Identification of essential genes and novel virulence factors of *Neisseria gonorrhoeae* by transposon mutagenesis

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Identifizierung von essentiellen Genen und neuen Virulenzfaktoren von *Neisseria gonorrhoeae* durch Transposonmutagenese

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Abstract

Neisseria gonorrhoeae is a human-specific pathogen that causes gonorrhea. It is defined as a super bacterium by the WHO due to the emergence of gonococci that are resistant to a variety of antibiotics and a rapidly increasing infection incidence. Genome-wide investigation of neisserial gene essentiality and novel virulence factors is urgently required in order to identify new targets for anti-neisserial therapeutics. To identify essential genes and new virulence factors, a high-density mutant library in *N. gonorrhoeae* MS11 was generated by *in vitro* transposon mutagenesis. The transposon library harbors more than 100,000 individual mutants, a density that is unprecedented in gonococcal research. Essential genes in *N. gonorrhoeae* were determined by enumerating frequencies of transposon insertion sites (TIS) with Illumina deep sequencing (Tn-seq). Tn-seq indicated an average distance between adjacent TIS of 25 bp. Statistical analysis unequivocally demonstrated 781 genes that were significantly depleted in TIS and thus are essential for *Neisseria* survival. A subset of the genes was experimentally verified to comprise essential genes and thus support the outcome of the study. The hereby identified candidate essential genes thus may constitute excellent targets for the development of new antibiotics or vaccines.

In a second study, the transposon mutant library was applied in a genome-scale "negative-selection strategy" to identify genes that are involved in low phosphate-dependent invasion (LPDI). LPDI is dependent on the *Neisseria* porin subtype $PorB_{IA}$ which acts as an epithelial cell invasin in absence of phosphate and is associated with severe pathogenicity in disseminated gonococcal infections (DGI). Tn-seq demonstrated 98 genes, which were involved in adherence to host cells and 43 genes involved in host cell invasion. E.g. the hypothetical protein NGFG_00506, an ABC transporter ATP-binding protein NGFG_01643, as well as NGFG_04218 encoding a homolog of mafI in *N. gonorrhoeae* FA1090 were experimentally verified as new invasive factors in LPDI. NGFG_01605, a predicted protease, was identified to be a common factor involved in $PorB_{IA}$, Opa_{50} and Opa_{57}-mediated neisserial engulfment by the epithelial cells. Thus, this first systematic Tn-seq application in *N. gonorrhoeae* identified a set of previously unknown *N. gonorrhoeae* invasive factors which demonstrate molecular mechanisms of DGI.

Zusammenfassung

Neisseria gonorrhoeae ist ein human-spezifisches Pathogen, das die Krankheit Gonorrhoe verursacht. Aufgrund der steigenden Anzahl antibiotikaresistenter Gonokokken und der damit verbundenen, rapide zunehmenden Anzahl von Infektionen erklärte die WHO Gonokokken 2012 zum Superbakterium. Daher ist eine genomweite Untersuchung der neisseriellen Genessentiatialität und neuer Virulenzfaktoren dringend erforderlich, um neue Ziele für die antineisserielle Therapie zu identifizieren. Hierzu wurde eine high-density Mutantenbibliothek in *N. gonorrhoeae* MS11 durch *in vitro* Transposonmutagenese generiert. Die Transposonbibliothek enthält mehr als 100.000 individuelle Mutanten - eine Dichte, die in der Gonokokken-Forschung beispiellos ist. Essentielle Gene von *N. gonorrhoeae* wurden durch die Ermittlung der Häufigkeit von Transposon insertion sites (TIS) mit Hilfe von Illumina deep sequencing (Tn-seq) bestimmt. Tn-seq ergab eine durchschnittliche Distanz von 25 Basenpaaren zwischen benachbarten TIS. Die statistische Analyse zeigte eindeutig 781 Gene, die signifikant weniger TIS aufwiesen und deshalb als essentiell für das Überleben der Neisserien verstanden werden können. Für ausgewählte Gene wurde experimentell bestätigt, dass sie essentielle Gene beinhalten, wodurch das Ergebnis der Tn-seq unterstützt wird. Die hierbei identifizierten essentiellen Gene könnten exzellente Targets für die Entwicklung neuer Antibiotika oder Impfstoffe darstellen.

In einer zweiten Studie wurde die Transposon Mutanten Bibliothek für eine genomweite „negative Selektionsstrategie“ bereitgestellt. Es sollten Gene identifiziert werden, die an der phosphatfreien Invasion (low phosphate-dependent invasion = LPDI) beteiligt sind. Die LPDI ist vom neisseriellen Porin Subtyp $PorB_{IA}$ abhängig, welches bei Epithelzellen in Abwesenheit von Phosphat als Invasin fungiert und mit einer schweren Pathogenität in disseminierenden Gonokokkeninfektionen (DGI) assoziiert ist. Tn-seq ergab 98 Gene, die an der Adhärenz an die Wirtszelle, und 43 Gene, die an der Wirtszellinvasion beteiligt waren. Zum Beispiel wurden das hypothetische Protein NGFG_00506, ein ABC Transporter, das ATP-bindende Protein NGFG_01643, wie auch NGFG_04218, das für ein Homolog von *mafI* in *N. gonorrhoeae* FA1090 kodiert, experimentell als neue Invasionsfaktoren in der LPDI verifiziert. NGFG_01605, bei dem angenommen wird, dass es sich um eine Protease handelt, wurde als ein allgemeiner Faktor

identifiziert, der an der $PorB_{IA}$-, Opa_{50}- and Opa_{57}-vermittelten Einstülpung der Membran von Epithelzellen beteiligt ist. Die erste systematische Anwendung von Tn-seq in *N. gonorrhoeae* identifizierte eine Reihe bisher unbekannter Invasionsfaktoren von *N. gonorrhoeae*, die molekulare Mechanismen der DGI zeigen.

1 Introduction

1.1 *Neisseria gonorrhoeae*

1.1.1 Pathogenesis of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae (also named as gonococcus or GC) is a Gram-negative, aerobic or facultative anaerobic diplococcus [1,2]. It is coffee bean-shaped with a diameter of 0.6–1 μm and was discovered by German physician Albert Neisser in 1879. The gonococci belong to the big genus *Neisseria*, commensal bacteria that colonize the mucosal surfaces of many animals. Among eleven species that colonize humans, two are pathogens, *N. gonorrhoeae* and *N. meningitidis* which cause bacterial meningitis and meningococcal septicemia.

The obligate and human-specific pathogenic bacterium *N. gonorrhoeae* is the causative agent of the second most common sexually transmitted disease, gonorrhea, which is colloquially known as “the clap”. With more than 106 million of the estimated 498 million new cases of curable sexually transmitted infections (STIs) that occur globally every year, the gonococcal infections remain a serious threat to world health [3]. The gonococci usually infect the urogenital tract and preferentially colonize the mucosal surface of the male urethra and the female cervix, but the rectum, pharynx and the conjunctiva of the eye can also be infected. The infection in men mostly causes urethritis, epididymitis, and prostatitis. While many infected women are asymptomatic. But, occasionally they have symptoms of vaginal and pelvic discomfort of dysuria and these infections may develop to ascending gonococcal infection and subsequently pelvic inflammatory disease (PID) which increases the risk of infertility and ectopic pregnancy [4,5]. One in ten women suffers from PID, of which *N. gonorrhoeae* contributes to 40% of all reported cases [4]. If the urogenital gonococcal infections are undiagnosed or untreated, *N. gonorrhoeae* will spread in the host body and cause disseminated gonococcal infection (DGI) which can lead to some serious conditions such as arthritis, endocarditis and meningitis. The gonococci transmit from person to person via intimate contact, especially sexual contact, and the most common transmission are sexual transmission and mother-to-child transmission during birth which may cause gonococcal

conjunctivitis of the neonate [6]. In addition, 10–30% of patients with gonorrhea were found with a concomitant *Chlamydia* infection [7]. The gonococcus is also found to be one of the significant cofactors for human immunodeficiency virus (HIV) transmission and gonococcal urethritis increases the risk of acquiring and transmitting HIV infection about three-fold [8].

1.1.2 Diagnosis and therapy

Urogenital gonococcal infections are usually diagnosed by culture tests, but other tests which are less labor-intensive have similar accuracy. For example, the new nonculture technique is the nucleic acid amplification test with 92–96% sensitivity and 94–99% specificity when compared with culture tests [9]. For the therapy strategy, the uncomplicated local gonococcal infections are usually treated by antibiotics, but reinfection is a common occurrence with gonorrhea. It may result from a lack of protective immune response to *Neisseria* enormous variations. The experimental infection in male human volunteers confirmed the initial infection would not protect against reinfection with the same *Neisseria* strain [10]. Many studies show that gonococcal mucosal infections can result in an immune response although very weak. Serum antibodies against different GC antigens are readily detected, decrease with time and disappear several months after infection treatment [11]. The sera obtained at the time of mucosal infection or from the convalescent-phase were bactericidal but did not prevent infection [12]. There are almost no naturally acquired immunity responses to gonococci after uncomplicated infection [13,14]. It was suggested that GC might be able to suppress the host immune response by as yet unknown mechanisms [14]. GC inhibit human CD4 T cells or B cells by binding to human CEACAM1 (carcinoembryonic antigen cellular adhesion molecule 1) on lymphocytes with gonococcal opacity-associated (Opa) proteins [15-17]. Mucosal infections with gonococci are characterized by the abundant influx of polymorphonuclear leukocytes (PMNs) to the inflammation site [18]. GC can resist non-oxidative antimicrobial factors secreted by adherent, IL-8-primed PMNs [19], suppress oxidative burst of PMNs [20], and also delay apoptosis in PMNs [21]. Furthermore, antigenic and phase variation of gonococcal outer membrane structures such as lipooligosaccharide (LOS), pili and Opa proteins constitutes an efficient mechanism to escape recognition by the host immune system.

1.1.2.1 Vaccine development

With no evidence for naturally acquired immunity after infection by the gonococcus, it is difficult to develop an effective vaccine for GC. In the 1970s, Greenberg et al. tried a crude whole cell vaccine made from killed gonococci in small but well controlled clinical trials. The vaccine gave an antibody response in over 90% of vaccine recipients and good tolerance with only mild reactions in humans but no efficacy in preventing gonorrhea [22]. The other vaccine which had entered into clinical trials was a pilus vaccine. Brinton et al. found that it protected human male volunteers against the experimental urethral infection by the homologous strain with parenteral immunization of isolated and purified pili [23]. The antibody response was detected in the serum and genital secretions, but the pilus vaccine failed to prevent infections with heterologous strains expressing antigenically variant pili [24]. Also it did not show resistance in a clinical trial [25] which might have been caused by antigenic variation of pili expressed in the gonococcus. Intranasal immunization of female mice with a gonococcal outer membrane vesicle (OMV) vaccine resulted in reduced colonization on the vaginal surface in infections with the homologous *N. gonorrhoeae* strain MS11 [26]. However, protection was not observed in subsequent infections with either MS11 or FA1090 OMV [13]. The individual outer membrane proteins are considered as promising vaccine targets, for example PorB (outer membrane porin protein B) [27] and TbpB (transferrin receptor protein) [28]. Immunization of BALB/C mice by intramuscular needle injection or epidermal gene gun bombardment with a DNA vaccine encoding *N. gonorrhoeae* PorB produced detectable levels of antigen-specific antibodies. The anti-PorB antibody levels are significantly increased with a boost of renatured recombinant (rr) PorB from *E. coli* or PorB expressed from viral replicon particles (VRPs) [27]. The resulting antibodies were shown to partly recognize the surface of the homologous strain *in vitro* but further experiments in mouse models will be required.

1.1.2.2 Antibiotic resistance

In the absence of effective vaccines, timely diagnosis and efficient antibiotic therapy remain the principal method to prevent epidemics and cure infections. The history of antibiotic treatment of

gonorrhea and evolution of resistance in *N. gonorrhoeae* in the United States demonstrates that *Neisseria* acquires antibiotic resistances (Fig. 1-1) [29]. For example, penicillin was introduced in the treatment of gonorrhea in 1943 [30] when sulfonamide-resistant gonococci became widespread. Penicillin was very effective in the treatment in the first 10-15 years after introduction. Then doses of penicillin had to be gradually increased due to developing resistance by sequential accumulation of chromosomal mutations [31]. In 1976, the plasmids containing β-lactamase were first reported in gonococci isolated from patients in Asia and Africa [32]. Alternative antibiotics, such as erythromycin, spectinomycin, tetracycline and fluoroquinolones were introduced in the treatment regimen, but resistant strains emerged soon due to chromosomal mutations or gene acquisitions. Today, the first-line antibiotics for treatment are third-generation cephalosporins (cefixime and ceftriaxone). Unfortunately, the first gonococcus with high-level resistance to ceftriaxone was identified in 2011 in Japan [33] and another clinical failure with cefixime treatment was recently reported in Europe [34]. *N. gonorrhoeae* thus evolved into a super bug and gonorrhea may become untreatable.

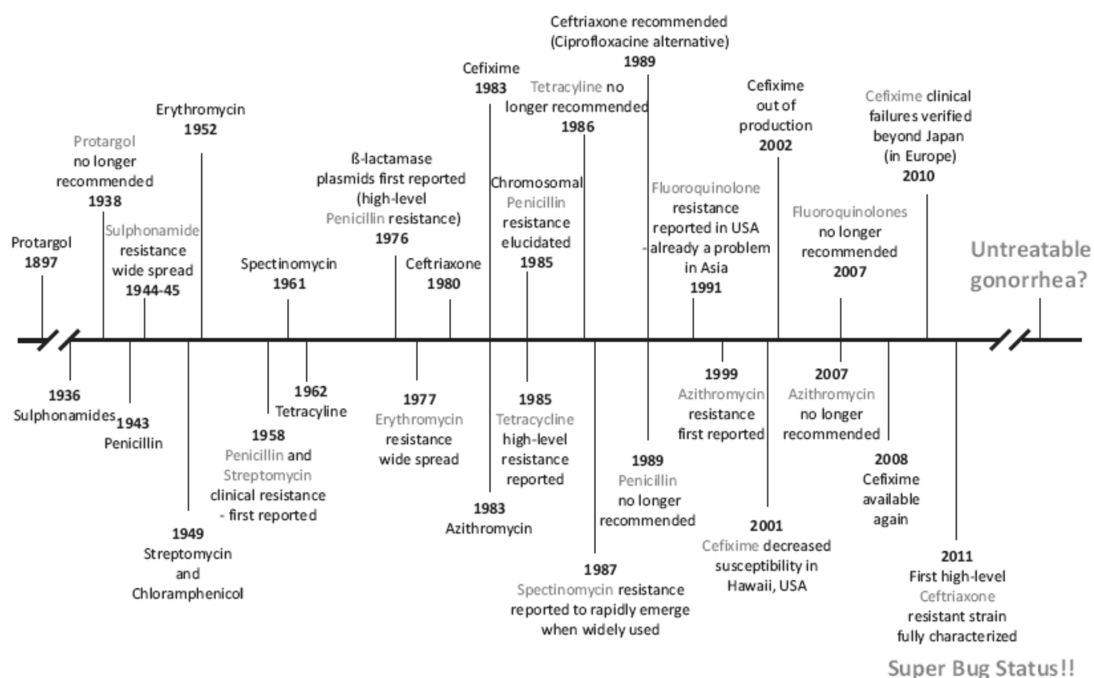

Fig. 1-1 History of antibiotic treatment of gonorrhea and evolution of resistance in *N. gonorrhoeae* in the United States. Figure modified from Unemo M and Shafer WM, 2011 [29].

1.1.3 *Neisseria* virulence factors

Neisseria gonorrhoeae expresses a set of virulence factors required for successful human infection, immune evasion, intracellular survival and transmission to a new host. Type IV pili (Tfp) induce the initial attachment to mucosal cells and enable the gonococci to form efficient colonization on the cell surface. Subsequently different Opa proteins trigger intimate binding and invasion into host cells, as well as transcellular transcytosis across polarized epithelial cell monolayers. Further, *Neisseria* Porin subtype $PorB_{IA}$ efficiently mediates bacterial attachment and internalization under the low phosphate condition mimicking the bloodstream. Porin is also capable to translocate from the bacterial outer membrane into host cell membranes and/or mitochondrial outer membranes of infected cells and modulates various cell processes, for example promotion of apoptosis and inhibition of phagosome maturation. Additionally, LOS, immunoglobulin A1 (IgA1) protease and factor H binding protein are important determinant to support the pathogenic potential of GC.

1.1.3.1 Type IV Pili

Pili are long filamentous structures on the surface of *Neisseria* and many other bacterial species. Type IV pili are important *Neisseria* virulence factors that mediate a set of functions, such as initial attachment to host cell, bacterial aggregation, twitching motility, microcolony formation and DNA uptake during natural transformation [35]. *Neisseria* pili are formed by non-covalent homopolymerization of major pilus subunit proteins, pilins, which are encoded by the gene *pilE.* Many pilus-associated proteins are involved in pilus assembly, extension and retraction. PilD, a bifunctional enzyme with peptidase and transmethylase activity, is responsible for pilus precursor maturation [36]. PilF is an ATPase that supplies the energy for pilus formation in the periplasm [37]. PilT is required for pilus disassembly and is dependent on ATP hydrolysis [38]. PilC is located at the tip of the pili and essential for pilus-mediated epithelial cell adherence and DNA transformation [39,40]. In addition, there are some minor pilus proteins, such as PilQ which forms a gated channel on the outer membrane for pilus extension [41]; PilV, which is found to be essential for adherence to the epithelial cells [42]; PilU and PilX, which are important for bacterial aggregation [43].

The antigenic and phase variation of *Neisseria* pili is one of the most effective strategies to evade host immune responses. *N. gonorrhoeae* possesses one pilin expression locus (*pilE*) but multiple silent pilin loci (*pilS*) located in the discrete locations in the genome [44]. The nonreciprocal homologous recombination between any silent *pilS* copy and the expressed *pilE* results in the expression of a new variant pilin leading to pilus antigenic variation [45]. The new variants can be fully functional, poorly expressed or not expressed and the last two situations exhibit the non-piliated colony morphology thus causing the pilus phase variation [46]. The guanine quartet (G4) structure on the upstream of *pilE* locus is required for the recombination [47] and regulated by a small non-coding RNA (sRNA) [48] and RecQ DNA helicase [49]. Additionally, the recombination is RecA-dependent and utilizes the RecF-like mechanism instead of RecBCD [50,51]. Besides the variation of PilE, the pilus adhesin PilC also has effects on pilus phase variation. Most gonococcal strains carry two copies of *pilC, pilC1* and *pilC2*. These two genes are not identical and thus produce two different forms of PilC. Usually *pilC1* is out of frame and *pilC2* is expressed. The expression of *pilC* is altered by frequent frameshift mutations within a series of guanine residues in the signal peptide encoding region. Since PilC is required for pilus assembly, the switch in the expression of PilC results in gonococcal pilus phase variation [52,53].

