Gene expression in the human pathogen *Neisseria meningitidis*: Adaptation to serum exposure and zinc limitation

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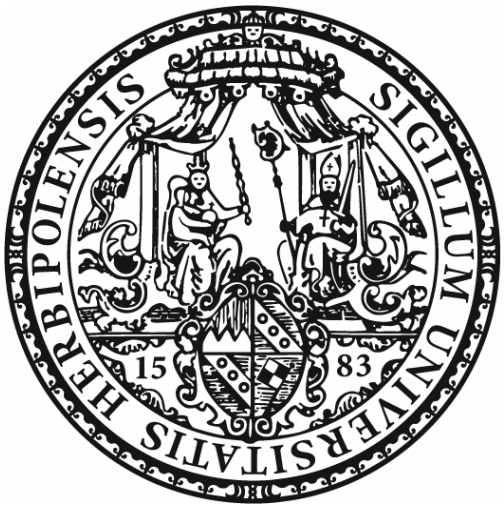

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AFFIDAVIT

I hereby confirm that my thesis entitled 'Adaptation of the human pathogen *Neisseria meningitidis* to serum exposure and zinc limitation' is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.
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Swansea, 20/04/2013

Marie-Christin Pawlik

Meiner lieben

Bischofswerdaer Oma

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1 ZUSAMMENFASSUNG

Neisseria meningitidis ist ein fakultatives Humanpathogen, welches mitunter sehr resistant gegenüber Serumkomplement-Exposition ist. Bereits beschriebene Faktoren, welche die Serumresistenz von Meningokokken fördern, sind beispielsweise die Kapsel, LPS-Sialylierung und Expression des fH-bindenden Proteins. Das Ziel dieser Arbeit war die Identifikation neuartiger Serumresistenzfaktoren, wobei ich zwei Ansätzen verfolgte: i) Die Analyse des Einflusses von globalen Regulatoren der Genexpression auf die Serumresistenz; und ii) eine vergleichenden Analyse von eng verwandten Stämmen, die sich hinsichtlich ihrer Serumresistanz unterschieden.

i) Von sechs untersuchten globalen Regulatoren der Genexpression, war die Komplementdeposition auf Meningokokken nur nach Mutation des Regulators der Zinkaufnahme, Zur, reduziert. Über Zur selbst und die regulatorischen Prozesse in Reaktion auf Zink war in Meningokokken wenig bekannt. Ich habe daher das bisher nicht bestimmte Zur-Regulon von Meningokokken aufgeklärt, wofür ich mittels Mikroarrays die transkriptionelle Antwort des *N. meningitidis*-Stammes MC58 unter Zink-Überfluss und Zink-Mangel zu vergleichen. Das Zur-Regulon von Meningokokken umfasst 17 Gene, von denen unter Zinküberfluss 15 reprimiert und zwei aktiviert wurden. Unter den Zur-reprimierten Genen fanden sich Gene, die in die Aufnahme von Zink, die Modikation von tRNAs und den Zusammenbau des Ribosoms involviert sind. Ein 23 bp langes Binde-Konsensusmotiv für Meningokokken-Zur (Zur-Box) mit einem konservierten zentralen Palindrom wurde ermittelt (TGTTATDNHATAACA) und in der Promotorregion aller regulierten Transkriptionseinheiten (Gene/Operons) detektiert. *In vitro*-Bindung des *N. meningitidis* Zur an die Zur-Box dreier ausgewählter Genen konnte mittels EMSAs erstmals gezeigt werden. Die Bindung von Zur an DNA war spezifisch abhängig von Zink, und Mutationen in der palindromischen Sequenz hemmten die Zur-Bindung an das DNA-Motiv.

ii) Drei eng verwandte Stämme des ST-41/44-Komplexes aus invasiver Erkrankung und Trägertum, die sich in ihrer Resistenz gegenüber Serumkomplement-Exposition unterschieden, wurden analysiert um neuartige Mediatoren der Serumresistenz zu identifizieren. Der Gengehalt der Stämme wurde mittels Mikroarray-Analyse verglichen. Dies offenbarte sechs Gene, die in den beiden Trägerstämmen vorhanden, aber in dem invasiven Isolat abwesend waren. Vier dieser Gene liegen innerhalb zweier Inseln horizontal transferierter DNA, d.h. IHT-B und –C. Weiterhin führte die Arbeitsgruppe eine Transkriptom- und Proteom-Analyse der drei Stämme sowie einen umfangsreichen Screening-Assay durch. Diese Ansätze führten zur Identifikation dreier Kandidaten-Proteine für die weitere Analyse. Ich wirkte daran mit, die Rolle dieser Proteine für die Serumresistenz von Meningokokken zu ermitteln: Das Adhäsin Opc vermittelt

Serumresistenz durch Bindung von Vitronektin, einem negativen Regulator des Komplementsystems; das hypothetische Protein NMB0865 trägt über einen bisher unbekannten Mechanismus geringfügig zur Serumresistanz bei; und NspA, für welches vor Kurzem erkannt wurde, dass es den negativen Komplementregulator Faktor H bindet, führte zu beträchtlich reduzierter Abtötung durch Komplement.

2 SUMMARY

Neisseria meningitidis is a facultative human pathogen that occasionally shows strong resistance against serum complement exposure. Previously described factors that mediate meningococcal serum resistance are for example the capsule, LPS sialylation, and expression of the factor H binding protein. I aimed for identification of novel serum resistance factors, thereby following two approaches, i) the analysis of the impact of global regulators of gene expression on serum resistance; and ii) a comparative analysis of closely related strains differing in serum resistance.

(i) Of six meningococcal global regulators of gene expression studied, only mutation of the zinc uptake regulator Zur reduced complement deposition on meningococci. Little was known about meningococcal Zur and regulatory processes in response to zinc. I therefore elucidated the yet unidentified meningococcal Zur regulon comparing the transcriptional response of the *N. meningitidis* strain MC58 under zinc-rich and zinc-deficient conditions using a common reference design of microarray analysis. The meningococcal Zur regulon comprises 17 genes, of which 15 genes were repressed and two genes were activated at high zinc condition. Amongst the Zur-repressed genes were genes involved in zinc uptake, tRNA modification, and ribosomal assembly. A 23 bp meningococcal consensus Zur binding motif (Zur box) with a conserved central palindrome was established (TGTTATDNHATAACA) and detected in the promoter region of all regulated transcriptional units (genes/operons). *In vitro* binding of meningococcal Zur to the Zur box of three selected genes was shown for the first time using EMSAs. Binding of meningococcal Zur to DNA depended specifically on zinc, and mutations in the palindromic sequence constrained Zur binding to the DNA motif.

ii) Three closely related strains of ST-41/44 cc from invasive disease and carriage which differed in their resistance to serum complement exposure were analysed to identify novel mediators of serum resistance. I compared the strains' gene content by microarray analysis which revealed six genes being present in both carrier isolates, but absent in the invasive isolate. Four of them are part of two *Islands of horizontally transferred DNA*, i.e. IHT-B and –C. The working group furthermore applied a comprehensive screening assay, a transcriptome and a proteome analysis leading to identification of three target proteins. I contributed to establish the role of these three proteins in serum resistance: The adhesin Opc mediates serum resistance by binding of vitronectin, a negative regulator of the complement system; the hypothetical protein NMB0865 slightly contributes to serum resistance by a yet unknown mechanism; and NspA, recently identified to bind the negative complement regulator factor H, led to considerable reduced complement-mediated killing.

3 INTRODUCTION

3.1 The pathogen *Neisseria meningitidis*

The gram negative β-proteobacterium *Neisseria meningitidis* was first discovered in 1887 by the Austrian physician Anton Weichselbaum in the cerebrospinal fluid of a meningitis patient (Weichselbaum, 1887). The prokaryote is pathogenic only in humans and is the major cause of bacteraemia and meningitis worldwide (Tan et al, 2010).

Meningococci are transmitted by direct contact with or inhalation of respiratory droplets. Asymptomatic colonisation (carriage) of the human nasopharynx by *N. meningitidis* may only be transient, or last days or even months; ending by natural clearing of the organism (Christensen et al, 2010; Stephens, 2007). However, carriage forms a reservoir for transmission (Claus et al, 2005). It furthermore can lead to invasive disease, for which an interplay of bacterial virulence factors (e.g. LPS), host susceptibility (e.g. due to absence of protective antibodies) and environmental factors (crowded living conditions, smoking, social gathering and intimate kissing) is thought to be responsible (Pace & Pollard, 2012; Stephens et al, 2007). Upon infection, meningococci penetrate the nasopharyngeal mucosal epithelium and invade the blood stream. Their massive proliferation and dissemination can cause septicaemia. When the diplococci eventually pass the blood-brain barrier, meningitis develops (Rouphael & Stephens, 2012).

3.2 Meningococcal population structure & epidemiology

The meningococcal population displays high variability due to extensive genomic recombination. Despite that, lineages have evolved which persist on a world-wide scale over decades (Budroni et al, 2011). For tracing transmission and detection of disease clusters, genetic typing of isolates is important (Elias et al, 2010). This is achieved by determination of the capsular serogroups, and antigen sequence typing of the major outer membrane porin PorA and the iron-regulated outer-membrane protein ferric enterochelin A (FetA). Furthermore, multilocus sequence typing (MLST), based on seven housekeeping genes, helps to assign the lineage by defining the sequence type (ST) and the clonal complex (cc). These classification systems ease epidemiological analysis and the surveillance of meningococcal disease (Jolley et al, 2007) and will in future be conducted by next generation sequencing approaches (Vogel et al, 2012a).

Carriage of *N. meningitidis* varies depending on setting and age. High rates of carriage for example have been reported in close contact persons of IMD patients (Kristiansen et al, 1998), military personnel (Pether et al, 1988) or Hajj pilgrims (Hahne et al, 2002). Prevalence of carriage is low in infants with 4.5%, increases during childhood and adolescence reaching a peak with 23.7% in 19-year olds, and decreases again during adulthood, e.g. 13.1% and 7.8% in 30- and 50-year olds, respectively (Christensen et al, 2010).

In contrast, invasive meningococcal disease (IMD) is most prevalent in infants and adolescents (Goldschneider et al, 1969a; Goldschneider et al, 1969b). IMD is of particular interest for public health due to the fast progress of infection, that may lead to death within 12 to 24 hours, and a high mortality of 10-15%, despite of the administration of suitable antibiotics (Brandtzaeg & van Deuren, 2012; Pace & Pollard, 2012). Furthermore, up to 20% of patients that survive the infection suffer from long-term sequelae as amputations, hearing loss, impaired motor skills and neuronal dysfunction (Bilukha & Rosenstein, 2005; Pace & Pollard, 2012).

IMD is sporadic in industrialised countries IMD with an average incidence in Europe of approximately 1/100,000 (Tan et al, 2010). In contrast, it is of special concern in non-industrialised regions like Africa. Especially serogroup A meningococci (MenA) periodically cause epidemics in the so called "African meningitis belt", a term first employed by Lapeyssonnie for the sub-Saharan region spanning from Ethiopia to Senegal, where large epidemics occur annually during the dry season, and devastating outbreaks caused thousands of deaths (Lapeyssonnie, 1963; Stephens et al, 2007). Additionally, MenW-135 and recently MenX caused local outbreaks in the meningitis belt, e.g. in Burkina Faso (Bertherat et al, 2002; Delrieu et al, 2011). MenW-135 also was associated with outbreaks among pilgrims during the Hajj (Decosas & Koama, 2002). MenY emerged as an IMD-causing group relatively recently in Northern America, for example in the USA (McEllistrem et al, 2004).

However, most cases of IMD in North and South America, Europe, Australia and Asia are caused by MenB and MenC (Bettinger et al, 2012; Gorla et al, 2012; Racloz & Luiz, 2010; Russell et al, 2008; Tribe et al, 2002; Xu et al, 2012). MenB thereby is associated with larger outbreaks and substantial morbidity and mortality, causing up to 80% of IMD in Europe (Rouphael & Stephens, 2012). In Germany, incidences of IMD in persons below 25 years were 0.32 and 0.10 per 100,000 due to MenB and MenC, respectively (Hellenbrand et al, 2013). More than half of MenB cases are caused by the clonal complexes ST-41/44 and ST-32. The large hypervirulent complex ST-41/44 cc is the most diverse one with more than 1000 STs, among these the geographically widespread and most common ST-41 and ST-42 (Racloz & Luiz, 2010). ST-41/44 cc caused

epidemics in The Netherlands and New Zealand (Dyet & Martin, 2006; Scholten et al, 1994) and was most prevalent in cases of IMD in Belgium, Italy and Ireland (Harrison et al, 2009; Racloz & Luiz, 2010). In Germany, ST-41/44 cc was responsible for a community outbreak in the Greater Aachen region (Elias et al, 2010).

3.3 Vaccine development against meningococcal disease

Extensive research regarding the immunogenicity of the meningococcal capsule led to development of quadrivalent meningococcal vaccines composed of MenA, C, W-135, and Y capsular polysaccharides already in the 1970s (Artenstein et al, 1970; Gold et al, 1975; Goldschneider et al, 1969a; Goldschneider et al, 1969b). However, polysaccharides only elicit a weak T cell-independent immune response with no immune memory in infants (Gold et al, 1975; Stein, 1992). Therefore, monovalent MenC meningococcal glyco-conjugate vaccines, in which the capsule polysaccharide was conjugated to a carrier protein, were introduced in the late 1990s that led to a robust T-cell dependent response and induction of immune memory (MacDonald et al, 1998). These vaccines reduced MenC infection rates and also asymptomatic carriage rates, thereby contributing to herd immunity (Maiden et al, 2008). This success also prompted development of quadrivalent meningococcal glycoconjugate vaccines against MenA, C, W-135 and Y, in which the MenACWY capsule polysaccharides are conjugated to diphtheria or tetanus toxoid (Dull & McIntosh, 2012).

In the sub-Saharan meningitis belt, 85% of IMD are caused by MenA (Burki, 2011). Therefore, in 2001 the *Meningitis Vaccine Project* was set up by the World Health Organisation (WHO) and the Program for Appropriate Technology for Health (PATH) to develop a low-priced vaccine against MenA (LaForce & Okwo-Bele, 2011). MenAfriVac, a monovalent MenA-tetanus toxoid conjugated vaccine, was introduced in three hyperendemic countries in December 2010, and vaccination is ongoing in the other 23 countries within the meningitis belt (Burki, 2011; Djingarey et al, 2012).

Development of protective vaccines against MenB was lagged, because the MenB capsule polysaccharide constitutes a molecular mimicry of human fetal NCAM (3.5.1) which results in poor immunogenicity and might cause autoimmune reactions (Finne et al, 1987). As a first suitable approach, vaccines comprising outer membrane vesicles (OMVs) of a particular IMD-causing strain were effective against MenB outbreaks, e.g. in Norway, Cuba and New Zealand (Bjune et al, 1991; Oster et al, 2005; Sierra et al, 1991). However, they could only induce a strain-specific immune response and short-

term protection. Using the novel approach of *reverse vaccinology* (Rappuoli, 2000), various surface-exposed proteins and secreted antigens were predicted as putative novel MenB vaccine targets [3.6.2.1] (Pizza et al, 2000). Based on this, the company Novartis designed the multi-component MenB vaccine Bexsero® (4CMenB) which contains the protein components Neisserial adhesin A (NadA), factor H binding protein (fHBP) and Neisserial heparin-binding antigen [NhbA, NMB2132; Welsch et al (2003)] that are fused to carrier proteins; and additionally OMVs (PorA P1.4) used in the New Zealand vaccine (Dull & McIntosh, 2012). All four main components are involved in immune evasion - whereas NhbA binds heparin leading to enhanced bacterial serum resistance (Gasparini et al, 2012), the actions of NadA, fhBP and PorA are discussed in more detail in chapter 3.5. This January, Bexsero® received EU approval by the European medicines agency (EMA) for use in persons from two months age and older (Novartis, 2013). The vaccine is predicted to cover 78% of MenB strains based on collections from five European countries (Vogel et al, 2013).

3.4 Meningococci and the human complement system

Meningococci evolved several mechanisms to avoid killing by human complement (Schneider et al, 2007), and serum resistance contributes invasive disease (Vogel & Frosch, 1999).

The complement system consists of more than 30 antimicrobial proteins and is an important component of the innate (unspecific) and the adapted (specific) immunity. The name derived from its function to 'complement' further mechanisms of defence by recognition and elimination of invading microorganisms [for review see Zipfel and Skerka (2009)].

3.4.1 The complement cascade

Activation of complement leads to a cascade of enzymatic reactions that can occur via three different pathways: The i) classical pathway (CP); ii) alternative pathway (AP); and ii) lectin pathway (LP) (Sarma & Ward, 2011). These pathways and their components are depicted in Figure 1. The activation cascade is summarised below according to various review articles (DiScipio, 1997; Sarma & Ward, 2011; Schneider et al, 2007; Zipfel & Skerka, 2009).

The CP is initiated by binding of the complement protein C1q to the bacterial surface, i.e. directly on lipid A or lipoteichoic acids (LTA) or indirectly on surface-bound antibodies (IgG, IgM). Binding of C1q activates C1r and C1s which leads to formation of the C1 complex. The products of C1s-mediated cleavage of C4 and C2 then form the C3 convertase of the CP (C4b2a). Likewise, the LP is initiated by binding of mannose-binding lectin (MBL) or ficolin to carbohydrates on the pathogen's surface. This induces autoactivation of MBL-associated proteins (Masp1/2) that then cleave C4 and C2 which leads to formation of the C3 convertase C4b2a. The C3 convertase cleaves C3 into C3a, a potent anaphylatoxin, and C3b. C3b can directly bind to bacterial surfaces leading to an amplification loop of complement activation via the AP. Upon binding, factor B is recruited and cleaved by factor D which results in the formation of a C3bBb complex, the C3 convertase of the AP. Furthermore, C3b interacts with the C3 convertases of CP/LP and AP to form the C5 convertases, C4b2a3b and C3bBbC3b, respectively. The C5 convertases cleave C5 into C5a, another anaphylatoxin, and C5b. C5b is the start point for the terminal pathway, initiating the assembly of the membrane attack complex (MAC). It first associates with C6 and C7 leading to the C5b-7 complex. If close to a phospholipid bilayer, this complex embeds to the outer leaflet. Upon binding of C8, the formed C5b-8 complex pierces the membrane, creating an initial pore. The C5b-8 complex initiates and accelerates polymerisation of $C9_n$. Eventually, the C5b-9 complex (MAC) forms a pore in the bacterial membrane which leads to cell lysis. Mature MAC can be detected by monoclonal antibodies specific to a neoepitope which is only exposed in this terminal complement complex (Mollnes & Harboe, 1987).

Besides MAC-mediated cell lysis, the complement system has two more important functions: Firstly, by binding to bacterial surfaces, C3b and its inactivated form iC3b also act as opsonins leading to phagocytosis of the respective bacterial cell. Secondly, anaphylatoxins derived from factors C3, C4 and C5 (i.e. C3a, C4a and C5a) are chemo-attractants for phagocytic cells (neutrophils, monocytes) to sites of injury or inflammation, induce release of histamines by mast cells and reactive oxygen species by neutrophils (oxidative burst), and cause vasodilation leading to increased blood flow. High levels of anaphylatoxins therefore contribute to an overshooting immune reaction upon meningococcal septic shock that might lead to multiorgan failure and death (Brandtzaeg & van Deuren, 2012).

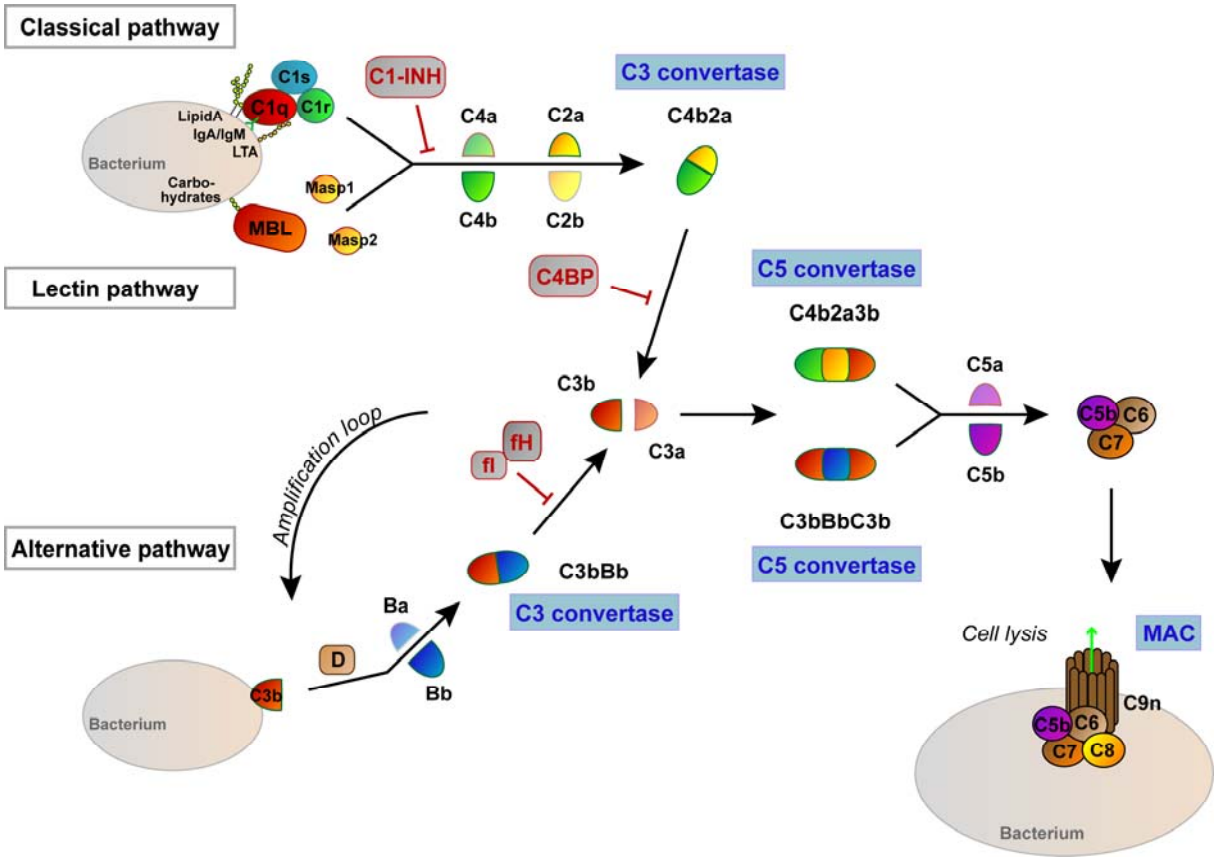

Figure 1. Schematic overview of the three pathways of the complement system and the points of action of important negative regulators. Binding of complement component C1q and MBL on bacterial surface structures (lipid A, IgA/IgM, LTA; Carbohydrates) initiates activation of complement via the classical (CP) and the lectin pathway (LP), respectively, leading to formation of the C3 convertase C4b2a. Cleavage of C3 into C3a and C3b induces an amplification loop of complement activation via the alternative pathway (AP) leading to formation of the C3 convertase C3bBb. C3b also interacts with each C3 convertase to form the C5 convertases of CP/LP and AP which cleave C5 into C5a and C5b. C5b associates with C6, C7, C8 and polymerised $C9_n$ to form of the MAC which causes pore formation and cell lysis. The negative regulators of complement, i.e. C1-INH, C4BP and fH (together with fI), downregulate complement activation. They inhibit C3 convertase formation and C3 cleavage, thereby interfering with the amplification loop and formation of the C5 convertases. *The size of the components does not correspond to their actual size.*

3.4.2 Negative regulators of complement

To prevent destruction of the body's own cells as well as overshooting reactions, complement activation is limited by various regulatory proteins that are either membrane-bound or soluble. Host membrane-bound regulators such as the membrane-cofactor protein (MCP or CD46) and the decay-accelerated factor (DAF or CD55) more generally regulate the three complement activation pathways, leading to inactivation of

C3 and accelerated decay of the C3 convertase, respectively. Soluble regulatory proteins such as C1 inhibitor (C1-INH), factor H (fH), factor I (fI) and C4 binding protein (C4BP) act more specifically on one component or pathway (Zipfel & Skerka, 2009). C1-INH inactivates C1r, C1s and a MBL-associated protein, thereby hampering cleavage of C2 and C4 and thus formation of the C3 convertase of the CP/LP (Davis et al, 2008). fH and C4BP have similar functions in the AP and CP/LP, respectively: fH inhibits the amplification loop of the AP at several steps. It binds C3b, thereby preventing the formation of the C3 convertase C3bBb; and furthermore causes dissociation of that complex. It also hinders spontaneous C3 activation in fluid phase or on cell surface by acting as cofactor for fI in the inactivation of C3b to iC3b (Ferreira et al, 2010). iC3b is then further proteolysed by fI into C3c and C3dg, which is finally cleaved by trypsin into C3d and C3g (Nilsson & Nilsson Ekdahl, 1997). Likewise, C4BP binds C4b, thereby preventing formation of the C3 convertase C4b2b of CP and LP, and also accelerates decay of this complex (Jarva et al, 2005). Additionally, C4BP co-acts with fI in cleavage of C4b into C4c and C4d (Blom, 2002; Sarma & Ward, 2011). The steps of the terminal pathway leading to MAC assembly are negatively regulated by the soluble proteins vitronectin and clusterin (Zipfel & Skerka, 2009). They block MAC assembly by binding to the C5b-7 complex, and furthermore inhibit C9 polymerisation and pore formation (Milis et al, 1993; Tschopp et al, 1993).

3.5 Meningococcal virulence factors and serum resistance

The meningococcal genome encodes an array of virulence factors that contribute to survival during infection, enable the bacteria to cope with complement proteins in saliva and normal human serum (NHS) (Andoh et al, 1997; Vogel & Frosch, 1999). Meningococcal factors most important for complement evasion and serum resistance are discussed in the following and a summarising overview is given in Figure 2.

3.5.1 Capsule

The most important virulence factor of meningococci is the polysaccharide capsule. It is essential for protection of the bacteria against complement-mediated killing and phagocytosis (Vogel et al, 1996). Hence, almost all isolates obtained from patient blood are encapsulated (Schneider et al, 2007). Thirteen capsular serogroups have been described. However, only six of them, namely A, B, C, W-135, Y and X, account for

almost all cases of disease (Tan et al, 2010). The capsule structures of these disease-associated serogroups have been extensively studied in the 1970s. Whereas capsules of serogroups A and X are built up of N-acetyl-mannosamine-phosphate and N-acetyl-glucosamine-phosphate, respectively, those of serogroups B, C, W-135 and Y are composed of derivatives of sialic acids (Bhattacharjee et al, 1975; Bhattacharjee et al, 1976; Bundle et al, 1973; Bundle et al, 1974; Liu et al, 1971a; Liu et al, 1971b). As mammalian cells also harbour sialic acid, these serogroups established a molecular mimicry. Especially the capsule of meningococci of serogroup B (MenB) is poorly immunogenic (Finne et al, 1987), because it consists of a (α2-8)-N-acetylneuraminic acid [NeuNAc] polymer which is also carried by the neural cell adhesion molecule (N-CAM) of mammals (Bitter-Suermann & Roth, 1987). Expression of the capsule prevents complement-mediated lysis and is essential for serum resistance of MenB (Kahler et al, 1998). Moreover, capsules of MenB and C were indirectly shown to limit C3 deposition (Jarvis & Vedros, 1987; Uria et al, 2008). Furthermore, sialic acid-containing cell surfaces have an increased affinity of fH, the negative regulator of the AP, to C3b (Pilatte et al, 1993). Both mechanisms protect the bacteria from complement attack.

3.5.2 Lipopolysaccharide

In an analysis of a mutant collection, all mutants with reduced serum resistance showed mutations in genes responsible for capsule or LPS synthesis which confirms the capsule and the lipopolysaccharide (LPS) being the most important meningococcal factors mediating serum resistance (Geoffroy et al, 2003).

The Lipopolysaccharide (LPS) is composed of: i) lipid A, which is anchored to the outer membrane; ii) an inner core of two heptose and two 2-Keto-3-Deoxy-D-manno-octonate (KDO) residues bound to the lipid A; and iii) variable oligosaccharide chains (α, β, γ) bound to the heptoses (Plant et al, 2006). Upon infection, lipid A and KDOs act as endotoxin up-regulating the immune response (Zughaier et al, 2006). LPS also is a target for complement component C4b which binds to phosphoethanolamine (PEA) residues on the second heptose of the inner core (Ram et al, 2003). Meningococci show a large variety in LPS structure and until now 12 different LPS immunotypes (designated L1 to L12) have been described (Mistretta et al, 2010). Types L1-L8 are found in MenB and C strains, L9 in MenA, B and C isolates, and L10-L12 only in MenA strains (Mandrell & Zollinger, 1977; Zollinger & Mandrell, 1980). However, several of these immunotypes can be expressed at the same time, resulting in e.g. the L3,7,9 immunotypes that prevails among invasive isolates (Jones et al, 1992; Vogel & Frosch, 1999). The IMD-associated LPS immunotypes L2, L3, L5, L7 and L9 contain a Lacto-N-neotetraose (LNT) epitope in the α-chain (Tsai & Civin, 1991). This LNT moiety constitutes a molecular mimicry as it is

identical to a human blood group antigen (Schneider et al, 2007). Furthermore, LNT can be terminally sialylated (Mandrell et al, 1991; Tsai et al, 2002). Sialylation of LPS was shown to block phagocytosis, possibly by disguising bacterial surface proteins involved in the process (Kurzai et al, 2005; Unkmeir et al, 2002a). Its effect on serum resistance, however, remains controversial: On the one hand, Vogel et al. only found a minor influence of sialylation on serum resistance and survival in an infant rat model (Vogel et al, 1997a; Vogel et al, 1999). On the other hand, LPS sialylation was shown to raise resistance to serum when testing strains with naturally occurring sialylation differences (Estabrook et al, 1997; Kahler et al, 1998).

3.5.3 Adhesins, OMPs and their role in complement defense

Meningococci express a variety of adhesins that besides their importance for adherence also support evasion of the immune response by interaction with immune cells or recruiting of regulators of complement (Hill & Virji, 2012; Schneider et al, 2007).
Pili are filamentous appendages sticking out of the bacterial surface (Soto & Hultgren, 1999). They enhance the meningococcal attachment to and invasion in nasopharyngeal cells and erythrocytes (Takahashi et al, 2012), and furthermore facilitate the selective uptake of DNA (Cehovin et al, 2013; Proft & Baker, 2009). Pili can be posttranslational modified at their pilin subunits, e.g. by O-linked glycosylation (Chen & Seifert, 2011). Some of these modifications are associated with reduced adherence or bacterial aggregation which in turn supports dissemination of *N. meningitidis* within or between hosts (Chamot-Rooke et al, 2011; Chen & Seifert, 2011; Marceau et al, 1998). Hubert el al recently discovered that enhanced serum resistance was associated with pilin conversion leading to increased autoaggregation, which most likely constitutes a physical barrier to complement (Hubert et al, 2012). Furthermore, binding of naturally occurring anti-Gal serum antibodies to terminal α-galactosyl residues of meningococcal pili was shown to interfere with complement-mediated lysis via the AP (Hamadeh et al, 1995). However, a direct interaction of type IV pili with complement components until date was only shown for gonococci: C4BP, an inhibitor of CP/LP, binds to the pilus-associated protein PilC which is assumed to interfere with complement activation (Blom et al, 2001).

