Functional and molecular characterization of FarR – a transcriptional regulator of the MarR family in *Neisseria meningitidis*

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Stephanie Schielke

aus Lörrach

Würzburg, 2010

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Thomas Dandekar

Gutachter : Prof. Dr. Oliver Kurzai

Gutachter: Prof. Dr. Roy Gross

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig angefertigt und nur die angegebenen Hilfsmittel verwendet habe. Von mir verwendete Quellen sind als solche gekennzeichnet. Diese Arbeit hat bisher in gleicher oder ähnlicher Form keinem anderen Prüfungsverfahren vorgelegen. Am 15.12.2006 hat mir die Universität Bonn den akademischen Grad der Diplom-Biologin verliehen. Weitere akademische Grade habe ich weder erworben noch versucht zu erwerben.

Würzburg, den 26.02.2010

_____

Stephanie Schielke

Publications

Parts of this work have been published in the following journals:

Schielke, S., Huebner, C., Spatz, C., Nägele, V., Ackermann, N., Frosch, M., Kurzai, O. and Schubert-Unkmeir, A. **(2009)** Expression of the meningococcal adhesin NadA is controlled by a transcriptional regulator of the MarR family. *Mol Microbiol* **72**: 1054-1067.

Schielke, S., Schmitt, C., Spatz, C., Frosch, M., Schubert-Unkmeir, A. and Kurzai, O. **(2010)** Lipopoly-saccharide composition but not FarR regulated expression of *farAB* is responsible for fatty acid resistance of *Neisseria meningitidis*. *In preparation.*

Schielke, S., Spatz, C., Schwarz, R., Joseph, B., Schoen, C., Schulz, S.M., Hubert, K., Frosch, M., Schubert-Unkmeir, A. and Kurzai, O. **(2010a)** Characterization of FarR as a highly specialized growth phase dependent, serum responsive transcriptional regulator in *Neisseria meningitidis. In preparation.*

Schielke, S., Frosch, M. and Kurzai, O. **(2010b)** Invited Review Article: Virulence determinants involved in differential host niche adaptation of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Med Microbiol Immunol. Special issue on Pathogen Variability and Host Response in Infectious Disease*. *In preparation.*

Nägele, V., Heesemann, J., Schielke, S., Jimenez-Soto, L., Kurzai, O., Hauck, C. R. and Ackermann, N. **(2010)** *Neisseria meningitidis* adhesin NadA targets β1 integrins: a trimeric autotransporter with functional similarity to *Yersinia* invasin. *In preparation.*

Villwock, A., Schmitt, C., Schielke, S., Frosch, M., and Kurzai, O. **(2008)** Recognition via the class A scavenger receptor modulates cytokine secretion by human dendritic cells after contact with *Neisseria meningitidis*. *Microbes Infect* **10**: 1158-1165.

Conferences

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

09/2009 **61st Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)**
Göttingen, Germany

Talk Contribution of the transcriptional regulator FarR to niche adaptation in pathogenic *Neisseriae*
S. Schielke, M. Frosch, O. Kurzai and A. Schubert-Unkmeir

Poster FarR is a growth phase dependent transcriptional regulator in *Neisseria meningitidis*
C. Spatz, S. Schielke, M. Frosch, A. Schubert-Unkmeir and O. Kurzai

06/2009 **3rd Congress of European Microbiologists (FEMS 2009)**
Gothenburg, Sweden

Poster The role of the transcriptional regulator FarR in the closely related human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis*
S. Schielke, C. Hübner, C. Spatz, M. Frosch, O. Kurzai and A. Schubert-Unkmeir

04/2009 **4th Congress of the EuroPathoGenomics Graduate Academy**
Palma de Mallorca, Spain

Talk The conserved transcriptional regulator FarR orchestrates divergent functions in the closely related human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis*
S. Schielke, C. Hübner, C. Spatz, M. Frosch, O. Kurzai and A. Schubert-Unkmeir

09/2008 **16th International Pathogenic Neisseria Conference (IPNC)**
Rotterdam, The Netherlands

Poster NadR, a negative transcriptional regulator of the *Neisseria meningitidis* adhesin NadA
S. Schielke[1], C. Hübner, M. Frosch, O. Kurzai and A. Schubert-Unkmeir

[1]supported by an IPNC travel grant

09/2008 **60th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)**
Dresden, Germany

Poster The transcriptional regulator NadR negatively controls expression of NadA in *Neisseria meningitidis*
S. Schielke[1], C. Hübner, M. Frosch, O. Kurzai and A. Schubert-Unkmeir

[1]supported by a DGHM travel grant

Table of content

1. Summary

Neisseria meningitidis is a facultatively pathogenic human commensal and strictly adapted to its niche within the human host, the nasopharynx. Not much is known about the regulatory processes required for adaptation to this environment. Therefore the role of the transcriptional regulator NMB1843, one of the two predicted regulators of the MarR family in the meningococcal genome, was investigated. As this gene displayed a high sequence homology to FarR, the **F**atty **a**cid **r**esistance **R**egulator in *N. gonorrhoeae*, we designated the meningococcal protein FarR (*Nm*FarR). Homology modeling of this protein revealed a dimeric structure with the characteristic winged helix-turn-helix DNA binding motif of the MarR family. *Nm*FarR is highly conserved among meningococcal strains and expression of *farR* during exponential growth is controlled post-transcriptionally, being highest in the late exponential phase. By means of electrophoretic mobility shift assays (EMSAs) the direct and specific binding of FarR to the *farAB* promoter region was shown, comparable to its homologue in gonococci. As FarR is involved in fatty acid resistance in *N. gonorrhoeae*, susceptibility assays with the medium chain lauric acid ($C_{12:0}$), the long chain saturated palmitic acid ($C_{16:0}$) and the long chain unsaturated linoleic acid ($C_{18:2\Delta9\Delta12}$) were performed, testing a wide variety of strains of both species. In contrast to the unusually susceptible gonococci, a high intrinsic fatty acid resistance was detected in almost all meningococcal isolates. The molecular basis for this intrinsic resistance in *N. meningitidis* was elucidated, showing that both a functional FarAB efflux pump system as well as an intact lipopolysaccharide (LPS) are responsible for palmitic acid resistance. However, even despite circumvention of the intrinsic resistance, FarR could not be connected with fatty acid resistance in meningococci. Instead, FarR was shown to directly and specifically repress expression of the *Neisseria* adhesin A (*nadA*), a promising vaccine candidate absent in *N. gonorrhoeae*. Microarray analyses verified these results and disclosed no further similarly regulated genes, rendering the FarR regulon the smallest regulon in meningococci reported until now. The exact FarR binding site within the *nadA* promoter region was identified as a 16 bp palindromic repeat and its influence on *nadA* transcription was proved by reporter gene fusion assays. This repression was also shown to be relevant for infection as *farR* deficient mutant strains displayed an increased attachment to epithelial cells. Furthermore, *farR* transcription was attested to be repressed upon contact with active complement components within human serum. Concluding, it is shown that FarR adopted a role in meningococcal host niche adaptation, holding the balance between immune evasion by repressing the highly antigenic *nadA* and host cell attachment via this same adhesin.

2. Zusammenfassung

Neisseria meningitidis ist ein fakultativ pathogener menschlicher Kommensale und eng an die Bedingungen seiner spezifischen Nische, den Nasopharynx, angepasst. Über die regulatorischen Mechanismen, die für diese Anpassung vonnöten sind, ist nicht viel bekannt. Daher wurde die Rolle des Transkriptionsregulators NMB1843 untersucht, eines der beiden prognostizierten Regulatoren der MarR Familie im Meningokokken-Genom. Aufgrund einer hohen Sequenzhomologie dieses Gens zu FarR, dem **F**atty **a**cid **r**esistance **R**egulator in *N. gonorrhoeae*, nannten wir das Meningokokken-Protein ebenfalls FarR (*Nm*FarR). Homologie-Modellierung dieses Proteins ergab eine dimere Struktur mit dem charakteristischen winged helix-turn-helix DNA-Bindemotiv der MarR Familie. Es wurde gezeigt, dass *Nm*FarR in Meningokokken-Stämmen hochkonserviert ist. Die Expression von *farR* wird während des exponentiellen Wachstums posttranskriptional kontrolliert und erreicht ihren Höchststand in der spätexponentiellen Phase. Wie bei seinem Homolog in Gonokokken konnte die direkte und spezifische Bindung von FarR an die *farAB* Promotorregion nachgewiesen werden. Da FarR in *N. gonorrhoeae* an der Fettsäureresistenz beteiligt ist, wurde die Suszeptibilität einer großen Auswahl von Stämmen beider Spezies gegenüber drei unterschiedlichen Fettsäuren getestet: Laurinsäure ($C_{12:0}$), Palmitinsäure ($C_{16:0}$) und Linolsäure ($C_{18:2\Delta9\Delta12}$). Im Gegensatz zu den ungewöhnlich sensitiven Gonokokken konnte eine hohe inhärente Fettsäureresistenz in fast allen Meningokokken-Isolaten beobachtet werden. Nach Analyse der molekularen Grundlage dieser Resistenz konnte gezeigt werden, dass sowohl eine funktionale FarAB Efflux-Pumpe als auch ein intaktes Lipopolysaccharid (LPS) für die Palmitinsäureresistenz verantwortlich sind. Trotz Umgehung der inhärenten Resistenz konnte keine Verbindung von FarR mit Fettsäureresistenz in Meningokokken hergestellt werden. Stattdessen reprimiert FarR direkt und spezifisch die Expression des *Neisseria* Adhäsins A (*nadA*), eines vielversprechenden Impfstoffbestandteils. Microarrays bestätigten diese Ergebnisse, zeigten aber keine weiteren ähnlich regulierten Gene auf. Somit ist das FarR-Regulon das bisher kleinste Regulon in Meningokokken. Die genaue FarR-Bindestelle innerhalb des *nadA* Promotors wurde als ein 16 bp Palindrom identifiziert und dessen Einfluss auf die Transkription von *nadA* mittels Reportergenanalysen gezeigt. Auch in Infektionsversuchen wurde die Relevanz dieser Repression deutlich, da ein *farR*-deletierter Stamm eine höhere Adhärenz an Epithelzellen aufwies. Die Transkription von *farR* sank nach Kontakt mit aktiven Komplementbestandteilen aus humanem Serum. Zusammenfassend wurde gezeigt, dass FarR eine Rolle in der Nischenadaptation von Meningokokken zukommt, indem er zwischen Immunevasion durch Repression des hoch-immunogenen *nadA* und Wirtszelladhäsion durch eben dieses Adhäsin vermittelt.

3. Introduction

3.1. *Neisseria meningitidis*

Neisseria meningitidis, the meningococcus, has first been isolated from patients with meningitis in 1887 by Anton Weichselbaum and was shortly afterwards recognized as the cause of meningococcal meningitis (Weichselbaum, 1887). The genus *Neisseria* belongs to the class of β-proteobacteria which are heterotrophic Gram-negative diplococci. *N. meningitidis* only metabolize carbohydrates in an oxidative way and are thus aerobic bacteria. Additionally, they are positive for oxidase and catalase, unable to form spores and amotile. Meningococci are exclusively adapted to the human host, possibly owing to their ability to acquire the necessary free iron from the human sources transferrin and lactoferrin (Weinberg, 1974, Irwin *et al.*, 1993, Gray-Owen & Schryvers, 1996). Consequently meningococci are no obligate pathogens; they mainly colonize the nasopharyngeal mucosa as commensals (carriage strains) without affecting the host. In non-endemic times, 5 – 10 % of healthy individuals carry *N. meningitidis* in the upper airway (Cartwright *et al.*, 1987, Stephens, 1999). However, the carriage rate varies with age being below 3 % in toddlers, reaching a peak of 10 – 35 % in adolescents from 15 – 24 years and declining to less than 10 % in older age groups (Cartwright *et al.*, 1987, Blackwell *et al.*, 1990, Caugant *et al.*, 1994). Moreover, high carriage rates prevail in population groups with close contact like military recruits or family members (Olcen *et al.*, 1981, Caugant *et al.*, 1992, Tyski *et al.*, 2001). Only in rare cases, the bacteria penetrate the mucosal membrane and enter the bloodstream. Subsequently, they can cause severe septicaemia and/or meningitis with a fatal outcome in 10 – 20 % of the cases (reviewed in Peltola, 1983, Tzeng & Stephens, 2000). The overall incidence of meningococcal disease in Europe is rated at 0.2 – 14 cases per 100 000 people (reviewed in Harrison *et al.*, 2009), in non-endemic times there are about 500 000 cases per year worldwide, resulting in more than 50 000 deaths (Tikhomirov *et al.*, 1997). However, during severe epidemics in the African "meningitis belt", the sub-Saharan area that extends from Senegal to Ethiopia (Lapeyssonnie, 1963), rates can increase to 1 000 cases per 100 000 people (reviewed in Yazdankhah & Caugant, 2004). Generally, 10 – 20 % of the survivors sustain permanent sequelae including deafness, mental retardation and amputation (reviewed in Tzeng & Stephens, 2000, Wheeler *et al.*, 2003).

3.2. Characterization of meningococcal strains

As meningococcal strains can cause epidemics, identification and characterization of the causative strains is of major importance. Almost all disease-associated strains of *N. meningitidis* are enveloped by a polysaccharide capsule, a major virulence factor. The capsules provide the bacteria with antiphagocytic and antibactericidal properties and thus enhance their survival in the bloodstream or the cerebrospinal fluid (Stephens *et al.*, 1993, McNeil *et al.*, 1994, Vogel & Frosch, 1999). The biochemical composition of this capsule determines the immunological classification of meningococcal strains into serogroups. Up to now, 12 distinct serogroups (A, B, C, 29E, H, I, K, L, W-135, X, Y and Z) have been described, but virtually all (> 90 %) of the isolates from invasive disease belong to the five serogroups A, B, C, W-135 and Y (reviewed in Peltola, 1983). While the capsules of serogroup A and X are composed of N-acetyl-D-mannosamine-6 phosphate and N-acetylglucosamine-1 phosphate, respectively (Dolan-Livengood *et al.*, 2003, Tzeng *et al.*, 2003), the capsules of the serogroups B, C, W-135 and Y are composed of sialic acid derivatives. Approximately 50 % of the strains isolated from healthy carriers lack the capsule and are thus not serogroupable by serological means (Claus *et al.*, 2002). Among these, there are strains genetically incapable of expressing capsular polysaccharides, either due to exchange of the capsule region of the chromosome by the capsule null locus (*cnl*) or to various genetic mechanisms inactivating capsule synthesis genes (Claus *et al.*, 2002, Weber *et al.*, 2006). For epidemiology, sub-capsular antigens are used to characterize *N. meningitidis* beyond the serogroup and provide the basis for serotyping (variation in PorB porin), serosubtyping (variation in PorA porin) and immunotyping (changes in lipopolysaccharide composition) (reviewed in Frasch *et al.*, 1985, Hitchcock, 1989). Additionally, multilocus sequence typing (MLST) determines the genetic variation between meningococcal strains with a high resolution (Maiden *et al.*, 1998). Briefly, the genetic variants at seven loci are determined and assigned an allele number, these numbers are then combined and result in an allelic profile, the sequence type (ST). According to their similarity to a central allelic profile, the genotype, the sequence types are grouped into clonal complexes defined by identity in four or more loci.

3.3. *Neisseria gonorrhoeae*

Beside *N. meningitidis*, the other human pathogenic *Neisseria* species is the closely related *Neisseria gonorrhoeae*, the gonococcus, which was first described in 1879 by Albert Neisser (Neisser, 1879). In almost all physiological aspects, gonococci are highly similar to meningococci but unlike the latter they cannot metabolize maltose. Furthermore, gonococci lack the capsule biosynthetic genes, whereas almost all virulent meningococcal strains are encapsulated. In contrast to meningococci, which rather accidentally lead to severe disease in some cases, gonococci are obligate pathogens causing gonorrhea. This sexually transmitted disease is rated at more than 60 million cases annually worldwide (Gerbase *et al.*, 1998, Fenton & Lowndes, 2004). *N. gonorrhoeae* infections affect the urethra in males and females as well as the endocervix, fallopian tubes and the uterus in females. The clinical entities in women range from local genital infection to disseminated bloodstream infections. A high percentage of endocervical infections remain unrealized, yet often entail grave consequences like pelvic inflammatory disease (PID). PID can lead to chronic pelvic pain or ectopic pregnancy and is a major cause of female infertility (Soper, 1994). Men with asymptomatic urethritis as well as women with asymptomatic infections are a major problem for unrecognized disease transmission.

3.4. The outer membrane structure of *Neisseria spp.*

Both *N. meningitidis* and *N. gonorrhoeae* are highly adapted to their human host, but despite their close relatedness they inhabit very distinct niches and cause different clinical entities. One difference in the phenotype is the capsule. Almost all virulent meningococcal strains are encapsulated whereas gonococci lack the capsule biosynthetic genes. Beneath the capsule, both Gram-negative bacteria show the typical outer membrane structure of these microorganisms. They have a cytoplasmic membrane followed by a periplasmic space which consists of a thin peptidoglycan layer and an outer membrane containing lipopolysaccharide (LPS), pili and other proteins, porins and phospholipids (Figure 1). Though the overall structure of the outer membrane of these two microorganisms is very similar, there are also differences. An atypical sensitivity of *N. gonorrhoeae* to hydrophobic compounds has been reported and was attributed to an increased permeability of the outer membrane caused by enriched phospholipid bilayer regions (Sarubbi *et al.*, 1975, Guymon & Sparling, 1975, Miller & Morse, 1977, Lysko & Morse, 1981). Normally, the outer membrane is a highly ordered structure and an effective permeability barrier against hydrophobic compounds like

fatty acids (C_{10} – C_{18}), hydrophobic antibiotics (for example macrolides, novobiocin, rifamycins or actinomycin D), dyes (eosine, methylene blue etc.) and detergents (Triton X-100, SDS, bile salts) (Sheu & Freese, 1973, Leive, 1974, Nikaido & Nakae, 1979, reviewed in Nikaido & Vaara, 1985).

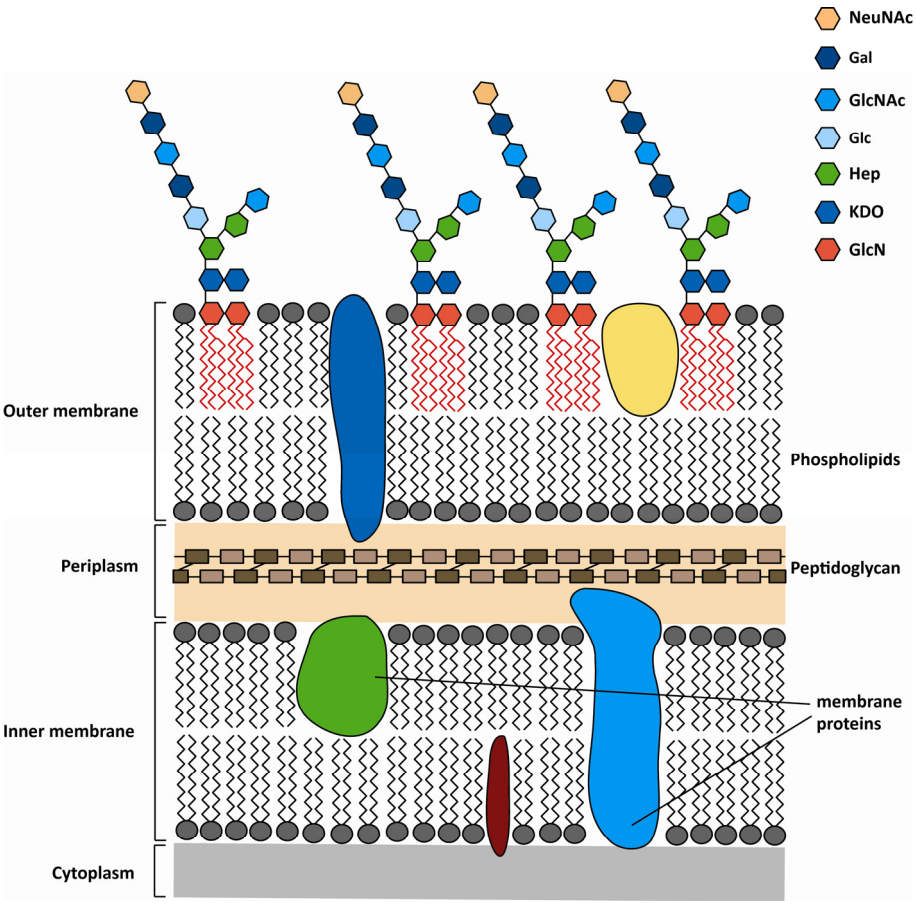


Figure 1. Schematic illustration of the outer membrane of *Neisseriae*. The outer leaflet of the outer membrane contains many lipopolysaccharide (LPS) molecules, the lipid A part anchoring these in the membrane is shown in red. Carbohydrate composition of the LPS is according to *N. meningitidis* strain MC58; gonococci possess an additional glucosyl transferase encoded by *lgtD*, which adds an *N*-acetylgalactosamine at the terminal galactose residue of the α-chain. GlcN, glucosamine; KDO, 2-keto-3-deoxyoctulosonic acid; Hep, Heptose; Glc, Glucose; GlcNAc, N-acetylglucosamine; Gal, Galactose; NeuNAc, N-acetylneuraminic acid.

The protection of Gram-negative bacteria from these antimicrobial compounds is not least due to the LPS molecules covering approximately 75 % of the outer membrane. Generally, LPS consists of four parts: first lipid A, a glucosamine disaccharide with multiple fatty acids which acts as membrane anchor, second the inner core oligosaccharide with the two unusual carbohydrates heptose and KDO (2-keto-3-deoxyoctulosonic acid), third the outer core oligosaccharide protruding from the bacterial surface and fourth the O-antigen, a repetitive glycan polymer. The LPS of the two human pathogenic *Neisseria* species varies significantly from the typical Gram-negative LPS as it lacks these O-antigen extensions and is therefore sometimes referred to as lipooligosaccharide (LOS). The two heptose

molecules in the inner core oligosaccharide carry only short oligosaccharide side chains (Pavliak *et al.*, 1993, reviewed in Verheul *et al.*, 1993). In addition, the lipid A part of the LPS of *Neisseria spp.* is clearly distinct from that of *Enterobacteriaceae* as the six acyl chains are symmetrically distributed among the disaccharide and are composed differently (Takayama *et al.*, 1986, Kulshin *et al.*, 1992). The LPS structure of *N. meningitidis* has been analyzed in detail (Pavliak *et al.*, 1993, Kogan *et al.*, 1997, Rahman *et al.*, 1998, Zughaier *et al.*, 2007, Tsai *et al.*, 2009), showing that the outer carbohydrate residues are highly diverse, leading to 12 different immunotypes (Zollinger & Mandrell, 1977, Scholten *et al.*, 1994).

3.5. Evasion of host immune mechanisms

Both *N. meningitidis* and *N. gonorrhoeae* are renowned successful human pathogens because they are able to evade the immune mechanisms by constantly varying their surface structures or by mimicry of host molecules (Segal *et al.*, 1986, Mandrell & Apicella, 1993). The capsule is the outermost structure of meningococci and is thought to protect the bacteria during transmission in aerosol droplets (reviewed in Diaz Romero & Outschoorn, 1994). Once inside the host, systemic spread of the bacteria is a prerequisite for serious infection and they therefore have to survive the host defenses within the blood like limitation of free iron or killing of pathogens by the complement system. However, meningococci and gonococci have evolved various mechanisms to survive these detrimental circumstances. Both sense low iron conditions and activate an adaptive stress response (Grifantini *et al.*, 2003, Ducey *et al.*, 2005). As mentioned earlier, *N. meningitidis* overcomes the iron restriction by seizing the required iron from human transferrin or lactoferrin and is even proposed to use the availability of these iron donors as niche indicators (Weinberg, 1974, Gray-Owen & Schryvers, 1996, Jordan & Saunders, 2009). The complement system is part of innate immunity and crucial in host defense against *Neisseria spp.* Patients with defects in complement components often suffer from recurrent bacteremia with both *Neisseriae* (Petersen *et al.*, 1979). This system comprises different biochemical pathways, leading to the proteolytic cleavage of complement factor C3 and attachment of C3b to the bacterial surface enabling opsonophagocytosis and assembly of the membrane attack complex (C5b through C9), culminating in membrane disruption and bacterial cell lysis. Meningococci are inherently more resistant against complement-mediated killing as they have evolved various mechanisms to evade complement attack (reviewed in Schneider *et al.*, 2007, Lo *et al.*, 2009). These mechanisms mainly rely on capsule expression, LPS composition and recruitment of negative complement regulators (Mackinnon *et al.*, 1993, Estabrook *et al.*, 1997, Kahler *et al.*, 1998,

Geoffroy *et al.*, 2003, Jarva *et al.*, 2005, Schneider *et al.*, 2006). With respect to immune evasion, the capsule assumes various roles - it prevents the bacteria from being recognized by the complement system, but also from detection by phagocytes and the adverse activity of antimicrobial peptides. The capsule was the first target for the development of a vaccine against *N. meningitidis*. Capsule-based vaccines are available for four of the five major disease associated serogroups A, C, W-135 and Y. The serogroup B capsule, however, is composed of α2-8 *N*-acetylneuraminic acid, a sialic acid homopolymer which mimics the glycosylated neuronal cell adhesion molecules NCAM-1 (Finne *et al.*, 1983) and is therefore poorly immunogenic. Albeit this proved to be a major drawback, by now two promising vaccines composed of sub-capsular antigens are being assessed in phase II and III clinical trials (Giuliani *et al.*, 2006, Jacobsson *et al.*, 2009). Another case of immune evasion by molecular mimicry can be seen in the LPS molecule, where the terminal carbohydrate residues comply with the tetrasaccharide lacto-*N*-neotetraose (Gal-GlcNAc-Gal-Glc) which is also present in paragloboside, the precursor of the ABH glycolipid antigens on human erythrocytes (Moran *et al.*, 1996). A single sialic acid molecule can be to added to this epitope (Mandrell *et al.*, 1991), contributing to resistance against complement-mediated lysis in gonococci and possibly also in meningococci, although the role of sialylation in the latter microorganism remains controversial (Wetzler *et al.*, 1992, Vogel *et al.*, 1997a, Estabrook *et al.*, 1997, Kahler *et al.*, 1998, Geoffroy *et al.*, 2003). But sialylation of the LPS plays a role in inhibition of phagocytosis in *N. meningitidis* (Unkmeir *et al.*, 2002, Kurzai *et al.*, 2005), possibly by masking proteins on the bacterial outer membrane. Besides the capsule and LPS, meningococci can additionally shield themselves from complement attack by recruiting the negative complement regulators C4BP and factor H (Jarva *et al.*, 2005, Madico *et al.*, 2006, Schneider *et al.*, 2006). Recent results indicate that the factor H binding protein fHBP is not only crucial for survival in the presence of human serum but also enhances the viability of meningococci in human blood and in the presence of LL-37, an antimicrobial peptide (Seib *et al.*, 2009).

3.6. Interactions with the host

In order to establish a persisting interaction with the host, *N. meningitidis* and *N. gonorrhoeae* must adhere to mucosal tissues. Therefore, they have a repertoire of adhesins allowing specific interaction (recently reviewed in Virji, 2009, Carbonnelle *et al.*, 2009). The first contact with human cells is mediated by the polymeric type IV pili, which are also hypothesized to be involved in host specificity (Rudel *et al.*, 1995, Kirchner & Meyer, 2005). The major adhesins in *Neisseria spp.* are the Opa proteins, β-barrel structures in the outer bacterial membrane (Virji *et al.*, 1992, Swanson, 1978),

undergoing antigenic variation due to phase variation and homologous recombination (Stern *et al.*, 1986, Hobbs *et al.*, 1998). While gonococci contain only Opa, some meningococci have an additional opacity protein (Opc). Due to their basic nature, these Opa proteins bind to negatively charged molecules within the human host like sialic acids or heparan sulphate proteoglycans (de Vries *et al.*, 1998, Achtman *et al.*, 1988). This interaction with sialic acid might explain the reduced attachment of encapsulated or sialylated bacteria with host cells (Moore *et al.*, 2005). In addition to these targets, most meningococcal and gonococcal isolates were shown to bind to CEACAM-1 (carcinoembryonic antigen-related cell adhesion molecule 1), a member of the immunoglobulin superfamily (Virji *et al.*, 1996), expressed on the surface of epithelial cells, endothelial vessels and hematopoietic cells (Obrink, 1997, Prall *et al.*, 1996). There are at least five more supplementary adhesins, so called "minor" adhesins, as they are less efficient in mediating *in vitro* host cell interactions than pili or Opa proteins (recently reviewed in Carbonnelle *et al.*, 2009, Virji, 2009). Among these, NhhA (*Neisseria hia* homologue A) and App (Adhesion penetration protein) are autotransporter proteins (Peak *et al.*, 2000, Serruto *et al.*, 2003, Scarselli *et al.*, 2006) whereas HrpA and HrpB (hemagglutinin/haemolysin related proteins A and B) build up a two-partner secretion system (Schmitt *et al.*, 2007, Tala *et al.*, 2008). Furthermore MspA (Meningococcal serine protease A) and NadA (*Neisseria* adhesin A) (Comanducci *et al.*, 2002, Turner *et al.*, 2006) were classified as members of the oligomeric coiled-coil family of adhesins (Oca-family) owing to a high homology to YadA of *Yersinia enterocolitica* and UspAs in *Moraxella catarrhalis* (reviewed in Cornelis *et al.*, 1998, Chen *et al.*, 1999). NadA was found through reverse vaccinology (Pizza *et al.*, 2000) as it elicits a strong bactericidal immune response and is included in a five component vaccine for serogroup B meningococci currently being tested in humans (Giuliani *et al.*, 2006).

Figure 2. Schematic illustration of the NadA molecule. The trimeric protein consists of a globular head domain, an internal region with high coiled-coil propensity and a β-barrel domain at the C-terminus, with which it is anchored in the outer meningococcal membrane.

The *nadA* gene clusters into three alleles and is overrepresented in three of the four hypervirulent lineages of *N. meningitidis* (ST-8, ST-11 and ST-32), but is absent in the ST-41/44 strains and underrepresented in commensal carrier strains; additionally it is neither present in *N. gonorrhoeae* nor in *N. lactamica* (Comanducci *et al.*, 2004). NadA is a trimeric protein with an N-terminal globular

head domain responsible for host cell interaction, a coiled-coil region and a C-terminal membrane anchor domain (Figure 2) that functions as autotransporter by organizing oligomerization of the adhesin and insertion in the outer bacterial membrane. The *nadA* gene harbors a tract with repetitive TAAA elements within its promoter region, which is responsible for phase variable expression of the adhesin due to slipped strand mispairing (Martin *et al.*, 2003, Martin *et al.*, 2005). It has been shown that NadA uses its head domain for interaction with human epithelial cells (Chang cells), whereas adhesion to human epithelial cells (HUVECs) could not be observed (Comanducci *et al.*, 2002, Capecchi *et al.*, 2005). A receptor for NadA has not yet been identified. Furthermore, NadA specifically binds to monocytes and promotes their differentiation into a macrophage-like phenotype (Franzoso *et al.*, 2008, Mazzon *et al.*, 2007), supporting the bactericidal potential of this adhesin.

3.7. Genetic basis of the differences between meningococci and gonococci

Despite the high level of sequence similarity between the genomes of the two human-specific pathogens *N. meningitidis* and *N. gonorrhoeae*, they are extremely adapted to their respective host niches and cause very differing clinical symptoms. This is probably due to differences in genetic content (Perrin *et al.*, 2002). The genome of *N. meningitidis* contains only about half the amount of genes as the genome of the commensal *E. coli,* but meningococci use this compact and highly flexible genome to their advantage, acquiring foreign DNA by being naturally competent for transformation (Tettelin *et al.*, 2000, reviewed in Davidsen & Tonjum, 2006). *N. meningitidis* harbors for example approximately 2000 copies of the DNA uptake signal sequence 5'-GCCGTCTGAA, indicating that free DNA with this signal was taken up and integrated into the genome (Kroll *et al.*, 1998, Smith *et al.*, 1999). Therefore, meningococci have no classic pathogenicity islands but acquired new traits by horizontal gene transfer from *Neisseria* species but also from other mucosal commensals it encountered (Schoen *et al.*, 2008, Tettelin *et al.*, 2000). Meningococci probably share a common ancestor with *N. gonorrhoeae* and *N. lactamica*, and evolved only later into encapsulated pathogens, presumably by acquiring the capsule genes from *Pasteurellaceae* using horizontal gene transfer (Schoen *et al.*, 2008). However, differences in gene expression between meningococci and gonococci can also result from transcriptional and translational regulation.

3.8. Transcriptional regulation

Transcriptional regulation can be achieved by various means. Alternative sigma factors provide promoter recognition specificity to the RNA-polymerase, two-component systems react to external stimuli and transcriptional regulators directly or indirectly influence transcription of their target genes. As for alternative sigma factors, none are used by *N. meningitidis*, whereas in *N. gonorrhoeae* two factors have been found which respond to oxidative damage or cell contact, respectively (Laskos *et al.*, 1998, Du *et al.*, 2005, Gunesekere *et al.*, 2006). Interestingly, this low number affirms the hypothesis that host-adapted microorganisms need fewer alternative sigma factors. The free-living *Streptomyces coelicolor* for instance contains 65 alternative sigma factors. There are four two-component systems encoded in the meningococcal genome, but only the PhoP/PhoQ (MisR/MisS) system has been shown to be functional so far, contributing to adaptation of the bacteria to host conditions by modulating their outer membrane structure (Tzeng *et al.*, 2004, Tzeng *et al.*, 2008, Jamet *et al.*, 2009). Compared to *E. coli* which displays more than 200 transcriptional regulators, a comparably low amount is found in the pathogenic *Neisseriae* (Pareja *et al.*, 2006) – 35 putative transcriptional regulators in *N. meningitidis* (strain MC58, GenBank AE002098) versus 34 in *N. gonorrhoeae* (strain FA1090, GenBank AE004969). Of these, meningococci as well as gonococci have each two transcriptional regulators of the MarR family.

3.9. The MarR family of transcriptional regulators

The MarR family of transcriptional regulators is widely distributed among bacteria as well as archaea and is thought to have evolved before the divergence of these two domains (Perez-Rueda & Collado-Vides, 2001). The regulators are characterized by a conserved winged helix-turn-helix DNA binding domain (Clark *et al.*, 1993). Most of the examined MarR-like transcriptional regulators show repressor activities by directly binding to their target sequences within the respective promoter region. Additionally, they appear as homodimers (Figure 3). The eponymous protein MarR was originally characterized as the repressor of the multiple antibiotic resistance operon *marRAB* in *E. coli* (Seoane & Levy, 1995). Indeed, members of this family enable the bacteria to survive adverse environmental conditions such as the presence of antibiotics, toxic chemicals, organic solvents or reactive oxygen species (Alekshun & Levy, 1999b, Wilkinson & Grove, 2004, Hong *et al.*, 2005, reviewed in Wilkinson & Grove, 2006). But these regulators can also play a crucial role in virulence factor expression, enabling the bacteria to adapt to host environments (Ellison & Miller, 2006, Wei *et*

al., 2007). This is particularly important for pathogenic bacteria like *Staphylococcus aureus* or *Pseudomonas aeruginosa*. In these microorganisms, the MarR-like transcriptional regulators MgrA and MexR, respectively, control efflux pump systems for broad antibiotic resistance (Lim *et al.*, 2002, Luong *et al.*, 2003, Truong-Bolduc & Hooper, 2007). Even in the hyperthermophilic archeon *Sulfolobus tokodaii* a MarR-like transcriptional regulator was found with ST1710, regulating a multiple drug resistance operon (Miyazono *et al.*, 2007, Lomovskaya *et al.*, 1995). In *Yersinia enterocolitica* and *Y. pseudotuberculosis*, RovA controls transcription of the adhesion factor invasin (Ellison *et al.*, 2004), and in *N. gonorrhoeae*, FarR controls expression of the fatty acid resistance operon *farAB* (Lee & Shafer, 1999, Lee *et al.*, 2003).

Figure 3. Schematic illustration of a MarR-like transcriptional regulator. The protein consists of a homodimer, the second dimer half is shown in light blue and binds to the promoter region of the target gene or operon, influencing its transcription.

Concluding, all transcriptional regulators of the MarR family share a high structural homology due to their biological function but are involved in a wide variety of tasks as they have an amino acid similarity of less than 25 % and can therefore recognize many different ligands and DNA sequences within promoter regions.

3.10. Objectives

The aim of this work was the molecular and functional characterization of NMB1843 (*Nm*FarR), a predicted transcriptional regulator of the MarR family in *Neisseria meningitidis*. Transcriptional regulation is indispensable for adaptation to changing conditions. As meningococci are closely adapted to the relatively stable environment of the human host, they contain only few specialized regulatory systems enabling them to survive in their specific host niche. However, not many of these systems have been characterized so far.