Neisserial pili mediate initial attachment of the bacteria to human cells, but the pilus receptor on the host cell surface is still controversial. Human CD46 (also termed membrane cofactor protein, MCP) is proposed as *Neisseria* pilus receptor [54]. Piliated, but not non-piliated gonococci bound to hamster cells expressing human CD46. The binding of piliated *Neisseria* to epithelial cells further was blocked by CD46 antibodies and a purified recombinant CD46 competitor [54]. However, Kirchner et al. found that the different binding efficiencies of piliated gonococci on human epithelial cells did not correlate with the level of surface-expressed CD46 and pilus-mediated binding was not reduced when CD46 expression was down-regulated by siRNA [55]. Alternatively, complement receptor 3 (CR3) was proposed as gonococcal pilus receptor on cervical epithelial cells [56] whereas an I-domain-containing integrin was demonstrated as pilus receptor on urethral epithelial cells (UECs) [57]. Recent research showed that after attachment, *Neisseria* pili prevented bacterial internalization by forming microcolonies and inducing anti-invasive signals trigged by caveolin-1 phosphorylation [58]. The natural loss of piliation

switches gonococcal local infection to a porin/scavenger receptor-triggered invasive infection [59] (1.1.3.3).

1.1.3.2 Opacity-associated (Opa) proteins

Neisseria encodes a family of phase-variable and antigenically distinct Opa proteins which mediate aggregation of gonococci by binding to LOS and bacterial intimate attachment to and efficient invasion into host cells during infection. In strain MS11, eleven different *opa* gene alleles have been identified and each of them has its own promoter [60]. *Opa* genes are constitutively transcribed but the expression is regulated by pentameric CTCTT repeat sequences within the leader peptide-coding sequences. The number of CTCTT determines if the coding regions are either in or out of frame [61,62]. The expression of each *opa* gene can be independently switched on or off, so bacteria derived from a single colony actually represent a mixture with respect to Opa proteins expression [60].

All Opa proteins are integral outer membrane proteins with different surface-exposed loops which show different host cell receptor binding specificities [62,63]. Opa proteins are grouped into two classes binding either *i*) heparan sulfate proteoglycans (HSPGs) or *ii*) carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs; previously CD66). The first class represented by Opa_{30}/Opa_{50} (encoded by *opaC* gene, for nomenclature of Opa proteins, see [63]) mediates invasion into epithelial cells by binding to HSPGs on the cell surface [64], but the subsequent host signaling pathway is dependent on cell line [64]. For example, the binding of Opa_{50} to the human conjunctiva epithelial cell line Chang cells stimulates two lipid hydrolysis enzymes, phosphatidylcholine-specific phospholipase C (PC-PLC) and acidic sphingomyelinase (ASM), which results in cytoskeletal rearrangements and bacterial uptake [65]. However, in other epithelial cell lines, like Chinese hamster ovary (CHO) cell, HeLa and Hep-2, the serum-derived extracellular matrix proteins vitronectin or fibronectin serve as a molecular bridge between an Opa-proteoglycan complex and host cell integrins [66-68].

The second class including most Opa proteins Opa_{51-60} interact with CEACAMs [69]. Among twelve different human CEACAMs, CEACAM1, 3, 5 and 6 have been described as Opa receptors

[70-73]. The interaction triggers various important cellular functions, such as neisserial engulfment by the epithelial cells [74], transcellular transcytosis across polarized epithelial cell monolayers [75], entry into endothelial cells [76], suppression of lymphocyte response [15] and bacterial engulfment and killing by neutrophils [70]. Opa-dependent phagocytosis is mediated by CEACAM3, a CEACAM family member exclusively expressed on polymorphonuclear granulocytes. Upon bacterial engagement, the cytoplasmic domain of CEACAM3 is phosphorylated by Src family kinases and then the phosphorylated cytoplasmic domain recruits the small GTPase Rac followed by actin rearrangements [77-80]. However, internalization of *N. gonorrhoeae* in epithelial cells via CEACAMs (CEACAM1, CEACAM5 or CEACAM6) is independent of cytoplasmic domain of epithelial CEACAMs but the endocytosis is involvement of cholesterol-rich membrane microdomains, phosphatidylinositol-3' kinase (PI3K) and phosphatidylinositol 3, 4, 5-phosphate [PI(3,4,5)P] [79,81,82].

1.1.3.3 Pore-forming proteins (Porin)

Porins are the major outer membrane proteins in *Neisseria* and account for over than 60% of the total proteins on the outer membrane. In *N. gonorrhoeae*, the dominant outer membrane protein is PorB, encoded by a single gene *por*. PorB has two related serotypes $PorB_{IA}$ and $PorB_{IB}$ encoded by alleles of *por* gene, *porA* and *porB* respectively. Most clinical isolates express $PorB_{IB}$ and only 20% express $PorB_{IA}$. Among the $PorB_{IA}$ expressing strains, 80% are isolated from disseminated infection cases [83-85]. Further, $PorB_{IA}$ but not $PorB_{IB}$ triggers efficient bacterial internalization in many different cell lines under low phosphate conditions. This invasion is independent of pili and Opa proteins [86]. The study of Zeth K et al. demonstrated that in $PorB_{IA}$ the amino acid at position 92 was conserved either as arginine or histidine, whereas $PorB_{IB}$ encoded a serine at the same position. Arg/His92 in $PorB_{IA}$ of disseminating gonococci was critical for phosphate-sensitive adherence and invasion [87]. Recently, the human heat shock glycoprotein Gp96 and the scavenger receptor expressed on endothelial cells I (SREC-I) were found as host receptor for *Neisseria* $PorB_{IA}$. The study indicated that the binding of $PorB_{IA}$ to Gp96 initiated a rapid and massive adherence in a phosphate-sensitive manner but blocked the invasion. The entry of gonococci to the host cell is trigged by the interaction of $PorB_{IA}$ with SREC-I [88].

$PorB_{IA}$-dependent bacterial uptake into epithelial cells requires the formation of membrane rafts and caveolin-1 phosphorylation. The underlying signaling cascade involves PI3K and phospholipase C ɣ1 (PLC ɣ1) [59].

PorB forms an anion-selective ion channel that is essential for neisserial viability. In addition, porin is able to translocate from the bacterial outer membrane into host cell membranes where it modulates the infection process and affects various cell functions. PorB pore formation is modulated by cytosolic purine nucleoside triphosphates, especially by ATP/GTP [89]. The translocation of neisserial porin causes rapid calcium influx from the extracellular milieu into target cells. The increase in cytosolic calcium subsequently induces apoptosis by activation of the calcium-dependent protease calpain as well as proteases of the caspase family [90]. Porins share similarity with mitochondrial voltage-dependent anion channels (VDAC) with respect to structure, function and the mechanism of ion flow across mitochondria membrane [89-92]. Porin can be selectively transported to the mitochondrial outer membranes of infected cells causing efflux of cytochrome c and loss of the mitochondrial membrane potential thus ultimately resulting in apoptosis [91,92]. By contrast, Binnicker MJ et al. demonstrated NF-κB activation by $PorB_{IB}$ and increased expression of host anti-apoptotic factors in UEC cells [93]. PorB is further supposed to inhibit phagosome maturation as was evidenced by the experimentation that more early endocytic markers and less late endocytic markers were detected in isolated phagosomes from macrophages incubated with purified PorB [94].

1.1.3.4 Other virulence factors

LOS is one of the important virulence determinants of *N. gonorrhoeae*. It contains three short oligosaccharide chains covalently linked through ketodeoxyoctonoic acid to a lipid A component which anchors in the outer membrane [95]. Contrasting lipopolysaccharide (LPS), which is often found in Gram-negative bacteria, LOS contains only a short oligosaccharide instead of polysaccharide o-chain repeats. LOS plays several key roles in gonococcal infection, immune evasion, tissue damage and the stimulation of bactericidal antibodies [96-98]. One *Neisseria* strain usually produces two to six different LOS molecules and the antigenic variation of LOS is mainly due to the types and numbers of carbohydrates in their LOS which are modulated by a frameshift

on the poly (G) tract of the coding sequence of the gene *lsi-2* [99].

Pathogenic *Neisseria* further can express an extracellular serine protease, the so called IgA1 protease, which specifically cleaves the principal mucosal antibody, immunoglobulin A1 (IgA1) [100]. IgA1 protease contains an amino-terminal leader, the protease and a carboxyl-terminal “helper” domain. The leader and the “helper” domains are required for the transport through the inner and outer membranes [101]. *Neisseria* IgA1 protease was shown to promote intracellular survival within epithelial cells by degradation of LAMP1 (lysosomal-associated membrane protein 1), a major integral membrane glycoprotein of late endosomes and lysosomes [102,103].

1.1.4 Disseminated gonococcal infection (DGI)

In most cases, *Neisseria gonorrhoeae* cause uncomplicated gonococcal infections, such as cervicitis and urethritis, but rarely, in 1–3% of patients infected with *N. gonorrhoeae*, the gonococci spread from the local infection sites to other organs of the host body and cause disseminated gonococcal infection (DGI) which commonly leads to joint pain, skin lesions and polyarthritis. If untreated, DGI may develop to some serious conditions, such as bacterial endocarditis, meningitis, and pneumonia [104].

DGI is three or four times more common in women than men. The higher frequency among women may be due to many infected women are asymptomatic which gives the gonococci the opportunity for systemic spread. Besides, menstruation, pregnancy or the initial postpartum period increases the risk of dissemination from the genitourinary tract. The congenital or acquired complement deficiencies of the complement C5-C8 are less common risk factor [105]. The complement-dependent bactericidal effect of normal human sera can efficiently prevent the dissemination of serum-sensitive gonococci. However, the gonococci develop many strategies to evade the killing by host immune responses. Sialylation of gonococcal LOS results in conversion of previously serum-sensitive strains to unstable serum resistance allowing the gonococci escape from bactericidal activity of the serum [106]. Besides, *Neisseria* porins play an important role in stable serum resistance of nonsialylated gonococci by binding to factor H or C4b-binding protein (C4bp) to inhibit complement activity [107,108]. In addition to resist the bactericidal activity of

the serum, the gonococci can utilize the component in the serum for invasion into the host cell. For example, *Neisseria* Opa_{50} mediates internalization in some epithelial cell lines with assistance of the serum-derived extracellular matrix proteins vitronectin or fibronectin as a molecular bridge of Opa-proteoglycan complex [66-68].

It is reported most gonococci isolated from patients with DGI are serum-resistant and AHU auxotype (Arg-Hyx-Ura auxotype) which means the gonococci require arginine, hypoxanthine, and uracil for growth on chemically defined medium [109]. Further, $PorB_{IA}$ expressing gonococci are frequently isolated from patients with disseminated infection [83-85]. It is found *Neisseria* $PorB_{IA}$ can trigger efficient invasion under a phosphate-sensitive condition mimicking the bloodstream [86]. In this process, the heat shock protein Gp96 and scavenger receptor SREC-1 serve as the host receptors for $PorB_{IA}$ [88]. The formation of membrane rafts, caveolin-1 phosphorylation and a series of activation of PI3K, PLCγ1, Rac1 (ras-related C3 botulinum toxin substrate) and PKD1 (PKCμ, protein kinase C μ) are involved in the subsequent signal transduction pathway which result in cytoskeletal rearrangements for membrane ruffling and then uptake of the gonococci [59]. However, little is known about *Neisseria* factors involved in this invasion process so far. It is likely that some other additional gonococcal factors may participate in $PorB_{IA}$-triggered invasion and these factors may be new targets for the development of anti-infectives against gonococci.

1.2 Transposon mutagenesis

1.2.1 Transposons

A transposable element (TE) or "transposon", is a DNA sequence that can move from one genomic location to another. Transposons first were described as jumping genes in maize by Barbara McClintock in 1948 [110,111]. TEs have been identified in almost all the prokaryotic and eukaryotic organisms and comprise a large proportion of the genome. For example, TEs make up approximately 12% of *Caenorhabditis elegans* genome [112,113], 37% of the mouse genome [114] , 50% of the human genome [115] and up to 90% of the maize genome [116]. Thus they play a significant role in changes of genome size during evolution and in genetic plasticity of

organisms [117-119]. The mobilization of TEs can cause insertion, excision, duplication or translocation at the site of integration and thus may positively or negatively influence gene expression and also induce gene deletions and illegitimate recombination. TEs are considered as selfish DNA and tend to widely spread throughout the whole genome. Since deleterious effects of transposons in essential genes will ultimately lead to a reduction in the fitness of the affected organism, most transpositions are identified in the nonessential regions of the genome [120,121]. Besides, transpositions are frequently found in the germ line or embryonic cell as the harmful mutations can be selected during the development [122,123]. On the other hand, the host have evolved several strategies to curtail TEs spread, such as DNA methylation to reduce the expression of TEs [124,125], RNA interference (RNAi) [126] or specific proteins mediated inhibition mechanism [127]. So the distribution of TEs in the genome is the balance result of host cells' defense against TE's expansive spread.

TEs are classified into two major groups. Class I TEs, also known as retrotransposon such as retroviruses, propagate through an RNA intermediate via a "copy and paste" mechanism. A reverse transcriptase is necessary for the transposition, which is encoded by the class I TE itself. Class I TEs produce RNA transcripts and the RNA transcripts can be reverse transcribed to DNA which is inserted into a new location of the genome. There are two major types of class I TEs: LTR retrotransposons with long terminal repeats (LTRs) and non-LTR TEs lacking these repeats such as long-interspersed nuclear elements (LINEs) or short-interspersed nuclear elements (SINEs). Classes I TEs occupy nearly 40% of the mammalian genome (reviewed in [117,118]).

By contrast Class II TEs are DNA transposons. Most class II TEs are excised from one position and reintegrated into another position within the genome using a "cut and paste" mechanism. Transposition of class II TE is catalyzed by transposase. Class II TEs are further characterized by 9 to 40 base pairs terminal inverted repeats (TIRs). The transposases recognize these TIRs and cut the whole DNA transposon from the excision site. Some transposases recognize and bind to specific DNA sequence as target site for insertion of the transposon. For example, the transposase of the Tc1/mariner element specifically recognizes TA dinucleotide in the genome and catalyzes a random insertion in any TA target site. Other transposases catalyze nonspecific transpositions in any target site. Transposases cut their respective target sites and generate sticky ends for the DNA

transposon. Subsequently, the two gaps of target site are filled up and closed by DNA polymerase and DNA ligase. Target site duplications (TSDs) comprise a unique hallmark of DNA transposons containing flanking direct repeats (LTRs in class I or TIRs in class II). After excision, these repeats are left behind as “footprint”. Class II TEs are also classified into different families depending on their sequences, such as Tc1/mariner, P elements, hAT superfamily (hobo/Ac/Tam3) and so on. Only few transposons utilize a replicative transposition mechanism instead of the “cut and paste” pattern. These include, for example, Helitron and Maverick transposons (reviewed in [119]).

Both class I and class II TEs contain autonomous and non-autonomous elements. The autonomous transposons encode the proteins required for their transposition and can move on their own. Non-autonomous transposons lack genes encoding reverse transcriptase or transposase and thus are dependent on autonomous transposons for their mobilization. For example, activator element (*Ac*) is an autonomous TE and dissociation element (*Dc*) is a non-autonomous TE which requires the presence of *Ac* for transposition [128].

Transposons can regulate gene expression and contribute to genome evolution, but they are used as a powerful molecular tool for both, single gene analysis and a wide variety of genomic studies in microorganism and higher eukaryotes. Transposon-based strategies for microbial functional genomics include gene sequencing, gene fusions, signature-tagged mutagenesis, and genetic footprinting (reviewed in [129]). Transposons have been rediscovered as efficient genetic tools in higher eukaryotes and even in vertebrates and are used, for example, for the generation of transgenic cells in tissue culture and transgenic animal models and even for therapy of genetic disorders in humans (reviewed in [130]).

1.2.2 DNA Transposon Tn5

Tn5 is one of the first identified transposons [131] and today one of the most frequently used transposition systems. It was isolated from Gram-negative bacteria and comprises a composite transposon which contains three antibiotic resistance genes, *kan* (kanamycin), *ble* (bleomycin) and *str* (streptomycin) flanked by two inverted insertion sequence IS50 elements. Each IS50 element is defined by two 19 bp end sequences (ES), the outside end (OE) and inside end (IE), which are

critical binding sites for transposase (Tnp). IE is methylated by deoxyadenosine methylase (Dam) in some bacteria, and this methylation strongly inhibits recognition by the transposase [132]. Moreover, both OE and IE are suboptimal for transposition. So the hyperactive version of the ES, which is called mosaic end (ME, CTGTCTCTTATACACATCT), is usually introduced in synthetic Tn5 transposon systems, which drastically increases the transposition efficiency. IS50R encodes the functional Tnp and the transposition inhibitor (Inh). Whereas IS50L is almost identical to IS50R, it only encodes truncated, inactive versions of Tnp and Inh (Fig. 1-2A, reviewed in [133,134]).

Tn5 utilizes a “cut and paste” mechanism for its transposition during which Tn5 is excised from the original site and then inserted into the target site. The whole transposition process requires three macromolecules, the donor DNA-containing transposon, the target DNA sequence and the 476 amino acid residues Tnp. In brief, the transposition process contains three steps: (1) Tnp recognizes and binds to the ES of Tn5 and a Tnp-transposon DNA synaptic complex is formed by dimerization of Tnp; (2) In the presence of Mg^{2+} or Mn^{2+}, the synaptic complex is catalytically cleaved off the donor DNA; (3) The released synaptic complex captures the target DNA sequence and Tnp catalyzes strand transfer (Fig. 1-2B, reviewed in [133,134]). The strand transfer leaves two 9 bp gaps on either end of the inserted Tn5 which are likely filled in and sealed by host. However, some details of these three steps are still unclear. The analysis of the target sequence from thousands of inserts indicates the preferred target sequence contains 19 bp with a 9 bp core sequence surrounded by 5 bp on either side [135]. The consensus 9 bp core sequence is A-GNTYWRANC-T (N = A/G/C/T , Y = T/C, W = A/T and R = A/G) [136]. Although there is a slight sequence bias of Tn5 insertion sites, almost any sequence can be chosen at some frequency, so the randomness is sufficient for most applications and the impact of the bias is negligible.