Furthermore, integral outer-membrane proteins (OMPs), i.e. porins (PorA and PorB) and opacity proteins (Opa and Opc), are involved in meningococcal virulence. PorA interacts with the laminin receptor on human brain microvascular endothelial cells which may cause a tropism to the central nervous system (Orihuela et al, 2009). It furthermore binds C4BP, the negative regulator of the CP/LP, which leads to evasion of complement-mediated killing and thus enhanced serum resistance (Jarva et al, 2005). PorB interacts with immune cells (e.g. B cells) and upregulates the immune response (Massari et al,

2003). Moreover, gonococcal PorB has been shown to mediate bacterial invasion and dissemination (Bauer et al, 1999; Zeth et al, 2013). The various Opa proteins mainly interact with host cell receptors of the CEACAM family, molecules that mediate cell-cell-adhesion, leading to adhesion to and invasion of endothelial cells and immune cells (neutrophils, T and B lymphocytes). On the one hand, they interfere with the immune response and confer enhanced resistance to complement-mediated killing (Sadarangani et al, 2011). On the other hand, they are a target structure for binding of complement proteins C3b and C4b on the bacterial surface that opsonise the cell for killing (Lewis et al, 2008). Opa proteins moreover interact with heparan-sulfate proteoglycans (HSPGs) on the cell surface (Chen et al, 1995). This was also shown for Opc, mediating invasion to epithelial cells (de Vries et al, 1998). Opc furthermore binds to the extracellular matrix proteins fibronectin and vitronectin to mediate invasion of endothelial cells (Sa et al, 2010; Unkmeir et al, 2002b; Virji et al, 1994).

Vitronectin is also bound by the meningococcal surface fibril (Msf, NhhA), an OMP and autotransporter, although binding occurs at different sites of the vitronectin molecule (Griffiths et al, 2011). Recruiting of vitronectin by Msf interferes with the terminal pathway of complement system, therefore enhancing meningococcal serum resistance (Griffiths et al, 2011; Singh et al, 2010). Another autotransporter, the *Neisseria meningitidis* adhesin A (NadA), promotes invasion of epithelial cells and stimulates immune cells, i.e. monocytes, macrophages and dendritic cells (Capecchi et al, 2005; Franzoso et al, 2008).

3.5.4 Recruitment of negative regulators of the host immune response

Meningococci are able to recruit negative regulators of the complement system (3.4.2) onto their surface (Schneider et al, 2007). Examples already mentioned are recruitment of C4BP, the key soluble regulator of CP/LP, by PorA, as well as binding of vitronectin, a soluble regulator of the terminal complement pathway, by Opc and Msf (3.5.3). Furthermore, the key soluble regulator of the AP, fH, is recruited to the meningococcal surface. In comparison to gonococci, where fH is bound by porins (Welsch & Ram, 2008), meningococci employ two membrane proteins, i.e. factor H binding protein (fHBP) (Madico et al, 2006) and Neisserial surface protein A (NspA) (Lewis et al, 2010).

The meningococcal outer membrane lipoprotein fHBP (NMB1870) was first identified through *reverse vaccinology* (Masignani et al, 2003). It was surface-exposed on all strains tested, and verified to act as a ligand for the complement protein fH, thereby enhancing meningococcal serum resistance (Fletcher et al, 2004; Madico et al, 2006). NspA was identified as a second fH-binding surface protein whose deletion leads to enhanced C3 deposition and hence complement-mediated killing (Lewis et al, 2010). Expression levels of each protein, fHBP and NspA, differ among meningococcal isolates

(Vogel et al, 2013) and correlate with the amount of bound fH (Lewis et al, 2010; Masignani et al, 2003). Furthermore, both proteins bind fH at the same site, i.e. the domains 6-7 (Lewis et al, 2010; Schneider et al, 2009). However, fHBP and NspA were found to cooperatively contribute to fH binding (Lewis et al, 2012). This is also supported by a recent study, where *fHbp* and *nspA* both were upregulated in MC58 after four-hour growth in human blood (Hedman et al, 2012).

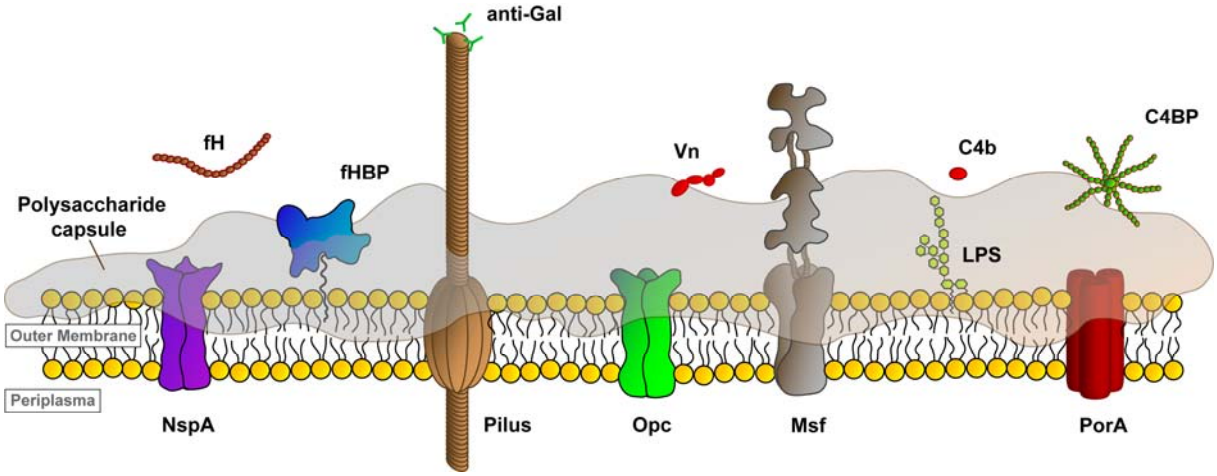

Figure 2. Meningococcal virulence factors contributing to serum resistance and complement evasion. Meningococcal structures and the host components they bind (anti-Gal antibodies in serum, complement component C4b) and negative regulators of complement they recruit (fH, factor h; Vn, vitronectin; C4BP, C4 binding protein). The polysaccharide capsule prevents MAC insertion and C3b deposition, and attracts binding of fH. NspA, neisserial surface protein A, and fHBP, factor H binding protein, recruit fH. The type IV pilus binds anti-Gal antibodies. Opc, Opacity protein C, and Msf, meningococcal surface fibril, recruit vitronectin. LPS, lipopolysaccharide binds C4b, and PorA, porin A, recruits C4BP. The illustration of the shown components is adapted from (Feavers & Pizza, 2009; Griffiths et al, 2011; Sa et al, 2010; Schneider et al, 2007; Serruto et al, 2012; Tan et al, 2010).

3.6 Genomic methods for the analysis of meningococcal serum resistance factors and vaccine candidates

A variety of genomic methods have been developed and applied to identify meningococcal serum resistance factors and screen for putative vaccine candidates. Basically, there are two approaches to investigate bacterial pathogenicity factors: Firstly, going from an occuring phenotype backwards to the genotype responsible; and secondly, analysing the genome to identify putative virulence genes.

3.6.1 From phenotype to genotype

Hereby, mutants derived from natural or artificial mutagenesis are screened for a phenotype that increases bacterial survival. Signature tagged mutagenesis (STM) for example uses transposons that carry each a unique DNA sequence for gene disruption (Hensel et al, 1995). Using STM, Sun et al constructed insertion mutants of *N. meningitidis* and analysed them in an infant rat model to identify genes essential for bacteraemic infection. Mutants attenuated in survival showed insertions within the genes coding for capsule, LPS, iron acquisition molecules as well as further genes involved e.g. in transport and metabolism (Sun et al, 2000). A genome-wide screen of a STM-derived mutant library for resistance to complement-mediated lysis again highlighted the importance of capsule and LPS for serum resistance of meningococci (Geoffroy et al, 2003). A colorimetric serum bactericidal assay developed by Perez and co-workers based on the visualisation of metabolic end products of surviving bacteria, made high throughput screening possible (Rodriguez et al, 2002; Rodriguez et al, 2003). An adaptation of this assay was used in our work group to screen for colonies with enhanced serum resistance, which led to identification of three classes of mutants with changes in *opc* expression, the LPS and the pilus in which I was partially involved (Hubert et al, 2012).

3.6.2 From genotype to phenotype

This approach to analyse meningococcal virulence factors became possible through the sequencing of genomes of various *N. meningitidis* strains within the last twelve years (Bentley et al, 2007; Parkhill et al, 2000; Tettelin et al, 2000). Comparing meningococcal strains of different serogroup and source, Schoen and colleagues defined genes that are present in all strains analysed. This so called meningococcal core genome comprised 1139 genes (~68%) which show a higher GC content in contrast to accessory genes that vary between different strains, and mainly encode housekeeping functions as the

metabolism (Joseph et al, 2011). Five large regions of atypical nucleotide composition were identified and designated *islands of horizontally transferred DNA* (IHT): IHT-A harbours the genes for capsule synthesis, IHT-B contains hypothetical proteins; IHT-C comprises homologues of toxins, putative virulence factors and mobile elements; IHT-D and IHT-E encode prophage elements and a transposon (Dunning Hotopp et al, 2006; Tettelin et al, 2000).

Genome-based methods that were used to search for pathogenicity factors and vaccine candidates include: i) *in silico* prediction of phase-variable genes or putative vaccine targets (reverse vaccinology); ii) functional genomics and iii) proteomics (Pajon et al, 2009).

3.6.2.1 *In silico* prediction

Meningococci optimise adaptation to new host environments by variable expression of virulence factors due to phase variation, a reversible mutation altering the length of DNA repeats which interferes with transcription or translation (Saunders et al, 2000). Moxon and colleagues identified 65 putative phase variable genes in the MC58 genome, several of whom were associated with known phase-variable structures, i.e. the capsule, LPS, pili and surface proteins (Parkhill et al, 2000; Tettelin et al, 2000). However novel phase-variable genes coded for e.g. LPS glycosyltransferases, restriction-modification systems and NadA (Martin et al, 2003; Saunders et al, 2000).

The availability of the MC58 genome sequence furthermore enabled *in silico* prediction of potentially surface-exposed proteins in an approach starting from the genome instead of the microorganism which became known as *reverse vaccinology* (Rappuoli, 2000; Serruto et al, 2009). Based on the genome sequence of the MenB strain MC58, ORFs coding for putative surface-exposed or exported proteins were predicted bioinformatically. Target genes were recombinantly expressed in *E. coli*, and surface exposure and bactericidal activity of these proteins were tested in mice. Accessibility to antibodies and conservation of the molecules were analysed using a representative collection of IMD-associated MenB isolates (Pizza et al, 2000). Gorringe and colleagues extended this *reverse vaccinology* approach in a pan-genome analysis of the genome sequences of strains Z2491 (MenA), MC58 (MenB) and FAM18 (MenC), and identified five additional novel surface antigens with vaccine potential (Pajon et al, 2009).

3.6.2.2 Functional genomics

Extensive research to determine virulence factors was achieved by means of whole-genome microarrays that were used for gene content comparisons of different neisserial strains and the analysis of transcriptional responses to different stimuli (Claus et al,

2007). Various microarray-based genome studies were conducted to define putative virulence genes that are specific for i) pathogenic Neisseria (*N. meningitidis* and *N. gomorrhoeae*) in comparison to commensal Neisseria (mainly *N. lactamica*); ii) meningococci compared to other *Neisseria spp.*; and iii) invasive meningococcal isolates versus carrier isolates. These studies concluded:

i) Pathogen-specific sequences resemble 3% of the Z2491 genome and are scattered in small islands within the genome (Perrin et al, 2002). 55 meningococcal pathogen-specific genes were identified when comparing MenB strains to various commensal species (Stabler et al, 2005), whereas 60 putative core pathogen-specific genes were found in a comparison of meningo- and gonococci to *N. lactamica* (Dunning Hotopp et al, 2006). However, only six of 127 putative virulence genes of *Neisseria spp.* were found in none of 13 *N. lactamica* strains analysed (Snyder & Saunders, 2006).

ii) Only 2% of Z2491 genes are meningococcus-specific (Perrin et al, 2002). Moreover, only 18 potential *N. meningitidis*-specific genes were present in all 48 meningococcal strains examined (Dunning Hotopp et al, 2006).

iii) A whole-genome comparison of strains of hyper- and non-invasive STs identified a chromosomally integrated bacteriophage designated *meningococcal disease associated island* (MDA) associated with disease-causing strains (Bille et al, 2005). However, Tettelin and co-workers did not find any genetic differences between invasive and carriage meningococcal isolates, and detected the MDA in only 60% of the invasive but 42% of the carriage strains tested (Dunning Hotopp et al, 2006). A whole-genome comparison of meningococcal strains confirmed this finding as only eleven genes were present in all disease and absent in all carriage isolates analysed. When only comparing listed candidate virulence genes, just one gene was elusively present in all disease strains, which had paralogues in the carriage strains (Schoen et al, 2008).

These studies highlight the similar genetic pool of different neisserial species and the extensive variability even between meningococcal strains. Virulence genes may have been transferred horizontally within genetic islands within meningococci, neisseria or even different respiratory colonizers (Dunning Hotopp et al, 2006). However, *in silico* predictions and whole-genome comparisons do not provide any information about expression levels of antigens during infection, which is of importance in light of extensive variation of protein expression levels due to phase variation, mutation, and DNA uptake (Frosch & Meyer, 1992; Hammerschmidt et al, 1996). Therefore, comparing gene expression levels by transcriptome analysis using DNA microarrays is crucial to identify genes essential for virulence and pathogenesis. First studies analysed transcriptional changes upon meningococcal interaction with epithelial or endothelial cells as a model of invasion (Dietrich et al, 2003; Grifantini et al, 2002a; Grifantini et al, 2002b). Two

hundred and seventy nine differentially regulated genes, e.g. encoding membrane proteins and transporters, were differentially expressed during meningococcal exposure to human serum as a model system for infection (Kurz et al, 2003). Upregulation of transcriptional regulators, transport and binding proteins, detoxifying enzymes and surface-exposed virulence factors [e.g. *fHbp* and *nspA*] was shown upon *ex vivo* growth of *N. meningitidis* in human blood (Echenique-Rivera et al, 2011). Moreover, due to the importance of iron during infection, the transcriptional regulation by the ferric uptake regulator Fur was studied in meningococci (Delany et al, 2006; Grifantini et al, 2003), which will be discussed in more detail in section 3.7.

3.6.2.3 Proteomics

The first systematically annotated meningococcal proteome map of a MenA strain Z4970 consisting of 273 proteins was the basis for a meningococcal proteome database (Bernardini et al, 2004). A subsequent protein inventory of MC58 comprised 238 proteins of which 83 had counterparts in the MenA proteome and 33 were previously unidentified (Mignogna et al, 2005). Only 1% of Men A/B proteins were classified having a function in pathogenicity, although also 7% and 6% of Z4970 and MC58 proteins, respectively, were hypotheticals (Bernardini et al, 2007). The development of outer membrane vesicle (OMV)-based vaccines (see 3.3) led to several proteome studies analysing the proteins in OMVs (Ferrari et al, 2006; Sanchez et al, 2005; Uli et al, 2006; Vaughan et al, 2006). Moreover, the immunoproteome of *N. meningitidis* was studied, i.e. proteins that are recognised by the host immmune response, leading to identification of novel antigens that are potential vaccine candidates (Mendum et al, 2009; Williams et al, 2009). Furthermore, comparative proteomics analyse differential protein expression at particular conditions, e.g. upon growth in a biofilm [as a model of carriage] compared to planktonic cells, 2% of meningococcal proteins were differentially expressed (van Alen et al, 2010).

3.7 Regulation of gene expression in meningococci

Regulators of gene expression in prokaryotes in principle are alternative sigma factors, two-component regulatory systems (TCS), and transcriptional regulators. These regulation mechanisms enable bacterial adaptation to changing environmental conditions (e.g. nutrients, oxygen level, pH, temperature), and also are often involved in expression of virulence genes (Finlay & Falkow, 1997).
Alternative sigma factors are initiation factors of transcription, mediating specific binding of the RNA polymerase to a promoter in response to stress (Gruber & Gross, 2003). In the meningococcal genome, three alternative sigma factors have been annotated, i.e. RpoH (σ32), RpoN (σ54) and RpoE (σE) (Gunesekere et al, 2006). The RpoE regulon was described in detail (Hopman et al, 2010; Huis in 't Veld et al, 2011), RpoN seems to be not functional (Laskos et al, 1998), and RpoH has been investigated more closely only in gonococci (Gunesekere et al, 2006). Two-component systems (TCS) consist of an histidin kinase sensing environmental stimuli and a transcriptional response regulator (Stephenson & Hoch, 2002). The magnesium-dependent MisR/MisS (PhoPQ) TCS seems to be the only functional in the meningococcal genome, mediating virulence control and modulation of the outer membrane structure (Newcombe et al, 2005; Tzeng et al, 2008).

According to current genome annotations, meningococci only express 35 global regulators in their genome - compared to 200 in *E. coli* (Pareja et al, 2006). Of these transcriptional regulators, until now only AsnC, CrgA, FNR, Fur (and NrrF), Hfq, NadR (FarR), NsrR, OxyR and Zur have been experimentally verified. The contact-regulated gene A (*crgA*) is expressed upon contact with epithelial cells and represses genes involved in biosynthesis of pilin, adhesin and capsule triggering adhesion (Deghmane et al, 2004; Deghmane et al, 2000). OxyR, the regulator of oxidative stress, tightly controls expression of genes for detoxification in response to intracellular hydrogen peroxide (Ieva et al, 2008; Sainsbury et al, 2010). The nitric oxide-sensitive repressor NsrR regulates an array of genes involved in synthesis and detoxification of nitric oxide (Heurlier et al, 2008; Rock et al, 2007). NadR very specifically represses expression of the meningococcal adhesin NadA which is one component of the 4CMenB vaccine (3.3) (Schielke et al, 2009; Schielke et al, 2011). Recently, the saliva metabolite 4-hydroxyphenylacetic acid (4HPA) was shown to attenuate NadR DNA-binding activity leading to derepression of NadA, yet corepression of MafA adhesins. Therefore, NadR is now supposed to more globally regulate the transcriptional response to signals sensed within human saliva (Fagnocchi et al, 2012; Montanari et al, 2012).

The ferric uptake regulator Fur acts as a global regulator in response to differing iron concentrations in the environment (Andrews et al, 2003). Its role in iron homoeostasis,

survival and pathogenesis has been extensively studied in meningococci (Delany et al, 2006; Delany et al, 2003; Delany et al, 2004; Grifantini et al, 2004; Grifantini et al, 2003). Binding of its cofactor Fe^{2+} activates Fur to bind to its specific target DNA sequence, designated Fur binding motif or Fur box (Escolar et al, 1999). Fur mainly acts as a repressor, but can also activate transcription (Delany et al, 2004; Grifantini et al, 2003). Repression of gene transcription is achieved by direct binding of Fur to the promoter of regulated genes, thereby blocking the entry of the RNA-Polymerase (Escolar et al, 1999; Lee & Helmann, 2007). Activation of gene transcription was shown to occur either i) directly, by binding to the promoter of the regulated gene as studied for e.g. *norB* and *nspA* (Delany et al, 2004; Shaik et al, 2007); or ii) indirectly, by repression of the small regulatory RNA (sRNA) NrrF, i.e. neisserial regulatory RNA responsive to iron, that otherwise binds to the *sdhA* and *sdhC* transcripts (Mellin et al, 2007). Genco and co-workers identified the meningococcal Fur binding motif (Fur box) and the iron-dependent Fur regulon by transcriptome comparison of iron-repleted and -depleted conditions in MC58, showing that several virulence-associated genes were upregulated under iron depletion (Grifantini et al, 2003). In a follow-up study, they analysed the MC58 Fur modulon comparing transcriptional changes in response to iron using the wild type as well as its *fur* deletion and *fur*-complemented mutant. The transcriptional regulators AsnC, FNR, Hfq and Zur; the sigma factor RpoH the small regulatory RNA NrrF were of stronger interest for this work, because their regulation of versatile targets may also influence bacterial serum resistance. An overview of the current knowledge about these global meningococcal regulators and their target structures is given in Table 1.

Table 1. Meningococcal regulators that might influence serum resistance.

Regulator (ID)	**Description**	**Source**
AsnC (NMB0573)	**Transcriptional activator** Adaptive **response to nutrient poor conditions** **Targets** cell surface proteins & components of aerobic metabolism	Ren et al (2007)
NrrF (between NMB2073 & NMB2074)	**N**eisserial **r**egulatory **R**NA responsive to iron [**F**e], Fur regulated **Targets** e.g. succinate dehydrogenase genes (*sdhA*, *sdhC*)	Mellin et al (2007)
Zur (NMB1266)	**Z**inc **U**ptake **R**epressor	Panina et al (2003)
	Regulator of a Zn^{2+} uptake system	Stork et al (2010)
FNR (NMB0380)	**F**umarate and **n**itrate **r**eduction regulator **Oxygen-sensitive activator**, adaptation to oxygen restrictive conditions **Targets** e.g. *aniA* (nitrate reductase), *galM* (sugar transporter), mapA (maltose phosphorylase); *fHbp*	Bartolini et al (2006)
Hfq (NMB0748)	**RNA chaperone**, regulates sRNA stability	Fantappie et al (2009)
	Involved in **post-transcriptional gene regulation of many stress-responsive genes** by sRNAs	Mellin et al (2010)
	Involved in iron-dependent and –independent **gene regulation of general metabolism, iron metabolism and virulence**	Pannekoek et al (2009)
RpoH (NMB0712)	RNA polymerase **sigma factor** Major **regulator of stress response** (e.g. heat shock) in γ-proteobacteria **Targets** in *N. gonorrhoeae* are e.g. chaperones as *groES*, *dnaK/J* and the transcriptional regulator *marR*	Gunesekere et al (2006)

3.8 Zinc homoeostasis and the Zinc-uptake regulator

Trace metals like zinc are essential cofactors of many enzymes and DNA-binding proteins (O'Halloran, 1993). Upon infection of the host, availability of transition metals like zinc is decreased for a pathogen, and its deprivation poses a challenge for a pathogen (Kehl-Fie & Skaar, 2010). On the contrary, at high concentrations, zinc can lead to production of toxic reactive oxygen species (Reyes-Caballero et al, 2011). Therefore, bacteria have to tightly regulate metal homeostasis by specialised metal-sensing transcriptional regulators. These so called metalloregulatory proteins undergo conformation changes upon binding of the specific ions (Chen & He, 2008). This induces either i) metal-mediated repression by binding to the activated regulator to the promoter of regulated genes, or ii) metal-mediated transcriptional derepression by regulator detachment (Reyes-Caballero et al, 2011).

Zinc uptake regulators (Zur) belong to a family of transcriptional regulators that also includes Fur, Mur and Nur which are sensors of iron, manganese and nickel, respectively (Lee & Helmann, 2007). Zinc-uptake systems and their regulation by Zur were investigated for various bacteria such as *Escherichia coli* (Patzer & Hantke, 1998), *Bacillus subtilis* (Gaballa & Helmann, 1998), *Listeria monocytogenes* (Dalet et al, 1999) and *Staphylococcus aureus* (Lindsay & Foster, 2001). Furthermore, the Zur regulons of *B. subtilis (Gaballa et al, 2002)*, *Mycobacterium tuberculosis* (11), *Yersinia pestis* (Li et al, 2009) and *Corynebacterium glutamicum* (Schröder et al, 2010) were characterised in the last ten years. Using comparative computational analysis, the promoter binding motifs for Zur were identified for γ-Proteobacteria, the *Agrobacteria* group, the *Rhodococcus* group, and Gram-positive bacteria, as well as for the streptococcal zinc repressor AdcR (Panina et al, 2003).

Over the last years, several genomes of *N. meningitidis* strains of different serogroups have been sequenced (Bentley et al, 2007; Parkhill et al, 2000; Tettelin et al, 2000), which was the basis for bioinformatic prediction of a Zur binding motif for β-Proteobacteria in the RegPrecise database (Novichkov et al, 2010). Recently, Tommassen and co-workers identified ZnuD, a meningococcal TonB-dependent outer membrane receptor involved in zinc acquisition upon zinc limitation (Stork et al, 2010), which gave the only experimental evidence for regulation by meningococcal Zur to date. They also discovered that expression of *znuC* and *znuD* is enhanced in a *zur* deletion mutant, and predicted Zur binding motifs upstream of both genes based on the *E. coli* Zur binding sequence (Stork et al, 2010). Subsequently, meningococcal *znuD* was observed being also regulated by Fur, and mediating heme capture on cell surface upon expression in *E. coli* (Kumar et al, 2012). However, so far binding of Zur to the promoter of any Zur-regulated gene (incl. *znuD*) has not been demonstrated by *in vitro* experiments.

3.9 Objectives

Within an ERA-NET PathoGenoMics project, the aim of this work was the identification of MenB factors involved in resistance to serum complement that could be useful as vaccine targets. The MenB genome contains over 2000 open reading frames (ORFs), a high percentage of which have not been assigned a biological function to date. It was therefore considered very likely that there exist further proteins besides the already known ones which mediate serum resistance. The development of vaccines against MenB is still ongoing and strain coverage of current vaccine formulations is predicted to be only 78% (Vogel et al, 2013). Hence, newly discovered proteins mediating serum resistance could have a valuable potential as vaccine targets and amend already existing vaccine formulations.
During my dissertation, I therefore followed three genomic approaches to unravel novel proteins mediating serum resistance of serogroup B meningococci:

(A) Impact of global regulators of gene expression on serum resistance

- Analysis of the serum resistance of global regulator mutants
- Analysis of the zinc-responsive regulon of *Neisseria meningitidis* MC58
  - Validation of growth conditions for differential expression of zinc-dependent genes
  - Microarray transcriptome analysis of the zinc-dependent genes leading to elucidation of the Zur regulon
  - *In silico* prediction of the promoter organisation of Zur-regulated genes and the meningococcal Zur binding motif
  - Establishment of electrophoretic mobility shift assays (EMSAs) to confirm binding of Zur to the predicted meningococcal Zur binding motif of selected Zur-regulated genes

(B) Analysis of genetically closely related strains with differing serum resistance

- Characterisation and analysis of serum resistance of strains from carriage and disease
- Gene content analysis of selected strains to unravel genetic differences leading to differed serum resistance of the strains
- Analysis of the contribution of identified target proteins to serum resistance

4 MATERIALS

4.1 Laboratory equipment

Table 2. Laboratory equipment used.

Laboratory Equipment	Manufacturer (Type)
Agarose gel electrophoresis chamber	Bio-Rad (SubCell GT)
Analytical balance	Sartorius (R 160 P)
Cell Density Meter	Biochrom (WPA CO8000)
Cell Disrupter	Thermo Savant (FastPrep FP120)
Centrifuges with refrigeration system	Sorvall (RC5B Plus); Heraeus (Megafuge 1.0 R)
Colony counter	Synoptics (PhotoCOL SR 92000)
Electrophoresis power supply	Bio-Rad (3000Xi)
Electroporation system	Fischer (EPI 2500)
ELISA reader	Thermo Labsystems (Multi Scan Ex)
Flow cytometer	BD (FACS Calibur™)
Gel documentation system	Herolab (UVT 28 MP)
Gel scanner	Bio-Rad (GS-800)
Heating block	VLM (1Q)
Hybridisation oven	Biometra (OV5)
Microarray hybstation	Tecan (HS4800 Pro)
Microarray scanner	Molecular Devices (Genepix 4200A)
Incubator	Thermo Scientific (Heraeus)
Incubator shaker	New Brunswick Scientific (G25); B. Braun Biotech (Certomat H/R/U); Edmund Bühler Labtech (TH30)
Magnetic stirrer	IKA (RH IKAMAG)
Microwave	Bauknecht (MW 1820 Duo)
PCR Thermocycler	MWG-Biotech (Primus 96 plus); Biometra (T3000)
pH meter	WTW (pH530)
Processor (X-ray films)	AGFA (Curix 60)
Real-Time PCR system	Applied Biosystems (StepOne Plus)
RNA electrophoresis system	Agilent (2100 Bioanalyzer)
SDS-PAGE chamber	Biometra (Mini Gel Twin)
Sequencer (DNA)	Applied Biosystems (3130)
Shaker	Biotech Fischer (Phero Shaker)
Table centrifuge	Heraeus (Biofuge 15)
Thermo shaker	Eppendorf (Thermomixer 5436)
Ultrasonic system	HTU (Soni 130)
UV Crosslinker	Stratagene (Stratalinker 1800)
UV-Vis spectrophotometer	Peqlab (NanoDrop 1000)
Vortex mixer	Stuart (SA8)
Water bath (shaker)	Julabo (SW20)
Western Blot module	Bio-Rad (Mini Trans Blot Cell)

4.2 Chemicals & consumables

Standard chemicals used were purchased from AppliChem, Merck, Roth, Roche, Sigma-Aldrich in p.a. quality. Standard consumables were supplied from Ambion, Biozym, Eppendorf, Greiner bio-one, Hartenstein, Nunc, Schleicher & Schuell and Sarstedt. All specific reagents and consumables used are listed in Table 3 and Table 4, respectively.

Table 3. Specific chemicals used and sources of supply.

Specific chemicals	*Source*
ABTS tablets and buffer	Roche
Acrylamide	Roth
Ammonium persulfate (APS)	Roth
Anti-Digoxigenin-AP Fab fragments (Anti-DIG-AP)	Roche
Bacteria Protect reagent	Qiagen
Barbital	Serva
β-mercaptoethanol (β-ME)	Roth
Bovine serum albumine (BSA)	AppliChem
Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA)	Sigma
Coomassie Brilliant Blue G-250	Merck
CSPD	Roche
Cy3 dCTP/ Cy5-dCTP, 25 nM	GE Healthcare
DIG DNA Labeling Mix (10x)	Roche
Dithioerythritol (DTE)	Applichem
Dithiothreitol (DTT)	Sigma-Aldrich
dNTPs	Invitrogen
Ethanol absolute	AppliChem
Hank's Balanced Salt Solution ($HBSS^{--}$, 1x)	Invitrogen
Hexanucleotide Mix (10x)	Roche
Nona primer	Sigma
Nuclease-free water	Ambion
PerfectHyb Plus Hybridization Buffer	Sigma
Phenol/Chloroform/Isoamylalcohol (25:24:1)	AppliChem
Phosphate-buffered saline (PBS Dulbecco, 10x)	Invitrogen
Pooled human complement preserved serum (Order no.: IPLA_CSER0; LOT: C10562)	Dunn Labortechnik
Polyacrylamid (PAA): Rotiphorese Gel 30	Roth
Power SYBR Green PCR Master Mix	Applied Biosystems
Poly-D-lysine hydrobromide	Sigma
RNA Protect Bacteria Reagent	Qiagen
RNase Zap	Ambion
Salmon sperm ssDNA	Sigma
Sequencing buffer (5x)	Applied Biosystems
Silver nitrate	AppliChem
Skim milk powder	Heirler Cenovis
Sodium dodecyl sulfate (SDS), ultra pure	AppliChem
Tetramethylethylendiamine (TEMED)	Merck
Triton-X 100%	Sigma

Table 4. Specific consumables used and sources of supply.

Specific consumables	*Source*
96 Well ELISA Microplates	Greiner bio-one
Chromatography paper (3 mm)	Whatman
Electroporation cuvettes (2 mm)	Eurogentec
Epoxy-coated microarray slides Nexterion E	Schott
Nitrocellulose transfer membrane (Protran, 0.45 µM)	Whatman
Nylon membrane (*porablot NY plus*, 0.45 µM)	Macherey-Nagel
Optical 96-well reaction plate (for qRT-PCR)	Applied Biosystems
RNA Nano Chips	Agilent Technologies
X-ray film (Super RX)	Fujifilm

4.3 Kits, antibodies, enzymes, protein & DNA ladders

Table 5. Laboratory kits, antibodies, enzymes and protein/DNA ladders used and sources of supply.

Kits	*Source*
Agilent RNA 6000 Nano Reagents	Agilent Technologies
DIG Gel Shift Kit, 2nd generation	Roche
Genomic-tip 100/G	Qiagen
illustra AutoSeq G-50 dye terminator removal kit	GE Healthcare
Pierce BCA Protein Assay Kit	Thermo Scientific
Pierce ECL Western Blotting Substrate	Thermo Scientific
Plasmid Midi kit	Qiagen
QIAamp DNA Mini Kit	Qiagen
QIAprep Spin Miniprep kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen
RNeasy Mini kit	Qiagen

Antibodies	*Source*
Monoclonal mouse anti-human C5b-9	Dako
Polyclonal rabbit anti-human C3d complement	Dako
AlexaFluor 488 goat anti-mouse IgG H+L	Invitrogen
AlexaFluor 488 chicken anti-rabbit IgG H+L	Invitrogen
Mouse anti-Opc (B306)	Mark Achtmann, Ireland
Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG+IgM (H+L)	Jackson Immuno Research
Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG, $(Fab')_2$ fragment specific	Jackson Immuno Research
Rabbit anti-znuD #1041	Martine Bos, The Netherlands
Mouse mAb735	Frosch et al. (1985)
L3,7,9	Wendell Zollinger, USA;
L8	Scholten et al (1994)
3F11	Sanjay Ram, USA

Enzymes	***Source***
Klenow	Roche
Lysozyme	Biomol
Phusion High Fidelity DNA Polymerase	Finnzymes
Proteinase K	Roth
Recombinant DNase I (RNase-free)	Invitrogen
Restriction endonucleases	New England Biolabs
RNase A	Sigma
RNase Out	Invitrogen
Superscript II	Invitrogen
T4 DNA ligase	New England Biolabs
Taq DNA polymerase	New England Biolabs

Protein/DNA ladders	***Source***
DIG III ladder / DIG VII ladder	Roche
Page Ruler prestained protein marker, 10-170 kDa	Fermentas
Smart ladder	Eurogentec
Agilent RNA 6000 Nano ladder	Agilent Technologies

4.4 Plasmids

Table 6. Vectors and plasmids used and in this study.