The main aspects to be addressed in this work were:

- Classification of FarR by sequence analysis and structure prediction
- Microarray analysis and RT-PCR validation of the FarR regulon
- Production of a specific antibody against FarR
- Establishment of electrophoretic mobility shift assays (EMSAs) to reveal the DNA binding activity as well as the binding sequence of FarR
- Generation of deletion and complementation mutant strains in *N. meningitidis* for the functional characterization of FarR
- Creation of reporter gene fusion constructs for expression profiling
- Establishment of infection experiments with the Chang epithelial cell line
- Elucidation of the role of FarR in meningococcal fatty acid resistance
- Identification of the molecular basis for the intrinsic fatty acid resistance of meningococci
- Implementation of experiments for the examination of the regulation of FarR

4. Experimental Procedures

4.1. Table of Chemicals and Kits

Chemicals and Kits	Company
ABTS tablets and buffer	Roche
Acetic acid	Roth
Agarose	Roth
Ammonium chloride (NH_4Cl)	Merck
Ampicillin	Ratiopharm
APS (ammonium persulfate)	Roth
Bacto-Agar	Becton-Dickinson GmbH
Barbital	Serva
BCA Protein Assay Kit	Pierce
Boric acid (BH_3O_3)	Merck
Bromophenol blue	Merck
BSA (Bovine serum albumine)	Applichem
Calcium chloride ($CaCl_2$)	Merck
Chloramphenicol	Sigma
Chloroform	Applichem
$CoCl_2$	Sigma
Coomassie Brilliant Blue R250	Merck
CSPD	Roche
$CuCl_2$	Merck, Darmstadt
Desferal (Deferoxamine mesylate salt)	Sigma
Diethylether	Merck
Difco™ GC Medium Base	Becton-Dickinson GmbH
DIG Gel Shift Kit, 2nd generation	Roche
Dimethylformamide (DMF)	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
Disodium Hydrogen Phosphate (Na_2HPO_4)	Merck
DMEM	Biochrom
DNA-*free* kit	Ambion
dNTP mixture	TaKaRa Bio Inc.
DTT	Applichem
EDTA	Serva
Erythromycin	Applichem
Ethanol, absolute	Roth
Ethanolamine	Sigma
Ethidiumbromide	Merck
FCS (Fetal Calf Serum)	Biochrom
Ferric nitrate ($Fe(NO_3)_3$)	Fluka AG

Formaldehyde (37 %)	Roth
Genomic Tips 100	Qiagen
Gentamicin	Biochrom
Glucose	Merck
Glutardialdehyde	Merck
Glycerin	Roth
Glycine	Roth
Guanidine-HCl	Roth
Hexadecane	Sigma
Hydrochloric acid (HCl)	Roth
Hydrogen peroxide (H_2O_2 , 30%)	Merck
Imidazole	Sigma
Isopropanol	Roth
Kanamycin	Sigma
Klenow enzyme	Pharmacia Biotech.
L-Arginine monohydrochloride	Sigma
Lauric acid	Sigma
LB-agar	Invitrogen
L-Cysteine	Sigma
L-Glutamic acid monosodium salt hydrate	Sigma
L-Glutamine	Schuchardt
Linoleic acid	Sigma
Luciferase	Promega
Lysozyme	Biomol
Magnesium chloride ($MgCl_2$)	Merck
Magnesium sulfate ($MgSO_4$)	Merck
Maleic acid	Merck
Methanol	Roth
Milk powder (low-fat)	Heirler Cenovis
$MnCl_2$	Merck
Nitric acid (HNO_3, 69%)	Applichem
PBS	Biochrom
Phosphoric acid (H_3PO_4, 85%)	Merck
Phusion Taq	Finnzymes
Platinum Taq Polymerase, High Fidelity	Invitrogen
Polyacrylamide	Roth
Poly-D-Lysine	Sigma
PolyViteX	bioMérieux
Potassium chloride (KCl)	Merck
Potassium dihydgrogen phosphate (KH_2PO_4)	Merck
Potassium hydroxide (KOH)	Riedel-de Haën
Prestained Protein Ladder	New England Biolabs
ProBond™ Nickel-Chelating Resin	Invitrogen

Protease inhibitor (Complete)	Roche
Proteinase K	Roth
Proteose-peptone	Becton-Dickinson GmbH
QIAGEN OneStep RT-PCR Kit	Qiagen
QIAGEN Plasmid Midi Kit	Qiagen
QIAmp DNA mini kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR purification kit	Qiagen
Random Nonamer Primer	Eurofins, MWG
Restriction enzymes	New England Biolabs
RNase	Roche
RNase out	Invitrogen
Smart Ladder	Eurogentec
Sodium acetate	Merck
Sodium carbonate (Na_2CO_3)	Roth
Sodium chloride (NaCl)	Roth
Sodium citrate	Applichem
Sodium dodecyl sulfate (SDS)	Applichem
Sodium hydrogen carbonate ($NaHCO_3$)	Merck
Sodium hydroxide (NaOH)	Roth
Sodium phosphate (NaH_2PO_4)	Merck
Sodium salicylate	Sigma
Sodium sulphate (Na_2SO_4)	Merck
Sodium thiosulfate($Na_2S_2O_3$)	Riedel-de Haën
Spectinomycin	Sigma
Starch	Fluka
StepOnePlus Mastermix (*Power* SYBR® Green)	Applied Biosystems
Superscript II Kit	Invitrogen
T4 DNA Ligase	New England Biolabs
Taq Polymerase	New England Biolabs
TEMED	Roth
Tris	Roth
Triton X-100	Roth
TRIzol® Reagent	Invitrogen
Trypsin/EDTA	Biochrom
Tween-20	Roth
Uracil	Sigma
Urea	Applichem
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	MP Biomedicals
yeast extract	DIFCO
$ZnCl_2$	Merck, Darmstadt
β-Mercaptoethanol	Merck

4.2. Table of meningococcal strains

WUE-ID*	Status	Strain	Serogroup	Sequence Type
2120	invasive	FAM18	C	11
2135	invasive	MC58	B	32
2137	invasive	Z2491	A	4
2370	invasive		C	11
2514	invasive	H44/76	B	32
3887	carrier		B	32
DE 6790	invasive		B	32
DE 6853	invasive		Y	23
DE 7080	invasive		C	8
DE 7100	invasive		W-135	11
DE 7671	invasive		Y	23
DE 8640	hypervirulent		B	41
DE 8840	invasive		C	8
DE 8910	invasive		C	8
DE 10173	invasive		B	42
Y 172	carrier		Y	166
α 7	carrier		29E	60
α 16	carrier		B	41/44
α 24	carrier		Y	23
α 42	carrier		cnl	53
α 153	carrier		29E	60
α 219	carrier		B	44
α 275	carrier		W-135	22
α 316	carrier		Z	22
α 580	carrier		cnl	845
α 710	carrier		B	136

*ID according to the IHM Würzburg strain collection

4.3. Table of gonococcal strains

NRZM-ID*	Species	Strain	Isolation site
38	*N. gonorrhoeae*		-
114	*N. gonorrhoeae*		-
154	*N. gonorrhoeae*	MS11	-
155	*N. gonorrhoeae*		blood
245	*N. gonorrhoeae*		-
308	*N. gonorrhoeae*		cervix
359	*N. gonorrhoeae*		-
441	*N. gonorrhoeae*		-
452	*N. gonorrhoeae*		vagina
458	*N. gonorrhoeae*		-
489	*N. gonorrhoeae*		-
522	*N. gonorrhoeae*		-
1092	*N. gonorrhoeae*		urethra
1146	*N. gonorrhoeae*		rectum
1225	*N. gonorrhoeae*		urethra
2136	*N. gonorrhoeae*	FA1090	-
2498	*N. gonorrhoeae*		-
2805	*N. gonorrhoeae*		oropharynx

*ID according to the NRZM Würzburg strain collection

4.4. Table of mutant strains

WUE-ID*	Description	Genotype	Resistance[#]	Reference
2390	Unencapsulated, parental strain MC58 (2135)	Δ*siaD*	Ery	(Unkmeir *et al.*, 2002a)
2425	Unencapsulated, parental strain MC58 (2135)	Δ*siaD*	Cm	(Frosch *et al.*, 1989)
3023	Inactivation of NMB0178 (*lpxA*), parental strain H44/76 (2514)	Δ*lpxA*	Kan	(Steeghs *et al.*, 1998)
3240	Unencapsulated, parental strain MC58 (2135)	Δ*siaD*	Cm	(Kurzai *et al.*, 2005)
3275	Unencapsulated, inactivation of NMB1929, the LPS glucosyl transferase A *lgtA*, parental strain H44/76 (2514)	Δ*siaD* Δ*lgtA*	Cm Kan	(Kurzai *et al.*, 2005)
3675	Inactivation of NMB1843, the transcriptional regulator *farR,* parental strain MC58 (2135)	Δ*farR*	Kan	This study (Schielke *et al.*, 2009)
3677	Inactivation of *farR*, parental strain MC58 Δ*siaD* (2390)	Δ*siaD* Δ*farR*	Ery Kan	This study (Schielke *et al.*, 2009)

WUE-ID*	Description	Genotype	Resistance#	Reference
3711	Inactivation of NMB0014, the 3-deoxy-D-manno-octulosonic-acid transferase *kdtA*, parental strain MC58 Δ*siaD* (3240)	Δ*siaD* Δ*kdtA*	Spec Kan	This study (Schielke *et al.*, 2010, in preparation)
3717	Inactivation of NMB1638, the phosphoethanolamine transferase *lptA*, parental strain MC58 Δ*siaD* Δ*lgtA* (3275)	Δ*siaD* Δ*lgtA* Δ*lptA*	Cm Kan	This study (Schielke *et al.*, 2010, in preparation)
3722	Inactivation of NMB1638, the phosphoethanolamine transferase *lptA*, parental strain MC58 Δ*siaD* Δ*kdtA* (3711)	Δ*siaD* Δ*kdtA* Δ*lptA*	Cm Spec Kan	This study (Schielke *et al.*, 2010, in preparation)
4206	In frame fusion of the *nadA* promoter with deleted FarR binding site to the reporter *lacZ*, parental strain MC58 (2135)	*nadA* deleted */lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4207	In frame fusion of the *nadA* promoter with transverted FarR binding site to the reporter *lacZ*, parental strain MC58 (2135)	*nadA* transverted/ *lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4208	In frame fusion of the *nadA* promoter to the reporter *lacZ*, parental strain MC58 (2135)	*nadA/lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4400	Complementation of the *farR* deletion, parental strain MC58 Δ*farR* (3675)	Δ*farR* (pAP1*farR*)	Kan Ery	(Schielke *et al.*, 2009)
4476	Inactivation of NMB1994, the *Neisseria* adhesin A *nadA*, parental strain MC58 (2135)	Δ*nadA*	Spec	This study (Schielke *et al.*, 2009)
4477	Inactivation of NMB1994, the *Neisseria* adhesin A *nadA*, parental strain MC58 Δ*farR* (3675)	Δ*farR* Δ*nadA*	Kan Spec	This study (Schielke *et al.*, 2009)
4498	Inactivation of NMB1994, the *Neisseria* adhesin A *nadA*, parental strain MC58 Δ*siaD* (3240)	Δ*siaD* Δ*nadA*	Cm Spec	This study (Nägele *et al.*, 2009, in preparation)
4512	Negative control, *lacZ* reporter gene construct without promoter, pSMS1 in parental strain MC58 (2135)	*/lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4513	In frame fusion of the *farR* promoter region to the reporter gene *lacZ*, parental strain MC58 (2135)	*farR/lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4514	In frame fusion of the *porA* promoter region to the reporter gene *lacZ* , pSMS5, parental strain MC58 (2135)	*porA/lacZ*	Kan	This study (Schielke *et al.*, 2010a, in preparation)
4515	Inactivation of NMB1638, the phosphoethanolamine transferase *lptA*), parental strain MC58 Δ*siaD* (2425)	Δ*siaD* Δ*lptA*	Cm Spec	This study (Schielke *et al.*, 2010, in preparation)

WUE-ID*	Description	Genotype	Resistance[#]	Reference
4516	Inactivation of NMB0014, the 3-deoxy-D-manno-octulosonic-acid transferase *kdtA*, parental strain MC58 Δ*farR* (3675)	Δ*farR* Δ*kdtA*	Spec Kan	This study (Schielke *et al.*, 2010, in preparation)
4520	Inactivation of NMB1418, the lauroyl transferase *lpxL1*, parental strain MC58 Δ*siaD* (3240)	Δ*siaD* Δ*lpxL1*	Cm Spec	This study (Schielke *et al.*, 2010, in preparation)
4521	Complementation of the natural 10 bp deletion within NMB1418, the lauroyl transferase *lpxL1*, parental strain α 24	intact *lpxL1*	-	This study (Schielke *et al.*, 2010, in preparation)
4534	Inactivation of the NMB0318/0319 operon (*farAB*), parental strain MC58 (2135)	Δ*farAB*	Spec	This study
4535	Inactivation of the NMB0318/0319 operon (*farAB*), parental strain MC58 Δ*siaD* Δ*kdtA* (3711)	Δ*siaD* Δ*kdtA* Δ*farAB*	Spec Kan	This study

*ID according to the IHM Würzburg strain collection

[#] Resistance: Amp: Ampicillin, Cm: Chloramphenicol, Ery: Erythromycin, Kan: Kanamycin, Spec: Spectinomycin

4.5. Table of plasmids

WUE-ID*	Description	Plasmid	Resistance[#]	Reference
3010	pTL1, pBluescript (Invitrogen, the Netherlands) containing the *Neisseria* uptake sequence	pTL1	Amp	(Unkmeir *et al.*, 2002)
3381	Meningococcal expression vector pAP1 with restriction sites for cloning of genes under control of the *porA* promoter	pAP1	Ery	(Lappann *et al.*, 2006)
3544	NMB0014 (*kdtA*) with EcoRI restriction sites in pTL1	pCS6	Amp	Corinna Schmitt (Schielke *et al.*, 2010, in preparation)
3556	NMB1638 (*lptA*) with EcoRI restriction sites in pTL1	pCS23	Amp	Corinna Schmitt (Schielke *et al.*, 2010, in preparation)
3563	Spectinomycin resistance cassette with AvrII and EcoRI restriction sites in TOPO BE	pCS31	Kan Spec	Corinna Schmitt (Schielke *et al.*, 2010, in preparation)
3564	Deletion plasmid for *kdtA,* insertion of a SmR cassette with AvrII restriction sites (3563) in *kdtA* from pCS6 (3544)	pCS32	Amp Spec	Corinna Schmitt (Schielke *et al.*, 2010, in preparation)

WUE-ID*	Description	Plasmid	Resistance#	Reference
3573	Deletion plasmid for *lptA*, insertion of a SmR cassette via AvrII restriction sites in *lptA* from pCS23 (3556)	pCS46	Amp Spec	Corinna Schmitt (Schielke *et al.*, 2010, in preparation)
3694	FarR expression plasmid, *farR* gene with flanking V5 epitope, His-Tag and thioredoxin-tag in pBad TOPO Thio Fusion (Invitrogen, Germany)	pFarR	Amp	This study (Schielke *et al.*, 2009)
3964	Promoterless *lacZ* gene in pHC6.7, a pBluescript (Invitrogen, the Netherlands) derivative flanked by the *hrt* locus for recombination followed by a KanR cassette	pSMS1	Amp Kan	Sabine M. Schulz (Claus *et al.*, 1998) (Schielke *et al.*, 2010a, in preparation)
4051	In frame fusion of the *porA* promoter with BglII sites in front of the *lacZ* gene of pSMS1 (3964)	pSMS5	Amp Kan	Sabine M. Schulz (Schielke *et al.*, 2010a, in preparation)
4201	*nadA* promoter with deleted FarR binding site, via BglII restriction sites in TOPO-TA	444, *nadA* deleted	Amp	MWG Eurofins, Germany
4202	*nadA* promoter with transverted FarR binding site, via BglII restriction sites in TOPO-TA	463, *nadA* trans-verted	Amp	MWG Eurofins, Germany
4203	In frame fusion of the *nadA* promoter with deleted FarR binding site via BglII restriction sites to the reporter gene *lacZ* of pSMS1	*nadA* deleted /*lacZ*	Amp Kan	This study (Schielke *et al.*, 2010a, in preparation)
4204	In frame fusion of the *nadA* promoter with transverted FarR binding site via BglII restriction sites to the reporter gene *lacZ* of pSMS1	*nadA* transverted /*lacZ*	Amp Kan	This study (Schielke *et al.*, 2010a, in preparation)
4205	In frame fusion of the unaltered *nadA* promoter region via BglII restriction sites to the reporter gene *lacZ* of pSMS1	*nadA* unaltered/ *lacZ*	Amp Kan	This study (Schielke *et al.*, 2010a, in preparation)
4302	*nadA* promoter, unaltered FarR binding site via StuI in pPCR-Script	Operon-1	Amp	Operon Biotech., Germany
4303	*nadA* promoter, deleted FarR binding site via StuI in pPCR-Script	Operon-2	Amp	Operon Biotech., Germany
4304	*nadA* promoter,transversed FarR binding site via StuI in pPCR-Script	Operon-3	Amp	Operon Biotech., Germany
4305	*nadA* promoter with pointmutation G7T, C10A via StuI in pBluescript II	Point-mutation1	Amp	MWG Eurofins, Germany
4306	*nadA* promoter with pointmutation T6G, A11C via StuI in pBluescript II	Point-mutation2	Amp	MWG Eurofins, Germany
4307	*nadA* promoter with pointmutation A5C, T12G via StuI in pBluescript II	Point-mutation3	Amp	MWG Eurofins, Germany
4308	*nadA* promoter with pointmutation T4G, A13C via StuI in pBluescript II	Point-mutation4	Amp	MWG Eurofins, Germany

WUE-ID*	Description	Plasmid	Resistance#	Reference
4309	*nadA* promoter with pointmutation A3C, T14G via StuI in pBluescript II	Point-mutation5	Amp	MWG Eurofins, Germany
4310	*nadA* promoter with pointmutation A2C, T15G via StuI in pBluescript II	Point-mutation6	Amp	MWG Eurofins, Germany
4311	*nadA* promoter with pointmutation T1G, A16C via StuI in pBluescript II	Point-mutation7	Amp	MWG Eurofins, Germany
4399	NMB1994 (*nadA*) with XbaI and XhoI restriction sites in pTL1	pTL1-*nadA*	Amp	This study (Schielke *et al.*, 2009)
4401	Meningococcal expression plasmid pAP1 with NMB1843 (FarR) cloned via EcoRI restriction sites behind the *porA* promoter	pAP1*farR*	Ery	This study (Schielke *et al.*, 2009)
4475	Deletion plasmid for *nadA*, insertion of a SmR cassette with AvrII sites (3563) in pTL1-*nadA* (4399)	pTL1-*nadA*-SmR	Amp Spec	This study (Schielke *et al.*, 2009)
4517	NMB1418 (*lpxL1*) with EcoRI restriction sites in pTL1	pTL1-*lpxL1*	Amp	This study (Schielke *et al.*, 2010, in preparation)
4518	Deletion plasmid for *lpxL1*, insertion of a SmR cassette with AvrII restriction sites (3563) in *lpxL1* from pTL1-*lpxL1* (4517)	pTL1-*lpxL1*-SmR	Amp Spec	This study (Schielke *et al.*, 2010, in preparation)
4531	Deletion plasmid for *farR*, insertion of a SmR cassette with AvrII restriction sites in pTL1	pTL1-*farR*-SmR	Spec Amp	This study
4532	Middle fragment of the NMB 0318/0319 operon (*farAB*) with EcoRI restriction sites in pTL1	pTL1-*farAB*	Amp	This study (Schielke *et al.*, 2010a, in preparation)
4533	Deletion plasmid for the *farAB* operon, insertion of a SmR cassette via AvrII restriction sites in *farAB* (4532)	pTL1-*farAB*-SmR	Spec Amp	This study (Schielke *et al.*, 2010a, in preparation)

*ID according to the IHM Würzburg strain collection

Resistance: Amp: Ampicillin, Kan: Kanamycin, Ery: Erythromycin, Spec: Spectinomycin, SmR: Spectinomycin resistance cassette

4.6. Table of oligonucleotides

AGSU-ID*	Name	Sequence (5' - 3')#
35	nadA9	CAG ATA TTA ATG CCG AAC TAC C
36	nadA10	GGC ATA TAT ACA ATA CAT TGT CGC
44	nadA3	ACC CAA CGA TAT TTT CAC CGC
45	nadA6	CTT GCA GTT AGG TAG TTC GGC
119	farR3	ACG CCC ACA GGC TTT CCC GAC
120	farR4	TGC TGC ACA CAA GGC ATC CCC
123	farR5	TAT CGG TCT GAT ACA GGC
124	farR6	CTC GGC AGT CAG CTT CAG
127	petB1	TGG TAT TGC TCG GCT TCC TT
128	petB2	TAC CAT ACC GGC GCA ATG TG
129	nadA4	CGG CCT TTT TGT CGG ATT TAG
130	nadA5	ATT CAG ACG GCA ACC CAT ATC
131	nadA7	CGT CCT CGA TTA CGA AGG CAA
132	nadA8	ATT CTG CCG GTG TCG TGT CTT
160	NMB2052 qRT1	CCA ATA GCA AAG CAA AAG CA
161	NMB2052 qRT2	TGA GCC GAA GAA ATA CCA GAA
191	farRcom2	GCG CGC GAA TTC CGT AAT ACG AGT TCA ACG CAT
196	farAB1	CGG TTC CGT ACT ATT TGT AC
197	farAB2	CAA AAA ACA ACG CTT CAG GC
214	FarR shift fw	GCC TGC CGA TGT AAT CCC T
215	FarR shift rev	GCA TCG GAC GAC TGG GTA TA
218	farRcom1	GCG CGC GAA TTC CTT GGA GAC TTA TAC AAT GCC
225	nadA11	GCG CGC TCT AGA AAC ACT TTC CAT CCA AAG
226	nadA12	GCG CGC CTC GAG TTA CCA CTC GTA ATT GAC G
227	SmR HincII fw	GCG CGC GTC GAC ATG CGC TCA CGC AAC TGG T
228	SmR HincII rev	GCG CGC CAG CTG TTA TTT GCC GAC TAC CTT GGT
229	NMB1842 fw	CAA ACT TCG TTC CGT GAT GC
230	NMB1842 rev	CCG ATT TCA TGT TTG CCG AC
231	NMB1842 rev seq	GGA TGC GTT GAA CTC GTA AT
236	M13 fw	GTA AAA CGA CGG CCA G
237	M13 rev	CAG GAA ACA GCT ATG AC
240	nadA13	CGA AGC CCT AGG CGA TAT CGC CGA TTC ATT GG
241	nadA14	CCC AAT CCT AGG GGC GTT GGT GGT TTC ATC CA
242	farR prom. fw	GCG CGC AGA TCT CTT TCC GAC TGT CAA CGA TG
244	hrtA fw	CCT GCC GGT GTA CCA AAT CG
245	hrtA rev	AAT ATT TGC CGA GCG TGC CC
246	porA prom. rev	CGA AAC ACC CGA TAC GTC TT
247	lacZ rev	AGA GAT TCG GGA TTT CGG CG
249	farR prom. rev	GCG CGC AGA TCT GTA GGC ATT GTT TAA GTC TC
250	nadA prom. fw	GCG CGC AGA TCT TGA CAA AAT TAA GAC ACG AC

AGSU-ID*	Name	Sequence (5' - 3')#
251	nadA prom. rev	GCG CGC AGA TCT AAG TGT TTC ATG CTC ATT AC
256	lpxL1.1	AAT CCT TCG GGG ATG CAG GT
257	lpxL1.2	TTT CGA CTC GAA ACG CCT GA
258	lpxL1.3	GCG CGC GAA TTC GAT CAT TTT TAC GTC GCC TC
259	lpxL1.4	GCG CGC GAA TTC GCC ATT TTC TAC GCT TTG C
260	lpxL1.5	CTG CCG CCT AGG ACC TGC ATC CCC GAA GGA TT
261	lpxL1.6	AAG ACA CCT AGG GCG AAC ATC CGG AAC AAT AT
262	lpxL1.7	CCG TGT TGA AAC A
263	lpxL1.8	AGG GTA GAA ATG C
268	farR ko fw	GCG CGC CCT AGG CCT ACC CAA TCA AAA CAT GCG
269	farR ko rev	GCG CGC CCT AGG TGC TGC ACA CAA GGC ATC C
270	NMB1034 qRT fw	GCA AAT CCT GTT GAC GCG TAA
271	NMB1034 qRT rev	GGT AAT GTC AAA TGT GAA CGT TTC G
286	NMB1710 qRT fw	CAA CTT CAG GTT TGG AAA TGA G
287	NMB1710 qRT rev	GAC TTT GCC GTA TTT CAG AC
288	NMB1299 qRT fw	GTT GAT TAC GAC CAT TCA GGA
289	NMB1299 qRT rev	GAC CAT AGC TCA GAA TGG AC
290	NMB0207 qRT fw	GTT TCC ATG GAC TTC AAC CA
291	NMB0207 qRT rev	CAT TCG TTG TCA TAC CAA GC
292	NMB0866 qRT fw	CCA CAC TTT CCT TAA AGA CGA
293	NMB0866 qRT rev	CGG ATC TAT GGC TTT GTA GG
302	farAB inv fw	GTC GCA CCT AGG GTT AAT GGC ATC CTA TCC GC
303	farAB inv rev	GGA CGG CCT AGG GCT GCA CCA CTT TAA TCC AG
306	farAB ko fw	GCG CGC GAA TTC GAC GAT AAT GAT GTG CTG GC
307	farAB ko rev	GCG CGC GAA TTC CAA TGA CGC CGA CGG TAA AA
	farR1	CCT ACC CAA TCA AAA CAT GCG
	farR2	TAT AAG GTC CAT GCT GCA CAC AAG GCA TCC
	farR7	CGA GTT CAA CGC ATC CTC
	nadA1	CAG ACA CTG ATG CCG CTT TA
	nadA2	GGC GAT ATC GTT GAA TGC TT
	AP1	GGC ATT TAT GAG ATA TTT GTT C
	AP2	GCT CAG AGC ATG GCT TTA TG

*ID according to the AG Schubert-Unkmeir oligonucleotide collection

Restriction sites are underlined

4.7. Buffers and solutions

Unless indicated otherwise, all buffers and solutions were prepared with distilled water.

Microarray blocking solution (2 x)

Tris	0.2 M
Ethanolamine	6.2 ml
pH 9.0	

50 x TAE

Tris	242 g
EDTA	37.2 g
Acetic acid (96%)	59.5 ml
ad 1000 ml	

GEBS

Glycerin (20%)	20 ml
Sarcosyl (0.5%)	5 ml
EDTA (50 mM)	10 ml
Bromophenol blue	0.05 %
ad 100 ml, pH 8.0	

10 x SDS buffer

Tris	60.6 g
Glycin	287.4 g
SDS (20 %)	50 ml
ad 2000 ml	

10 x Laemmli buffer

Tris	60 g
Glycin	288 g
ad 2000 ml, pH 8.7	

Western Blot buffer

10 x Laemmli buffer	200 ml
Methanol	400 ml
ad 2000 ml	

Coomassie Staining Solution

Acetic acid	40 ml
Ethanol (96 %)	200 ml
Coomassie Brilliant Blue	200 mg
ad 400 ml	

Coomassie Destaining Solution

Methanol	400 ml
Acetic acid	200 ml
ad 2000 ml	

$CaCl_2$-solution

$CaCl_2$	60 mM
PIPES, pH 7.0	10 mM
Glycerin	15 %
filtered sterile, stored at 4°C	

PBS-T

PBS (1 x)	
Tween-20	0.05 %

VBS/BSA

Barbital	5 mM
NaCl	145 mM
$MgCl_2$	0.5 mM
$CaCl_2$	0.15 mM
BSA	0.5 %
adjusted to pH 7.4 in 1 x PBS	

X-Gal

X-Gal	200 mg
ad 5 ml with DMF	

5 x Sample Solution

Glycerine	50 %
Tris (250 mM, pH 6.8)	25 %
β-Mercaptoethanol	25 %
SDS (w/v)	10 %

Buffer B1

Na_2EDTA	1.86 g
Tris	0.61 g
Tween-20	5 ml
Triton X-100	5 ml
ad 100 ml, pH 8.0	

Buffer B2

Guanidine-HCl	28.7 g
Tween-20	20 ml
ad 100 ml	

Electroelution-Buffer

Tris	25 mM
Glycin	192 mM
SDS	0.025 %

4.8. Culture Media and Agar Plates

Proteose-Peptone-Medium (PPM)

Proteose-peptone	1.5%
NaCl	0.5 %
Starch	0.05 %
KH_2PO_4	0.4 %
K_2HPO_4	0.1 %
pH 7.8	

Supplemented Proteose-Peptone-Medium (PPM^+)

PPM	50 ml
$MgCl_2$ (2 M)	250 µl
$NaHCO_3$ (8.4 %)	250 µl
PolyViteX	500 µl

LB Medium

Bactotryptone	1 %
yeast extract	1 %
NaCl	1 %
Bacto-Agar	1.5 %
(for agar plates)	

SOC-Medium

Bactotryptone	2 %
yeast extract	0.5 %
NaCl	10 mM
KCl	2.5 mM
Glucose	20 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM

Neisseria Defined Medium (NDM)

NDM (Archibald & DeVoe, 1978), modified by Martin Lappann

L-Glutamic acid	10 mM
D-Glucose	10 mM
L-Cysteine	1 mM
Uracil	1 mM
L-Arginine	1 mM
Tris	40 mM
NaCl	140 mM
NaH_2PO_4	1 mM
Na_2SO_4	2.3 mM
$CaCl_2$	0.5 mM
$MgSO_4$	0.2 mM
KCl	2 mM
NH_4Cl	10 mM
$ZnCl_2$	20 nM
$CuCl_2$	20 nM
$CoCl_2$	20 nM
$MnCl_2$	20 nM
$FeNO_3$	1 mg
ad 1000 ml, pH 7.6	
$NaHCO_3$	5 mM
autoclave	
PolyViteX	1 %

GC Agar plates

Difco™ GC Medium Base	36 g
ad 1000 ml, autoclave	
PolyViteX	1 %

4.9. Materials for Electrophoretic Mobility Shift Assays

TEN-Buffer

Tris	10 mM
EDTA	1 mM
NaCl	0.1 M
pH 8.0	

10 x TBE

Tris	890 mM
Boric acid	890 mM
EDTA	20 mM
pH 8.0	

Maleic acid Buffer

Maleic acid	0.1 M
NaCl	0.15 M
pH 7.5, adjusted with solid NaOH	

Washing Buffer

Maleic acid buffer	
Tween-20	0.3 %

Detection Buffer

Tris	0.1 M
NaCl	0.1 M
pH 9.5	

Blocking solution (10 x)

Maleic acid Buffer	
Blocking reagent (w/v)	10 %
solved in the microwave, autoclaved and stored at -20°C	

Antibody solution

Blocking solution (1 x)	
Anti DIG-AP	1:10000

CSPD working solution

Detection buffer	
CSPD	1:250

SSC (20 x)

NaCl	3 M
Sodium citrate	0.3 M
pH 7.0	

Native polyacrylamide gel (6 %)

Aqua dest.	14.9 ml
TBE (10x)	1 ml
Polyacrylamide	4 ml
APS (16%)	90 µl
TEMED	20 µl

4.10. Cultivation of bacteria

Neisseria meningitidis was inoculated from freezer stocks onto Columbia Agar Plates with 5 % sheep blood (COS plates) and incubated over night at 37°C with 5 % CO_2. When using recombinant strains, they were cultivated on gonococcal (GC) Agar Plates supplemented with PolyViteX and the corresponding antibiotic, kanamycin (100 µg/ml), erythromycin (7 µg/ml), chloramphenicol (7 µg/ml) or spectinomycin (125 µg/ml). For cultivation in liquid culture, the strains were inoculated in either supplemented proteose peptone medium (PPM^+) or *Neisseria* defined medium (NDM) with cotton swabs and incubated for the appropriate time at 37°C and 200 rpm. *Neisseria gonorrhoeae* was inoculated from freezer stocks onto GC agar plates. For cultivation in liquid culture, strains were grown in PPM^+. *E. coli* was streaked onto Lennox L (LB) agar plates, optionally with the corresponding antibiotic, kanamycin (30 µg/ml), erythromycin (30 µg/ml) or spectinomycin (75 µg/ml). In liquid culture, strains were inoculated into LB Medium.

4.11. Determination of bacterial cell number

For adjusting a defined number of bacteria, strains were grown in liquid culture and at the designated time point, 1 ml was transferred to a cuvette and the absorption at 600 nm was measured with a photometer against the culture medium. For calculation of the cell number, 1 OD_{600} is equivalent to approximately $1 \cdot 10^9$ meningococci or gonococci. For *E. coli*, 1 OD_{600} corresponds to approximately $2 \cdot 10^8$ bacteria.

4.12. Generation of chemically competent *E. coli* TOP10 cells

Chemically competent TOP10 cells were obtained from Invitrogen and multiplied for further usage. 10 µl TOP10 bacteria were inoculated into 100 ml LB-medium and grown over night at 37°C and 200 rpm. 2 ml of this culture were transferred into 250 ml fresh LB Medium and grown until an OD_{600} of 0.5 was reached. Then they were incubated for 10 minutes on ice in a pre-cooled centrifuge tube. After pelleting for 10 minutes at 4°C and 3752 g, bacteria were resuspended in 50 ml ice-cold 0.1 M $MgCl_2$ solution. They were kept for 20 minutes on ice, pelleted for 5 minutes at 0°C and 1351 g, resuspended in 5 ml ice-cold $CaCl_2$ solution, distributed in fresh caps à 200 µl and stored at -80°C.

4.13. Transformation of *E. coli*

Chemically competent *E. coli* TOP10 cells (chapter 4.12) were thawed slowly on ice. The cells were incubated for 30 minutes on ice with the vector - 10 µl ligation reaction or 1 µl purchased vector. Subsequently the cells were heat-shocked at 42°C for 45 seconds. They were immediately put back on ice and kept there for 5 minutes. Subsequently, 250 µl SOC Medium was added and the cells were incubated for 45 minutes at 37°C shaking horizontally. Afterwards, transformants were plated onto LB agar plates with the required antibiotic.

4.14. Transformation of *N. meningitidis*

As *Neisseria meningitidis* are naturally competent organisms, no heat shock is necessary. Bacteria were grown in PPM^+ for 90 minutes at 37°C and 200 rpm, then $2 \cdot 10^8$ bacteria were transferred to a 15 ml tube and adjusted with PPM^+ to a final volume of 1 ml. Of the transforming DNA (Plasmid Midi preparation, chapter 4.18), 10 µg were added and the cultures were incubated for 5 hours at 37°C and 200 rpm. Subsequently, bacteria were pelleted at 4000 rpm for 5 minutes at room temperature. 800 µl of the supernatant were discarded and the pellet was resuspended in the remaining medium. Finally, the bacteria were plated onto GC agar plates supplemented with the required antibiotic.

4.15. Preparation of meningococcal genomic DNA

Meningococci were streaked onto two agar plates and grown over night. The whole bacterial material was inoculated into 5 ml 1 x PBS with a cotton swab. The bacterial cell number was determined (chapter 4.11) and $1.5 \cdot 10^{10}$ bacteria were transferred to a fresh cap and pelleted at 4000 rpm for 10 minutes at 4°C. The pellet was redissolved in 7 ml buffer B1 and 14 µl RNase-solution (100 mg/ml). After addition of 160 µl lysozyme (100 mg/ml), the mixture was incubated for 30 minutes at 37°C prior to freezing for 60 minutes at -80°C. Subsequently the solution was thawed at 50°C and 2.4 ml buffer B2 as well as 200 µl proteinase K solution (20 mg/ml) were added. The solution was mixed thoroughly and incubated for 60 minutes at 50°C. 15 ml buffer QBT was added and the DNA was extracted using the Qiagen genomic tip-100 kit. The resulting purified DNA pellet was dried and resuspended in 100 µl distilled water. The DNA concentration was measured using a NanoDrop 1000 (Peqlab) and stored at -20°C.

4.16. Polymerase Chain Reaction (PCR)

PCR was used to amplify specific DNA products or to prove mutagenesis of candidate mutant strains. Generally, either 60 ng genomic DNA (chapter 4.15) or 22.5 µl of a suspension of mutant colonies (cloning reactions see chapter 4.19) was used as template DNA. Oligonucleotides were added at a concentration of 10 µM. After addition of 2 µl dNTPs (dATP, dCTP, dGTP and dGTP at a concentration of 4 mM each), 3 µl polymerase buffer and 0.5 µl Taq polymerase the final volume was adjusted to 30 µl with distilled water, mixed thoroughly and centrifuged briefly. The probes were transferred to

the thermal cycler as soon as the latter reached the denaturing temperature of 95°C. The PCR program was initiated with a denaturing sequence at 95°C for 10 minutes (genomic DNA) or 15 minutes (mutant test PCR), followed by step 2, a 30 second denaturing step at 95°C. The annealing temperature for the respective oligonucleotides was kept for 1 minute; the elongation step was set at 72°C for 1 minute. The PCR was performed for 35 cycles between the last step and step 2. Afterwards the PCR was cooled down to 15°C prior to further examination.

4.17. Visualization and Purification of PCR products

Detection of the PCR products was carried out using agarosegel electrophoresis. Therefore, agarose was weighed and added to 1 x TAE buffer at a final concentration of 1 % (w/v). The mixture was solved in a microwave, cooled down and poured into a gel-chamber. The PCR products were mixed with 3 µl GEBS buffer, 5 µl SmartLadder were used as standard and subsequently the probes were subjected to electrophoresis in 1 x TAE buffer at 170 V. Afterwards the gel was incubated in a 0.1 % ethidiumbromide bath for 15 minutes prior to visualization with a UV-table. Depending on the continuing treatment, the PCR products were either purified using the QIAquick PCR Purification Kit or extracted from the gel with the QIAquick Gel Extraction Kit and eluted in 30 µl distilled water.

4.18. Plasmid preparation – Midi

This plasmid preparation was carried out with the QIAGEN Plasmid Midi Kit in modification from the manufacturer's protocol. The recombinant *E. coli* strain was inoculated in two separate 50 ml LB Medium cultures supplemented with the respective antibiotic. The cultures were incubated over night at 37°C and 200 rpm, and then pelleted at 4000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellets were pooled and resuspended in 4 ml pre-cooled buffer P1 by inverting the tube 5 times. Then the reaction was incubated for 5 minutes at room temperature prior to addition of 4 ml ice-cool buffer P3. The reaction was mixed again by inverting the tube, then the reaction was incubated for 15 minutes on ice. Subsequently the liquid was distributed into six 2 ml caps and centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant was transferred to fresh caps and centrifuged again for 15 minutes at 14000 rpm at 4°C. QIAGEN Tip-100 columns were equilibrated with 4 ml buffer QBT, and then the supernatant was added followed by two washing steps with 10 ml buffer QC. The DNA was eluted into a fresh tube with 5 ml buffer QF and mixed with

3.5 ml isopropanol. This mixture was transferred to five 2 ml caps and the DNA was pelleted at 14000 rpm for 30 minutes at 4°C. The supernatant was removed; the DNA washed with 500 µl 70 % ethanol and the supernatant discarded. The pellet was dried by vacuum concentration in the Savant SpeedVac®Plus (Thermo Scientific) for 5 minutes and redissolved in 20 µl distilled water.

4.19. Construction of mutant strains

4.19.1. Construction of translational *lacZ* fusions

Reporter gene constructs were generated by fusing the promoter region of the respective gene and the first coding triplets in frame to codon no. 10 of the *lacZ* gene (Von Loewenich *et al.*, 2001). To clone the *farR* promoter region in front of the *lacZ* gene, a PCR product was amplified from genomic DNA of MC58 with the oligonucleotides 242 and 249 (see Table 4.6) containing BglII sites. The DNA fragment was digested for 2 hours at 37°C and purified with the QIAGEN PCR Purification Kit. Ligation was prepared with the T4 DNA Ligase with 50 ng pSMS1 (kindly provided by Sabine Marita Schulz and Prof. U. Vogel, Würzburg; for description see Table 4.5) and 150 ng PCR product in a final volume of 20 µl and incubated over night at 16°C. The resulting plasmid was transformed into *E. coli* TOP10 cells (see chapter 4.13) and transformants were selected on LB-agar plates supplemented with 30 µg/ml kanamycin, 100 µg/ml ampicillin and 500 µM X-Gal. For analysis, one colony was picked with a sterile toothpick and inoculated into 50 µl distilled water. 22.5 µl of this suspension was applied to a colony PCR (chapter 4.16) to verify positive clones. Subsequently the remaining bacterial suspension was inoculated into 4 ml LB-medium with kanamycin and ampicillin and incubated at 37°C at 200 rpm over night prior to purification (chapter 4.18). Afterwards, the plasmid was used to transform the parental strain MC58 (chapter 4.14). Correct integration of the *farR* promoter region was verified by PCR using the oligonucleotides 242 and 247, 244 and 249. As positive control an MC58 strain with the constitutively expressed *porA* gene (NMB1429) in front of *lacZ* was generated with pSMS5 (kindly provided by Sabine Marita Schulz and Prof. U. Vogel, Würzburg; described in Table 4.5). As negative control, strain MC58 was transformed with the promoterless *lacZ* gene construct pSMS1. For generating the *nadA* promoter/*lacZ* fusion products, the unaltered *nadA* promoter fragment was amplified by PCR using the oligonucleotides 250 and 251. The *nadA* promoter fragments with deleted or transversed FarR binding site were obtained from Eurofins MWG Operon. The promoter fragments were cloned in frame in front of the *lacZ* gene as described above and transformed into strain MC58. All candidate mutants were verified by PCR and direct sequencing.

4.19.2. Construction of *nadA* deletion strains

To create the insertional mutation to abolish transcription of the *nadA* gene (NMB1994), the *nadA* gene was amplified using the oligonucleotides 225 and 226, cleaved with Xba I and Xho I and cloned into the pTL1 vector, resulting in pTL1-*nadA*. An inverse PCR was conducted with the oligonucleotides 240 and 241 using the High Fidelity Platinum Taq Polymerase, modifying the PCR program (chapter 4.16) to 68°C for the elongation temperature. The plasmid containing the spectinomycin cassette was extracted via plasmid Midi preparation (chapter 4.18) from *E. coli* strain #3563. Both the PCR product and the plasmid were digested with AvrII for 3 hours at 37°C, purified and ligated as described above. The resulting vector pTL1-*nadA*-SmR was transformed into *E. coli* TOP10 cells (see chapter 4.13); positive transformants were selected, verified and finally inoculated for plasmid Midi preparation. Afterwards, the parental strains MC58 and MC58 Δ*farR* were transformed (chapter 4.14) with 10 µg of plasmid pTL1-*nadA*-SmR and selected on GC agar plates supplemented with spectinomycin. All mutant strains were confirmed by PCR, direct sequencing and Western blot analysis. Additionally, the presence of Opa, Opc and pili was verified by Western blot analysis.

4.19.3. Construction of an unencapsulated *lptA* mutant strain

Parental strain MC58 Δ*siaD* (#2425) was transformed with plasmid pCS46 harboring a spectinomycin resistance cassette within the ORF of the *lptA* gene (NMB1638). Inactivation of *lptA* was confirmed by PCR and DNA-sequencing.

4.19.4. Construction of a *kdtA farR* double deletion strain

For construction of the *kdtA* deletion mutant, parental strain MC58 Δ*farR* (Schielke *et al.*, 2009) was transformed with plasmid pCS32 harboring a spectinomycin resistance cassette within the ORF of the *kdtA* gene (NMB0014). Inactivation of *kdtA* was confirmed by PCR and direct sequencing. Additionally, the LPS immunotype was analyzed by ELISA (see chapter 4.33).