Tn5 transposition has been used as a powerful tool for molecular genetics by enclosing cargo DNA, such as antibiotic markers, genes for fluorescent proteins or therapeutic genes with ME [137]. Wild-type Tnp is low active because frequent transpositions easily lead to lethal genetic mutation of the host which will cause the loss of Tn5. Goryshin et al. greatly increased the transposition efficiency of Tnp by introducing mutations that render Tnp hyperactive and enable

an *in vitro* Tn5 transposition system [138]. Transposition can be conditionally regulated by the providing Tnp for the transposition reaction.

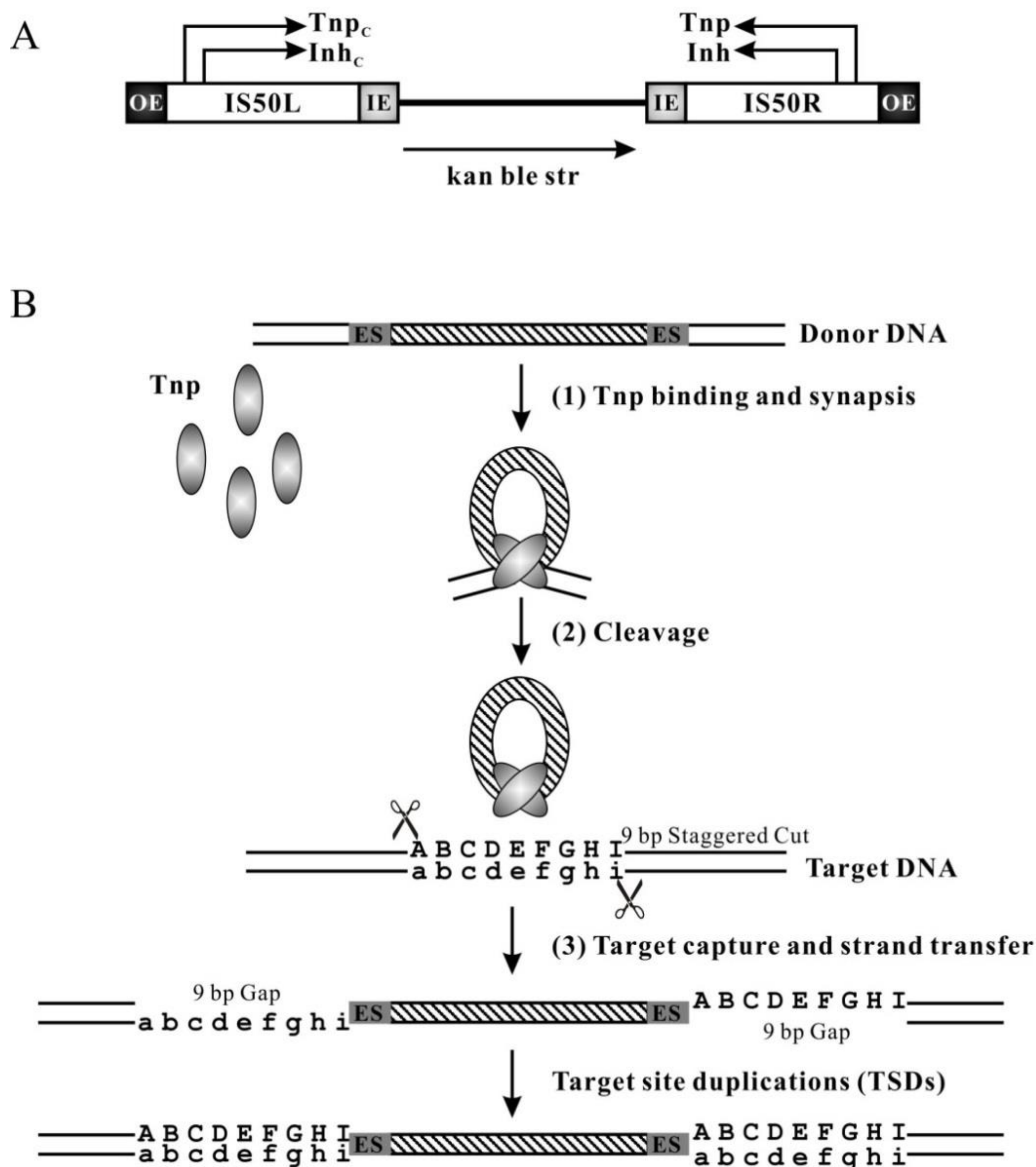

Fig. 1-2 Tn5 structure and transposition mechanism.

(A) Tn5 structure. Two IS50 elements bracket three antibiotic resistance genes. IS50R encodes transposase (Tnp) and transposase inhibitor (Inh). IS50L encodes C-terminal truncated, inactive versions of Tnp and Inh. IS50 elements are defined by transposon end sequences (ES), outside end sequence (OE) and inside end sequence (IE).

(B) Tn5 transposition mechanism. Tnp recognizes and binds to ES to form a Tnp-DNA synaptic complex which is cleaved from the donor DNA. The released complex captures the target DNA and Tn5 is then inserted into target DNA by strand transfer. The 9 bp gaps at both ends of the insertion site are repaired by host cell proteins.

1.3 Next generation sequencing (NGS) technologies

Next generation sequencing [NGS; also called second generation sequencing, deep sequencing, or massively parallel sequencing (MPS)] became available only a few years ago, but since then the technology has been broadly applied in genomics, transcriptomics and epigenomics.

The first-generation sequencing, known as Sanger sequencing, applies chain-termination method for DNA sequencing [139] (Fig. 1-3A). Briefly, during primer elongation, the random insertion of fluorophores labeled ddNTPs (dideoxynucleotides) instead of dNTPs terminates the synthesis of the chain. The products including all possible lengths of chains are separated on the capillary gel where the fluorophores are detected by an imaging system to identify the base and then the sequence is analyzed by computer. Compared with Sanger dideoxynucleotide terminator sequencing, NGS can perform massively parallel sequencing of millions of DNA fragments in a single sequencing run and thus is much cheaper, about one hundred thousandth of the expenses of the traditional sequencing technologies, and faster, since hundreds of Gbp can be readily acquired. Another advantage of NGS is that the sequencing library can be constructed and amplified *in vitro* rather than in *E. coli* [140].

Several NGS platforms have been developed, such as Illumina HiSeq 2500, NextSeq and MiSeq, Life Technologies SOLID4 and Ion Torrent Personal Genome Machine (PGM), Roche 454 GS-FLX and GS Junior [141]. One of the most commonly used platforms is Illumina HiSeq 2000. Illumina sequencing is performed by synthesis (Fig. 1-3B). Bridge amplification of DNA fragments enclosed by specific adapters generates up to 1,000 identical copies of each single DNA template in a very small area (diameter of 1 micron or less) on a flow cell. Sequencing is performed by chain synthesis with fluorescently-labeled nucleotides (FI-dNTPs) that contain a removable terminator. After FI-dNTP incorporation, the emitted fluorescence from each cluster is imaged to identify the incorporated nucleotide. Then the fluorescent dye terminator is enzymatically cleaved from the nucleotide exposing a hydroxyl group that enables the incorporation of the next labelled nucleotide base and reinitiating the procedure. With additional repeats of the sequencing cycles, the base sequence in the DNA amplicon is determinated. Subsequently, the image-based raw data is transformed into sequence reads by several

computational analysis steps mainly including removing adaptor sequences and low quality reads, mapping to the reference sequence and bioinformatics analysis of the compiled sequence [142].

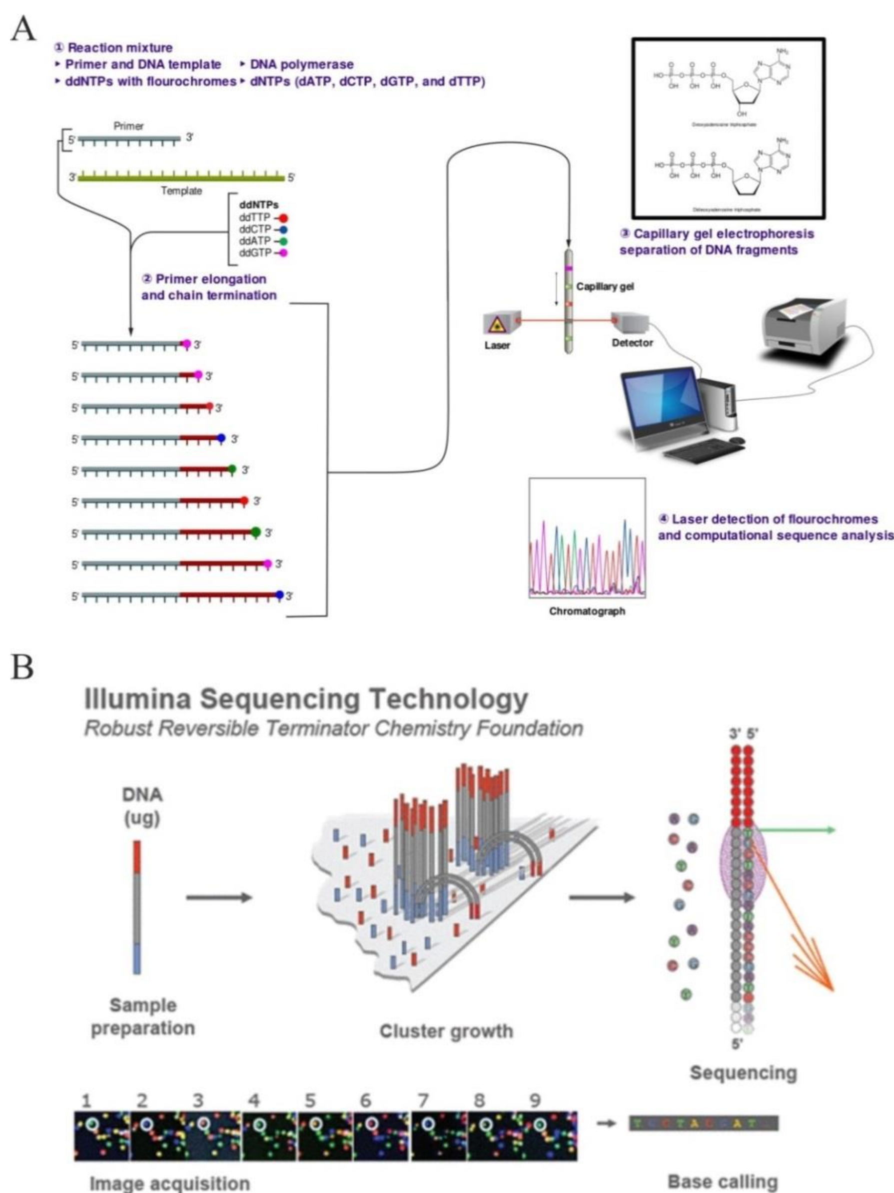

Fig. 1-3 Schematic diagram of sequencing process.

(A) Sequencing process of the first generation sequencing Sanger sequencing (http://en.wikipedia.org/wiki/File:Sanger-sequencing.svg).

(B) Sequencing process of Illumina sequencing, an example of the next generation sequencing (http://openwetware.org/wiki/BioMicroCenter:Sequencing).

1.4 Transposon insertion sequencing (Tn-seq)

With the advent of NGS, the microbial genomes have flooded the database; however, the knowledge of gene function has greatly lagged behind gene discovery. Approximately 30-40% of genes are unknown in a new sequenced microorganism [143-145]. In order to reveal genotype-phenotype relationships in a high-throughput manner, the techniques combined NGS with traditional transposon mutagenesis has been recently developed, such as transposon sequencing (Tn-seq), high-throughput insertion tracking by deep sequencing (HITS), insertion sequencing (INSeq) and transposon-directed insertion site sequencing (TraDIS) [146-150]. In these similar approaches, a high-density transposon mutant library in which nearly all the non-essential genes contain insertions is applied to grow in a defined condition, *in vitro* growth conditions or *in vivo* infection of the host. The contribution of each gene in this condition is determined by comparing the relative frequency of each mutant in the population during the growth which can be quantified by massively parallel sequencing (MPS) of the transposon junctions. Such methods had been used to identify essential genes in different bacteria including *Salmonella Typhi* [147], *Salmonella Typhimurium* [151], *Caulobacter crescentus* [152], *Mycobacterium tuberculosis* [153], *Porphyromonas gingivalis* [154], *Streptococcus pneumonia* [148] and so on. Besides, these approaches were applied to identify virulence genes in some pathogens, such as *Haemophilus influenza* genes required in mouse lung infection model [146] and *Pseudomonas aeruginosa* genes for resistance functions [155]. In addition, the emerging applications of this technique are for the identification of sRNAs required for pathogenesis [156] and for the elucidation of genetic interactions [157] in *Streptococcus pneumonia*. Owing to the wide activity of the Mariner and Tn5 transposons which were used in the studies mentioned above, the transposon insertion sequencing (Tn-seq) has the potential to contribute to the exploration of complex pathways across many different species [158].

1.5 Aims of this study

The human-specific pathogen *Neisseria gonorrhoeae* might develop to an untreated super bacterium in the near future. It is urgent to develop novel strategy to control the infections and cure the disease as recently appealed by WHO (World Health Organization) [3]. Therefore, identification of essential genes and virulence factors will be an effective approach to find out promising targets for vaccine or drug development.

Genome sequencing and preliminary annotation have been completed for some *N. gonorrhoeae* strains, but little is known about gene essentiality and the contribution of genes to neisserial virulence. This is in part due to the lack of straight forward transposon mutagenesis as transposons are usually inactive in gonococci. The first aim of this study thus was to construct a high-density transposon mutant library in *N. gonorrhoeae.* Then the distribution of transposon insertion sites (TIS) was to be analyzed by deep sequencing (Tn-seq) in order to identify the essential genes required for gonococcal survival and growth. Further, the transposon mutant library was to be used to screen for virulence factors involved in gonococcal disseminated infection (DGI), a severe systemic infection that occurs in about 1-3% of gonococcal infections.

2 Material and methods

2.1 Material

2.1.1 Bacterial strains

All the gonococcal strains used and constructed in this study are derived from *Neisseria gonorrhoeae* MS11 and are listed in Table 2.1. *Escherichia coli* strains XL1 Blue and DH5α were used for amplification of plasmids. *E. coli* strains BL21 and soluBL21 (a gift from Dr. Rosalia Deeken, Department of Botany I, University of Wuerzburg) were used for protein expression.

Table 2.1 *N. gonorrhoeae* strains used in this study

Stain Identifier	Phenotype	Genotype/Plasmid	Source
MS11	$PorB_{IB}$, P^+, Opa^-	*porB*	Our lab
N2009	$PorB_{IA}$, P^+, Opa^-	MS11, *porB*::*porA*	[159]
N219	$PorB_{IB}$, P^-	MS11-B1 (P^s) [160], ptetM25.2 [161]	[162]
N220	N219, pTH10a	N219, pTH10a	Our lab
N931	N219, Opa_{50}	N219, pTH6a (Opa_{50})	[63]
N313	N219, Opa_{57}	N219, pTH6a (Opa_{57})	[63]
N2020	N2009 Δ01605, Opa_{50}	N2009, NGFG_01605::Kan^R, pTH6a(Opa_{50})	[163]
N2021	N2009 Δ01605, Opa_{57}	N2009, NGFG_01605::Kan^R, pTH6a(Opa_{57})	[163]
N2022	N2009, Opa_{50}	N2009, pTH6a(Opa_{50})	This study
N2023	N2009, Opa_{57}	N2009, pTH6a(Opa_{57})	This study
N2024	MS11, Kan-P_{trc}-00442	MS11, (promoter of NGFG_00442)::(Kan^R-P_{trc})	This study
N2025	MS11, Kan-P_{trc}-00442, $lacI^q$	MS11, (promoter of NGFG_00442)::(Kan^R-P_{trc}), (pTH10a)	This study
N2026	MS11, Kan-P_{trc}-04144	MS11, (promoter of NGFG_04144)::(Kan^R-P_{trc})	This study
N2027	MS11, Kan-P_{trc}-04144, $lacI^q$	MS11, (promoter of NGFG_04144)::(Kan^R-P_{trc}), pTH10a	This study

N2028	MS11, Kan-P_{trc}-02103	MS11, (promoter of NGFG_02103)::(Kan^R- P_{trc})	This study
N2029	MS11, Kan-P_{trc}-02103, $lacI^q$	MS11, (promoter of NGFG_02103)::(Kan^R- P_{trc}), pTH10a	This study
N2030	MS11, Kan-P_{trc}-00007	MS11, (promoter of NGFG_00007)::(Kan^R- P_{trc})	This study
N2031	MS11, Kan-P_{trc}-00007, $lacI^q$	MS11, (promoter of NGFG_00007)::(Kan^R- P_{trc}), pTH10a	This study
N2032	N2009Δ00599	N2009, NGFG_00599::Kan^R	This study, done by Weitner, H
N2033	N2009Δ00859-00860	N2009, NGFG_00859-00860::Kan^R	This study, done by Weitner, H
N2034	N2009 Δ01489	N2009, NGFG_01489::Kan^R	This study
N2035	N2009 Δ01393	N2009, NGFG_01393::Kan^R	This study
N2036	N2009 Δ02032	N2009, NGFG_02032::Kan^R	This study
N2037	N2009 Δ00042	N2009, NGFG_00042::Kan^R	This study
N2038	N2009 Δ01836	N2009, NGFG_01836::Kan^R	This study
N2039	N2009 Δ04218	N2009, NGFG_04218::Kan^R	This study
N2040	N2009 Δ01605	N2009, NGFG_01605::Kan^R	[163]
N2041	N2009 Δ00072	N2009, NGFG_00072::Kan^R	[163]
N2042	N2009 Δ01266	N2009, NGFG_01266::Kan^R	[163]
N2043	N2009 Δ01643	N2009, NGFG_01643::Kan^R	[163]
N2044	N2009 Δ00506	N2009, NGFG_00506::Kan^R	[163]
N2045	N2009 Δ00827	N2009, NGFG_00827::Kan^R	This study