Plasmid	***E. coli* DH5α (WUE-ID)**	**Description**	**References**
pBluescript II SK (pBS)	315	Cloning vector	Stratagene
pCR-XL-Erm1	19	Vector comprising the gene encoding rRNA methyltransferase conferring erythromycin resistance (Erm^R cassette)	Claus, H; unpublished
pHp45Ω	2963	Vector carrying the Ω-fragment of plasmid R100.1 with the *aadA* gene conferring spectinomycin resistance ($Spec^R$ cassette)	Prentki & Krisch (1984)
pUC4K	58	Vector comprising gene encoding an amino-glycoside 3'-phosphotransferase conferring kanamycin resistance (Kan^R cassette)	GE Healthcare
pQE-60	2269	Vector for expression of recombinant protein, with a C-terminal HIS tag and Amp^R	Qiagen
pEWoc2a	2738	Knockout construct for *opc* of *N.m.* B1940 in pUC18 with central insertion of Kan^R cassette from pUC4K	Unkmeir et al. (2002b)
pGH7	2326	Knockout construct for *lst* of *N.m.* B1940 in pCR-Script with central insertion of Kan^R cassette from pUC4K	Vogel et al. (1997a)
pHC47	4343	Overexpression construct for *nmb1053* (*opc*) of MC58 in pAP1 (Erm^R)	Hubert et al (2012)

pIK3	4082	Knockout construct for *znuD (nmb0964)* of *N.m.* H44/76 in pBS, exchange of *znuD* against Kan^R cassette from pUC4K	Hubert et al, unpublished
pIK6	4288	Overexpression construct for *znuD* (*nmb0964)* of H44/76 in pAP2-1 ($Spec^R$)	Hubert et al, unpublished
pMF32.35::T5	43	Knockout construct for *siaD* of *N.m.* B1940 with a Tn1725 insertion in pMF32.35 *siaD*	Vogel et al. (1997b)
pMP1	4170	Knockout construct for *fhbp* of *N.m.* MC58 in pBS, exchange of *fhbp* against $Spec^R$ cassette from pHp45Ω	this work
pMP2	4658	Knockout construct for *asnC* of MC58 in pBS, exchange of *asnC* against Erm^R cassette from pCR-XL-Erm1	this work
pMP3	4664	Knockout construct for *nrrF* of MC58 in pBS, exchange of *nrrF* against Erm^R cassette from pCR-XL-Erm1	this work
pMP4	4670	Knockout construct for *zur* of MC58 in pBS, exchange of *zur* against Erm^R cassette from pCR-XL-Erm1	this work
pMP5	4811	Knockout construct for *zur* of MC58 in pBS, exchange of *zur* against Kan^R cassette from pUC4K	this work
pMP12	4892	Overexpression construct of *zur* of MC58 in pQE-60 for recombinant protein expression	this work

4.5 Oligonucleotides

Table 7. Oligonucleotides used.

ID	Sequence (5'-3')	Target region (gene)	Amplicon length [bp]
Cloning *(Underlined bases indicate restriction sites.)*			
MP1	GCTCTAGACCAGCCACGCGCATAC	fhbp up* (nmb1869)	525
MP6	GCGCGCCAATTGGACGGCATTTTGTTTACAGG		
MP4	CCCGCTCGAGCAGCGTATCGAACCATGC	fhbp down* (nmb1871)	567
MP7	GCGCGCCAATTGCGCCAAGCAATAACCATTG		
MP8	GCGCGCTCTAGATTGGCAATGCTGTCTTCGC	asnC up (nmb0572)	533
MP9	GCGCGCGAATTCTGCCCTTCCGATTTTCCC		
MP10	GCGCGCGAATTCAAAAGTTATACCGTCATCCGGGAC	asnC down (nmb0573 down)	429
MP11	GCGCGCCTCGAGTTTGTTCAGATGCCGCCC		
MP12	GCGCGCTCTAGAATGCCGTCTGAAGGCTTCAG	nrrF up (nmb2073)	478
MP13	GCGCGCGAATTCCTGTCCGCCGATGTTTGTG		
MP14	GCGCGCGAATTCACTTGAGGTTCAGTTTGCCG	nrrF down (nmb2074)	381
MP15	GCGCGCCTCGAGCACGTTGCCAGCAGGAG		
MP16	GCGCGCTCTAGAGATGGCGGAATACATTTTG	zur up (nmb1267)	470
MP17	GCGCGCGAATTCTTCTCTGTTTATGCCGTCTG		

MP18	GCGCGCGAATTCATAAGCCTTTCGAAAGGAGC	zur down (nmb1264/65)	475
MP19	GCGCGCCTCGAGCGTTTTCACCGATAAGGAAC		
MP183	GCGCGCTCATGAAAACAAATTTCAAACAGAAAATTA	nmb1266 (zur)	469
MP184	GCGCGCAGATCTCTGCTGACATTTTTTACAAATC		
Verification of genomic microarrays			
MP46	GACGCAGCAAGGGCAGTC	nmb0500	646
MP47	GCCCACACCTTATGCATGG		
MP48	GACATTAGCAGGGCAAGGG	nmb0501	496
MP49	TGGAACGCGTGCGTGCTG		
MP52	GCTGGCGGTTTTGGGCAAC	nmb1043	543
MP53	CCTACGCGAGACTGACGAC		
MP64	GCTGCTGACGCTGCATGG	nmb0495	653
MP65	CTTCCGCCCTGACCCGTA		
MP80	TGCCATTGCCGATCTCGCT	nmb1755/1756	629
MP81	CACGGGGTACGCTCAGCA		
qRT-PCR			
MP126	GGTATTCGACAGCAGCAAAGC	nmb1567	72
MP127	GTCCAACCCGGAATCACTTG		
MP117	CCGTTCCCCGGTTTTGA	nmb0964 (znuD)	80
MP118	CTGCATCGCCTGCTTTTTC		
MP141	GCAAATCCCTGAAACTCTACCTCTT	nmb0317	72
MP142	TGATGTTGACGCAGTCTTCATG		
MP143	CGGCGTTCCGAATACCTTT	nmb0525	66
MP144	GCGTAAATCGCGGCATAGAG		
MP145	GCAACTACCAAATGCAGCTCAA	nmb0586 (znuA)	69
MP146	CAGCAGGGACGGCATTAAAT		
MP147	GGCTTTGGCACGTCCTCTT	nmb0587 (znuB)	72
MP148	GTGTGCCGAGGGCTTGAA		
MP149	TCGCCCGCGAAAAAATT	nmb0588 (znuC)	66
MP150	CTTGGGCGAGGTAGGGTTCT		
MP151	AGGATGTCCTGAATGTACTCGAAAT	nmb0817	80
MP152	CTGTCTCTGTGTCCTCCAACCTAA		
MP153	CCCTCCTCATCCTCGACACA	nmb0819	69
MP154	CAGCACCTGCGGCAGTAA		
MP155	CCGCCAAAGCCCTAAACA	nmb0820	72
MP156	GGTTGTCGGGATTTGAACGT		
MP157	CCGCTGTTTTCGCTGGATA	nmb0942	71
MP158	GTGTTGACGTTGCGCTGTTT		
MP159	TCGGACAAAACTTGGAAATCG	nmb1475	68
MP160	TTTGAAGCGTTCGCCTACGT		
MP161	TGCCCAACATCCAAGAAATGT	nmb1497	68
MP162	CTGGTTTTAAGGCGGTGTGAA		
MP163	ACGACGGCGGTCATCTTTAC	nmb2142	68
MP164	CGCCGTATGATGCACCATT		
MP165	CTGCCGCCGGAATCG	nmb0546	75
MP166	GACCAAAGAGCCGACCACTTC		
MP171	TGCCATTATCGCGCTTGTC	nmb0990	81
MP172	GCCTGCTGCTTCGCAAAT		
MP179	CGGGCGCGGATAACG	nmb0577	61
MP180	AGGCAGACGCTCCGCATA		

EMSA			
MP185 MP186	TAAAAAATGTAATGTTATATAATAACAAACTTTT TTTCAAAAGTTTGTTATTATATAACATTACATTT	nmb0964 (unaltered)	34
MP187 MP188	CGATACCTATTTTGTTATAACATAACAAAATCTT GTTAAAGATTTTGTTATGTTATAACAAAATAGGT	nmb1475 (short)	34
MP189	TCTTCACACGATACCTATTTTGTTATAACATAACA AAATCTTTAACCCACA	nmb1475 (long)	51
MP190	CTCGTGTGGGTTAAAGATTTTGTTATGTTATAACA AAATAGGTATCGTGTG		
MP191 MP192	CTTTCCAAGATGTTATAATATAACATATAATCTAT AAATATAGATTATATGTTATATTATAACATCTTGG	nmb0546	35
MP193 MP194	TAAAAAATGTAATGTTATATAACGATGAACTTTT TTTCAAAAGTTCATCGTTATATAACATTACATTT	nmb0964 (TA/CA)	34
MP195 MP196	TAAAAAATGTAATGTTATATAATAATGAACTTTT TTTCAAAAGTTCATTATTATATAACATTACATTT	nmb0964 (CA)	34
MP197 MP198	TAAAAAATGTAATGTTATATAACGACAAACTTTT TTTCAAAAGTTTGTCGTTATATAACATTACATTT	nmb0964 (TA)	34
MP199 MP200	TAAAAAATGTAATGTTATATAATAATAAACTTTT TTTCAAAAGTTTATTATTATATAACATTACATTT	nmb0964 (C)	34
General primers			
AP1 AP2	GGCATTTATCAGATATTTGTTC CGTCAGAGCATGGCTTTATG	Amplification of an insert within pAP1	variable
GH155 ermR	GAACATGATGAGTGATCGTTA GAGGTCGACGGTATCGATTC	Amplification of Erm^R cassette	~1000
Kana1	CACGAGGCAGACCTCAA	Amplification of region down of Kan^R cassette	variable
Kana2	GATTTTGAGACACAACGTGG	Amplification of region up of Kan^R cassette	variable
GSK2R KH9	AGCGTTTGTTGCGCAGGTC TAATCAACACACCCCTCCTC	nmb0964 (znuD)	~1000
KB9 329	AATACGACTCACTATAGGGC ACCATGATTACGCCAAGC	Amplification of an insert within pBS	variable
SpecF SpecR	ATGCGCTCACGCAACTGGT TTATTTGCCGACTACCTTGGT	Amplification of $Spec^R$ cassette	~1000
Que-F Que-R	CGGATAACAATTTCACACAG GTTCTGAGGTCATTACTGG	Amplification of an insert within pQE-60	variable

**Note:* up, upstream; down, downstream

4.6 Bacterial strains and mutants

The *E. coli* strain DH5α was used for general cloning (Hanahan, 1983). *E coli* M15 [pREP4] was used for overexpression of Zur (5.2.6.2).

Table 8. Meningococcal wild type strains used.

ID	Epidemiology	ST	cc	PorA-VR1	PorA-VR1	FetA	penA	PorB	Reference
α16	Carriage	41	41/44	7-2	4	1-5	1	3-1	German reference laboratory for
α528	Carriage	41	41/44	7-2	15-39	1-5	1	3-301	meningococci (NRZM); Bavarian
DE9686	Disease	42	41/44	7-2	4	1-5	1	3-1	carriage study (Claus et al, 2005)
MC58	Disease	74	32	7	16-2	1-5	3	3-24	Richard Moxon, Oxford; McGuiness et al. (1991)

Table 9. Meningococcal mutant strains used.

WUE-ID[1]	Parental strain	Genotype	Resistances[2]
2344	MC58 (WUE2135)	Δlst	Kan
4209	α16	ΔsiaD	Cm
4240	DE9686	ΔsiaD Δfhbp	Cm, Spec
4294	α16	ΔsiaD Δlst Δfhbp	Cm, Kan, Spec
4297	α528	ΔsiaD Δlst Δfhbp	Cm, Kan, Spec
4300	DE9686	ΔsiaD Δlst Δfhbp	Cm, Kan, Spec
4353	WUE4294	ΔsiaD Δlst Δfhbp fnr+	Cm, Kan, Spec, Erm
4320	WUE4294	ΔsiaD Δlst Δfhbp hfq+	Cm, Kan, Spec, Erm
4547	WUE4294	ΔsiaD Δlst Δfhbp rpoH+	Cm, Kan, Spec, Erm
4659	WUE4294	ΔsiaD Δlst Δfhbp ΔasnC	Cm, Kan, Spec, Erm
4665	WUE4294	ΔsiaD Δlst Δfhbp ΔnrrF	Cm, Kan, Spec, Erm
4671	WUE4294	ΔsiaD Δlst Δfhbp Δzur	Cm, Kan, Spec, Erm
4684	WUE4209	ΔsiaD znuD+	Cm, Spec
4686	WUE4209	ΔsiaD ΔznuD	Cm, Kan
4712	WUE4240	ΔsiaD Δfhbp ΔopcA	Cm, Spec, Kan
4747	WUE4240	ΔsiaD Δfhbp Δnmb0865	Cm, Spec, Kan
4748	WUE4712	ΔsiaD Δfhbp ΔopcA opcA+	Cm, Spec, Kan, Erm
4792	WUE4240	ΔsiaD Δfhbp ΔnspA	Cm, Spec, Kan
4812	MC58 (WUE2135)	Δzur	Kan

[1] According to strain collection of the Institute for Hygiene and Microbiology (IHM) Würzburg;
[2] Cm, chloramphenicol; Kan, kanamycin; Spec, spectinomycin; Erm, erythromycin

4.7 Culture media

GC medium

Difco™ GC Medium Base [Becton Dickinson]	36 g
De-ionised H_2O	ad 990 ml
→ Autoclave	
PolyVitex [bioMerieux]	10 ml

LB medium

Difco™ LB agar Lennox [Becton Dickinson]	35 g
De-ionised H_2O	ad 1000 ml

Proteose peptone medium (PPM)

Bacto™ proteose peptone [Becton Dickinson]	15 g
NaCl [Roth]	5 g
Starch from potatoes [Fluka]	0.5 g
KH_2PO_4 [Roth]	4 g
K_2HPO_4 [Roth]	1 g
De-ionised H_2O	ad 1000 ml
	→ pH 7.8

PPM was supplemented with 500 µl of 2 M $MgCl_2$, 500 µl of 8.4% $NaHCO_3$ and - shortly before use - 1 ml PolyVitex [bioMerieux] per 100 ml to obtain PPM^+, the final medium used for cultivation.

SOC medium for electroporation

Bacto tryptone [Becton Dickinson]	2%
Bacto™ Yeast extract [Becton Dickinson]	0.5%
NaCl [Roth]	10 mM
KCl [Roth]	2.5 mM

SOC medium was prepared freshly by adding 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM Glucose to SOB medium.

For agar plates, 1.5% agar was added to the medium before autoclaving.

Additional solid media used were purchased from Becton Dickinson, i.e. Columbia agar plates with 5% sheep blood and GC chocolate agar plates. Furthermore, RPMI medium supplied from Invitrogen was used.

Medium for cryopreservation of bacteria

Standard nutrient broth [Merck]	5 g
Glycerol	20%
De-ionised H_2O	ad 200 ml

4.8 Buffers and solutions

If not stated elsewise, all buffers and solutions were prepared with de-ionised water.

4.8.1 For microbiological methods

4.8.1.1 Serum bactericidal assay

VBS/BSA buffer

Barbital	5 mM
NaCl	145 mM
MgCl2	0.5 mM
CaCl2	0.15 mM
BSA	0.5 %
→ pH 7.4	
→ storage at -20°C	

4.8.2 For molecular methods

4.8.2.1 Isolation of chromosomal DNA

Buffer B1

$Na_2EDTA \cdot 2\ H_2O$	50 mM
Tris	50 mM
Tween 20	5%
Triton X-100	5%
→ pH 8.0	
→ storage at 4°C	

Buffer B2

Guanidin-HCl	0.33 M
Tween 20	20%
→ storage at 4°C	

TE

Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

4.8.2.2 Electrophoretic separation of DNA in agarose gels

GEBS

Glycerol	20%
EDTA	50 mM
N-Lauroylsarcosine	0.5%
Bromophenol blue	0.05%
→ pH 8.0	

1xTBE

Tris/HCl	100 mM
Boris acid	100 mM
EDTA	2.5 mM
→ pH 8.3	

4.8.2.3 Southern blot

Denaturing buffer

NaOH	0.5 M
NaCl	1.5 M

Neutralising buffer

Tris/HCl, pH 7.0	1 M
NaCl	3 M

SSPE (20x)

NaCl	3.6 M
NaH_2PO4	0.1 M
Na_2HPO4	0.1 M
EDTA	20 mM

'High SDS' hybridisation buffer

SDS	7%
Formamide	50%
SSC	5x
Sodium phosphate buffer, pH 7.0	50 mM
N-Lauroylsarcosine	0.1%
Blocking reagent	2%

→ storage at -20°C

Maleic acid buffer

Maleic acid	0.1 M
NaCl	0.15 M

→ pH 7.5

Blocking solution (10x)

Maleic acid buffer	
Blocking reagent (w/v)	10 %

→ autoclave & store at -20°C

Detection buffer

Tris/HCl	0.1 M
NaCl	0.1 M

→ pH 9.6

SSC (20x)

NaCl	3 M
Sodium citrate	0.3 M

→ pH 7.0

Stringent washing buffer 1

SSC	2x
SDS	0.1%

Stringent washing buffer 2

SSC	0.1x
SDS	0.1%

Washing buffer

Maleic acid buffer	
Tween 20	0.3%

Antibody solution

Blocking solution	1x
Anti-DIG-AP	1:10,000

→ storage at 4°C

CSPD working solution

Detection buffer	
CSPD	1:250

→ storage at 4°C

4.8.2.4 Microarrays (cDNA and gDNA)

Blocking solution

Ethanolamine	50 mM
Tris/HCl, pH 9.0	0.1 M
SDS	0.1%

Labelling buffer (10x)

Tris/HCl, pH 7.2	0.5 M
$MgCl_2$	0.1 M
DTE	1 mM
BSA	0.2%

dNTP buffer (10x)

dATP/dGTP/dTTP	each 1 mM
dCTP	0.5 mM
Tris/HCl, pH 8.0	0.1 mM
EDTA, pH 8.0	10 mM

4.8.3 For protein analysis

4.8.3.1 SDS-PAGE

'Lower Tris' buffer

Tris/HCl, pH 8.8	1.5 M
SDS	2%

'Upper Tris' buffer

Tris/HCl, pH 6.8	0.5 M
SDS	2%

Solution for separation gel (12.5%)

30% PAA	2.5 ml
'Lower Tris'	1.5 ml
dH_2O	2 ml
TEMED	10 µl
10% APS	30 µl

Solution for loading gel

30% PAA	0.375 ml
'Upper Tris'	0.625 ml
dH_2O	1.625 ml
TEMED	10 µl
10% APS	17.5 µl

Sample Solution (1x)

β-ME	5%
SDS	2%
Glycerol	12.5%
Tris/HCl, pH 6.8	0.5 M
Bromophenol blue	spatula tip

Electrophoresis buffer

Tris	25 mM
SDS	0.05%
Glycine	50 mM

4.8.3.2 Staining of SDS-polyacrylamide gels

Coomassie staining solution

Methanol	50%
Acetic acid	10%
Coomassie Brilliant Blue G250	0.275%

Destaining solution (Coomassie)

Acetic acid	7.5%
Methanol	20%

Fixing solution (Silver staining)

Methanol	50%
Acetic acid	10%

Silver staining solution

Silver nitrate	0.1%
Formaldehyde	0.027%

Developing solution

Sodium carbonate	6%
Formaldehyde	0.018%

4.8.3.3 Western blot

Blot Buffer

Tris	0.3%
Glycine	1.44%
Methanol	20%

PBS-T

PBS (1x)	
Tween 20	0.1%

4.8.3.4 LPS analysis

Lysis buffer

SDS	2%
β-ME	4%
Glycerol	10%
Tris/HCl, pH 6.8	1 M
Bromophenol blue	spatula tip

→ storage at -20°C

Proteinase K/Lysis buffer

Proteinase K	2.5 mg
Lysis buffer	1 ml

→ storage at -20°C

AA/BA solution for separation gel

Acrylamid	46.5%
Bisacrylamid	3%
SERDOLIT	1.5%

AA/BA solution for loading gel

Acrylamid	48%
Bisacrylamid	1.5%
SERDOLIT	1.5%

Tricine Sample Solution (2x)

Glycerin	24%
SDS	8%
Tris/HCl, pH 6.8	0.1 M
β-ME	4%
Coomassie Brilliant Blue G250	0.02%

→ storage at -20°C

Gel buffer

Tris/HCl, pH 8.45	3 M
SDS	0.3 M

Solution for tricine separation gel

AA/BA solution for separation gel	2.5 ml
Gel buffer	2.5 ml
dH_2O	1.5 ml
Glycerol (86%)	1 ml
TEMED	7.5 µl
10% APS	75 µl

Solution for tricine loading gel

AA/BA solution for loading gel	0.25 ml
Gel buffer	0.78 ml
dH_2O	2.1 ml
TEMED	3 µl
10% APS	30 µl

Cathode buffer

Tris/HCl	0.1 M
Tricine	0.1 M
	→ pH 8.25
SDS	0.2 M

Anode buffer

Tris/HCl, pH 8.9	0.2 M

Fixer (LPS silver staining)

Ethanol	40%
Acetic acid	5%

Oxidiser (LPS silver staining)

Ethanol	40%
Acetic acid	5%
Periodic acid	0.7%

LPS silver staining solution

NaOH	0.47 M
Silver nitrate	0.27%
Ammonia	0.33%

Developer (LPS silver staining)

Citric acid	1.3 M
Formaldehyde	0.25%

4.8.3.5 Flow cytometry analysis

HBSS^{++}/BSA

HBSS^{--}	1x
$MgCl_2$	1 mM
$CaCl_2$	0.15 mM
BSA	1 %

→ storage at 4°C

4.8.3.6 Expression and purification of recombinant protein

Lysis buffer

NaH_2PO_4	50 mM
NaCl	0.3 M
Imidazole	10 mM

→ pH 8.0

Wash buffer

NaH_2PO_4	50 mM
NaCl	0.3 M
Imidazole	20 mM

→ pH 8.0

Elution buffer

NaH_2PO_4	50 mM
NaCl	0.3 M
Imidazole	0.25 M

→ pH 8.0

4.8.3.7 Electrophoretic Mobility Shift Assay (EMSA)

TEN buffer

Tris/HCl	10 mM
EDTA	1 mM
NaCl	0.1 M

→ pH 8.0

TB buffer (10x)

Tris/HCl	890 mM
Boric acid	890 mM

→ pH 8.0

Binding buffer (5x)

Tris/HCl, pH 8.0	0.1 M
KCl	0.25 M
DTT	5 mM
Glycerol	25%

Native polyacrylamide gel (8%)

TB buffer (10x)	1 ml
30% PAA	5.3 ml
dH_2O	13.6 ml
TEMED	20 µl
10% APS	90 µl

5 METHODS

5.1 Microbiological Methods

5.1.1 Cultivation

Meningococci were cultivated o/n on GC agar (supplemented with PolyVitex) or GC Chocolate agar plates at 37°C/5% CO_2 in an incubator. For selection of recombinant clones, the solid medium was supplemented with chloramphenicol (7 µg/ml), erythromycin (7 µg/ml), kanamycin (100 µg/ml), spectinomycin (125 µg/ml) or combinations of these antibiotics. For liquid cultures either proteose peptone medium supplemented with PolyVitex (PPM^+) or defined RPMI media supplemented with 100 µM $FeCl_3$ was used. Meningococcal strains were grown at 37°C while shaking at 200 rpm in an incubator shaker.

Escherichia coli were cultivated o/n on LB agar in an incubator at 37°C or in LB media at 37°C while shaking at 200 rpm in an incubator shaker. For growth of recombinant strains the solid or liquid medium was supplemented with ampicillin (100 µg/ml), chloramphenicol (30 µg/ml), erythromycin (250 µg/ml), kanamycin (30 µg/ml) or spectinomycin (75 µg/ml).

To adjust bacterial suspensions or cultures to a defined cell count, the optical density was defined in an UV-Vis spectrophotometer. For this purpose, 1 ml of the bacterial suspension was pipetted in a cuvette and the absorption at 600 nm was measured. An OD_{600} 1.0 approximately equates to a cell count of $1x10^9$ cells/ml.

5.1.2 Generation of electrocompetent *E. coli* DH5α

An o/n culture of E. coli DH5α was used to inoculate 700 ml of LB medium to an OD_{600} 0.1. The culture was grown to exponential growth phase (OD_{600} ~0.6) at 37°C/200 rpm. Bacterial growth was stopped by incubation of the culture in ice water for 10 min. Cells were pelleted at 4°C and 5000 rpm for 15 min (centrifuge RCB5, rotor GS3). The bacterial precipitate was washed twice in 500 ml and 200 ml of 4°C cold dH_2O, respectively, and once with 20 ml of 4°C cold 10% glycerol. Cells were centrifuged at 4°C/5000 rpm for 15 min each again. Finally, the bacterial precipitate was resuspended in 1.5 ml of cold 10% glycerol. Aliquots of the suspension were frozen immediately in liquid nitrogen and stored at -70°C.

5.1.3 Transformation of *E. coli*

An aliquot of the electrocompetent E.coli DH5α was thawed slowly on ice. To the bacteria, 1 µl of plasmid DNA obtained from ligation (5.2.5) was added. The sample was incubated on ice for 5 min until it was pipetted into a pre-cooled electroporation cuvette. The cuvette then was put into the electroporation system which was set at 2.5 kV, 25 µF and 400 Ω. The short electric pulse destabilises the bacterial membrane of specially prepared *E. coli* which enables small DNA fragments to pass. After electroporation, bacteria were immediately transferred into 37°C warm SOC medium and incubated at 37°C/200 rpm for an hour. Following, bacteria were plated on LB agar supplemented with the suitable antibiotic(s) for selection of correct transformants and incubated o/n at 37°C.

5.1.4 Transformation of *N. meningitidis*

Meningococci are naturally competent. The strain to be transformed was grown o/n on GC agar plate at 37°C/5% CO_2. Bacterial suspensions were adjusted to an OD_{600} between 0.2 and 0.5 in 5 ml of PPM^+. The culture was grown at 37°C/200 rpm for one hour and then diluted to OD_{600} 0.1. For transformation, 200 ng to 1 µg of the plasmid DNA was added to 1 ml the diluted bacterial culture. The culture was incubated for five to six hours at 37°C/200 rpm. Following, 100 µl and 900 µl of the transforming culture were plated on GC agar supplemented with the required antibiotic for selection of correct transformants. Plates were incubated o/n at 37°C/5% CO_2.

5.1.5 Serum killing assay

Serum killing assays (serum bactericidal assays, SBAs) were conducted in modification as described previously (Vogel et al, 1997b). Meningococci were grown o/n at 37°C/5% CO_2 on GC agar plates. Under the safety cabinet, bacteria from the third fraction were resuspended in VBS/BSA buffer (4.8.1.1) and diluted to 10^6 cfu/ml. Each serum stress reaction was prepared by firstly placing (360-x) µl of VBS/BSA buffer in a 1.5 ml cap on ice, followed by adding of 40 µl of the thoroughly vortexed bacterial suspension. Finally x µl of freshly thawed pooled human complement serum (normal human serum, NHS), e.g. 40 µl=10% NHS, were added to the sample. As negative control, a sample without any NHS or with addition of an equal amount of heat-inactivated serum (HIS) was used. HIS was obtained by incubation of NHS at 50°C for at least 30 min. The samples were incubated at 37°C/700 rpm on a thermo shaker, usually for 30 min. Afterwards the serum reactions were stopped by adding 400 µl cold 1xPBS. The amount of bacteria that survived the serum exposure was determined by plating of serial dilutions of each sample

on Columbia agar with 5% sheep blood. Plates were incubated o/n at 37°C/5% CO_2. Colony forming units (cfu) were counted using a colony counter.

5.2 Molecular methods

5.2.1 Isolation of plasmid DNA and chromosomal DNA

For preparation of plasmid DNA either the QIAprep Spin Miniprep kit or the Qiagen Plasmid Midi kit was used after the manufacturer's protocol.

Isolation of chromosomal DNA was achieved at small scale by using the QIAamp DNA Mini Kit after the manufacturer's instructions.
If higher amounts of DNA were needed, Genomic-tips 100/G were used. Meningococci were cultivated o/n at 37°C/5% CO_2 on GC agar plates. Under the safety cabinet, a cell count of $1.5x10^{10}$ cfu was adjusted in 5 ml 1xPBS. The suspension was centrifuged at 4000 rpm for 10 min. The bacterial precipitate was resuspended in 7 ml buffer B1 (4.8.2.1) and 14 µl RNase (100 mg/ml) were added. After vortexing, 160 µl of lysozyme (100 mg/ml) were added. The solution was incubated for 30 min at 37°C in an incubator and then frozen at -70°C for one hour. After thawing in a 50°C water bath, 200 µl proteinase K (20 mg/ml) and 2.4 ml buffer B2 were added to the solution, which was vortexed and incubated in the 50°C water bath for one hour. Proceeding now on the work bench, 15 ml of buffer QBT of the Genomic-tips 100/G kit were added. Further steps of the isolation were done according to the manufacturer's protocol. The resulting DNA precipitate was dried and 100 µl TE were added to slowly resuspend DNA o/n at 4°C. Concentration and purity of the DNA were measured using the NanoDrop, DNA was stored at -20°C.

5.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reactions (PCR) were used to specifically amplify DNA fragments and to screen for correct clones after a transformation (5.1.3, 5.1.4). Thereby, for cloning purposes Phusion polymerase was used for PCR because of its proof-reading activity. Other than that, the Taq polymerase was used. PCR samples were prepared according to the manufacturer's protocol as follows:

Taq polymerase (NEB)		Phusion Polymerase (Finnzymes)
5 µl	10x NEB buffer/ Phusion HF buffer	10 µl
5 µl	dNTPs [2 mM]	5 µl
0.5 µl	Fw primer [20 µM]	1.25 µl
0.5 µl	Rev primer [20 µM]	1.25 µl
0.1 µl	Taq / Phusion	0.5 µl
ad 50 µl	dH_2O	ad 50 µl

As a template, either 20 ng of chromosomal DNA, 10 ng of plasmid DNA or a part of a bacterial colony were used.The PCR programmes were adjusted to the following conditions:

Taq polymerase (NEB)		Phusion Polymerase (Finnzymes)	
95°C, 1 min	Initial Denaturation	98°C, 30 sec	
95°C, 1 min	Denaturation	98°C, 10 sec	} 35 x
Ta_{NEB}, 1 min	Annealing	($Ta_{Finnzymes}$), 20 sec	
72°C, 1 min/kb	Extension	72°C, 30 sec/kb	
72°C, 10 min	Final Extention	72°C, 10 min	
16°C	*Cooling*	*16°C*	

The annealing temperatures (Ta) of the NEB Taq polymerase were obtained by calculating the melting temperatures (Tm) of each primer with the formula [4×(G+C)+2×(A+T)], and subtracting 4°C of the lower Tm calculated. For Phusion polymerase, the Tm was determined using the Finnzymes online calculator (http://www.finnzymes.fi/tm_determination.html). If primers were not longer than 20 nucleotides, the Ta equated the lower Tm given; for primers with more than 20 nucleotides, 3°C were added to the lower Tm given by the calculator.