4.19.5. Construction of an insertional mutation in the *lpxL1* gene

For construction of the *lpxL1* deletion strain, the *lpxL1* gene (NMB1418) was amplified with 500 bp flanking on both sides using the oligonucleotides 258 and 259. The PCR product was digested with EcoRI and cloned into pTL1, resulting in the plasmid pTL1-*lpxL1*. An inverse PCR with the oligonucleotides 260 and 261 generated a linearized product which was digested with AvrII and

ligated with the spectinomycin cassette (for details see chapter 4.19.2). The resulting plasmid pTL1-*lpxL1*-SmR was used for transformation of the parental strain MC58 Δ*siaD* (#3240). Inactivation of *lpxL1* was confirmed by PCR and direct sequencing.

4.19.6. Complementation of a naturally occurring mutation in the *lpxL1* gene

For complementation of the ten base pair deletion within the *lpxL1* gene in strain α 24, the parental strain was transformed with the abovementioned plasmid pTL1-*lpxL1* and plated onto GC agar plates supplemented with palmitic acid. Transformants were picked and screened for complementation by PCR using the oligonucleotides 262 and 263, of which the latter encompassed the 10 bp deletion. Of the candidate complemented strains, the *lpxL1* gene was amplified and sequenced with the oligonucleotides 258 and 259. All mutant strains were checked for expression of Opa, Opc and pili by Western blot analysis.

4.19.7. Complementation of the insertional *farR* deletion

For complementation of the *farR* deletion, the *farR* gene (NMB1843) was amplified with the oligonucleotides 218 and 191 and digested with EcoRI along with the meningococcal expression vector pAP1 (Lappann *et al.*, 2006) for 2 hours at 37°C. After ligation as described above, the plasmid was transformed into *E. coli* TOP10 cells (chapter 4.13), amplified via plasmid Midi preparation and used to transform the parental strain MC58 Δ*farR*. Transformants were selected on GC agar plates supplemented with erythromycin, finally leading to the complemented strain MC58 Δ*farR* (pAP1*farR*). Candidate mutant strains were confirmed by PCR, direct sequencing and Western blot analysis. Additionally, the presence of Opa, Opc and pili was verified by Western blot analysis.

4.19.8. Construction of an insertional mutation in the *farAB* operon

For construction of the *farAB* deletion strain, a 1700 bp fragment of the *farAB* operon (NMB0318/0319) was amplified using the oligonucleotides 306 and 307. The PCR product was digested with EcoRI and cloned into pTL1, resulting in the plasmid pTL1-*farAB*. An inverse PCR with the oligonucleotides 302 and 303 generated a linearized product which was digested with AvrII and ligated with the spectinomycin cassette (for details see chapter 4.19.2). The resulting plasmid pTL1-*farAB*-SmR was used for transformation of the parental strains MC58 (#2135) and MC58 Δ*siaD* Δ*kdtA* (#3711). Inactivation of *farAB* was confirmed by PCR and direct sequencing.

4.20. Reverse Transcription PCR

Reverse transcription PCR was applied to verify the transcription of the downstream genes of FarR (NMB1843) despite its deletion in the mutant strain MC58 Δ*farR*. Therefore, RNA of this strain was prepared (method see chapter 4.28.1) and stored at -80°C. A reverse transcription reaction was prepared with the OIAGEN OneStep RT-PCR Kit according to manufacturer's instructions, utilizing 4 µl template RNA and the oligonucleotides 229 and 230 for the *farR*-adjacent gene NMB1842. The resulting PCR mastermix was split into two fresh caps and submitted to PCR or RT-PCR, respectively. As the control PCR showed no signal, the RNA preparation was DNA free and the resulting product in RT-PCR transcript of the NMB1842 gene.

4.21. Preparation of meningococcal whole cell lysates

Meningococci were streaked on plates and inoculated into PPM+ the next day. These liquid cultures were kept shaking at 200 rpm for 90 minutes (unless indicated otherwise) at 37°C. Afterwards $2 \cdot 10^8$ bacteria were pelleted at 13000 rpm for 5 minutes at room temperature and redissolved in 25 µl 5 x sample solution. This mixture was heated to 100°C for 10 minutes and stored at -20°C up to separation via SDS gel electrophoresis (chapter 4.23).

4.22. Quantification of protein

Quantification of protein in meningococcal lysates (chapter 4.21) or of purified protein (chapter 4.25) was performed using the BCA Protein Assay Kit, which utilizes bichinchoninic acid for protein detection. A standard dilution series of BSA was prepared according to manufacturer's instructions. Protein dilution series were prepared at 1:100 and 1:1000. 25 µl of each probe was transferred in triplicate (standard) or in duplicate (probes) to a 96 well plate. Distilled water served as negative control. The working reagent was prepared and 200 µl added to each well. The plate was shaken briefly and incubated for 30 minutes at 37°C. After cooling to room temperature, the absorption of the solutions was measured at 570 nm.

4.23. SDS PAGE

Separation of protein probes was accomplished with denaturing polyacrylamide gel electrophoresis. Therefore, 12.5 % gels were cast consisting of a separation gel and a loading gel. The separation gel was prepared of 2.5 ml Tris (1.5 M, pH 8.8), 50 µl SDS (20 %), 3.23 ml H_2O, 4.17 ml polyacrylamide, 60 µl APS (16 %) and 10 µl TEMED. This gel was left to dry for 30 minutes, covered with a layer of distilled water. After the polymerization time and removal of the excess water, the loading gel was cast on top, consisting of 1.25 ml Tris (0.5 M, pH 6.8), 25 µl SDS (20 %), 700 µl polyacrylamide, 3 ml H_2O, 30 µl APS (16 %) and 5 µl TEMED. After polymerization, the gel was kept in humid atmosphere at 4°C until usage. For gel electrophoresis, the gel was loaded with the protein probe (10 mg whole cell lysate with known protein concentration, see chapter 4.21 or purified protein, see chapter 4.25.2) and 10 µl prestained protein ladder was used as standard. The electrophoresis was performed in 1 x SDS buffer at 160 V.

4.24. Western Blot analysis and antibody detection

Subsequently the gel was transferred to a Western Blot tank to be blotted onto a nitrocellulose membrane in Western Blot buffer at 300 mA for 60 minutes. Then the membrane was washed for 60 minutes in blocking solution (5 % low-fat milk powder (w/v) in PBS-T) prior to incubation over night in blocking solution with the respective antibody. The immune serum against NadA was utilized at a dilution of 1:2000, the antibody against FarR at 1:5000 and the antibody against PorA at 1:2000. Mutant strains were analyzed for expression of pili (mouse α pili 1:4000), Opc (mouse α Opc 1:1000) and Opa (mouse α Opa 1:1000). The membrane was washed twice with PBS-T, and then incubated for one hour with secondary antibody directed against the IgGs of the primary antibody, coupled with horseradish peroxidase (HRP). Anti-rabbit-HRP or anti-mouse-HRP antibodies were used at a dilution of 1:5000 for 60 minutes in PBS-T. After another two washing steps, the membrane was covered for 5 minutes with 1 ml ECL Western Blot detection Kit substrate. An X-ray film was then exposed to the membrane packed in plastic wrap, followed by signal detection.

4.25. Expression and purification of the recombinant protein FarR

4.25.1. Expression and isolation of FarR

E. coli strain #3694, containing a plasmid with the *farR* gene in front of a His_6 tag, was grown on LB agar supplemented with ampicillin, inoculated into 25 ml LB Medium with 100 µg/ml ampicillin and cultivated at 37°C and 200 rpm over night. 10 ml of this suspension were transferred to 1 liter fresh LB Medium with ampicillin and grown at 37°C and 220 rpm until an OD_{600} of 0.5 was reached. Protein expression was induced with 0.002 % arabinose and the cultures were incubated over night at 20°C shaking with 220 rpm. Bacteria were pelleted at 4000 rpm for 30 minutes at 4°C and resuspended in 10 ml ice-cold 1 x PBS supplemented with 1 % Triton X-100. The lysate was separated and 5 ml each were transferred to 50 ml tubes. A spattle tip lysozyme was added and the mixture incubated for 10 minutes on ice. For ultrasound treatment, a Sonifier 450 (Branson) was pre-cooled to 4°C, set to Duty Count 50 and Output Control 4-5. The bacterial lysate was treated 4 times for 30 seconds with ultrasound, interrupted each time with 30 seconds incubation on ice. Afterwards the cell debris was removed by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and stored at -20°C.

4.25.2. Purification of FarR

For purification of the protein, 2 ml nickel beads (ProBond™ Resin, Invitrogen, 50 % slurry in 20 % ethanol) were washed three times with 2 ml 1 x PBS containing 1 % Triton X-100, in between being settled down at 500 rpm for 1 minute. After these washing steps, the culture supernatant was mixed with the beads and incubated over night at 4°C with slight movement. The beads were filled into a column and the flow-through was captured, mixed with 10 % glycerin and stored at -20°C. The beads were washed twice with 10 ml 1 x PBS containing 1 % Triton X-100 adjusted to a pH of 8.0 by slow inverting of the stoppered column. The washing fluids were captured and stored as described. A imidazole stock solution was prepared with 3 M imidazole in 1 x PBS with 1 % Triton X-100 and adjusted to a pH of 6.0. Then the first elution step was performed with 10 ml 5 mM imidazole solution. Subsequently, 1 ml of 20, 40, 60, 80, 100, 200, 500, 750, 1000 and 1500 mM imidazole were added to the column, captured separately and stored at -20°C. The different elution steps were applied to 12.5 % SDS gels (preparation see chapter 4.23) and stained for 30 minutes with Coomassie Blue. After destaining for 60 minutes with multiple buffer exchanges the protein bands became visible and were excised to undergo electroelution.

4.25.3. Electroelution and dialysis of FarR

The BIOTRAP chamber BT 1000/5 (Schleicher & Schuell) was prepared for electroelution according to manufacturer's protocol and filled with electroelution-buffer. The small polyacrylamid-gel-pieces were transferred to the chamber and covered with buffer. Electrophoresis was performed at 200 V for 4 hours, then at 100 V over night. To detach the protein from the BT1 membrane, the polarity of the current was changed for 20 seconds at 200 V. The probe (0.8 – 1 ml) was extracted from the trap and transferred to a Visking dialysis tube (14 kDA exclusion, Roth), which had previously been cooked for 10 minutes at 100°C. After addition of the protein solution, the tube was closed very tightly and incubated in 5 liters 0.5 x TBE over night at 4°C. The buffer was exchanged and dialysis was allowed to proceed for another 2 hours. Subsequently the protein was transferred to a fresh cap and the protein concentration was measured (see chapter 4.22).

4.26. Generation of an affinity purified antibody against FarR

A purified antibody against FarR was obtained from immunoGlobe® Antikörpertechnik GmbH (Himmelstadt). For this purpose, 1 mg of the dialyzed protein (for extraction and purification see chapter 4.25) was used to immunize a rabbit. The resulting serum was affinity purified by immunoGlobe®, using another 1 mg of the dialyzed protein.

4.27. Electrophoretic mobility shift assays (EMSAs)

The electrophoretic mobility shift assay or gel mobility shift assay was employed to visualize interaction of the transcriptional regulator FarR with target DNA sequences. The DNA sequence is labeled with digoxigenin and can thus be visualized by chemiluminescence. Upon addition of the purified protein, interaction can occur and direct binding leads to a "shift" as the DNA-protein-complex moves slower through the gel. For examination of the specificity of this reaction, excess amounts of competitor probes are added. These are either specific competitors (the respective DNA probe but unlabeled) or unspecific competitors (unlabeled DNA probes of another sequence). Incubation of the protein-labeled-DNA-complex with a specific competitors leads to a "reversal" of the shift as the protein binds to the abundant unlabeled DNA probe and the shift is therefore no longer visible in the gel.

4.27.1. Labeling of oligonucleotides and determination of labeling efficiency

DNA fragments containing overlapping parts of the *nadA* promoter region were generated by PCR from genomic DNA of MC58 using the oligonucleotides (sequences listed in 4.1) 44 and 129 (fragment 1), 130 and 45 (fragment 2), 131 and 132 (fragment 3), 44 and 45 (fragment 4) and 35 and 36 (fragment 5). The *farAB* promoter region was generated using the oligonucleotides 196 and 197. DNA probes of 100 bp length were obtained within a recombinant vector (listed in Table 4.5), transformed into *E. coli* TOP 10 cells, digested with StuI and purified, resulting in recovery of the DNA probes. The purified PCR products and DNA probes, respectively, were 3' end-labeled with digoxigenin using the DIG Gel Shift Kit. For this purpose, sterile distilled water and 100 ng PCR product were adjusted to a final volume of 10 µl in a 1.5 ml reaction vial. For the control reaction, 1 µl oligo (vial 6) was handled accordingly. The oligonucleotides were heated for 10 minutes at 95°C, and then cooled on ice. The following reagents were added on ice.

Labeling Buffer	(Vial 1)	4 µl
$CoCl_2$-Solution	(Vial 2)	4 µl
DIG-ddUTP-Solution	(Vial 3)	1 µl
Terminal Transferase	(Vial 4)	1 µl

The reaction was mixed and centrifuged briefly. Subsequently it was incubated at 37°C for 15 minutes prior to addition of 2 µl EDTA (0.2 M, pH 8.0) on ice for stopping the reaction. Addition of 3 µl sterile, distilled water led to a final concentration of 4 ng/µl DIG-labeled oligonucleotide. The efficiency of the labeling reaction was checked by comparison of spotted dilution series with the labeled control-oligonucleotide (vial 7) on a positively charged nylon membrane. The nucleic acids were fixed to the membrane by cross linking with UV-light (120 mJ, Stratalinker, Stratagene) followed by a 2 minute incubation in washing buffer at room temperature. The membrane was then submerged face up in blocking solution for 30 minutes before incubation with the antibody solution for another 30 minutes. The membrane was washed two times for 15 minutes in washing buffer and equilibrated for 5 minutes in detection buffer. 500 µl CSPD working solution was spread equally over the surface and incubated in the dark for 5 minutes. Afterwards the membrane was wrapped tightly in plastic wrap and excess liquid was squeezed out. After incubation at 37°C for 10 minutes the membrane was exposed to an X-ray film. The 4 pg spots should be visible in the control and in the labeled oligonucleotides.

4.27.2. Gel Shift Reaction

A 6 % polyacrylamide gel was cast the previous day and stored at 4°C in humid atmosphere. It was pre-electrophoresed at 160 V in 0.5 x TBE buffer. The labeled oligonucleotides were diluted to a final concentration of 0.4 ng/µl and mixed with the following reagents on ice.

Binding buffer	(Vial 5)	2 µl
poly [d(I-C)]	(Vial 9)	0.5 µl
poly L-Lysine	(Vial 11)	0.5 µl
FarR protein		150 ng – 2 µg
Competitor DNA		400 ng – 2 µg
H_2O		ad 10 µl

The reaction was mixed, centrifuged briefly and incubated for 15 minutes at room temperature. Afterwards, 2.5 µl loading buffer (Vial 13) was added on ice and the probes were immediately applied to the gel. Electrophoresis was performed at 160 V for approximately 30 minutes, then the DNA was blotted with the standard protocol for electroblotting onto a nylon membrane at 300 mA for 60 minutes in 0.5 x TBE buffer. The membrane was put onto a Whatman-paper soaked in 2 x SSC and subjected to UV-crosslinking. After incubation for 20 minutes in washing buffer, the chemiluminescence detection was performed as described above.

4.28. Microarray Analysis

4.28.1. RNA preparation

RNA preparation for microarray analysis was performed by inoculating MC58 and MC58 Δ*farR* in PPM^+ and growing them for 60 minutes at 37°C and 200 rpm. Subsequently, the OD_{600} was adjusted to 0.1 in 6 ml PPM^+ for each strain and the cultures were incubated shaking for 2.5 hours to mid log phase. Bacteria were pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C, resuspended in 1 ml TRIzol® Reagent and transferred to FastRNA Blue caps (BIO 101, USA) followed by mechanical disruption for 45 s at a speed of 6.5 in a FastPrep®-24 system (MP Biomedicals, USA). The supernatant was centrifuged for 5 minutes at 13000 rpm at room temperature. The resulting supernatant was transferred to a fresh cap prior to addition of 200 µl chloroform and incubation for 2 minutes at room temperature. The mixture was vortexed thoroughly and centrifuged for 10

minutes at 13000 rpm at 4°C. The upper phase was carefully transferred to a fresh cap and mixed with 500 µl ice-cold isopropanol. The RNA was precipitated by freezing the solution for 30 minutes at -20°C. Subsequently the RNA was pelleted for 15 min at 13000 rpm at 4°C and washed with ice-cold ethanol (70 %) followed by another centrifugation step for 10 minutes at 13000 rpm and 4°C. The supernatant was discarded and the pellet dried for 5 minutes in the SpeedVac®Plus without heating. The RNA was redissolved for 10 minutes at 50°C in 100 µl RNase free water. Remaining DNA was digested using the DNA-*free* kit, with 95 µl RNA and 12 µl buffer, 5 µl DNase, 1 µl RNase out and 7 µl RNase free water. This mixture was incubated at 37°C for 60 minutes. Afterwards, 20 µl DNase inactivation reagent was added, incubated with shaking for 2 minutes at room temperature and pelleted for 2 min at 13000 rpm at room temperature. The supernatant was stored at -80°C.

4.28.2. Transcription and Hybridization

The quantity of the resulting RNA was measured using a NanoDrop and the quality was assured on RNA Nano Chips with an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. The RNA of three independent RNA isolations was then used for cDNA transcription, each with dye-swap pairs resulting in 6 microarray slides. Briefly, 10 µg RNA was used to synthesize cDNA differentially labeled with Cy3-dCTP and Cy5-dCTP in a first-strand reverse transcription reaction with Superscript II RNase H− reverse transcriptase and 10 µg of random nonamer primer. Thus, for each replicate, 10 µg RNA was taken up in duplicate on ice in 13 µl RNase free water. 2 µl labeled standard RNA of *Saccharomyces cerevisiae*, one with Cy3 and one with Cy5, was added to the corresponding replicate. To each probe, 1 µl Luciferase (10 ng/µl) and 2 µl Random Nonamers were added and the resulting solution was incubated for 5 minutes at 70°C. After cooling for 5 minutes on ice, 20 µl of the following mastermix was added to each probe:

RNase free water	5.8 µl
SSC II Buffer	8 µl
dATP (20 mM)	10 µl
dTTP (20 mM)	10 µl
dGTP (20 mM)	10 µl
dCTP (10 mM)	0.8 µl
DTT	0.4 µl
RNase Out	1 µl
SuperScript II	1 µl

Subsequently, 2 µl Cy3 dCTP was added to the Cy3 probes and 2 µl Cy5 dCTP to the Cy5 probes, resulting in a final volume of 40 µl. For further procedures the probes were kept protected from light. The caps were vortexed and centrifuged briefly, then incubated for 10 minutes at room temperature. The transcription was performed for two hours at 42°C followed by inactivation of the enzyme for 15 minutes at 70°C. Remaining RNA was digested by addition of 2 µl RNase and incubation for 45 minutes at 37°C.

4.28.3. Preparation of the Microarray Slides

Transcriptome analyses were performed using whole genome DNA microarrays. Oligonucleotides (70mers) covering the genomes of MC58, Z2491, FAM18 and α14 were designed and synthesized by Eurofins MWG Operon, Germany and spotted onto epoxy coated Nexterion E slides using a BioRobotics MicroGrid II spotter at the University of Freiburg (Dr. Anke Becker). Each oligonucleotide was spotted four times on the slide. Slides were prehybridized according to manufacturer´s protocol: they were bathed for 5 minutes in 0.1 % Tx-100, then two times for 2 minutes in 1 mM HCl solution, followed by 10 minutes in 100 mM KCl solution. Slides were washed with distilled water and incubated in 1 x blocking solution for 15 minutes at 50°C. After another wash step in distilled water, slides were put in a 50 ml tube and dried at 1600 rpm for 5 minutes at room temperature.

4.28.4. Purification of the labeled cDNA

The labeled cDNA was purified using illustra AutoSeq™ G50 columns (GE Healthcare). Therefore, the column was vortexed thoroughly, the tip was severed and the column was placed in a fresh cap without lid. The column was centrifuged for 2 minutes at 5000 rpm. The remaining liquid at the column top was removed; the column placed in a fresh cap and 40 µl of the labeled cDNA was added. The labeled cDNA was eluted for 2 minutes at 5000 rpm. Subsequently, in each case, 40 µl of the differentially labeled cDNA was combined and diluted in 3 x SSC supplemented with 0.1 % SDS. The probes were heated for 2 minutes to 95°C, vortexed and centrifuged briefly.

4.28.5. Hybridization and data analysis

The probes were hybridized to the microarray using the Hybridization Station HS 4800™ Pro (Tecan) and incubated at 65°C for 16 h. The slides were scanned using the Axon GenePix® Pro 4200A (Molecular Devices) and analyzed using the GenePix Pro software. Spots were flagged and eliminated

from analysis when the signal-to-noise ratio was less than three or in obvious cases of high background or stray fluorescent signals. Data analysis was performed by Roland Schwarz (Cambridge Research Institute, UK) and is described in detail in (Schielke *et al.*, 2010a). The logFC values were converted to expression change rates, meaning that a logFC value of 1.0 is equivalent to a 2-fold upregulation, whereas a logFC value of 0.5 is equivalent to a 2-fold downregulation.

4.29. Quantitative RT-PCR

For validation of the Microarray data, quantitative real-time PCR was performed.

4.29.1. Reverse Transcription

Total RNA from meningococci was prepared as described in chapter 4.28.1. Of this RNA, 2 µg was used for reverse transcription with the SuperScript II Kit (Invitrogen). To this end, the RNA was adjusted to a final volume of 20 µl in distilled water using 2.5 µg Random Nonamers and incubated for 5 minutes at 70°C. Afterwards, the reaction was kept on ice and a mastermix of the following components was prepared:

dATP (10 mM)	1 µl
dCTP (10 mM)	1 µl
dGTP (10 mM)	1 µl
dTTP (10 mM)	1 µl
First Strand Buffer (5 x)	8 µl
DTT (0.1 M)	0.4 µl
Nuclease-free water	5.6 µl
RNase out	1 µl
SuperScript II	1 µl

This mastermix was then vortexed, centrifuged briefly and added to the RNA solution, mixed thoroughly and incubated for 10 minutes at room temperature. Reverse transcription was performed at 42°C for 2 hours and the enzymes were inactivated for 15 minutes at 70°C. The resulting cDNA was cleansed of residual RNA using 2 µl RNase and incubation at 37°C for 45 minutes prior to purification with the QIAGEN PCR Purification Kit according to manufacturer's instructions. The cDNA was eluted in 40 µl water and stored at -20°C, resulting in a final, arbitrary cDNA concentration of 50 ng/µl.

4.29.2. qRT-PCR reaction

Quantitative real-time PCR was carried out using a StepOnePlus™ system (Applied Biosystems) utilizing the *Power* SYBR® Green Master Mix (Applied Biosystems). Primers were used in a concentration of 45 nM and are listed in Table 0. Primer pairs were tested by PCR (chapter 4.16) to result in just one clear band. The expression levels of the target genes were normalized to *leuD* expression (NMB1034) as a housekeeping control gene. A relative standard curve experiment was performed for each primer pair beginning at 10 ng cDNA with a dilution series in five steps covering 1 : 5 dilutions, each in triplicate. The efficiency of amplification was determined and only primer pairs with a comparable efficiency to the housekeeping gene (> 93 %, R^2 > 0.990) were used for further experiments. These were conducted with 1 ng cDNA and a control without template was run to exclude primer dimer formation. Each assay was performed in triplicate and cDNA from two independent RNA preparations was used. Relative expression values for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4.30. Fatty acid resistance

The fatty acid resistance was estimated by an efficiency-of-plating (EOP) analysis as described by Rouquette-Loughlin and colleagues and performed in modification as described by Lee and colleagues (Rouquette-Loughlin *et al.*, 2002, Lee *et al.*, 2003). For preparation of the GC-agar plates, the fatty acids were solved in absolute ethanol. The final concentration in 16 ml agar/plate was adjusted to 10 µM for lauric and linoleic or to 585 µM (150 µg/ml) for palmitic acid in 100 µl absolute ethanol. The corresponding volume (100 µl) of absolute ethanol was used for the control plates. The plates were filled with 16 g GC-agar with supplement. For the palmitic acid plates, the plates were cast individually by hand to avoid disproportionate distribution due to precipitation of the palmitic acid in aqueous solutions. Bacteria were grown over night on the appropriate agar plate. Each strain was then inoculated with a cotton swab into 10 ml PPM^+. The liquid cultures were incubated at 37°C and 200 rpm for 90 minutes. The OD_{600} of these cultures was measured and a dilution series in PBS was adjusted to a final concentration of 500 bacteria per 100 µl suspension. For each strain, 100 µl suspension was plated in triplicate onto GC agar control plates supplemented with ethanol, as well as GC plates supplemented with lauric, palmitic or linoleic acid, respectively. This experiment was performed at least three times for each strain. Colony forming units (CFU) were counted and EOP values calculated by division of the mean of the CFU on the fatty acid plates by the mean of the CFU

on the control plates. For data presentation, resistance was expressed in percent by multiplying EOP values by one hundred.

4.31. β-galactosidase Assay

Reporter gene assays were performed to measure the transcriptional activity of meningococcal promoter regions. This assay was first described by J. H. Miller in 1972 and was performed with slight modifications: for quantitation of gene expression, the respective strain containing the *lacZ* reporter fusion construct (for generation see 4.19.1) was grown over night on GC agar plates supplemented with 100 µg/ml kanamycin. Strains were inoculated into PPM^+, grown for 60 minutes at 37°C shaking with 200 rpm before the OD_{600} was adjusted to 0.1 for all strains. Subsequently they were incubated in liquid culture for the indicated time points. Then the OD_{600} was measured, noted and adjusted to 0.2, the required volume was noted as well. Bacteria were pelleted at 4000 rpm for 5 minutes at room temperature and subsequently washed two times with 1 ml 0.85 % NaCl solution. After pelleting, the bacteria were resuspended in 500 µl 0.85 % NaCl and 500 µl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-Mercaptoethanol; pH 7.0). After addition of 20 µl 0.1 % SDS and 40 µl chloroform the probes were mixed thoroughly and heated for 2 minutes to 30°C in a thermo shaker. 200 µl prewarmed (30°C) ONPG (4 mg/ml) were added and the probes were incubated at 30°C in the Thermomixer 5436 (Eppendorff) at level seven for 4 – 5 minutes and the exact time was noted. The reaction was stopped by addition of 500 µl 1 M Na_2CO_3 solution. The probes were incubated for another 5 minutes at 30°C, then centrifuged at 13000 rpm for 5 minutes at room temperature to avoid scattering. 200 µl of each probe were transferred to a 96 well plate and analyzed at 414 nm and 540 nm in an ELISA reader. For data analysis, Miller Units were calculated using the formula: $1000 \cdot [(A_{414} – (1.75 \cdot A_{540}))/t \cdot v \cdot A_{600}]$ with t being the reaction time and v the initial bacterial volume. Expression was set in proportion to expression of the *porA*/*lacZ* construct and stated in per cent. All experiments were performed in duplicate, measured in triplicate and repeated at least three times.

4.31.1. Depletion and repletion of iron

Differences in FarR expression under iron limited or replete conditions were tested in *Neisseria* Defined Medium (NDM). Desferal, an iron chelator with a stability of 10^{31} for iron was added for iron depletion whereas ferric nitrate was added in excess for iron repletion. Meningococci were grown in

PPM^+ for 60 minutes; the OD_{600} was adjusted to 0.1 in NDM for control reactions and in NDM supplemented with 25 µM Desferal for stress reactions (Desai *et al.*, 1995, Grifantini *et al.*, 2003) and with 100 µM ferric nitrate for iron repletion (Grifantini *et al.*, 2003, Shaik *et al.*, 2007). Subsequently the cultures were allowed to reach the mid log phase. After 2.5 hours, $2 \cdot 10^8$ bacteria were transferred to a cap, pelleted and the protocol continued with the washing steps with 0.85 % NaCl solution as described above.

4.31.2. Effect of human serum

Serum stress reactions were performed in modification as described previously (Gordon *et al.*, 1988, Jarvis, 1994). Briefly, bacteria were grown in PPM^+ for 90 minutes, then $2 \cdot 10^8$ bacteria were transferred to a cap each for the serum-free, 40 % and 80 % serum reactions. The complement preserved pooled human serum (Dunn Laboratories, Germany) was either employed in its native state or heat-inactivated by incubation at 56°C for 30 minutes. The bacteria were pelleted and resuspended in 250 µl VBS/BSA buffer with or without the respective amount of native or heat-inactivated serum. The stress experiment was incubated for 90 minutes at 37°C. Subsequently the bacteria were pelleted and the protocol continued with the washing steps with 0.85 % NaCl solution as described above.

4.32. Dot Blot analysis

For dot blot analysis, chromosomal DNA was prepared using the QIAmp DNA mini kit, 200 ng samples were spotted onto nylon membranes and fixed with UV-crosslinking. The probe specific for the meningococcal *farR* gene was generated by PCR using the primer pair 123 and 124 and the resulting PCR product was purified employing the QIAquick PCR purification kit and eluted in 50 µl distilled water. This volume was reduced to 15 µl in the SpeedVac®Plus and heated to 95°C for 10 minutes. After cooling on ice, the DNA was labeled with digoxigenin-11-ddUTP using a random primer digoxigenin kit: 2 µl hexanucleotides, 2 µl dNTP and 1 µl Klenow-enzyme were added. This mixture was incubated over night at 37°C. Then the reaction was stopped by addition of 2 µl EDTA (0.2 M, pH 8.0) and the DNA precipitated by adding 2.5 µl sodium acetate (3 M, pH 4.5) and 75 µl ice-cold ethanol (absolute). The DNA was incubated for 30 minutes at -80°C, pelleted at 13000 rpm for 15 minutes at 4°C and washed with 1 ml 70 % ethanol, pelleted again and subsequently dried for 10 minutes in the SpeedVac®Plus. The purified DNA was redissolved in 50 µl distilled water for 5

minutes at 37°C. For quantification, dilution series of the labeled DNA and a control DNA with known quantity were spotted onto a nylon membrane, fixed with UV-crosslinking and the digoxigenin was detected with CSPD. This procedure is the same as for the electrophoretic mobility shift assays (see chapter 4.27). The nylon membranes were prehybridized for 30 – 60 minutes at 42°C with 10 ml hybridization solution in a hybridization oven. By means of the control DNA, the concentration of the labeled DNA was estimated and the appropriate volume for 100 ng transferred to a final volume of 10 ml hybridization solution (high SDS). This mixture was heated for 10 minutes to 100°C and then incubated over night with the prehybridized membrane at 42°C. Subsequently the membrane was washed twice for 5 minutes with 2 x SSC containing 0.1 % SDS, followed by another two washing steps with preheated 0.1 x SSC containing 0.1 % SDS at 68°C. Digoxigenin was detected with CSPD.

4.33. ELISA

Determination of LPS immunotype, examination of encapsulation or detection of *nadA* expression was performed by an enzyme linked immunosorbent assay (ELISA). Each well of a 96 well ELISA plate was coated with 50 µl poly-D-lysine (25 µg/ml in 1 x PBS) and the plates were incubated for 30 minutes at 37°C. After three washing steps with 1 x PBS, the plates were dried and stored at -20°C until usage. Meningococci were streaked onto agar plates and grown over night. Colonies were inoculated into 1 ml 1 x PBS with a cotton swab. The bacterial cell number was determined (chapter 4.11) and adjusted to $1.5 \cdot 10^8$ bacteria in 1 ml 1 x PBS. As negative control for the capsule ELISA, unencapsulated isogenic strains were used. As controls for immunotype ELISAs, strain #3077 was used for L8 and strain #2390 for immunotype L3,7,9. All probes were tested in triplicate. Blanks were prepared with 1 x PBS. For whole cell ELISA experiments (*nadA* expression, capsule expression), 20 µl bacterial suspension was added per well, the plate was incubated for 60 minutes at 37°C and subsequently the bacteria were fixed with 100 µl 0.05 % glutardialdehyde for 10 minutes at 37°C. For immunotype ELISA, bacteria were killed with heat prior to addition to the plate. Bacterial suspensions were heated to 110°C for 10 minutes, transferred with 20 µl per well to the ELISA plate and incubated for 60 minutes at 37°C. The following steps were performed identically for all applications. The plate was washed three times with 1 x PBS, 150 µl blocking solution (1 % BSA in 1 x PBS) was added per well and the plate incubated for 30 minutes at 37°C. After another three washing steps, 20 µl primary antibody [α-*nadA* 1:500 (rabbit), α-capsule B 1:4000 (mouse), α-L8 1:1000 (mouse), α-L3,7,9 1:100 (mouse)] in blocking solution was added and the plate incubated for 60 minutes at 37°C. The plate was washed thrice with 1 x PBS, then 20 µl horse radish peroxidase

coupled secondary antibody (α-rabbit 1:2500, α-mouse 1:2500) in blocking solution was added per well. After another three washing steps with 1 x PBS, 20 µl substrate (ABTS, 1 mg/ml) was added per well. After a reaction time of 10 minutes at 37°C, the absorption was measured at 414 nm.

4.34. Infection experiments

Chang epithelial cells (ATCC CCL20.2; Wei *et al.*, 1974) were cultured in DMEM with 15 mM L-glutamine and 10 % FCS and kept at 37°C with 5 % CO_2. Prior to an infection experiment, $1 \cdot 10^5$ cells per well were seeded into 24 well plates and grown for three days at 37°C in 5 % CO_2. Bacteria were streaked onto agar plates and incubated over night. They were inoculated into PPM^+ and grown for 90 minutes at 37°C with 200 rpm. The OD_{600} was measured and the bacterial cell number determined (chapter 4.11). One well with Chang cells was washed with 1 x PBS, 1 ml trypsine/EDTA was added and after a short incubation time, cells were harvested and counted in a Neubauer chamber using the formula

$$\frac{\text{mean number of cells counted}}{\text{volume}} \cdot 10^4 \cdot \text{dilution factor}$$

The bacterial cell number was adjusted to a multiplicity of infection (MOI) of 10, meaning that ten times as many bacteria were added per well as Chang cells were present. Infection was performed in 300 µl DMEM with 1 % FCS for 3 hours at 37°C with 5 % CO_2. For measuring the bacteria in the supernatant, the latter was transferred to a fresh cap, a serial dilution series prepared in 1 x PBS and plated in triplicate on blood agar plates. CFU were counted using a ProtoCol automated colony counter (Synbiosis). For counting the adherent bacteria, Chang cells were washed twice with 1 ml 1 x PBS and 300 µl medium supplemented with 1 % saponin was added for 15 minutes at 37°C. Then the cells and bacteria were scraped from the wells, dilution series were prepared and plated, CFU were counted and CFU of the invasive bacteria were subtracted to result in adherent bacteria. For invasion assays, separate probes were prepared as described, the supernatant was removed, the cells washed twice with 1 x PBS and 1 ml medium with 200 µg/ml gentamicin was added. After incubation for 2 hours at 37°C with 5 % CO_2, cells were washed three times, lysed with medium with 1 % saponin as described above and dilution series were plated.

4.35. FACS analysis

The ability of palmitic acid to penetrate the cell wall and accumulate at the cytoplasmic membrane was measured by a fluorescence assay (Kasurinen, 1992). Bacteria grown over night on GC agar plates were resuspended in PBS and adjusted to an OD_{600} of 0.3 in 1 ml. Fluorescently labeled palmitic acid (BODIPY® FL C_{16}; 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid) was solved at a concentration of 1 µg/µl in absolute ethanol. 100 µl bacterial suspension was mixed with 0.5 µl of this fluorescent lipid, mixed and incubated for 5 min on ice. Bacteria were pelleted at 4000 rpm for 5 minutes at room temperature, washed with 1 x PBS and fixed for 60 min in 1 % formaldehyde in PBS. Subsequently bacteria were pelleted again, resuspended in 1 ml 1 x PBS and transferred to FACS tubes. Fluorescence of 25000 bacteria within the gate per experiment was measured in a flow cytometer at 530 nm (FACSCalibur, BD Biosciences).

4.36. Computational analysis

Data analysis was performed with Microsoft® Office Excel version 2007. Two-tailed Student's t-test was used to calculate statistical significance (*P*-values).

4.36.1. DNA

DNA Sequences were analyzed using BioEdit version 7.0.5.3 (Hall, 1999) and multiple alignments were facilitated with ClustalW (Thompson *et al.*, 1994). Conserved residues were identified with the help of BioEdit. Retrieval of the palindromic sites within the *nadA* promoter region was accomplished using "Einverted" of Jemboss version 1.5 (Rice *et al.*, 2000). The FarR binding site was visualized with Weblogo version 3.0 (Schneider & Stephens, 1990, Crooks *et al.*, 2004). The meningococcal genome was searched for similar binding sites using Artemis version 11 (Rutherford *et al.*, 2000).

4.36.2. Protein

For modeling the secondary structure and the most probable biological molecule for FarR, secondary protein structure prediction was performed using the Protein Homology/analogY Recognition Engine Phyre version 0.2 (Kelley & Sternberg, 2009). The fold recognition tool within Phyre identified PA4135 of *P. aeruginosa*, whose crystal structure is accessible in the PDB archive (accession code

2FBI). Homology modeling using HOMER version 1.5 (Tosatto, 2009, The VICTOR Package for 3D Protein Structure Modelling, http://protein.cribi.unipd.it/homer) produced a prediction for the FarR monomer. This monomer was utilized to generate a dimer model using the GRAMM-X Protein Docking Web Server version 1.2.0 (Tovchigrechko & Vakser, 2006). As control, the crystal structures of the monomers of ST1710 (PDB-ID 2EB7) and PA4135 (PDB-ID 2FBI) were subjected to protein docking as well, resulting in very similar models to the published molecules. The results were visualized and edited using Jmol (Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org).

4.36.3. LPS and other chemical structures

The schematic model of the meningococcal lipopolysaccharide and all other chemical structures were drawn with ACD/ChemSketch Freeware Product Version 12.01 (2009) by ACD/Labs and refined with Adobe Illustrator CS, version 11.0.0 by Adobe Systems Incorporated.

5. Results

5.1. Characterization of the transcriptional regulator NMB1843 (*Nm*FarR)

5.1.1. Sequence alignment and classification

NMB1843 and NMB1585 are the two transcriptional regulators of the MarR family predicted in *N. meningitidis* (Tettelin *et al.*, 2000). Although its function remains to be elucidated, the crystal structure of NMB1585 revealed a dimeric protein with the characteristic MarR family winged helix-turn-helix (wHTH) DNA binding domain (Nichols *et al.*, 2009). The open reading frame of the other MarR-like protein, NMB1843, comprises 441 base pairs which might result in a protein of 146 amino acids with a molecular weight of 16.583 kDa. For classification of this protein, the amino acid sequence was compared with other virulence-associated members of this family (Figure 4A). The highest sequence similarity (98.40 %) was detected with the **f**atty **a**cid **r**esistance **R**egulator FarR of *N. gonorrhoeae*, and therefore NMB1843 will be referred to as FarR (*Nm*FarR) further on. Furthermore, the sequence alignment revealed an overall very low sequence identity (less than 25 %), but a high structural homology was observed by secondary structure prediction. The wing motif is formed by β2, W and β3, whereas the helices α1, 5 and 6 are involved in dimerization. To elucidate the three-dimensional structure of the FarR protein, a search for similar MarR proteins with known crystal structure was performed. The transcriptional regulator PA4135 of *Pseudomonas aeruginosa* revealed the highest sequence identity (46.43 %) to FarR. Using the PA4135 crystal structure, which was solved at a resolution of 2.1 Å (Lunin *et al.*, 2009, DOI:10.2210/pdb2fbi/pdb), a model of the FarR monomer (Figure 4B) was fashioned via homology modeling with HOMER (Tosatto, 2009, The VICTOR Package for 3D Protein Structure Modelling, http://protein.cribi.unipd.it/homer). Indicated in a color coding matching the secondary structure elements (Figure 4A), the resulting FarR monomer closely resembled other proteins of the MarR family despite their sequence variety (Kumarevel *et al.*, 2009). As MarR-like regulatory proteins bind the DNA mainly as homodimers, the dimeric biological FarR molecule (Figure 4C) was modeled using the GRAMM-X Protein Docking Web Server (Tovchigrechko & Vakser, 2006). And indeed, the dimeric FarR showed close homology to the molecules of MarR (PDB-ID 1JGS), NMB1585 (PDB-ID 3G3Z) and PA4135 (PDB-ID 2FBI), affirming the hypothesis that FarR acts as a dimer (Figure 4D), using the residues within the flexible wing domains for interaction with the DNA.