2.1.2 Cell lines

Table 2.2 Cell lines

Cell line	Properties	Media	Source
Chang T	Human conjunctiva epithelial cells	RPMI 1640, 10% FCS	ATCC CCL-20.2
HFF	Human Foreskin Fibroblast cells	DMEM, 10% FCS	ATCC SCRC-1041
NIH 3T3	Mouse embryonic fibroblast cell line	DMEM, 10% FCS	ATCC CRL-1658
HeLa 229	Human epithelial cervical carcinoma cells	RPMI 1640, 10% FCS	ATCC CCL-2.1
HeLa CEA	Human cervix carcinoma epithelial cell expressing CEACAM 1	RPMI 1640, 10% FCS	Our lab

2.1.3 Plasmids

Table 2.3 Plasmids used in this study

Plasmid	Properties	Source
pGEM®-T-Easy	Cloning vector	Promega
pGEM-T-Ptrc	P_{trc} promoter cloned in pGEM-T-Easy	This study
pGEM-T-kan-Ptrc	Kanamycin cassette cloned in pGEM-T-P_{trc} (SpeI/SacI)	This study
pCR2.1®-Topo	Cloning vector	Invitrogen
pCR2.1-Tn5	Tn5 cassette cloned in pCR2.1®-Topo	This study
pCR2.1-Tn5-DUS	DUS sequence inserted in pCR2.1-Tn5 by site-directed mutagenesis	This study
pET28b	Expression vector, mutation in 244-239 from GGCAGC to GGATCC	Novagen, Lab of Dr. Rosalia Deeken, Department of Botany I, University of Wuerzburg
pET28b-AIF1	ORF of NGFG_01605 cloned in pET28b (BamHI/HindIII)	This study
pET28b-AIF1$_{185\text{-}451aa}$	DNA sequence encoding AIF1$_{185\text{-}451aa}$ cloned in pET28b (BamHI/HindIII)	This study

2.1.4 Oligonucleotides

Table 2.4 Oligonucleotides for generation and sequencing of the libraries

Primer name	Oligonucleotide Sequence (5' → 3')	Comment
Adaptor sense	p-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG	Y-type adaptor
Adaptor antisense	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T	
EZ-Tn5-Kan2-DUS-F	TGGCGGATGCCGTCTGAAGATCCTCTAGAGTCGACC	Insert DUS in the Tn5
EZ-Tn5-Kan2-DUS-R	GGATCTTCAGACGGCATCCGCCACGGTTGATGAGAGC	
Ez-Tn5 Amplify primer	CTGTCTCTTATACACATCTCAACC	Amplify Tn5-DUS
P5-ME	biotin-AATGATACGGCGACCACCGAGATCTACGGTTGAGATGTGTATAAGAGACAG	
Antisense Input	CAAGCAGAAGACGGCATACGAGAT**ACACGT**CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC	Library 1

TnSeq-PE-Index-YX1	CAAGCAGAAGACGGCATACGAGAT**ACACGT**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	438_C
TnSeq-PE-Index-YX2	CAAGCAGAAGACGGCATACGAGAT**GTACAC**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	438_D
TnSeq-PE-Index-YX3	CAAGCAGAAGACGGCATACGAGAT**CATGAC**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	438_E
TnSeq-PE-Index1	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	438_F
TnSeq-PE-Index2	CAAGCAGAAGACGGCATACGAGAT**ACATCG**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	Library 2 or 438_A
TnSeq-PE-Index3	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**CGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATC	Library 3 or 438_B
TnSeq Primer	ACCGAGATCTACGGTTGAGATGTGTATAAGAGACA G	Sequencing TIS
TnSeq Index SP	GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGAC CG	Sequencing barcode

p: phosphorylation; *: phosphothioate bond; biotin: biotin-TEG modification; the *Neisseria* DNA Uptake Sequence (DUS) is underlined; bold: library specific barcode.

Table 2.5 Oligonucleotides for conditional knockout assays

Primer name	Oligonucleotide Sequence (5’➔3’)
Kan-SpeI-F	CGACTAGTATCATCGATGAATTGTGTCTC
Kan-SacI-R	TAGAGCTCCTGAAGCTTGCATGCCTG
Ptrc-F	GCGCCGACATCATAACGGTTCTG
Ptrc-R	CATGGTCTGTTTCCTGTGTGAAATTG
Kan-cassette-R	CTGAAGCTTGCATGCCTGCA
rib-up-f	GTGCGTTTAATCAGTGAGTCAGGC
rib-up-r	TGCAGGCATGCAAGCTTCAGGCAATCGGAGTAAGCGGAAAA
rib-down-f	CACACAGGAAACAGACCATGCCTAAAATGAAAACCAAGTCTAGCG
rib-down-r	CGGCTTTATCGAACACGGCC
PorB-up-f	CTTCGCCGCACTGATTCAAGAAC
PorB-up-r	TGCAGGCATGCAAGCTTCAGGATGTGCATTTTGAAGGACGG
PorB-down-f	CACACAGGAAACAGACCATGAAAAAATCCCTGATTGCCCTGAC
PorB-down-r	GCGTATTGTACGCTGCCGCTG
01315-up-F	ATTGTTTGCCTACGAACCGCCG
01315-up-R	TGCAGGCATGCAAGCTTCAGCGTTACTTCAAACCGGCTTGC
01315-down-F	CACACAGGAAACAGACCATGTTTATCCCTGCCGCCCTGC
01315-down-R	CAGCATATTCCTCAATCCGGCACG
04144-up-F	GAAGCCGTTGACCGGTGGATAC
04144-up-R	TGCAGGCATGCAAGCTTCAGTTAATCTCCTAAACCTGTTTTAACAATG

	CC
04144-down-F	CACACAGGAAACAGACCATGGCATCATATGTTTCCATCAAAGGATGG
04144-down-R	GCCAACCTACGCTTACTGAAAACCA
02103-up-F	GAAGACGAAGCGGGCAAGC
02103-up-R	TGCAGGCATGCAAGCTTCAGCGGATTTGTTCTTTAACCCATTGGG
02103-down-F	CACACAGGAAACAGACCATGAACCCAACCAAACAATCCAAAAAAAG C
02103-down-R	CATACACCCTTCAGGGAACTCTTATC
00007-up-F	ACCAAACTGTAACTTATCCTGCGACT
00007-up-R	TGCAGGCATGCAAGCTTCAGCGAGCCTGTTTTACTTTTATTCCG
00007-down-F	CACACAGGAAACAGACCATGAACATCGTTAAAAAATACGCTGTAAAA GC
00007-down-R	GAACAGAATTAGAACCGTCGAACCGA
00686-up-F	CCGTTTCCCATACCGTCTGAATC
00686-up-R	TGCAGGCATGCAAGCTTCAGGATTTAAGAAGGAAGGTCAGCAGC
00686-down-F	CACACAGGAAACAGACCATGTCCGAACAACCCGAAAAACACC
00686-down-R	CGCCGATGTGGATGGGTTCTTTA

Table 2.6 Oligonucleotides for genetic footprinting assays

Primer name	Oligonucleotide Sequence (5’➔3’)
01048-up-f	GCCGTCTGAACAGCCGATTCATAGACGAAATGCC
01052-down-r	CTTCGTATGCTTGGCGGTGGC
1063-up-f	GCCGTCTGAACGGCATAAAAGTCAGTGAGTTGGCG
1068-down-r	GAGTGACGAAAGGCGGGAACAAC
Tn ME sequence	GGTTGAGATGTGTATAAGAGACAG

Neisseria DNA Uptake Sequence (DUS) is underlined.

Table 2.7 Oligonucleotides for construction of genes knockout mutants

Primer name	Oligonucleotide Sequence (5’➔3’)
00599-up-forward	GCCGTCTGAATTTGGGCGCAAACCGTTTC
00599-up-reverse	TGAGACACAATTCATCGATGATGTTTCATGGCGGTGGTGTTC
00599-down-forward	TGCAGGCATGCAAGCTTCAGAATCAGGACAAGGCGACGAA
00599-down-reverse	GGATTTGGCGAGGTGGGAGAG
01674-up-forward	GCCGTCTGAAACTTGAACGACAAAACCCGC
01674-up-reverse	TGAGACACAATTCATCGATGATCTCATGATAACCTCGCTGTTGG
01674-down-forward	TGCAGGCATGCAAGCTTCAGTGATTCCGCAAAGCCGC
01674-down-reverse	TGACGACGGGTTGGACGAACA
01912-up-forward	GCCGTCTGAAGCGAAGCCGAAGTAGATGCT
01912-up-reverse	TGAGACACAATTCATCGATGATCTGTGTCATGGGATACCTTGC
01912-down-forward	TGCAGGCATGCAAGCTTCAGGATGATTGACCATAGGGTCGG
01912-down-reverse	CGCGAGAGTGCAGGGGCATTAA

01489-up-forward	GCCGTCTGAATTCCTCAACGGCTACCGTTT
01489-up-reverse	TGAGACACAATTCATCGATGATGAGCGAGTTCATGTAGCCGT
01489-down-forward	TGCAGGCATGCAAGCTTCAGAACCATGCCGTCTGAAAAATACCTG
01489-down- reverse	GGTGTGGCAGCGTAGGTAATGCTG
01393-up-forward	GCCGTCTGAAACCTCCAGCTTCCCTATGTC
01393-up-reverse	TGAGACACAATTCATCGATGATGTATAAGGCGGGTTTCAGCC
01393-down-forward	TGCAGGCATGCAAGCTTCAGTAATCGGCTCGCGATGCC
01393-down-reverse	GACGGTATCCAGCCCGCAC
02032-up-forward	GCCGTCTGAAGGGAAACGGAAGAAGCCAT
02032-up-reverse	TGAGACACAATTCATCGATGATCTTCGCTGTCGATAAAGTCGG
02032-down-forward	TGCAGGCATGCAAGCTTCAGCCGAATCCATGCCCGAAA
02032-down-reverse	AATCGGGCCGCAATCCAGCT
00042-up-forward	GCCGTCTGAATTCCAAGCGTTTGACGACGA
00042-up-reverse	TGAGACACAATTCATCGATGATCGTCCTTTTGAGTGTATGAAGGG
00042-down-forward	TGCAGGCATGCAAGCTTCAGAACGCACATCCCGAAAAAATGC
00042-down-reverse	CCGATGATGATGAGCTGCGGC
01836-up-forward	GCCGTCTGAAAAACGCTCTCCAAACCTTCG
01836-up-reverse	TGAGACACAATTCATCGATGATAAAAAGATGGTTTCGGGCGG
01836-down-forward	TGCAGGCATGCAAGCTTCAGAACCGGCAAAACAATGCCG
01836-down-reverse	TGTCGAACGCAGGCGGTATGTG
00860-00859-up-forward	GCCGTCTGAACCCGTCTCTTCAGGATAAGC
00860-00859-up-reverse	TGAGACACAATTCATCGATGATGGGTTGGTTGATACTACTCAGAGA
00860-00859-down-forward	TGCAGGCATGCAAGCTTCAGTCTAATCGCGGCGATATGCC
00860-00859-down-reverse	GGCACGAAGCGCGCGATGAT
04218-up-forward	GCCGTCTGAACTACGCCGGTCTGCAAAAAC
04218-up-reverse	TGAGACACAATTCATCGATGATCCGTTTGAAGTGGCGTTCAG
04218-down-forward	TGCAGGCATGCAAGCTTCAGATCCTAGTTGTCCAGGACGG
04218-down-reverse	CGATAATCCCCCATCCCGCC
00072-up-forward	GCCGTCTGAAGTGTTGGCGGCGATTCTGTTTG
00072-up-reverse	TGAGACACAATTCATCGATGATTCCTTGAATATCCGATGTTCCGC
00072-down-forward	TGCAGGCATGCAAGCTTCAGAGCCGCAGAATAAACATACACATCC

00072-down-reverse	GCGGTACACGGTAACCAGGCTC
01605-up-forward	GCCGTCTGAACGCCATCATGTCCCTGAC
01605-up-reverse	TGAGACACAATTCATCGATGATGGCGGGCAATAAGAGTTCGG
01605-down-forward	TGCAGGCATGCAAGCTTCAGGAACCCCTGAGCCACAATG
01605-down-reverse	TGCCAAAGTAGCTGTGGAAGCCG
01266-up-forward	GCCGTCTGAACAACCTCAGCAAACAAAGCACG
01266-up-reverse	TGAGACACAATTCATCGATGATCTTGATGGTTGCGTACTCGGTT
01266-down-forward	TGCAGGCATGCAAGCTTCAGCGGACGGTATTTCCACAACAG
01266-down-reverse	GGCCCGCCAATTCTTTTGACAGG
01643-up-forward	GCCGTCTGAATTATTTGGTTTTGCCACTGCGGA
01643-up-reverse	TGAGACACAATTCATCGATGATCGGGACTCGAACCAGGAAAATA
01643-down-forward	TGCAGGCATGCAAGCTTCAGCTCAGCGAACACGTCGAGT
01643-down-reverse	GATTTGCCCATACCGCTTTGTCCG
00506-up-forward	GCCGTCTGAAGATGCGGGCGACAAGATTTTC
00506-up-reverse	TGAGACACAATTCATCGATGATGTTACGCCCGACATTATAAAATCCC
00506-down-forward	TGCAGGCATGCAAGCTTCAGCCAAAAATGTTTGCTCTTGCCGC
00506-down-reverse	TAATGCCCTGCCAGCGGTCG
00827-up-forward	GCCGTCTGAATGATGTTTCAAGTCGCTTTCG
00827-up-reverse	TGAGACACAATTCATCGATGATTGAAATGAAGCATCATAATCTAAAGG
00827-down-forward	TGCAGGCATGCAAGCTTCAGGAAATGCCGTCTGAAACACCT
00827-down-reverse	GGCTTCAGACGGCATTTTGCC
op_kan_s	ATCATCGATGAATTGTGTCTCAAAATCTCTGAT
op_kan_hfq-mut_as	CTGAAGCTTGCATGCCTGCA
01393-seq2	GGTTCGCTGATTCTGACCGC
02032-seq2	GCAACTGCCGCTCTTTGAAACC
01836-seq2	ACGGGAATAAGGTACAGCAGCC
00042-seq2	GCCCAAGGTTACGCGCAC
00860-00859-seq2	TCTCTTCCTGCGTCCACTGA
00599-seq2	CCGGCTTCAAACTCAGCC
01674-seq2	CGTATCATCGCGTCGATGCC
01912-seq2	AGGCAACTTCGACAAAGCCG
01489-seq2	GTCCGCTGAAGGCAAACAGC
seq2-04218-f	GGCGAGGCGATGATGGCATTC
seq-01605-sense	TCAAGCCTTCCCGTTCCACA

seq-01266-sense	TTCTTTCCCTTTTCGCCTCC
seq-00072-sense	ATCCTTCGGCAGTATCACGCTG
seq-01643-sense	CGAAATCGTCAAAAACGGACAGGA
seq-00506-sense	GACCTGATTCCGACTGCCAA
seq-00827-f	ATCTGGTCGAATACGCTTCGTGG

Neisseria DNA Uptake Sequence (DUS) is underlined.

Table 2.8 Oligonucleotides for RT-PCR

Primer name	Oligonucleotide Sequence (5’➔3’)
RNase P-F	CGGAAAGTGGAACAGAAAGC
RNase P-R	GTTTGGTCTTGCTCCGAATG
Rt-00506-forwards	AGAAAAGTTACGAAGTGCCCA
Rt-00506-reverse	GTTTCGTTGCTCTCGTTCCTC
Rt-01605-forwards	TTCGTTGCCGACATGGAGCC
Rt-01605-reverse	TTTGAGGCTGTCCACACCG
Rt-00072-forwards	CGGTTGCCTTTCTCGCTTTC
Rt-00072-reverse	CCCTCAGCGTTTTTCTCGGC
Rt-01266-forwards	CACCGATACAAACGGGCTGC
Rt-01266-reverse	GATGTCCCACGGCATTTCGG
Rt-01643-forwards	TGGGCAAAATCGTAGAGTGGC
Rt-01643-reverse	GACTGCTTGGCATAGACGG

Table 2.9 Oligonucleotides for NGFG_01605

Primer name	Oligonucleotide Sequence (5’➔3’)
01605-BclI-F	CG**TGATCA**ATGAAAGCACCCGAACTCTTATTGC
01605-HindIII-R	CCC**AAGCTT**TCAGGGGTTCAACACGCG
01605-HindIII-flag-R	CCC**AAGCTT**TTACTTATCGTCGTCATCCTTGTAATCGGGGTTCAACACGCGTGC
01605-BamHI-185-F	CG**GGATCC**AACCACCGCGATCCCAAC
L-01605-rt	CCGATCCATCTGTCCGTACA
R-01605-rt	ATCGCGGTGGTTGAAATAGC

Bold: restriction enzyme cutting sites

Table 2.10 siRNA Oligonucleotides

Primer name	Sequence source
siLuciferase	ON-TARGETplus Non-targeting Pool, D-001810-10-05, Thermo Fisher Scientific™ Dharmacon™
siGp96	ON-TARGETplus HSP90B1 siRNA, LU-006417-00-0002, Thermo Fisher ScientificTM DharmaconTM

2.1.5 Buffers, solutions and media

Table 2.11 Media and solutions for cell culture

Medium/Chemical	Source
RPMI 1640	GIBCO
DMEM	Sigma Aldrich
Opti-MEM® I Reduced Serum Medium	GIBCO
DPBS	GIBCO or Sigma Aldrich
Tryple™ Express	GIBCO
Fetal calf serum (FCS)	PAA

Table 2.12 Bacterial culture media and buffers

Medium/Buffer	Ingredients
Cell Stocking Medium	70% FCS, 10% DMSO, 20% cell medium (DMEM or RPMI 1640)
LB Medium (1L)	10 g tryptone, 5 g yeast extract, 10 g NaCl
LB Agar (1L)	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar
SOC medium	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
GC Agar (1L)	36.23 g GC agar base, after autoclaving add 1% vitamin mix
PPM Medium (1L)	15 g proteose peptone, 5 g NaCl, 0.5 g soluble starch, 1 g KH_2PO_4, 4 g KH_2PO_4. Adjust to pH 7.2. Sterilize by sterile filtration
Vitamin Mix	combine Vitamin Mix Solution I and II (add dH_2O up to 2 L)
Vitamin Mix Solution I	200 g D(+)-glucose, 20 g L-glutamine, 0.026 g 4-aminobenzoic acid, 0.2 g cocarboxylase, 0.04 g iron(III) nitrate nonahydrate, 0.006 g thiamine hydrochloride (vitamin B1), 0.5 g NAD, 0.02 g vitamin B12, 52 g L-cysteine hydrochloride monohydrate; add 1 L dH_2O
Vitamin Mix Solution II	2.2 g L-cystine, 0.3 g L-arginine monohydrochloride, 1 g uracil, 0.06 g guanine-hydrochloride, 2 g adenine hemisulfate, add 600 mL dH_2O, 30 mL 32% HCl
Neisseria Growth or Conjugation Medium	PPM medium supplemented with 1% vitamin mix and 0.5% $NaHCO_3$
Neisseria Transformation Medium	PPM medium supplemented with 1% vitamin mix, 0.5% $NaHCO_3$, 10 mM $MgCl_2$
HEPES Medium (phosphate free medium)	50 ml solution I, 10 ml solution II, 200 µl solution III, 3 ml solution IV/V, 5 ml solution VI, 50 ml solution VII, 50 ml solution VIII Up to 500 ml H_2O, regulate pH 7.3, filter sterilization
HEPES -Solution I	0.1% L-Alanine, 0.15% L-Arginine, 0.025% L-Asparagine, 0.025% Glycine, 0.018% L-Histidine, 0.05% L-Lysine, 0.015%

