ATP-binding protein PotA-1		NMA0816	
NMB0615	+2.3	0.4	Ammonium transporter AmtB, putative		NMA0820	
NMB0663	−2.2	0.5	Outer membrane protein NspA		NMA0862	
NMB0696	−1.8	0.2	Amino acid ABC transporter, ATP-binding protein		NMA0900	
NMB0707	−1.7	0.1	Rare lipoprotein B, putative		NMA0912	
NMB0709	−2.3	0.4	Hypothetical protein		NMA0914	
NMB0762	+2.9	1.0	Putative integral membrane protein		NMA0973	
NMB0768	−1.8	0.2	Twitching motility protein PilT	+	NMA0979	
NMB0787	+2.9	1.3	Amino acid ABC transporter, periplasmic amino acid-binding protein		NMA0997	
NMB0887	+2.1	0.5	Type IV pilus assembly protein PilV, putative		NMA1107	
NMB0889	+2.3	0.4	Putative membrane protein		NMA1109	
NMB0890	+1.9	0.5	Type IV pilin-related protein	+	NMA1110	
NMB1051	+2.7	0.8	ABC transporter, ATP-binding protein AbcZ		NMA1249	
NMB1271	−2.0	0.5	Mercury transport periplasmic protein, putative		NMA1476	
NMB1313	−1.7	0.3	Trigger factor Tig		NMA1526	
NMB1369	−2.2	0.7	Putative membrane lipoprotein		NMA1581	
NMB1429	+2.5	0.1	Outer membrane protein PorA	+	NMA1642	+
NMB1468	+2.2	0.3	Putative membrane protein		NMA1680	
NMB1623	−2.4	0.5	Major anaerobically induced outer membrane protein, nitrite reductase AniA		NMA1887	
NMB1646	−1.6	0.1	Hemolysin, putative	+	NMA1900	
NMB1707	+3.1	0.6	Sodium- and chloride-dependent transporter		NMA1961	
NMB1714	−2.0	0.2	Multi-drug efflux pump channel protein MtrE	+	NMA1968	
NMB1715	−3.9	0.7	Multiple transferable resistance system protein MtrD	+	NMA1969	
NMB1716	−1.8	0.2	Membrane fusion protein MtrC		NMA1970	
NMB1730	+2.1	0.4	TonB protein	+	NMA1985	
NMB1732	+2.2	0.1	Transporter, putative		NMA1988	
NMB1808	−1.6	0.3	PilM protein	+	NMA0654	
NMB1809	−2.1	0.5	PilN protein	+	NMA0653	
NMB1810	−1.6	0.4	PilO protein	+	NMA0652	
NMB1811	−1.6	0.3	PilP protein	+	NMA0651	
NMB1812	−2.4	0.4	PilQ protein	+	NMA0650	
NMB1820	−1.7	0.3	Pilin glycosylation protein PglB		NMA0639	
NMB1847	+1.7	0.4	PilC1	+	NMA0609	+
NMB1966	+1.8	0.1	ABC transporter, ATP-binding protein		NMA0485	
NMB1989	−1.7	0.2	Iron(III) ABC transporter, periplasmic-binding protein FetB	+	NMA0452	
NMB2020	+1.7	0.3	Putative integral membrane protein		NMA0420	
NMB2078	+1.8	0.1	Conserved hypothetical protein		NMA0353	
NMB2136	+1.9	0.4	Peptide transporter		NMA0222	

[a] ORF numbers in the published *N. meningitidis* serogroup B genome sequence [12] are given.
[b] ORF numbers in the published *N. meningitidis* serogroup A genome sequence [11] are given.

A high proportion (21) of the ORFs upregulated after interaction with HEp-2 cells encode membrane proteins and transporters as well as secreted proteins (*iga*, *frpC*, *opa*, *fimT*, *porA*, *alaR*, *tonB*, *pilN*, *pilO*, *cysW*, *cysT*, *exbD*, *secG*, *fbpB*, *lbpB*, NMB0177, NMB0364, NMB0787, NMB0788, NMB0889, NMB1017). Of these, 10 are previously identified virulence genes: genes encoding IgA protease (*iga*), several iron uptake systems (*tonB*, *exbD*, *lbpB*, *fbpB*), type IV pilus assembly proteins (*pilN*, *pilO*), invasins (*opa*, *porA*) and potential toxins (*frpC*). A number of these genes had previously been shown to be involved in the interaction of pathogenic *Neisseriae* with epithelial and endothelial cells [15,20,26]. Of the proteins encoded by the upregulated genes, some represent previously assessed vaccine candidates like PorA and LbpB. While PorA probably has a lower priority as a vaccine antigen due to its variability, iron acquisition plays an important role in meningococcal virulence [25]. Hence surface-exposed proteins being part of iron transport systems are interesting vaccine candidates. The upregulation of multiple iron acquisition systems upon cell adherence supports the subunit vaccine approaches employing proteins such as the lactoferrin binding protein LbpB [27].