5.2.3 Visualisation and Purification of PCR products

PCR products were electrophoretically separated in an agarose gel using TBE buffer (4.8.2.2). Depending on the size of the DNA fragment and the subsequent application, concentrations of agarose gels varied between 0.8% and 3.5%.

GEBS buffer was added to the PCR probes at the rate of 1:5, and the samples were pipetted into the slots of the gel. The Smart ladder was used as molecular weight marker. DNA probes were separated at 170 to 200 V. After gel electrophoresis, the gel was incubated in 0.001% ethidium bromide solution for 10 to 20 min. DNA bands are visualised by UV light due to intercalation of ethidium bromide into the DNA.

For sequencing or cloning procedure, DNA probes were then purified directly from the remaining PCR samples using the QIAquick PCR Purification kit. DNA was eluted in 30 µl of buffer EB (of the kit), mixed with dH_2O at the rate of 1:4.

5.2.4 Sequencing of PCR products and plasmids

Sequencing was performed with the ABI Prism 3130 DNA sequencer by the DNA laboratory of our institute. Sequencing reactions were prepared to a final volume of 9 µl with dH_2O, with 1 µl of the respective primer [10 µM] and 2 µl of 5x sequencing buffer. As template DNA, either purified PCR products (100 ng) or plasmid DNA (400 ng) was used.

5.2.5 Cloning procedure: Digest of DNA fragments, Ligation and Purification for electroporation

For cloning, purified PCR products were enzymatically digested using different restriction endonucleases. In a final volume of 20 µl, 1 µl of each enzyme [20 U/µl], 2 µl of the suitable buffer (NEB buffers 1-4), and if necessary 2 µl of 10xBSA, were filled up with the purified PCR product or plasmid (vector) DNA [1-2 µg] and dH_2O. The mixture was incubated at 37°C for one to two hours. Next, digested DNA fragments were purified using the QIAquick PCR Purification kit. Opened vector DNA was then applied to a 0.8% agarose gel (5.2.3), sliced out of the gel and extracted using the QIAquick Gel Extraction kit according to the manufacturer's instructions. If two enzymes had to be used, but buffers were not combinable, each digest was done separately with a purification step in between.

Ligation of purified digested DNA fragments and the opened vector was achieved using the T4 DNA ligase. For that purpose, the correct proportion of inserts to the vector was calculated with the following formula:

$$\frac{\frac{\text{Amount of vector [ng]}}{\text{Size of vector [kb]}}}{} \times 3 = \frac{\text{Amount of insert [ng]}}{\text{Size of insert [kb]}}$$

For each ligation sample, 100 ng of the purified vector DNA was mixed on ice with the respective amount of each insert. Then, 1 µl of the ligase buffer [10x] and 1 µl of T4 DNA ligase [400 U/µl] was added. If necessary, dH_2O was added to adjust a final volume of 10 µl. Samples were incubated o/n at 16°C.

5.2.6 Construction of mutant strains

5.2.6.1 Construction of deletion mutants

Deletion mutants were constructed for *fhbp*, *asnC*, *nrrF* and *zur* (Table 10). For this purpose, a fragment upstream and a fragment downstream of the gene to be deleted were amplified by PCR from *N. m.* MC58 with primers introducing restriction sites. Fragments were enzymatically digested and purified. A suitable resistance cassette was chosen and excised from its vector. We either used: A) a polar spectinomycin resistance cassette ($Spec^R$) with a terminator excised from vector pHp45Ω using EcoRI; B) a polar erythromycin resistance cassette (Erm^R) with a terminator gained by EcoRI digest of vector pCR-XL-Erm1; or C) a kanamycin resistance cassette (Kan^R) excised from vector pUC4K with EcoRI. The cloning vector pBluescript II SK (pBS) was opened with XbaI/XhoI (suitable for the outer restriction sites of the upstream and downstream fragment). All three fragments - upstream fragment, downstream fragment and the resistance cassette - were ligated with the opened vector (5.2.5). The ligation sample was purified and transformed into *E. coli* DH5α. Antibiotic-resistant clones were screened by PCR with cloning primers, as well as primers binding within the resistance cassette (SpecF+SpecR for $Spec^R$; GH155+ermR for Erm^R; Kana1+Kana2 for $Kana^R$) and within the vector (KB9+329). Correct ligation was confirmed by sequencing. Finally, the resulting plasmid was isolated from *E. coli* DH5α and transformed into mutants of *N. m.* strains MC58, α16, α528 or DE9686. Deletion of the gene was then confirmed by PCR and Southern blot using a probe generated with the outer cloning primers (5.2.7).

5.2.6.2 Construction of a mutant overexpressing a recombinant Zur protein

The entire coding region of *zur* gene without start and stop codon (469 bp) was amplified from MC58 using primers MP183 and MP184 harbouring a BspHI and a BglII restriction site, respectively. The PCR product was digested with BspHI and BglII (5.2.5). The chosen expression vector pQE-60 contained an IPTG-inducable promoter, a C-terminal HIS tag, as well as own start and stop codons. pQE-60 was opened at its NcoI and BglII sites. Next, the opened vector and the digested *zur* fragment were ligated (5.2.5). *E. coli* M15 [pREP4] was transformed with the gained recombinant vector pMP12. Clones resistent to kanamycin and ampicillin were selected, and correctness of the plasmid was verified by DNA sequencing. The resulting Zur-overexpressing *E. coli* strain was designated WUE4892.

5.2.7 Generation of Digoxigenin-tagged DNA probes

The respective gene (e.g. *zur*) that has been deleted in meningococcal strains and the surrounding region was amplified using the Phusion polymerase with the primers also used for generation of the deletion constructs. Following PCR purification and elution in 15 µl EB:H_2O (1:4), the DNA was denatured by incubation in a water bath at 95°C for 10 min and then immediately cooled down on ice. To the denatured DNA, 2 µl of Hexanucleotide Mix [10x], 2 µl DIG DNA Labeling Mix [10x] and 1 µl of Klenow enzyme were added. The samples were incubated o/n at 37°C in an incubator. Addition of 2 µl EDTA [0.2 M, pH 8.0] stopped the reaction, and 2.5 µl sodium acetate [3 M, pH 4.5] and 75 µl of cold ethanol [96%] were added to precipitate the DNA. Samples were incubated at -70°C for 30 min. After centrifugation at 4°C and 13,000 rpm for 15 min in the Heraeus Megafuge 1.0 R, precipitates were washed with cold ethanol [70%], centrifuged again, dried in vacuum and finally resuspended in 50 µl TE buffer (4.8.2.1). For quantification, 1 µl of serial dilutions of the tagged DNA and a tagged control oligonucleotide were pipetted onto a nylon membrane. The DNA was cross linked to the nylon membrane by UV light. DNA was detected by chemiluminescence (5.2.8.2) and concentration was determined based on the comparison with the control DNA of known concentration.

Table 10. Generation of deletion constructs.

Gene	Gene size [bp]	Upstream fragment primers & restriction sites	Upstream fragment size [bp]	Downstream fragment primers & restriction sites	Down-stream fragment size [bp]	Resistance cassette[1]	Resulting plasmid	Southern Blot probe	Reference
fhbp (nmb1870)	963	MP1 (XbaI)+ MP6 (MfeI)	525	MP7 (MfeI)+ MP4 (XhoI)	567	$Spec^R$	pMP1	MP1+MP4	Masignani et al. (2003)
asnC (nmb0573)	564	MP8 (XbaI)+ MP9 (EcoRI)	533	MP10 (EcoRI)+ MP11 (XhoI)	429	Erm^R	pMP2	MP8+MP11	Ren et al. (2007)
nrrF (between nmb2073-74)	223	MP12 (XbaI)+ MP13 (EcoRI)	478	MP14 (EcoRI)+ MP15 (XhoI)	381	Erm^R	pMP3	MP12+MP15	Metruccio et al. (2009)
zur (nmb1266)	477	MP16 (XbaI)+ MP17 (EcoRI)	470	MP18 (EcoRI)+ MP19 (XhoI)	475	Erm^R	pMP4	MP16+MP19	Masignani et al. (2003)
						Kan^R	pMP5		

[1] Erm, erythromycin; Kan, kanamycin; Spec, spectinomycin

5.2.8 Southern blot

5.2.8.1 Digest, electrophoresis, capillary transfer and hybridisation

Chromosomal DNA was isolated of the respective mutant strains and the wild type strain and digested with a suitable restriction enzyme (5.2.5). DNA probes as well as the DIG-tagged ladders III or VII (for subsequent size determination of DNA fragments) were applied to a 0.8% agarose gel. The digested DNA was separated at 170 V for ~2.5 h (5.2.3) and the gel was stained to monitor correct DNA digest and separation. The DNA was depurinated by incubation of the agarose gel in 0.25 M HCl for 20 min, denatured in suitable buffer (4.8.2.3) for 30 min, and finally neutralised in respective buffer for another 30 min. The "transfer sandwich" was build up at RT. The flow of 10xSSPE buffer led to capillary transfer of DNA from the agarose gel to the positivated nylon membrane. After transfer for 4 h or o/n, the nylon membrane was dried and cross linked.

The nylon membrane was pre-hybridised in 10 ml of 'High SDS' hybridisation buffer (4.8.2.3) for at least 30 min at 42°C in a hybridisation oven. Meanwhile, 100 ng of the DIG-tagged DNA probe (5.2.7) were added to 10 ml of hybridisation buffer. Next, the mixture was incubated in a water bath at 95°C for 10 min and immediately cooled down on ice. The pre-hybridisation buffer was exchanged against the DNA-buffer solution in which the membrane was incubated o/n at 42°C.

5.2.8.2 Detection of DIG-tagged DNA by chemiluminescence

The next day the DNA-buffer solution was poured off and stored at -20°C for repeated use. The nylon membrane was washed two times each in Stringent washing buffer 1 (4.8.2.3) at RT for 5 min, and in Stringent washing buffer 2 at 68°C for 15 min. Subsequent steps were carried out at RT on a shaker. The membrane was first incubated in Washing buffer for 5 min, then in 1xBlocking solution for 30 min, an finally in Antibody solution for another 30 min. The membrane was washed two times in Washing buffer for 15 min each, and equilibrated in Detection buffer for 5 min. Next, CSPD working solution was poured onto the membrane for another 5 min. The blot was wrapped into cling film and left in an incubator at 37°C for 15 min. An X-ray film was placed onto the membrane for 20 min to 1 h.

For subsequent hybridisation with a different DNA probe, the nylon membrane was stripped by shaking it two times in 0.2 M NaOH/0.1% SDS at 37°C for 15 min. After washing in 2xSSC and pre-hybridisation, the membrane was pre-hybridised and hybridisation with another DIG-tagged DNA probe was carried out.

5.2.9 RNA preparation

5.2.9.1 RNA isolation from liquid culture

For RNA preparation from liquid culture strain MC58 and its *zur* deletion mutant WUE4812 were grown on GC agar o/n at 37°C/5% CO_2. Bacteria from the third fraction were suspended into 20 ml RPMI medium supplemented with 100 µM $FeCl_3$, and an OD_{600} 0.2 was adjusted. Under this low zinc condition, cultures were incubated at 37°C while shaking at 200 rpm until middle exponential growth phase (OD_{600}~1.0). Cultures were then diluted in fresh RPMI+$FeCl_3$ to OD_{600} 0.2 and the MC58 culture was divided into two cultures. High zinc condition was applied to the first culture by adding 0.5 µM $ZnSO_4$ to the medium according to Stork et al. (2010). The second culture and the MC58 Δ*zur* culture were further grown under low zinc condition. All three cultures were grown at 37°C/200 rpm for another 2 h leading to an OD_{600}~1.0 again.

In 2 ml caps, 500 µl of the bacterial cultures were directly added to 1 ml RNAprotect Bacteria Reagent (4.2). The mixture was vortexed and incubated at RT for 5 min previous to centrifugation in a Heraeus Biofuge 15 at 6,000 rpm for 10 min. The bacterial pellet was resuspended in 200 µl TE buffer containing 15 mg/ml Proteinase K and 4 mg/ml lysozyme, vortexed and incubated on a thermo shaker at RT for 10 min. RLT buffer of the Qiagen RNeasy kit (4.3) was mixed 1:100 with β-mercaptoethanol. To each sample, 700 µl RLT/β-ME and 500 µl of 96% ethanol were added, and gently mixed by inverting the cap. The sample was then applied to a Qiagen column of the RNeasy Mini kit. The next steps were carried out in slight modification to the manufacturer's protocol. The column was washed with buffer 700 µl buffer RW1 and 700 µl buffer RPE, followed by washing twice with 800 µl of 80% ethanol to rinse guanidine salts. Finally, the column was dried by centrifugation in a Heraeus Biofuge 15 at 13,000 rpm for 3 min. RNA was eluted twice with 45 µl Nuclease-free H_2O each, followed by incubation for 1 min and centrifugation at 13,000 rpm for 1 min. DNA remains were digested by adding 2 µl (4 U) of RNase-free recombinant DNase I (rDNase I) to each ~43 µl RNA sample gained, and 5 µl of 10x rDNase I buffer. After incubation at 37°C for 1 h, the two digested samples of the same RNA eluate were reunified (~100 µl) and mixed with 350 µl RLT/β-ME and 250 µl of 96% ethanol. Samples were purified and concentrated using another Qiagen RNeasy column, and finally eluted by applying 10 µl Nuclease-free H_2O twice. Of the final RNA sample yielded, an aliquot of 4 µl was saved for quantity measurement and quality controls, and RNA samples were stored at -70°C.

5.2.9.2 Determination of RNA quantity and quality

RNA samples were analysed with the NanoDrop 1000. RNA was considered pure with a 260 nm/280 nm ratio above 2.0, because lower ratios imply contamination with DNA or protein remains. Additionally, a 260 nm/230 nm ratio above 2.0 indicated no contamination with organic chemicals as EDTA. Furthermore, RNA concentration was determined.

To ensure complete digest of all DNA remains, a control PCR was set with 1 µl of the RNA sample, and primers KH9 and GSK2R (4.5). As a positive control, chromosomal DNA of a serogroup B meningococcal strain was used.

The Agilent 2100 Bioanalyzer was used to determine RNA integrity of RNA samples by electrophoretic separation and subsequent laser induced fluorescence detection. With the Agilent RNA 6000 Nano Reagents kit (4.3) an RNA gel was prepared in the RNA Nano Chip. Aliquots of all RNA samples to be tested were incubated at 70°C for 2 min, and immediately set on ice. One microliter of each RNA sample was pipetted in a sample well of the RNA Nano Chip. The RNA chip was placed in the instrument and bioanalysis was performed using the Agilent 2100 software with the programme 'Prokaryote total nano series II'. For RNA quality assessment, the Bioanalyzer software calculates a RNA Integrity Number (RIN) for every RNA sample based on its entire electrophoretic trace including degradation products. The RIN obtained should be ≥8.0 for microarray or qRT-PCR analysis.

5.2.10 Microarray Analysis

5.2.10.1 cDNA microarray

For each microarray analysis, at least three biological (independent bacterial cultures) with each two technical replicates (independent transcription including dye swap) were conducted which resulted in at least six microarray slides.

5.2.10.1.1 Transcription of RNA in cDNA

Nonamer random primers were purchased from Sigma-Aldrich at a concentration of 1 µmol, and were adjusted with Nuclease-free H_2O to a final concentration of 5 µg/µl. For microarray comparison of two RNA samples, each sample was labelled twice with different dyes (dye swap). For each sample, 10 µg of the isolated RNA (0) were adjusted with Nuclease-free H_2O to a final volume of 13 µl (for a Cy3 sample) or 12 µl (for a Cy5 sample). Then, 2 µl of the nonamer primer were added. The mixture was incubated at 70°C for 10 min and set on ice. Meanwhile, a dNTP mix was prepared as follows:

Reagent	Volume	Final concentration
dCTP [10 mM]	0.6 µl	200 µM
dATP [20mM]	0.8 µl	500 µM
dGTP [20mM]	0.8 µl	500 µM
dTTP [20mM]	0.8 µl	500 µM
DTT [0.1 M]	1.5 µl	5 mM
5x First Strand Buffer	6 µl	1x
RNase Out	1 µl	2 U
Superscript II	2 µl	4 U

Of this dNTP mix, 13.5 µl were pipetted to each sample. Finally either 1.5 µl Cy3-dCTP or 2.5 µl Cy5-dCTP were added to each sample. To avoid bleaching of the dyes all incubation steps were performed in the dark. After vortexing, quick centrifugation and incubation at RT for 10 min, samples were incubated for 2 h at 42°C on a heating block for transcription of RNA into cDNA. Samples were then heated at 70°C for 15 min to inactivate the Superscript II transcriptase and set on ice. The samples were incubated for 45 min at 37°C with 2 µl DNase-free RNase to digest the RNA template. The labeled cDNA was purified using the illustra AutoSeq G-50 dye terminator removal kit (4.3) according to the manufacturer's instructions which yielded in 40 µl of purified Cy3- or Cy5-labelled cDNA.

5.2.10.1.2 Prehybridisation of microarray slides

Microarray analysis was performed using whole-genome DNA microarrays. Seventy-mer oligonucleotides covering four meningococcal genomes (MC58, Z2491, FAM18, and α14) were spotted in quadruplicate onto epoxy-coated Nexterion E slides as described previously (Schwarz et al, 2010). Slides were prehybridised according to the manufacturer's protocol. For this purpose, four microarray slides were set into a rack and incubated in the following solutions: 1) in 0.1% Triton X-100 for 5 min at RT; 2) two times in 1 mM HCl for 2 min at RT; 3) in 0.1 M KCl for 10 min at RT; 4) in dH_2O for 1 min at RT; 5) in Blocking Solution (4.8.2.4) for 15 min at 50°C; and 6) in dH_2O for 1 min at RT. For all incubation steps except of 5) the rack which was placed on a stirrer to keep the solutions in movement. Slides were placed in a 50 ml Cellstar tube and dried by centrifugation in the Heraeus Megafuge 1.0 R at 1600 rpm for 5 min.

5.2.10.1.3 Hybridisation

One Cy3- and one Cy5-labeled cDNA sample were combined and dH_2O was added to a final volume of 97.5 µl. Then, 13 µl 1% SDS and 19.5 µl 20xSSC were added. The resulting 130 µl hybridisation probe was incubated for 5 min at 95°C, shortly cooled down on ice and finally loaded onto the prehybridised microarray slide. The slide was then placed into the hybridisation chamber. Used liquids in the channels (Ch) were as

follows: 1) 2xSSC, 0.2% SDS; 2) 2xSSC; 3) 0.2xSSC; 4) 0.1xSSC; 5) dH_2O. Hybridization was carried out at the Tecan HS4800 Pro hybridization station using the HS Pro Control Manager software and the following hybridisation programme:

1 - WASH: 60°C, Ch:1, Runs:1, Wash time: 30 sec
2 - SAMPLE INJECTION: 60°C, Agitation
3 - HYBRIDISATION: 52°C, Agitation frequency: High, Time: 17 h
4 - WASH: 60°C, Ch:1, Runs: 2, Wash time: 1 min
5 - HYBRIDISATION: 52°C, Agitation frequency: High, Time: 1 min
6 - WASH: 23°C, Ch:2, Runs: 1, Wash time: 1 min, Soak time: 1 min
7 - HYBRIDISATION: 23°C, Agitation frequency: High, Time: 1 min
8 - WASH: 23°C, Ch:3, Runs: 2, Wash time: 1 min
9 - HYBRIDISATION: 23°C, Agitation frequency: High, Time: 30 sec
10 - WASH: 23°C, Ch:4, Runs: 2, Wash time: 30 sec
11 – SLIDE DRYING: 23°C, Ch5, Time: 2 min, Final manifold cleaning

5.2.10.1.4 Data analysis

Hybridised slides were scanned with the Genepix 4200 scanner. Using Genepix Pro 4.0, raw data files for each microarray slide were generated and further processed with the Limma package (Smyth, 2005). Data normalisation was done by Dr. Biju Joseph. After implementing data in R (The-R-Development-Core-Team, 2008), only genes with a false discovery rate (FDR)<0.01 and a B statistic value>0 were considered for further analysis.

5.2.10.2 gDNA microarray

Microarray comparative genomic hybridisation (mCGH) analysis with chromosomal DNA (genomic DNA, gDNA) was carried out in modification to Joseph et al (2011). Chromosomal DNA was isolated using Genomic Tip 100/G (5.2.1) from *N. m.* strains α16, α528 and DE9686. Four micrograms of gDNA were adjusted with TE buffer (4.8.2.1) to a final volume of 12 µl. The sample was incubated at 95°C for 10 min and immediately set on ice. A master mix each was prepared for Cy3- and Cy5-labelling:

Reagent	Volume
10xdNTP	2 µl
Nonamer primer	2 µl
10x Labelling buffer	2 µl
Klenow	1 µl
Cy3- or Cy5-dCTP	1 µl

The master mix was vortexed, shortly centrifuged and pipetted to the DNA sample. The samples were incubated o/n at 37°C in an incubator in the dark. The next day, reactions

were stopped by adding 2 µl of 0.2 M EDTA (pH 8). DNA was precipitated by addition of 2 µl 4 M sodium acetate (pH 4.5) and 75 µl cold 96% ethanol and subsequent centrifugation in the Heraeus Megafuge 1.0 R at 13,000 rpm for 15 min at 4°C. After washing with 1 ml of 70% cold ethanol, centrifugation was repeated. The DNA precipitate was dried and dissolved in 2 µl dH_2O. One Cy3- and one Cy5-labelled probe were combined, and 3 µl salmon sperm ssDNA and 113 µl PerfectHyb Plus hybridization buffer (4.2) were added to yield a 120 µl gDNA hybridisation probe. The probe was incubated at 95°C for 5 min in the dark, briefly cooled down on ice and applied to a prehybridised microarray slide (5.2.10.1.2) which was placed in the hybridisation chamber. Liquids were used in the channels (Ch) as follows: 1) 2xSSC, 0.2% SDS; 2) 2xSSC; 3) 0.2xSSC; 4) 0.1xSSC; 5) dH_2O. The hybridisation was conducted at the Tecan HS4800 Pro hybridization station using the following hybridisation programme:

1 - WASH: 60°C, Ch: 1, Runs:1, Wash time: 30 sec
2 - SAMPLE INJECTION: 60°C, Agitation
3 - HYBRIDISATION: 50°C, Agitation frequency: High, Time: 17 h
4 - WASH: 60°C, Ch: 1, Runs: 2, Wash time: 1 min
5 - HYBRIDISATION: 50°C, Agitation frequency: High, Time: 1 min
6 - WASH: 23°C, Ch: 2, Runs: 1, Wash time: 1 min, Soak time: 1 min
7 - HYBRIDISATION: 23°C, Agitation frequency: High, Time: 1 min
8 - WASH: 23°C, Ch: 3, Runs: 2, Wash time: 1 min
9 - HYBRIDISATION: 23°C, Agitation frequency: High, Time: 30 sec
10 - WASH: 23°C, Ch: 4, Runs: 2, Wash time: 30 sec
11 – SLIDE DRYING: 23°C, Ch: 5, Time: 2 min, Final manifold cleaning

5.2.11 Quantitative real-time PCR (qRT-PCR)

qRT-PCR analysis was performed as recently described using the StepOnePlus™ system (Schielke et al, 2011). Total RNA was extracted (0) and reversely transcribed into cDNA (5.2.10.1.1). Primers were designed using the Primer Express 3.0 software. Each primer pair was first tested in PCR with chromosomal DNA as well as cDNA. If PCR products displayed the expected size, primer efficiency and unique melting temperature were tested using relative standard curve experiments of the StepOne software v2.0. Additionally, the best cDNA concentration for the subsequent analysis was determined.
qRT-PCR reactions comprised the Power SYBR Green Master Mix, 15 ng cDNA and primers at a final concentration of 900 nM. *nmb1567*, coding for a putative membrane-associated peptidyl-prolyl isomerase, was used as endogenous control, and expression levels of the target genes were normalised to *nmb1567* expression. Negative controls without cDNA template were added. In each assay, triplicates of each sample were

conducted. Furthermore, each gene was analysed in three independent qRT-PCRs (technical replicates) based on RNA from independent bacterial cultures (biological replicates). Data analysis was done with the StepOnePlus™ software based on the ΔΔcT method. For each gene, RQ (relative quantity) values were obtained that represented the fold change of expression of the investigated gene.

5.3 Protein analysis

5.3.1 Preparation of whole cell lysate protein from *N. meningitidis*

Bacteria were grown o/n on GC agar plates. OD_{600} 0.2 was adjusted in 1xPBS, bacterial cells were pelleted and resuspended in 50 µl 1x Sample Solution (4.8.3.1).

5.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were cast on the previous day, composed of a 10% or 12.5% separation gel and a loading gel (4.8.3.1). Prior to electrophoresis, protein samples (5.3.1) were incubated at 95°C for 10 min and cooled down on ice. For Silver gels and Western blots, 5 µl of protein samples were loaded on the gel. For Coomassie gels, 20 µl of protein samples were used. For determination of the protein size, 5 µl of the Page Ruler prestained protein marker were pipetted in one slot. Separation of proteins was carried out for approximately 1.5 h in Electrophoresis buffer. Thereby, 10 mA/gel were applied first until samples passed the border to the separation gel, followed by 20 mA/gel until the dye front completely ran off.

5.3.3 Staining of SDS-Polyacrylamide Gels

Following SDS-PAGE, gels were stained either by Coomassie or Silver staining. For Coomassie staining, the gels were shaken in Coomassie staining solution (4.8.3.2) for 30 min. The gel was then incubated in Destaining solution for at least 2 h until protein bands became visible against the background.

Silver staining was performed in modification to Blum et al. (1987). Gels were incubated in the following solutions: 1) Fixing solution for 30 min; 2) 50% ethanol for 10 min; 3) 10% ethanol for 10 min; 4) 0.02% sodium thiosulfate for exactly 1 min to block unspecific binding sites; 5) dH_2O three times for 20 sec each to wash and 6) freshly prepared Silver staining solution for 20 min. Then, the gel was washed twice with dH_2O for 20 sec each. By subsequent shaking in Developing solution for few minutes protein bands became visible. Developing procedure was stopped with 10% acetic acid, in which

the gel then was incubated for another 15 min. Gels were kept in dH_2O until scanning on the gel scanner and preservation in plastic film.

5.3.4 Western Blot

Western blots were used for antibody-mediated detection of specific proteins. Proteins separated in SDS-polyacrylamide gels were transferred from the gel to a nitrocellulose membrane by electroblotting for 1 h at 300 mA in Blot buffer (4.8.3.3). Non-specific binding sites were saturated by incubation of the membrane in 5% low fat milk in PBS-T for 1 h. The blot was then incubated o/n at 4°C in a dilution of the primary antibody (4.3) in 1% low fat in PBS-T. The membrane was washed twice for 5 min each in PBS-T, and the horse reddish peroxidase-conjugated secondary antibody diluted in PBS-T was applied for 1 h at RT. After washing in PBS-T three times for 5 min each, the blot was developed with the Pierce ECL chemiluminescence detection system according to the manufacturer's instructions. An X-ray film was placed for usually 10 sec to 2 min.
For subsequent incubation with another primary antibody, the nitrocellulose membrane was incubated in Stripping buffer (2% SDS; 20 mM Tris/HCl, pH 6.8; 0.7% β-ME) for 3 h at 50°C or o/n at RT. After washing in PBS-T three times for 10 min each and blocking of unspecific binding sites, another primary antibody could be used.

5.3.5 Analysis of the Lipopolysaccharide (LPS)

5.3.5.1 LPS preparation

Preparation of the Lipopolysaccharide (LPS) was done in modification to Hitchcock & Brown (1983). Meningococcal strains were grown o/n at 37°C/5% CO_2 on GC agar plates with CMP-NANA [0.5% in Tris/HCl]. Supplementation with CMP-NANA (Cytidine-5'-monophospho-N-acetylneuraminic acid), an additional substrate for sialyltransferases, ensures full LPS sialylation if the strain is capable of it. OD_{600} 0.2 was adjusted in 1xPBS, bacteria were pelleted in a Heraeus Biofuge 15 at 13,000 rpm for 2 min and resuspended in 50 µl Lysis buffer (4.8.3.4). After incubation in a water bath at 95°C for 10 min and at 60°C for another 10min, 10 µl Proteinase K/lysis buffer was added and incubation was continued at 60°C for 1 h. Samples were stored at -20°C.

5.3.5.2 LPS SDS-PAGE

To more efficiently separate low-molecular proteins and LPS, Tricine-sodium dodecyl sulfate-polyacrylamide gels were used (Schägger & von Jagow, 1987). For this purpose, tricine gels were cast analogous to SDS-polyacrylamide gels, but made of the Solution for tricine separation gel (4.8.3.4), and a loading gel made of the Solution for tricine loading gel. The inner chamber of the SDS-PAGE system was filled with Cathode buffer, the outer one with Anode buffer. Then, a mixture of 1.5 µl LPS preparation and 1.5 µl 2x Tricin Sample Solution was pipetted into each slot of the tricine gel. No molecular weight marker was needed. Samples were separated for approximately 4.5 h, first at 30 V until they passed the border to the separation gel, and then at 110 V.

5.3.5.3 LPS silver staining

For LPS silver staining after Tsai & Frasch (1982) all buffers were prepared with distilled water in plastic material, and for each step a new plastic container was used. The gel first was incubated in: 1) Fixer (4.8.3.4) for at least 1 h or o/n; 2) Oxidiser (4.8.3.4) for 5 min; 3) dH_2O three times for 15 min each; 4) freshly prepared LPS silver staining solution for 10 min; and 6) in dH_2O three times for 10 min each. The gel was then shaken in Developer for approximately 2 min until the LPS bands became visible. Developing procedure was stopped by adding 5% acetic acid in which the gel was incubated for another 15 min. Then, the gel was put into dH_2O, scanned on a gel scanner and preserved in plastic film.

5.3.6 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were used to analyse meningococcal capsule and LPS structure. All incubation steps were performed at 37°C in an incubator. Meningococci grown o/n on GC agar plates were adjusted to an OD_{600} 0.1 in 1xPBS. A 96 well ELISA microplate was coated with 50 µl of poly-D-lysine (25 mg/l) for 30 min to promote the adhesion of bacterial cells to the microplate. Following three times of washing of the microplate with 1xPBS, 20 µl of each bacterial suspension were pipetted in duplicates into the wells and incubated for 1 h. Adherent bacteria were fixed to the surface by addition of 100 µl 0.05% glutaraldehyde/PBS to each well and incubation for 10 min. The wells were washed three times with 1xPBS. Non-specific binding sites were blocked by addition of 150 µl of 1% BSA/PBS for 30 min. Next, 20 µl of primary antibody solution were added to each well and incubated for 1 h. As a primary antibody against the B capsule, mouse monoclonal antibody (mAb) 735 was used and diluted 1:4,000 in 1% BSA/PBS. For LPS ELISA, different murine antibodies were used in different dilutions in 1% BSA/PBS, i.e. L3,7,9 (1:100); L8 (1:1,000) and 3F11 (undiluted). After washing three times with

1xPBS the secondary antibody, a Peroxidase-conjugated goat anti-mouse IgG+IgM antibody (4.3), was diluted 1:2,500 in 1%BSA/PBS and 20 µl were added to each well. After 45 min of incubation, the wells were washed another three times with 1xPBS. Finally, 20 µl ABTS [1 mg/ml] were pipetted in each well. ABTS acts a substrate to the peroxidase, resulting in a colour reaction that was measured photometrically after 10 min in an ELISA reader at 414 nm.

5.3.7 Flow cytometry analysis

Flow cytometry was used to analyse complement deposition on the meningococcal surface. For this purpose, deposition of the complement components C3 or C5b-9 on the meningococcal surface was detected by incubation with adequate antibodies and subsequent measurement in a flow cytometer.