	L-Methionine, 0.05% Proline, 0.05% L-Serine, 0.05% L-Threonin, 0.061% L-Cysteine, 0.036% L-Cysteine, 0.05% L-Glutamine, 0.046% reduced Glutathione (GSH), 0.0032% Hypoxanthine, 0.008% Uracil, 0.004% D-Biotin, add 18% 1 N NaOH and 82% H_2O
HEPES -Solution II	375 g/L Glucose
HEPES -Solution III	10 g/L $Fe(NO_3)_3 \cdot 9H_2O$
HEPES -Solution IV/V	0.33% Nicotinamide adenine dinucleotide (NAD), 0.33% Carboxylase, 0.33% Thiamin, 0.33% Ca-Pantothenate, 0.188% $CaCl_2 \cdot 2H_2O$, 4.17% Na-Lactate, 15.33% Glycerin, 3.33% Oxaloacetate
HEPES -Solution VI	50 g/L $MgCl_2 \cdot 7H_2O$
HEPES -Solution VII	50 g NaCl, 34 g Na-Acetat in 1 L H_2O
HEPES -Solution VIII	23.8 g/L Hepes

Table 2.13 Buffers for agarose gel electrophoresis, SDS-PAGE, western blotting, Immunofluorescence

Buffers	Ingredients/Source
10x TAE (1L)	48.5 g Tris, 11.4 mL glacial acetic acid, 20 ml 0.5 M EDTA (pH 8.0)
10x TBE (1L)	54 g Tris, 27.5 g Boric acid, 20 ml 0.5 M EDTA (pH 8.0)
4x SDS upper buffer (1 L)	0.5 M Tris/HCl (pH 6.8), 0.4% (w/v) SDS
4x SDS lower buffer (1 L)	1.5 M Tris/HCl (pH 8.8), 0.4% (w/v) SDS
8% SDS lower gel solution	for 10 mL: 2.7 mL 30% acrylamide, 2.5 mL 4x lower buffer, 4.8 mL H_2O, 100 µL 10% (w/v) APS, 10 µL TEMED
10% SDS lower gel solution	for 10 mL: 3.3 mL 30% acrylamide, 2.5 mL 4x lower buffer, 4.1 mL H_2O, 100 µL 10% (w/v) APS, 10 µL TEMED
12% SDS lower gel solution	for 10 mL: 4 mL 30% acrylamide, 2.5 mL 4x lower buffer, 3.6 mL H_2O, 100 µL 10% (w/v) APS, 10 µL TEMED
3% SDS upper gel solution	for 3.3 mL: 330 µL 30% acrylamide, 825 µL 4x upper buffer, 2.1 mL H_2O, 40 µL APS, 4 µL TEMED
2x Laemmli buffer	4% (w/v) SDS, 20% (v/v) glycerol, 120 mM Tris/HCl (pH 6.8), 0.2 mg/mL bromophenol blue, 0.1 M DTT
10x SDS Electrophoresis buffer (1 L)	30.25 g Tris, 144 g glycine, 10 g SDS
Coomassie Staining solution	44% methanol, 11% acetic acid, 0.2% Coomassie blue R250
Coomassie Destaining solution	20% methanol, 7% acetic acid

10x semi dry buffer (1 L)	24 g Tris, 113 g glycine, 2 g SDS
Semi dry transfer buffer	1x semi dry buffer, 20% (v/v) methanol
10x TBS-T (1 L)	48.5 g Tris, 175 g NaCl, 10 mL Tween-20, adjust to pH 7.5 with HCl
Blocking solution for WB	1x TBS-T, 3% (w/v) BSA
Restore™ Plus Western Blot Stripping Buffer	Thermo Fisher Scientific
4% paraformaldehyde (PFA, 400 mL)	40 ml 10x PBS, 16 g PFA, 80 µL 10 N NaOH, 320 mL H_2O, regulate pH to 7.4
Blocking solution for IF	1x PBS, 1% (w/v) BSA
Permeabilization solution	1x PBS, 0.1% (v/v) Triton X-100
Mowiol mounting medium	2.4 g Mowiol 4-88, 6 g glycerol, 6 mL H_2O, 12 mL 0.2 M Tris/HCl (pH 8.5)

Table 2.14 Buffers for neisserial RNA and DNA isolation

Buffers	Ingredients/Source
GTE buffer	50 mM glucose, 25 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0)
TE buffer	10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0)
Lysis buffer for RNA isolation	TE buffer + 1 µL/10 mL Ready Lyse
3M Sodium acetat solution pH 5.2	Sigma

Table 2.15 Buffers for protein purification, antibody purification and inclusion bodies' isolation

Buffers	Ingredients
Binding buffer	50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0
Lysis buffer	Binding buffer, 10 mM imidazol, 1% Triton X-100, 1% NP40
Washing buffer	Binding buffer, 20 mM imidazol
Elution buffer for protein purification	Binding buffer, 250 mM imidazol
0.2 M Carbonate buffer pH8.9	10 mL 0.1 M Na_2CO_3, 90 mL 0.1 M $NaHCO_3$
Elution buffer for antibody purification	0.2 M acetic acid (pH 2.7), 500 mM NaCl
Lysis buffer for isolation of inclusion bodies	10% saccharose, 50 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0)
NTE buffer	50 mM NaCl, 50 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0)

Table 2.16 Annealing buffer for adaptor oligonucleotides

Buffers	Ingredients
10x Annealing buffer	100 mM Tris/HCl (pH 7.5), 1 M NaCl, 10 mM EDTA (pH8.0)

2.1.6 Antibodies

Table 2.17 Primary antibodies for western blotting (WB) and immunofluorescence staining (IF)

Antibody	Origin	Application/Dilution	Source
N. gonorrhoeae	polyclonal rabbit	IF 1:100	US Biological N0600-02
$PorB_{1A}$	polyclonal mouse	WB 1:500	Our lab
AIF1 (NGFG_01605)	polyclonal rabbit	WB 1:500	This study, ImmunoGlobe
Hsp60	monoclonal mouse	WB 1:500	Santa Cruz sc-57840

Table 2.18 Secondary antibodies

Antibody	Origin	Application/Dilution	Source
ECL™ anti-mouse IgG HRP linked	Goat	WB 1:3000	Santa Cruz sc2005
ECL™ anti-rabbit IgG HRP-linked	Goat	WB 1:3000	Santa Cruz sc2004
Anti-rabbit IgG Cy2-linked	Goat	IF 1:100	Dianova
Anti-rabbit IgG Cy3-linked	Goat	IF 1:100	Dianova

2.1.7 Kits

Table 2.19 Kits

Kit	Manufacturer
Agilent High Sensitivity DNA Kit	Agilent Technologies
AxyPrep™ Plasmid Miniprep Kit	Axygen
EZ-Tn5™ <kan> insertion Kit	Epicentre Biotechnologies
GeneJet™ Gel Extraction Kit	Fermentas
NEBNext® dA-Tailing Module	New England BioLabs
NEBNext® End Repair Module	New England BioLabs
NucleoSpin Tissue Kit	Machery-Nagel
QIAquick Gel Extraction Kit	Qiagen
RevertAid™ First Strand cDNA Synthesis Kit	Fermentas

2.1.8 Chemicals

2.1.8.1 Antibiotics

Table 2.20 Final antibiotic concentrations used in this study

Antibiotics	*E. coli* (µg/mL)	*N. gonorrhoeae* (µg/mL)
Ampicillin	100	----
Chloramphenicol	30	15
Gentamicin	----	50
Kanamycin	50	40
Erythromycin	250	7
Tetracycline	----	10

Ampicillin and kanamycin were dissolved in H_2O and sterile-filtered. Chloramphenicol, erythromycin and tetracycline were dissolved in 100% ethanol.

2.1.8.2 Markers

Table 2.21 DNA and protein markers used in this study

Size standard	Application	Manufacturer
GeneRuler™ 100 bp plus DNA ladder	DNA agarose gels	Fermentas
GeneRuler™ 1 kb DNA ladder	DNA agarose gels	Fermentas
GeneRuler™ 50 bp DNA ladder	DNA agarose gels	Fermentas
O'GeneRuler™ 100 bp DNA ladder	DNA agarose gels	Fermentas
PageRuler™ Prestained Protein Ladder	Protein PAGE	Thermo Fisher Scientific

2.1.8.3 Enzymes

Table 2.22 Enzymes

Enzyme	Manufacturer
DNase I	Fermentas
Klenow Fragment	Fermentas
Phusion Polymerase	Thermo Fisher Scientific
Restriction enzymes	Fermentas
RNase A	Fermentas
T4 DNA ligase	Fermentas
T4 DNA Polymerase	Fermentas
Taq Polymerase	Genaxxon

2.1.8.4 Fine chemicals

Table 2.23 Fine chemicals

Chemicals	Supplier
Acrylamid Rotiphorese Gel 30 (37.5:1)	Roth
Ammonium persulfate (APS)	Merck
Bacto™ Proteose Peptone No. 3	BD
Bovine serum albumin (BSA)	Roth
Coomassie R250	Roth
GC Agar base	Oxoid
GlycoBlue	Ambion
Lipofectamine™ 2000	Invitrogen
PerfeCTa™ SYBR® Green FastMix™, ROX	Quanta Bioscience
Phalloidin 555	Invitrogen
Ready Lyse	Epicentre® Biozym
Roti®Aqua Phenol (pH 4.5-5)	Roth
Roti®Phenol/Chloroform/Isoamylalcohol (pH 7.5-8)	Roth
Saponin	Sigma
Sodium deoxycholate (DOC)	Merck
Soluble starch	Riedel-deHaen
Tetramethylethylenediamine (TEMED)	Fluka Analytical
Trichloroacetic acid (TCA)	Roth

All other chemicals were purchased from Roth, Sigma Aldrich, Serva or Merck Chemicals if not stated otherwise.

2.1.9 Technical equipment

Table 2.24 Technical equipment used in this study

Equipment	Supplier
Agilent 2100 Bioanalyzer	Agilent Technologies
Automated Colony Counter	New Brunswick Scientific
Avanti™ J-25T centrifuge	Beckman Coulter
Balance ABS-80-4	Kern
Balance EW 1500-2M	Kern
Binocular SMZ-168	Motic
C6 Flow Cytometer	Accuri
Centrifuge CT15RE	VWR

Chemiluminescence camera system	Intas
DMIL light microscope	Leica
Hera Cell 240i incubator	Thermo
Hera Safe sterile bench	Thermo
Magnetic stirrer RMO	Gerhardt
Megafuge 1.0R centrifuge	Heraeus
MicroPulser^{TM} Electroporation Apparatus	Biorad
NanoDrop 1000 spectrophotometer	Peqlab Biotechnology
Optima Max-xp Ultra centrifuger	Beckman Coulter
PerfectBlue Semi-Dry Elektroblotter	Peqlab Biotechnology
Plate reader infinite 200	TECAN
Scanjet G4010	HP
SenTix pH Electrode	WTW
Shaker TR125	Infors HT
Sonifier 250	Branson
Sonorex RK 255S	Bandelin
Step One Plus real-time PCR system	Applied Biosystems
TCS SPE confocal microscope	Leica
Thermal cycler 2720	Applied Biosystems
Thermal cycler GS1	G-STORM
Thermo mixer comfort	Eppendorf
Ultrospec 3100 pro Spectrophotometer	Amersham Bioscience
Vortex shaker Reax 2000	Heidolph

2.1.10 Software

Office 2010 (Microsoft), NCBI blast (http://blast.ncbi.nlm.nih.gov), ClustalX2 [164], CorelDraw X6 (Corel Corporation), ABI StepOne v2.3 (Applied Biosystems), DNAMAN 6.0 (Lynnon Corporation), LAS AF confocal microscopy software (Leica), ChemoStar Imager software (Intas), ImageJ (http://imagej.nih.gov), Notepad++ (http://notepad-plus-plus.org/), ApE v2.0 (A plasmid Editor by M. Wayne Davis), Artemis 14.0 (Wellcome Trust Sanger Institute), Integrated Genome Browser (IGB) v7.0.4 (BioViz), Vector NTI (Life Technologies), Agilent 2100 Bioanalyzer Expert Software (Agilent Technologies) and Primer3web (http://primer3.ut.ee/) and EndNote X7 (Thomson Reuters).

2.2 Methods

2.2.1 Bacterial culture methods

2.2.1.1 Cultivation of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae MS11 derivatives and transposon mutant library used in this study were grown on GC agar base plates supplemented with 1% vitamin mix at 37 ℃ in 5% CO_2 in a humidified incubator. *Neisseria* were grown on GC agar plates for no longer than 16–18 h to avoid *Neisseria* autolysis. The appropriate antibiotic (Table 2.20) was added to the GC agar plates for selection of antibiotic resistant *Neisseria* mutants. The piliation and opacity phenotypes were distinguished and selected by colony morphology under a stereo microscope [165].

2.2.1.2 Determination of *Neisseria gonorrhoeae* growth curves

Neisseria grown on GC agar plates for 16–18 h were inoculate in 10 ml pre-warmed (37 ℃) *Neisseria* growth medium at an optical density (OD_{550}) of 0.15. For equalization of the growth stages of different strains, the primary culture was grown at 190 rpm at 37 ℃ to an OD_{550} of 0.4–0.5. Then the pre-culture was inoculated in 20 ml *Neisseria* growth medium at an OD_{550} of 0.1. *Neisseria* were then shaken at 190 rpm at 37 ℃ and the OD_{550} was measured every 30 min for a total of 180 min.

2.2.1.3 Cultivation of *Escherichia coli*

Escherichia coli strains were cultured overnight on LB agar plates supplemented with appropriate antibiotics (Table 2.20) at 37 ℃ and 5% CO_2 in a humidified incubator. For plasmid DNA preparation or recombinant protein expression, *E. coli* strains were grown overnight in LB medium containing appropriate antibiotics under agitation on a rotary shaker (190 rpm) at 37 ℃.

2.2.1.4 Bacterial stocks

Neisseria strains grown on GC agar plate for 16–18 h were suspended in 1 mL PPM medium in a 2 mL cryo tube whereas for *E. coli* stocks, 1 ml of overnight culture was transferred to cryo tubes. The bacterial cultures were then mixed with 350 µL 100% glycerol to a final concentration of 25% (v/v) and stored at −80 ℃.

2.2.2 Transformation of bacteria

2.2.2.1 *Neisseria* transformation

Piliated gonococci are naturally competent and readily take up DNA fragments containing *Neisseria* DNA uptake sequence (DUS) [166,167]. For transformation, piliated gonococci were selected and grown on GC agar plate for 16–18 h. Bacteria were collected in pre-warmed *Neisseria* transformation medium. OD_{550} was determined. 5×10^6 bacteria were suspended in 50 µL *Neisseria* transformation medium and mixed with 10 ng PCR product by gentle pipetting. The mixture was dropped on a GC agar plate and incubated at 37 ℃ and 5% CO_2 for 24 h. The resulting colony was resuspended in PPM medium and plated on antibiotics-supplemented GC agar plates for selection (Table 2.20).

2.2.2.2 *Neisseria* conjugation

Neisseria strains were grown on GC plates for 16–18 h and collected in pre-warmed *Nesseria* growth medium. 10^7 donor and recipient *Neisseria* were mixed in 50 µL *Nesseria* growth medium and dropped on GC agar plate for incubation at 37 ℃ for 6–8 h until forming a visible colony. Colonies were collected in 1 mL PPM medium, and then serial dilutions were plated on selective GC plates and incubate for 24 h at 37 ℃.

2.2.2.3 Transformation of *E. coli*

Preparation of chemically competent *E. coli* DH5α

E. coli DH5α was inoculated in 4 mL LB medium and cultured at 37 ℃, 190 rpm overnight. The *E. coli* overnight culture was diluted 1:100 in 120 mL of LB medium and grown at 37 ℃ and 250 rpm to an OD_{600} of 0.4. Bacteria were incubated on ice for 15 min and split into pre-cooled 50 ml Polypropylene tubes (Greiner) and collected by centrifugation at 4000 rpm and 4 ℃ for 10 min. The bacterial pellet was washed with 10 mL of ice-cold 0.1 M $CaCl_2$ twice, and resuspended in 10 mL of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Subsequently, the bacteria were centrifuged at 4 ℃ at 4000 rpm for 5 min. The pellet was resuspended in 2 mL ice-cold 0.1 M $CaCl_2$ containing 20% (v/v) glycerol. 100 μL aliquots were stored at −80 ℃.

Transformation of chemically competent *E. coli*

An aliquot of chemo-competent *E. coli* strain was thawed on ice followed by addition of 0.68 μL β-mercaptoethanol and additional 10 min incubation on ice. DNA was added and incubated for 30 min on ice. The cells were heat-shocked for 90 s at 42 ℃, and incubated for 2 min on ice. Immediately 800 μL of pre-warmed SOC medium was added to the mixture and the bacteria were incubated for 1 h at 37 ℃ and 150 rpm. Bacteria were plated on selective LB agar plates supplemented with appropriate antibiotics (Table 2.20).

Preparation of electro-competent *E. coli* BL21

200 mL LB medium was inoculated with 1/100 volume of a fresh overnight *E. coli* culture and grown at 37 ℃ and 250 rpm to an OD_{600} of 0.5. The bacteria were chilled on ice for 20 min and harvested by centrifugation at 4000 g for 15 min at 4 ℃. Afterwards, the bacterial pellet was washed with 200 mL ice-cold 10% glycerol, followed by 80 mL ice-cold 10% glycerol and 20 mL ice-cold 10% glycerol. Finally, the bacterial pellet was resuspended in 2 mL ice-cold 10% glycerol and stored at −80 ℃ in aliquots of 100 μL.