We also identified two ORFs encoding membrane proteins which are downregulated: NMB0177 and *fetA*. This result is astonishing especially for *fetA* (previously *frpB*), since the siderophore receptor encoded by this gene is an intensively investigated vaccine candidate [28].

3.3. Analysis of the transcriptome of HBMEC cell surface-associated meningococci

HBMEC were infected with *N. meningitidis* strain MC58 *siaD* and RNA isolated from cell-adherent bacteria. As previously described [22], infection of HBMEC with *N. meningitidis* strain MC58 *siaD* revealed significant transcriptional changes for 48 ORFs, equivalent to 2.2% of all ORFs present in strain MC58. Forty-two of these were upregulated, six were downregulated. Four upregulated genes encode membrane proteins and transporter components: *tonB*, *secY*, NMB1468 and NMB1646. Of these, the iron uptake protein TonB and the putative hemolysin encoded by NMB1646 are involved in meningococcal virulence. Thirteen of the ORFs (27%) differentially regulated upon cell contact showed excellent agreement in HEp-2- and HBMEC-adherent meningococci. Interestingly, most virulence-associated genes differentially regulated in HBMEC-adherent meningococci were also induced during HEp-2 interaction, suggesting that they have a general function for host cell interaction. Accordingly, they should be valuable vaccine candidates. The non-concordant differentially regulated genes may in contrast be specifically required during infection of epithelial or endothelial cells, respectively. For example, most iron-regulated genes, pilus and capsule synthesis genes and *iga* are induced only in HEp-2-adherent bacteria. The different transcription patterns of HEp-2- and HBMEC-adherent meningococci may be caused by bacterial binding to different receptors on the surface of the host cells.

Surprisingly, we found a widely contrasting pattern when comparing genes differentially regulated in human serum with those differentially regulated upon host cell contact, only 39 genes were differentially regulated in human serum as well as during cell adherence. Of these, three (*mdaB*, *tonB*, NMB1575) were differentially regulated in all three systems, 23 only in serum and during HBMEC adherence, 13 only in serum and during HEp-2 adherence. Ten genes were even regulated inversely, (induced upon cell adherence and repressed in serum or vice versa) (data not shown).

Quantitative RT-PCR was employed as an independent method to assess the differential regulation of selected ORFs in all three experimental systems and confirmed the microarray-based observations for all genes analyzed ([22] and data not shown).

The differentially regulated genes identified in this study exhibit a mainly random distribution throughout the meningococcal genome (Fig. 2). The majority of the differentially regulated genes is also present in the genome of *N. meningitidis* serogroup A strain Z2491. Vaccination

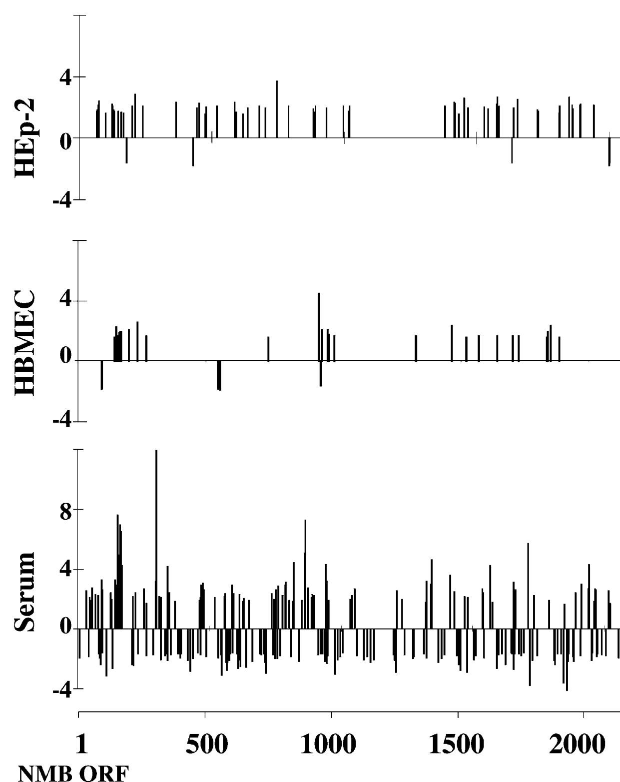

Fig. 2. Genomic distribution of differentially regulated genes throughout the *N. meningitidis* strain MC58 genome. Differential regulation of genes of *N. meningitidis* adherent to HEp-2 cells, HBMEC and exposed to human serum is shown. The position of differentially transcribed ORFs within the MC58 genome [12] is shown on the *x*-axis, the level of deregulation is shown on the *y*-axis.