5.3.7.1 Serum killing assay for flow cytometry

Neisseria meningitidis strains were grown on GC chocolate agar plates o/n (~16 h) at 37°C/ 5% CO_2. An OD_{600} 0.2 of bacteria of the third fraction was adjusted in VBS/BSA buffer (4.8.1.1) in a 1.5 ml reaction tube. Bacteria were pelleted and resuspended in (400-x) µl VBS/BSA buffer. NHS was thawed on ice and x µl (e.g. 40 µl=10%) of NHS were added to the bacterial suspension. For each strain in the assay, a negative control was set with HIS (5.1.5) instead of NHS. All samples were incubated at 37°C and 200 rpm in an incubator shaker for 10 min up to 30 min. The serum stress was stopped by setting the reaction tube on ice and adding 400 µl of cold $HBSS^{++}$/BSA (4.8.3.5).

5.3.7.2 Antibody binding

All centrifugation steps following were carried out at 11,000 rpm/7 min in a Heraeus Biofuge 15. Bacteria were pelleted, washed with 700 µl $HBSS^{++}$/BSA, and centrifuged again. The bacterial pellet was resuspended of in 50 µl of a 1:50 dilution of the monoclonal mouse anti-human C5b9 antibody (4.3) or the polyclonal rabbit anti-human C3d complement antibody in $HBSS^{++}$/BSA. One sample served as an antibody negative control and was resuspended in 50 µl $HBSS^{++}$/BSA only. All samples were incubated for 30 min at 37°C/700 rpm on a thermo shaker. Bacteria were pelleted and washed with 700 µl $HBSS^{++}$/BSA. The bacterial pellet was then resuspended in 50 µl of a 1:200 dilution of the AlexaFluor 488 goat anti-mouse IgG antibody or the AlexaFluor 488 chicken anti-rabbit IgG antibody in $HBSS^{++}$/BSA. After incubation for 30 min at 37°C/700 rpm, samples were pelleted and washed with 700 µl $HBSS^{++}$/BSA. Pellets were resuspended in 1 ml of 1% formaldehyde in 1xPBS for fixation and killing of the

meningococci. After incubation at 4°C in the dark for at least 1 h or o/n, bacterial cells were pelleted at 11,000 rpm for 10 min and resuspended in 400 µl PBS.

5.3.7.3 Flow cytometry

Samples were transferred into FACS tubes and measured with a flow cytometer at the following settings:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E02	3.50	lin
P2	SSC	350	1.00	log
P3	FL1	650	1.00	log

After measurement of the negative control without primary antibody, a gate was defined corresponding to the densest bacterial population. Measurement was adjusted to 20,000 counts of bacteria within the defined gate. Histogram graphs of data were generated using Weasel software (http://www.wehi.edu.au).

5.3.8 Expression and Purification of recombinant protein Zur

Protein expression was carried out according to the QIAexpressionist kit manual (Qiagen). Purification of the recombinant protein Zur was performed under native conditions. One liter of LB medium supplemented with ampicillin and kanamycin (5.1.1) was inoculated with a 20 ml o/n culture of WUE4892 (0) harbouring pMP12 (5.2.6.2). Bacteria were grown to an OD_{600} 0.6 at 37°C shaking at 250 rpm. Protein expression was induced by adding 1 mM IPTG to the culture and incubation was continued for another 4.5 h at 37°C. After harvesting of cells (10,000xg at 4°C for 30 min), bacterial pellets were resuspended in Lysis buffer (4.8.3.6). Cells were lysed by adding 1 mg/ml lysozyme for 30 min on ice and subsequent sonification in an ice water bath. Using an ultrasonic homogenizer, six 30 sec bursts at 130 W, with a minute cooling period in between each burst, were applied. The lysate was then centrifuged at 10,000xg at 4°C for 30 min, and the supernatant was saved on ice. The supernatant was loaded on a HIS GraviTrap Ni sepharose column equilibrated with Lysis buffer. After washing three times with Wash buffer, the HIS-tagged recombinant protein was eluted in Elution buffer. The Zur eluate was dialysed at 4°C four times for 12 h each against 5 l of 0.5xTB buffer each (4.8.3.7) to remove imidazol and nickel. Protein concentration was determined (5.3.9) and purity was verified by SDS-PAGE with Coomassie staining. Protein samples were stored in aliquots of 200 µl at -20°C.

5.3.9 Quantification of protein

Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufacturer's protocol. Briefly, dilution series of BSA as a standard and of the protein were prepared. Of every sample 25 µl were pipetted in duplicates into the wells of a 96-well ELISA microplate, and the dilution reagent was used as a blank (e.g. 1xPBS). Then, 200 µl of Working reagent were added to each well and the plate incubated at 37°C for 30 min. Absorption was measured with an ELISA reader at 570 nm. The protein concentration was calculated based on the BSA standard curve.

5.3.10 Electrophoretic Mobility Shift Assay (EMSA)

For electromobility shift assays (EMSAs) the DIG Gel Shift Kit (4.3) was used. Short complementary oligonucleotides of 34-51 bp with sticky ends, comprising the Zur boxes of three chosen genes, were designed using the NCBI Primer BLAST. Oligonucleotides were resuspended in TEN (10 mM Tris, 1 mM EDTA, 0.1 M NaCl; pH 8.0) to a final concentration of 100 µM. To generate dsDNA fragments, 300 µl each of the 100 µM complementary oligonucleotides were mixed and annealed by incubation for 10 min at 95°C and subsequent slow cool down to RT. Obtained dsDNA fragments were labelled with digoxigenin (5.2.7).

Binding reactions of the labelled dsDNA fragments and the purified recombinant protein (5.3.8) were performed in 5x Binding buffer (4.8.3.7) in modification to Shin et al. (2007), supplemented with poly (dI-dC) [1 µg/µl] and poly-L lysine [0.1 µg/µl] that are included in the DIG gel shift kit. Standard reactions were pipetted as follows:

Reagent	Volume	Final concentration
Binding buffer (5x)	2 µl	1x
poly (dI-dC) [vial9]	0.5 µl	0.1 µg/µl
poly-L lysine [vial 11]	0.5 µl	0.01 µg/µl
Labelled dsDNA fragment	1 µl	0.4 ng (15.5 fmol)
Purified protein (Zur)	1 µl	15 pmol (250 ng)
$ZnSO_4$	1 µl	100 µM
dH_2O	ad 10 µl	

As zinc was required for Zur-DNA interaction, standard reactions were supplemented with $ZnSO_4$ to reach a final concentration of 100 µM according to Gaballa & Helmann (1998). To furthermore avoid chelation of zinc, EDTA was omitted from the binding and the running buffer. A native 8% polyacrylamide gel was cast the previous day (4.8.3.7). The gel was pre-run with 4°C cold 0.5xTB buffer at 120 V and 4°C for at least 30 min.

Meanwhile, binding reactions were pipetted and incubated at RT for 20 min. Next, 2.5 µl loading buffer [vial 13] was added on ice and samples were applied to the gel and separated at 120 V at 4°C for 110 min in 0.5xTB buffer. Proteins were electroblotted on a nylon membrane (5.3.4) in 0.5xTB buffer, and chemiluminescent detection of the bound dsDNA fragments was carried out.
For analysis of the influence of ions other than zinc, the salts $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnSO_4$ or $NiSO_4$ were added at a final concentration of 100 µM. For specific chelation of zinc in some reactions, EDTA and TPEN (N,N,N',N'-*tetrakis*-(2-pyridyl-methyl)ethylenediamine) were added at a final concentration of 312.5 µM. For competition of binding, 125- to 1000-fold excess of the unlabelled dsDNA probe was added to the reactions.

5.4 Computational Analysis

Microsoft Excel 2003 was used for general data analysis and evaluation of data obtained by ELISA, Flow cytometry, microarray, SBA, qRT-PCR and protein quantification.

The DNAStar Lasergene 8 software package was used for a variety of applications, e.g. MegAlign for alignment of gene sequences of different strains; SeqBuilder for planning of digests with restriction endonucleases for cloning and Southern blots; SeqMan for assembling of partial sequences to one sequence. BioEdit 7.0.9 (Hall, 1999) was applied for sequence visualization, analysis and alignments.
The Primer BLAST of the National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/tools/primer-blast] and Primer Express 3.0 (Applied Biosystems) were used for designing oligonucleotides.
The NCBI BLAST programme of the National Center for Biotechnology Information (NCBI) [http://blast.ncbi.nlm.nih.gov/Blast.cgi] was used for similarity searches.

Sequences of genes of strain MC58 were retrieved from NeMeSys (Rusniok et al, 2009) and the J. Craig Venter Institute [http://cmr.jcvi.org]. Annotation data were retrieved from NeMeSys (Rusniok et al, 2009) and Uniprot (UniProt-Consortium, 2012) databases.
Operons predictions were kindly provided by Dr. Biju Joseph, based on RNAseq data (Joseph et al., unpublished) and the DOOR database (Mao et al, 2009). Promoter predictions were carried out using the online software tools NNPP (Reese, 2001) and BPROM [http://linux1.softberry.com/berry.phtml]. Conserved motifs within upstream sequences were searches using the GIBBS Motif Sampler (Thompson et al, 2007). The meningococcal Zur binding motif was visualised using WebLogo 3.2 (Crooks et al, 2004).

6 RESULTS

6.1 Impact of global regulators of gene expression on serum resistance

During this thesis I aspired to identify novel serum resistance factors of serogroup B meningococci. Initially, I tried to adapt meningococci to higher levels of serum resistance by alteration of various environmental conditions, i.e. growth under microaerophilic conditions, hydrogen peroxide stress, exposure to very high serum concentrations and repeated passage in serum. However, all these attempts failed. I therefore used mutants of meningococcal global regulators to investigate the impact of their regulons on serum resistance. The global regulators analysed in this study are presented in Table **1** (3.7). These global regulators were either deleted or overexpressed to simulate specific environmental conditions and modify target expression. All mutations were carried out in a mutant of carriage strain α16 deficient in expression of capsule and fHBP as well as LPS sialylation (α16 Δ*siaD* Δ*lst* Δ*fhbp*).

I constructed deletion mutants of *asnC*, *nrrF* and *zur* by transformation of the generated plasmids pMP2, pMP3 and pMP4, respectively (5.2.6.1). The mutant strains were designated WUE4659, WUE4665, and WUE4671. Deletion of AsnC simulates reduced nutrient levels (amino acid deficiency) leading to reduced expression of major outer membrane proteins which may emphasise the role of minor surface proteins. Deletion of the sRNA NrrF results in constant expression of its target mRNAs independently of Fur and iron. Deletion of Zur simulates zinc starvation and leads to de-repression of its target genes, resulting e.g. in enhanced zinc uptake.

Furthermore, overexpressing mutants of RpoH (WUE4547), Fnr (WUE4353) and Hfq (WUE4320) were generated by Dr. Kerstin Hubert of our work group using the plasmids pAB1, pAB2 and pAB3, respectively. Overexpression of Fnr simulates oxygen restrictive conditions in the cell leading to constant expression of its target genes involved in anaerobic metabolism. Overexpression of the RNA chaperon Hfq simulates increased environmental stress, resulting in enhanced sRNA stability and regulation of their mRNA targets. Overexpression of RpoH, the major regulator of stress response, simulates environmental stress such as a heat shock, which results in increased expression of target genes, e.g. chaperones.

Flow cytometry measurement of complement deposition on surface of these mutants in comparison to their parental strain was conducted after treatment with 10% NHS for 10 min (5.3.7). MAC deposition on surface of all three overexpression mutants equalled that of the parental strain (Figure 3A). Therefore, overexpression of the fumarate and nitrate

reduction regulator Fnr, the RNA chaperone Hfq and the major regulator of stress response RpoH had no influence on serum resistance.

Similarly, deletion of AsnC, a transcriptional activator at poor nutrition, and of the small regulatory RNA NrrF had no effect (Figure 3B). However, the knockout mutant of Zur, the regulator of zinc uptake, showed reduced binding of complement components C5b-9 (Figure 3B).

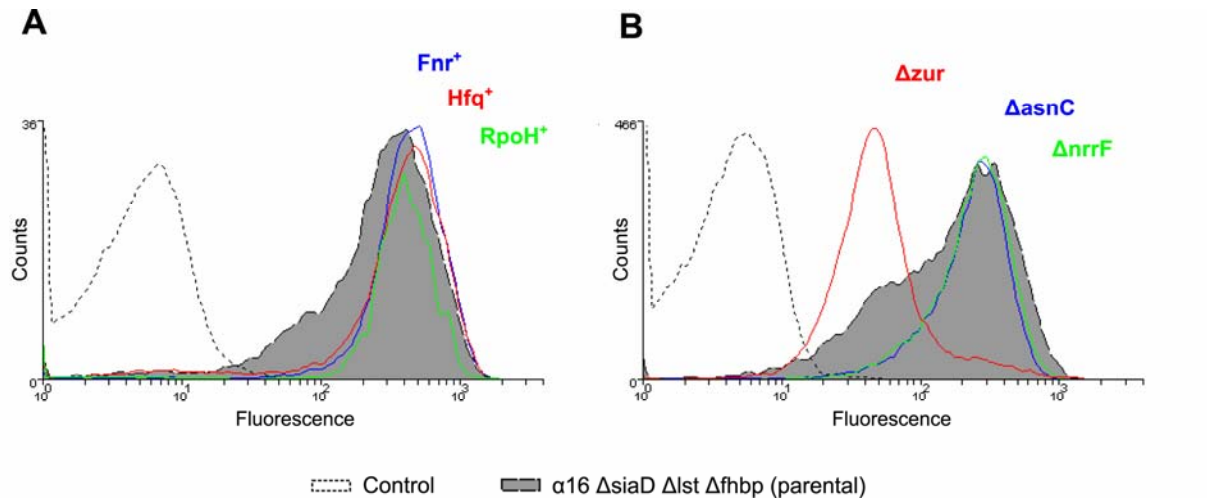

Figure 3. FACS analysis of MAC deposition of different global regulators mutants. MAC deposition was detected with the mAb mghC5b-9 after incubation with 10% NHS for 10 min. Representative results are shown as seen in independent experiments. **A) Overexpression mutants**: MAC deposition on surface of Fnr, Hfq and RpoH mutants was similar to the parental strain α16 Δ*siaD* Δ*lst* Δ*fhbp*. **B) Deletion mutants**: MAC deposition on surface of Δ*asnC* and Δ*nrrF* mutants did not differ compared to the parental strain. Deletion of *zur* led to reduced MAC deposition.

Analysis of deposition of complement component C3d let to same results for all mutants. Compared to the parental strain, C3d deposition was only reduced on surface of the *zur* knockout mutant (data not shown). We therefore assumed that deletion of *zur* led to changes in regulation that might remodel the meningococcal surface and thus affect the interaction with serum complement.

6.1.1 Analysis of the Zur-regulated protein ZnuD with regard to mediation of serum resistance

Zur is known as a regulator of zinc uptake in different bacterial species (Hantke, 2001), and the meningococcal gene *nmb1266* was identified as a homologue of the *E. coli zur* (Stork et al, 2010). Furthermore, Stork et al demonstrated that ZnuD, a TonB-dependent

outer membrane receptor, is involved in the uptake of zinc at zinc-limited conditions. They also identified a putative Zur responsive element in the promoter region of *znuD*, and showed that deletion of the meningococcal *zur* homologue resulted in increased ZnuD expression (Stork et al, 2010). Besides of that, nothing was known about the meningococcal Zur and the regulatory processes in response to zinc.

As shown above, deletion of Zur reduced complement deposition indicating enhanced serum resistance. Hence, we wanted to know if the reason for this might be increased expression of ZnuD on the meningococcal surface. We constructed a *znuD* overexpressing and a *znuD* knockout mutant by transformation with plasmids pIK6 and pIK3, respectively. The resulting *znuD*$^+$ and Δ*znuD* strains were WUE4684 and WUE4686, respectively (Table 9). Due to the combination of resistance cassettes, this was performed in a α16 Δ*siaD* background. The parental strain and both mutants were treated with 10% or 20% NHS for 10 minutes and MAC deposition on surface was measured by FACS analysis. After 10% NHS treatment, the *znuD* knockout mutant was covered only marginally more with MAC than the parental strain and the *znuD* overexpressing strain. Incubation with 20% NHS led to equal MAC deposition on surface of all three strains (Figure 4).

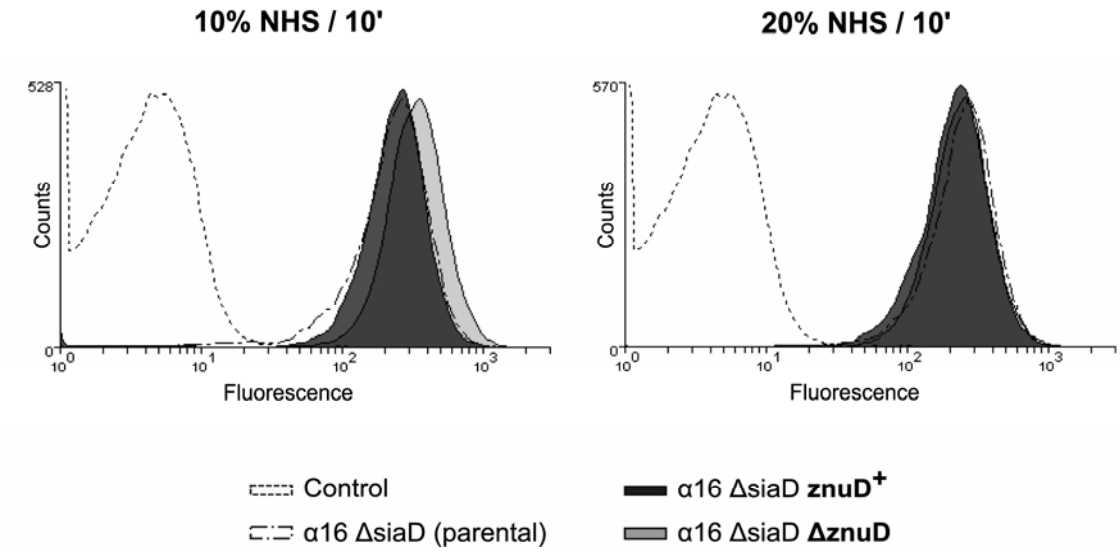

Figure 4. FACS analysis of MAC deposition on surface of *znuD* overexpression and deletion mutants. Deposition of C5b-9 (MAC) was detected using the mAb mαhC5b-9 after incubation with 10% and 20% NHS for 10 min. Exemplary graphic presentation of comparable data obtained in three independent experiments. Deposition on surface of the *znuD*+ and the Δ*znuD* mutant in comparison to the parental strain α16 Δ*siaD* were similar after 10% NHS for 10 min, and equalled after 20% NHS for 10 min.

Thus, ZnuD did not seem to be the key player leading to the considerably reduced complement deposition on surface of the *zur* deletion mutant. In order to identify the potential serum resistance factor, we decided to elucidate first the zinc-dependent Zur regulon which had not been studied before.

6.2 The zinc-responsive regulon of *Neisseria meningitidis*

To address the Zur regulon of *N. meningitidis*, two decisions were made: (1) I selected strain MC58 for further analysis because of the availability of microarrays covering the genome of this strain (Schwarz et al, 2010). (2) In order to avoid the transcriptional analysis of genetically engineered mutants and possible indirect effects, the addition of $ZnSO_4$ was selected as stimulus.

Zur was shown to mainly act as a repressor in other bacteria (Gaballa et al, 2002; Maciag et al, 2007; Schröder et al, 2010). Therefore, mRNA of most Zur-regulated genes is expected to be present in a *zur* knockout strain. We selected for a common reference design of microarray analysis which also offered the possibility for future extension of the dataset (Yang & Speed, 2002). Deletion of *zur* in MC58 was achieved using the generated plasmid pMP5 (5.2.6.1). The resulting Δ*zur* strain was designated WUE4812 and used as common reference. Gene expression was compared at low and high zinc condition.

As outlined in Figure 5, cDNA obtained from strain MC58 grown with or without zinc supplementation (i.e. low and high zinc conditions, respectively) was hybridised separately along with the common reference, i.e. cDNA obtained from strain MC58 Δ*zur* grown at low zinc condition. Data analysis finally led to genes differentially regulated between low and high zinc condition.

This study has been published in Pawlik et al (2012).

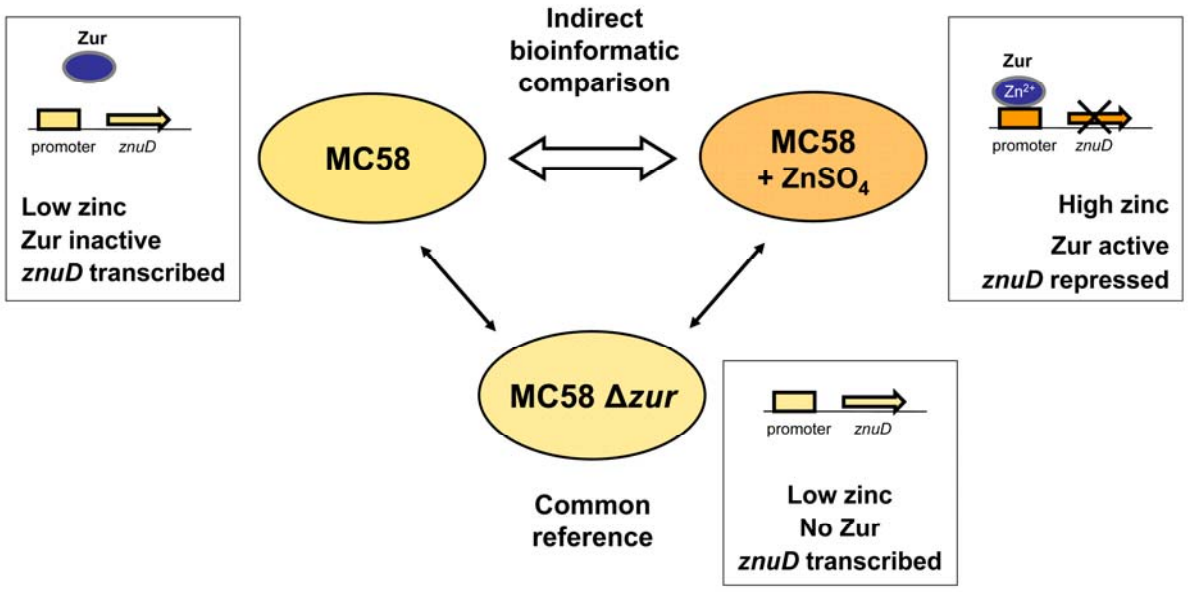

Figure 5. Common reference design of microarray comparison. cDNA obtained from MC58 grown at high low and high zinc conditions were hybridised along with cDNA of the common reference, a MC58 zur deletion strain grown at low zinc condition. The zinc-dependent promoter repression by Zur is visualised by cartoons using the example of znuD. In modification from Pawlik et al (2012).

6.2.1 Validation of growth conditions

Growth of the *zur* deletion strain in the chemically defined medium RPMI supplemented with $FeCl_3$ was indistinguishable from its parental strain MC58 (data not shown). I next conducted experiments to select the best possible time point of harvesting bacteria for RNA extraction. Western blot analysis and qRT-PCR of znuD, which was recently shown to be increased in a *zur* knockout mutant (Stork et al, 2010), was used as a marker. MC58 and MC58 Δ*zur* were grown at low zinc condition in RPMI medium supplemented with $FeCl_3$ until exponential phase. The MC58 culture was split in half and all three cultures were diluted in fresh medium. For one of the MC58 cultures, 0.5 µM $ZnSO_4$ were added to the medium. Incubation was continued for one, two and three hours, respectively. At each time point, samples of the three cultures were taken for analysis of protein and RNA levels in Western blot (5.3.4, Figure 6A) and qRT-PCR (5.2.11, Figure 6B), respectively.

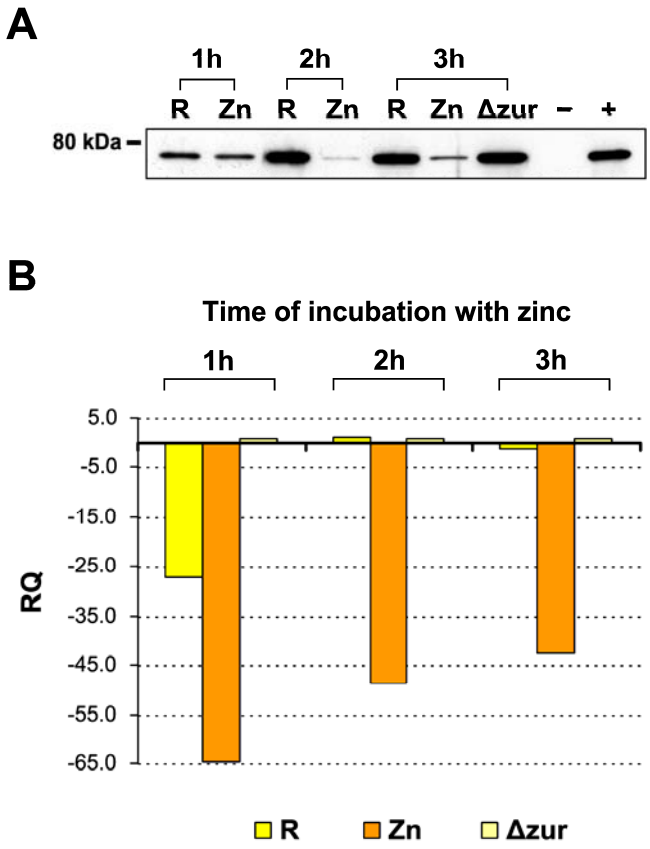

Figure 6. Validation of growth conditions by monitoring *znuD* expression by Western blot and qRT PCR. RNA was extracted after growth of MC58 with or without supplementation with $ZnSO_4$ for one, two and three hours, respectively. R, RPMI; Zn, RPMI+0.5 µM ZnSO4; Δ*zur*, MC58 Δ*zur* grown in RPMI was used for comparison. **A) Western blot analysis of ZnuD expression.** Protein detection using a rabbit anti-*znuD* antibody #1041. (-), *znuD* deletion strain (+), *znuD* overexpressing strain. ZnuD expression between RPMI (low zinc) and RPMI+$ZnSO_4$ (high zinc) condition was most differential after 2h. ZnuD expression of MC58 and MC58 Δ*zur* grown at low zinc condition is comparable after 2h. **B) qRT-PCR analysis of *znuD* expression.** Relative quantity (RQ) of *znuD* expression in MC58 grown at low or high zinc relative to MC58 Δ*zur*. After 2h, MC58 and MC58 Δ*zur* grown at low zinc showed equal expression of *znuD*, whereas *znuD* expression was strongly repressed in MC58 grown at high zinc.

After one hour, ZnuD expression in Western blot was comparable for MC58 grown at low and high zinc, but less for MC58 Δ*zur* (Figure 6A). This was confirmed in qRT-PCR where MC58 grown with zinc addition showed reduced *znuD* expression compared to MC58 Δ*zur*. However, expression of *znuD* was still reduced in MC58 grown at low zinc, probably due to intracellular zinc remains of the overnight culture on GC agar (Figure 6B). After two and three hours, protein levels of MC58 (RPMI) were comparable to MC58 Δ*zur* (RPMI), whereas the ZnuD band almost vanished after two hours of zinc addition, but was visible again after three hours (Figure 6A). Also in qRT-PCR, *znuD* expression was most differential between MC58 grown at high and low zinc, but most equal between

MC58 and MC58 Δ*zur* grown at low zinc condition after two hours (Figure 6B). Based on this data, zinc exposure for two hours was selected as suitable condition for the microarray comparison.

6.2.2 Transcriptome analysis of zinc-dependent genes

Strain MC58 was grown at low zinc (only RPMI) and high zinc conditions (RPMI + 0.5 µM $ZnSO_4$) as defined by Western blot and qRT-PCR of *znuD*; strain MC58 Δ*zur* was grown at low zinc condition to yield a common reference. RNA was then extracted from all three cultures (5.2.9.1) and transcriptome analysis was carried out as described in 5.2.10.1. Gene expression profiles of MC58 at high and low zinc condition were compared by Dr. Biju Joseph using MC58 Δ*zur* as the common reference (Figure 5). The analysis revealed that sixteen genes were up-regulated, whereas three genes were down-regulated in MC58 grown at low zinc compared to high zinc condition. We checked all genes of this dataset for operon structures by using RNAseq data (Joseph et al, unpublished) and the DOOR database (Mao et al, 2009). For confirmation of microarray analysis, I performed qRT-PCR for all single genes and each first gene of predicted operon structures. This confirmed all genes except of two to be differentially expressed (Table 11).

The regulon verified in this study comprised 15 genes up-regulated and only two down-regulated in MC58 grown at low zinc compared to high zinc (Table 11). Nine genes were organised in four transcriptional units: *nmb0317-nmb0316* coding for a 7-cyano-7-deazaguanine reductase and an integral membrane protein, respectively; *nmb0817-nmb0818*, encoding hypothetical proteins that belong to the DUF723 family and may have a role in DNA-binding (UniProt-Consortium, 2012); *nmb0942-nmb0941*, coding for paralogues of 50S ribosomal proteins; and *nmb0588-nmb0587*, together with one single gene, *nmb0586*, encoding the components of the putative ABC transporter for zinc, ZnuCBA (Stork et al, 2010). Proteins encoded by the single genes *nmb0819* and *nmb0820* contain putative DNA-binding helix-turn-helix motifs. The highly differential expression of *znuD* (*nmb0964)*, the TonB-dependent outer membrane receptor involved in zinc uptake (Stork et al, 2010), represents a positive control for our analysis as *znuD* was used for optimisation of our microarray conditions as mentioned above.

Table 11. Differentially expressed genes in *N. meninigitidis* MC58 observed by comparison of low to high zinc conditions.

Locus	Gene	Predicted function	Predicted localization	Size [bp]	Differential gene expression Array[1]	qRT-PCR[2]
NMB0546	*adhP*	Alcohol dehydrogenase, propanol-preferring	Cytoplasmic	1047	**-4.1**	-21.7
NMB0577	-	NosR-related protein	Unknown	351	**-1.8**	-1.8
NMB0588	*znuC*	ABC transporter, ATP-binding protein	Cytoplasmic	756	**1.4**	4.4
NMB0586	*znuA*	Putative ABC transporter substrate-binding protein	Cytoplasmic Membrane	915	**1.5**	6.3
NMB0820	-	Hypothetical protein	Unknown	198	**1.6**	1.9
NMB1497	-	Putative TonB-dependent receptor	Outer Membrane	2766	**1.6**	1.8
NMB0818	-	Hypothetical protein	Unknown	411	**1.9**	-
NMB0817	-	Hypothetical protein	Unknown	384	**2.0**	3.1
NMB0587	*znuB*	Putative ABC transporter permease protein	Cytoplasmic Membrane	876	**2.1**	3.3
NMB0942	*rpmE2*	50S ribosomal protein L31 type B	Cytoplasmic	276	**2.2**	41.6
NMB0819	-	Hypothetical protein	Unknown	393	**2.3**	1.8
NMB0525	*queC*	7-cyano-7-deazaguanine synthase	Cytoplasmic	660	**2.5**	3.9
NMB0941	*rpmJ*	50S ribosomal protein L36	Cytoplasmic	126	**2.7**	-
NMB0317	*queF*	NADPH-dependent 7-cyano-7-deazaguanine reductase	Cytoplasmic	474	**3.4**	5.8
NMB1475	-	Conserved hypothetical periplasmic protein	Periplasmatic	807	**3.6**	96.4
NMB0964	*znuD*	TonB-dependent receptor	Outer Membrane	2277	**4.4**	51.1
NMB0316	-	Conserved hypothetical integral membrane protein	Cytoplasmic Membrane	687	**4.6**	-

Gene information were retrieved from the online databases NeMeSys (Rusniok et al, 2009) and Uniprot (UniProt-Consortium, 2012). Genes are in order of their differential expression in microarray analysis; [1] Fold change values obtained by cDNA microarray hybridisation; [2] Relative quantification (RQ) values obtained by qRT-PCR. Table taken from (Pawlik et al, 2012).

Expression of another putative TonB-dependent receptor of yet unknown function, *nmb1497*, was also increased at low zinc. Furthermore, expression of *nmb0525* (*queC*) and *nmb1475*, encoding a zinc-binding 7-cyano-7-deazaguanine synthase and a conserved hypothetical periplasmic protein with similarities to the acetate kinase AckA of *Bacillus spp.*, respectively, were strongly increased at low zinc. Only two genes showed enhanced expression at low zinc: *nmb0546 (adhP)*, encoding a zinc-containing alcohol dehydrogenase, and *nmb0577* that shows similarities to the *H. influenzae pfkA* coding for a 6-phosphofructokinase.