Electroporation of electro-competent *E. coli*

An aliquot of electro-competent *E. coli* strain was thawed on ice, mixed with 5 µL DNA and incubated on ice for 1 min. The mixture was transferred to the bottom of the pre-cooled 0.2 cm electroporation cuvette. The electroporation setting “Ec2” of the MicroPulser™ Electroporation Apparatus was used. Immediately, 1 mL pre-warmed SOC medium (37 ℃) was added to the cuvette, the cells were gently resuspended, transferred to a sterile tube and incubated at 37 ℃ at 225 rpm for 1 h. Afterwards, the bacteria were plated on selective LB agar plates supplemented with appropriate antibiotics (Table 2.20).

2.2.3 Cell culture methods

2.2.3.1 Cultivation of cells

All cell lines used in this study were cultured in 75 cm² cell culture flasks at 37 ℃ and 5% CO_2, and passaged every two to three days before reaching 100% confluency. For passaging, the cells were washed with DPBS and incubated with 1 mL trypsin to detach the cells. After ~5 min incubation at 37 ℃, pre-warmed complete cell culture medium was added to the flask to stop trypsin digestion. The cell suspension was either seeded in multi-well plates for experiments or transferred to a new 75 cm² cell culture flask for further cultivation.

2.2.3.2 Cell stocks

Cells were grown in 75 cm² cell culture flasks to 80–90% confluency. The cells were washed with DPBS once and then detached by incubation in 1 mL trypsin at 37 ℃ for ~5 min. After that, 4 mL of complete cell culture medium was added and the cell suspension was transferred to 15 mL falcon tubes. The cells were pelleted by centrifugation at 600 g for 5 min at room temperature. The cell pellet was resuspended in 5 mL pre-cooled cell stocking medium and transferred into cryo tubes in 1 mL aliquots. Immediately the cryo tubes were stored at −80 ℃ in a cell freezing container to cool down gradually with rate of −1 ℃/min. For longer storage, the tubes were subsequently transferred to a liquid nitrogen tank.

2.2.4 DNA techniques

2.2.4.1 Isolation of *Neisseria* genomic DNA

Pelleted *Neisseria* was resuspended in 500 µL GTE buffer containing 200 µg/mL RNase A and 0.1% SDS and was incubated at 42 ℃ for 10 min until the solution was clear. The lysates were transferred to a Phase Lock Gel™ tube (5 PRIME GmbH) and mixed with one volume of phenol-chloroform (1:1). After shaking vigorously for 30 s, the mixture was centrifuged at 15,000 rpm for 5 min. The aqueous phase was transferred to a new Phase Lock Gel™ tube and phenol-chloroform extraction was repeated. The upper phase was then transferred to a new tube and DNA was precipitated by adding 2.5 volumes of cold 100% ethanol and 0.1 volumes of 3 M NaAc (pH 5.2) followed by 1 h incubation at −20 ℃. The DNA was pelleted by centrifugation at 15,000 rpm and 4 ℃ for 15 min and washed with 2 volumes of ice-cold 75% ethanol. After centrifugation (15,000 rpm, 4 ℃, 5 min) the pellet was air dried and dissolved in 100 µL distilled H_2O.

2.2.4.2 Construction of recombinant vector

To construct a recombinant vector in *E. coli*, the inserted DNA fragment was amplified from recombinant plasmid or *Neisseria* genomic DNA with PCR (2.2.4.3) and purified with an appropriate method (2.2.4.5). The amplified DNA fragments and the target vector were digested with appropriate restriction enzymes (2.2.4.6). After purification, the inserts and the vector were ligated at a suitable ratio at 16 ℃ overnight (2.2.4.6). Subsequently, the ligation reaction was transformed into competent *E. coli* DH5α or XL1-blue (2.2.2.3) and plated on appropriate selective agar plates. Bacterial colonies were verified by cPCR to contain the recombinant DNA (2.2.4.7). Afterwards, plasmids from cPCR-positive colonies were isolated (2.2.4.8) and the inserted DNA fragment was confirmed by sequencing (2.2.4.9).

2.2.4.3 Polymerase Chain Reaction (PCR)

A standard PCR reaction was performed in a 0.5 mL PCR tube with 50–100 ng template DNA, 10 nmol dNTP, 10 pmol forward and reverse primers and 1 U Taq polymerase or 1 U Phusion polymerase in a total volume of 50 µL and using the following temperature profile: 30 s at 98 ℃ for Phusion or at 95 ℃ for Taq, 30 cycles of 10 s at 98 ℃ for Phusion or at 95 ℃ for Taq, 20 s at 56–62 ℃ (depending on the melting temperature of the primer pairs) and at 72 ℃ for the time based on the length of the template [30 s/1 kb (Phusion); 1 min/1 kb (Taq)], and then 10 min at 72 ℃. PCR products were analyzed on agarose gels and, if required, purified using a PCR purification kit.

2.2.4.4 Agarose gel electrophoresis

DNA samples were mixed with 6× loading dye (Fermentas) and separated on agarose gels (in 1× TAE buffer or 0.5× TBE buffer) containing ethidium bromide (Roth) or Intas HD Green (Intas) by applying an electric field of 12 V/cm for about 45 min. DNA fragments shorter than 500 bp were separated on 2% agarose gels, longer fragments (500-3000 bp) on 1% agarose gels. DNA bands were visualized under UV light.

2.2.4.5 DNA purification

DNA fragments from PCR or DNA restriction enzymes digestion were separated on agarose gels, were excised with a scalpel under UV light and the DNA was recovered using the GeneJet^{TM} Gel Extraction Kit (Fermentas) according to the manufacture's protocol. The DNA was eluted with 30 µL dH_2O and stored at −20 ℃. The concentration of recovered DNA was quantified by a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnology).

2.2.4.6 Restriction and Ligation

Amplified DNA and target vector were digested with appropriate Type II restriction enzymes (Fermentas). If there was no optimal buffer or working temperature for two restriction enzymes,

digestions were conducted serially. Generally 1 μg DNA was digested with 1 U of the respective restriction enzyme in the buffer system suggested by the supplier for 2–4 h at 37 ℃. The digested DNA fragments and the linearized vectors were purified using GeneJet™ Gel Extraction Kit (Fermentas). The DNA fragments and linearized vectors were quantified by a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnology) for ligation.

In the ligation reaction mix, the optimal molar ratio of DNA fragments to linearized vectors was from 3:1 to 8:1 in a total volume of 20−30 μL. The ligation reaction was performed by T4 DNA ligase (Fermentas) at 16 ℃ overnight.

2.2.4.7 Colony Polymerase Chain Reaction (cPCR)

Colony PCR is a convenient high-throughput method for verifying recombinant plasmids directly from *E. coli* colonies or gene knockout mutants from *Neisseria* colonies. Each single colony was numbered and picked into 20 μL dH_2O. Then the bacteria cells were lysed by heating to 95 ℃ for 10 min followed by 5 min incubation on ice. The solution was spun down and 2 μL of the supernatant was used as the cPCR template. Following the schematic for a standard PCR reaction 80 μL reaction mixture was aliquoted into ten PCR tubes (8 μL/tube) and 2 μL templates was added. The program for cPCR was the same as the standard PCR program. cPCR products were analyzed by agarose gel electrophoresis.

2.2.4.8 Plasmid extraction from *E. coli*

Plasmids were isolated from bacterial overnight cultures using AxyPrep™ Plasmid Miniprep Kit (Axygen) or NucleoBond® PC 100 plasmid midiprep Kit (Macherey-Nagel) following the instructions of the manufacturer.

2.2.4.9 DNA sequencing and analysis

All sequencing reactions based on the dideoxy chain termination method according to Sanger [139] were performed by Seqlab Biotech. 15 μL sequencing sample containing either 1.2 μg plasmid or

22.5 ng/100 bp of PCR products was supplemented with 30 pmol sequencing primer. Sequencing data was analyzed by DNAMAN Version 6 (Lynnon Corporation).

2.2.4.10 DNA analysis by Agilent 2100 Bioanalyzer

To test the size range of DNA fragments and the amount of DNA fragments in each range, DNA fragments were measured on the Agilent 2100 Bioanalyzer using Agilent High Sensitivity DNA chip, suitable for separation and detection of DNA segments with size range of 50–7000 bp and quantitative range of 5–500 pg/μL. DNA concentrations were first measured with a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnology) and then diluted to a concentration of 5–500 pg/μL before measurement on the Bioanalyzer. The Agilent High Sensitivity DNA chip then was loaded with DNA gel matrix containing DNA dye. 5 μL of marker and 1 μL of DNA sample were added into each well, followed by electrophoresis and measurement according to the manufacturer's instructions.

2.2.4.11 Site-directed mutagenesis

In order to introduce the *Neisseria* DNA uptake sequence (DUS, 5'-atgccgtctgaa-3') into the Tn5 transposon, the Tn5 transposon was first inserted into pCR2.1-Topo (Invitrogen). The Tn5 transposon from EZ-Tn5^{TM} <kan> insertion kit (Epicentre Biotechnologies) was dA-tailed by NEB's dA-tailing module and subsequently cloned into pCR2.1-Topo (Invitrogen) according to the manufacturer's instructions. The DUS was introduced to BamHI site of Tn5 transposon by site-directed mutagenesis with the primers EZ-Tn5-Kan2-DUS-F and EZ-Tn5-Kan2-DUS-R. Briefly, 25 μL PCR reactions with single primer EZ-Tn5-Kan2-DUS-F or EZ-Tn5-Kan2-DUS-R was performed with Phusion Polymerase using the following temperature profile: 30 s at 98 ℃, followed by 5 cycles of 10 s at 98 ℃, 20 s at 50 ℃ and 2 min at 72 ℃, and 5 min at 72 ℃. Both reactions were combined and a second PCR was performed: 30 s at 98 ℃, followed by 18 cycles of 10 s at 98 ℃, 20 s at 60 ℃ and 2 min at 72 ℃, followed by 10 min at 72 ℃. The methylated and non-mutated parental DNA templates in the PCR products were digested with 20 U DpnI for 3 h at 37 ℃ followed by 20 min at 80 ℃ for DpnI denaturation. 5 μL of the resulting preparation

was transformed to chemically competent *E. coli* DH5α and the successful mutagenesis was checked by sequencing.

2.2.5 RNA techniques

2.2.5.1 Isolation of *Neisseria* total RNA

Pelleted bacteria (1 mL of OD_{550} = 1.5) were resuspended in 800 µL lysis buffer containing 1 µL/10 mL Ready Lyse. 80 µL 10% SDS was added to a final concentration of 1% (w/v) and was incubated at 64 ℃ for 2 min. The samples were supplemented with 88 µL (0.1 volumes) of 1 M NaAc (pH 5.2) and 1 mL (one volume) Roti®Aqua Phenol (pH 4.5–5) and were incubated at 64 ℃ for 6 min while inverting every 40 s. After chilling on ice for 2 min, samples were centrifuged at 21,000 g and 4 ℃ for 5 min. The aqueous layer was transferred to a 2 mL Phase Lock Gel™ tube and mixed with 1 mL (one volume) of chloroform and centrifuged at 21,000 g and 4 ℃ for 5 min. The aqueous phase was then transferred to two new 2 mL tubes and mixed with 1 µL GlycoBlue (Ambion), 40 µL (0.1 volumes) of 1 M NaAc (pH 5.2) and 1 mL (2.5 volumes) of 100% ice cold ethanol and incubated at −80 ℃ overnight. The RNA was pelleted by centrifugation at 21,000 g and 4 ℃ for 25 min and washed with 1 mL (2.5 volumes) of ice cold 80% ethanol. The RNA pellets were air-dried and resuspended in 15.5 µL RNase-free H_2O and treated with 2 µL (2 U) DNase I (Fermentas), 0.5 µL (20 U) RiboLock RNase Inhibitor (Thermo Fisher Scientific) and 2 µL 10× DNase I buffer for 30 min at 37 ℃. The solution was filled up with 100 µL H_2O and 120 µL (one volume) Roti®Phenol Chloroform Isoamylalcohol, mixed extensively and centrifuged at 13,000 g for 20 min at 4 ℃. The aqueous phase was transferred to a new 1.5 mL tube, mixed with 12 µL (0.1 volumes) of NaAc (pH 5.2) and 300 µL (2.5 volumes) of 100% ice cold ethanol and incubated for 2 h at −80 ℃, and then centrifuged at 13000 g and 4 ℃ for 30 min. The pellet was washed with 70% ice cold ethanol, air dried and resuspended in 50 µL RNase-free H_2O followed by incubation for 2 min at 56 ℃. The RNA quantity and quality was checked by measurements at the NanoDrop 1000 Spectrophotometer (Peqlab Biotechnology) or 1% agarose gel, respectively. The total RNA was directly used for first-strand cDNA synthesis or stored at −80 ℃.

2.2.5.2 First strand cDNA (complementary DNA) synthesis

First strand cDNA synthesis was performed according to the manufacturer's instructions from RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific). Briefly, 2 μg isolated RNA was added to 12 μL reaction mix containing 1 μL Random Hexamer primer, incubated at 65 ℃ for 5 min and chilled on ice. Then 20 U RiboLock RNase Inhibitor, 1 mM dNTP and 200 U RevertAid M-MuLV Reverse Transcriptase were added to the mixture and were incubated in a thermocycler with the following temperature profile: 5 min at 25 ℃, 60 min at 45 ℃ and 5 min at 70 ℃. Controls without the enzyme RevertAid M-MuLV Reverse Transcriptase were prepared simultaneously. The cDNA was diluted 1:10 and was used as template in RT-PCR (2.2.5.3) or qRT-PCR (2.2.5.4).

2.2.5.3 Reverse transcription PCR (RT-PCR)

To check the expression of the associated gene in the knockout strains, RT-PCR was performed. 1 μl of a 1:10 dilution of the synthesized cDNA (2.2.5.2) was added to the 50 μL PCR mixture containing 10 nmol dNTPs, 10 pmol of gene specific forward and reverse primers, and 1 U Phusion polymerase. PCR reactions were performed with an initial denaturation step at 98 ℃ for 30 s followed by 25 cycles of 98 ℃ for 20 s, 55 ℃ for 20 s and 72 ℃ for 15 s and a final incubation for 10 min at 72 ℃. The constitutively expressed RNase P gene was used as positive control. To check for contaminating genomic DNA within the cDNA sample, the reaction was repeated using the Reverse Transcriptase-mock treated control sample (2.2.5.2). PCR products were checked on 1.5% agarose gel supplemented with 5% HD Green DNA dye.

2.2.5.4 Quantitative real-time PCR (qRT-PCR)

To test transcription levels of some genes, qRT-PCR was performed according to the manufacturer's protocol using PerfeCTa® SYBR® Green FastMix®, Rox. Briefly, the synthesized cDNA (2.2.4.2) was diluted 1:10, of which 2 μL was used for 20 samples mixed with 98 μL dH_2O and 200 μL PerfeCTa® SYBR® Green FastMix®, Rox. Primers designed by Primer3web were diluted to a final concentration of 1 μM. 5 μL primers mix and 15 μL cDNA mix constitute final

amplification mix and were added in one well of 96 well PCR plate. Each sample was measured in triplicates. PCR reactions were performed in a Step ONE Plus real-time PCR apparatus with holding phase of 95 ℃ for 30 s and 40 cycles of 95 ℃ for 10 s and 60 ℃ for 1 min. This was followed by determination of melt curves by incubating at 95 ℃ for 15 s, 60 ℃ for 1 min, and a gradual temperature increase to 95.3 ℃ with a rate of 0.3 ℃/s followed by an incubation at 95.3 ℃ for 15 s. In order to normalize the amount of input cDNA for different samples, the constitutively expressed *5sRNA* was determined in each sample as internal standard. Resulting data were analyzed by StepOne v2.3.

2.2.6 Protein techniques

2.2.6.1 Expression of recombinant proteins in *E. coli*

The full length of NGFG_01605 with stop codon was amplified from *N. gonorrhoeae* MS11 genomic DNA with primers 01605-BclI-F and 01605-HindIII-R (Table 2.9) and cloned into pET28b at BamHI and HindIII restriction enzymes sites to express His-tag N-terminal fusion protein. The recombinant plasmid was transformed to *E. coli* SoluBL21strain and selected on LB agar plates with kanamycin. Single colonies were picked into 5 mL LB medium containing kanamycin and incubated overnight (200 rpm, 37 ℃). Overnight bacterial cultures were used to inoculate fresh kanamycin-containing LB medium at a dilution of 1:20 dilution and were incubated at 250 rpm at 37 ℃ until on OD_{600} of 0.4–0.6 was reached. Then the cultures were induced by addition of a final concentration of 0.25 mM IPTG and were incubated at 25 ℃ and 200 rpm for 4 h. Afterwards, the bacterial pellet was collected by centrifugation at 4,000 rpm and 4 ℃ for 15 min. The pellet was either used directly for purification or stored at −20 ℃.

2.2.6.2 Ni-NTA purification of recombinant proteins from *E. coli*

The bacteria pellets (2.2.6.1) were resuspended in 5.4 mL pre-cooled lysis buffer containing 1 mg/mL lysozyme and incubated on ice for 30 min. The bacterial suspension was sonicated on ice for 10 min (Branson Sonifier 250; 50% duty cycle, output 4), the lysate was transferred in 12 ml polypropylene tubes (Greiner bio-one) and centrifuged at 10,000 g and 4 ℃ for 25 min. The pellet

was suspended in Laemmli buffer, whereas the supernatant was used for Ni-NTA His•Bind Resin purification. 500 µL Ni-NTA beads were equilibrated with 5 mL lysis buffer and incubated with the supernatant at 4 ℃ for 1 h on a rotary mixer. A purification column was equilibrated once with lysis buffer, the Ni-NTA beads were loaded on the column and the residual liquid was drained by gravity flow. The flow through was collected. Then the column was washed three times with 5 mL washing buffer. Afterwards, 1 mL elution buffer was added to the column to elute the protein. Elution was repeated five times. The purified protein was mixed with 100% glycerol to a final concentration of 20% and was stored at −20 ℃.