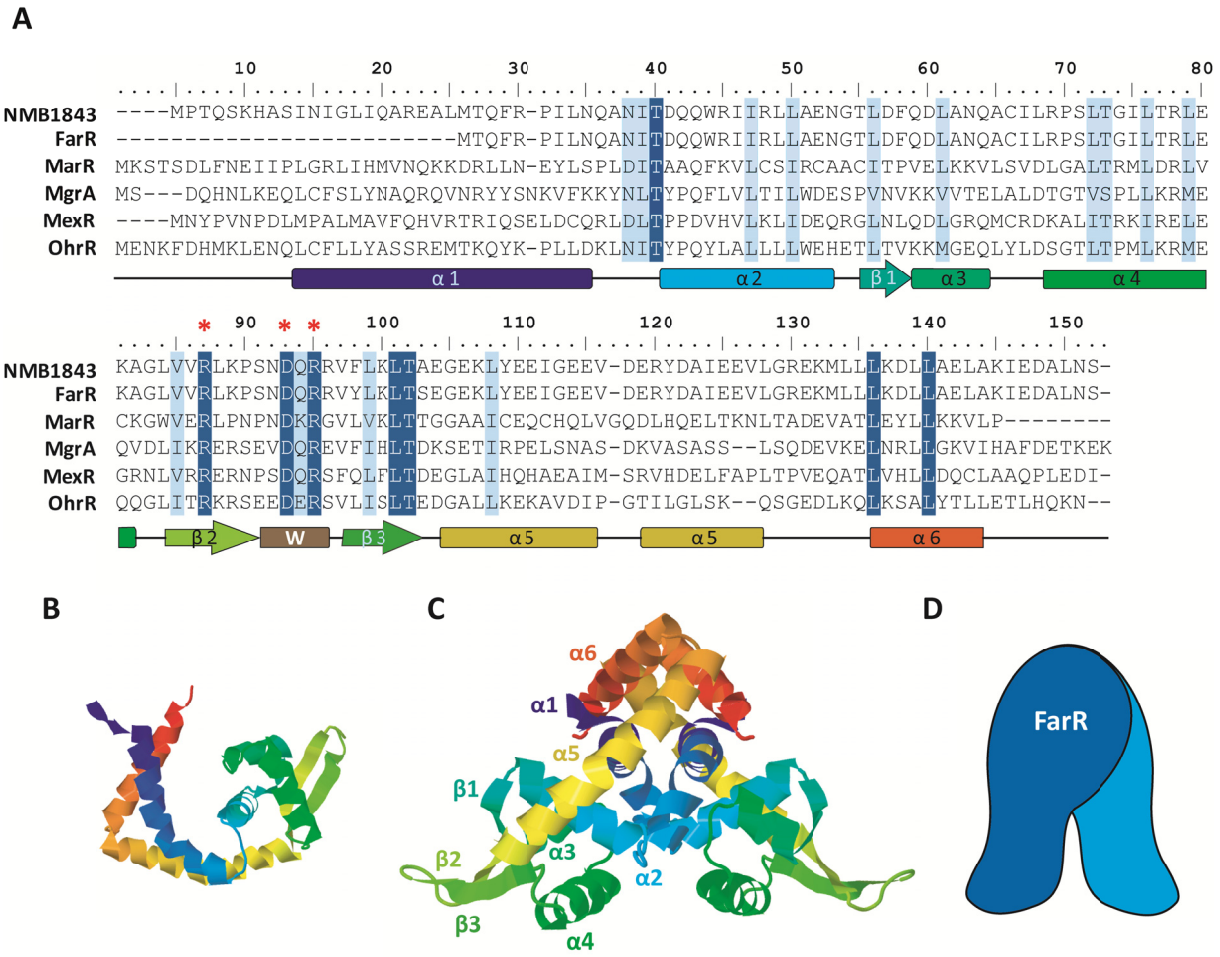


Figure 4. Classification of NMB1843 (*Nm*FarR). (A) Multiple alignment of NMB1843 with representative members of the MarR family: FarR (*N. gonorrhoeae*), MarR (*E. coli*), MgrA (*S. aureus*), MexR (*P. aeruginosa*) and OhrR (*Bacillus subtilis*). The alignment was generated using ClustalW. Light and dark blue shading indicates > 85% similarity or identity at that position, respectively. Residue numbering is according to the entire alignment. The secondary structure elements based on the MarR crystal structure (Alekshun *et al.*, 2001) show the conservation of the wHTH motif and are illustrated as boxes (α-helices), arrows (β-sheets) and lines (coils). The single wing domain (W) is indicated. DNA contacting sites are marked by red asterisks. **(B)** The FarR monomer, predicted by homology modeling with HOMER, coloring is according to the respective secondary structure elements. **(C)** Dimer model of the FarR protein, predicted with GRAMM-X. The domains of the left monomer are indicated on the basis of the *E. coli* MarR protein (Alekshun *et al.*, 2001). **(D)** Simplified model of the FarR transcriptional regulator. Figure 4A, B and C in modification from Schielke *et al.*, 2009, Schielke *et al.*, 2010a, in preparation.

5.1.2. Distribution and conservation within the species

In order to analyze the distribution of FarR within the species *N. meningitidis*, Dot Blot hybridization analyses were performed with 800 carrier isolates of the Bavarian meningococcal carrier strain collection (Claus *et al.*, 2002, Claus *et al.*, 2005), verifying the presence of FarR in some ambiguous cases by PCR analysis. In addition, 26 invasive isolates covering the hypervirulent ST complexes ST-8,

ST-11, ST-32 and ST-41/44 and ET-15 strains (see Table 4.2) as well as *N. lactamica* were tested by PCR analysis. Genomic DNA of *Saccharomyces cerevisiae* served as negative control. The *farR* gene was detected in all analyzed isolates and additionally in *N. lactamica* (Figure 5A). Therefore, sequence analysis of selected carrier and invasive isolates was conducted to identify possible mutations. The resulting amino acid sequences showed a similarity of > 99% (Figure 5B).

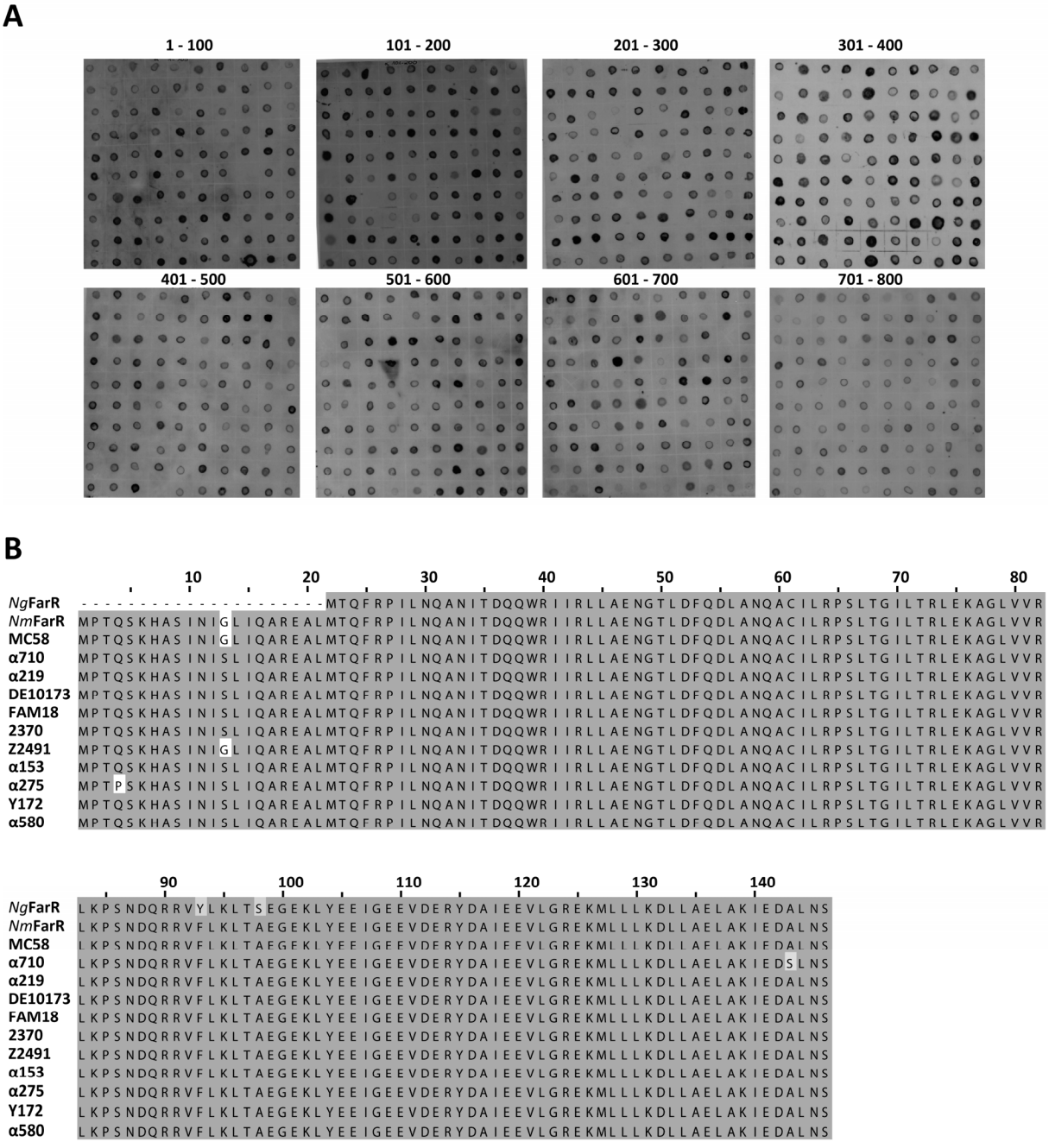


Figure 5. Distribution and conservation of *Nm*FarR. (A) Dot Blot analyses of 800 strains of the Bavarian meningococcal carrier strain collection (Claus *et al.*, 2002) to verify the presence of FarR. **(B)** Amino acid sequence alignment of representative carrier and invasive strains (Table 4.2) showing the high conservation of FarR within the species. Amino acid exchanges are indicated by lighter shading.

The rare point mutations leading to single amino acid exchanges were located beyond the predicted DNA binding site and are thus unlikely to be involved in DNA recognition. In conclusion, FarR is highly conserved and widely distributed throughout the species *N. meningitidis*, as it was detected in all tested strains regardless of their status as invasive or carrier isolates.

5.1.3. Interaction of FarR with the *farAB* promoter region in *N. meningitidis*

The transcriptional regulator FarR in *N. gonorrhoeae* is involved in the negative control of the *farAB* efflux pump system providing resistance against long chain fatty acids (Lee *et al.*, 2003, Lee & Shafer, 1999). This repression is mediated by direct interaction of FarR with the promoter region of the *farAB* operon (Lee *et al.*, 2003). Due to the close homology of *Nm*FarR with the gonococcal FarR the target DNA region was examined. Like in gonococci, there is a 99 % confidence that the genes *farA* and *farB* (NMB0318/0319) are organized in an operon in meningococci (Ermolaeva *et al.*, 2001). To assess whether *Nm*FarR directly interacts with the *farAB* promoter region, electrophoretic mobility shift assays (EMSAs, chapter 4.27) were conducted. Therefore, the FarR protein was natively purified (chapter 4.25) and a digoxigenin labeled DNA probe containing the *farAB* promoter region was generated by PCR (chapter 4.27). This probe was then incubated with or without 500 ng FarR protein, separated on a native polyacrylamide gel and detected by chemiluminescence. Indeed, direct binding of *Nm*FarR to the *farAB* promoter region was shown. This interaction was also proven to be specific as no shift could be observed after addition of the unlabeled DNA probe as specific competitor (Figure 6).

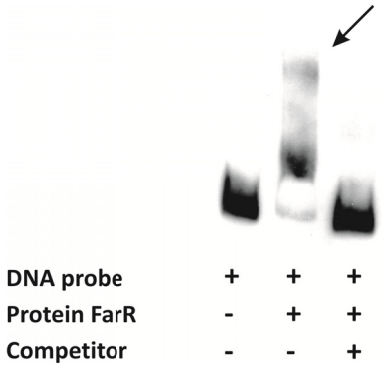


Figure 6. Interaction of FarR with the *farAB* promoter region. EMSA with DIG-labelled DNA probe of the *farAB* promoter, free or with 500 ng purified FarR protein or additionally with an excess amount of unlabeled specific DNA probe as competitor. Arrow indicates band shift. Figure taken from Schielke *et al.*, 2009.

5.1.4. *farR* mutant strains and a FarR specific antibody

For further analyses of the transcriptional regulator FarR, *farR* deletion mutants were constructed in the wild type strain MC58 as well as in the isogenic unencapsulated MC58 Δ*siaD*. Unchanged transcription of the downstream gene NMB1842 was assured by qualitative RT-PCR (see chapter 4.20). Additionally, the *farR* deletion was complemented by ectopic expression of *farR* under control of the *porA* promoter in the meningococcal expression vector pAP1 (Lappann *et al.*, 2006) resulting in the strain MC58 Δ*farR* (pAP1*farR*). By means of the purified FarR protein, an affinity purified antibody against FarR was generated in corporation with immunoGlobe® Antikörpertechnik GmbH (see chapter 4.26). This antibody was employed to verify the presence/absence of FarR expression in the mutant strains (Figure 7).

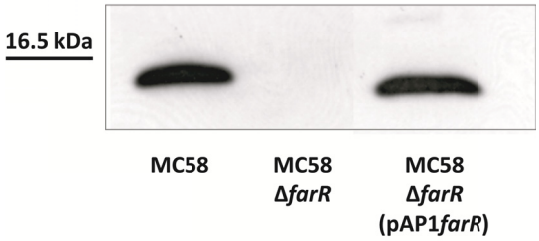


Figure 7. Western blot detection of FarR expression. Whole cell lysates of the wild type strain, the *farR* deletion strain and the complemented *farR* mutant strain were checked for the presence of FarR.

5.2. Control of FarR expression in *N. meningitidis*

5.2.1. FarR and antibiotics

To examine the control of FarR expression in meningococci, this protein was compared with other members of the MarR family. The eponymous protein of this family, MarR of *E. coli*, is inducible by tetracycline and chloramphenicol, thereby initiating multidrug resistance (George & Levy, 1983, Alekshun & Levy, 1999b). The effect of nine different antibiotics was tested, covering the β-lactams, tetracyclines, fluorchinolones, macrolides and ansamycins on the MC58 wild type and *farR* deletion mutant strain by E-tests. Each strain was tested in at least three independent experiments. The resulting minimal inhibitory concentration for each of the antibiotics tetracycline, chloramphenicol, imipenem, erythromycin, penicillin G, cefotaxim, ciprofloxacin, levofloxacin and rifampicin was not

significantly altered in the *farR* deletion strain (Figure 8), indicating that contact of FarR with these antimicrobials does not lead to a multidrug resistant phenotype in meningococci.

	MICs [µg/ml]								
Strain	TC	IP	Pen G	CT	CF	LF	RF	CM	EM
MC58	0.168 ± 0.04	0.064 ± 0.00	0.053 ± 0.01	0.003 ± 0.00	0.003 ± 0.00	0.004 ± 0.00	0.028 ± 0.02	0.917 ± 0.14	0.337 ± 0.08
MC58 Δ*siaD*	0.125 ± 0.06	0.058 ± 0.01	0.058 ± 0.01	0.003 ± 0.00	0.003 ± 0.00	0.004 ± 0.00	0.026 ± 0.03	1.000 ± 0.00	n.d.
MC58 Δ*farR*	0.138 ± 0.05	0.058 ± 0.01	0.047 ± 0.00	0.002 ± 0.00	0.003 ± 0.00	0.004 ± 0.00	0.019 ± 0.02	0.917 ± 0.14	0.420 ± 0.07
MC58 Δ*siaD* Δ*farR*	0.115 ± 0.07	0.058 ± 0.01	0.048 ± 0.02	0.003 ± 0.00	0.003 ± 0.00	0.004 ± 0.00	0.007 ± 0.01	1.000 ± 0.00	n.d.

Figure 8. Role of FarR in antibiotic susceptibility of *N. meningitidis*. Determination of the minimal inhibitory concentrations (MICs) of antibiotics of wild type MC58, the *farR* deficient MC58 Δ*farR* as well as the unencapsulated isogenic mutants. TC, Tetracycline; IP, Imipenem; Pen G, Penicillin G; CT, Cefotaxim; CF, Ciprofloxacin; LF, Levofloxacin; RF, Rifampicin; CM, Chloramphenicol; EM, Erythromycin; n.d., not determined.

5.2.2. FarR and oxidative stress

Another well-characterized member of the MarR family, OhrR of *B. subtilis*, is induced upon contact with reactive oxygen species, giving rise to transcription of the organic hydroperoxide resistance gene *ohrA* (Fuangthong *et al.*, 2001, Hong *et al.*, 2005). To test whether oxidative stress acts inducing upon FarR as well, stress reactions were performed in liquid culture with wild type and *farR* deletion strains. To exclude any shielding function by the capsule, isogenic unencapsulated mutant strains were included. For that purpose, the strains were inoculated into PBS for the control reactions and into PBS with either 1 mM of the redox cycling reagent Paraquat (releasing superoxide, O_2^-, as well as hydroxyl radicals, ·OH) or 0.88 mM hydrogen peroxide (H_2O_2) for the stress reactions and incubated shaking at 37°C. After 60 minutes a dilution series was plated, the resulting CFU were counted. Data were expressed in percent in relation to untreated control reactions at time point zero (Figure 9). The *farR* deficient strains showed no significantly higher survival under these conditions; furthermore, no difference was observed between encapsulated and unencapsulated strains. These results indicate that FarR is not induced upon contact with reactive oxygen species.

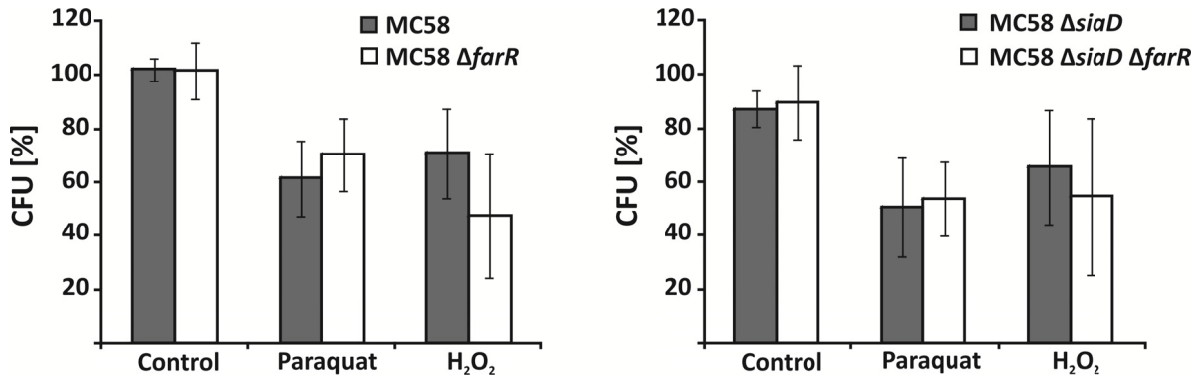


Figure 9. FarR and oxidative stress. MC58, the *farR* deletion strain MC58 Δ*farR* as well as the relative unencapsulated strains were tested for survival under oxidative stress conditions for 60 minutes with Paraquat or H_2O_2. Values are displayed in percent of untreated control reactions at time point zero. Error bars indicate the standard deviation of at least three independent experiments.

5.2.3. FarR and growth phase dependent expression

MarR-like transcriptional regulators may also be involved in virulence gene expression (Ellison & Miller, 2006), which again can be dependent on the growth phase. *Helicobacter pylori*, for example, is most virulent in the late logarithmic phase (Thompson *et al.*, 2003). To examine the expression of FarR in different growth phases of meningococci, the growth behavior of the mutant and wild type strains was tested in early (1 h), mid (2.5 h) and late (6 h) exponential growth phase. No significant growth differences between the strains were observed, as depicted exemplary (Figure 10A) for MC58, the unencapsulated MC58 Δ*siaD* and the complemented *farR* mutant strain MC58 Δ*farR* (pAP1*farR*). Expression of *farR* was tested by Western blot analysis of whole bacterial lysates adjusted to 10 mg protein per probe. In comparison to the constitutively expressed *porA* gene, FarR was expressed to a higher extent in mid and late exponential growth phase (Figure 10B). To assure these results, reporter gene fusion construct were generated by cloning the *farR* promoter in frame to the *lacZ* gene encoding the β-galactosidase (chapter 4.19.1). Thus, upon expression of *farR*, β-galactosidase is produced and able to cleave the substrate ONPG (o-nitrophenyl-β-D-galactoside) into the yellow o-nitrophenol and the colorless galactose. The yellow compound was then quantified at 414 nm in an ELISA reader. By means of this assay, expression of *farR* was displayed in comparison to the constitutively expressed *porA* gene. Indeed, as set in relation to the stable *porA* expression, a significant increase of *farR* transcript was measurable, being highest in the late exponential growth phase (Figure 10C). Interestingly, this growth phase dependent *farR* expression was also visible in the complemented strain MC58 Δ*farR* (pAP1*farR*) in the Western blots (Figure 10B), where the *farR* gene is under control of the *porA* promoter. This might be due to a promoter independent mechanism of transcriptional supervision, possibly a post-transcriptional control or modification.

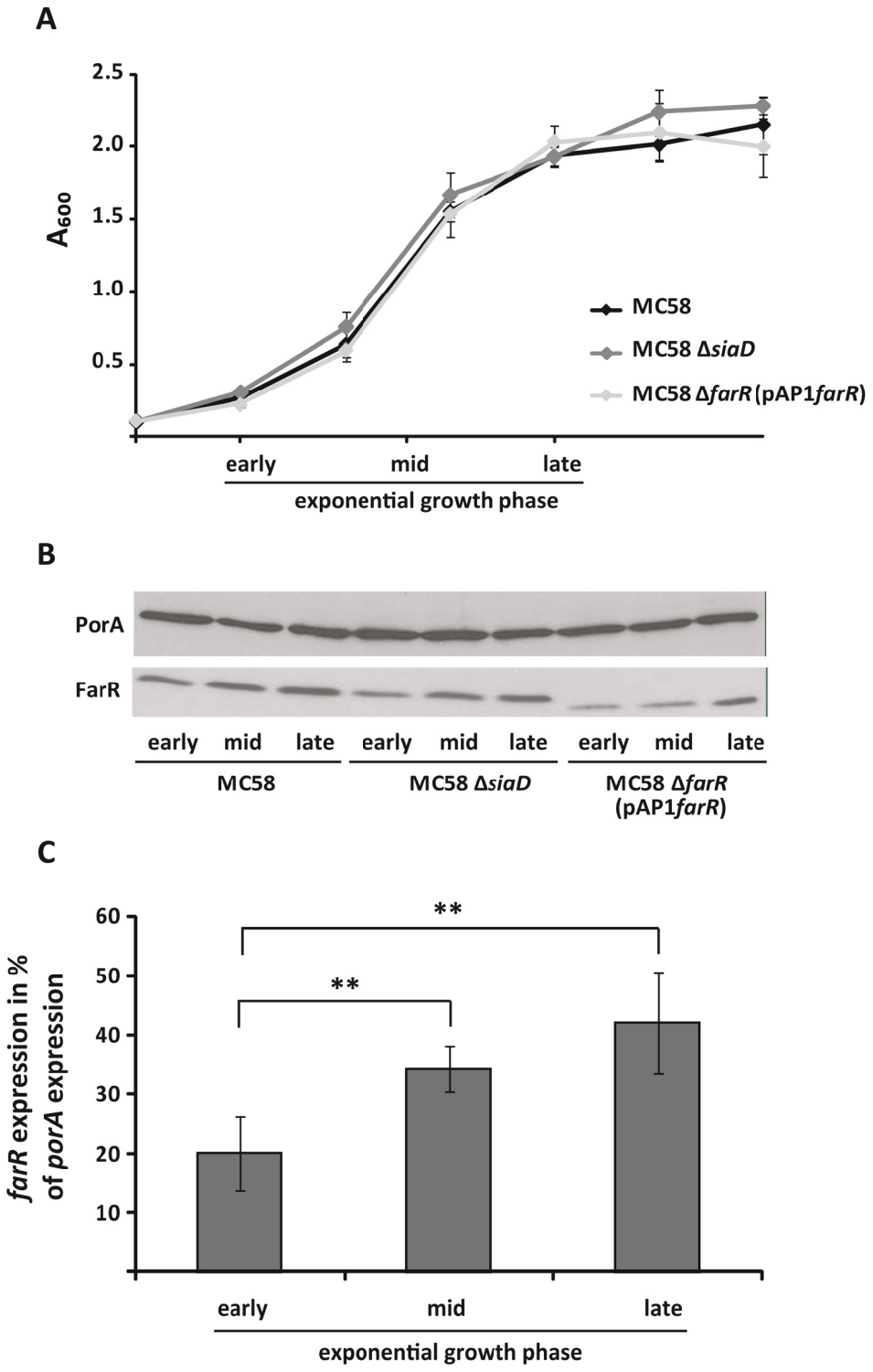


Figure 10. Growth phase dependent expression of *farR*. (A) The optical density of wild type strain MC58, the unencapsulated MC58 Δ*siaD* and the *farR* complemented strain MC58 Δ*farR* (pAP1*farR*) was recorded every hour at 600 nm. Early, mid and late exponential phase are indicated at the time points 1 h, 2.5 h and 4 h, respectively. Error bars represent the standard deviation of at least three independent experiments. **(B)** Western blot analysis of *porA* and *farR* expression in these three growth phase sections. **(C)** Quantification of *farR* expression in relation to the stably expressed *porA* gene. Error bars represent the standard deviation of at least three independent experiments. **, $P < 0.01$. Figure in modification from Schielke *et al.*, 2010a, in preparation.

5.2.4. FarR and autoregulation

As more than half of the characterized members of the MarR family are autoregulatory, including FarR of *N. gonorrhoeae*, OhrR of *B. subtilis*, SlyA of *Salmonella typhimurium* and HucR of *Deinococcus radiodurans* (Fuangthong *et al.*, 2001, Stapleton *et al.*, 2002, Lee *et al.*, 2003, Wilkinson & Grove, 2004), it was examined whether FarR in meningococci influences its own transcription. For this purpose, a DNA probe was generated by PCR using the oligonucleotides 214 and 215 (Table 4.6) and labeled with digoxigenin. In EMSAs with up to 1 µg of purified FarR protein, no direct interaction of FarR with its promoter region was observed. In conclusion, FarR is not autoregulatory.

5.2.5. FarR and depletion or repletion of iron

So far, the transcriptional regulator FarR is not comparable to other well-characterized members of the MarR family. As meningococci are one of the few bacterial species able to survive and replicate within the human body, the expression of *farR* under infection-like conditions was examined. One of the first obstacles invasive bacteria have to overcome in order to spread systemically is the survival within human blood. There, the level of free iron is kept at about 1 nM (Chipperfield & Ratledge, 2000), far below that required for bacterial growth (Weinberg, 1974, reviewed in Weinberg, 1978). Upon recognition of low iron concentrations, meningococci start an adaptive response (Grifantini *et al.*, 2003), which may possibly be sustained by FarR. To test this hypothesis, the *farR* expression was examined in *Neisseria* defined medium (Archibald & DeVoe, 1978, Lappann *et al.*, 2006) supplemented with 25 µM of the iron chelator Desferal (Desai *et al.*, 1995, Grifantini *et al.*, 2003).

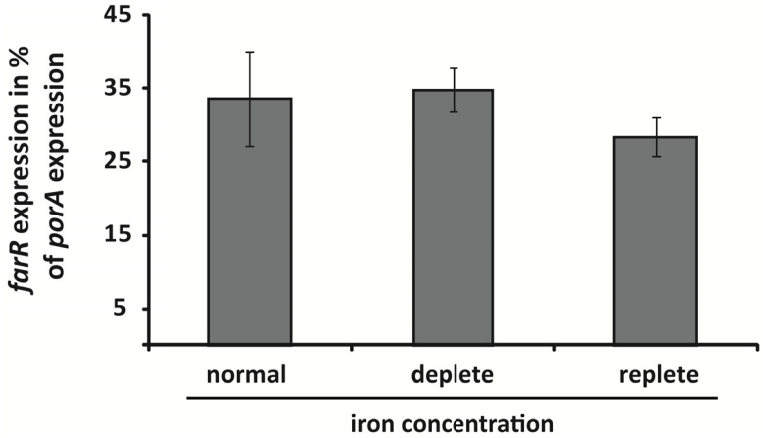


Figure 11. Effect of iron stress on *farR* expression. Quantification of *farR* expression in *Neisseria* defined medium under normal, iron deplete or replete conditions, displayed in relation to *porA* expression. Error bars indicate the standard deviation of at least three independent experiments. Figure taken from Schielke *et al.*, 2010a, in preparation.

In order to test *farR* expression under conditions with abundant iron, an iron-replete control reaction was performed with addition of 100 µM ferric nitrate (Grifantini *et al.*, 2003, Shaik *et al.*, 2007). However, FarR expression was not changed under these iron stress conditions (Figure 11), suggesting that this transcriptional regulator is not involved in the regulation of iron acquisition mechanisms needful for meningococcal survival.

5.2.6. FarR and complement preserved human serum

Another defense mechanism against invading pathogens within human blood is the complement system; a cascade of proteolytic components culminating in killing of the invading microorganisms. In order to examine the effect of serum on *farR* expression, stress assays were performed with 40 % and 80 % complement preserved human serum (Dunn Laboratories, Germany) with the MC58 reporter gene fusion strains. To prove that all assayed strains show a similar viability, survival of these strains in serum was assured by plating a dilution series at the measuring point. All strains survived in similar amounts, whereas the isogenic unencapsulated strain MC58 Δ*siaD* was killed swiftly. Ensuing β-galactosidase assays revealed a significant repressing effect of human serum on *farR* expression. With respect to the constitutively expressed *porA*, expression of the transcriptional repressor was significantly down-regulated (Figure 12).

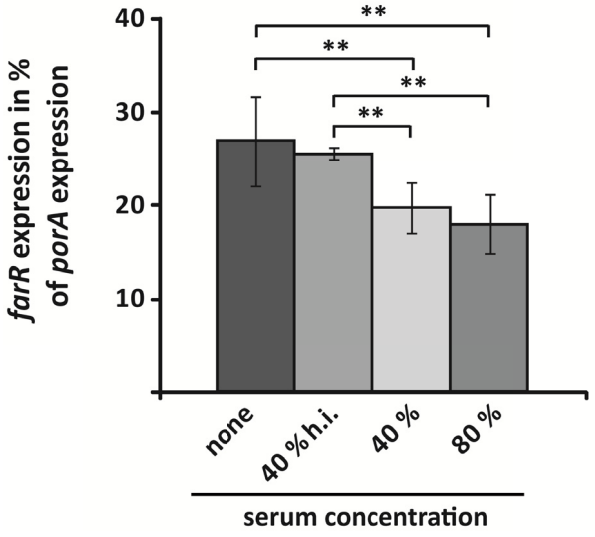


Figure 12. Effect of serum stress on *farR* expression. Quantification of *farR* expression in relation to *porA* expression under stress with complement preserved human serum. Error bars indicate the standard deviation of at least three independent experiments; h.i., heat inactivated. **, $P < 0.01$.

This effect became obvious upon treatment with 40 % and slightly increased with 80 % serum. However, the difference between 40 % and 80 % was not considerable. To check whether this effect was caused by active complement components, control reactions were performed with 40 % heat-inactivated serum. And indeed, this inactivated serum had no effect on *farR* expression, indicating that active complement components are crucial for the down-regulation of *farR* expression.

5.3. Fatty acid resistance in *N. meningitidis* and *N. gonorrhoeae*

In gonococci, the transcriptional regulator FarR is involved in resistance against long chain fatty acids. These are well-known for their antimicrobial activity (Kabara & Marshall, 2005), although the reason for this action has not yet been precisely pinpointed. They have been reported to block electron transport, inhibit oxygen and amino acid uptake, uncouple the oxidative phosphorylation as well as inhibit bacterial fatty acid synthesis (Sheu & Freese, 1973, Miller *et al.*, 1977, Zheng *et al.*, 2005). The human host maintains homeostasis of the colonizing microflora by secretion of antimicrobial compounds, whereof free fatty acids act non-selectively against a broad range of microorganisms (Wertz & Michniak, 2000, Stevens & Lowe, 2000, Drake *et al.*, 2008). The lipid content in human saliva, for example, is about 4 – 20 mg/100 ml and includes both saturated and unsaturated long chain fatty acids (Slomiany *et al.*, 1989, Tomita *et al.*, 2008). Interestingly, the antimicrobial activity of long chain fatty acids is mainly restricted to Gram-positive bacteria (Freese *et al.*, 1973, Kabara *et al.*, 1972), whereas Gram-negative bacteria seem to be shielded by the composition of their outer membrane (Galbraith & Miller, 1973, Sheu & Freese, 1973).

5.3.1. Effect of fatty acids on *N. gonorrhoeae*

In order to test the effect of fatty acids on gonococci, three different fatty acids were employed: the medium chain saturated lauric acid (dodecanoic acid, $C_{12:0}$), the long chain saturated palmitic acid (hexadecanoic acid, $C_{16:0}$) and the long chain unsaturated linoleic acid (9, 12-octadecadienoic acid $C_{18:2\Delta9\Delta12}$). The resistance of the bacteria was tested by modified efficiency of plating (EOP) assays (Rouquette-Loughlin *et al.*, 2002, Schielke *et al.*, 2009) as described in chapter 4.30. Gonococci were grown to mid-log phase in liquid culture, plated and the resulting CFU were set in relation to those on control plates. Two reference strains (MS11, FA1090) were tested as well as 16 clinical isolates of *N. gonorrhoeae*, isolated from patients with various gonococcal afflictions covering localized as well as disseminated infections (Table 4.3).

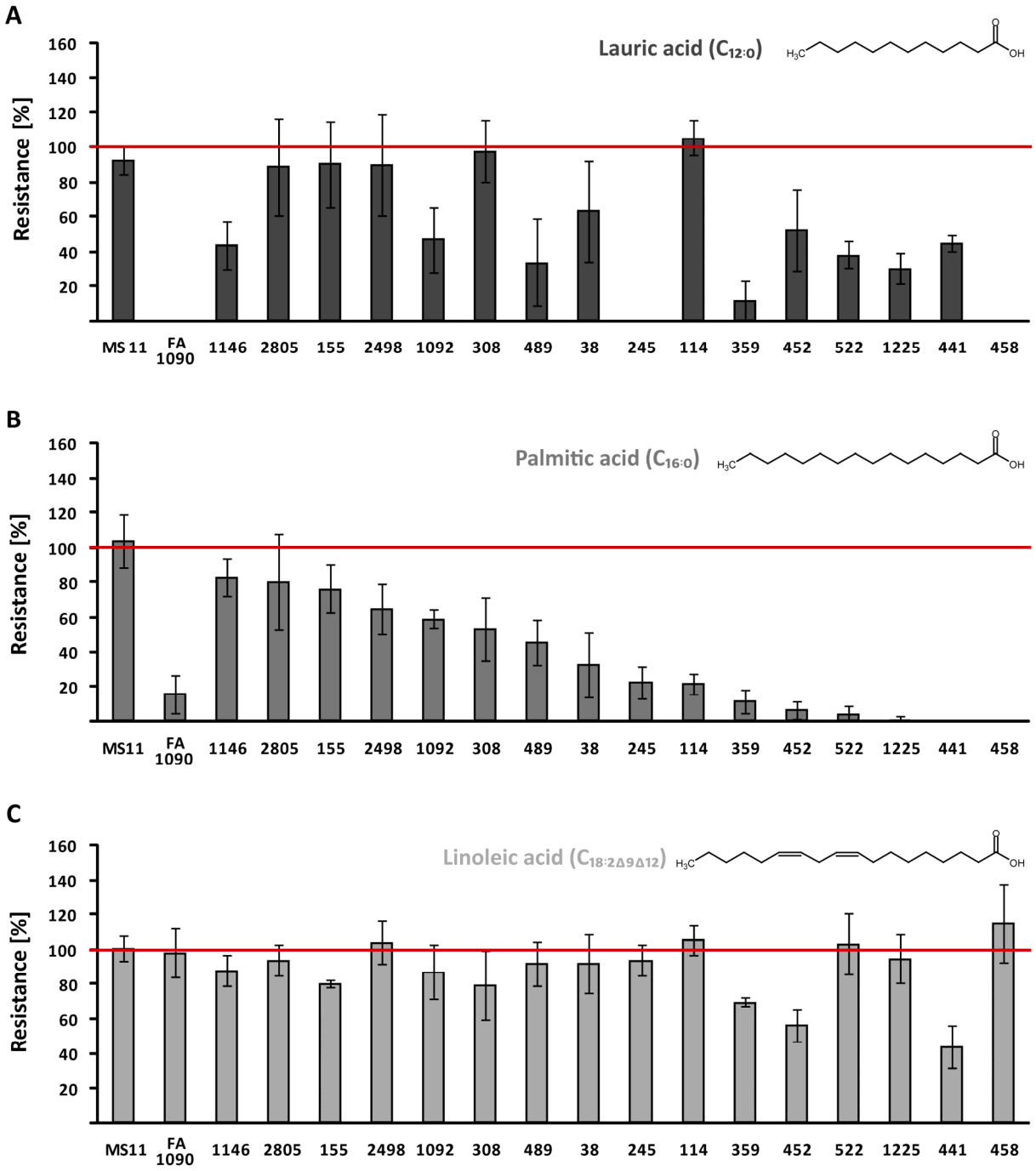


Figure 13. Fatty acid resistance in *N. gonorrhoeae*. Resistance of various gonococcal strains against **(A)** lauric or **(B)** palmitic or **(C)** linoleic acid. Resistance is shown as the ratio of growth on agar plates with supplementation compared to control plates in percent of at least three independent experiments. The error bars indicate the standard deviation. Figure 13 B and C taken from Schielke *et al.*, 2010, in preparation.

The experiments revealed a wide variety of results with resistances ranging from 0 to 100 percent against lauric and palmitic acid (Figure 13A and B) confirming the reports about the unusual susceptibility of gonococci against fatty acids (Ley & Mueller, 1946). Contrary to the findings by Miller and colleagues (Miller *et al.*, 1977), all tested strains showed a generally high resistance against the long chain unsaturated linoleic acid with only three strains below 80 % resistance (Figure 13C). The resistance results against these three fatty acids could not be correlated, indicating different modes of action depending on the chain length and saturation.

5.3.2. Effect of fatty acids on *N. meningitidis*

In order to test the fatty acid resistance of *N. meningitidis*, a variety of meningococcal strains were chosen including invasive and carrier isolates of both hypervirulent and non-virulent clonal complexes in addition to several reference strains. These 23 strains (listed in Table 4.2) covering the serogroups A, B, C, 29E, W-135, Y, Z and capsule null locus (cnl) as well as various sequence types (ST) were tested for their resistance against the abovementioned three fatty acids. In contrast to gonococci, nearly all meningococcal isolates displayed a high intrinsic resistance between 80 and 100 percent against these fatty acids (Figure 14). This intrinsic resistance seems to be influenced by the fatty acid chain length, as none of the strains were significantly susceptible to linoleic acid. Furthermore, no correlation between the strains susceptible against lauric acid (Figure 14A) and those sensitive against palmitic acid (Figure 14B) could be found. Interestingly, three strains of serogroup Y belonging to ST-23 displayed an unusual sensitivity to palmitic acid. The percentage of resistance for these three strains ranged from 0 to 16 % (average 10 % +/- 9) in contrast to 76 – 107 % (mean 92 % +/- 9) for all other strains ($P < 0.01$). Despite this sensitivity, these strains were resistant against lauric or linoleic acid. The susceptibility of these strains was also not due to expression of the serogroup Y capsule, as another serogroup Y strain (ST-166) showed full resistance against palmitic acid.