6.2.3 *In silico* prediction of promoter organisation and the Zur binding motif

Stork et al (2010) previously postulated Zur binding to a palindromic sequence upstream of *znuD*. This sequence resembles the *in silico* predicted Zur binding motif for β-proteobacteria of RegPrecise database (Novichkov et al, 2010). Based on this motif, I scanned the promoter regions of all confirmed zinc-responsive genes for a conserved Zur binding motif. I found homologies in the upstream regions of all regulated transcription units, i.e. genes or operons. We therefore assumed that we at least partially elucidated the Zur regulon with our approach, and that zinc treatment of the bacteria did not deregulate genes other than Zur-controlled ones.

The consensus motif deduced from all putative Zur-binding motifs found was graphically visualised by WebLogo 3.2 (Figure 7A). The meningococcal Zur binding motif (Zur box) consists of 23 bp and has a central palindromic part that comprises hexameric inverse repeats separated from each other by three nucleotides. This part is present in the predicted motif for *znuD* published by Stork et al (2010).

I conducted an alignment of all putative Zur boxes found. The output indicated that the strength of gene regulation by Zur was dependent on the precision and length of the palindrome. If nucleotides on both sides of the central three nucleotides formed pairs leading to a true palindrome, relative expression differences of the respective gene were considerably higher in qRT-PCR than if less nucleotide pairs were present (Figure 7B). This observation was confirmed when mismatches in the palindromic part of each Zur box were counted in comparison to the perfect palindrome of the *znuD* Zur box: Occurrence of at least one mismatch in the palindrome of the putative Zur box reduced the extent of zinc-dependent gene regulation (Figure 7C).

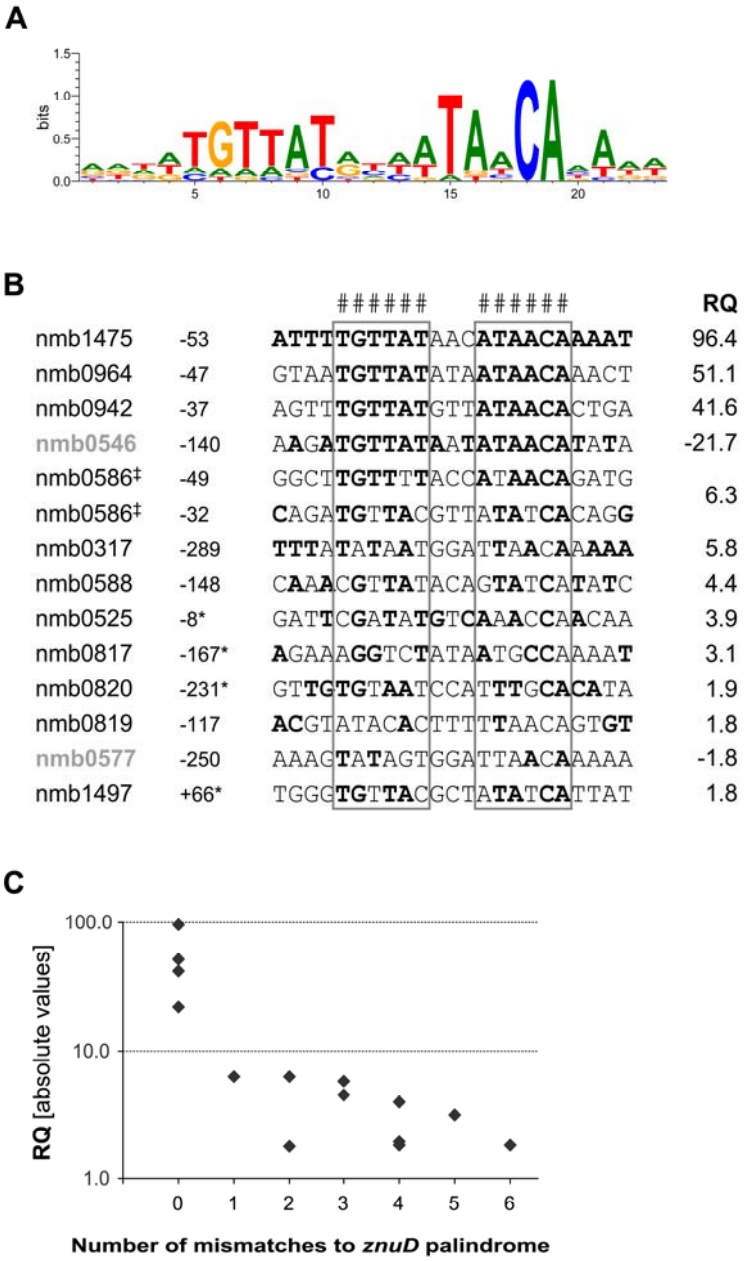

Figure 7. Prediction of the putative meningococcal Zur box. A) Graphical display generated with WebLogo 3.2 of the meningococcal Zur box based on consensus sequences of predicted Zur binding sites for all zinc-responsive genes. **B)** Nucleotide sequence alignment of putative Zur boxes upstream of zinc-regulated genes. For each gene, distances of the Zur motif to the start codon of translation and expression differences determined by qRT-PCR (RQ; low to high zinc) are provided. Motifs are ordered by RQ values. The two genes upregulated by Zur are marked in grey, bold letter. The highly conserved palindrome is marked (#) and boxed for all motifs. Bold nucleotides highlight complementary nucleotide pairs between both sides of each motif. Symbols indicate: * alternative start for the gene possible; ‡ two differing putative Zur motifs were found in the same promoter region. **C)** Comparison of gene expression (absolute RQ) with the number of mismatches in the palindrome sequence of each Zur box with reference to the *znuD* (*nmb0964*) palindrome. Gene expression changes were significantly higher in genes with a perfect Zur box compared to genes with at least one mismatch in the Zur box. In modification from Pawlik et al, (2012).

Differential gene expression was calculated from the absolute $\Delta\Delta c_T$ values obtained by qRT-PCR. It was significantly higher in genes with a perfect Zur box compared to genes with imperfect Zur boxes having at least one mismatch (Wilcoxon rank sum test with continuity correction, $p<0.01$). Some genes showed a palindromic sequence with extended length, e.g. *nmb0317* or n*mb0588*, which might partly compensate for mismatches in the central palindrome. The longest palindrome with a decameric inverse repeat was found for *nmb1475*. This probably represented the perfect Zur box as this gene showed the highest repression of gene expression upon zinc exposure (RQ=96.4). Interestingly, we detected two putative Zur boxes upstream of *nmb0586*. However, the reason for that remains unclear as this duplication did not seem to affect the strength of gene expression.

Promoter predictions were carried out using two software tools available online: NNPP (Reese, 2001), applying a minimum promoter score of 0.8, and BPROM [http://linux1.softberry.com]. I then analysed the position of the Zur boxes relative to the predicted promoter sequences which was surprisingly flexible (Figure 8).

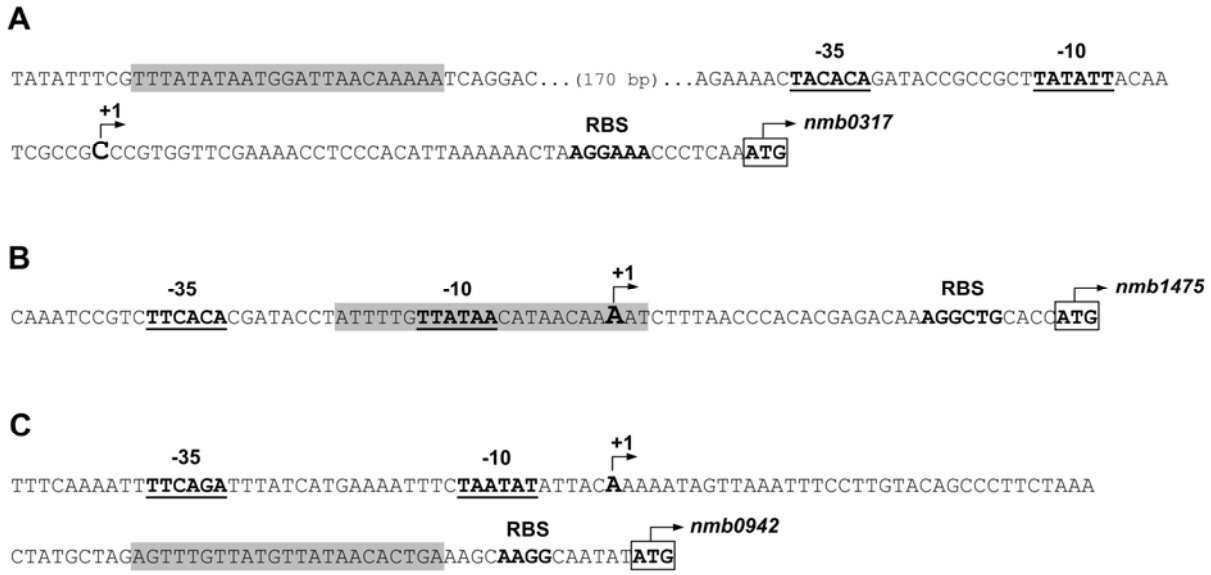

Figure 8. Promoter organisation of representative Zur-regulated genes. Predicted transcription start sites (+1) are given in bold letter with larger font; underlined, bold nucleotides show the predicted -10 and -35 regions. Putative ribosomal binding sites (RBS) are indicated in bold letter; the start codons are boxed and name the referring genes. Zur boxes are shaded in grey. **A) Zur box situated upstream** of the -10/-35 region. **B) Zur box overlapping** the -10/-35 region. **C) Zur box located downstream** of the -10/-35 region.

Zur boxes were either located upstream of the promoter (Figure 8A; i.e. *nmb0317* and *nmb0819*), or directly overlapping the -10/-35 elements (Figure 8B; i.e. *nmb0546*, *nmb0586*, *nmb0588*, *nmb0964* and *nmb1475)*, or downstream of the promoter (Figure 8C; i.e. *nmb0525*, *nmb0577*, *nmb0817*, *nmb0820*, *nmb0942* and *nmb1497*). Nevertheless, for most of the genes the Zur box was either overlapping with or located downstream of the promoter region. The Zur boxes of three out of four genes with high RQ values were directly overlapping with the promoter elements. However, only three of the five genes with overlapping Zur box showed high RQ values. Therefore, due to the limited number of regulated genes, a statistical proven association of the Zur box location with the extent of regulation of the respective gene was not possible. Genetically engineered strains will be needed to address this question.

6.2.4 Correia elements in the vicinity of Zur-regulated genes

I found insertions of Correia elements (CEs) in the surrounding regions of seven Zur-regulated genes (Figure 9). These transposon-like repeat elements are found in numerous copies within the meningococcal genome (Liu et al, 2002). CEs comprise two terminal inverted repeats (TIR) that harbour promoter element-like sequences (-10, -35 and TATA box). Eight different subtypes of CEs were defined, built up of four types of TIRs, i.e. α, α', β and β' (Siddique et al, 2011). Insertions of the following CEs were found in the vicinity of the Zur-regulated genes: CEs of the α-α subtype were inserted upstream and downstream of the *nmb0316-317* operon, as well as upstream of *nmb1475* and *nmb1497*. CEs with an internal deletion, also comprising the IHF-binding site, were noticed downstream of *nmb0586* (α-α‘ subtype), as well as upstream and downstream of *nmb0577* and *nmb0941* (α-β' subtypes), respectively (Figure 9).

However, I could not identify a pattern of CE insertion as they occur in low as well as highly differential expressed genes upon comparison of low to high zinc conditions; in Zur-repressed genes as well as one Zur-activated gene; and upstream as well as downstream of the Zur motif and the promoter elements or between those elements.

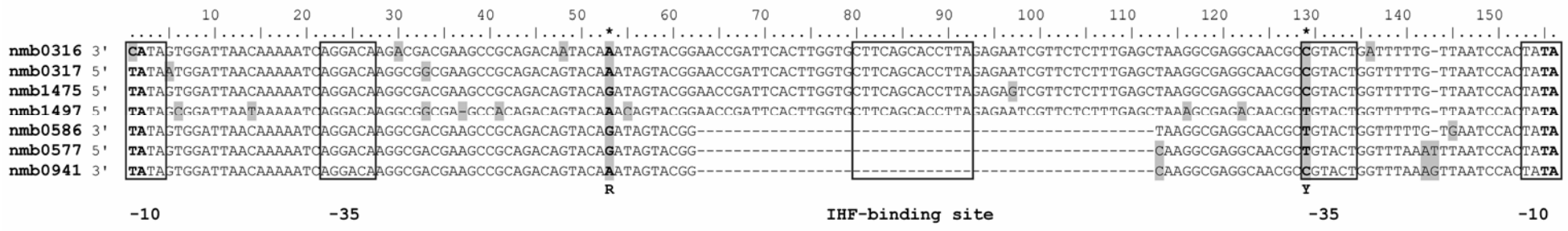

Figure 9. Nucleotide sequence alignment of Correia elements inserted upstream or downstream of seven Zur-regulated genes. Randomly scattered point mutations are shaded in grey, dashes indicate gaps. The two sites of polymorphic consensus nucleotides (R-53 and Y-129) are marked with asterisks; boxes indicate predicted –10 and –35 boxes within the Terminal inverted repeats (TIR) and the position of the IHF binding site [according to Siddique et al (2011)].

6.2.5 Verification of Zur binding to predicted DNA motifs by EMSA

In a next step, I analysed whether Zur binds to the *in silico* predicted DNA motifs. For that purpose, I aimed to perform EMSAs with a HIS-tagged recombinant Zur protein and synthetic dsDNA fragments. To yield recombinantly expressed Zur protein, the *zur* gene of MC58 was cloned into the expression vector pQE-60 (5.2.6.2), and the protein was overexpressed in *E. coli* and purified (5.3.8). Purified protein was analysed by SDS-PAGE. Only one distinct band was seen at approximately 17.5 kDa which verified protein purity (Figure 10).

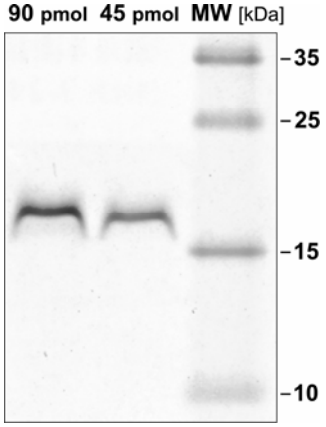

Figure 10. Coomassie-stained 15% SDS-PAGE of the purified HIS-tagged Zur protein. The 17.5 kDa protein (NMB1266) of *N. meningitidis* MC58 was recombinantly expressed in *E. coli* M15 [pREP4] and purified using HIS nickel sepharose columns. Lane 1 and 2: ~90 pmol (1.5 µg) and ~45 pmol (750 ng) of Zur protein, respectively; MW: molecular weight ladder.

Three of the Zur-regulated genes were selected for generation of short digoxygenin-labelled dsDNA fragments containing their Zur box (5.3.10). These labelled dsDNA fragments were incubated with the recombinant Zur protein and EMSAs were conducted (5.3.10).

The 34 bp dsDNA fragment comprising the *nmb0964* Zur box showed a mobility shift upon incubation with Zur. This shift was competed by 125-fold excess of the unlabelled *nmb0964* dsDNA fragment (Figure 11A). Thus, the binding of Zur to the Zur box seems to be specific.

I could only observe a band shift when 100 µM $ZnSO_4$ was added to the reactions, whereas other ions as $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnSO_4$ and $NiSO_4$ did not support binding of Zur to the dsDNA fragment. This indicates that zinc is specifically needed to mediate the Zur-DNA interaction (Figure 11B). The effect of zinc also could be blocked by addition of EDTA and TPEN due to chelation. That underlines the importance of zinc (Figure 11B).

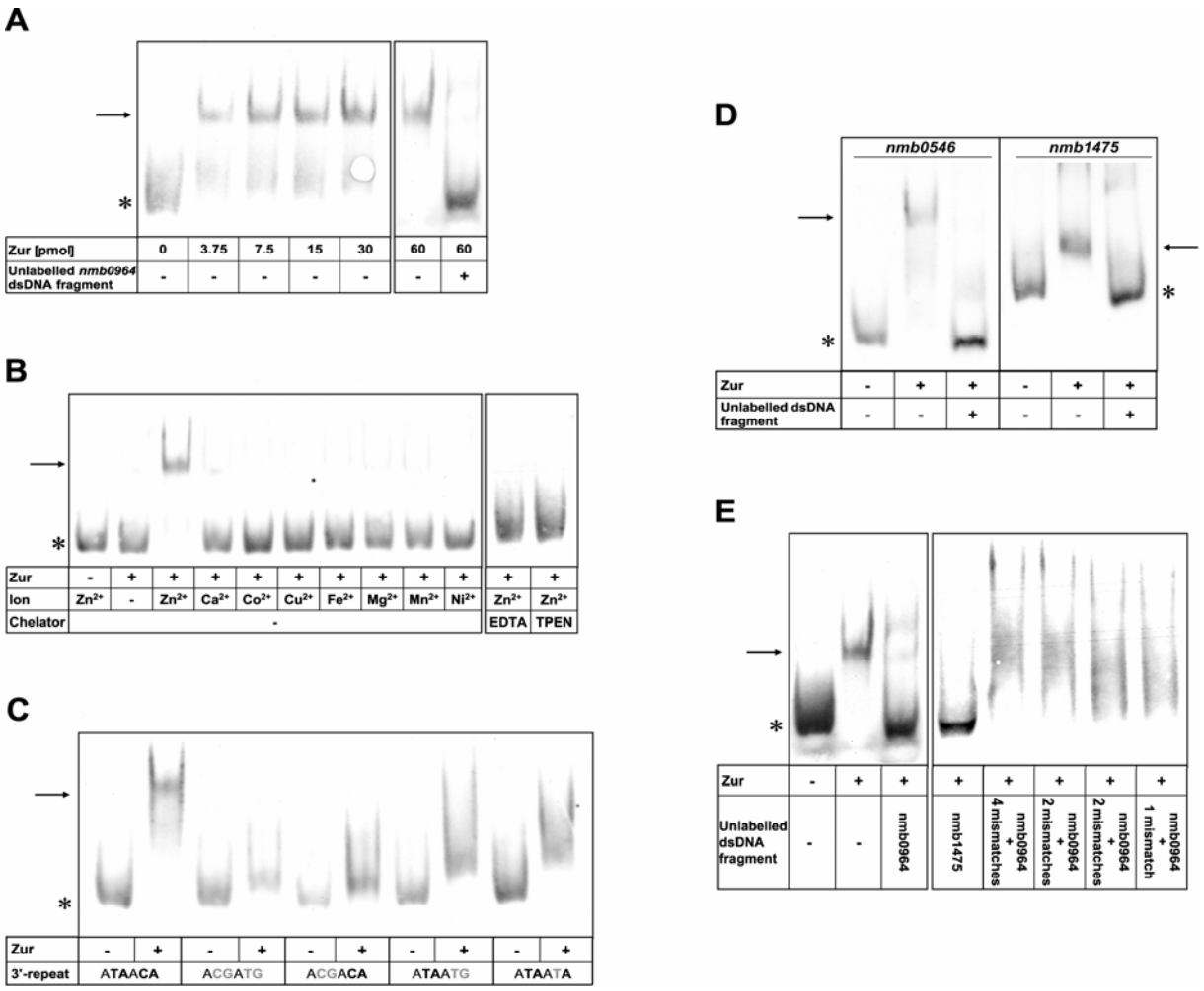

Figure 11. EMSAs of Zur binding to dsDNA fragments. Arrows indicate band shifts, asterisk unbound dsDNA fragments. **A) Zur binding to a dsDNA fragment comprising the Zur box of *nmb0964*.** Different amounts of Zur (3.75 pmol to 60 pmol) were added to the 34-mer dsDNA fragment, leading to band shifts. Addition of the unlabeled *nmb0964* dsDNA fragment at a 125-fold excess specifically competed the band shift. **B) Effect of divalent ions and chelators on Zur binding to the *nmb0964* dsDNA fragment.** Reactions were supplemented with 100 µM of different divalent ions. Zur binding to the *nmb0964* dsDNA fragment was only mediated by Zn^{2+}. Band shifts were abrogated by addition of 312.5 µM EDTA or TPEN. **C) Mutational analysis of the Zur box.** Band shift were inhibited by mutation of conserved nucleotides in the 3'-inverse repeat of the palindrome of the *nmb0964* Zur box. In bold the four highly conserved nucleotides are shown, in grey mutated nucleotides are highlighted. **D) Binding of Zur to dsDNA fragments containing the *nmb0546* and *nmb1475* Zur boxes.** Zur binding to the 34-mer *nmb0546* dsDNA and the 51-mer *nmb1475* dsDNA fragment comprising the respective Zur boxes resulted in band shifts. Addition of the respective unlabelled dsDNA fragment in 1000-fold excess competed the shifts. **E) Competition studies using the *nmb0964* dsDNA fragment.** Zur binding to the nmb0964 dsDNA fragment was competed by incubation with unlabelled *nmb0964* and *nmb1475* dsDNA fragments (125-fold excess). Addition of unlabelled mutated *nmb0964* dsDNA fragments harbouring mismatches within the palindrome (see Figure 11C) in 125-fold excess did not fully compete the shift. In modification from Pawlik et al (2012).

Mutations of either one, two or all four of the four most conserved nucleotides of the palindrome (ATAACA) were introduced in the 3'-inverse repeat of the palindrome of the nmb0964 dsDNA fragment. Zur binding was almost completely abrogated upon deletion of all four or the inner two nucleotides (Figure 11C). However, upon mutation of the marginal nucleotides or only a single nucleotide, interference with the band shifts was less clear. This indicates that i) all four conserved nucleotides of the palindrome are important for binding of Zur; ii) however, the inner nucleotides (TA) are more essential than marginal ones (CA); and iii) only one mutated nucleotide is not sufficient to abrogate the shift.

Binding of Zur to *in silico* predicted Zur boxes of two more genes was investigated. The first gene selected was *nmb0546*, one of the two genes activated in response to zinc. Incubation of Zur led to a clear band shift of the *nmb0546* dsDNA fragment which could be inhibited by addition of the unlabelled *nmb0546* dsDNA fragment. The second gene chosen was *nmb1475*, the one harbouring a Zur box with an extended perfect palindrome. I surprisingly could not observe any shift with a 34-mer dsDNA fragment (data not shown) as I did for the other two genes. I therefore extended the surrounding region by 8 and 9 bp on each side of the extended perfect palindrome, leading to a 51-mer dsDNA fragment. Upon incubation with Zur, this longer fragment then also showed a clear shift (Figure 11D). The Zur protein may probably need more flanking DNA sequence for binding to this perfect oversized palindrome that might stabilise the DNA structure.

As these results indicated that binding specifity mainly depends on the palindrome, I wanted to confirm this by competition experiments. EMSAs with the *nmb0964* dsDNA fragment and different unlabelled dsDNA fragments were carried out. The unlabelled *nmb0964* dsDNA fragment was used for a specific competition, whereas the *nmb1475* and the mutated *nmb0964* dsDNA fragments comprising mismatches within the 3'-inverse repeat palindrome (see above) were implemented for a more unspecific competition. EMSAs affirmed that the unlabelled *nmb0964* dsDNA fragment itself as well as the unlabelled *nmb1475* dsDNA fragment comprising the same palindrome abrogated the band shift. However, the mutated *nmb0964* dsDNA fragments were not completely able to compete the shift (Figure 11E). Hence, the palindrome seems to be of considerable importance for Zur binding.

6.3 Analysis of closely related strains differing in serum resistance

6.3.1 Selection and analysis of suitable strains

A second approach for identification of novel factors involved in meningococcal serum resistance during this thesis was the comparison of genetically closely related MenB strains that however differed in their resistance to serum complement attack.
Various strains from strain collections of the affiliated German reference laboratory for meningococci (NRZM) and a Bavarian carrier study (Claus et al, 2005) were reviewed. Of these, eight strains originating from different sources, i.e. from carriage or disease, were analysed in more detail. The fine type of each strain was determined by the NRZM using PCRs for PorA, PorB and FetA. In addition, the sequence type (ST) and clonal complex (cc) of each strain were identified using MLST (Maiden et al, 1998).
The polysaccharide capsule is the most important virulence factor of meningococci and protects the bacteria against complement-mediated killing and phagocytosis (Virji, 2009). To confirm the capsular serogroup of our chosen strains I performed an ELISA (5.3.6) with mAb735. All strains were meningococcal isolates of serogroup B (MenB).
Because meningococci show a large variety in LPS structure, an ELISA was performed to determine the LPS immunotype of the chosen ST-41/44 cc strains. Of the 12 different LPS immunotypes described, MenB strains display types L1-L9 (Mandrell & Zollinger, 1977; Zollinger & Mandrell, 1980). All strains expressed an LPS reactive with the L3,7,9 mAb or the L8 mAb. L3, 7 and 9 comprise a Lacto-N-neotetraose (LNT) moiety which can be sialylated (Tsai et al, 2002).
Meningococci circumvent the immune defence of their host by recruitment of negative regulators of complement like fH onto their surface (Schneider et al, 2007). Binding of fH by the factor H binding protein (fHBP) was shown to enhance serum resistance (Madico et al, 2006). Therefore, PCR and sequencing was conducted for *fhbp* (*nmb1870*) for all selected strains. All strains expressed *fHBP* of variant 1 and allele 14 or 371.
I also analysed the strains for possession of the *MDA*, a chromosomal islands that has been associated with strains causing disease (Bille et al, 2005). I furthermore determined conducted flow cytometry analysis (5.3.7) of MAC deposition on the strains' surface after serum exposure.

Three of these eight strains differed in only one of the fine type characteristics and belonged to the same clonal complex. Although closely related, they remarkably differed in their resistance to serum complement exposure. Therefore, they were finally chosen

for this study. Their sources and characteristics are listed in Table 12. The colouring in blue, green and red will be followed throughout this section to ease comparison of data.

Table 12. Closely related *N. meningitidis* strains used in this study.

Strain	Source	cc	ST	Fine type		LPS	fhbp	MDA
α16	Carriage	41/44	41	B.P1.7-2,4:	F1-5:PorB3-**301**	L3,7,9	1.14	+
α528	Carriage	41/44	41	B.P1.7-2,**15-39**:	F1-5:PorB3-1	L3,7,9	1.14	+
DE9686	Disease	41/44	**42**	B.P1.7-2,4:	F1-5:PorB3-1	L3,7,9	1.14	+

P1, PorA with its variable regions 1 and 2; **F**, FetA (single variable region); **ST**, multilocus sequence type; **cc**, clonal complex; **MDA**, meningococcal disease associated island. Different characteristics are marked in bold.

Strain DE9686 is a clinical isolate from the cerebrospinal fluid of a four year-old female from North Rhine-Westphalia that caused meningitis and sepsis. Strains α16 and α528 are non-invasive carrier isolates from a 19-year old male from a Bavarian military base and a 14-year old female from a Bavarian school, respectively.

The three strains are of sequence types (ST)-41 and -42 which differ in only one single nucleotide polymorphism (SNP) within the seven gene fragments analysed by MLST and belong to the same clonal complex, i.e. ST-41/44 cc (Table 12). The invasive isolate DE9686 did not show any MAC deposition on surface, whereas the carrier isolates α16 and α528 were considerably loaded with MAC (Figure 12).

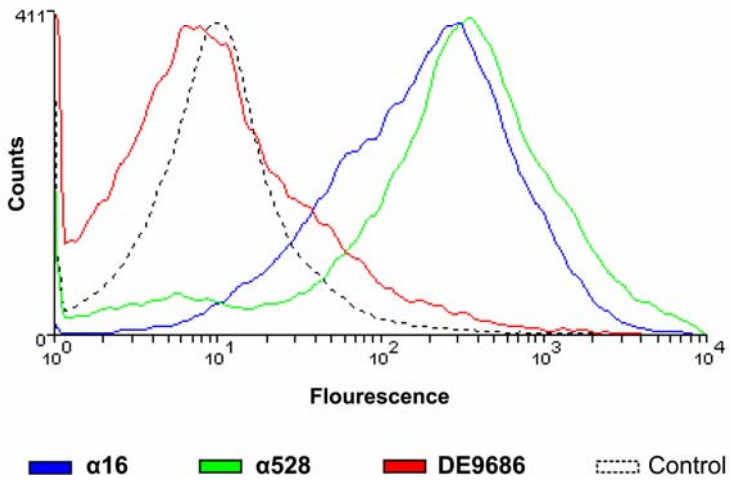

Figure 12. Flow cytometry analysis of complement deposition for the three selected strains. Incubation with mAb mαhC5b-9 after exposure to 20% NHS for 10 min. C5b-9 (MAC) deposition is clearly reduced on surface of the invasive isolate DE9686 in comparison to the carrier isolates α16 and α528. Graphical display of a representative experiment; control: merged curves of HIS controls for all strains.

6.3.2 Mutation of selected strains

To exclude factors that were already known to influence the resistance of meningococci to serum exposure, the respective genes for the capsule polysialyltransferase (*siaD*), the lipopolysaccharide sialyltransferase responsible for sialylation of LPS (*lst*), and fHBP (*fhbp*) were knocked out in the three strains using plasmids pMF32.35::T5, pGH7 and pMP1, respectively (4.4, 5.2.6.1).

All three mutations were genetically verified by sequencing and Southern blot, and those for capsule and LPS were also phenotypically confirmed: A capsule ELISA (5.3.6) with mAb735 verified unencapsulation of the mutants, and the *lst* mutation was confirmed by LPS gels (5.3.5). As shown for mutants of strain α528, the LPS band of a *lst* mutant runs below that one of the wild type strain, because the missing NeuNAc molecule leads to a LPS of reduced molecular weight (Figure 13). The gained triple mutants α16 Δ*siaD* Δ*lst* Δ*fhbp*, α528 Δ*siaD* Δ*lst* Δ*fhbp* and DE9686 Δ*siaD* Δ*lst* Δ*fhbp* were designated WUE4294, WUE4297 and WUE4300, respectively.

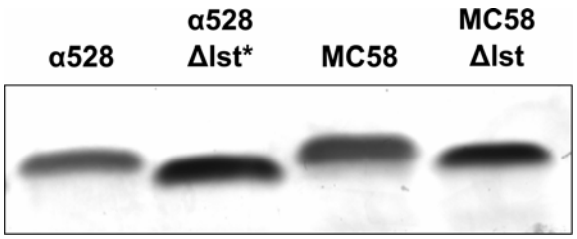

Figure 13. Tricine SDS-PAGE of LPS preparations. MC58 and MC58 Δ*lst* were applied for comparison. The LPS of α528 and the α528 triple mutant (*=Δ*fhbp* Δ*siaD* Δ*lst*) run at same molecular weight as LPS of MC58 and MC58 Δ*lst*, respectively.

6.3.3 Analysis of serum resistance in the mutants

These ST-41/44 mutants were then again compared in their resistance to serum exposure. Survival was measured in an SBA (5.1.5) with 5% NHS (Pooled human complement preserved serum, 4.2) at 10 min of incubation (Figure 14A). Approximately 94% of cells of the DE9686 triple mutant survived the serum stress (reduction from $\log_{10}$ 5.9 cfu/ml to $\log_{10}$ 5.5 cfu/ml). In comparison, triple mutants of the carrier isolates α16 and α528 were less serum resistant: Cell counts of α528 triple and a16 triple fell from initial $\log_{10}$ 5.9 cfu/ml to $\log_{10}$ 4.3 cfu/ml and $\log_{10}$ 2.6 cfu/ml, respectively.

Furthermore, MAC deposition on the bacterial surface after exposure with 20% serum for 10 min was measured using flow cytometry analysis (5.3.7) with an mαhC5b-9 monoclonal antibody (4.3). There was clearly less MAC deposition on surface of the invasive isolate than of the carrier isolates (Figure 14B).

These results suggested that the elevated serum resistance of the disease isolate DE9686 is not only dependent on the already described dominant factors, i.e. capsule, LPS sialylation and recruitment of fH.