2.2.6.3 Bradford assay

Concentration of proteins in solution was determined with the Bradford assay. A calibration curve was established by a series of BSA samples (0, 1.25, 2.5, 5.0 and 7.5 µg/mL respectively) in a final volume of 80 µL in a 96 well plate. 1 µL of the unknown protein samples were mixed with 79 µL H_2O. Every sample was prepared in duplicates. Then 20 µL of Bradford reagent was added to each well and mixed gently by pipetting without introducing air bubbles. The plate was incubated in the dark for 15 min and then analyzed by measuring the absorption at 595 nm in a plate reader (Tecan).

2.2.6.4 SDS-PAGE and Western blotting

Protein samples were resuspended in 2× Laemmli buffer and incubated at 95 ℃ for 5 min to denature the proteins. Then the samples were spun down and supernatants were loaded on the 10% SDS-polyacrylamide gels and electrophoresed at 12V/cm for 2 h.

For Coomassie staining, gels were incubated in Coomassie brilliant blue staining buffer at room temperature for 45 min and subsequently washed with destaining buffer until the protein bands appeared without background.

For Western blotting, gels were transfered on PVDF membranes using a semi-dry blotting chamber (Peqlab Biotechnology). Each PVDF membrane was activated by incubation in 100% methanol for 15 s. Then the transfer sandwich was assembled air bubble-free and contained (from

cathode to anode) 1 sheet of Whatman paper, the PVDF membrane, the polyacrylamide gel followed by 2 sheets of Whatman paper. Proteins were transferred at 0.8 mA/cm^2 for 2 h. Subsequently, the membrane was blocked for 1 h at room temperature in 1× TBST with 3% BSA. Blocking buffer was discarded and the membrane was then incubated overnight with diluted primary antibody in 3% BSA/ 1× TBST at 4 ℃, followed by three 10 min washing steps. The membrane was then treated with secondary antibody (usually diluted 1:3000 in 1× TBST containing 5% non-fat dry milk) at room temperature for 1 h. The membrane was washed three times with 1 x TBST for 10 min., Equal volumes of ECL solutions 1 and 2 (Thermo Fisher Scientific) were mixed and added to the membrane. Chemiluminescence was detected by an INTAS Imager digital system and proteins were quantified by ImageJ (http://imagej.nih.gov).

To reutilize the membranes for subsequent detections, the PVDF membrane was reactivated by incubation in 100% methanol for 5–10 s and washed with 1× TBST for 10 min for 3 times followed by incubation in stripping buffer (Thermo Fisher Scientific) for 15 min at room temperature. Afterwards, the membrane was washed three times with 1× TBST for 10 min and then blocked in 3% BSA in 1× TBST as described above.at room temperature for 1 h. The membrane was incubated with the new primary antibody.

2.2.6.5 Protein precipitation

In order to precipitate proteins from the supernatant, 1/100 volumes of 2% sodium deoxycholate (DOC) was added to the supernatant and the sample was incubated on ice for 30 min. Afterwards, 1/5 volumes of 72% trichloroacetic acid (TCA) was added to a final concentration of 14.4% and the sample was incubated at 4 ℃ overnight. Then, the sample was centrifuged at 15,000 g at 4 ℃ for 30 min and the resulting protein pellet was washed twice with one volume of ice-cold 100% acetone, and re-pelleted for 10 min by centrifugation at 15,000 g and 4 ℃. The pellet was air-dried and resuspended in a small volume of 2× Laemmli buffer for SDS-PAGE. If sample acidification by TCA resulted in a yellow color of the Laemmli buffer, 1 N NaOH or 1 M Tris/HCl pH 8.5 was used to neutralize the sample. Before SDS-PAGE analysis, the samples were mixed for 15 min at 65 ℃ and 650 rpm and spun down.

2.2.6.6 Isolation of bacterial inclusion bodies for immunization

To isolate inclusion bodies from *E. coli* protein expression strains, bacteria were harvested by centrifugation, the pellet was resuspended in 10 volumes of lysis buffer containing a final concentration of 0.5 mg/mL lysozyme and incubated on ice for 30 min. A final concentration of 0.2% Triton X-100 was added to the sample and again incubated on ice for 30 min. Afterwards, the bacterial suspension was lysed by sonication on ice with 3 pulses of 30 s (BRANSON SONIFIER 250; 50% duty cycle, output 5) and pelleted by centrifugation at 17,000 rpm at 4 ℃ for 30 min. The pellet was resuspended in 1 M urea in NTE buffer and the sonication and centrifugation was repeated. Then, the pellet was resuspended in 7 M urea in NTE buffer. After an additional sonication step, the suspension was centrifuged in an ultracentrifuge (Optima Max-xp Ultra centrifuger, MLA-80-Rotor, 16×64 mm centrifuge tube) at 80,000 rpm and 4 ℃ for 15 min. The supernatant contained the inclusion bodies. The concentration of proteins within purified inclusion bodies was measured by Bradford assay (2.2.6.3). Electro-elution of the purified inclusion bodies was performed to remove residual urea. Briefly, 3 mg of the purified inclusion bodies was separated in 8% SDS-PAGE and stained with Coomassie brilliant blue for 10 min followed by rinsing in destaining buffer for 20 s and three 10 min washes in 1 M Tris/HCl pH 7.5. The band was cut out of the gel with a clean scalpel. Immunization of rabbits was performed by ImmunoGlobe. Residual amounts of purified inclusion bodies were stored as aliquots at −20 ℃.

2.2.6.7 Antibody purification

Antibody was purified from rabbit serum by binding to and elution from immobilized antigen. First, an immobilized antigen column was generated by coupling of the recombinant protein (2.2.6.6) to cyanogen bromide-activated sepharose (CNBr; Sigma). 0.4 mg of recombinant protein used for immunization was re-buffered in 2 mL 0.2 M carbonate buffer pH 8.9 by a Vivaspin 6 column (10,000 MWCO, Sartorius Stedim Biotech GmbH) according to the manufacturer's instructions. 50 mg of CNBr beads (Sigma) were swelled in 10 mL of 1 mM HCl for 20 min, washed once with 0.2 M carbonate buffer and then immediately incubated with the recombinant protein at room temperature for 1 h and at 4 ℃ overnight. The beads were washed twice with 0.2 M carbonate buffer and then incubated in 100 mM ethanolamine for 1 h at room temperature to

block all the remaining coupling sites. Then the beads were washed three times with 0.2 M carbonate buffer and equilibrated with 500 mM NaCl in PBS. Next, the beads were incubated with 10 mL serum and 15 mL PBS in a 50 mL polypropylene tube (Falcon) at 4 ℃ overnight with slow rotation. The beads were harvested by centrifugation at 4,000 g and 4 ℃ for 10 min and washed two times with 30 mL 500 mM NaCl in PBS. Then the beads were transferred to a column and washed with 500 mM NaCl in PBS continuously until no protein was detected in the flow through. For that purpose 1 µL of the wash was spotted onto a piece of nitrocellulose membrane and was stained with 0.1% Ponceau red in acetic acid. Subsequently, 1 mL elution buffer (0.2 M acetic acid, pH 2.7, 500 mM NaCl) was applied to the column and the eluted antibody was collected in tubes containing 200 µL 1 M Tris/Base to neutralize the pH. The presence of eluted antibody was checked by Ponceau red staining of 1 µl elute. Eluted antibody was re-buffered in PBS and concentrated with a Vivaspin 6 column to a volume of 100 µL. Purified antibody was mixed with 87% glycerol to a final concentration of 45% and stored in aliquots at −80 ℃.

2.2.7 Transposon library construction

N. gonorrhoeae N2009 genomic DNA (gDNA) was extracted using the NucleoSpin Tissue Kit (Machery-Nagel). 0.5 µg gDNA was mutagenized *in vitro* with 0.12 pmol Tn5 transposon (EZ-Tn5^{TM} <KAN-2> Insertion Kit, Epicentre Biotechnologies) and purified by phenol extraction and ethanol precipitation. Gaps within the DNA were closed by 20 min incubation with 1 U T4 DNA polymerase (Fermentas) and 2 nmol dNTPs at 11 ℃ for 20 min followed by heat inactivation at 75 ℃ for 10 min. After phenol-chloroform extraction and ethanol precipitation, nicks in the mutagenized DNA were ligated by treatment with 5 U T4 DNA ligase (Fermentas) at 16 ℃ overnight. After precipitation, 0.1 µg mutagenized DNA was mixed with 50 µL *N. gonorrhoeae* N2009 suspension (OD_{550} = 0.32) and incubated for 24 h on GC agar plates. Resulting colonies were transferred to GC agar plates supplemented with kanamycin and were incubated for 48 h at 37 ℃. The colonies were harvested in PPM medium and stored at −80 ℃.

2.2.8 DNA sequencing sample preparation and Illumina sequencing

Recombinant *Neisseria* gDNA was isolated as described (2.2.4.1) and sheared by sonication (Bandelin Sonorex RK 255S) with 10 pulses of 60 seconds duration followed by pauses of 30 s. Sheared DNA was blunted and A-tailed by NEB's end repair and dA-tailing modules according to the manufacturer's instructions. Custom adapters were produced by annealing a final concentration of 90 µM "Adaptor sense" and "Adaptor antisense" oligonucleotides (Table 2.4) in 1x Oligo annealing buffer. The mixture was heated for 5 min at 94 ℃ and was allowed to cool to room temperature over 1 h. 0.4 nmol of adaptors were ligated to 0.5 µg A-tailed DNA with T4 DNA ligase (Fermentas) at 16 ℃ overnight. The ligation products in a range of 250–400 bp were size-selected by gel extraction using QIAquick Gel Extraction Kit (Qiagen).

Enrichment of DNA fragments containing parts of the transposon was performed by PCR with primers complementary to the adaptor and to the transposon mosaic end sequence ("Antisense Input" and "P5-ME"; Table 2.4). First, a PCR was performed with 4 nmol dNTPs, 0.05 µg DNA, 4 pmol P5-ME primer, 2% DMSO, 0.4 U Phusion polymerase (Thermo Fisher Scientific) in a total volume of 20 µL and using the following temperature profile: 30 s at 98 ℃, followed by 10 cycles of 10 s at 98 ℃, 20 s at 45 ℃ and 30 s at 72 ℃, and 10 min at 72 ℃. In a second PCR, the 30 µL reaction volume contained 10 pmol of each primer "P5-ME" and "Antisense Input", 6 nmol dNTPs, 2% DMSO, and 0.6 U Phusion. The following temperature profile was used: 30 s at 98 ℃, followed by 10 cycles of 10 s at 98 ℃, 20 s at 50 ℃ and 30 s at 72 ℃ and then 10 min at 72 ℃, followed by 30 s at 98 ℃, 18 cycles of 10 s at 98 ℃, 20 s at 55 ℃ and 30 s at 72 ℃ and a final incubation of 10 min at 72 ℃. PCR products of 250–300 bp were size-selected and gel purified prior to sequencing.

Illumina sequencing was performed at the Max Planck Genome Centre Köln by Dr. Bruno Huettel and Dr. Richard Reinhardt. DNA was sequenced on an Illumina HiSeq 2000 sequencer using 101 bp sequence cycles with a sequence primer that binds to the transposon mosaic end (TnSeq; Table 2.4). The library specific barcode was sequenced by "TnSeq index SP", which binds to the adaptor sequence next to barcode (Table 2.4).

2.2.9 Conditional knockout analysis

Conditional knockout assays [168-170] were performed to validate a subset of candidate essential genes. For that purpose we exchanged the promoter of each candidate gene with the isopropyl-D-thiogalactopyranoside (IPTG)-inducible P_{trc} promoter [171] flanked by a kanamycin cassette. P_{trc} originated from a Hermes-10 vector [171], was PCR-amplified with primers Ptrc-F and Ptrc-R and was subsequently cloned into pGEM-T (Promega) thereby yielding pGEM-T-Ptrc. The kanamycin cassette containing a *Neisseria* DNA uptake sequence (DUS; 5'-atgccgtctgaa-3') [166,167] was amplified from pCR2.1-Tn5-DUS (DUS was introduced in a BamHI site of the Tn5 kanamycin cassette by site-directed mutagenesis; see section 2.2.4.11) using oligonucleotides kan-SpeI-F and kan-SacI-R. The PCR fragment was restricted with the endonucleases SpeI and SacI and was inserted in the accordingly restricted pGEM-T-Ptrc resulting in pGEM-T-kan-Ptrc. The kan-Ptrc cassette was amplified from pGEM-T-kan-Ptrc by the primers Ptrc-R and kan-cassette-R. Approximately 500 bp long regions upstream and downstream of the targeted promoter were combined with the kan-Ptrc cassette via fusion PCR. The oligonucleotides rib-up-f and rib-up-r as well as rib-down-f and rib-down-r were used for amplification of the upstream and downstream region, respectively (Table 2.5). The resulting PCR fragment was purified and used in transformation of *N. gonorrhoeae* MS11. Bacteria were plated on selective GC-plates containing with 40 μg/mL kanamycin. Successful promoter replacement was checked by amplifying the genomic region via PCR and sequencing of the respective PCR products. Subsequently, the mutants were conjugated with *N. gonorrhoeae* N220, a strain which encodes lac^q on the plasmid pTH10a (our unpublished results). Conjugants were selected on the GC-plates containing 40 μg/mL kanamycin, 7 μg/mL erythromycin and 0.5 mM IPTG. Essentiality of the gene was tested by comparing bacterial growth in presence and absence of IPTG in GC-plates.

2.2.10 Genetic footprinting

Genetic footprinting on *Neisseria* transposed DNA fragments from *in vitro* and *in vivo* libraries was performed to validate a subset of the candidate identified essential genes as described before [146,172-174]. First, the predicted essential gene regions were amplified from chromosomal DNA

of *N. gonorrhoeae* N2009 by PCR. Purified PCR products were transposed *in vitro* with the EZ-Tn5 transposon and the gaps in transposed products were repaired as described above (chapter 2.2.7). An aliquot of *in vitro* transposed DNA was used as control for PCR-based footprinting. Then, transposed DNA was transformed into *N. gonorrhoeae* N2009 and the mutants were selected on GC agar plates supplemented with kanamycin (GC-kan). The mutants were collected in PPM medium supplemented with 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$, and incubate with 1 U/mL DNase I (Fermentas) at 37 ℃ for 30 min to remove remaining extracellular DNA. Subsequently, the mutants were passaged to a new GC-kan plate. After several passages on these selective plates, genomic DNA of the mutant pool was isolated by phenol-chloroform extraction and ethanol precipitation as described above (chapter 2.2.4.1). PCR-based genetic footprinting was carried out as described [146,172-174] by using a transposon-specific primer (Tn ME sequence; Table 2.6) and primers specific to each chromosomal region (Table 2.6). PCR reactions consisted of 200 ng transposed DNA fragments from *in vitro* or *in vivo*, 50 pmol of each primer, 10 nmol dNTPs, 2.5 U Taq DNA polymerase (Genaxxon), and 0.4 U Phusion DNA polymerase in 1× buffer S in a 50 µL reaction. The PCR program was as follows: 30 sec at 95 ℃; 30 cycles of 94 ℃ for 30 sec, 58 ℃ for 30 sec and 68 ℃ for 30 sec + 10 sec per cycle. PCR products were analyzed by gel electrophoresis on 1% agarose gel.

2.2.11 Screening for virulence factors

Glycerol stocks of the mutant library were recovered by growth on GC agar plates for 16–18 hours. 4×10^7 Chang cell were seeded in 6-well cell culture plates and grown to a confluency of 80–90% for infection. Chang cells were washed with HEPES medium twice and incubated in HEPES medium for 30 min before infection. Bacteria were collected in warm HEPES medium and added to the cells at an MOI of 100. After centrifugation for 3 min at 600 g, the infected cells were incubated for 1 h. To select for adherent and invasive cells (“output I library”), infected host cells were washed with HEPES medium three times and were lysed by treatment with 1% saponin for 15 min and subsequent plating on GC agar plates. To select for invasive bacteria (“output II library”), infected host cells were incubated with 100 µg/mL gentamicin for 2 h prior to saponin lysis and plating. After 16–20 h, the selected mutants were collected from the agar plates,

resuspended in PPM medium and aliquots were prepared as glycerol stocks. Another aliquot of each library was washed with HEPES medium and used in another round of infection. In total, three subsequent infections were performed with each library.

2.2.12 Quantification of total cell-associated and intracellular colony forming units

Chang cells were grown in 24-well cell culture plates to 80–90% confluency and infected with gonococcal strains at an MOI of 50 in HEPES medium for 30 min. For quantification of total cell-associated colony forming units (CFU), the monolayers were washed with HEPES medium to remove the non-adherent bacteria and then lysed by incubated with 1% saponin for 7 min. Serial dilution of the lysates were plated on GC agar plates and the CFU were determined after 24 h incubation at 37 ℃ and 5% CO_2. To quantify the intracellular viable bacteria, the infected monolayers were incubated with 50 µg/mL gentamicin for 2 hours prior to lysing in 1% saponin and plating. Gentamicin protection assay were performed at least three times in duplicate.

2.2.13 Construction of gene knockout mutants in *Neisseria*

Approximately 500 bp long regions upstream and downstream of the targeted gene were PCR-amplified from *Neisseria* genomic DNA (primers are listed in Table 2.7) and combined with a kanamycin cassette via overlap PCR (Fig. 2-1). The kanamycin cassette was amplified from Tn5 transposon (EZ-Tn5^{TM} <kan> insertion kit, Epicentre Biotechnologies) with the primers op_kan_s and op_kan_hfq-mut_as (Table 2.7). The overlap PCR fragment was purified and transformed into the wild type strain *N. gonorrhoeae* N2009. After homologous recombination, bacteria were selected for successful homologous recombination events on GC plates containing 40 µg/mL kanamycin. The gene deletion was verified by amplifying the genomic region via PCR and by sequencing of the respective PCR products.