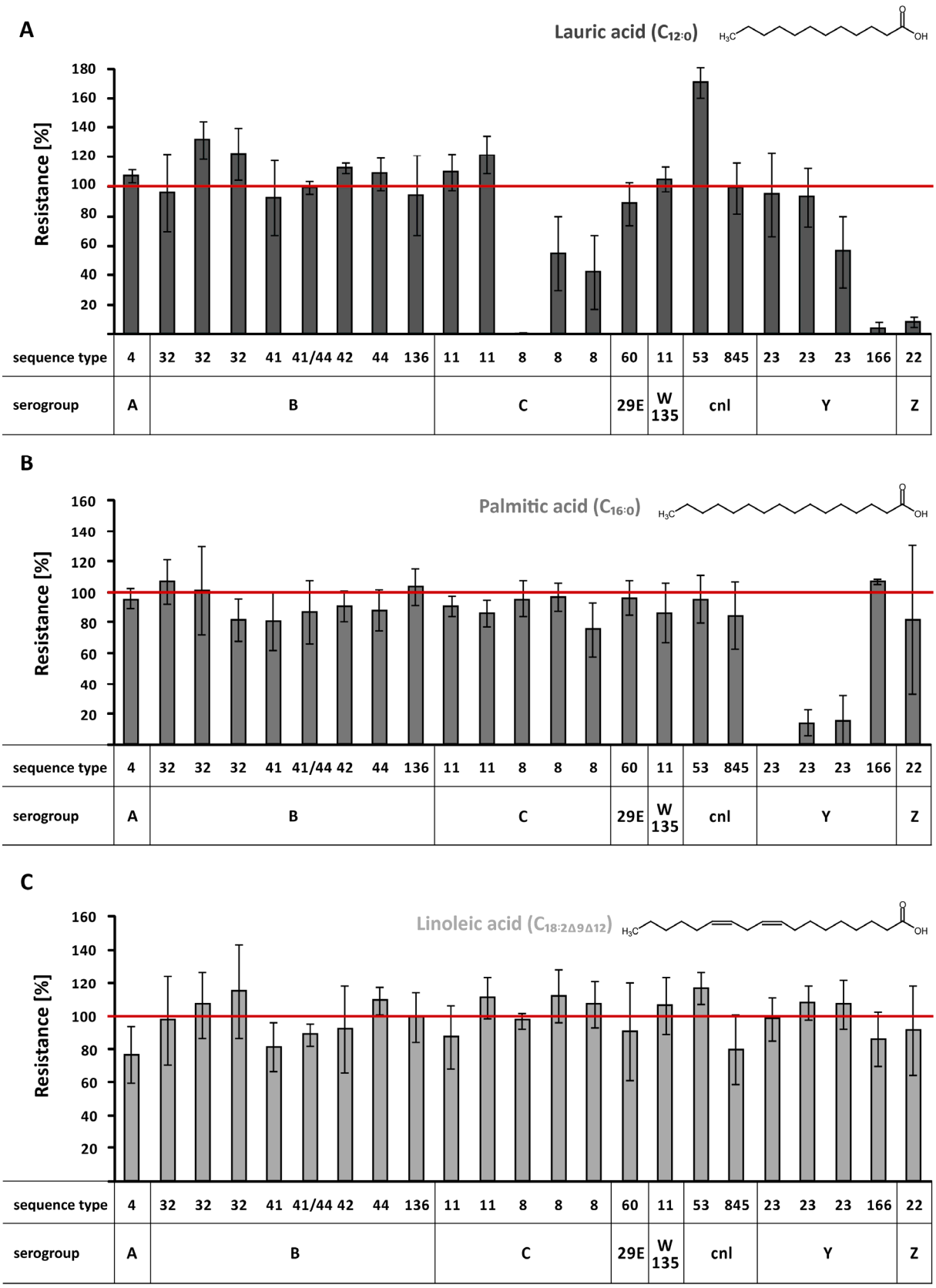


Figure 14. Fatty acid resistance in *N. meningitidis*. Various isolates covering seven serogroups were tested for their susceptibility against **(A)** lauric or **(B)** palmitic or **(C)** linoleic acid. Resistance is shown as the ratio of supplemented compared to control plates in percent of at least three independent experiments. The error bars indicate the standard deviation. Figure B and C taken from Schielke *et al.*, 2010, in preparation.

5.3.3. Meningococcal LPS composition and fatty acid resistance

As yet, the results indicate that the intrinsic resistance is neither due to serogroup nor correlated with virulence. Also the polysaccharide capsule has no influence on this resistance, as the cnl strains showed the same resistance as encapsulated strains (Figure 14). It has been shown for *E. coli* and *S. typhimurium* that LPS shields these Gram-negative bacteria from the antimicrobial activity of fatty acids (Sheu & Freese, 1973). However, the LPS of the *Neisseria* species is significantly different from enterobacteriaceal LPS as depicted in a structural model (Figure 15) integrating recent research and NMR analysis (Tsai *et al.*, 2009, Rahman *et al.*, 1998, Pavliak *et al.*, 1993, Zughaier *et al.*, 2007, Kogan *et al.*, 1997). The neisserial LPS is anchored in the outer bacterial membrane by lipid A. Two heptoses (HEP I and HEP II) branch from KDO I (2-keto-3-deoxyoctulosonic acid) carrying the short oligosaccharide side chains (Pavliak *et al.*, 1993, reviewed in Verheul *et al.*, 1993).

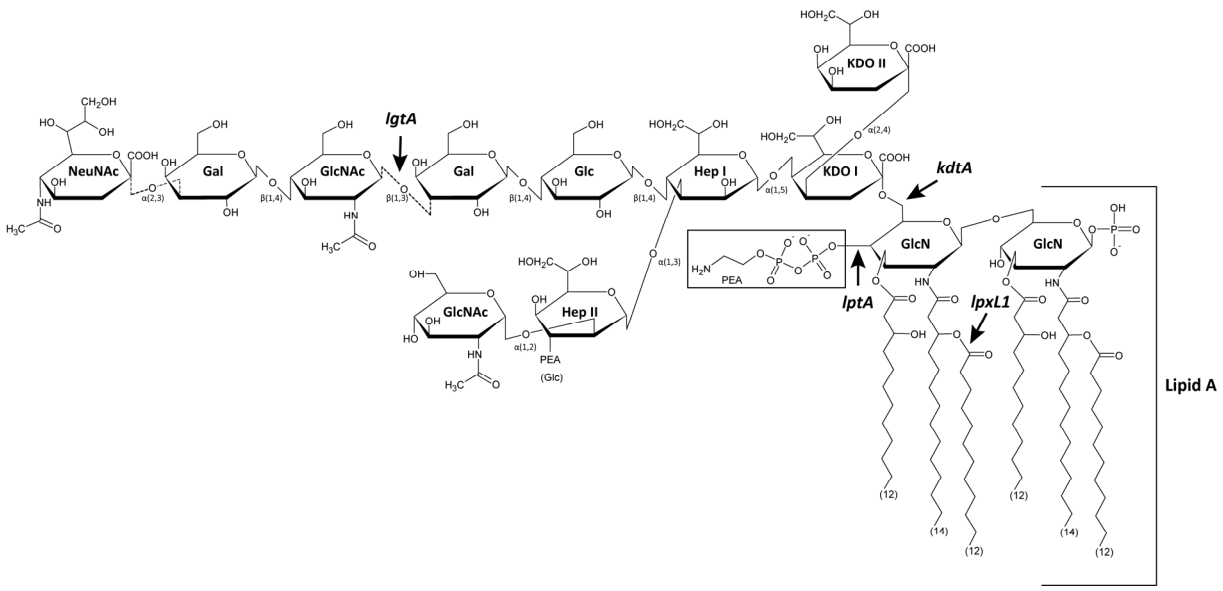


Figure 15. Structural model of meningococcal LPS. Depicted is the chemical structure of the LPS of MC58 (serogroup B, ST-32, immunotype L3). Genes responsible for the additional molecules beyond the particular linkage are indicated and labeled with an arrow. The numbers in brackets denote the number of carbon atoms of the acyl chain and dashed connections indicate phase variability. The PEA at the 3' position of Hep II can be replaced phase variably by an α(3,1)-linked glucose residue. GlcN, glucosamine; KDO, 2-keto-3-deoxy-octulosonic acid; Hep, Heptose; Glc, Glucose; GlcNAc, *N*-acetylglucosamine; Gal, Galactose; NeuNAc, *N*-acetylneuraminic acid; PEA, Phosphoethanolamine. Figure taken from Schielke *et al.*, 2010, in preparation.

To determine whether the meningococcal LPS is the reason for the intrinsic fatty acid resistance against long chain fatty acids, wild type and LPS mutant strains were tested for their susceptibility against palmitic and linoleic acid. As meningococci are viable without LPS, in contrast to other Gram-negative bacteria, a Δ*lpxA* mutant in strain H44/76 was tested, which is not able to produce any LPS (Steeghs *et al.*, 1998). And indeed, this strain was not able to grow on plates supplemented with

palmitic acid (Figure 16A) indicating a complete loss of the intrinsic resistance. The resistance against linoleic acid was significantly reduced but not completely abolished (Figure 16B). Thus, LPS plays a major role in fatty acid resistance. In order to elucidate the responsible LPS components, mutant strains with stepwise truncations in the oligosaccharide moiety of the LPS were generated (see Table 4.4). The sites of these truncations are indicated with the respective gene names in the structural LPS model (Figure 15). Neither deletion of the phosphoethanolamine (PEA) residue of lipid A in a Δ*lptA* mutant strain nor truncation of the α-chain behind the galactose residue in a Δ*lgtA* mutant strain led to fatty acid susceptibility. However, deletion of both these genes entailed a 50 % reduction in resistance against palmitic acid (Figure 16A).

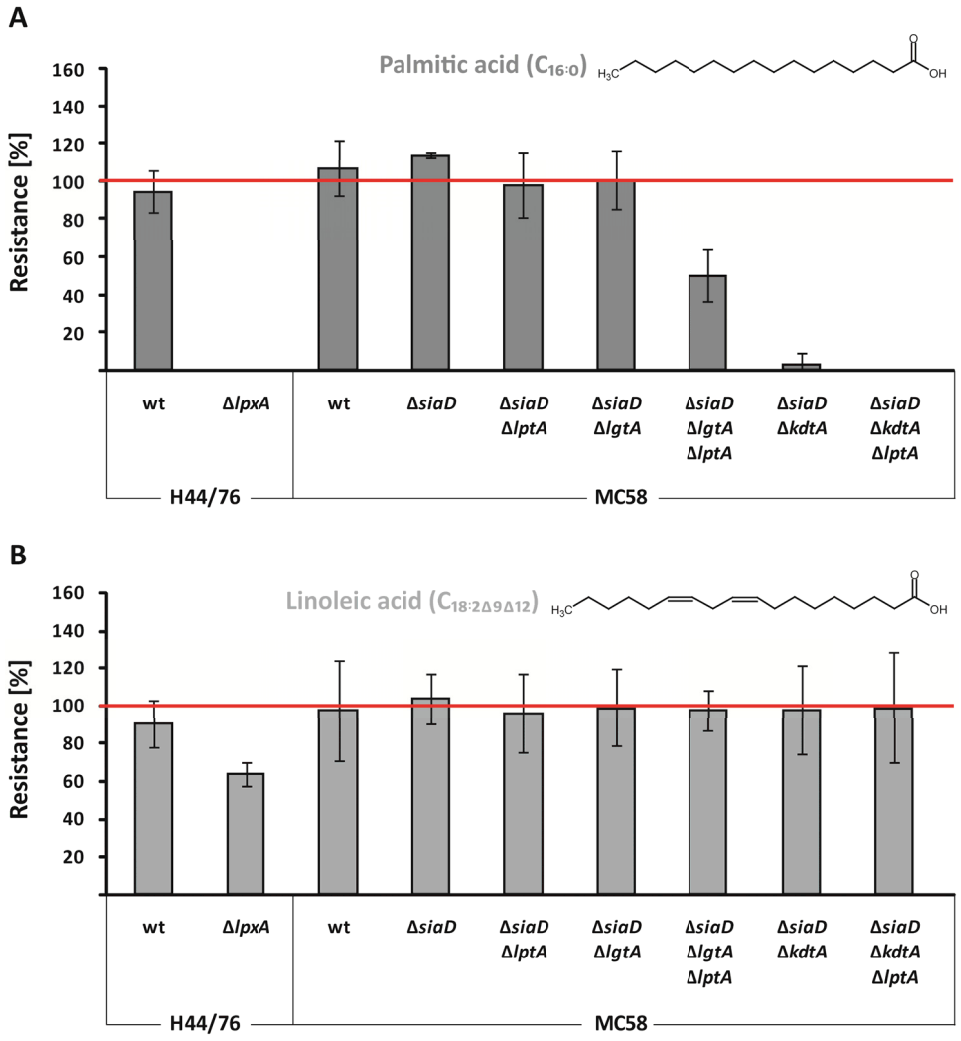


Figure 16. Influence of LPS composition on meningococcal fatty acid resistance. Serogroup B, ST-32 wild type strains (MC58 and H44/76), the unencapsulated MC58 Δ*siaD* and unencapsulated, LPS truncated mutant strains were tested for their susceptibility against **(A)** palmitic acid or **(B)** linoleic acid. Resistance is shown as the ratio of growth on agar plates with supplementation compared to control plates in percent of at least three independent experiments. The error bars indicate the standard deviation. Figure taken from Schielke *et al.*, 2010, in preparation.

Finally, deletion of the whole carbohydrate structure branching from lipid A in a Δ*kdtA* mutant strain reduced the palmitic acid resistance to a minimum and in combination with deletion of *lptA,* reversed it completely. Concluding these results, we state that truncation of the oligosaccharide moieties of the LPS reverses the high intrinsic resistance of meningococci to palmitic acid but not to linoleic acid. Thus composition of the meningococcal LPS is the major determinant of intrinsic palmitic acid resistance.

5.3.4. Hexaacylation of lipid A and the intrinsic palmitic acid resistance

Although the results show that the carbohydrate composition of the LPS is responsible for palmitic acid resistance in meningococci, such mutations are very unlikely to occur accidentally in wild type meningococci. Thus the unusual sensitivity of the Y, ST-23 strains cannot be explained by such mutations. However, a recent publication states that a high percentage of meningococcal isolates carry mutations in the *lpxL1* gene leading to penta-acylated and thus low-activity lipid A (Fransen *et al.*, 2009). As shown in Figure 15, normal meningococcal lipid A is anchored in the outer membrane via six acyl chains. The *lpxL1* gene is responsible for addition of the last lauroyl chain to the 2' end of the glucosamine disaccharide (van der Ley *et al.*, 2001). In order to determine whether these mutations also play a role in fatty acid resistance of any of the tested meningococcal strains, the *lpxL1* gene of all 23 strains was sequenced and checked for the seven different mutations that render the gene inactive (Fransen *et al.*, 2009). And indeed, each of the three serogroup Y, ST-23 strains with increased susceptibility to palmitic acid harbored an inactivating mutation (Figure 17A). Whereas strain DE 6853 contained a type V mutation, a deletion of an adenosine in a stretch of 7 adenosines, strains α 24 and DE 7671 had a deletion of 10 nucleotides and thus a type VI mutation (Fransen *et al.*, 2009). Deviant of the description of this type VI mutation, the latter two deletion mutants had one point mutation changing the adenosine at position 707 to a guanosine and the ten base pair deletion was shifted one nucleotide downstream. In the 20 palmitic acid resistant isolates, no mutations within the *lpxL1* gene were found. To prove that these mutations and thus the penta-acylated lipid A are responsible for the unusual sensitivity of these serogroup Y, ST-23 strains, an *lpxL1* deletion mutant was generated in the unencapsulated MC58 Δ*siaD* strain and subjected to susceptibility assays. Indeed, the absence of the sixth acyl chain totally abolished the resistance against the C_{16} fatty acid (Figure 17B), whereas the resistance against linoleic acid was unchanged. Additionally, strain α 24 showed fully restored resistance against palmitic acid upon reintegration of the wild-type *lpxL1* copy into the native chromosomal locus (Figure 17B).

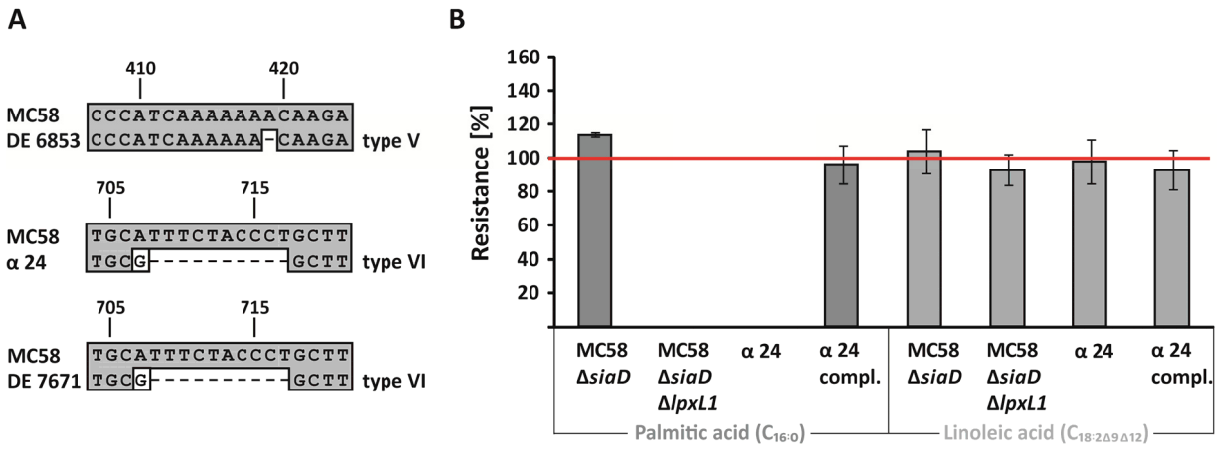


Figure 17. Hexaacylated lipid A and fatty acid resistance in meningococci. (A) The inactivating mutations found in the three serogroup Y, ST-23 strains. Alignment with the reference strain MC58 displayed a type V mutation in strain DE 6853 and type VI mutations in strains α 24 and DE 7671 (Fransen *et al.*, 2009). The position within the gene is indicated above, sequence accordance is shown in gray, nucleotide deletions are indicated by hyphens and the point mutation is boxed. **(B)** The unencapsulated strain MC58 Δ*siaD* and a corresponding *lpxL1* deletion mutant as well as strain α 24 and a *lpxL1*-complemented α 24 derivative were tested for their susceptibility to palmitic or linoleic acid. Resistance is shown as the ratio of growth on supplemented plates compared to control plates in percent of at least three independent experiments. The error bars indicate the standard deviation. Figure in modification from Schielke *et al.*, 2010, in preparation.

Looking for a molecular reason for these differences in fatty acid resistance, we examined whether there is a differential accumulation of palmitic acid on the bacterial cell wall or incorporation into the cytoplasmic membrane as described for fatty acid sensitive *S. aureus* strains (Kohler *et al.*, 2009). For this purpose, a fluorescence assay using BODIPY® labeled palmitic acid was performed. Analysis by flow cytometry resulted in no different fluorescence acquisition of the MC58 Δ*siaD* parental or the MC58 Δ*siaD* Δ*lpxL1* strain (Figure 17C). In conclusion, irrespective of lipid uptake or accumulation, hexaacylation of lipid A is necessary for the intrinsic resistance of meningococci against palmitic acid.

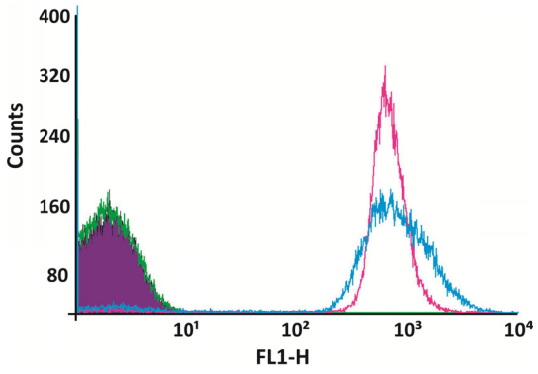


Figure 18. Accumulation of palmitic acid at the bacterial cell wall. FACS analysis of the incorporation of BODIPY® labeled palmitic acid to MC58 Δ*siaD* (pink) and MC58 Δ*siaD* Δ*lpxL1* (blue). Unlabeled bacteria were used as control (wild type; purple, Δ*lpxL1* mutant; green). Figure in modification from Schielke *et al.*, 2010, in preparation.

5.3.5. FarR and fatty acid resistance in meningococci

The unusual sensitivity of gonococci against fatty acids determined the development of mechanisms for adaptation. Few clinical isolates of *N. gonorrhoeae* with increased resistance against fatty acids have been reported and this resistance was attributed to the *farAB* encoded efflux pump (McFarland *et al.*, 1983, Lee & Shafer, 1999). Transcription of this *farAB* operon is negatively regulated by the transcriptional repressor FarR (Lee *et al.*, 2003) via direct interaction with the *farAB* promoter region (Figure 19A and B). This repression is supported by the heterodimeric integration host factor (IHF), which also binds to the *farAB* promoter region, stabilizes the FarR-DNA binding and is probably responsible for bending of the target DNA (Lee *et al.*, 2006). Upon deletion of *farR* the *farAB* operon is transcribed, resulting in the membrane fusion protein FarA and the cytoplasmic membrane transporter protein FarB (Figure 19B). After assembly of these proteins, MtrE takes its role as outer membrane channel and long chain fatty acids are exported from the bacterial cell (Delahay *et al.*, 1997, Lee & Shafer, 1999).

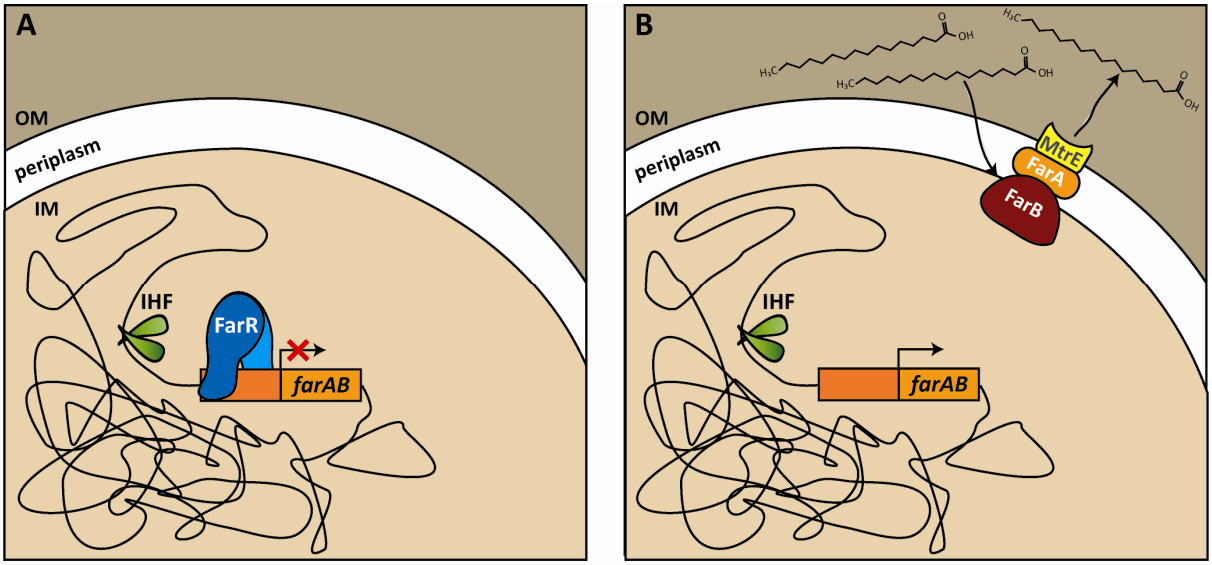


Figure 19. Schematic model of the role of FarR in gonococcal fatty acid resistance. (A) FarR, supported by the heterodimeric IHF, is bound to the *farAB* promoter region, repressing its transcription. **(B)** In the presence of antimicrobial fatty acids, the repressor dissociates from the *farAB* promoter, the RNA polymerase is able to bind and the efflux pump consisting of the FarB membrane transporter protein and the FarA membrane fusion protein is expressed at the cell surface. MtrE acts as outer membrane protein channel. OM, outer membrane; IM, inner membrane. Figure in modification from Schielke *et al.*, 2010, in preparation.

In meningococci, the transcriptional repressor *Nm*FarR binds directly to the promoter region of the *farAB* operon (see chapter 5.1.3). In order to assess whether FarR is also involved in fatty acid resistance in *N. meningitidis*, susceptibility assays with a *farR* deletion mutant strain were performed.

However, no effect of this mutation was observed (Figure 20). The lack of a fatty acid resistance phenotype might be due to the high intrinsic resistance of *N. meningitidis* preventing experimental quantification of genetic mutations resulting in enhanced resistance. To test this hypothesis, a *farR* deletion mutant strain was constructed in the palmitic acid susceptible strain MC58 Δ*siaD* Δ*kdtA* lacking all carbohydrates beyond lipid A and resistance assays were conducted. However, deletion of the transcriptional repressor FarR did not alter the susceptibility of MC58 Δ*siaD* Δ*kdtA* against palmitic acid (Figure 20), indicating that FarR is not involved in fatty acid resistance in meningococci. To exclude the possibility that the deployed concentration of palmitic acid of 150 µg/ml is too low to trigger a FarR dependent effect, the minimal inhibitory concentration (MIC) of palmitic acid for strain MC58 and the corresponding *farR* deletion strain was determined. In accordance with the hitherto results, no differences were observed; the MIC for MC58 and MC58 Δ*farR* was reached at 1500 µg/ml, confirming that FarR cannot be implicated in fatty acid resistance.

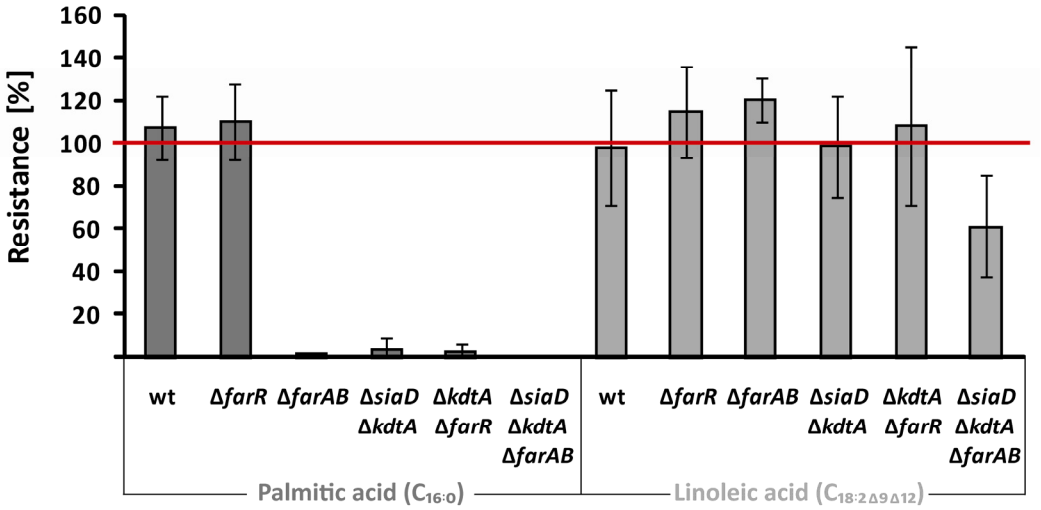


Figure 20. Role of FarR in fatty acid resistance in *N. meningitidis.* The wild type strain MC58, a Δ*farR* strain, a Δ*kdtA* strain without the polysaccharide moiety of LPS or a Δ*kdtA* Δ*farR* double mutant strain as well as a *farAB* deletion strains in MC58 and a Δ*farAB* strain in an unencapsulated, LPS truncated parental strain were tested for their susceptibility to palmitic or linoleic acid. Resistance is shown as the ratio of growth on agar plates with supplementation compared to control plates in percent of at least three independent experiments. The error bars indicate the standard deviation. Figure in modification from Schielke *et al.*, 2010, in preparation.

To further analyze the importance of the *farAB* efflux pump system for meningococcal resistance, *farAB* deficient strains were constructed in the wild type MC58 as well as in the palmitic acid sensitive MC58 Δ*siaD* Δ*kdtA*. Interestingly, inactivation of this operon completely abolished the palmitic acid resistance of the wild type strain, whereas the survival rate was unchanged with linoleic acid (Figure 20). This indicates that the palmitic acid resistance of meningococci is maintained by active efflux of this compound and additionally depends on LPS structural integrity.

5.4. Role of FarR in *N. meningitidis*

Besides direct interaction with the *farAB* promoter region, no functional similarities between *Ng*FarR and *Nm*FarR have been observed. To identify differentially expressed proteins, protein expression profiles of the wild type strain MC58 were compared to those of the *farR* deficient mutant strain MC58 Δ*farR*. An additional band with high molecular weight (≈ 210 kDA) was detected in the lysates of the mutant strains and the protein was analyzed by mass spectrometry (Hübner, 2004). Subsequently this protein with repressed expression in the wild type strain was identified to be the *Neisseria* adhesin A (NadA). This adhesin (NMB1994) is a 37 kDa surface exposed trimeric protein, a so called "minor adhesin" (Comanducci *et al.*, 2002).

5.4.1. Effect of *nadA* repression on NadA protein levels

In order to confirm the FarR-dependent repression of *nadA* in *N. meningitidis*, Western blot analysis was performed with whole cell lysates (see chapter 4.21) of the wild type strain MC58, the *farR* deficient mutant strain MC58 Δ*farR* as well as with the complemented strain MC58 Δ*farR* (pAP1*farR*). A polyclonal antiserum against NadA was obtained from N. Ackermann (Munich, Germany), which was generated as described previously (Schielke *et al.*, 2009). Western blot analysis revealed an almost total repression of *nadA* expression in the wild type strains, regardless of the presence of the capsule (Figure 21A). This repression was abolished in the *farR* deficient mutant strains and restored

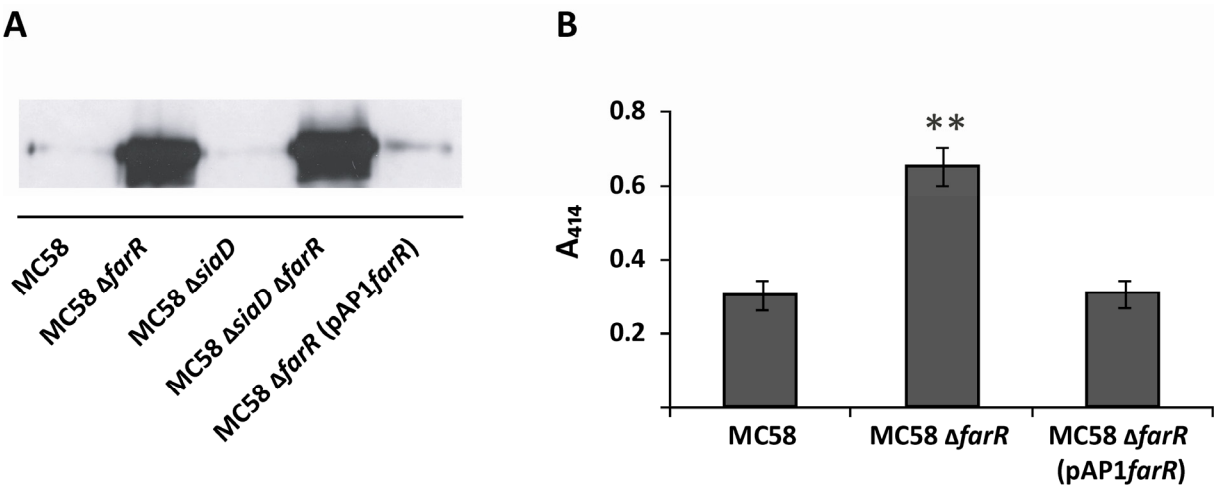
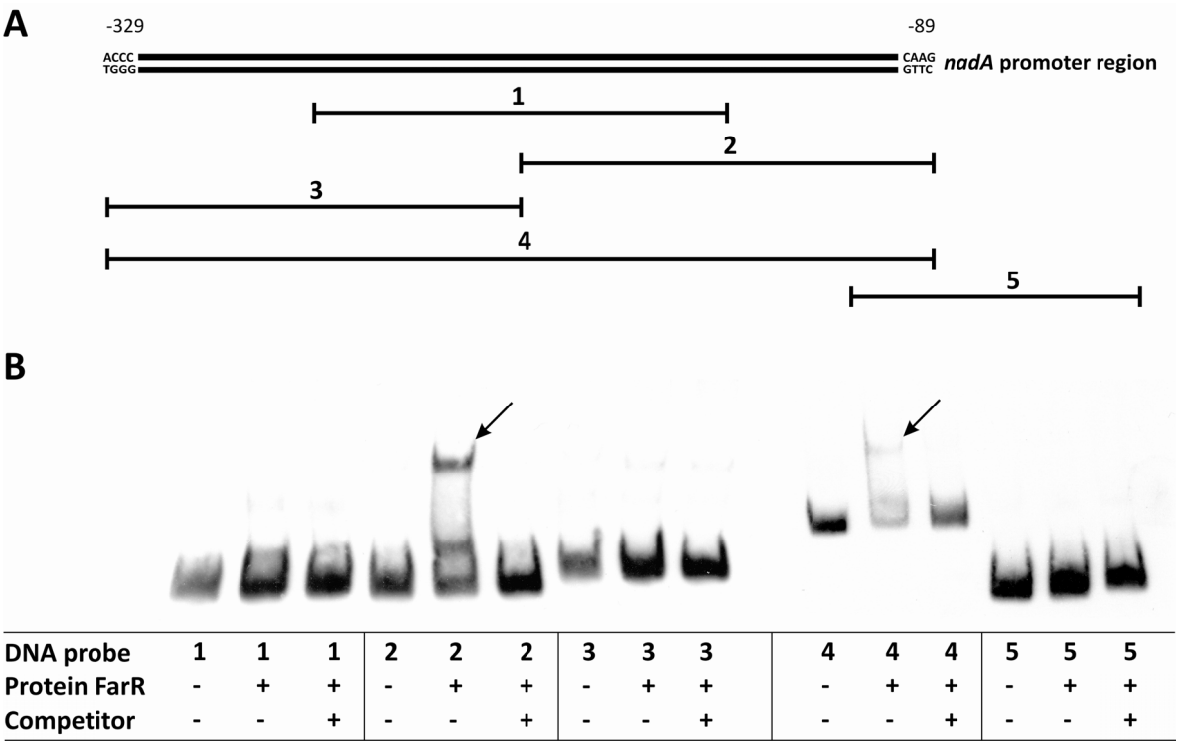


Figure 21. Effect of *nadA* repression on NadA protein levels. (A) Western blot analysis of NadA protein levels in MC58 wild type, *farR* deficient or complemented strain as well as in the isogenic unencapsulated mutant strains. **(B)** Whole-cell ELISA using a polyclonal NadA antiserum. The absorbance was measured at 414 nm. Error bars indicate the standard deviation of at least three independent experiments. **, $P < 0.001$. Figure in modification from Schielke *et al.*, 2009.

in the *farR* complemented mutant strain. In order to verify the presence of NadA on the bacterial cell surface, ELISA experiments were conducted (see chapter 4.33). Indeed, the presence of NadA on the cell surface differed significantly, being very low in the wild type and the *farR* complemented strain whereas significantly increased upon deletion of *farR* (Figure 21B).

5.4.2. Interaction of FarR with the *nadA* promoter region

These results indicated that FarR is involved in the negative regulation of *nadA* expression. In order to specify whether this involvement is based on a direct interaction, the binding activity of FarR with the *nadA* promoter region was examined by electrophoretic mobility shift assays (see chapter 4.27). To locate the FarR binding site, DNA probes were generated containing overlapping fragments of the *nadA* promoter (Figure 22A) covering the nucleotides -329 to -23 relative to the transcription start site of *nadA*. The purified FarR protein showed a specific binding to the whole promoter region (fragment 4, 240 bp, -329 to -89) as well as to fragment 2 (113 bp, -201 to -89), whereas no shift was observed with the fragments 1, 3 or 5 (Figure 22B). This binding was proved to be specific by competitive EMSAs, using an excess amount of the respective unlabeled DNA probe.



DNA probe	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5
Protein FarR	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
Competitor	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+

Figure 22. Gel Shift assays with FarR and the *nadA* promoter region. (A) Schematic representation of the overlapping DNA probes 1 – 5, covering 306 base pairs of the *nadA* promoter region (-329 to -23) relative to the transcription start site. **(B)** EMSAs with digoxigenin-labeled DNA probes, in each set: first lane, probe alone, second lane, with addition of 1 µg purified FarR protein, third lane, with specific competitor DNA probe. Arrows indicate band shifts. Figure taken from Schielke *et al.*,2009.

5.4.3. Identification of the exact FarR binding site within the *nadA* promoter

Analysis of the previous results limited the putative binding site of FarR within the *nadA* promoter to the base pairs -148 to -120, the 28 base pair region of DNA probe 2 minus the overlapping regions of DNA probes 1 and 5. The *nadA* promoter region, its transcription start site and the binding sites of the transcription factors IHF and Fur were characterized previously (Martin *et al.*, 2003).

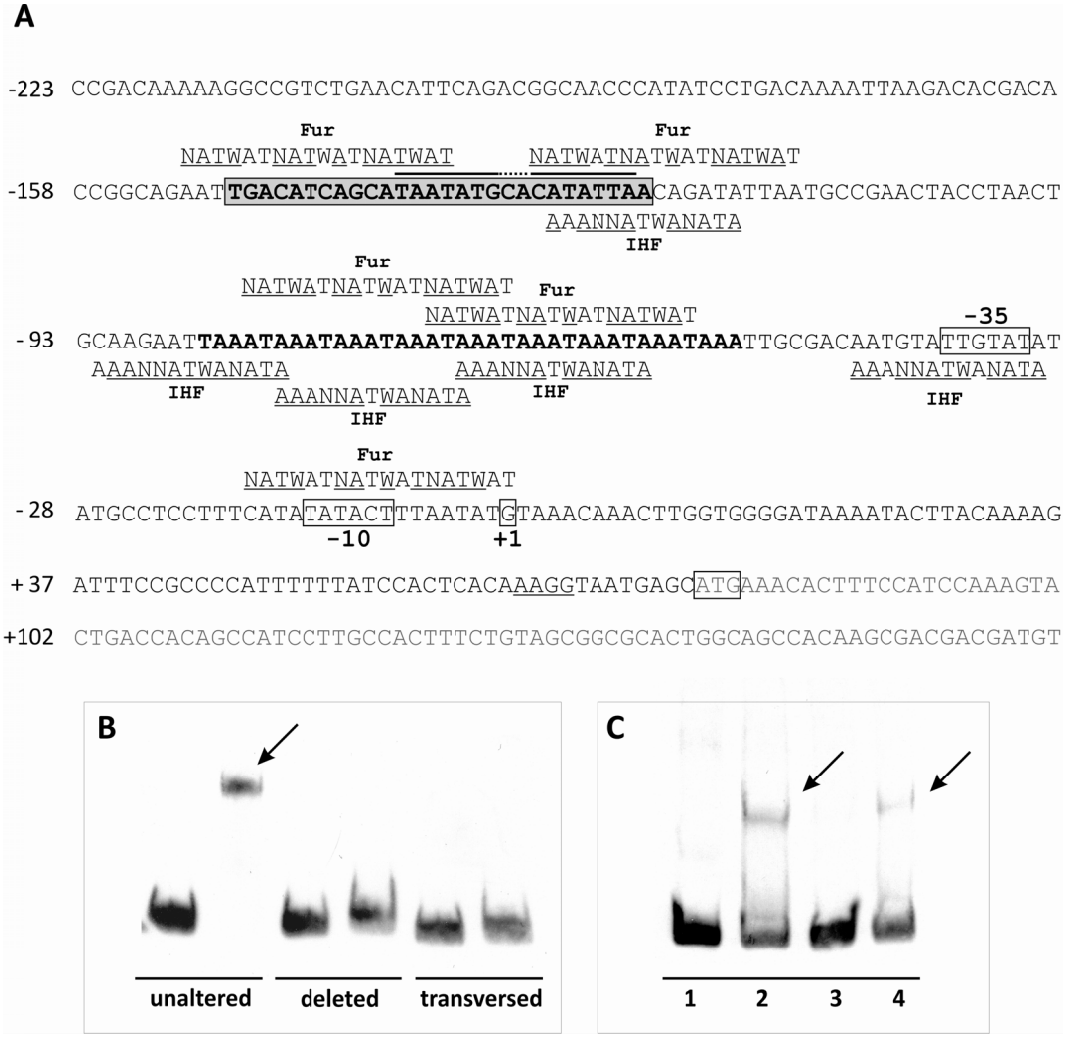


Figure 23. Localization of the FarR binding site within the *nadA* promoter region. (A) The sequence of the *nadA* promoter as well as the first nucleotides of its open reading frame, shown in gray (Martin *et al.*, 2003). The transcription start site (+1), the -10 and -35 element as well as the translation initiation codon are boxed, the ribosome binding site is underlined and the repeat tract is indicated in bold. The core binding site sequences of *Neisseria* Fur and IHF are indicated above and below the box, respectively, and the matching base pairs are underlined (Martin *et al.*, 2005). N: A, T, C or G; W:A, T. The 28 bp putative FarR binding site is boxed shaded and indicated in bold. The palindromic sequence (TAATATGNNCATATTA) is indicated above the box. **(B)** EMSAs with 100 bp fragments containing the 16 bp palindromic region either in its unaltered form, deleted or with transversed base pairs. The first lane in each pair represents the fragment without protein added; the second lane displays the fragments with addition of 1 µg FarR. **(C)** Competitive EMSAs with FarR. Lane 1, probe alone; lane 2, probe and 500 ng FarR; lane 3, additionally specific; lane 4, non-specific competitor DNA. Arrows indicate band shifts. Figure taken from Schielke *et al.*, 2009.