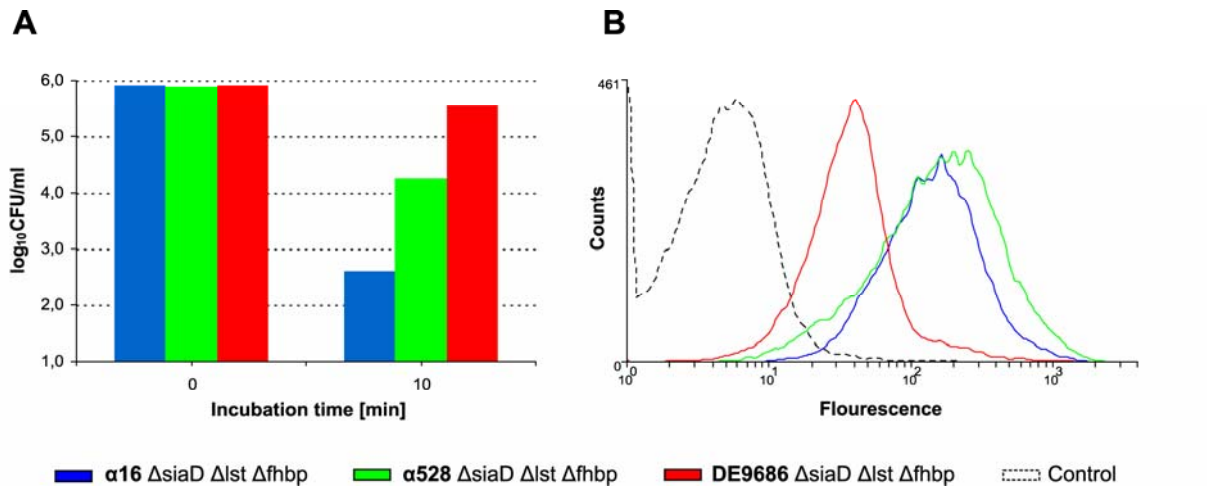

Figure 14. Comparison of serum response of the closely related strains. Triple mutants of the invasive isolate DE9686 and the carrier isolates α16 and α528 were exposed to serum complement. Experiments were repeated three times yielding comparable results. **A) Serum Killing Assay.** Survival after exposure to 5% NHS for 10 min. DE9686 showed enhanced serum resistance compared to α16 and α528. **B) Flow cytometry analysis.** Incubation with mAb mαhC5b-9 after exposure to 5% NHS for 10 min. Control: merged curves of HIS controls for all strains. C5b-9 deposition (MAC) is reduced in DE9686 compared to α16 and α528.

6.3.4 DNA microarray-based comparative genomic hybridisation (mCGH) of the three selected strains

To elucidate novel factors behind the differential serum resistance, I compared the gene content of the three ST-41/44 cc wild type strains by microarray analysis (5.2.10.2) using slides covering four meningococcal genomes, i.e. Z2491, MC58, FAM18, α14 (Schwarz et al, 2010). Labelled gDNA of α16 as well as α528 was separately hybridised against gDNA of DE9686. An intersection of the two data sets obtained yielded gene differences of both carrier strains compared to the disease isolate, i.e. genes present in α16 as well as α528, but absent in DE9686 and vice versa. Microarray results were verified by PCR and genome alignment with the DE9686 genome that was recently

sequenced in cooperation of our work group with Dag Harmsen using the Ion Torrent PGM sequencing technology of Life Technologies (Vogel et al, 2012b).

I did not encounter any gene that was exclusively present in the invasive isolate. In fact, the analysis confirmed that the invasive isolate DE9686 lacked six genes that were present in the genomes of the carrier isolates α16 and α528 (Table 13). Five out of these six genes encode proteins with unknown location and function according to the MC58 genome annotation in the NeMeSys database (Rusniok et al, 2009). None of the genes absent in DE9686 were predicted to be membrane-located.

Table 13. Genes present in α16 and α528, but absent in DE9686.

Locus	Predicted function	Location	Gene size [bp]	Protein size [aa]	Gene content differences[1] DE9686/ α16	DE9686/ α528
NMB0495	Replication initiation protein	Cytoplasm	984	327	**-16.6**	**-14.7**
NMB0500	Hypothetical protein	Unknown	378	125	**-11.7**	**-5.9**
NMB0501	Hypothetical protein	Unknown	375	124	**-24.4**	**-18.1**
NMB1043	Hypothetical protein	Unknown	207	68	**-5.8**	**-4.1**
NMB1755	Hypothetical protein	Unknown	345	114	**-47.4**	**-19.7**
NMB1756	Hypothetical protein	Unknown	195	64	**-200.3**	**-71.0**

[1] Fold change values obtained by gDNA microarray hybridisation; Genes belonging to the IHT-B and –C are shaded in light and dark grey, respectively.

Furthermore, all six genes genes seemed to be single transcripts according to RNA sequence data obtained by Dr. Biju Joseph (unpublished data, personal communication). According to the MC58 genome annotation, four of these six genes belong to *putative Islands of Horizontally Transferred DNA* [IHT] (Tettelin et al, 2000). NMB0500 and NMB0501 are within the IHT-B that contains hypothetical proteins. They share 36% protein identity with each other and have duplications in NMB1773 and NMB1774, respectively (Rusniok et al, 2009). None of the four protein sequences contains conserved domains. The NMB0500 amino acid sequence is 34% similar to *E. coli* AdiA, a biodegradative arginine decarboxylase with functions in the amino acid metabolism and the intracellular pH elevation in response to acids. NMB0501 has 37.5% similarity to *Lactococcus lactis* UvrC, a protein of the UvrABC system involved in repair of DNA lesions (UniProt-Consortium, 2012). NMB1755 and NMB1756 belong to the IHT-C which harbours homologues of toxins, putative virulence factors and mobile elements (Tettelin et al, 2000). Sequences of both proteins do not comprise any conserved domain. NMB1755 shows 37% similarity to *Pseudomonas aeruginosa* AccA, the carboxyl transferase of the Acetyl-coenzyme A carboxylase (ACC) complex involved in lipid and fatty acid

biosynthesis (UniProt-Consortium, 2012). NMB1756 does not show any similarity to proteins of bacteria other than Neisseria.
Two more genes were absent in the DE9686 genome, i.e. NMB0495 and NMB1043. In the Uniprot database, NMB0495 is annotated in MC58 as replication initiation protein and shows 27% protein similarity to *E. coli* RepE, a replication initiation protein for plasmid copy control. NMB1043 has 56.5% similarity to a probable ABC transporter permease of *Haemophilus influenzae*, and 42% similarity to the cell volume regulation protein A (CvrA) of *Salmonella spp.* that transports potassium ions over the inner membrane (UniProt-Consortium, 2012).

6.3.5 Further analysis of the strains within the Eranet consortium

As within the gene content of the three isolates I did not find any explanation for enhanced serum resistance of DE9686, within the Eranet consortium the three strains were furthermore compared using transcriptome and proteome analysis (unpublished). The transcriptome analysis particularly revealed a stronger expression of *nmb0865* in the more serum resistant isolate DE9686 compared to both carrier isolates (unpublished). Proteome analysis revealed enhanced protein levels of the fH-binding protein NspA (Lewis et al, 2010) in DE9686 compared to α16 and α528.
Another approach within the Eranet consortium was the screening of individual colonies for increased serum resistance (Hubert et al, 2012). The strain used for this study was a DE9686 mutant deficient in LPS sialylation and expression of capsule, fHBP, and MutS (a protein involved in missmatch repair). In the majority of serum resistant mutants, expression of the adhesin Opc (Virji et al, 1993) was increased. Differential expression was due to phase variation in the *opc* promoter (Hubert et al, 2012). The *opc* promoter sequence contains a variable number of cytidine residues that may cause slipped strand mispairing leading to incorrect translation which is associated with no, intermediate or strong protein expression (Sarkari et al, 1994).
Tracing back to the transcriptome analysis of the three ST41/44 strains conducted within the Eranet consortium, we noticed that Opc was more abundant on translational level in DE9686 compared to α16, but not to α528. Differential expression of Opc in the three strains was confirmed in Western Blot analysis (5.3.4) with whole cell extracts (data not shown).

We hence wanted to know which impact these three interesting candidates - Opc, NMB0865 and NspA – do have on the resistance to serum complement exposure.

6.3.6 Impact of Opc on serum resistance

As increased expression of Opc was the most important phenotype of serum resistant mutants within the screening approach, and the invasive isolate DE9686 showed enhanced Opc expression compared to the carrier isolates, it seemed likely that Opc expression is of advantage for meningococci to cope with serum complement exposure.
To test this hypothesis, I deleted *opc* in the DE9686 Δ*siaD* Δ*fhbp* background using pEWoc2a leading to strain WUE4712. The deletion was then complemented with pHC47 (*opc*$^{+}$) resulting in strain WUE4748. I analysed complement deposition on surface of the parental strains and both mutants by FACS after incubation with 20% NHS for 10 min (Figure 15).

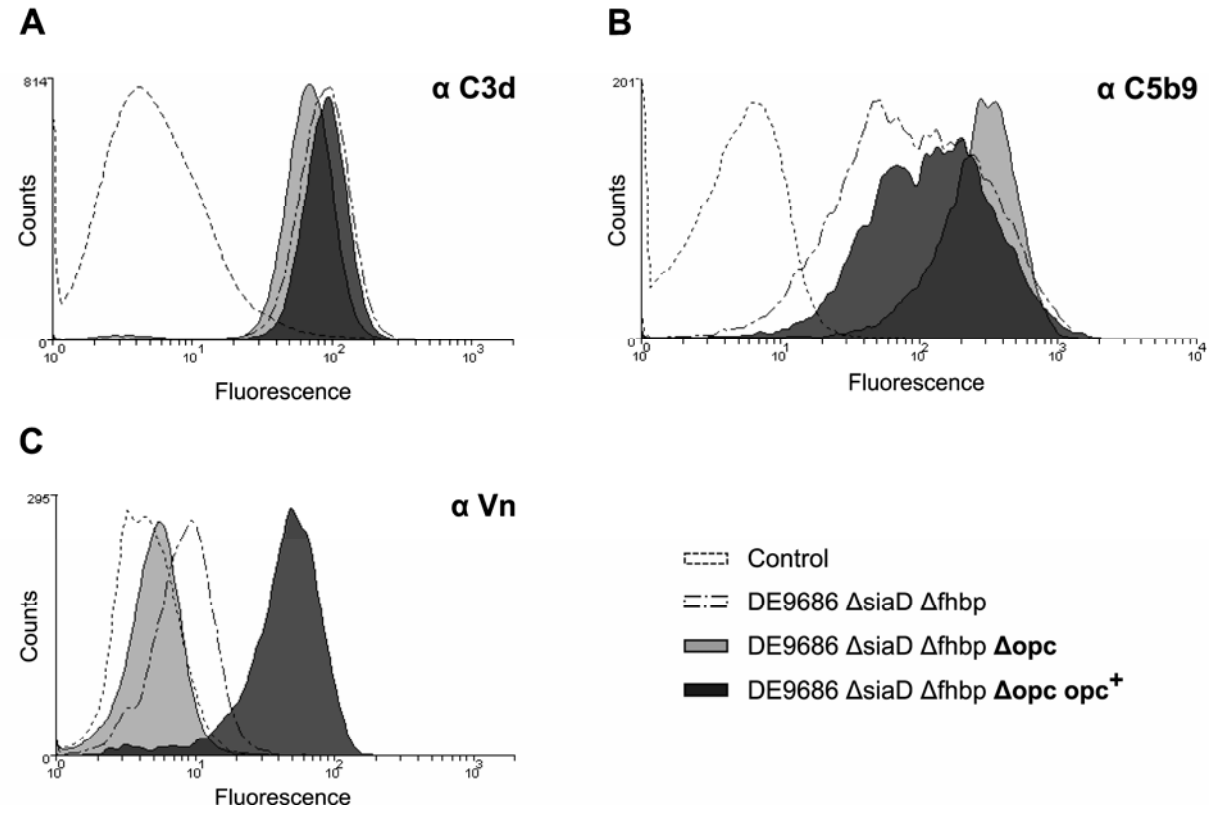

Figure 15. FACS analysis of isogenic *opc* mutants of DE9686 Δ*siaD* Δ*fhbp*. Control: merged HIS controls. The graphical displays are each based one representative result of three repeated experiments. **A) C3d deposition** is equal on the parental strain and the mutants after incubation with 20% NHS for 10 min and detection with pAb rαhC3d. **B) MAC deposition** on the *opc* deletion mutant is enhanced in comparison to the Opc-positive parental strain and the *opc* complemented strain DE9686 Δ*siaD* Δ*fHbp* Δ*opc opc*$^{+}$. **C) Vitronectin binding** is considerably enhanced in the complemented mutant compared to the parental strain and the Δ*opc* mutant. Vitronectin analysis was conducted by Dr. Hanna Jarva, Helsinki. In modification from Hubert et al (2012).

Deposition of C3d on the *opc* deletion mutant was comparable to the parental and the *opc*-complemented strain (Figure 15A). However, enhanced MAC deposition was noticed upon deletion of *opc*. This effect could be restored by complementation (Figure 15B). Thus, Opc blocks MAC insertion.

It is moreover well known that Opc binds vitronectin to invade human brain endothelial cells (Sa et al, 2010), and that vitronectin is a negative regulator of the complement system interfering with membrane attack complex deposition (Milis et al, 1993). Therefore, our Finish Eranet Pathogenomics partners performed vitronectin binding assays using the same set of mutants. They showed that deletion of *opc* reduced vitronectin binding to the surface of the meningococcal strains. After complementation of *opc*, binding of vitronectin to the complemented mutant again increased and was even stronger than to the parental strain (Figure 15C). This confirmed that expression of Opc enhances vitronectin binding. Taken together, the data demonstrate that Opc interacts with vitronectin that blocks MAC insertion, which results in slightly reduced serum killing.

6.3.7 Contribution of NMB0865 and NspA to serum resistance

In a next step, our work group constructed deletion mutants of *nmb0865* and *nspA* in an acapsulated, fHBP-negative background of DE9686, designated WUE4747 and WUE4792, respectively. I conducted FACS analysis of MAC deposition with these mutants.

MAC deposition on the surface of the *nmb0865* knockout mutant in DE9686 background was slightly, but reproducibly enhanced compared to the parental strain, though only after exposure to a higher serum concentration of 20% NHS for 10 minutes (Figure 16, right side). This shifted the extent of complement deposition towards MAC deposition on surface of the α16 background strain. NMB0865 therefore seems to at least partially contribute to the enhanced serum resistance of DE9686 compared to the carrier strains. Unfortunately, during the course of this thesis the work group did not succeed to recombinantly express NMB0865 in meningococci.

MAC deposition on surface of the *nspA* knockout mutant was strongly enhanced compared to the parental strain DE9686 Δ*siaD* Δ*fhbp*. Interestingly, MAC deposition on the surface of the DE9686 Δ*siaD* Δ*fhbp* Δ*nspA* mutant was comparable to strain α16 Δ*siaD* Δ*fhbp* (Figure 16, left side). This suggests that the level of NspA expression indeed is the major factor leading to differed serum resistance between the three closely related strains. Studies are ongoing in the work group to analyse mechanisms and impact of the findings.

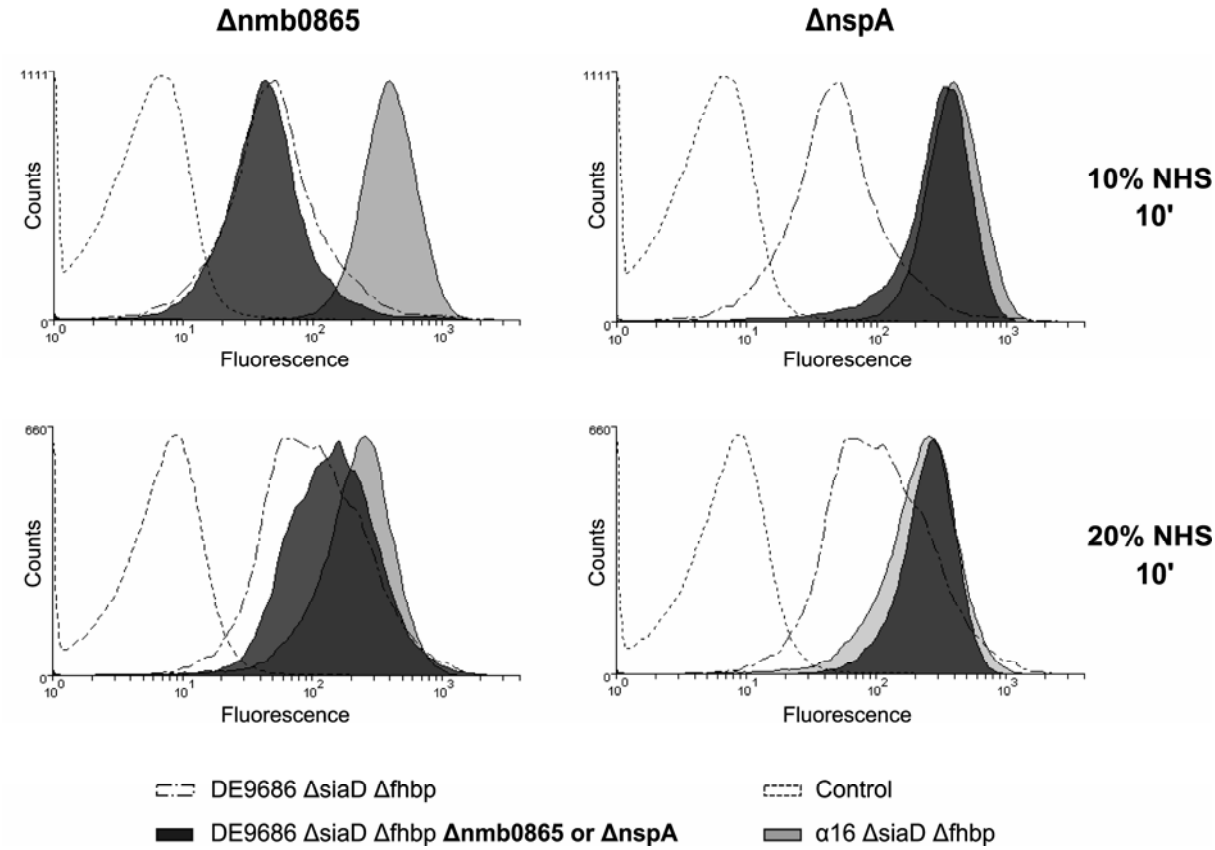

Figure 16. FACS analysis of MAC deposition of *nmb0865* and *nspA* deletion mutants. Incubation with mAb mαhC5b-9 after exposure to 10% or 20% NHS for 10 min. Control: merged curves of HIS controls for all strains. Graphical displays are representative results out of three repetitions. After incubation with 20% NHS, MAC deposition is slightly enhanced on surface of DE9686 Δ*nmb0865* compared to the parental strain. MAC deposition on surface of DE9686 Δ*nspA* is clearly enhanced compared to the parental strain and equals MAC deposition on α16 Δ*siaD* Δ*fhbp* Δ*nspA*.

7 DISCUSSION

Neisseria meningitidis is a major cause of bacteraemia and meningitis worldwide (Tan et al, 2010). Learning more about meningococci and their pathogenicity is therefore of high importance to counter devastating IMD. One characteristic of meningococci is their resistance to serum exposure and complement-mediated killing. *N. meningitidis* developed effective mechanisms to circumvent the host immune system which contributes to the fulminant course of disease and high-level bacteraemia (Schneider et al, 2007; Vogel & Frosch, 1999). Researchers therefore aim to identify virulence factors mediating meningococcal serum resistance. Using a high-throughput screening, Sun et al. generated mutants by STM and analysed those with reduced survival in an infant rat model. They showed deficiencies in genes encoding the capsule, LPS or iron acquisition molecules (Sun et al, 2000). The importance of capsule and LPS for meningococcal serum resistance was also confirmed by another STM study screening for resistance to complement-mediated lysis (Geoffroy et al, 2003). Furthermore, the impact of meningococcal adhesins has been highlighted in several studies. Among those are integral outer membrane proteins like PorA (Jarva et al, 2005; Orihuela et al, 2009) and Opc (de Vries et al, 1998; Sa et al, 2010). The recruitment of negative regulators of complement by specific outer membrane proteins, especially fHBP and NspA, is another way to mediate to serum resistance (Lewis et al, 2010; Madico et al, 2006). Although complement regulator binding proteins have been exploited for vaccine development (Johnson et al, 2012; Pajon et al, 2012), the expected number of outer membrane proteins without assigned functions initiated the search for further candidates. Minor OMPs may be attractive from a vaccine point of view, as they are not immunodominant and potentially less variable. An intriguing work of Poolman and colleagues studied the effect of minor OMPs (e.g. NspA, Msf) on eliciting bactericidal antibodies in a mouse model. Efficient complement-mediated killing was seen if at least two different minor but well-conserved OMPs were overexpressed in a PorA-deficient mutant. Adding further OMPs led to even higher bactericidal titres. The group developed the hypothesis that the more OMPs are targeted the closer are bactericidal antibodies on the bacterial surface, which is necessary to allow the first step of the classical pathway of complement cascade (Weynants et al, 2007).

We therefore developed two hypotheses that were investigated within this work:
i) Mutation of major global regulators of gene expression might have an impact on the composition of the outer membrane and bacterial surface structures and thereby affect serum resistance of meningococci;

ii) There are further OMPs not yet related to serum resistance which ought to be identified by genomic approaches.

7.1 Impact of global regulators of gene expression on serum resistance

Upon infection, meningococci have to face the host immune defences which include the action of antimicrobial proteins, phagocytes, and the complement system (Gasparini et al, 2012; Lo et al, 2009) and face changing environmental conditions. In the human body, bacteria have to cope with microaerophilic conditions and oxidative stress due to aerobic respiration and interaction with phagocytes (Seib et al, 2004), as well as limitation of essential transition metals like iron, zinc, manganese (Kehl-Fie & Skaar, 2010). At the beginning of this thesis I therefore experimentally applied a variety of conditions, and aimed for selection of clones with enhanced serum resistance. There were two lines of thought behind these approaches: Firstly, application of certain environmental conditions, for example growth under microaerophilic conditions or hydrogen peroxide stress, might change gene expression and leads to altered expression of proteins that may be of advantage for coping with serum stress. Secondly, analogue to bacteria becoming resistant to antimicrobial agents by mutation and selection (Tenover, 2006), I tried to select for meningococci carrying natural mutations that are of advantage for survival. I applied repeated passage in serum over hours, days and even weeks; alternatively, I treated meningococci with very high serum concentrations up to 80% NHS. However, using these approaches I did not gain clones with enhanced serum resistance. A possible explanation might be that resistance to serum is due to interplay of multiple factors and that enhanced resistance of a single clone due to one or more mutation events would likely stay unnoticed within a bacterial culture.

Since global regulators of gene expression respond to a variety of environmental conditions and influence the transcription on a range of genes, they are ideal targets to create a differential bacterial surface structure. To date, in meningococci only few global regulators, i.e. ten transcriptional regulators, three alternative sigma factors and one functional TCS, have been described (3.7). According to literature, few of these might have the potential to either directly or indirectly influence the outer membrane structure (3.7, Table **1**). We analysed the influence of overexpression of the fumarate and nitrate reduction regulator Fnr, the major stress regulator RpoH and the RNA chaperon Hfq on deposition of complement on cell surface. All three mutations did not alter complement deposition on the meningococcal surface, which was also true for deletion of the

transcriptional regulator AsnC or the small regulatory RNA NrrF. However, the Zur knockout showed a considerable decrease in deposition of complement components C3 and MAC. We hypothesised that elimination of the global regulator Zur leads to remodelling of the bacterial surface which affects the interaction with components of the complement system.

Zur was described as the regulator of zinc uptake in different bacterial species, such as *E. coli*, *B. subtilis* and *M. tuberculosis* (Hantke, 2001). Tommassen and colleagues identified the meningococcal homologue to the *E. coli zur* gene, i.e. *nmb1266;* as well as homologues of the putative *znuCBA* operon encoding a zinc uptake transporter, i.e. *nmb0588-86* (Stork et al, 2010). The group analysed in detail the meningococcal TonB-dependent outer membrane receptor ZnuD, encoded by *nmb0964*, which is expressed under zinc limitation and involved in zinc uptake. They showed that deletion of *zur* resulted in enhanced expression of ZnuD (Stork et al, 2010). If increased expression of ZnuD was the reason for reduced complement deposition on surface of the *zur* knockout strain, we postulated that overexpression of *znuD* would also lead to reduced deposition. However, deposition of C3 and MAC on meningococcal mutants overexpressing *znuD* was comparable to the wild type strain. Furthermore, the other way around, deletion of *znuD* did not lead to enhanced complement deposition as we would have expected if ZnuD was the Zur-dependent key player influencing serum resistance.

In summary, of six global regulators analysed, only Zur influenced serum resistance of the α16 mutant strain. The TonB-dependent outer membrane receptor ZnuD was not the key component mediating enhanced serum resistance of the zur deletion mutant. Neither there was information available about further genes whose transcription is regulated by Zur, nor had the Zinc uptake regulator of meningococci been studied in more detail. We therefore aimed to elucidate the meningococcal response to zinc by transcriptome analysis.

7.2 The zinc-responsive regulon of *N. meningitidis*

By analysing the transcriptional response of meningococci upon exposure to human serum and whole blood, microarray studies identified various differentially expressed genes encoding components that are potentially involved in serum resistance (Echenique-Rivera et al, 2011; Kurz et al, 2003). As described above, we discovered that deletion of the meningococcal regulator Zur let to reduced complement deposition on the bacterial surface. In order to elucidate the factors behind, we wanted to know more about the meningococcal Zur and the genes under its control, i.e. the Zur regulon. In the

past, Zur regulons of a variety of bacteria have been analysed, but not the meningococcal one. Anyway, the meningococcal ferric uptake regulator Fur which is closely related to Zur has been studied in detail using transcriptome microarrays. Genco and colleagues analysed the Fur regulon, comprising iron-activated and –repressed Fur-dependent genes, by comparison of gene expression upon iron-repletion and –depletion. They also identified a Fur binding motif (Grifantini et al, 2003). Three years later, Scarlato and co-workers used a transcriptional analysis comparing the response to different iron levels in wild type, *fur* knockout and *fur* complemented mutant to define the meningococcal Fur modulon (Delany et al, 2006).

In accordance with these studies, we comparably analysed the gene expression at low and high zinc condition using the MC58 wild type and its *zur* deletion mutant. In various studies of the iron response, iron was depleted for one condition using specific iron chelators (Delany et al, 2006; Grifantini et al, 2003; Shaik et al, 2007). In contrast, we deliberately abstained from using a zinc chelator such as TPEN to deplete zinc, because it could not be excluded that TPEN chelates ions other than zinc and interferes with the membrane integrity. Rather than zinc depletion, we therefore used a low zinc condition, yielded by meningococcal growth in RPMI medium which does not contain any source of zinc according to the manufacturer. Growth in RPMI promotes the expression of *znuD* indicative of Zur derepression.

Microarray studies elucidating the Zur regulon of bacteria such as *B. subtilis*, *Y. pestis*, *M. tuberculosis and C. glutamicum* directly compared a wild type strain with its *zur* knockout mutant at the same condition (Gaballa et al, 2002; Li et al, 2009; Maciag et al, 2007; Schröder et al, 2010). In contrast to that, in our common reference approach we also analysed the response to zinc by comparing the wild type strain at low and high zinc condition. The advantage is that pleiotropic effects of a constitutive *zur* knockout are most likely rejected.

The meningococcal Zur regulon we deduced from the transcriptome data and the consecutive Zur box analysis indeed is remarkably small. We identified 15 genes downregulated and two genes upregulated at high zinc using microarray analysis and qRT-PCR. A Zur binding motif (Zur box) was found for all transcriptional units. The entity of 17 regulated genes is comparable to the number found in microarray analyses of Zur regulons of other bacteria, i.e. 18 genes and 32 genes upregulated in a *zur* deletion mutant of *C. glutamicum* (Schröder et al, 2010) and *M. tuberculosis* (Maciag et al, 2007), respectively. The only exception is a comparative microarray analysis of a *Y. pestis* wild type strain and its *zur* deletion mutant, both grown at zinc-rich conditions, where 154 Zur-regulated genes were found (Li et al, 2009). However, only four of those harboured

a Zur box, which suggests that regulation of most genes reflects a general transcriptional response rather than a direct result of Zur binding to the promoters.

Two of the 17 genes regulated upon exposure to zinc were upregulated, i.e. *nmb0546* and *nmb0577*. Gene activation by Zur has only been reported in two other studies: A microarray analysis of the *B. subtilis* Zur regulon identified two genes upregulated in the wild type compared to its *zur* deletion mutant. However, Zur motifs could not be detected in the genes' regulatory regions, so that upregulation presumably was due to indirect effects of the altered zinc homeostasis (Gaballa et al, 2002). This can be excluded in our study as we identified Zur boxes in the upstream regions of both Zur-activated genes. Only in one other study of the phytopathogen *Xanthomonas campestris*, Zur acted as a repressor of zinc-uptake systems, and also directly activated expression of one gene encoding a zinc efflux pump by binding to two distinct DNA motifs different from each other (Huang et al, 2008). Yet, in our study, the same Zur boxes were detected upstream of upregulated and downregulated genes. Furthermore, Zur binding to the *nmb0546* motif was demonstrated by EMSA**.**

Nevertheless, the ferric uptake regulator Fur has been described to activate gene expression in meningococci, either indirectly by repression of sRNAs, i.e. NrrF (Mellin et al, 2007) or directly by binding to the promoter of regulated genes, i.e. *norB* and *nspA* (Delany et al, 2004; Shaik et al, 2007). However, it has been proposed that the position of the Fur box relative to the promoter may be crucial to determine if a gene is repressed or activated upon Fur binding, i.e. Fur activates and represses transcription if the binding motif is upstream of and overlapping with the promoter, respectively (Delany et al, 2004).

7.2.1 Analysis of the promoter region of zinc-regulated genes

Based on the prediction of the Zur binding motif for *znuD* by Stork et al (2010), I analysed the promoter regions of all genes that were found to be zinc-regulated in our study and identified the binding motif for meningococcal Zur with the palindromic sequence TGTTATDNHATAACA. This sequence is identical to the Zur binding motif proposed for *znuD* (Stork et al, 2010) and consistent with the palindrome in the bioinformatically predicted motif for the related γ-proteobacteria (Panina et al, 2003). An ideal palindrome sequence of the Zur box as shown above only was found in the promoter regions of *nmb0546*, *nmb0942*, *znuD* and *nmb1475*. These genes also showed the highest expression differences between low and high zinc condition. Zur boxes in the promoter regions of the other regulated genes constitute variations of the ideal palindrome with a varying number of mismatches when compared to the ideal palindrome. The number of mismatches was inversely correlated with the extent of gene

regulation. Mutations introduced into the palindrome led to abrogation of the full shift in EMSAs which has also been shown for the Zur binding motif of *C. glutamicum* (Schröder et al, 2010). We hypothesise that mismatches in the palindrome part of the Zur binding motifs may lead to reduced binding strength of Zur to the DNA. Nevertheless, at some circumstances the surrounding region of the Zur binding motif might be important for binding of Zur to DNA as only the 51-mer *nmb1475* dsDNA fragment led to a shift in EMSA. However, this seemed to be an exception of the rule.

Zur boxes of most of the meningococcal Zur-regulated genes were found to be either directly overlapping or downstream of the -10/-35 region which might interfere with the RNA polymerase (Madan Babu & Teichmann, 2003; Payankaulam et al, 2010). However, for two regulated genes, the motif was located upstream of the -10/-35 region. Therefore, the position of the Zur box relative to the promoter is more flexible in meningococci compared to other bacteria (Gaballa et al, 2002; Li et al, 2009; Maciag et al, 2007; Schröder et al, 2010; Shin et al, 2007). The Zur box location also did not determine whether a gene was repressed or activated. Local DNA topology, bending or supercoiling might be involved in activation of genes by high zinc and Zur binding, but detailed investigations were beyond the scope of this work.
The zinc-activated gene *nmb0546* was previously shown to be Hfq-repressed in *N. meningitidis* (Mellin et al, 2010; Pannekoek et al, 2009). The RNA chaperone Hfq stabilises small regulatory RNAs (sRNAs) and mediates their binding to their mRNA targets, which leads to subsequent repression of mRNA translation (Mellin et al, 2010). Therefore, regulation by a sRNA is feasible but needs to be investigated. Assuming that *nmb0546* and *nmb0577* were targets of a yet unknown sRNA regulated by zinc and Zur, comparable to the iron- and Fur-regulated sRNA NrrF (Mellin et al, 2007), one could hypothesize that activation of the genes occurred indirectly due to Zur-mediated repression of the putative sRNA that otherwise leads to degradation of the mRNAs.
Therefore, we postulate that meningococcal Zur, like Fur, can directly activate and repress gene expression by binding to the same DNA motif. However, the mechanism determining the mode of action remains to be discovered.