against the proteins encoded by these genes may therefore lead to cross-protection, provided their immunodominant epitopes are conserved. However, 24 ([22], Tables 1 and 2) differentially transcribed ORFs are absent from serogroup A meningococci. Future experiments should reveal whether these ORFs either contribute to specific virulence mechanisms of serogroup B strains or whether they are just dispensable for (serogroup A) meningococcal pathogenicity. Vaccination approaches based on the encoded proteins will certainly lead to highly specific immune responses against serogroup B meningococci. A total of 10 differentially regulated ORFs are phase variable. Phase variation is an important meningococcal mechanism for constant surface structure modulation and immune evasion. Therefore, proteins encoded by phase variable genes are unlikely to be good vaccine candidates.

While the DNA microarrays used in the present study allow the detection of transcriptional changes, these may not necessarily result in an induction or repression of protein expression. We are currently trying to extend our data from RNA analyses and determine the protein levels of selected candidates. Another important point to consider is that the current analysis averages transcriptional gene regulation in all cell-adherent or serum-treated bacteria. There may be heterogeneous populations of bacteria exhibiting different degrees of invasiveness or being at different stages of infection, which may not be detected with the present experimental systems. Additionally, we will construct mutant strains which should allow us to further analyze the in vivo roles of the differentially regulated ORFs. Nevertheless, the fact that the differentially regulated genes identified in this study comprise a wide range of virulence genes or ORFs that were previously shown to be essential in the infant rat model of meningococcal bacteremic infection by signature-tagged mutagenesis [17] demonstrates the physiological relevance of our approach.

Hence, DNA microarray-based transcriptome screening has the potential to provide vaccine researchers with important information for candidate selection. In contrast to most antigen identification techniques, it yields information on the in vivo expression level of all antigens encoded by the genome of a pathogenic microorganism. This may form a much better basis for considering specific antigens as vaccine candidates. This is particularly important for a disease like serogroup B meningococcal sepsis and meningitis, for which no accepted surrogate markers of protection or appropriate animal models exist [2]. For example, many surface-exposed proteins were identified as being upregulated during infection of human host cells or meningococcal exposure to human serum. These candidates are now being assessed for their immunogenicity and their potential to induce bactericidal immune responses. Provided they are immunogenic, the present data strongly suggest that the selected antigens are indeed expressed during human infection, making them ideal vaccine candidates. The fact that antibodies against PorA, Opa, IgA1 protease and Lbp have been shown to be present in the sera of convalescent patients validates our approach [7,27,29].

Proteins which are essential for the in vivo virulence and pathogenicity of meningococci should represent highly suitable candidate antigens since they are less likely to be subject to evolutionary changes due to immunologic pressure. Extension of our studies to a set of strains with broad epidemiological coverage will yield information on the general in vivo expression levels of these proteins and help to identify a set of antigens providing universal protection.

Another aspect is that a substantial proportion of the ORFs in the meningococcal genome have no known function. A high number of ORFs for which no specific role has been assigned so far are induced upon cell contact and in human serum, suggesting that they are induced during infection and may contribute to meningococcal virulence. Transcriptome-based antigen identification may provide a suitable technology platform to unravel the role of ORFs for which no biological function has previously been found and thus lead to the identification of novel vaccine candidates so far unavailable through classic screening technologies.

On the other hand, our data also shed new light on well-investigated vaccine candidates such as FrpB [28] and NspA [30]. The downregulation found in the model systems of meningococcal infection assessed in this work may have important implications for vaccine research and can help vaccinologists to prioritize antigens. However, microarray-based data should certainly not be the only basis for the exclusion of an antigen since particularly NspA and FrpB are known to be present during infection as demonstrated by the fact that antibodies against both proteins are present in convalescent sera [31,32].

The current study clearly demonstrates that bacterial transcriptome analysis during interaction with the human host is a suitable model system for the natural colonization and infection by bacterial pathogens like *N. meningitidis*. The knowledge of the components involved in meningococcal crossing of epithelial and endothelial borders as well as dissemination in human blood may form the basis for the development of novel strategies to prevent infection by *N. meningitidis* and the selection of appropriate vaccine candidates.

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