The 28 base pair putative binding region is upstream of the -10 and -35 elements as well as the microsatellite region with TAAA repeats (Figure 23A). Within this region, a 16 base pair palindromic sequence was located using Jemboss Einverted (Rice *et al.*, 2000). To determine whether this inverted repeat serves as FarR binding site, DNA probes of 100 bp length were generated, in which the inverted repeat TAATATGcaCATATTA was either present in its unaltered form, deleted or each base pair was transversed (GCCGCGTacACGCGGC). These probes were incubated with 1 µg purified FarR and analyzed by EMSAs. Addition of FarR led to a shift of the unaltered DNA probe, but this shift was not observed upon deletion of the 16 bp sequence (Figure 23B). Likewise, transversal of these 16 base pairs abolished the shift, excluding the possibility that the deletion changed the DNA conformation and thus inhibited binding of the transcriptional regulator. This interaction of FarR with the *nadA* promoter was also shown to be specific as the shift was reversed upon addition of excess amounts of specific but not of unspecific competing DNA probes (Figure 23C).

5.4.4. Molecular characterization of the FarR binding site

Hitherto the direct and specific binding of FarR to the 16 bp palindromic repeat within the *nadA* promoter region was shown. To elucidate the specific role of each base within this repeat, DNA probes with point mutations were generated (Figure 24A). Subsequently conducted EMSAs showed a complete shift of the DNA probe with the unaltered probe as well as with probes 1, 3 and 7 (Figure 24B). This indicates that the accordant exchanged base pairs are only of minor importance to the DNA recognition of FarR. With the probes 2 and 4, two shifted bands were visible, possibly due to inefficient binding of FarR indicating that the changed base pairs are important for a tight interaction of FarR with its cognate DNA sequence. This interaction was completely abolished with the probes 5 and 6, revealing that these nucleotides are indispensable for the specific interaction of FarR with the *nadA* promoter. Integrating the results obtained with the *farAB* promoter (see chapter 5.1.3), the *farAB* promoter region was screened for a similar binding sequence, finding a pattern with only five mismatches regarding the binding site within the *nadA* promoter. Subsequently, a minimal binding sequence for FarR was generated (Figure 24C) and visualized with WebLogo (Schneider & Stephens, 1990, Crooks *et al.*, 2004).

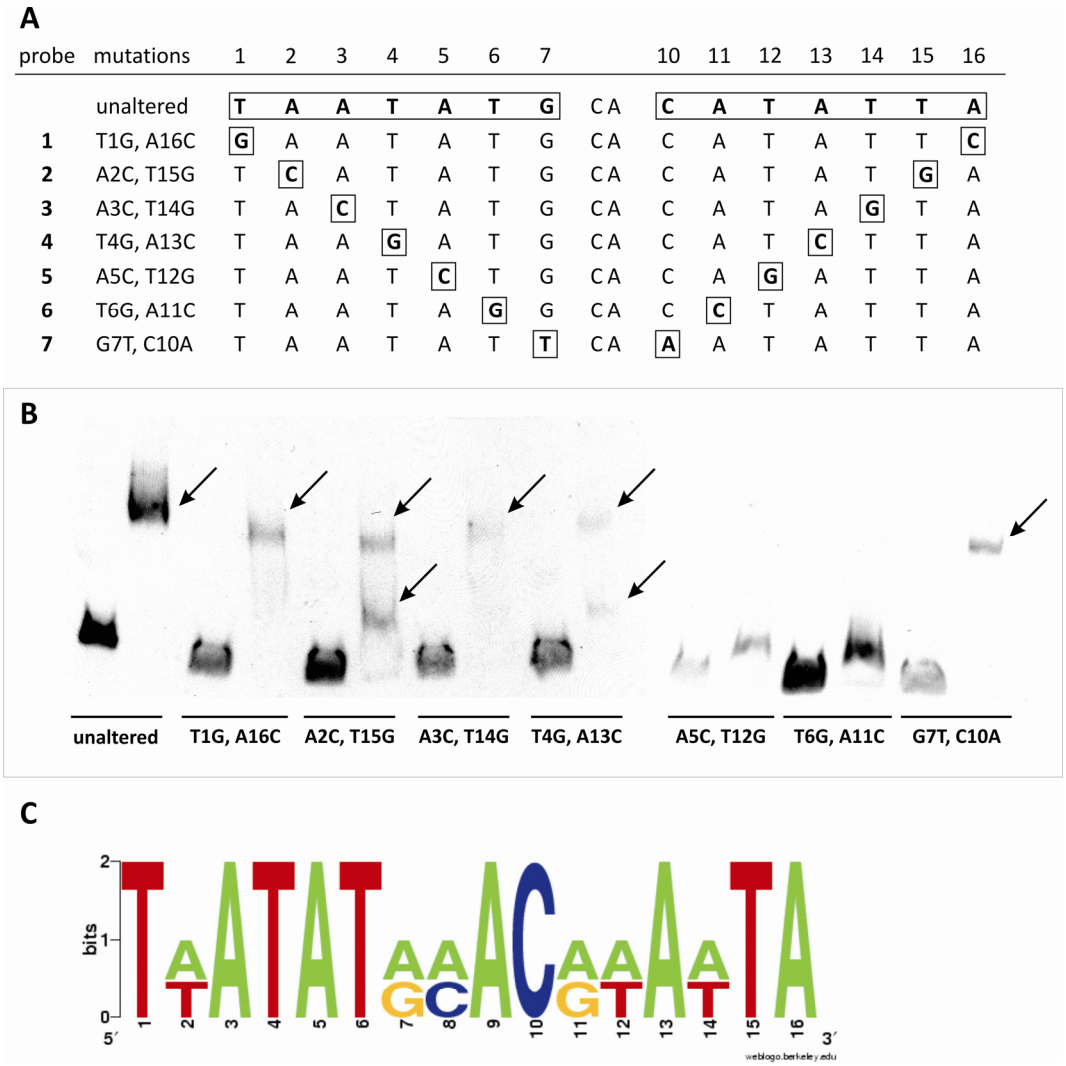


Figure 24. Mutational analysis of the FarR binding site within the *nadA* promoter. (A) Schematic display of the putative FarR binding site. The probable dimer binding site corresponding seven base pairs of the inverted repeat in the native constitution are boxed and shown in bold. The point mutations of each DNA probe are boxed and indicated in bold. **(B)** EMSAs with FarR and 100 bp probes containing the putative binding sequence either unaltered or with point mutations corresponding to the indicated positions within the dimer binding sites. First lane, DNA probe alone; second lane with 1 µg FarR. Arrows indicate band shifts. **(C)** Graphical display of the FarR minimal binding site, generated with WebLogo. Figure modified from Schielke *et al.*, 2009.

5.4.5. Role of FarR during interaction of *N. meningitidis* with epithelial cells

To examine the functional relevance of the FarR-dependent *nadA* repression, infection experiments were conducted with wild type strain MC58 as well as with the *farR* deficient strain MC58 Δ*farR* and the complemented strain MC58 Δ*farR* (pAP1*farR*). Infection experiments were performed with Chang epithelial cells because NadA was previously shown to promote adhesion and invasion into these cells (Comanducci *et al.*, 2002, Capecchi *et al.*, 2005). Monolayers of Chang cells were infected for three hours before dilution series were plated to determine the CFUs (see chapter 4.34).

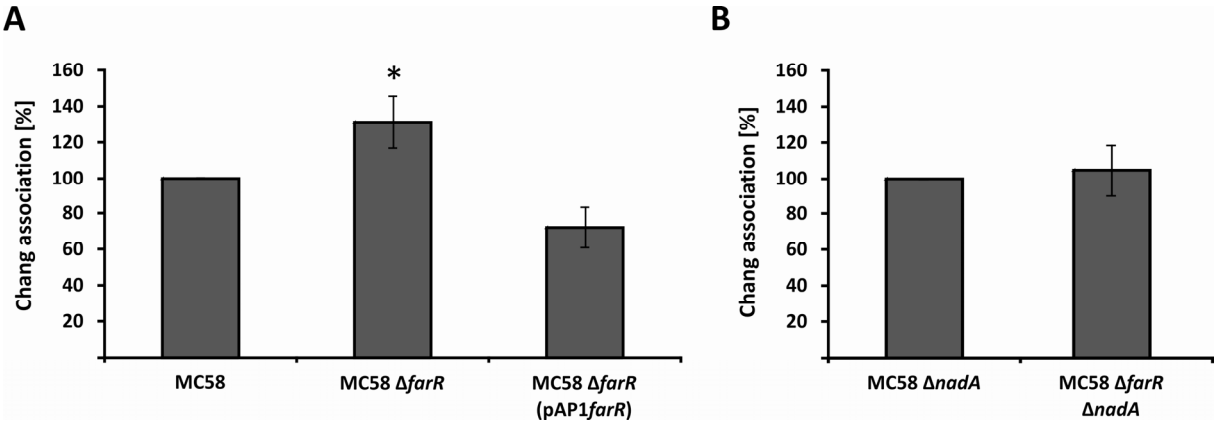


Figure 25. Adhesion of *N. meningitidis* to Chang epithelial cells. (A) Association of MC58, MC58 Δ*farR* and the complemented strain MC58 Δ*farR* (pAP1*farR*) to Chang epithelial cells. Cell-associated bacteria are displayed in relation to the wild type. **(B)** Adherence of MC58 Δ*nadA* and MC58 Δ*nadA* Δ*farR* to Chang epithelial cells. Data for MC58 **(A)** or MC58 Δ*nadA* **(B)** were used for normalization to exclude interexperimental variation. Error bars show the standard error of the mean of more than three experiments. *, $P < 0.02$. Figure in modification from Schielke *et al.*, 2009.

Bacteria associated with the epithelial cells are displayed in relation to the wild type strain (Figure 25A), revealing that the *farR* deficient strain showed considerably more adhesion. This difference, which was restored to wild type level in the complemented mutant strain, is possibly due to the de-repression of the adhesin NadA. To confirm this hypothesis, the adherence of the *nadA* deficient strain MC58 Δ*nadA* was compared to that of a *farR nadA* double mutant strain MC58 Δ*nadA* Δ*farR*. And indeed, no difference in adherence between these two strains was observed (Figure 25B), approving the role of *nadA* as a minor adhesin as well as the role of FarR in its repression.

5.4.6. Competition of FarR interaction with the *nadA* promoter

Capitalizing on the FarR-dependent strong and direct repression of *nadA,* the binding affinity of the transcriptional regulator was examined under competition conditions. For this purpose, fragment 2 (see chapter 5.4.2) of the *nadA* promoter region was employed as DNA probe. Salicylic acid as a phenolic compound has been shown to inhibit the direct interaction of the eponymous transcriptional regulator MarR in *E. coli* with its target DNA sequence (Cohen *et al.*, 1993a, Martin & Rosner, 1995). The effect of sodium salicylate on the interaction of FarR with the *nadA* promoter was examined using competitive EMSAs, but no reduction in FarR binding affinity was noted (Figure 26A).

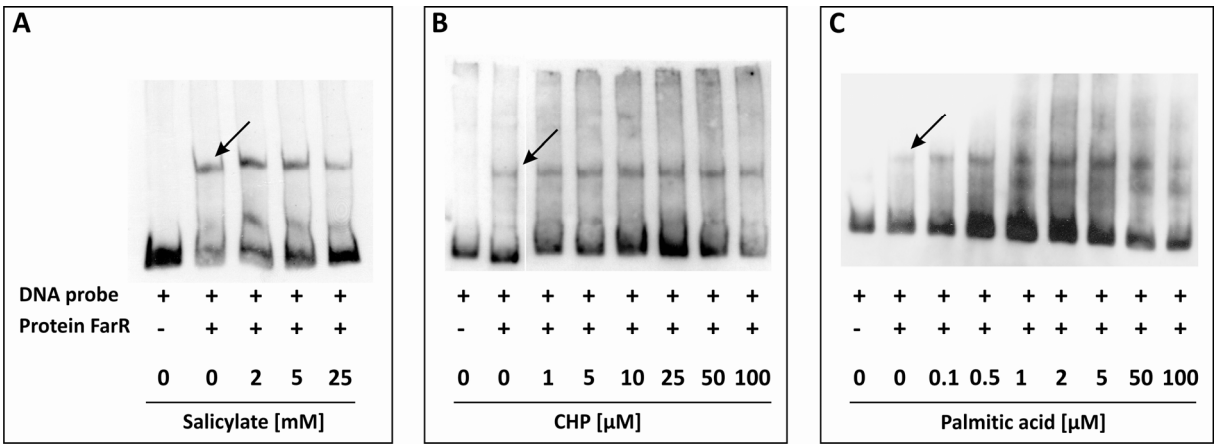


Figure 26. Competitive EMSAs with FarR and the *nadA* promoter. (A) EMSA with fragment 2 of the *nadA* promoter, addition of 150 ng FarR and increasing amounts of sodium salicylate **(B)** Interaction of 500 ng FarR with the *nadA* promoter upon addition of increasing amounts of CHP or **(C)** palmitic acid as competitors. Arrows indicate band shifts.

Furthermore, the aforementioned MarR-like regulators MexR, MgrA and OhrR all contain conserved cysteine residues responsible for oxidation-sensing; they dissociate from their target DNA upon treatment with CHP, cumene hydroperoxide (Chen *et al.*, 2006, Chen *et al.*, 2008, Eiamphungporn *et al.*, 2009). In order to determine the influence of CHP on the interaction of FarR with the *nadA* promoter competitive EMSAs were performed – with no visible attenuation of FarR binding, even with increasing CHP concentrations (Figure 26B). Although we demonstrated that FarR is not involved in fatty acid resistance in meningococci, competitive EMSAs with palmitic acid assured that the DNA binding capacity of FarR is unchanged in the presence of this hydrophobic compound (Figure 26C). Taken together, these experiments showed that FarR dissociation from the *nadA* promoter is not induced by salicylic acid, reactive oxygen species or fatty acids.

5.4.7. Promoter fusion constructs and *nadA* expression

Seeing the results so far, FarR represses *nadA* transcription by direct interaction with a 16 bp consensus sequence in the *nadA* promoter region and the NadA protein levels were significantly raised in a *farR* deficient mutant strain. To assure that this consensus sequence and thus the binding of FarR is really crucial for *nadA* expression promoter fusion constructs of the *nadA* promoter with the *lacZ* gene were generated (chapter 4.31). Furthermore, variants of this promoter containing either the unaltered, the deleted or transversed FarR binding site were constructed (in accordance to the experiments in chapter 5.4.3). Indeed, expression of *nadA* was completely repressed in the

strains with the unaltered *nadA* promoter, whereas deletion or alteration of the FarR binding site resulted in a strong increase of *nadA* expression (Figure 27A).

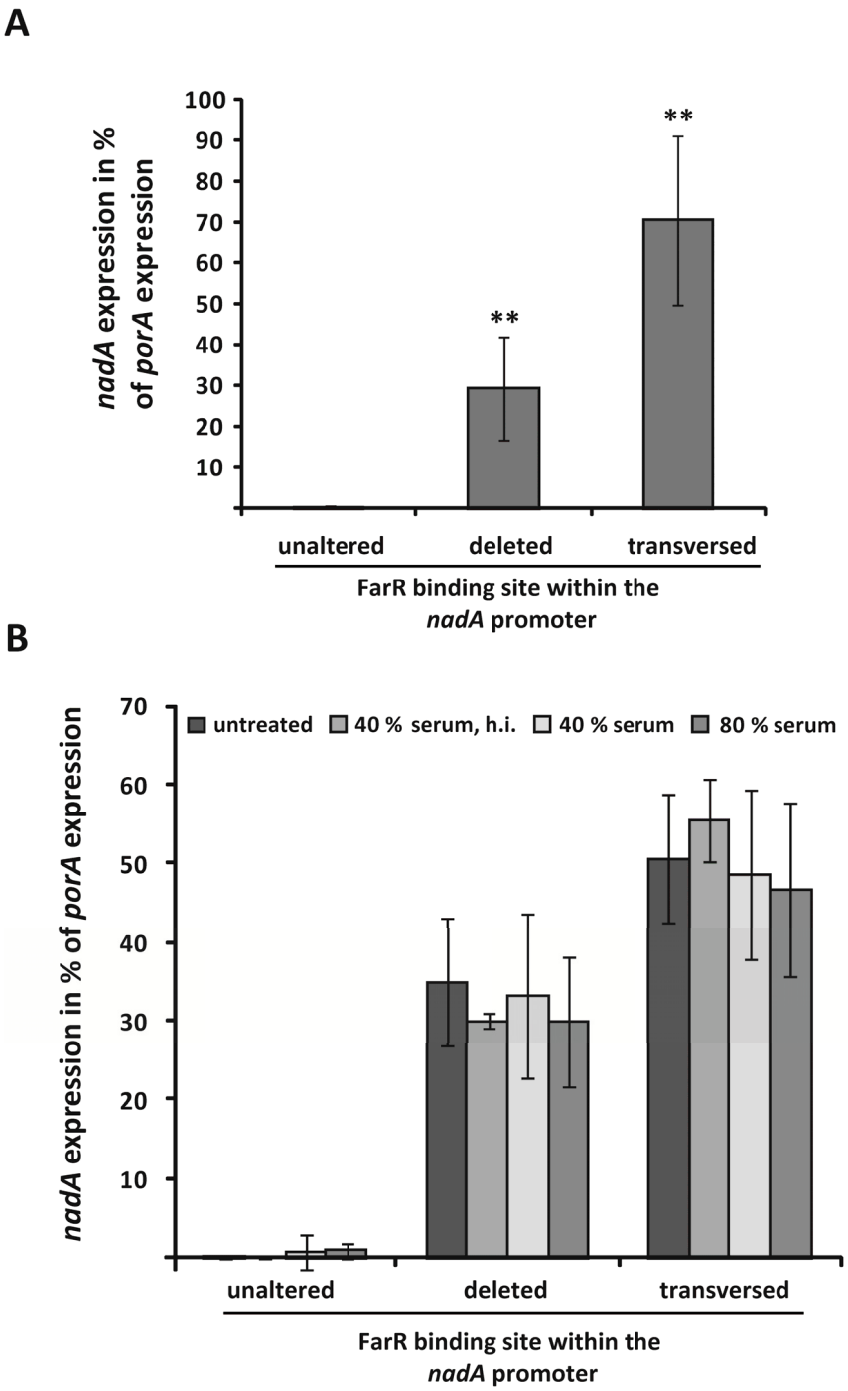


Figure 27. Effect of FarR binding on *nadA* expression. (A) Reporter gene fusion assays with *nadA* promoter constructs containing either the unaltered, deleted or transversed FarR binding site. Expression of *farR* is displayed in relation to the stably expressed *porA* gene. Error bars represent the standard deviation of at least six independent experiments. **, $P < 0.01$ **(B)** Quantification of *nadA* expression dependent on FarR binding in the presence or absence of complement preserved human serum. Error bars represent the standard deviation of at least three independent experiments displayed in relation to the stably expressed *porA* gene. Figure in modification from Schielke *et al.*, 2010a, in preparation.

Interestingly, *nadA* expression was significantly higher with the transversed than with the deleted FarR binding site. This might be caused by conformational changes in the DNA induced by the 16 bp deletion, which again might impede efficient binding of the RNA polymerase to this promoter fragment. As explained before, expression of *farR* is significantly reduced in the presence of complement preserved human serum. To test whether this downregulation of *farR* might entail an increased expression of *nadA*, the aforementioned *nadA* promoter fusion constructs were employed in serum stress experiments (chapter 4.31.2). However, in this experimental setup, only a weak rise in *nadA* transcription was detectable upon contact with active human serum whereas the strains containing promoter constructs with altered FarR binding site showed no significant differences in *nadA* expression (Figure 27B).

5.5. The FarR regulon in *N. meningitidis*

In order to identify further regulated genes of the transcriptional regulator FarR, a genome wide search for the consensus sequence (shown in Figure 24C) was performed using Artemis version 11 (Rutherford *et al.*, 2000). However, neither this sequence nor the exact FarR binding site within the *nadA* promoter region were found at another position in the genome of *N. meningitidis* MC58. Allowing for multiple mismatches with the search pattern NWNTATNNNNRWANTN 83 hits in putative promoter regions were located. Gel shift assays with a select few did not prove any direct interaction of FarR (Carolin Spatz, 2010, MD thesis). So as to characterize the regulon of FarR, DNA Microarray analysis was performed with the wild type strain MC58 and the *farR* deletion strain MC58 Δ*farR* (experimentation see chapter 4.28). The microarray results revealed only six significantly regulated genes in the *farR* deficient strain, whereof three showed an expression rate below the two-fold change threshold (Figure 28). However, the strong regulation of the adhesin NadA was confirmed with an almost thirty-fold higher expression upon deletion of *farR*. Besides *nadA*, the expression of three further genes was found to be upregulated in the wild type strain, NMB0866, NMB1299 and NMB0207. While NMB0866 encodes for a hypothetical protein repressed upon iron stress conditions (Grifantini *et al.*, 2003), NMB1299 (degenerate sodium/chloride dependent transporter) is a pseudogene with multiple premature stop codons in all sequenced meningococcal strains. Expression of the third gene, NMB0207 (*gapA-1*, GAPDH) was indicated to be significant although the change is not even two-fold. The gene product GAPDH is involved in the carbohydrate metabolism catalyzing the conversion of D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl

phosphate in the glycolysis and gluconeogenesis and was found to be upregulated upon iron depletion (van Ulsen *et al.*, 2009).

Locus	**Function**	**Expression change** ***fold increase***	***P* - value**
NMB1994	adhesin/invasin, *nadA*	29,02	$6{,}44 \cdot 10^{-19}$
NMB1299	sodium/chloride-dependent transporter, pseudogene	2,11	$1{,}58 \cdot 10^{-3}$
NMB0866	hypothetical protein, iron repressed (Grifantini *et al.*, 2003)	2,25	$2{,}06 \cdot 10^{-3}$
NMB0207	glyceraldehyde 3-phosphate dehydrogenase, *gapA-1*	1,80	$2{,}06 \cdot 10^{-3}$

Locus	**Function**	**Expression change** ***fold decrease***	***P* - value**
NMB1843	transcriptional regulator, *farR*	3,41	$1{,}73 \cdot 10^{-5}$
NMB0025	large pilS cassette	1,88	$7{,}01 \cdot 10^{-3}$
NMB1710	NADP-specific glutamate dehydrogenase, *gdhA*	1,75	$7{,}01 \cdot 10^{-3}$

Figure 28. Microarray results with significant gene expression alterations. Tabular display of the genes up- or downregulated in MC58 Δ*farR* in comparison to the wild type strain MC58. Expression changes are arithmetic means of three independent RNA isolations resulting with dye-swap pairs in six microarray slides. *P*-values were multiple-testing-corrected using fdr adjustment (Benjamini & Hochberg, 1995). Figure taken from Schielke *et al.*, 2010a, in preparation.

Besides the regulator itself, two genes were found to be significantly but only slightly (less than two-fold) downregulated in the wild type strain, NMB1710 and NMB0025. NMB1710 (*gdhA*) is involved in the amino acid metabolism as deaminating $NADP^+$ oxidoreductase converting L-glutamate to 2-oxoglutarate and NMB0025 encodes for the large *pilS* cassette, one of multiple silent *pilE* homologues (Perry *et al.*, 1988), which are not expressed in *N. meningitidis*. To verify these partly marginal microarray results, quantitative RT-PCR analyses were performed (see chapter 4.29). As for the upregulated genes upon *farR* deletion, expression of NMB1299 and NMB0207 was affirmed to be slightly more than two-fold increased, whereas the differential expression of NMB0866 could not be confirmed (Figure 29).

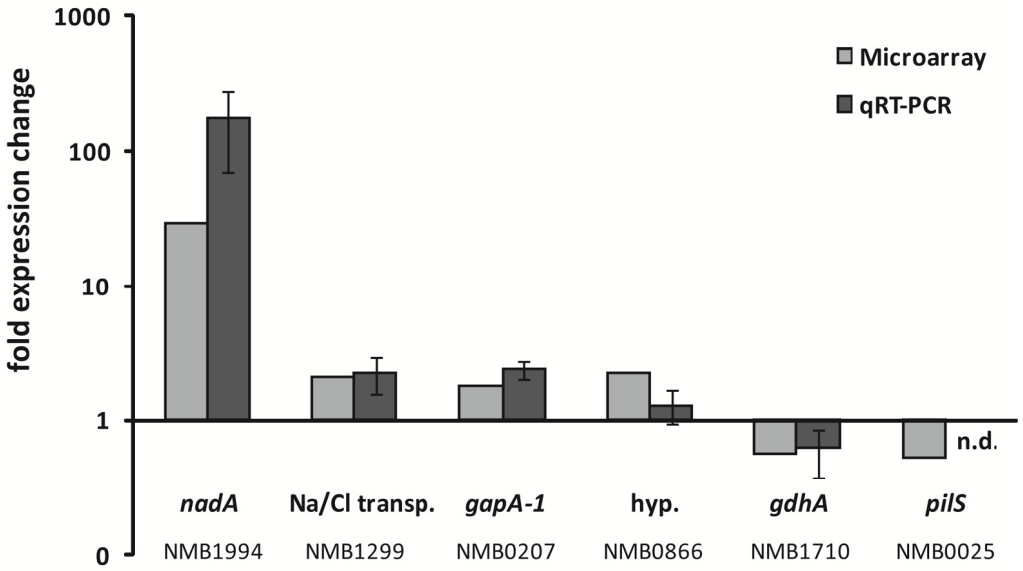


Figure 29. Comparison of FarR regulated genes by microarray and qRT-PCR analysis. Comparative display of the expression changes of the five significantly FarR-regulated genes (see Figure 28) by microarray and qRT-PCR analysis. Data are presented as fold expression changes with a logarithmic scale. Error bars indicate the standard deviation of two independent experiments, each performed in triplicate. n.d., not determined. Figure taken from Schielke *et al.*, 2010a, in preparation.

Of the downregulated genes NMB1710 and NMB0025, the latter could not be verified by qRT-PCR analysis as no single PCR product could be generated. Nevertheless, the results for NMB1710 were verified to represent no considerable expression change upon *farR* deletion. Taken together, these results assured the repressory action of FarR with the adhesin NadA as its major target and NMB0207 (*gapA-1*, GAPDH) as only other slightly but significantly regulated gene that is regularly expressed in *N. meningitidis*.

6. Discussion

6.1. Characterization of the transcriptional regulator FarR

Neisseria meningitidis is exclusively adapted to a specific niche in the human host. Little is known about the regulatory pathways responsible for this adaptation – no alternative sigma factors and only one two-component system have been described (Tzeng *et al.*, 2008, Jamet *et al.*, 2009). Furthermore, so far only 5 of the predicted 35 transcriptional regulators have been characterized: CrgA is upregulated upon contact with human epithelial cells repressing *crgA* and *pilC1* (Deghmane *et al.*, 2000, Deghmane *et al.*, 2004), NMB0573 (annotated as AsnC) is a global regulator controlling response to poor nutrient conditions (Ren *et al.*, 2007) and the ferric uptake regulator Fur is involved in the response to iron (Delany *et al.*, 2004, Delany *et al.*, 2006). Adaptation to oxygen limited conditions is mediated by FNR (Bartolini *et al.*, 2006) as well as by the nitric oxide sensor and repressor NsrR (Rock *et al.*, 2007, Heurlier *et al.*, 2008). Here NMB1843, one of the two transcriptional regulators of the MarR family in *N. meningitidis* (Tettelin *et al.*, 2000) was identified and characterized. Dot Blot and sequence analysis revealed that this regulator is highly conserved and present in all tested strains, regardless whether they were carriage or disease isolates. Additionally, homologues of this gene were also found in the commensal *N. lactamica* and in the closely related pathogen *N. gonorrhoeae*. An amino acid sequence alignment with other members of the MarR family involved in virulence gene expression in human pathogens disclosed a very low sequence similarity despite a high predicted structural homology. The highest amino acid sequence homology (98.40 %) was detected with FarR (the **f**atty **a**cid **r**esistance **R**epressor) of *N. gonorrhoeae* and NMB1843 was therefore named FarR (*Nm*FarR). Homology modeling and dimer prediction revealed a dimeric DNA binding protein with a winged helix-turn-helix motif characteristic for the MarR family of transcriptional regulators. This dimer probably interacts with the target DNA sequence utilizing the flexible wing domains.

6.2. Control of FarR expression in *N. meningitidis*

The MarR family of transcriptional regulators was named for controlling multiple antibiotic resistance in *E. coli* and has gathered increased attention in health care as clinical strains with mutations within this regulator were found to contribute to multidrug resistance (George & Levy, 1983, Maneewannakul & Levy, 1996). This so-called *mar* phenotype was demonstrated to be inducible by structurally unrelated lipophilic or phenolic compounds like salicylic acids, chloramphenicol or tetracycline (Hachler *et al.*, 1991, Cohen *et al.*, 1993a, Cohen *et al.*, 1993b, Ariza *et al.*, 1994, Seoane & Levy, 1995). Antibiotics act as inactivating ligands for the eponymous protein MarR in *E. coli* as well as for MexR in *P. aeruginosa* and MgrA in *S. aureus*, thereby inducing expression of multidrug efflux pumps conveying resistance to antibiotics (Lim *et al.*, 2002, Luong *et al.*, 2003, Truong-Bolduc & Hooper, 2007). The effect of nine antibiotics on *N. meningitidis* MC58 wild type and *farR* deletion strains were tested. However, the resulting minimal inhibitory concentration for each antimicrobial did not differ significantly between these two strains, indicating that there is no antibiotic resistant phenotype in meningococci upon inactivation of the repressor FarR. It has been shown that the bactericidal activity of antibiotics is additionally due to the generation of oxidative stress (Kohanski *et al.*, 2007). Indeed, not only MexR and MgrA, but also the organic hydroperoxide resistance gene regulator OhrR of *B. subtilis* are induced upon contact with reactive oxygen species released by treatment with H_2O_2, CHP (cumene hydroperoxide) or the redox cycling reagent paraquat (Chen *et al.*, 2006, Chen *et al.*, 2008, Fuangthong & Helmann, 2002). We examined whether oxidative stress exerted a similar inducing activity on FarR in meningococci but observed no significant differences between the wild type and the *farR* deficient strain over a time course of sixty minutes. Therefore, FarR does not seem to be responsive to oxidation. Unlike many other MarR-like regulators, FarR is also not autoregulatory as no direct interaction of FarR with the *farR* promoter region was observed. Virulence gene expression can be dependent on the growth phase, as has been published for *Helicobacter pylori* which is most virulent in the late logarithmic phase (Thompson *et al.*, 2003). In other Gram-negative bacteria, alternative sigma factors like RpoS (σ^S) respond very sensitively to growth rate (Teich *et al.*, 1999), but in *N. meningitidis*, no functional alternative sigma factors have been found so far. In order to examine expression of the transcriptional regulator during growth of *N. meningitidis*, reporter gene fusion constructs were generated with the *farR* promoter and *lacZ*, the gene encoding β-galactosidase. Indeed, a significant increase in *farR* transcript was observed during exponential growth, being highest in late exponential phase. This increase was verified by western blot analysis. The promoter region of the constitutively expressed *porA* gene served as control and showed no differential expression in any of the two assays. However, a growth phase dependent

expression of FarR was also visible in the complemented *farR* strain where the ectopic *farR* gene is under control of the *porA* promoter. This might indicate that *farR* expression is subject to post-transcriptional control or modification. Expression of the *Yersinia* virulence regulator RovA, as well a member of the MarR family, is also subject to growth phase control, independent of the native promoter region (Herbst *et al.*, 2009). In this case, the post-transcriptional regulation proved to be due to small non-coding carbon storage regulator system (Csr-type) RNAs (Heroven *et al.*, 2008). In conclusion, the results indicate that the transcriptional regulator FarR in *N. meningitidis* is differentially expressed during exponential growth but neither autoregulated nor affected by a challenge with antibiotics or oxidative stress. Therefore, FarR seems not comparable to any other described member of the MarR family. As meningococci are highly adapted to their specific niche within the human host, the expression of FarR was tested under infection-like conditions, similar to those the bacteria encounter within their natural habitat. One of the most challenging situations during infection is the iron restriction because the level of free iron in human blood is far below that required for growth (Weinberg, 1974, reviewed in Weinberg, 1978). This iron starvation signal was shown to induce transcription of virulence-associated genes in meningococci dependent on the stage of colonization (Grifantini *et al.*, 2003, Shaik *et al.*, 2007, Jordan & Saunders, 2009, Basler *et al.*, 2006). Consequently, the transcription of *farR* was determined in a reporter fusion assay under iron-depleted or -replete conditions, respectively. The expression of the transcriptional regulator was constant, indicating that FarR is not involved in this specific stress response. Besides limitation of free iron, the bactericidal activity of normal human serum is an important defense mechanism of the human host. This activity is mainly attributed to the complement system culminating in assembly of the membrane attack complex and thus in bacterial lysis. The effect of complement preserved normal human serum was examined on the expression of *farR* in meningococci. The bacteria survived the presence of even 80 % serum over a time course of six hours, whereas the isogenic unencapsulated strain was killed swiftly. This is in accordance with the literature describing that the capsule as well as LPS sialylation are essential for meningococcal serum resistance (Mackinnon *et al.*, 1993, Vogel *et al.*, 1997b). Expression of *farR* is significantly reduced in the presence of 40 % serum compared to control reactions. Doubling of the serum concentration led to noticeably less transcript but not to a significant difference to the results with 40 % serum. This reduction in *farR* transcription seems to depend on active complement components rather than on the nutrient availability within the serum as no differences in expression were observed regarding heat-inactivated serum.

6.3. Fatty acid resistance in *N. meningitidis* and *N. gonorrhoeae*

Within their respective mucosal niches, both meningococci and gonococci are confronted with antimicrobial fatty acids secreted by the epithelia. Saliva as well as vaginal excretions are rich in saturated and unsaturated fatty acids acting as broad-band antimicrobials (Paavonen, 1983, Slomiany *et al.*, 1989, Tomita *et al.*, 2008). Therefore, both *Neisseriae* species had to evolve mechanisms to avoid being killed by these compounds. In gonococci, there are two efflux pump systems relevant for the survival on the urogenital mucosa, firstly the MtrCDE system exporting hydrophobic agents like bile salts, antibacterial peptides (Protegrin-1 and LL-37) and steroidal hormones (Hagman *et al.*, 1995, Hagman *et al.*, 1997) and secondly the FarAB-MtrE system responsible for the export of long chain fatty acids (McFarland *et al.*, 1983, Lee & Shafer, 1999). These systems are composed of three components, a membrane fusion protein (MtrC, FarA) and a cytoplasmic membrane transporter protein (MtrD, FarB) which seize and transport the hydrophobic agents to the outer membrane, whereas the MtrE protein functions as outer membrane channel for both systems (Hagman *et al.*, 1995, Delahay *et al.*, 1997, Lee & Shafer, 1999). As the expression of such elaborate systems is energy-draining, they are kept under strict transcriptional control (Nikaido, 1996). Indeed, both systems have been shown to be repressed by the transcriptional regulator MtrR, the *mtrCDE* system directly (Hagman *et al.*, 1995, Hagman & Shafer, 1995), and the *farAB-mtrE*-system indirectly via MtrR-dependent repression of the repressor *farR* (Lee & Shafer, 1999, Lee *et al.*, 2003). FarR in *N. gonorrhoeae* was shown to interact specifically with the *farAB* promoter region and deletion of the repressor gene *farR* in gonococci led to increased fatty acid resistance due to elevated transcription of the *farAB* efflux pump system (Lee *et al.*, 2003). The first step holds also true for meningococci – direct and specific binding of FarR to the *farAB* promoter region was shown by electrophoretic mobility shift assays. To test whether the fatty acid resistance in meningococci was influenced by FarR as well, their susceptibility to the medium chain lauric acid ($C_{12:0}$), the long chain saturated palmitic acid ($C_{16:0}$) and the long chain unsaturated linoleic acid ($C_{18:2\Delta9\Delta12}$) was tested. Interestingly, all evaluated meningococcal strains, including the *farR* deletion mutant, proved to be highly resistant against all of these fatty acids. Consequently, these analyses were extended to examine the fatty acid resistance in clinical isolates covering the major disease-associated serogroups as well as carriage isolates and unencapsulated strains. All of these strains were shown to display a generally high resistance; the only exceptions with a high susceptibility to palmitic acid were three strains belonging to serogroup Y and sequence type 23. For most *N. meningitidis* strains, a higher resistance against linoleic acid was observed, but the susceptibilities against lauric, linoleic or palmitic acid were seemingly not correlated. Gonococcal clinical isolates, on the other hand,

displayed a highly variable lauric and palmitic acid resistance, in a pattern that could not be correlated with their isolation site or other strain attributes. The atypical sensitivity of gonococci to hydrophobic compounds has been described before and was attributed to an increased permeability of the outer membrane (Sarubbi *et al.*, 1975, Guymon & Sparling, 1975, Miller & Morse, 1977), which was hypothesized to be due to enriched phospholipid bilayer regions on the gonococcal outer membrane (Lysko & Morse, 1981). As the intrinsic fatty acid resistance of meningococci could mask any effect of an efflux pump system, the molecular basis for this resistance was examined. Bacterial susceptibility to free fatty acids depends in most cases on organization of the cell wall with Gram-negative bacteria being more resistant than Gram-positives (Wille & Kydonieus, 2003). The outer membrane composition and the presence of LPS contribute to the natural protection of Gram-negative bacteria against hydrophobic substances (Galbraith & Miller, 1973, Sheu & Freese, 1973). To assess the role of LPS in the intrinsic resistance of *N. meningitidis*, we generated stepwise truncated lipopolysaccharide mutants in meningococci. Additionally, an LPS deficient mutant strain was tested, which is viable in meningococci, while this mutation is lethal in most other Gram-negative bacteria, including *N. gonorrhoeae* (Steeghs *et al.*, 1998, Bos & Tommassen, 2005). This LPS deficiency led to palmitic acid susceptibility. In *E. coli* and *S. typhimurium*, the absence of the core oligosaccharide in a *kdtA* mutant strain also led to fatty acid susceptibility, which was correlated with the fatty acid chain length and not existent with the long chain linoleic acid (Sheu & Freese, 1973). We generated a *kdtA* mutant strain in meningococci and obtained similar results: these mutant strains were sensitive to palmitic but not to linoleic acid. In conclusion, the carbohydrate moiety is important for resistance against palmitic acid in meningococci. However, the susceptibility of the serogroup Y, ST-23 strains is not likely to be explained by this as deep mutations within the LPS carbohydrate moiety hardly occur in nature. On the contrary, mutations within the lipid A part of LPS have recently been described to appear naturally (Fransen *et al.*, 2009). These mutations affect the *lpxL1* gene, resulting in penta- instead of hexaacylated lipid A (Fransen *et al.*, 2009). The lipid A part of the LPS with its six acyl chains anchoring it in the bacterial outer membrane is causative of the endotoxin activity of LPS as it is recognized by TLR4 (reviewed in Beutler & Rietschel, 2003). The last acyl chain added by *lpxL1* is necessary for induction of full inflammatory responses in both *N. meningitidis* and *N. gonorrhoeae* (Ellis *et al.*, 2001, Mogensen *et al.*, 2006, Fransen *et al.*, 2009) and seems to be important for intracellular survival of gonococci (Post *et al.*, 2002). Indeed, by analyzing the *lpxL1* sequence of the three serogroup Y, ST-23 strains different inactivating mutations could be identified. By complementing such a mutation, the resistance to palmitic acid was fully restored, indicating that the hexaacylation of lipid A plays a crucial role in susceptibility to fatty acids. In *S. aureus*, fatty acid susceptibility is correlated with accumulation of fatty acids at the cytoplasmic membrane (Kohler *et*

al., 2009). To examine whether the unusual fatty acid sensitivity of the strains with mutations within the *lpxL1* gene is due to the same mechanism, FACS analyses with fluorescently labeled palmitic acid were performed. However, the *lpxL1* deletion strain showed no increased binding of this hydrophobic compound in comparison to the parental strain. The hypothesis for the role of *lpxL1* in fatty acid susceptibility is not conferrable to *N. gonorrhoeae* as the sequenced strain FA1090 showed a high susceptibility but none of the described inactivating mutations (Fransen *et al.*, 2009). Concluding, fatty acid resistance in meningococci is based on LPS carbohydrate composition and independently, also on lipid A acylation. In contrast to gonococci where deletion of the transcriptional repressor *farR* led to increased expression of the *farAB* encoded efflux-pump and thus to resistance against fatty acids (Lee & Shafer, 1999, Lee *et al.*, 2003), deletion of *farR* in meningococci had no influence on fatty acid resistance. However, it is possible that the intrinsic fatty acid resistance of *N. meningitidis* disguised any effect generated by an upregulation of the *farAB* efflux pump system due to the deletion of *farR*. Therefore we generated a *farR* deletion mutant strain in a fatty acid sensitive *kdtA* mutant strain and repeated the susceptibility experiment. This strain also displayed no increased resistance against palmitic or linoleic acid. To exclude the possibility that FarR is only induced upon a high amount of palmitic acid, the minimal inhibitory concentration (MIC) for palmitic acid for wild type and *farR* deletion strains was determined. However, the MICs were found to be 1500 µg/ml, independent of the *farR* deletion and resistance was also not changed in a *kdtA farR* double mutant. In order to analyze whether the *farAB* efflux pump system is indeed insignificant for meningococcal resistance, *farAB* deficient strains were generated in the wild type as well as in the palmitic acid sensitive unencapsulated LPS-truncated strain Δ*siaD* Δ*kdtA*. Interestingly, inactivation of this operon completely abolished the palmitic acid resistance, indicating that resistance against palmitic acid is maintained by active efflux of this compound, which is not negatively controlled by FarR. Additionally, an intact LPS molecule is indispensable for resistance as truncation of the oligosaccharide moiety or incomplete acylation of lipid A rendered the bacteria susceptible irrespective of the presence of *farAB*. Such interdependency between the permeability barrier of the outer membrane and efflux pumps has also been described for the MtrCDE system in gonococci (Lucas *et al.*, 1995, Nikaido, 1994). Possibly, intact LPS is necessary for correct insertion of the efflux pump systems into the outer membrane (Ried *et al.*, 1990). Furthermore, the *lpxL1* deletion and thus the absence of one out of six acyl chains may change the outer membrane of meningococci as a decrease in the number of acyl chains linked to lipid A may destroy the integrity of the outer membrane and thus result in defects of the permeability barrier maintained by the polyanionic LPS (Fisseha *et al.*, 2005, Murray *et al.*, 2001, Snyder & McIntosh, 2000). Losing this important barrier function, LPS may not be able to maintain its

protection of the efflux pump proteins from exposure as it has been shown for porins (Judd & Shafer, 1989, Lucas *et al.*, 1995). This is yet another indication for evolution of different concepts of transcriptional control in the closely related *N. meningitidis* and *N. gonorrhoeae*, as they contain the same efflux pump systems responsible for the export of hydrophobic compounds. In both species, these systems are dependent on an unperturbed permeability barrier and thus an intact LPS for their functionality. While gonococci rely on the MtrRCDE efflux pump system for survival in the urogenital tract (Shafer *et al.*, 1995, Jerse *et al.*, 2003), meningococci have a functional MtrCDE system but no functional repressor MtrR (Pan & Spratt, 1994, Abadi *et al.*, 1996, Rouquette-Loughlin *et al.*, 2004). However, natural downregulation of *mtrC* expression by IHF-dependent insertions of Correia Elements prevents a constitutively active MtrCDE efflux pump in meningococci (Correia *et al.*, 1986, Rouquette-Loughlin *et al.*, 2004). Furthermore, gonococci need the FarRAB system to survive highly hydrophobic environments like the rectum (McFarland *et al.*, 1983, Morse *et al.*, 1982) and meningococci harbor a functional FarAB system that is not controlled by FarR, but rather constitutively active and thus responsible for the overall high resistance against fatty acids.