Insertions of Correia elements (CEs), transposon-like repeat elements, were observed in the vicinity of seven Zur-regulated genes, namely *nmb0316-317*, *nmb0577*, *nmb0586*, *nmb0941*, *nmb1475* and *nmb1497*. In the MC58 genome, 261 CEs have been identified representing approximately 2% of the genome (Liu et al, 2002; Siddique et al, 2011). Comparable numbers were found for MenA strain Z2491 and MenC strain FAm18 (Bentley et al, 2007; Schoen et al, 2009). Two of the eight defined CE subtypes were shown to encode strong promoters that may affect the expression of adjacent genes (Siddique et

al, 2011). All of the CEs found in proximity of Zur-regulated genes, harboured the left α-repeat that has been associated with especially high transcription levels (Siddique et al, 2011). The CE inserted upstream of *nmb1497* even contained both, the left α-repeat and the right α-repeat with Y-130-T, also found to strongly drive transcription (Siddique et al, 2011).

As CEs often are located close to genes involved in virulence, metabolism and transport, their abundance in Neisseria genomes may pose a significant role in the control of gene expression. Furthermore, conversion events occurring between different meningococcal strains during evolution led to subtype switch of CEs. This might represent a novel phase-variation mechanism as gene expression could be modulated depending on the strength of the encoded CE promoter (Liu et al, 2002; Siddique et al, 2011). However, Correia element insertion in the proximity of our Zur-regulated genes might rather be a coincidence, because it was not associated with gene expression.

7.2.2 Functions of zinc-regulated genes

The meningococcal genes *nmb0588-nmb0587-nmb0586,* previously identified as homologues to the *E. coli znuCBA* operon encoding an ABC transporter for high-affinity zinc uptake, and *nmb0964 (znuD)* coding for a TonB-dependent receptor mediating zinc uptake at low zinc concentration (Stork et al, 2010), were shown to be repressed at high zinc in our study. This finding is reminiscent to *E. coli* (Patzer & Hantke, 2000), *M. tuberculosis* (Maciag et al, 2007), *B. subtilis* (Gaballa et al, 2002), *C. glutamicum* (Schröder et al, 2010), *Y. pestis* (Li et al, 2009) and *Streptomyces coelicolor* (Shin et al, 2007).

In our study, *nmb1475* was most strongly regulated which encodes a conserved hypothetical periplasmic protein with 34% similarity to the acetate kinase AckA of *Bacillus spp.* This enzyme is involved in the conversion of acetate to acetyl-CoA (UniProt-Consortium, 2012) and its *E. coli* homologue was shown to bind zinc (Katayama et al, 2002). The protein furthermore harbours a conserved domain, similar to the CbiK (COG5266) domain of the periplasmic component of an ABC-type Co^{2+} transport system, as identified by NCBI Blast. Thus, NMB1475 may be involved in the uptake of zinc and/or other transition metals.

Zinc-uptake systems most likely are important for bacterial survival and pathogenicity during infection as access to zinc is limited within the human host (Hantke, 2001; Stork et al, 2010). Indeed, *nmb0586* (*znuA)* was shown to contribute to *in vivo* pathogenicity of *Salmonella enterica* (Ammendola et al, 2007). In addition, ZnuABC and ZnuD help *E. coli* (Gabbianelli et al, 2011) and *N. meningitidis* (Kumar et al, 2012), respectively, to adhere to epithelial cells. Interestingly, van Alen et al found expression of NMB0586 (in this study named MntC instead of ZnuA) and NMB1475 being increased in biofilms, and

showed that deletion of *nmb0586* reduces biofilm formation (van Alen et al, 2010). Extenuated biofilm formation also was previously seen upon deletion of *znuA* in gonococci (Lim et al, 2008) and *znuB* in *E. coli* (Gunasekera et al, 2009). A link between the availability of zinc and biofilm formation has also been reported for staphylococci where zinc chelation prevented biofilm formation because the intercellular adhesion is dependent on zinc-containing proteins (Conrady et al, 2008). A similar mechanism may apply for meningococci, resulting in increased expression of zinc-uptake proteins upon reduced zinc availability within the biofilm and extenuated biofilm formation upon zinc limitation.

Furthermore, *nmb1497* encoding a putative TonB-dependent receptor was identified to be Zur-regulated gene in a zinc-dependent manner in this study. It also was defined as one of 60 putative neisserial core pathogen-specific genes in a microarray study comparing *N. meningitidis* and *N. lactamica* strains (Dunning Hotopp et al, 2006). This protein might therefore contribute to pathogenicity of the meningococcus.

Two meningococcal genes repressed at high zinc and harbouring an upstream Zur box, i.e. *nmb0317* and *nmb0525*, are most likely involved in queuosine biosynthesis as determined by protein similarity analysis. *nmb0525* codes for QueC, a zinc-binding 7-cyano-7-deazaguanine synthase, and *nmb0317 en*codes QueF, a 7-cyano-7-deazaguanine reductase (UniProt-Consortium, 2012). Until now queuosine biosynthesis in bacteria is still not fully understood, but the pathways have been studied in *E. coli* and *B. subtilis* (Gaur & Varshney, 2005; Lee et al, 2007). Queuosine is a highly complex modified nucleoside which is incorporated at the wobble position of a subset of tRNAs (Durand et al, 2000). Such modification of tRNAs improves efficiency and correctness of translation (Durand & Bjork, 2003). Varshney and colleagues demonstrated that a natural defect in queuosine biosynthesis of *E. coli* leads to reduced fitness under nutrient limitation (Dineshkumar et al, 2002). Furthermore, lack of queuosine attenuated *Shigella flexneri* virulence (Durand & Bjork, 2003; Durand et al, 2000). Queuosine biosynthesis in meningococci seems to be considerably Zur-regulated since two enzymes of the biosynthesis are up-regulated at zinc-limiting conditions (Kehl-Fie & Skaar, 2010). The question arises if increased queuosine modification of tRNAs may be a result of zinc depletion and contributes to the elevated expression of virulence factors which could support fitness of *N. meningitidis* during infection.

Another interesting finding was that the expression of several genes encoding ribosomal proteins (r-proteins) was also Zur-repressed in *N. meningitidis*. This is in accordance with other bacteria such as *E. coli* (Graham et al, 2009; Panina et al, 2003), *Y. pestis* (Li et al,

2009), *M. tuberculosum* (Maciag et al, 2007), *S. coelicolor* (Shin et al, 2007) and *B. subtilis* (Akanuma et al, 2006). Several bacterial genomes encode two paralogous forms of r-proteins (Makarova et al, 2001). The first form (C+) contains a metal-binding Zn^{2+}-ribbon usually consisting of four conserved cysteines, whereas in the second form (C-) this zinc ribbon is degenerated (Sankaran et al, 2009). The paralogous pair of the L31 r-protein in *B. subtilis* is RpmE (C+) and YtiA (C-). YtiA (C-), whose expression is repressed by Zur, liberates RpmE (C+) from the ribosome upon lack of zinc (Akanuma et al, 2006; Li et al, 2009). Candidate binding sites for several zinc repressors have been identified upstream of genes coding for (C-) paralogues of the r-proteins L31, L33, L36 and S14 in a variety of bacterial species. Thereby, Gelfand and co-workers confirmed that upon existence of a (C+) and a (C-) copy of the r-protein the gene encoding the (C-) copy is regulated by a zinc-dependent repressor (Panina et al, 2003). The replacement of zinc-containing r-proteins by non-zinc containing paralogues upon zinc-depletion might liberate zinc for maintenance of zinc homeostasis and may enhance bacterial survival when facing zinc-restrictive conditions *in vivo* (Kehl-Fie & Skaar, 2010; Panina et al, 2003). Paralogous forms of r-proteins with different zinc content may have evolved because ribosomal assembly needs to be maintained at zinc-restrictive conditions in any case. In meningococci, the 50S ribosomal proteins L31 and L36 are encoded by paralogous gene pairs. Based on sequence similarity to *B. subtilis* RpmE and Yti, the paralogous meningococcal proteins are NMB1956 (C+) and NMB0942 (C-) for L31, as well as NMB0164 (C+) and NMB0941 (C-) for L36 (Makarova et al, 2001; Rusniok et al, 2009). In this study, we showed that the *nmb0942-nmb0941* (*rpmEJ*) operon is repressed at high zinc, and furthermore identified a Zur box upstream of *nmb0942*. We therefore presume that also in meningococci the r-protein without zinc ribbon (C-) takes over the function of the C+ protein at zinc-deficient condition.

7.2.3 *In vitro* binding of Zur to Zur boxes of regulated genes

The meningococcal Zur binding motif (Zur box) and its variants are described above. Using qRT-PCR it was previously shown that Zur possibly represses the genes *znuD* and *znuA* in meningococci (Stork et al, 2010). However, binding of meningococcal Zur to the Zur box has not been demonstrated before this study. In *E. coli* and *B. subtilis*, Zur has been shown to be active as a dimer, and in the dimeric complex each monomer binds two zinc ions (Ma et al, 2011; Patzer & Hantke, 2000). In case of *E. coli* Zur, dimerisation appeared to be zinc-independent, whereas DNA binding required presence of zinc (Patzer & Hantke, 2000). In this study, EMSAs proved that binding of meningococcal Zur to its Zur binding motif is specifically dependent on zinc and cannot be mediated by any of the other bivalent ions tested. Studies of other bacterial Zinc uptake regulators also directly demonstrated by EMSA that activity of Zur is dependent on the presence of zinc (Gaballa

& Helmann, 1998; Li et al, 2009; Maciag et al, 2007; Schröder et al, 2010; Shin et al, 2007), and for some bacteria it was shown that also manganese is able to mediate Zur interaction with the DNA (Gaballa & Helmann, 1998; Maciag et al, 2007; Schröder et al, 2010).

7.2.4 Comparison to other regulons

Recently, Serruto and colleagues analysed the transcriptomic response of *N. meningitidis* upon exposure to whole human blood. In their study, some of the genes were deregulated that had also altered expression upon zinc exposure in our study. The genes *nmb0964* and *nmb1497*, encoding TonB-dependent receptors, as well as *nmb0546*, coding for an alcohol dehydrogenase, were increasingly upregulated with the duration of meningococcal growth in human blood (Echenique-Rivera et al, 2011). This finding suggests that zinc depletion upon transfer of bacteria from liquid culture to whole blood is responsible for a part of the transcriptional changes observed. However, in our study in contrast to the whole blood study, *nmb0546* was downregulated at low zinc condition rather than upregulated, which might be due to further factors influencing gene expression in a more complex *in vivo* situation. Furthermore, the finding of Echenique-Rivera et al also reinforces that changes of zinc concentration mimic an important environmental signal encountered by bacteria during pathogen-host interaction.

Several of the genes that are Zur-regulated in this work, were also differentially regulated in a microarray study comparing transcriptional profiles of *N. meningitidis* grown with different host iron binding proteins, i.e. haemoglobin, transferrin and lactoferrin, as a sole iron source. Upon exposure to lactoferrin (compared to haemoglobin or transferrin) *nmb0941*, *nmb0942*, *nmb1475* and *nmb1497* were downregulated, and *nmb0546* was upregulated (Jordan & Saunders, 2009). Lactoferrin is present in secretions and on mucosal surfaces of the human host, and bacteria are able to acquire iron from the protein using a lactoferrin receptor (Morgenthau et al, 2012). A possible explanation for the seen overlap of the zinc and lactoferrin regulons is that lactoferrin also was reported to loosely bind zinc besides iron (Ainscough et al, 1980). It therefore might be an additional source of zinc for meningococci which then acts as a cofactor for Zur, resulting in Zur-mediated regulation of the genes mentioned above as it was seen in our study.

However, a second explanation for the lactoferrin-dependent regulation of zinc-regulated genes might be that upon lactoferrin exposure regulation of these genes is not only accomplished in zinc-dependent, but also in iron-dependent manner. This hypothesis is supported by expression of *znuD*. This gene recently was shown to be repressed by zinc but also induced by iron, because its promoter site not only contains a Zur box but also a

Fur box (Kumar et al, 2012). I confirmed *in vitro* binding of Zur to the *znuD* Zur box in this study [also see Pawlik et al (2012)], whereas *in vitro* Fur binding to its Fur box was shown by Kumar et al (2012). However, we neither detected a Fur box upstream of *nmb0942-nmb0941*, *nmb1475*, *nmb1497*, and *nmb0546*, nor has *znuD* been regulated upon lactoferrin exposure in the work of Jordan & Saunders (2009).
Nevertheless, another study again supports the hypothesis of a link between iron-dependent regulation by Fur and zinc-dependent regulation by Zur: Genco and colleagues analysed the transcriptional response to iron in strain MC58 and identified Fur-regulated genes. Among these also were *nmb0316* and *nmb0317*, encoding a conserved hypothetical integral membrane protein and QueF, respectively, both involved in queuosine biosynthesis. They predicted a Fur box in the *nmb0317* promoter region and showed *in vitro* Fur binding to it in an EMSA (Grifantini et al, 2003), whereas we determined zinc-dependent regulation of the *nmb0316-nmb0317* operon in our transcriptome analysis and identified a Zur binding motif upstream of *nmb0317*. In line with hypotheses mentioned previously, lack of various trace metals might be sensed by meningococci as an indication for the host environment and may lead to coordinated action of the different respective transcriptional regulators resulting in elevated expression of virulence factors that support meningococcal survival. Increased modification of tRNAs with queuosine may be one factor contributing to this.

7.2.5 Zur regulon and serum resistance

Initially we found Zur to be connected with increased serum resistance since a *zur* deletion mutant of the carriage strain α16 showed reduced complement deposition on surface in comparison to its parental strain. We decided to analyse the Zur regulon in the MC58 rather than the α16 background since the α16 genome was neither represented on the available microarray nor was a genome sequence available of this strain. Of the 17 genes found to be regulated by Zur in MC58, none was previously described to be involved in serum resistance. Our first target protein was the TonB-dependent receptor ZnuD (NMB0964), which we however determined to not be responsible for the effect of *zur* deletion on serum resistance using the same serum source. Nevertheless, the second TonB-dependent receptor regulated by Zur, NMB1497, might be a promising target for future studies. Since lack of zinc in the human host poses a challenge for bacteria during infection (Hantke, 2001), it would furthermore be worth to study the influence of the ABC transporter ZnuCBA on serum resistance. Moreover, NMB1475 may be a promising candidate as it was most strongly regulated in this study and may also be involved in zinc uptake.

Future studies should analyse the outer membrane location of members of the Zur regulon, their binding of bactericidal antibodies and the individual contribution to serum resistance.

7.3 Analysis of genetically closely related strains with differing serum resistance

In another approach during this dissertation, we identified closely related meningococcal strains that differed in serum resistance. In collaboration with the research group, these strains were analysed by a variety of genomic methods and a screening approach.
The three selected strains belonged to the same clonal complex (ST-41/44 cc). The invasive isolate DE9686 showed enhanced resistance to complement exposure and reduced complement deposition of surface in comparison to the carrier isolates α16 and α528. Gene content comparison of the three strains using microarrays and PCR led to a total of only six genes that are present in the carrier isolates, but absent in the invasive isolate. One of these genes, *nmb0495*, encodes a replication initiation protein that might be involved in plasmid copy control due to 27% protein similarity to *E. coli* RepE. Plasmids generally are not essential for bacterial survival and rather represent an advantage in the competition with other microorganisms (Actis et al, 1999). However, plasmid copies have to be regulated as plasmids probably constitute a slight metabolic burden for their host. Therefore, a possible explanation for sorting out NMB0495 may be that another protein accomplishes the same function rendering the protein redundant. Candidates may be NMB1076, NMB1543 or NMB1634 that are all annotated to be involved in DNA replication initiation in MC58 (UniProt-Consortium, 2012). Alternatively, DE9686 may have evolved towards a protein-independent mechanism of plasmid copy control, i.e. achieved only by action of an antisense RNA (del Solar & Espinosa, 2000). A second gene, *nmb1043*, shows similarity to genes coding for membrane transport proteins, e.g. 56.5% similarity to a probable ABC transporter permease of *Haemophilus influenzae*. Therefore, *nmb1043* might be an OMP that is a target for complement attack and absence of the gene in DE9686 may have been evolved to evade complement attack.

Three of the remaining four genes absent in DE9686 show similarities to proteins of different bacterial species that have functions in metabolism and DNA repair. The first gene, *nmb0500* codes for a protein with 34% similarity to *E. coli* AdiA, a biodegradative arginine decarboxylase with functions in the amino acid and glutamate metabolism and the intracellular pH elevation in response to acids. The second protein encoded by *nmb1755* shows 37% similarity to *Pseudomonas aeruginosa* AccA, the carboxyl transferase of the Acetyl-coenzyme A carboxylase (ACC) complex involved in lipid and

fatty acid biosynthesis. The data suggest that the metabolom of the isolates should be analysed, also with respect to a possible association with resistance to serum complement. It has for example been shown that lactate supports meningococcal colonisation and enhances resistance to complement-mediated killing (Exley et al, 2005a; Exley et al, 2005b). DE9686 may therefore have evolved to focus on specific metabolic pathways that are important for pathogenicity.
The third gene, *nmb0501*, encodes a protein with 37.5% similarity to the *Lactococcus lactis* UvrC, a member of the UvrABC system. Regulated by SOS response, the UvrABC proteins recognise and cleave damaged DNA, one mechanism of DNA repair that is known as Nucleotide Excision Repair (NER) (Truglio et al, 2006). However, in MC58 *nmb1326* is annotated to encode UvrC (UniProt-Consortium, 2012). Therefore, NMB0501 might be a related protein contributing to DNA repair but may have been sorted out in DE9686 in order to dispose unnecessary additional genes.

All four proteins are hypotheticals belonging to the IHT-B or IHT-C. This is concordant with a study in which a comparison of a carrier strain of ST-41/44 cc and the invasive isolate MC58 (ST-32 cc) showed considerable variation in IHT-B and IHT–C (Joseph et al, 2010). IHTs most likely have been obtained by horizontal gene transfer between species. The IHT-B and IHT-C encode numerous hypothetical proteins that may also be putative virulence factors (Tettelin et al, 2000). Both IHTs are variable in their presence within diverse mCGH groups which suggests that the islands have been gained and lost multiple times, probably due to the recombinant nature and transformability of *Neisseria spp.* (Dunning Hotopp et al, 2006; Feil et al, 2001).

We therefore hypothesise that the invasive isolate DE9686 displays signs of reductive microevolutionary events. Microevolution is crucial for the pathogenesis of infectious diseases. In contrast to long-term processes termed macroevolution that lead to development of new species, microevolution describes a short-term development happening within days or weeks leading to new variants of a (sub)species. Both processes are important for the development of pathogenic microorganisms and are conditioned by horizontal transfer of mobile genetic elements. Plasmids, bacteriophages and pathogenicity islands strongly contribute to microevolution. Moreover, insertion sequences and transposons play an important role, which is also supported by acquisition of point mutations and genetic rearrangements as well as phase variation and antigenic variation, especially of OMPs or surface structures such as pili (Morschhauser et al, 2000). Meningococci are naturally highly competent to take up DNA, and it has been reported that microevolution occurs frequently in *N. meningitidis*, leading to different variants which may disappear again within months or years. Especially microevolution of

serogroup A meningococci was highlighted since clonally related isolates originating from a common ancestor diversified at numerous loci within the last decades (Achtman, 1998; Morelli et al, 1997). Furthermore, Vogel et al reported diverseness of two invasive MenC strains isolated from a patient compared to carrier isolates isolated from healthy contact persons and reference strains of the same clonal complex (Vogel et al, 1998). This case study illustrates within-host microevolution of a specific strain during infection without further spread of this variant. It also shows the increased invasive potential of certain strains due to genetic adaptation to the host.

Our study emphasises within-host microevolution in serogroup B meningococci. The three closely related isolates α16, α528 and DE9686 are most probably descendants from a common ancestor. Yet, in contrast to the carrier strains, the invasive isolate DE9686 seems to have adapted to the human host during passages. Due to gene duplications or dispensability of certain genes within the human host, these genes may not have been necessary anymore and only represented 'ballast' for successful invasion. This might have led to genomic reduction over generations as identified in our analysis.

We did not identify genes that are exclusively present in the invasive isolate, but absent in both carrier isolates. Therefore, enhanced serum resistance of strain DE9686 cannot be explained by exclusive possession of a certain gene encoding a virulence factor that mediates serum resistance, e.g. an additional regulator or an OMP like fHBP. We cannot rule out that the hypothetical genes may encode OMPs as *in silico* predictions of protein locations are unknown. However, this scenario seems to be less likely since for meningococci the benefits of OMP expression are possibly higher than the benefits of non-expression. Furthermore, importance of genetic islands for serum resistance is questionable as presented in work of Tinsley and colleagues. They analysed eight large genetic islands that are present in *N. meningitidis* but absent in *N. gonorrhoeae* regarding their impact on virulence *in vitro* and *in vivo*. Deletion of these regions did not result in an alteration of the bacterial resistance to serum (Klee et al, 2000).

However, for gene content analysis we used microarrays that covered the genomes of four meningococcal strains but neither the genome of α16 nor α528 or DE9686. Therefore, DE9686 could possess genes that are not covered by the microarray used and that may be absent in the carrier strains. This question could be addressed bioinformatically by performing an *in silico* hybridisation of DE9686 with MC58, which now would be possible since the DE9686 genome was sequenced in cooperation with Dag Harmsen (Vogel et al, 2012b). Furthermore, non-coding small regulatory RNAs (sRNAs) could influence gene expression and modulate protein activity by binding to mRNAs and proteins, respectively (Storz et al, 2011). Due to their small size, however, they cannot

be detected in the genomes of the strains analysed using a mCGH or transcriptome microarray.

By employing a screening approach, the work group identified upregulation of Opc as a mechanism of serum resistance (Hubert et al, 2012). Furthermore, enhanced expression of Opc was also detected in the invasive isolate DE9686.
In the early 1990s, Opc was recognised as a virulence factor involved in adhesion, colonisation and invasion of epithelial and endothelial cells (Sarkari et al, 1994; Virji et al, 1993; Virji et al, 1992). Recently, Virji and co-workers identified that Opc binds to the extracellular matrix protein and serum factor vitronectin and uses them to anchor to integrin receptors of human brain endothelial cells (Sa et al, 2010). Vitronectin in turn is known as a negative regulator of the terminal pathway of complement system by blocking assembly and insertion of the MAC and hindering pore formation (Milis et al, 1993).
This study is the first to demonstrate the link between expression of the adhesin Opc and meningococcal serum resistance: Deletion of *opc* in DE9686 background had a slight impact on serum resistance. Serum exposure led to enhanced MAC deposition on the *opc* knockout strain, whereas deposition of C3 was comparable. Using the same set of mutants, our Finnish project partners identified considerably increased binding of vitronectin in the *opc* complemented mutant in DE9686 background which overexpressed Opc. We therefore summarise that Opc binds the negative complement regulator vitronectin which reduces MAC deposition on the bacterial surface which contributes to serum resistance of meningococci (Hubert et al, 2012). Therefore, binding of vitronectin by Opc does not only conduce to the interaction with integrin receptors to attach to endothelial cells, but also to escape complement attack.

Moreover, another target, i.e. NspA, was identified by a comparative proteome analysis of the three ST41/44 strains. Expression of NspA was increased in DE9686 compared to α16 and α528. Deletion of *nspA* in an acapsulated, LPS sialylation-deficient DE9686 strain resulted in considerably enhanced deposition of MAC on the bacterial surface, comparable to complement deposition levels of the acapsulated, LPS sialylation-deficient α16. This is consistent with findings of Ram and colleagues who identified NspA as a second protein binding fH, a negative regulator of the complement system (Lewis et al, 2010). fH blocks the amplification loop of the AP of complement system by hindering the formation of the C3 convertase and acting as a cofactor for the C3b-inactivating fI (Ferreira et al, 2010). Deletion of *nspA* leads to increase of C3b deposition, resulting in a boost of the AP, leading to enhanced MAC deposition, as seen after serum treatment of our mutants, and complement-dependent killing (Lewis et al, 2010).

Since NspA levels in the invasive isolate DE9686 were higher than in both carrier isolates, we assume that NspA is also most likely the major factor conferring serum resistance in our set of strains that lacked capsule, LPS sialylation and fHBP. The view of NspA as a mediator of serum resistance is supported by a recent study of Kroll and colleagues in which *nspA* was upregulated after meningococcal growth in human blood for four hours (Hedman et al, 2012). Another study showed that meningococcal deletion mutants lacking expression of NspA, Fur or the transferrin-binding protein TbpB are sensitive to killing in human blood (Echenique-Rivera et al, 2011).
NspA is widely distributed among meningococcal strains of different serogroups and elicits a bactericidal response. It was therefore suggested as a meningococcal vaccine candidate (Martin et al, 1997). We are currently investigating mechanisms of gene regulation and general differences between carriage and IMD isolates.

Comparative transcriptome analysis of the three strains especially highlighted an increased expression of *nmb0865*, coding for a hypothetical integral membrane protein, in the invasive isolate DE9686 compared to α16 and α528. We could show within this study that NMB0865 at least to some extend contributes to serum resistance of DE9686, as MAC deposition was increased in the *nmb0865* deletion mutant which shifted the extent of complement deposition towards levels seen in the α16 mutant (acapsulated and deficient in LPS sialylation). The protein does not contain any putative conserved domain, and only shows similarities to the CrcB protein family conferring camphor resistance. Further research on the mechanism of action of NMB0865 and its role in mediating serum resistance in meningococci is currently conducted within the work group.

In summary, we analysed a set of genetically closely related strains from carriage and disease. We aimed to identify virulence factors that mediate the enhanced serum resistance of the invasive isolate, even if dominant effects of capsule LPS sialylation and fHBP were excluded. A gene content analysis highlighted signs of reductive microevolution in the invasive isolate DE9686 that probably represents better adaptation to the human host. By means of transcriptomic and proteomic analyses, we identified three proteins that influence serum complement deposition and thus serum resistance of DE9686, i.e. NMB0865, Opc and NspA.
The data support the view that a large number of proteins contribute to serum resistance of meningococci. Which repertoire is used under a given situation and whether different lineages utilise different components to achieve the same goal needs to be studied in detail.

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9 ANNEX

9.1 Abbreviations

AP	alternative pathway of complement system
APS	ammonium persulfate
β-ME	beta-mercaptoethanol
bp	base pairs
BSA	bovine serum albumine
C4BP	complement component 4 binding protein
CEACAM	carcino-embryonic antigen-related cell adhesion molecule
cc	clonal complex
cDNA	copy DNA, obtained by transcription of RNA
CE	Correia element
cfu	colony forming units
CP	classical pathway of complement system
DIG	digoxygenine
dH_2O	distilled water
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
dsDNA	double-stranded DNA
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
et al	*et altera* (and others)
fH	factor H
fHBP	factor H binding protein
fI	factor I
FNR	Fumarate and nitrate reduction regulator
gDNA	genomic DNA (chromosomal DNA)
HBSS	Hank's balanced salt solution
HIS	heat-inactivated serum
IHF	integration host factor
IHT	island of horizontally transferred DNA
IMD	invasive meningococcal disease
kb	kilo base pairs
kDa	kilo Dalton
KDO	3-deoxy-2-keto-D-manno-octulosonic acid
LNT	lacto-N-neotetraose

LP	lectin pathway of complement system
LPS	lipopolysaccharide
M	molar (mol/litre)
mAb	monoclonal antibody
MAC	membrane attack complex
Men[A]	meningococci [of serogroup A]
MLST	multilocus sequence typing
Msf	meningococcal surface fibril
N-CAM	neural cell adhesion molecule
NeuNAc	(α2-8)-N-acetylneuraminic acid
NHS	normal human serum; herein: Dunn Lab pooled human complement serum
N.m.	Neisseria meningitidis
NrrF	neisserial regulatory RNA responsive to iron
NspA	neisserial surface protein A
OD_{600}	optical density measured at an absorption of 600 nm
o/n	over night
OMV	outer membrane vesicle
Opa/Opc	opacity protein A/C
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PorA/B	porin A/B
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
rpm	rounds per minute
RpoH	RNA polymerase sigma factor
rRNA	ribosomal RNA
RT	room temperature
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sg	serogroup
SDS	sodium-dodecyl-sulfate
SNP	single nucleotide polymorphism
SSC	saline-sodium-citrate buffer
SSPE	saline-sodium-phosphate-EDTA buffer
ST	sequence type
STM	signature tagged mutagenesis
TEMED	tetramethylethylenediamine
TIR	terminal inverted repeat
TPEN	N,N,N',N'-*tetrakis*-(2-pyridyl-methyl)-ethylenediamine
Tris	trishydroxymethylaminomethane
U	units

9.2 Publications

Parts of this work have been published in:

Pawlik MC, Hubert K, Joseph B, Claus H, Schoen C, Vogel U. The zinc-responsive regulon of *Neisseria meningitidis* comprises 17 genes under control of a Zur element. J Bacteriol. 2012. 194(23):6594-6603.

Hubert K, Pawlik MC, Claus H, Jarva H, Meri S, Vogel U. Opc expression, LPS immunotype switch and pilin conversion contribute to serum resistance of unencapsulated meningococci. PLoS ONE. 2012. 7(9): e45132.

During my doctoral research I also contributed to the following publications:

Claus H, Jördens MS, Kriz P, Musilek M, Jarva H, Pawlik MC, Meri S, Vogel U. Capsule null locus meningococci: typing of antigens used in an investigational multicomponent meningococcus serogroup B vaccine. Vaccine. 2012. 5;30(2):155-60.

Parts of this work have been presented at the following international conferences:

09/2012 XVIIIth International Pathogenic Neisseria Conference (*IPNC*) – Würzburg, Germany

Poster The Zinc-responsive Regulon of Neisseria meningitidis Comprises 17 Genes under Control of a Zur element. Pawlik MC, Hubert K, Joseph B, Claus H, Schoen C, Vogel U.

10/2011 6th International Student Symposium of the Graduate School of Life Sciences - "BioBang: the Xpanding Space of Life Science" - Würzburg, Germany

Poster Studies on meningococcal gene regulation by the Zinc uptake regulator Zur. Pawlik MC, Hubert K, Vogel U.

05/2011 1st Mol Micro Meeting - Würzburg, Germany

Poster Establishment of genomic approaches to unravel meningococcal serum resistance factors. Pawlik MC, Hubert K, Claus H, Vogel U.

04/2011 FEMS-Leopoldina Symposium on "Emerging Topics in Microbial Pathogenesis" - Würzburg, Germany

Poster Establishment of genomic approaches to unravel meningococcal serum resistance factors. Pawlik MC, Hubert K, Claus H, Vogel U.

04/2011 Annual Conference of the Association for General and Applied Microbiology (VAAM) - Karlsruhe, Germany

Poster Establishment of genomic approaches to unravel meningococcal serum resistance factors. Pawlik MC, Hubert K, Claus H, Vogel U.

Parts of this work have been presented at the following international meetings:

11/2011 3rd Meeting of the CoMeVac Consortium - Frankfurt/Main, Germany

Talk Studies on meningococcal gene regulation by the Zinc uptake regulator Zur. Pawlik MC, Hubert K, Vogel U.

11/2010 2nd Meeting of the CoMeVac Consortium - Würzburg, Germany

Talk Analysis of the adaptive response of meningococci to serum complement exposure. Pawlik MC, Hubert K, Claus H, Vogel U.

02/2010 1st Meeting of the CoMeVac Consortium - Paris, France

Talk Characterization and mutation of ST-32 cc and ST-41/44 cc strains from France and Germany. Pawlik MC, Hubert K, Claus H, Vogel U.