6.4. Role of FarR in *N. meningitidis*

As FarR obviously assumed a different role in meningococci than in gonococci, further FarR-regulated genes were to be identified in *N. meningitidis*. Protein expression profiles of MC58 wild type and a *farR* deficient strain identified the adhesin *nadA* as a strongly repressed target gene of FarR. This adhesin is a surface exposed trimeric protein that is highly antigenic, present in most hypervirulent strains and therefore part of a promising serogroup B vaccine, which is entering phase III clinical trials (Pizza *et al.*, 2000, Comanducci *et al.*, 2002, Bowe *et al.*, 2004, Giuliani *et al.*, 2006). ELISA and Western Blot analyses confirmed the FarR modulated *nadA* repression to such an extent that the NadA protein was almost undetectable on the surface of the wild type strain. This strong repression might explain the divergent adhesion results when recombinant NadA is expressed in *E. coli* compared to wild type *Neisseriae*. In meningococci, no strong increase in adhesion was observed with NadA present on the surface, as it is repressed by FarR, which again is not present in this form in *E. coli* (V. Nägele and N. Ackermann, personal communications). The *nadA* gene has a lower GC-content than the average neisserial genome and was probably acquired by horizontal gene transfer. This hypothesis is sustained by the fact that NadA is distributed irregularly among meningococcal strains, being underrepresented in commensal carrier strains and absent in the closely related gonococci (Comanducci *et al.*, 2002, Comanducci *et al.*, 2004). Additionally, the *nadA* gene is

frequently inactivated by the insertion element *IS1301* in strains of the ET-15 clonal complex (Elias & Vogel, 2007). When the gene is present, however, FarR interacts directly and specifically with the *nadA* promoter region. The binding sequence of FarR within the *nadA* promoter region was narrowed to a 16 bp palindromic repeat upstream of the -35 and -10 promoter elements as well as the microsatellite, comprising the base pairs -136 to -121 relative to the transcription start site of *nadA* (Martin *et al.*, 2003). Phase variation due to slipped strand mispairing as well as the transcription factors IHF and Fur have been implicated with transcription of the *nadA* gene and it was even proposed that a transcriptional regulator might be involved (Saunders *et al.*, 2000, Martin *et al.*, 2003, Martin *et al.*, 2005). Furthermore, reporter gene fusion assays demonstrated that alterations of the FarR binding site within the *nadA* promoter are sufficient to induce *nadA* transcription. This regulation of *nadA* by FarR is indeed functionally relevant as a *farR* deletion strain adhered significantly more to Chang epithelial cells than the wild type strain. A complemented mutant strain, where the repressor was restored and *nadA* thus expressed to a lower extent, reconstituted the wild type phenotype. This might hint at a clinically relevant role for FarR: it can contribute to immune evasion of the *nadA* expressing virulent meningococci, making sure that *nadA* is not expressed and thus shielding the bacteria from the host innate immune answer to this highly immunogenic surface component. No data concerning the transcriptional regulation of another member of the Oca-family of adhesins have been published so far, indicating that these results are the first to describe a concerted action of a transcriptional repressor with phase variability for this group of adhesins. For further analysis, this strong and direct repression of *nadA* expression by FarR was employed and the *nadA* promoter utilized as a tool to examine yet other functions of FarR. However, the phenolic compound sodium salicylate, which induces the transcriptional regulator MarR in *E. coli* (Cohen *et al.*, 1993a, Martin & Rosner, 1995), caused no reduction in FarR DNA binding affinity as measured in competitive EMSAs with the *nadA* promoter region. Although a *farR* deletion mutant in *N. meningitidis* showed no changed resistance against fatty acids, we assured that the DNA binding capacity of FarR is unchanged in the presence of palmitic acid. As the aforementioned MarR-like regulators MexR, MgrA and OhrR all dissociate from their target DNA upon treatment with CHP due to conserved cysteine residues responsible for oxidation-sensing (Chen *et al.*, 2006, Chen *et al.*, 2008, Eiamphungporn *et al.*, 2009), the influence of CHP on the interaction of FarR with the *nadA* promoter was tested. No visible attenuation of FarR binding was observed, even with increasing CHP concentrations. Therefore, FarR does not seem to be responsive to oxidation and harbors none of the described cysteine residues as shown in an alignment with MexR, MgrA and OhrR. Taken together, these experiments show that FarR dissociation from the *nadA* promoter is not induced by salicylic acid, fatty acids or reactive oxygen species. Recently, 4-hydroxyphenylacetic acid has been

identified as FarR inducing ligand, a nitrated phenolic that may be generated in human saliva (Takahama *et al.*, 2003, Metruccio *et al.*, 2009). As discussed earlier, expression of *farR* is significantly reduced in the presence of complement preserved human serum. This downregulation of *farR* might entail an increased expression of the adhesin NadA. Using *nadA* promoter fusion constructs, a weak rise in *nadA* transcription was detectable in our experimental setup. A possible explanation for this weak reaction may be that despite downregulation of *farR* transcript, FarR protein is not induced and still tightly bound to the *nadA* promoter to inhibit its transcription. Possibly, the situation *in vivo* needs an additional trigger not present in our *in vitro* assay. However, the tendency to *farR* downregulation and upregulation of the adhesin *nadA* in the presence of human complement is obvious. Interestingly, NhhA, the second trimeric autotransporter adhesin in *N. meningitidis*, has recently been connected with serum resistance (Sjolinder *et al.*, 2008). NhhA resembles NadA in multiple respects: likewise, it was identified to be a surface-exposed antigen, it triggers a bactericidal antibody response and it facilitates adhesion to human epithelial cell lines *in vitro* (Pizza *et al.*, 2000, Scarselli *et al.*, 2006). For NadA, no results regarding serum resistance have been published so far, but as NhhA impedes the assembly of the membrane attack complex (Sjolinder *et al.*, 2008), a similar role for the structurally related NadA may well be possible.

6.5. The FarR regulon in *N. meningitidis*

The results up to now indicated that the transcriptional regulator FarR is involved in adaptation to the host environment, tightly controlling expression of the adhesin *nadA*. To identify other effector genes microarray analyses were performed to reveal the regulon of FarR. Indeed, the repression of *nadA* was clearly evident with an at least 30-fold induction upon deletion of *farR*. Besides *nadA*, only five genes were significantly regulated, of which only two showed a slightly higher regulation than a two-fold change. All significant results were validated by qRT-PCR but only two of these five genes were found to be significantly regulated. These two genes, a degenerate sodium/chloride-dependent transporter (NMB1299) and *gapA-1* (GAPDH), a key enzyme in the carbohydrate metabolism (NMB0207), were barely repressed to the two-fold expression change threshold. Although the promoter region of NMB1299 contains a putative degenerate binding site for FarR with six matches compared to the consensus sequence, this gene is a pseudogene in all sequenced meningococcal strains containing multiple premature stop codons. Thus the only functional regulated gene was *gapA-1*, making FarR a transcriptional repressor with a regulon of two genes – *nadA* and *gapA-1*. These results agree with previous findings showing that *N. meningitidis*, which colonizes a highly

specific niche within the human host, displays a diminished need for global transcriptional regulation. For example, the regulon of the nitric oxide sensing repressor NsrR in meningococci was shown to be very compact, comprising only four genes and one pseudogene compared to the equivalent regulon in *E. coli*, which controls approximately 40 genes (Filenko *et al.*, 2007, Rock *et al.*, 2007, Heurlier *et al.*, 2008). This discrepancy holds also true for FNR, the regulator of the adaptive oxygen limitation response: the regulon in *N. meningitidis* was shown to consist of nine transcriptional units, whereas FNR in *E. coli* controls more than 100 operons (Bartolini *et al.*, 2006, Overton *et al.*, 2006). The only known exception of these highly specialized meningococcal regulators is Fur, whose regulon comprises more than 200 genes (Grifantini *et al.*, 2003). In conclusion, FarR is a highly specialized transcriptional repressor of the adhesin NadA. No other identical copy of the FarR binding sequence was found within the MC58 genome. However, this analysis of the controlled genes is a snap-shot of the exponential growth phase in rich medium and thus it cannot be ruled out that FarR controls further genes under different circumstances – for instance under *in vivo* conditions. Furthermore, FarR might act indirectly via post-transcriptional regulators like small RNAs or the RNA chaperone Hfq (Fantappie *et al.*, 2009). In conclusion, meningococcal FarR is extremely specialized on repression of *nadA*.

6.6. The role of FarR in the divergent evolution of pathogenic *Neisseriae*

In the genus *Neisseria*, *N. meningitidis* and *N. gonorrhoeae* are the only relevant potentially human pathogenic microorganisms and they are closely related. Interestingly, their common history and thus the time span for co-evolution is probably very short. Incidences of gonorrhoea can be traced back to passages in the Old Testament of the bible (4th book of Moses 25: 1-9) and are already described in detail by Procopius in the 6th century, a historian of the late classic period (Procopius Caesariensis, *Historia arcana/Anecdota*). The first account of meningococcal meningitis, on the other hand, was recorded in 1805 in Switzerland (Vieusseaux, 1805) and recurrent epidemics were registered and charted throughout the 19th century (Hedrich, 1931). Although cases of meningococcal meningitis or septicaemia could have passed unrecognized due to an ambiguous disease pattern with other pathogens, these written reports might indicate that meningococcal disease emerged only recently. Indeed, the confirmation that *N. meningitidis* and *N. gonorrhoeae* are divided into two distinct species was a long time coming, hindered by their natural competence for DNA uptake and thus the high rate of horizontal gene transfer within each and the somewhat lower recombination between these species (Vazquez *et al.*, 1993, Smith, 1994). The theory for a late split

in the phylogenetic tree has been supported by genomic data showing that *N. meningitidis* probably evolved from a common ancestor together with *N. gonorrhoeae*, *N. lactamica* and the unencapsulated meningococcal carrier strain α14 (Schoen *et al.*, 2008). As an additional difference to gonococci, some meningococci acquired the ability to cause severe and highly lethal afflictions for their human host during their evolution within the nasopharynx. This virulence trait was conferred by the genes for capsule synthesis, which were probably acquired via horizontal gene transfer from *Pasteurellaceae* (Elias *et al.*, 2006, Schoen *et al.*, 2008). Expression of the capsule provided survival during aerosol transmission, smoothing the way for spread by airborne infections but also shielding the meningococci from recognition by the human immune system. Although reports exist about seeming niche-switches with gonococci causing pharyngitis, septicaemia and even meningitis (Fiumara *et al.*, 1967, Metzger, 1970, Rice *et al.*, 1986) as well as meningococci involved in urogenital infections (Beck *et al.*, 1974, McKenna *et al.*, 1993), both *Neisseria* species are highly adapted to their specific host niche. Gonococci are specialized on survival of the abundant hydrophobic compounds in the urogenital tract (Willcox, 1981) and therefore rely on the MtrR-FarRAB system for controlled efflux of these antimicrobials (McFarland *et al.*, 1983, Lee & Shafer, 1999, Lee *et al.*, 2003). Intensified by the ecological isolation from gonococci (Vazquez *et al.*, 1993), meningococci used their relatively short evolution time for adaptation of their lifestyle to the nasopharynx. The constitutive downregulation of *mtrC* expression indicates the redundancy of this efflux pump system for hydrophobic compounds (Rouquette-Loughlin *et al.*, 2004). The remaining efflux pump system FarRAB, on the contrary, is functional and active in meningococci, taking care of the active efflux of palmitic acid. As the transcriptional repressor FarR is not involved in this resistance, FarAB is probably constitutively active, providing the bacteria with the high intrinsic fatty acid resistance. While losing its function in control of fatty acid resistance, the transcriptional regulator FarR has adopted another role in *N. meningitidis.* A similar case has been reported for *Yersinia enterocolitica* and *Yersinia pestis*, where the MarR-like transcriptional regulator RovA is present in both strains but the regulated genes show little overlap (Cathelyn *et al.*, 2007). Interestingly, most of the differentially regulated genes in these bacteria have no orthologues in the respective other strain, indicating that RovA established control over these genes after they had been acquired by horizontal gene transfer and thus after divergence of these two species (Cathelyn *et al.*, 2007). Likewise, *N. meningitidis* acquired the adhesin gene *nadA* by horizontal gene transfer facilitating attachment of the bacteria to epithelial cells as a first step of invasive infection (Capecchi *et al.*, 2005). But this adhesin also triggers a strong immune reaction in the host (Comanducci *et al.*, 2002). Therefore it is possible that the transcriptional regulator FarR was assigned the negative control over *nadA* expression in meningococci, probably mediating immune evasion and thus allowing for a persistent colonization

rather than a short-term lethal infection, which would mean a dead-end to these bacteria (Meyers *et al.*, 2003). In accordance with this hypothesis is the downregulation of FarR upon contact with active human complement. Thus, meningococci might utilize human complement as trigger for the adaptation to the host circulation system, decreasing expression of the repressor *farR* and thus increasing expression of the adhesin *nadA*, virtually getting ready for attachment during their abidance in human blood. In conclusion, FarR plays a different role in the closely related *N. meningitidis* and *N. gonorrhoeae*, providing – if required – fatty acid resistance for survival of the latter in the urogenital tract and enabling the former with inducible attachment to host epithelia. Therefore, FarR is exquisitely involved in host niche adaptation of the two human pathogenic *Neisseria* species.

7. References

Abadi, F. J., P. E. Carter, P. Cash & T. H. Pennington, (1996) Rifampin resistance in Neisseria meningitidis due to alterations in membrane permeability. *Antimicrob Agents Chemother* **40**: 646-651.

Achtman, M., M. Neibert, B. A. Crowe, W. Strittmatter, B. Kusecek, E. Weyse, M. J. Walsh, B. Slawig, G. Morelli, A. Moll & et al., (1988) Purification and characterization of eight class 5 outer membrane protein variants from a clone of Neisseria meningitidis serogroup A. *J Exp Med* **168**: 507-525.

Alekshun, M. N. & S. B. Levy, (1999b) The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends Microbiol* **7**: 410-413.

Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton & J. F. Head, (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. *Nat Struct Biol* **8**: 710-714.

Archibald, F. S. & I. W. DeVoe, (1978) Iron in Neisseria meningitidis: minimum requirements, effects of limitation, and characteristics of uptake. *J Bacteriol* **136**: 35-48.

Ariza, R. R., S. P. Cohen, N. Bachhawat, S. B. Levy & B. Demple, (1994) Repressor mutations in the marRAB operon that activate oxidative stress genes and multiple antibiotic resistance in Escherichia coli. *J Bacteriol* **176**: 143-148.

Bartolini, E., E. Frigimelica, S. Giovinazzi, G. Galli, Y. Shaik, C. Genco, J. A. Welsch, D. M. Granoff, G. Grandi & R. Grifantini, (2006) Role of FNR and FNR-regulated, sugar fermentation genes in Neisseria meningitidis infection. *Mol Microbiol* **60**: 963-972.

Basler, M., I. Linhartova, P. Halada, J. Novotna, S. Bezouskova, R. Osicka, J. Weiser, J. Vohradsky & P. Sebo, (2006) The iron-regulated transcriptome and proteome of Neisseria meningitidis serogroup C. *Proteomics* **6**: 6194-6206.

Beck, A., J. L. Fluker & D. J. Platt, (1974) Neisseria meningitidis in urogenital infection. *Br J Vener Dis* **50**: 367-369.

Benjamini, Y. & Y. Hochberg, (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* **57**: 289-300.

Beutler, B. & E. T. Rietschel, (2003) Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* **3**: 169-176.

Blackwell, C. C., D. M. Weir, V. S. James, W. T. Todd, N. Banatvala, A. K. Chaudhuri, H. G. Gray, E. J. Thomson & R. J. Fallon, (1990) Secretor status, smoking and carriage of Neisseria meningitidis. *Epidemiol Infect* **104**: 203-209.

Bos, M. P. & J. Tommassen, (2005) Viability of a capsule- and lipopolysaccharide-deficient mutant of Neisseria meningitidis. *Infect Immun* **73**: 6194-6197.

Bowe, F., E. C. Lavelle, E. A. McNeela, C. Hale, S. Clare, B. Arico, M. M. Giuliani, A. Rae, A. Huett, R. Rappuoli, G. Dougan & K. H. Mills, (2004) Mucosal vaccination against serogroup B meningococci: induction of bactericidal antibodies and cellular immunity following intranasal immunization with NadA of Neisseria meningitidis and mutants of Escherichia coli heat-labile enterotoxin. *Infect Immun* **72**: 4052-4060.

Capecchi, B., J. Adu-Bobie, F. Di Marcello, L. Ciucchi, V. Masignani, A. Taddei, R. Rappuoli, M. Pizza & B. Arico, (2005) Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. *Mol Microbiol* **55**: 687-698.

Carbonnelle, E., D. J. Hill, P. Morand, N. J. Griffiths, S. Bourdoulous, I. Murillo, X. Nassif & M. Virji, (2009) Meningococcal interactions with the host. *Vaccine* **27 Suppl 2**: B78-89.

Cartwright, K. A., J. M. Stuart, D. M. Jones & N. D. Noah, (1987) The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica. *Epidemiol Infect* **99**: 591-601.

Cathelyn, J. S., D. W. Ellison, S. J. Hinchliffe, B. W. Wren & V. L. Miller, (2007) The RovA regulons of Yersinia enterocolitica and Yersinia pestis are distinct: evidence that many RovA-regulated genes were acquired more recently than the core genome. *Mol Microbiol* **66**: 189-205.

Caugant, D. A., E. A. Hoiby, P. Magnus, O. Scheel, T. Hoel, G. Bjune, E. Wedege, J. Eng & L. O. Froholm, (1994) Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population. *J Clin Microbiol* **32**: 323-330.

Caugant, D. A., E. A. Hoiby, E. Rosenqvist, L. O. Froholm & R. K. Selander, (1992) Transmission of Neisseria meningitidis among asymptomatic military recruits and antibody analysis. *Epidemiol Infect* **109**: 241-253.

Chen, D., V. Barniak, K. R. VanDerMeid & J. C. McMichael, (1999) The levels and bactericidal capacity of antibodies directed against the UspA1 and UspA2 outer membrane proteins of Moraxella (Branhamella) catarrhalis in adults and children. *Infect Immun* **67**: 1310-1316.

Chen, H., J. Hu, P. R. Chen, L. Lan, Z. Li, L. M. Hicks, A. R. Dinner & C. He, (2008) The Pseudomonas aeruginosa multidrug efflux regulator MexR uses an oxidation-sensing mechanism. *Proc Natl Acad Sci U S A* **105**: 13586-13591.

Chen, P. R., T. Bae, W. A. Williams, E. M. Duguid, P. A. Rice, O. Schneewind & C. He, (2006) An oxidation-sensing mechanism is used by the global regulator MgrA in Staphylococcus aureus. *Nat Chem Biol* **2**: 591-595.

Chipperfield, J. R. & C. Ratledge, (2000) Salicylic acid is not a bacterial siderophore: a theoretical study. *Biometals* **13**: 165-168.

Clark, K. L., E. D. Halay, E. Lai & S. K. Burley, (1993) Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* **364**: 412-420.

Claus, H., M. Frosch & U. Vogel, (1998) Identification of a hotspot for transformation of Neisseria meningitidis by shuttle mutagenesis using signature-tagged transposons. *Mol Gen Genet* **259**: 363-371.

Claus, H., M. C. Maiden, R. Maag, M. Frosch & U. Vogel, (2002) Many carried meningococci lack the genes required for capsule synthesis and transport. *Microbiology* **148**: 1813-1819.

Claus, H., M. C. Maiden, D. J. Wilson, N. D. McCarthy, K. A. Jolley, R. Urwin, F. Hessler, M. Frosch & U. Vogel, (2005) Genetic analysis of meningococci carried by children and young adults. *J Infect Dis* **191**: 1263-1271.

Cohen, S. P., H. Hachler & S. B. Levy, (1993b) Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in Escherichia coli. *J Bacteriol* **175**: 1484-1492.

Cohen, S. P., S. B. Levy, J. Foulds & J. L. Rosner, (1993a) Salicylate induction of antibiotic resistance in Escherichia coli: activation of the mar operon and a mar-independent pathway. *J Bacteriol* **175**: 7856-7862.

Comanducci, M., S. Bambini, B. Brunelli, J. Adu-Bobie, B. Arico, B. Capecchi, M. M. Giuliani, V. Masignani, L. Santini, S. Savino, D. M. Granoff, D. A. Caugant, M. Pizza, R. Rappuoli & M. Mora, (2002) NadA, a novel vaccine candidate of Neisseria meningitidis. *J Exp Med* **195**: 1445-1454.

Comanducci, M., S. Bambini, D. A. Caugant, M. Mora, B. Brunelli, B. Capecchi, L. Ciucchi, R. Rappuoli & M. Pizza, (2004) NadA diversity and carriage in Neisseria meningitidis. *Infect Immun* **72**: 4217-4223.

Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory & I. Stainier, (1998) The virulence plasmid of Yersinia, an antihost genome. *Microbiol Mol Biol Rev* **62**: 1315-1352.

Correia, F. F., S. Inouye & M. Inouye, (1986) A 26-base-pair repetitive sequence specific for Neisseria gonorrhoeae and Neisseria meningitidis genomic DNA. *J Bacteriol* **167**: 1009-1015.

Crooks, G. E., G. Hon, J. M. Chandonia & S. E. Brenner, (2004) WebLogo: a sequence logo generator. *Genome Res* **14**: 1188-1190.

Davidsen, T. & T. Tonjum, (2006) Meningococcal genome dynamics. *Nat Rev Microbiol* **4**: 11-22.

de Vries, F. P., R. Cole, J. Dankert, M. Frosch & J. P. van Putten, (1998) Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol Microbiol* **27**: 1203-1212.

Deghmane, A. E., D. Giorgini, L. Maigre & M. K. Taha, (2004) Analysis in vitro and in vivo of the transcriptional regulator CrgA of Neisseria meningitidis upon contact with target cells. *Mol Microbiol* **53**: 917-927.

Deghmane, A. E., S. Petit, A. Topilko, Y. Pereira, D. Giorgini, M. Larribe & M. K. Taha, (2000) Intimate adhesion of Neisseria meningitidis to human epithelial cells is under the control of the crgA gene, a novel LysR-type transcriptional regulator. *Embo J* **19**: 1068-1078.

Delahay, R. M., B. D. Robertson, J. T. Balthazar, W. M. Shafer & C. A. Ison, (1997) Involvement of the gonococcal MtrE protein in the resistance of Neisseria gonorrhoeae to toxic hydrophobic agents. *Microbiology* **143 (Pt 7)**: 2127-2133.

Delany, I., R. Grifantini, E. Bartolini, R. Rappuoli & V. Scarlato, (2006) Effect of Neisseria meningitidis fur mutations on global control of gene transcription. *J Bacteriol* **188**: 2483-2492.

Delany, I., R. Rappuoli & V. Scarlato, (2004) Fur functions as an activator and as a repressor of putative virulence genes in Neisseria meningitidis. *Mol Microbiol* **52**: 1081-1090.

Desai, P. J., R. Nzeribe & C. A. Genco, (1995) Binding and accumulation of hemin in Neisseria gonorrhoeae. *Infect Immun* **63**: 4634-4641.

Diaz Romero, J. & I. M. Outschoorn, (1994) Current status of meningococcal group B vaccine candidates: capsular or noncapsular? *Clin Microbiol Rev* **7**: 559-575.

Dolan-Livengood, J. M., Y. K. Miller, L. E. Martin, R. Urwin & D. S. Stephens, (2003) Genetic basis for nongroupable Neisseria meningitidis. *J Infect Dis* **187**: 1616-1628.

Drake, D. R., K. A. Brogden, D. V. Dawson & P. W. Wertz, (2008) Thematic review series: skin lipids. Antimicrobial lipids at the skin surface. *J Lipid Res* **49**: 4-11.

Du, Y., J. Lenz & C. G. Arvidson, (2005) Global gene expression and the role of sigma factors in Neisseria gonorrhoeae in interactions with epithelial cells. *Infect Immun* **73**: 4834-4845.

Ducey, T. F., M. B. Carson, J. Orvis, A. P. Stintzi & D. W. Dyer, (2005) Identification of the iron-responsive genes of Neisseria gonorrhoeae by microarray analysis in defined medium. *J Bacteriol* **187**: 4865-4874.

Eiamphungporn, W., S. Soonsanga, J. W. Lee & J. D. Helmann, (2009) Oxidation of a single active site suffices for the functional inactivation of the dimeric Bacillus subtilis OhrR repressor in vitro. *Nucleic Acids Res* **37**: 1174-1181.

Elias, J., D. Harmsen, H. Claus, W. Hellenbrand, M. Frosch & U. Vogel, (2006) Spatiotemporal analysis of invasive meningococcal disease, Germany. *Emerg Infect Dis* **12**: 1689-1695.

Elias, J. & U. Vogel, (2007) IS1301 fingerprint analysis of Neisseria meningitidis strains belonging to the ET-15 clone. *J Clin Microbiol* **45**: 159-167.

Ellis, C. D., B. Lindner, C. M. Anjam Khan, U. Zahringer & R. Demarco de Hormaeche, (2001) The Neisseria gonorrhoeae lpxLII gene encodes for a late-functioning lauroyl acyl transferase, and a null mutation within the gene has a significant effect on the induction of acute inflammatory responses. *Mol Microbiol* **42**: 167-181.

Ellison, D. W., M. B. Lawrenz & V. L. Miller, (2004) Invasin and beyond: regulation of Yersinia virulence by RovA. *Trends Microbiol* **12**: 296-300.

Ellison, D. W. & V. L. Miller, (2006) Regulation of virulence by members of the MarR/SlyA family. *Curr Opin Microbiol* **9**: 153-159.

Ermolaeva, M. D., O. White & S. L. Salzberg, (2001) Prediction of operons in microbial genomes. *Nucleic Acids Res* **29**: 1216-1221.

Estabrook, M. M., J. M. Griffiss & G. A. Jarvis, (1997) Sialylation of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. *Infect Immun* **65**: 4436-4444.

Fantappie, L., M. M. Metruccio, K. L. Seib, F. Oriente, E. Cartocci, F. Ferlicca, M. M. Giuliani, V. Scarlato & I. Delany, (2009) The RNA chaperone Hfq is involved in stress response and virulence in Neisseria meningitidis and is a pleiotropic regulator of protein expression. *Infect Immun* **77**: 1842-1853.

Fenton, K. A. & C. M. Lowndes, (2004) Recent trends in the epidemiology of sexually transmitted infections in the European Union. *Sex Transm Infect* **80**: 255-263.

Filenko, N., S. Spiro, D. F. Browning, D. Squire, T. W. Overton, J. Cole & C. Constantinidou, (2007) The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol* **189**: 4410-4417.

Finne, J., M. Leinonen & P. H. Makela, (1983) Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet* **2**: 355-357.

Fisseha, M., P. Chen, B. Brandt, T. Kijek, E. Moran & W. Zollinger, (2005) Characterization of native outer membrane vesicles from lpxL mutant strains of Neisseria meningitidis for use in parenteral vaccination. *Infect Immun* **73**: 4070-4080.

Fiumara, N. J., H. M. Wise, Jr. & M. Many, (1967) Gonorrheal pharyngitis. *N Engl J Med* **276**: 1248-1250.

Fransen, F., S. G. Heckenberg, H. J. Hamstra, M. Feller, C. J. Boog, J. P. van Putten, D. van de Beek, A. van der Ende & P. van der Ley, (2009) Naturally occurring lipid A mutants in neisseria meningitidis from patients with invasive meningococcal disease are associated with reduced coagulopathy. *PLoS Pathog* **5**: e1000396.

Franzoso, S., C. Mazzon, M. Sztukowska, P. Cecchini, T. Kasic, B. Capecchi, R. Tavano & E. Papini, (2008) Human monocytes/macrophages are a target of Neisseria meningitidis Adhesin A (NadA). *J Leukoc Biol*.

Frasch, C. E., W. D. Zollinger & J. T. Poolman, (1985) Serotype antigens of Neisseria meningitidis and a proposed scheme for designation of serotypes. *Rev Infect Dis* **7**: 504-510.

Freese, E., C. W. Sheu & E. Galliers, (1973) Function of lipophilic acids as antimicrobial food additives. *Nature* **241**: 321-325.

Frosch, M., C. Weisgerber & T. F. Meyer, (1989) Molecular characterization and expression in Escherichia coli of the gene complex encoding the polysaccharide capsule of Neisseria meningitidis group B. *Proc Natl Acad Sci U S A* **86**: 1669-1673.

Fuangthong, M., S. Atichartpongkul, S. Mongkolsuk & J. D. Helmann, (2001) OhrR is a repressor of ohrA, a key organic hydroperoxide resistance determinant in Bacillus subtilis. *J Bacteriol* **183**: 4134-4141.

Fuangthong, M. & J. D. Helmann, (2002) The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc Natl Acad Sci U S A* **99**: 6690-6695.

Galbraith, H. & T. B. Miller, (1973) Effect of long chain fatty acids on bacterial respiration and amino acid uptake. *J Appl Bacteriol* **36**: 659-675.

Geoffroy, M. C., S. Floquet, A. Metais, X. Nassif & V. Pelicic, (2003) Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. *Genome Res* **13**: 391-398.

George, A. M. & S. B. Levy, (1983) Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in Escherichia coli: involvement of a non-plasmid-determined efflux of tetracycline. *J Bacteriol* **155**: 531-540.

Gerbase, A. C., J. T. Rowley, D. H. Heymann, S. F. Berkley & P. Piot, (1998) Global prevalence and incidence estimates of selected curable STDs. *Sex Transm Infect* **74 Suppl 1**: S12-16.

Giuliani, M. M., J. Adu-Bobie, M. Comanducci, B. Arico, S. Savino, L. Santini, B. Brunelli, S. Bambini, A. Biolchi, B. Capecchi, E. Cartocci, L. Ciucchi, F. Di Marcello, F. Ferlicca, B. Galli, E. Luzzi, V. Masignani, D. Serruto, D. Veggi, M. Contorni, M. Morandi, A. Bartalesi, V. Cinotti, D. Mannucci, F. Titta, E. Ovidi, J. A. Welsch, D. Granoff, R. Rappuoli & M. Pizza, (2006) A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci U S A* **103**: 10834-10839.

Gordon, D. L., J. Rice, J. J. Finlay-Jones, P. J. McDonald & M. K. Hostetter, (1988) Analysis of C3 deposition and degradation on bacterial surfaces after opsonization. *J Infect Dis* **157**: 697-704.

Gray-Owen, S. D. & A. B. Schryvers, (1996) Bacterial transferrin and lactoferrin receptors. *Trends Microbiol* **4**: 185-191.

Grifantini, R., S. Sebastian, E. Frigimelica, M. Draghi, E. Bartolini, A. Muzzi, R. Rappuoli, G. Grandi & C. A. Genco, (2003) Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of Neisseria meningitidis group B. *Proc Natl Acad Sci U S A* **100**: 9542-9547.

Gunesekere, I. C., C. M. Kahler, C. S. Ryan, L. A. Snyder, N. J. Saunders, J. I. Rood & J. K. Davies, (2006) Ecf, an alternative sigma factor from Neisseria gonorrhoeae, controls expression of msrAB, which encodes methionine sulfoxide reductase. *J Bacteriol* **188**: 3463-3469.

Guymon, L. F. & P. F. Sparling, (1975) Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive mutants of Neisseria gonorrhoeae. *J Bacteriol* **124**: 757-763.

Hachler, H., S. P. Cohen & S. B. Levy, (1991) marA, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in Escherichia coli. *J Bacteriol* **173**: 5532-5538.

Hagman, K. E., C. E. Lucas, J. T. Balthazar, L. Snyder, M. Nilles, R. C. Judd & W. M. Shafer, (1997) The MtrD protein of Neisseria gonorrhoeae is a member of the resistance/nodulation/division protein family constituting part of an efflux system. *Microbiology* **143 (Pt 7)**: 2117-2125.

Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd & W. M. Shafer, (1995) Resistance of Neisseria gonorrhoeae to antimicrobial hydrophobic agents is modulated by the mtrRCDE efflux system. *Microbiology* **141 (Pt 3)**: 611-622.

Hagman, K. E. & W. M. Shafer, (1995) Transcriptional control of the mtr efflux system of Neisseria gonorrhoeae. *J Bacteriol* **177**: 4162-4165.

Hall, T. A., (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Harrison, L. H., C. L. Trotter & M. E. Ramsay, (2009) Global epidemiology of meningococcal disease. *Vaccine* **27 Suppl 2**: B51-63.

Hedrich, A. W., (1931) The movements of epidemic meningitis, 1915 - 1930. *Pub Health Rep* **46**: 2709 - 2726.

Herbst, K., M. Bujara, A. K. Heroven, W. Opitz, M. Weichert, A. Zimmermann & P. Dersch, (2009) Intrinsic thermal sensing controls proteolysis of Yersinia virulence regulator RovA. *PLoS Pathog* **5**: e1000435.

Heroven, A. K., K. Bohme, M. Rohde & P. Dersch, (2008) A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of Yersinia pseudotuberculosis through RovM. *Mol Microbiol* **68**: 1179-1195.

Heurlier, K., M. J. Thomson, N. Aziz & J. W. Moir, (2008) The nitric oxide (NO)-sensing repressor NsrR of Neisseria meningitidis has a compact regulon of genes involved in NO synthesis and detoxification. *J Bacteriol* **190**: 2488-2495.

Hitchcock, P. J., (1989) Unified nomenclature for pathogenic Neisseria species. *Clin Microbiol Rev* **2 Suppl**: S64-65.

Hobbs, M. M., B. Malorny, P. Prasad, G. Morelli, B. Kusecek, J. E. Heckels, J. G. Cannon & M. Achtman, (1998) Recombinational reassortment among opa genes from ET-37 complex Neisseria meningitidis isolates of diverse geographical origins. *Microbiology* **144 (Pt 1)**: 157-166.

Hong, M., M. Fuangthong, J. D. Helmann & R. G. Brennan, (2005) Structure of an OhrR-ohrA operator complex reveals the DNA binding mechanism of the MarR family. *Mol Cell* **20**: 131-141.

Hübner, C., (2004) Dissertation. *http://www.opus-bayern.de/uni-wuerzburg/volltexte/2005/1353/*.

Irwin, S. W., N. Averil, C. Y. Cheng & A. B. Schryvers, (1993) Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, tbpA and tbpB, from Neisseria meningitidis. *Mol Microbiol* **8**: 1125-1133.

Jacobsson, S., S. T. Hedberg, P. Molling, M. Unemo, M. Comanducci, R. Rappuoli & P. Olcen, (2009) Prevalence and sequence variations of the genes encoding the five antigens included in the novel 5CVMB vaccine covering group B meningococcal disease. *Vaccine* **27**: 1579-1584.

Jamet, A., C. Rousseau, J. B. Monfort, E. Frapy, X. Nassif & P. Martin, (2009) A two-component system is required for colonization of host cells by meningococcus. *Microbiology* **155**: 2288-2295.

Jarva, H., S. Ram, U. Vogel, A. M. Blom & S. Meri, (2005) Binding of the complement inhibitor C4bp to serogroup B Neisseria meningitidis. *J Immunol* **174**: 6299-6307.

Jarvis, G. A., (1994) Analysis of C3 deposition and degradation on Neisseria meningitidis and Neisseria gonorrhoeae. *Infect Immun* **62**: 1755-1760.

Jerse, A. E., N. D. Sharma, A. N. Simms, E. T. Crow, L. A. Snyder & W. M. Shafer, (2003) A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. *Infect Immun* **71**: 5576-5582.

Jordan, P. W. & N. J. Saunders, (2009) Host iron binding proteins acting as niche indicators for Neisseria meningitidis. *PLoS One* **4**: e5198.

Judd, R. C. & W. M. Shafer, (1989) Topographical alterations in proteins I of Neisseria gonorrhoeae correlated with lipooligosaccharide variation. *Mol Microbiol* **3**: 637-643.

Kabara, J. J. & D. L. Marshall, (2005) Medium-chain fatty acids and esters. In: Antimicrobials in Food. P. M. Davidson, J. N. Sofos & A. L. Branen (eds). Taylor and Francis Group, LLC, pp. 328 - 352.

Kabara, J. J., D. M. Swieczkowski, A. J. Conley & J. P. Truant, (1972) Fatty acids and derivatives as antimicrobial agents. *Antimicrob Agents Chemother* **2**: 23-28.

Kahler, C. M., L. E. Martin, G. C. Shih, M. M. Rahman, R. W. Carlson & D. S. Stephens, (1998) The (alpha2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B Neisseria meningitidis to resist the bactericidal activity of normal human serum. *Infect Immun* **66**: 5939-5947.

Kasurinen, J., (1992) A novel fluorescent fatty acid, 5-methyl-BDY-3-dodecanoic acid, is a potential probe in lipid transport studies by incorporating selectively to lipid classes of BHK cells. *Biochem Biophys Res Commun* **187**: 1594-1601.

Kelley, L. A. & M. J. Sternberg, (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**: 363-371.

Kirchner, M. & T. F. Meyer, (2005) The PilC adhesin of the Neisseria type IV pilus-binding specificities and new insights into the nature of the host cell receptor. *Mol Microbiol* **56**: 945-957.

Kogan, G., D. Uhrin, J. R. Brisson & H. J. Jennings, (1997) Structural basis of the Neisseria meningitidis immunotypes including the L4 and L7 immunotypes. *Carbohydr Res* **298**: 191-199.

Kohanski, M. A., D. J. Dwyer, B. Hayete, C. A. Lawrence & J. J. Collins, (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797-810.

Kohler, T., C. Weidenmaier & A. Peschel, (2009) Wall teichoic acid protects Staphylococcus aureus against antimicrobial fatty acids from human skin. *J Bacteriol* **191**: 4482-4484.

Krakat, N., A. Westphal, S. Schmidt & P. Scherer, (2010) Anaerobic digestion of renewable biomass - thermophilic temperature governs population dynamics of methanogens. *Appl Environ Microbiol*.

Kroll, J. S., K. E. Wilks, J. L. Farrant & P. R. Langford, (1998) Natural genetic exchange between Haemophilus and Neisseria: intergeneric transfer of chromosomal genes between major human pathogens. *Proc Natl Acad Sci U S A* **95**: 12381-12385.

Kulshin, V. A., U. Zahringer, B. Lindner, C. E. Frasch, C. M. Tsai, B. A. Dmitriev & E. T. Rietschel, (1992) Structural characterization of the lipid A component of pathogenic Neisseria meningitidis. *J Bacteriol* **174**: 1793-1800.

Kumarevel, T., T. Tanaka, T. Umehara & S. Yokoyama, (2009) ST1710-DNA complex crystal structure reveals the DNA binding mechanism of the MarR family of regulators. *Nucleic Acids Res* **37**: 4723-4735.

Kurzai, O., C. Schmitt, H. Claus, U. Vogel, M. Frosch & A. Kolb-Maurer, (2005) Carbohydrate composition of meningococcal lipopolysaccharide modulates the interaction of Neisseria meningitidis with human dendritic cells. *Cell Microbiol* **7**: 1319-1334.

Lapeyssonnie, L., (1963) [Cerebrospinal Meningitis in Africa.]. *Bull World Health Organ* **28**: SUPPL:1-114.

Lappann, M., J. A. Haagensen, H. Claus, U. Vogel & S. Molin, (2006) Meningococcal biofilm formation: structure, development and phenotypes in a standardized continuous flow system. *Mol Microbiol* **62**: 1292-1309.

Laskos, L., J. P. Dillard, H. S. Seifert, J. A. Fyfe & J. K. Davies, (1998) The pathogenic neisseriae contain an inactive rpoN gene and do not utilize the pilE sigma54 promoter. *Gene* **208**: 95-102.

Lee, E. H., S. A. Hill, R. Napier & W. M. Shafer, (2006) Integration Host Factor is required for FarR repression of the farAB-encoded efflux pump of Neisseria gonorrhoeae. *Mol Microbiol* **60**: 1381-1400.

Lee, E. H., C. Rouquette-Loughlin, J. P. Folster & W. M. Shafer, (2003) FarR regulates the farAB-encoded efflux pump of Neisseria gonorrhoeae via an MtrR regulatory mechanism. *J Bacteriol* **185**: 7145-7152.

Lee, E. H. & W. M. Shafer, (1999) The farAB-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. *Mol Microbiol* **33**: 839-845.

Leive, L., (1974) The barrier function of the gram-negative envelope. *Ann N Y Acad Sci* **235**: 109-129.

Ley, H. L. & J. H. Mueller, (1946) On the Isolation from Agar of an Inhibitor for Neisseria gonorrhoeae. *J Bacteriol* **52**: 453-460.

Lim, D., K. Poole & N. C. Strynadka, (2002) Crystal structure of the MexR repressor of the mexRAB-oprM multidrug efflux operon of Pseudomonas aeruginosa. *J Biol Chem* **277**: 29253-29259.

Livak, K. J. & T. D. Schmittgen, (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408.

Lo, H., C. M. Tang & R. M. Exley, (2009) Mechanisms of avoidance of host immunity by Neisseria meningitidis and its effect on vaccine development. *Lancet Infect Dis* **9**: 418-427.

Lomovskaya, O., K. Lewis & A. Matin, (1995) EmrR is a negative regulator of the Escherichia coli multidrug resistance pump EmrAB. *J Bacteriol* **177**: 2328-2334.

Lucas, C. E., K. E. Hagman, J. C. Levin, D. C. Stein & W. M. Shafer, (1995) Importance of lipooligosaccharide structure in determining gonococcal resistance to hydrophobic antimicrobial agents resulting from the mtr efflux system. *Mol Microbiol* **16**: 1001-1009.

Luong, T. T., S. W. Newell & C. Y. Lee, (2003) Mgr, a novel global regulator in Staphylococcus aureus. *J Bacteriol* **185**: 3703-3710.

Lysko, P. G. & S. A. Morse, (1981) Neisseria gonorrhoeae cell envelope: permeability to hydrophobic molecules. *J Bacteriol* **145**: 946-952.

Mackinnon, F. G., R. Borrow, A. R. Gorringe, A. J. Fox, D. M. Jones & A. Robinson, (1993) Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for Neisseria meningitidis using an infant mouse intranasal infection model. *Microb Pathog* **15**: 359-366.

Madico, G., J. A. Welsch, L. A. Lewis, A. McNaughton, D. H. Perlman, C. E. Costello, J. Ngampasutadol, U. Vogel, D. M. Granoff & S. Ram, (2006) The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. *J Immunol* **177**: 501-510.

Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman & B. G. Spratt, (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**: 3140-3145.

Mandrell, R. E. & M. A. Apicella, (1993) Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. *Immunobiology* **187**: 382-402.

Mandrell, R. E., J. J. Kim, C. M. John, B. W. Gibson, J. V. Sugai, M. A. Apicella, J. M. Griffiss & R. Yamasaki, (1991) Endogenous sialylation of the lipooligosaccharides of Neisseria meningitidis. *J Bacteriol* **173**: 2823-2832.

Maneewannakul, K. & S. B. Levy, (1996) Identification for mar mutants among quinolone-resistant clinical isolates of Escherichia coli. *Antimicrob Agents Chemother* **40**: 1695-1698.

Martin, P., K. Makepeace, S. A. Hill, D. W. Hood & E. R. Moxon, (2005) Microsatellite instability regulates transcription factor binding and gene expression. *Proc Natl Acad Sci U S A* **102**: 3800-3804.

Martin, P., T. van de Ven, N. Mouchel, A. C. Jeffries, D. W. Hood & E. R. Moxon, (2003) Experimentally revised repertoire of putative contingency loci in Neisseria meningitidis strain MC58: evidence for a novel mechanism of phase variation. *Mol Microbiol* **50**: 245-257.

Martin, R. G. & J. L. Rosner, (1995) Binding of purified multiple antibiotic-resistance repressor protein (MarR) to mar operator sequences. *Proc Natl Acad Sci U S A* **92**: 5456-5460.

Mazzon, C., B. Baldani-Guerra, P. Cecchini, T. Kasic, A. Viola, M. de Bernard, B. Arico, F. Gerosa & E. Papini, (2007) IFN-gamma and R-848 dependent activation of human monocyte-derived dendritic cells by Neisseria meningitidis adhesin A. *J Immunol* **179**: 3904-3916.

McFarland, L., T. A. Mietzner, J. S. Knapp, E. Sandstrom, K. K. Holmes & S. A. Morse, (1983) Gonococcal sensitivity to fecal lipids can be mediated by an Mtr-independent mechanism. *J Clin Microbiol* **18**: 121-127.

McKenna, J. G., R. J. Fallon, A. Moyes & H. Young, (1993) Anogenital non-gonococcal neisseriae: prevalence and clinical significance. *Int J STD AIDS* **4**: 8-12.

McNeil, G., M. Virji & E. R. Moxon, (1994) Interactions of Neisseria meningitidis with human monocytes. *Microb Pathog* **16**: 153-163.

Metruccio, M. M., E. Pigozzi, D. Roncarati, F. Berlanda Scorza, N. Norais, S. A. Hill, V. Scarlato & I. Delany, (2009) A novel phase variation mechanism in the meningococcus driven by a ligand-responsive repressor and differential spacing of distal promoter elements. *PLoS Pathog* **5**: e1000710.

Metzger, A. L., (1970) Gonococcal arthritis complicating gonorrheal pharyngitis. *Ann Intern Med* **73**: 267-269.

Meyers, L. A., B. R. Levin, A. R. Richardson & I. Stojiljkovic, (2003) Epidemiology, hypermutation, within-host evolution and the virulence of Neisseria meningitidis. *Proc Biol Sci* **270**: 1667-1677.

Miller, R. D., K. E. Brown & S. A. Morse, (1977) Inhibitory action of fatty acids on the growth of Neisseria gonorrhoeae. *Infect Immun* **17**: 303-312.

Miller, R. D. & S. A. Morse, (1977) Binding of progesterone to Neisseria gonorrhoeae and other gram-negative bacteria. *Infect Immun* **16**: 115-123.

Miyazono, K., M. Tsujimura, Y. Kawarabayasi & M. Tanokura, (2007) Crystal structure of an archaeal homologue of multidrug resistance repressor protein, EmrR, from hyperthermophilic archaea Sulfolobus tokodaii strain 7. *Proteins* **67**: 1138-1146.

Mogensen, T. H., S. R. Paludan, M. Kilian & L. Ostergaard, (2006) Two neisseria meningitidis strains with different ability to stimulate toll-like receptor 4 through the MyD88-independent pathway. *Scand J Immunol* **64**: 646-654.

Moore, J., S. E. Bailey, Z. Benmechernene, C. Tzitzilonis, N. J. Griffiths, M. Virji & J. P. Derrick, (2005) Recognition of saccharides by the OpcA, OpaD, and OpaB outer membrane proteins from Neisseria meningitidis. *J Biol Chem* **280**: 31489-31497.

Moran, A. P., M. M. Prendergast & B. J. Appelmelk, (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* **16**: 105-115.

Morse, S. A., P. G. Lysko, L. McFarland, J. S. Knapp, E. Sandstrom, C. Critchlow & K. K. Holmes, (1982) Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. *Infect Immun* **37**: 432-438.

Murray, S. R., D. Bermudes, K. S. de Felipe & K. B. Low, (2001) Extragenic suppressors of growth defects in msbB Salmonella. *J Bacteriol* **183**: 5554-5561.

Nägele, V., J. Heesemann, S. Schielke, L. Jimenez-Soto, O. Kurzai, C. R. Hauck & N. Ackermann, (2009) *Neisseria meningitidis* adhesin NadA targets β1 integrins: a trimeric autotransporter with functional similarity to *Yersinia* invasin. *In preparation*.

Neisser, A., (1879) Über eine der Gonorrhoe eigenthümliche Micrococcenform. *Centralbl Med Wiss* **28**: 497–500.

Nichols, C. E., S. Sainsbury, J. Ren, T. S. Walter, A. Verma, D. K. Stammers, N. J. Saunders & R. J. Owens, (2009) The structure of NMB1585, a MarR-family regulator from Neisseria meningitidis. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **65**: 204-209.

Nikaido, H., (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**: 382-388.

Nikaido, H., (1996) Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* **178**: 5853-5859.

Nikaido, H. & T. Nakae, (1979) The outer membrane of Gram-negative bacteria. *Adv Microb Physiol* **20**: 163-250.

Nikaido, H. & M. Vaara, (1985) Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**: 1-32.

Obrink, B., (1997) CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. *Curr Opin Cell Biol* **9**: 616-626.

Olcen, P., J. Kjellander, D. Danielsson & B. L. Lindquist, (1981) Epidemiology of Neisseria meningitidis; prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. *Scand J Infect Dis* **13**: 105-109.

Overton, T. W., L. Griffiths, M. D. Patel, J. L. Hobman, C. W. Penn, J. A. Cole & C. Constantinidou, (2006) Microarray analysis of gene regulation by oxygen, nitrate, nitrite, FNR, NarL and NarP during anaerobic growth of Escherichia coli: new insights into microbial physiology. *Biochem Soc Trans* **34**: 104-107.

Paavonen, J., (1983) Physiology and ecology of the vagina. *Scand J Infect Dis Suppl* **40**: 31-35.

Pan, W. & B. G. Spratt, (1994) Regulation of the permeability of the gonococcal cell envelope by the mtr system. *Mol Microbiol* **11**: 769-775.

Pareja, E., P. Pareja-Tobes, M. Manrique, E. Pareja-Tobes, J. Bonal & R. Tobes, (2006) ExtraTrain: a database of Extragenic regions and Transcriptional information in prokaryotic organisms. *BMC Microbiol* **6**: 29.

Pavliak, V., J. R. Brisson, F. Michon, D. Uhrin & H. J. Jennings, (1993) Structure of the sialylated L3 lipopolysaccharide of Neisseria meningitidis. *J Biol Chem* **268**: 14146-14152.

Peak, I. R., Y. Srikhanta, M. Dieckelmann, E. R. Moxon & M. P. Jennings, (2000) Identification and characterisation of a novel conserved outer membrane protein from Neisseria meningitidis. *FEMS Immunol Med Microbiol* **28**: 329-334.

Peltola, H., (1983) Meningococcal disease: still with us. *Rev Infect Dis* **5**: 71-91.

Perez-Rueda, E. & J. Collado-Vides, (2001) Common history at the origin of the position-function correlation in transcriptional regulators in archaea and bacteria. *J Mol Evol* **53**: 172-179.

Perrin, A., S. Bonacorsi, E. Carbonnelle, D. Talibi, P. Dessen, X. Nassif & C. Tinsley, (2002) Comparative genomics identifies the genetic islands that distinguish Neisseria meningitidis, the agent of cerebrospinal meningitis, from other Neisseria species. *Infect Immun* **70**: 7063-7072.

Perry, A. C., I. J. Nicolson & J. R. Saunders, (1988) Neisseria meningitidis C114 contains silent, truncated pilin genes that are homologous to Neisseria gonorrhoeae pil sequences. *J Bacteriol* **170**: 1691-1697.

Petersen, B. H., T. J. Lee, R. Snyderman & G. F. Brooks, (1979) Neisseria meningitidis and Neisseria gonorrhoeae bacteremia associated with C6, C7, or C8 deficiency. *Ann Intern Med* **90**: 917-920.

Pizza, M., V. Scarlato, V. Masignani, M. M. Giuliani, B. Arico, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C. L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, E. Blair, T. Mason, H. Tettelin, D. W. Hood, A. C. Jeffries, N. J. Saunders, D. M. Granoff, J. C. Venter, E. R. Moxon, G. Grandi & R. Rappuoli, (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* **287**: 1816-1820.

Post, D. M., N. J. Phillips, J. Q. Shao, D. D. Entz, B. W. Gibson & M. A. Apicella, (2002) Intracellular survival of Neisseria gonorrhoeae in male urethral epithelial cells: importance of a hexaacyl lipid A. *Infect Immun* **70**: 909-920.

Prall, F., P. Nollau, M. Neumaier, H. D. Haubeck, Z. Drzeniek, U. Helmchen, T. Loning & C. Wagener, (1996) CD66a (BGP), an adhesion molecule of the carcinoembryonic antigen family, is expressed in epithelium, endothelium, and myeloid cells in a wide range of normal human tissues. *J Histochem Cytochem* **44**: 35-41.

Rahman, M. M., D. S. Stephens, C. M. Kahler, J. Glushka & R. W. Carlson, (1998) The lipooligosaccharide (LOS) of Neisseria meningitidis serogroup B strain NMB contains L2, L3, and novel oligosaccharides, and lacks the lipid-A 4'-phosphate substituent. *Carbohydr Res* **307**: 311-324.

Ren, J., S. Sainsbury, S. E. Combs, R. G. Capper, P. W. Jordan, N. S. Berrow, D. K. Stammers, N. J. Saunders & R. J. Owens, (2007) The structure and transcriptional analysis of a global regulator from Neisseria meningitidis. *J Biol Chem* **282**: 14655-14664.

Rice, P., I. Longden & A. Bleasby, (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* **16**: 276-277.

Rice, R. J., W. O. Schalla, W. L. Whittington, Y. JeanLouis, J. W. Biddle, M. Goldberg, W. DeWitt, C. A. Pasquariello, E. Abrutyn & R. Swenson, (1986) Phenotypic characterization of Neisseria gonorrhoeae isolated from three cases of meningitis. *J Infect Dis* **153**: 362-365.

Ried, G., I. Hindennach & U. Henning, (1990) Role of lipopolysaccharide in assembly of Escherichia coli outer membrane proteins OmpA, OmpC, and OmpF. *J Bacteriol* **172**: 6048-6053.

Rock, J. D., M. J. Thomson, R. C. Read & J. W. Moir, (2007) Regulation of denitrification genes in Neisseria meningitidis by nitric oxide and the repressor NsrR. *J Bacteriol* **189**: 1138-1144.

Rouquette-Loughlin, C., I. Stojiljkovic, T. Hrobowski, J. T. Balthazar & W. M. Shafer, (2002) Inducible, but not constitutive, resistance of gonococci to hydrophobic agents due to the MtrC-MtrD-MtrE efflux pump requires TonB-ExbB-ExbD proteins. *Antimicrob Agents Chemother* **46**: 561-565.

Rouquette-Loughlin, C. E., J. T. Balthazar, S. A. Hill & W. M. Shafer, (2004) Modulation of the mtrCDE-encoded efflux pump gene complex of Neisseria meningitidis due to a Correia element insertion sequence. *Mol Microbiol* **54**: 731-741.

Rudel, T., I. Scheurerpflug & T. F. Meyer, (1995) Neisseria PilC protein identified as type-4 pilus tip-located adhesin. *Nature* **373**: 357-359.

Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream & B. Barrell, (2000) Artemis: sequence visualization and annotation. *Bioinformatics* **16**: 944-945.

Sarubbi, F. A., Jr., P. F. Sparling, E. Blackman & E. Lewis, (1975) Loss of low-level antibiotic resistance in Neisseria gonorrhoeae due to env mutations. *J Bacteriol* **124**: 750-756.

Saukkonen, K., C. Cabellos, M. Burroughs, S. Prasad & E. Tuomanen, (1991) Integrin-mediated localization of Bordetella pertussis within macrophages: role in pulmonary colonization. *J Exp Med* **173**: 1143-1149.

Saunders, N. J., A. C. Jeffries, J. F. Peden, D. W. Hood, H. Tettelin, R. Rappuoli & E. R. Moxon, (2000) Repeat-associated phase variable genes in the complete genome sequence of Neisseria meningitidis strain MC58. *Mol Microbiol* **37**: 207-215.

Scarselli, M., D. Serruto, P. Montanari, B. Capecchi, J. Adu-Bobie, D. Veggi, R. Rappuoli, M. Pizza & B. Arico, (2006) Neisseria meningitidis NhhA is a multifunctional trimeric autotransporter adhesin. *Mol Microbiol* **61**: 631-644.

Schielke, S., C. Huebner, C. Spatz, V. Nagele, N. Ackermann, M. Frosch, O. Kurzai & A. Schubert-Unkmeir, (2009) Expression of the meningococcal adhesin NadA is controlled by a transcriptional regulator of the MarR family. *Mol Microbiol* **72**: 1054-1067.

Schielke, S., C. Schmitt, C. Spatz, M. Frosch, A. Schubert-Unkmeir & O. Kurzai, (2010) Lipopolysaccharide composition but not FarR regulated expression of *farAB* is responsible for fatty acid resistance of *Neisseria meningitidis*. *In preparation*.

Schielke, S., C. Spatz, R. Schwarz, B. Joseph, C. Schoen, S. M. Schulz, H. Claus, M. Frosch, A. Schubert-Unkmeir & O. Kurzai, (2010a) Characterization of FarR as a highly specialized growth phase dependent, serum responsive transcriptional regulator in *Neisseria meningitidis*. *In preparation*.

Schmitt, C., D. Turner, M. Boesl, M. Abele, M. Frosch & O. Kurzai, (2007) A functional two-partner secretion system contributes to adhesion of Neisseria meningitidis to epithelial cells. *J Bacteriol* **189**: 7968-7976.

Schneider, M. C., R. M. Exley, H. Chan, I. Feavers, Y. H. Kang, R. B. Sim & C. M. Tang, (2006) Functional significance of factor H binding to Neisseria meningitidis. *J Immunol* **176**: 7566-7575.

Schneider, M. C., R. M. Exley, S. Ram, R. B. Sim & C. M. Tang, (2007) Interactions between Neisseria meningitidis and the complement system. *Trends Microbiol* **15**: 233-240.

Schneider, T. D. & R. M. Stephens, (1990) Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res* **18**: 6097-6100.

Schoen, C., J. Blom, H. Claus, A. Schramm-Gluck, P. Brandt, T. Muller, A. Goesmann, B. Joseph, S. Konietzny, O. Kurzai, C. Schmitt, T. Friedrich, B. Linke, U. Vogel & M. Frosch, (2008) Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in Neisseria meningitidis. *Proc Natl Acad Sci U S A* **105**: 3473-3478.

Scholten, R. J., B. Kuipers, H. A. Valkenburg, J. Dankert, W. D. Zollinger & J. T. Poolman, (1994) Lipo-oligosaccharide immunotyping of Neisseria meningitidis by a whole-cell ELISA with monoclonal antibodies. *J Med Microbiol* **41**: 236-243.

Segal, E., P. Hagblom, H. S. Seifert & M. So, (1986) Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proc Natl Acad Sci U S A* **83**: 2177-2181.

Seib, K. L., D. Serruto, F. Oriente, I. Delany, J. Adu-Bobie, D. Veggi, B. Arico, R. Rappuoli & M. Pizza, (2009) Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37. *Infect Immun* **77**: 292-299.

Seoane, A. S. & S. B. Levy, (1995) Characterization of MarR, the repressor of the multiple antibiotic resistance (mar) operon in Escherichia coli. *J Bacteriol* **177**: 3414-3419.

Serruto, D., J. Adu-Bobie, M. Scarselli, D. Veggi, M. Pizza, R. Rappuoli & B. Arico, (2003) Neisseria meningitidis App, a new adhesin with autocatalytic serine protease activity. *Mol Microbiol* **48**: 323-334.

Shafer, W. M., J. T. Balthazar, K. E. Hagman & S. A. Morse, (1995) Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of Neisseria gonorrhoeae that are resistant to faecal lipids. *Microbiology* **141 (Pt 4)**: 907-911.

Shaik, Y. B., S. Grogan, M. Davey, S. Sebastian, S. Goswami, B. Szmigielski & C. A. Genco, (2007) Expression of the iron-activated nspA and secY genes in Neisseria meningitidis group B by Fur-dependent and -independent mechanisms. *J Bacteriol* **189**: 663-669.

Sheu, C. W. & E. Freese, (1973) Lipopolysaccharide layer protection of gram-negative bacteria against inhibition by long-chain fatty acids. *J Bacteriol* **115**: 869-875.

Sjolinder, H., J. Eriksson, L. Maudsdotter, H. Aro & A. B. Jonsson, (2008) Meningococcal outer membrane protein NhhA is essential for colonization and disease by preventing phagocytosis and complement attack. *Infect Immun* **76**: 5412-5420.

Slomiany, B. L., V. L. Murty, I. D. Mandel, G. Zalesna & A. Slomiany, (1989) Physico-chemical characteristics of mucus glycoproteins and lipids of the human oral mucosal mucus coat in relation to caries susceptibility. *Arch Oral Biol* **34**: 229-237.

Smith, H. O., M. L. Gwinn & S. L. Salzberg, (1999) DNA uptake signal sequences in naturally transformable bacteria. *Res Microbiol* **150**: 603-616.

Smith, J. M., (1994) Estimating selection by comparing synonymous and substitutional changes. *J Mol Evol* **39**: 123-128.

Snyder, D. S. & T. J. McIntosh, (2000) The lipopolysaccharide barrier: correlation of antibiotic susceptibility with antibiotic permeability and fluorescent probe binding kinetics. *Biochemistry* **39**: 11777-11787.

Soper, D. E., (1994) Pelvic inflammatory disease. *Infect Dis Clin North Am* **8**: 821-840.

Stapleton, M. R., V. A. Norte, R. C. Read & J. Green, (2002) Interaction of the Salmonella typhimurium transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. *J Biol Chem* **277**: 17630-17637.

Steeghs, L., R. den Hartog, A. den Boer, B. Zomer, P. Roholl & P. van der Ley, (1998) Meningitis bacterium is viable without endotoxin. *Nature* **392**: 449-450.

Stephens, D. S., (1999) Uncloaking the meningococcus: dynamics of carriage and disease. *Lancet* **353**: 941-942.

Stephens, D. S., P. A. Spellman & J. S. Swartley, (1993) Effect of the (alpha 2-->8)-linked polysialic acid capsule on adherence of Neisseria meningitidis to human mucosal cells. *J Infect Dis* **167**: 475-479.

Stern, A., M. Brown, P. Nickel & T. F. Meyer, (1986) Opacity genes in Neisseria gonorrhoeae: control of phase and antigenic variation. *Cell* **47**: 61-71.

Stevens, A. & J. S. Lowe, (2000) *Pathology*. Mosby.

Swanson, J., (1978) Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. *Infect Immun* **21**: 292-302.

Takahama, U., S. Hirota, T. Nishioka & T. Oniki, (2003) Human salivary peroxidase-catalyzed oxidation of nitrite and nitration of salivary components 4-hydroxyphenylacetic acid and proteins. *Arch Oral Biol* **48**: 679-690.

Takayama, K., N. Qureshi, K. Hyver, J. Honovich, R. J. Cotter, P. Mascagni & H. Schneider, (1986) Characterization of a structural series of lipid A obtained from the lipopolysaccharides of Neisseria gonorrhoeae. Combined laser desorption and fast atom bombardment mass spectral analysis of high performance liquid chromatography-purified dimethyl derivatives. *J Biol Chem* **261**: 10624-10631.

Tala, A., C. Progida, M. De Stefano, L. Cogli, M. R. Spinosa, C. Bucci & P. Alifano, (2008) The HrpB-HrpA two-partner secretion system is essential for intracellular survival of Neisseria meningitidis. *Cell Microbiol* **10**: 2461-2482.

Teich, A., S. Meyer, H. Y. Lin, L. Andersson, S. Enfors & P. Neubauer, (1999) Growth rate related concentration changes of the starvation response regulators sigmaS and ppGpp in glucose-limited fed-batch and continuous cultures of Escherichia coli. *Biotechnol Prog* **15**: 123-129.

Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D. Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, H. Cittone, E. B. Clark, M. D. Cotton, T. R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H. O. Smith, C. M. Fraser, E. R. Moxon, R. Rappuoli & J. C. Venter, (2000) Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. *Science* **287**: 1809-1815.

Thompson, J. D., D. G. Higgins & T. J. Gibson, (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680.

Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee & S. Falkow, (2003) Gene expression profiling of Helicobacter pylori reveals a growth-phase-dependent switch in virulence gene expression. *Infect Immun* **71**: 2643-2655.

Tikhomirov, E., M. Santamaria & K. Esteves, (1997) Meningococcal disease: public health burden and control. *World Health Stat Q* **50**: 170-177.

Tomita, Y., N. Miyake & S. Yamanaka, (2008) Lipids in human parotid saliva with regard to caries experience. *J Oleo Sci* **57**: 115-121.

Tovchigrechko, A. & I. A. Vakser, (2006) GRAMM-X public web server for protein-protein docking. *Nucleic Acids Res* **34**: W310-314.

Truong-Bolduc, Q. C. & D. C. Hooper, (2007) The transcriptional regulators NorG and MgrA modulate resistance to both quinolones and beta-lactams in Staphylococcus aureus. *J Bacteriol* **189**: 2996-3005.

Tsai, C. M., E. Jankowska-Stephens, R. M. Mizanur & J. F. Cipollo, (2009) The fine structure of Neisseria meningitidis lipooligosaccharide from the M986 strain and three of its variants. *J Biol Chem* **284**: 4616-4625.

Turner, D. P., A. G. Marietou, L. Johnston, K. K. Ho, A. J. Rogers, K. G. Wooldridge & D. A. Ala'Aldeen, (2006) Characterization of MspA, an immunogenic autotransporter protein that mediates adhesion to epithelial and endothelial cells in Neisseria meningitidis. *Infect Immun* **74**: 2957-2964.

Tyski, S., W. Grzybowska, G. Dulny, L. Berthelsen & I. Lind, (2001) Phenotypical and genotypical characterization of Neisseria meningitidis carrier strains isolated from Polish recruits in 1998. *Eur J Clin Microbiol Infect Dis* **20**: 350-353.

Tzeng, Y. L., A. Datta, K. Ambrose, M. Lo, J. K. Davies, R. W. Carlson, D. S. Stephens & C. M. Kahler, (2004) The MisR/MisS two-component regulatory system influences inner core structure and immunotype of lipooligosaccharide in Neisseria meningitidis. *J Biol Chem* **279**: 35053-35062.

Tzeng, Y. L., C. M. Kahler, X. Zhang & D. S. Stephens, (2008) MisR/MisS two-component regulon in Neisseria meningitidis. *Infect Immun* **76**: 704-716.

Tzeng, Y. L., C. Noble & D. S. Stephens, (2003) Genetic basis for biosynthesis of the (alpha 1-->4)-linked N-acetyl-D-glucosamine 1-phosphate capsule of Neisseria meningitidis serogroup X. *Infect Immun* **71**: 6712-6720.

Tzeng, Y. L. & D. S. Stephens, (2000) Epidemiology and pathogenesis of Neisseria meningitidis. *Microbes Infect* **2**: 687-700.

Unkmeir, A., U. Kammerer, A. Stade, C. Hubner, S. Haller, A. Kolb-Maurer, M. Frosch & G. Dietrich, (2002) Lipooligosaccharide and polysaccharide capsule: virulence factors of Neisseria meningitidis that determine meningococcal interaction with human dendritic cells. *Infect Immun* **70**: 2454-2462.

Unkmeir, A., K. Latsch, G. Dietrich, E. Wintermeyer, B. Schinke, S. Schwender, K. S. Kim, M. Eigenthaler & M. Frosch, (2002a) Fibronectin mediates Opc-dependent internalization of Neisseria meningitidis in human brain microvascular endothelial cells. *Mol Microbiol* **46**: 933-946.

van der Ley, P., L. Steeghs, H. J. Hamstra, J. ten Hove, B. Zomer & L. van Alphen, (2001) Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect Immun* **69**: 5981-5990.

van Ulsen, P., K. Kuhn, T. Prinz, H. Legner, P. Schmid, C. Baumann & J. Tommassen, (2009) Identification of proteins of Neisseria meningitidis induced under iron-limiting conditions using the isobaric tandem mass tag (TMT) labeling approach. *Proteomics* **9**: 1771-1781.

Vazquez, J. A., L. de la Fuente, S. Berron, M. O'Rourke, N. H. Smith, J. Zhou & B. G. Spratt, (1993) Ecological separation and genetic isolation of Neisseria gonorrhoeae and Neisseria meningitidis. *Curr Biol* **3**: 567-572.

Verheul, A. F., H. Snippe & J. T. Poolman, (1993) Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. *Microbiol Rev* **57**: 34-49.

Vieusseaux, M., (1805) Mémoire sur la maladie qui a régné à Genève au printemps de 1805. *J Med Chir Pharmacol* **11**: 163.

Virji, M., (2009) Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nat Rev Microbiol* **7**: 274-286.

Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, J. Sarkari & E. R. Moxon, (1992) Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by Neisseria meningitidis. *Mol Microbiol* **6**: 2785-2795.

Virji, M., K. Makepeace, D. J. Ferguson & S. M. Watt, (1996) Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol Microbiol* **22**: 941-950.

Vogel, U., H. Claus, G. Heinze & M. Frosch, (1997a) Functional characterization of an isogenic meningococcal alpha-2,3-sialyltransferase mutant: the role of lipooligosaccharide sialylation for serum resistance in serogroup B meningococci. *Med Microbiol Immunol* **186**: 159-166.

Vogel, U. & M. Frosch, (1999) Mechanisms of neisserial serum resistance. *Mol Microbiol* **32**: 1133-1139.

Vogel, U., A. Weinberger, R. Frank, A. Muller, J. Kohl, J. P. Atkinson & M. Frosch, (1997b) Complement factor C3 deposition and serum resistance in isogenic capsule and lipooligosaccharide sialic acid mutants of serogroup B Neisseria meningitidis. *Infect Immun* **65**: 4022-4029.

Von Loewenich, F. D., E. Wintermeyer, M. Dumig & M. Frosch, (2001) Analysis of transcriptional control mechanisms of capsule expression in Neisseria meningitidis. *Int J Med Microbiol* **291**: 361-369.

Weber, M. V., H. Claus, M. C. Maiden, M. Frosch & U. Vogel, (2006) Genetic mechanisms for loss of encapsulation in polysialyltransferase-gene-positive meningococci isolated from healthy carriers. *Int J Med Microbiol* **296**: 475-484.

Wei, C. I., J. Y. Chen, I. Y. Kao & I. H. Pan, (1974) Chromosome study and sex determination of Chang human conjunctival cell line. *Taiwan Yi Xue Hui Za Zhi* **73**: 267-276.

Wei, K., D. J. Tang, Y. Q. He, J. X. Feng, B. L. Jiang, G. T. Lu, B. Chen & J. L. Tang, (2007) hpaR, a putative marR family transcriptional regulator, is positively controlled by HrpG and HrpX and involved in the pathogenesis, hypersensitive response, and extracellular protease production of Xanthomonas campestris pathovar campestris. *J Bacteriol* **189**: 2055-2062.

Weichselbaum, A., (1887) Über die aetiologie der akuten meningitis cerebro-spinalis. *Fortschr. Med.* **5**: 573–583.

Weinberg, E. D., (1974) Iron and susceptibility to infectious disease. *Science* **184**: 952-956.

Weinberg, E. D., (1978) Iron and infection. *Microbiol Rev* **42**: 45-66.

Wertz, P. W. & B. B. Michniak, (2000) *Sebum. Cosmeceuticals: Drugs vs. Cosmetics*. Marcel Dekker Inc., NY.

Wetzler, L. M., K. Barry, M. S. Blake & E. C. Gotschlich, (1992) Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. *Infect Immun* **60**: 39-43.

Wheeler, J. S., B. J. Anderson & T. M. De Chalain, (2003) Surgical interventions in children with meningococcal purpura fulminans--a review of 117 procedures in 21 children. *J Pediatr Surg* **38**: 597-603.

Wilkinson, S. P. & A. Grove, (2004) HucR, a novel uric acid-responsive member of the MarR family of transcriptional regulators from Deinococcus radiodurans. *J Biol Chem* **279**: 51442-51450.

Wilkinson, S. P. & A. Grove, (2006) Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins. *Curr Issues Mol Biol* **8**: 51-62.

Willcox, R. R., (1981) The rectum as viewed by the venereologist. *Br J Vener Dis* **57**: 1-6.

Wille, J. J. & A. Kydonieus, (2003) Palmitoleic acid isomer (C16:1delta6) in human skin sebum is effective against gram-positive bacteria. *Skin Pharmacol Appl Skin Physiol* **16**: 176-187.

Yazdankhah, S. P. & D. A. Caugant, (2004) Neisseria meningitidis: an overview of the carriage state. *J Med Microbiol* **53**: 821-832.

Zheng, C. J., J. S. Yoo, T. G. Lee, H. Y. Cho, Y. H. Kim & W. G. Kim, (2005) Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Lett* **579**: 5157-5162.

Zollinger, W. D. & R. E. Mandrell, (1977) Outer-membrane protein and lipopolysaccharide serotyping of Neisseria meningitidis by inhibition of a solid-phase radioimmunoassay. *Infect Immun* **18**: 424-433.

Zughaier, S. M., B. Lindner, J. Howe, P. Garidel, M. H. Koch, K. Brandenburg & D. S. Stephens, (2007) Physicochemical characterization and biological activity of lipooligosaccharides and lipid A from Neisseria meningitidis. *J Endotoxin Res* **13**: 343-357.

8. Danksagung

Ich danke Prof Dr. Matthias Frosch für das in mich gesetzte Vertrauen, die Unterstützung sowie fachliche Diskussionen und das Aufzeigen vieler Möglichkeiten für die Zukunft.

Prof Dr. Oliver Kurzai möchte ich für seine stete Förderung, die wertvollen Diskussionen und den Ansporn mit seinem unglaublichen Fachwissen danken. Vielfältige Anregungen, hohe Ansprüche aber auch die Freiheit, eigene Ideen einzubringen haben mir eine sehr erfolgreiche Zeit in diesem Projekt ermöglicht. Danke, ich habe so viel von Dir gelernt!

Ich danke auch PD Dr. Alexandra Schubert-Unkmeir für die spannende Aufgabenstellung und das rege Interesse an neuen Experimenten, die Motivation, die „medizinische“ Sichtweise auf oftmals zu „biologisch“ angelegte Versuche und das sorgfältige Korrekturlesen aller entstandenen Dokumente.

Prof. Dr. Roy Gross danke ich für die freundliche Übernahme des Koreferats.

Als „Gemeinschaftsprojekt“ der AGs Kurzai/Schubert-Unkmeir habe ich viele nette Leute kennengelernt, die das Arbeiten im großen Labor immer mit viel Spaß und hilfreichen Diskussionen angenehm gemacht haben. Ein besonderer Dank geht an Nina Trzeciak und Sabrina Hebling – für die hervorragende Hilfe beim Gießen der Fettsäure-Platten, bei Klonierungen und den β-Galaktosidase-Assays. Die gute Arbeitsatmosphäre mit Sabrina Hebling, Nina Trzeciak und Corinna Schmitt wurde anfangs noch von Iwona Wozniok und Andrea Villwock unterstützt, später dann von Carolin Spatz, Heiko Slanina und Alexandra König bereichert. Zum Glück hat sich unsere Zusammenarbeit nicht nur auf das Labor beschränkt - besonders unsere Kochabende und die „Wohnungsbesichtigungstour“ werden mir immer in Erinnerung bleiben!

Es hat mir sehr viel Spaß gemacht, in unserem Institut zu arbeiten – nicht zuletzt, weil ich bei jeglichen ausgefallenen Ideen oder Experimenten immer freundliche Unterstützung erhalten habe – sei es in der Diagnostik oder in anderen Forschungsgruppen:

- AG Schoen – ein Dankeschön an Dr. Dr. Christoph Schoen für wertvolle Diskussionen und Anregungen rund um das Meningokokken-Genom. Außerdem danke ich Kathi Engelhard für tolle Fotos, die sonnige Stimmung im Labor und Dr. Biju Joseph für die Hilfe bei den Microarrays.
- AG Vogel – ich danke Prof. Dr. Ulrich Vogel und PD Dr. Heike Claus für hilfreiche Diskussionen sowie die Bereitstellung von Meningokokken-Stämmen.
- AG Brehm – danke, dass ich als „Echi-Plus“-Mitglied immer dazugehören durfte! Außerdem bin ich froh, dass ich Schafkopf gelernt (danke, Moni!) und beim Spielen mit Moni Bergmann, Sabine Lorenz, Sarah Hemer und Tessa van Alen so viele entspannte Mittagspausen verbracht habe!

Ich bin froh, dass ich hier in Sabine Lorenz und Tessa van Alen so gute Freunde gefunden habe – unsere vielen Gespräche, eure Hilfsbereitschaft und die oftmals dringend notwendigen Kaffeepausen mit Motivase hätte ich nicht missen wollen!

Meinem Freund Nic danke ich ganz herzlich für seine Hilfsbereitschaft und seinen unerschütterlichen Optimismus. Vielen Dank für Deine liebevolle Unterstützung, vor allem in den stressigen Phasen!

Meine Familie hat ein ganz besonderes Dankeschön verdient – für die unermüdliche Unterstützung, den Rückhalt und die erholsamen Wochenenden. Ohne Euch hätte ich das nicht geschafft!