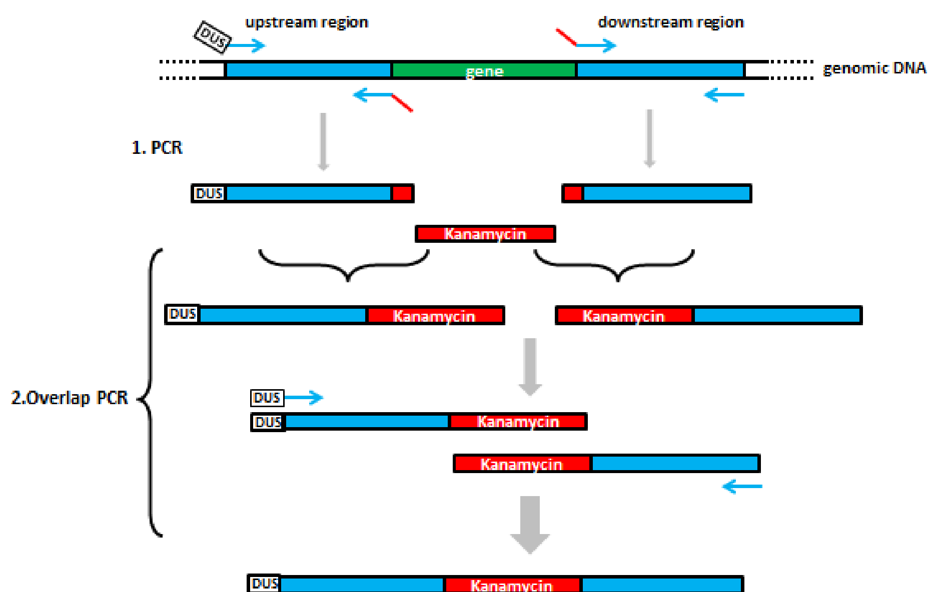

Fig. 2-1 Overview of the overlap PCR procedure for targeted gene knock-out in *N. gonorrhoeae*. PCR-amplified upstream and downstream homology regions of the interested gene are combined with a kanamycin cassette. The combined fragments are assembled and amplified via overlap PCR.

As shown in Fig 2-1, the upstream and downstream regions and the kanamycin resistance cassette were amplified separately. 50 µL reaction mixture contained 10 nmol dNTP, 10 pmol forward and reverse primers, 50–200 ng *Neisseria* genomic DNA or Tn5 transposon and 1 U Phusion Polymerase. The program started with an initial step at 98 ℃ for 30 sec, followed by 30 cycles of 10 sec at 98 ℃, 15 sec at 55–65 ℃ (depends on the melting temperature of each paired primers) and 20 sec at 72 ℃, and ended with an final step at 72 ℃ for 10 min. The PCR products were purified prior to fusion PCR. The upstream or downstream region was assembled with the kanamycin cassette by 10 PCR cycles without primers. The reaction mix of 20 µL was composed of 4 nmol dNTP, 25–100 ng upstream or downstream region, an equal molarity of kanamycin cassette, 0.4 U Phusion Polymerase and 0.5 U Taq Polymerase. The program had an initial step at 98 ℃ for 30 s, 10 cycles at 98 ℃ for 10 s, 50 ℃ for 20 s and 72 ℃ for 20 s and a final step at 72 ℃ for 10 min. Then the two reactions were mixed, aliquoted into two PCR vials and the PCR program was repeated. Then the assembled whole fragment was amplified by PCR by adding 30 µL reaction mixture containing 10 pmol "upstream forward" primer and "downstream reverse" primer, 6 nmol dNTP, 0.6 U Phusion Polymerase and 0.5 U Taq Polymerase. PCR reactions were performed with an initial denaturation step at 98 ℃ for 30 s, 20 cycles at 98 ℃ for 20 s, 60 ℃ for

20 s and 72 ℃ for 1 min and a final elongation step at 72 ℃ for 10 min. The fusion PCR products were separated on 1% agarose gel and gel purified. The products were verified by sequencing (with “upstream forward” and “downstream reverse” primers, respectively) and the fusion fragments were transformed into *Neisseria*.

2.2.14 Differential immunofluorescence staining

Chang cells were grown on round glass coverslips (12 mm diameter, VWR) in 12 well cell culture plates to 60–70% confluency and infected with *Neisseria* strains in HEPES medium at an MOI of 10 for 30 min. After several washes with HEPES medium, the cells were fixed with 4% Paraformaldehyd (PFA) for 15 min at room temperature. After washing with phosphate-buffered saline (PBS) unspecific antibody-binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. To stain the extracellular bacteria, the coverslips were incubated with a polyclonal rabbit anti-*N. gonorrhoeae* antibody (1:100 in 1% BSA, US Biological NO600-02) for 1 h, washed with PBS and incubated with a Cy2 conjugated goat anti-rabbit immunoglobulin G (1:100 in 1% BSA, Dianova) for 1 h. After extensive washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. Detection of extra- and intracellular bacteria was performed as described above, with the exception that a Cy5-conjugated goat anti-rabbit immunoglobulin G (1:100 in 1% BSA, Dianova) was used for labeling. To stain host cell actin, cells were incubated with Alexa 555-conjugated phalloidin (1:70 in PBS; Invitrogen) for 20 min. Subsequently, the coverslips were washed with PBS, mounted on glass slides with Mowiol (Roth) and analyzed by confocal fluorescence microscopy on a Leica TCS SPE (Leica) using a 63x oil immersion objective (numerical aperture 1.4). Lasers used for detection of Cy2, Cy5 and Alexa 555 were with the excitation wavelengths of 488 nm, 532 nm and 635 nm. Band-pass filters were set 520-550 nm, 570-600 nm and 660-710 nm respectively.

2.2.15 GP96 knock-down in Chang by RNAi

Chang cells were grown to 60–70% confluency in 12 well cell culture plates. Before transfection, the medium was changed to Opti-MEM® media. The transfection mix was prepared with 100 μL Opti-MEM® media, 25 nM siRNA (siluci or siGP96) and 3 μL HiPerFect transfection reagent

(Qiagen) in a sterile Eppendorf tube. The sample was mixed by vortexing and incubated at room temperature for 5–10 min to allow the formation of transfection complexes. The transfection complexes were added to cells dropwise and were distributed by gently swirling the plates. The transfected cells were sub-cultured after 24 h and transfection efficiency was analyzed by collecting cells at 24 h, 48 h and 72 h post transfection in order to determine the appropriate time of gene silencing.

3 Results

3.1 Construction and sequencing of a transposon mutant library in *Neisseria gonorrhoeae*

3.1.1 Construction of a high-density transposon mutant library in *N. gonorrhoeae*

With completion of the gonococcal genome sequence (Supplementary Table 6.1) [175,176], the study of *Neisseria gonorrhoeae* has entered the post genomic era. Many of the sequenced and predicted genes are still of unknown function and thus genome wide strategies are required to aid in annotation of the genome to identify novel drug and vaccine targets as well as virulence factors in the pathogen. In this study, a genome-wide high-density random transposon mutagenesis library was successfully established in *N. gonorrhoeae* strain MS11 with Tn5 transposon containing a kanamycin resistance marker (Fig. 3-1A).

Two steps, transposition and transformation, are critical for construction of a high density transposon mutant library. Since efficient transformation of *N. gonorrhoeae* depends on the presence of a DNA uptake sequence (DUS), an attempt was undertaken to introduce a synthetic DUS into a modified Tn5 transposon (chapter 2.2.4.11). However, usage of the so-called Tn5-DUS did not significantly increase the frequency of insertion events (data not shown). Further it was observed that DNA isolation by phenol extraction (chapter 2.2.4.1) strongly inhibited the *in vitro* transposition. In order to obtain high-quality chromosomal DNA without phenol contamination, *Neisseria* chromosomal DNA was isolated from MS11 derivative strain N2009 by a silica column-based method (chapter 2.2.7). Subsequently *Neisseria* genomic DNA was subjected *in vitro* to Tn5 transposon mutagenesis.

Since *in vitro* transposition of Tn5 leaves 9 bp gaps flanking each side of the inserted transposon, the gaps were filled and covalently closed by treatment with T4 DNA polymerase and T4 DNA ligase, respectively. Mutagenized DNA was transformed into *N. gonorrhoeae* N2009 and

recombinant bacteria were selected by kanamycin. A single reaction thereby produced about 20,000 kanamycin-resistant colonies. After a total of six independent repetitions, more than 100,000 individual colonies were obtained and subsequently pooled.

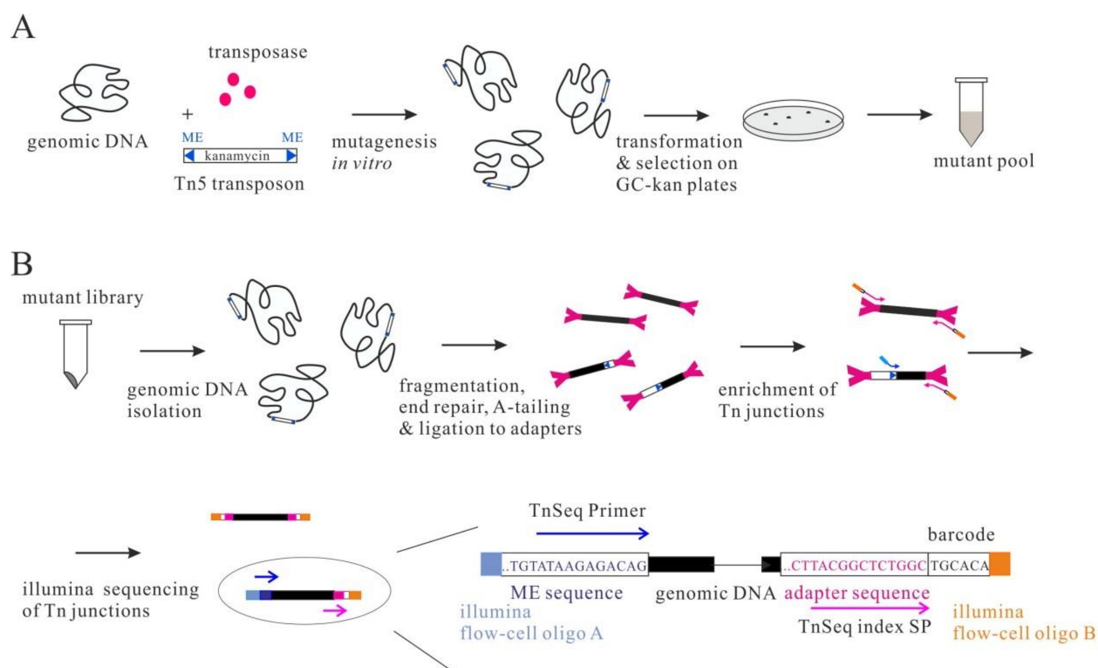

Fig. 3-1 Generation and sequencing of a transposon mutant library.
(A) Construction of a genome-wide random transposon mutant library. *Neisseria* genomic DNA is mutagenized *in vitro* by Tn5 transposon containing a kanamycin resistance marker, and then transformed naturally into a *Neisseria* population. The bacteria are selected on GC agar plates with kanamycin and subsequently pooled.
(B) Preparation of Illumina sequencing sample. Genomic DNA from the pool is extracted, fragmented and ligated to Illumina PE adaptors. Transposon junctions are amplified with the primers complementary to transposon mosaic ends (ME) and adaptors. The DNA fragments carrying terminuses (orange, blue) compatible to the Illumina flow-cell are amplified on a cluster station and then sequenced specifically with the sequencing primer (TnSeq Primer) that binds to transposon mosaic ends and the library specific barcodes are sequenced by TnSeq index SP.

3.1.2 Sequencing the transposon mutant library

To identify transposon insertion sites (TIS) in the mutant library, the DNA sequences adjacent to TIS were amplified and sequenced by Illumina sequencing (Fig. 3-1B). *Neisseria* genomic DNA from the mutant pool (chapter 2.2.4.1) was extracted and sheared by a water bath sonication to achieve a fragment size distribution of 300–500 bp. The electrophoresis of fragmented *Neisseria* genomic DNA is depicted in Fig. 3-2A (Lane 2, 4 and 6). The results were further confirmed by

measurement with an Agilent 2100 Bioanalyzer (Fig. 3-3A). The sheared DNA was blunted and A-overhangs were added prior to the ligation of Illumina PE adaptors (chapter 2.2.8). The adaptors contained a T-overhang and non-complementary regions resulting in a Y-shaped conformation to avoid self-ligation of the adaptors. Selective amplification of the DNA sequences adjacent to transposon insertion sites was performed by PCR with primers complementary to the adaptor and to the transposon mosaic end sequence (P5-ME and Antisense input, Table 2.4). In order to test the specificity of the amplification, control reactions were performed containing either only the adaptor-specific primer or only the transposon-specific primer. However, non-specific amplification products were not observed (Fig. 3-2B). Further, PCR products were cloned into pCR2.1-TOPO vector by TA cloning. The resulting plasmids were verified by EcoRI digestion and subsequently the inserts from the recombinant plasmids were sequenced by Sanger sequencing (SeqLab). The sequencing results indicated that the inserts were indeed *Neisseria* genomic DNA sequence containing a transposon mosaic end sequence on one end (data not shown). PCR products were further analyzed by agarose gel electrophoresis (Fig. 3-2B) and on an Agilent 2100 Bioanalyzer (Fig. 3-3B). The gels and electropherograms demonstrated that most DNA fragments were in the size range from 200 to 400 bp. The PCR products were size-selected by gel extraction and the fragments between 250 and 300 bp were purified and sent for sequencing on an Illumina HiSeq 2000 next-generation sequencing platform (Max Planck genome Centre, Cologne) using a custom sequencing primer that binds to the transposon mosaic end. Illumina sequencing cluster generation was enabled by addition of sequences to the PCR primers that were complementary to two specific capture oligonucleotides on the Illumina flow cell. The library specific barcodes were sequenced by TnSeq index SP thus enabling multiplexing of the libraries.[1]

[1] Dr. Richard Reinhardt and his colleagues (Max Planck Genome Centre, Köln) performed the Illumina sequencing and preliminary quality control of the sequencing reads (Fig. 3-4).

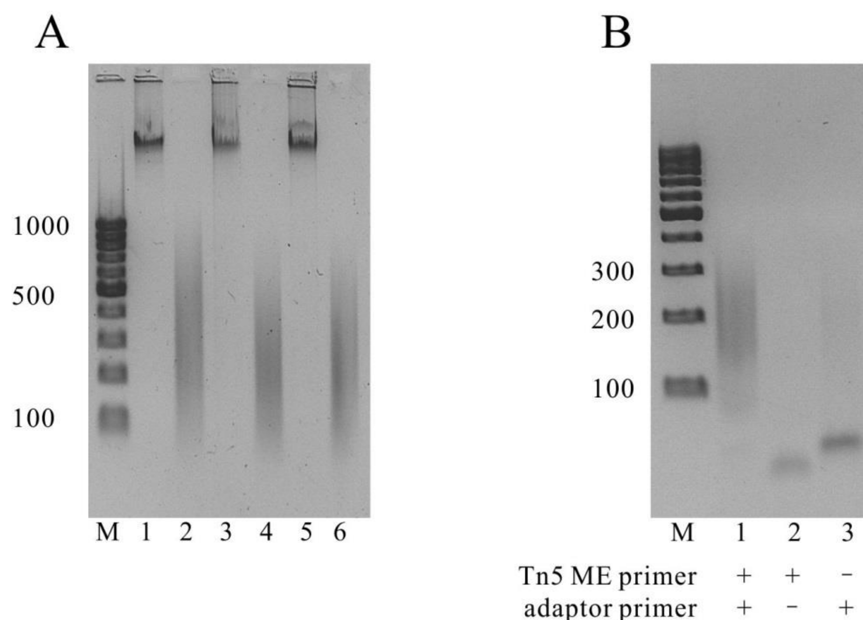

Fig. 3-2 Quality of DNA fragments obtained in the process of sequencing sample preparation was tested by agarose gel electrophoresis.

(A) Electropherogram of isolated *Neisseria* genomic DNA before and after sonication. Lane 1, 3 and 5 indicate complete *Neisseria* genomic DNA. Lane 2, 4 and 6 indicate the results of fragmentation of *Neisseria* genomic DNA after sonication.

(B) Enrichment of transposon-chromosomal junctions was performed by PCR amplification with primers complementary to transposon mosaic ends (ME) sequence and adaptors sequence (Lane 1). Lane 2 indicates the PCR amplification products only with transposon-specific primer. Lane 3 indicates the PCR amplification products only with adaptor-specific primer. M: 100 bp DNA ladder.

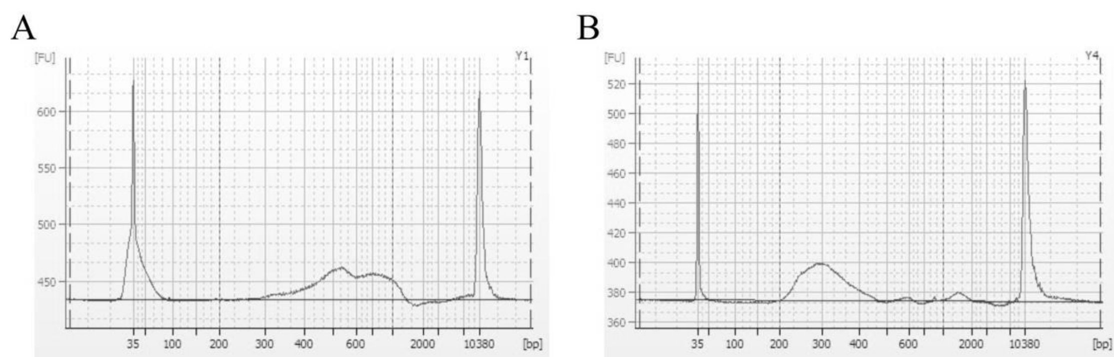

Fig. 3-3 Size range and the amount of the DNA fragments measured by Agilent 2100 Bioanalyzer. The X-axis represents the product size in bp and the Y-axis is the arbitrary fluorescence intensity in fluorescence unit (FU). The DNA markers present are the lower marker at 35 bp (left peak) and the upper marker at 10,380 bp (right peak). (A) DNA fragmentation. (B) PCR enrichment of transposon-chromosomal junctions.

3.2 Analysis of sequencing data

A single sequencing run yielded more than 30 million raw sequencing reads. Firstly, the quality of the sequencing reads was checked by the software Fast QC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The evaluation report indicated the library was random and diverse (Fig. 3-4). For example, 101 bases sequencing reads were obtained with 94 bases high quality and the last 7 bases good quality (Fig. 3-4A). It indicated the quality of each base in the sequencing reads was credible. The percent of G, A, T and C appeared in each base of the reads should be nearly 25%. The report of Per Base Sequence Content (Fig. 3-4B) indicated the percent of G, A, T and C appeared in most bases of the reads except the first 9 bases was nearly the same, about 25%. The first 9 bases were the duplication sequences caused by transposition, which indicated the position of a transposon insertion site. The analysis report showed there may be insertion preference of Tn5 or bad sequencing quality at the beginning of the sequencing. Another evaluation index is the GC distribution over all sequences. The GC count per read in red nearly coincided with the theoretical distribution shown in Fig. 3-4C.

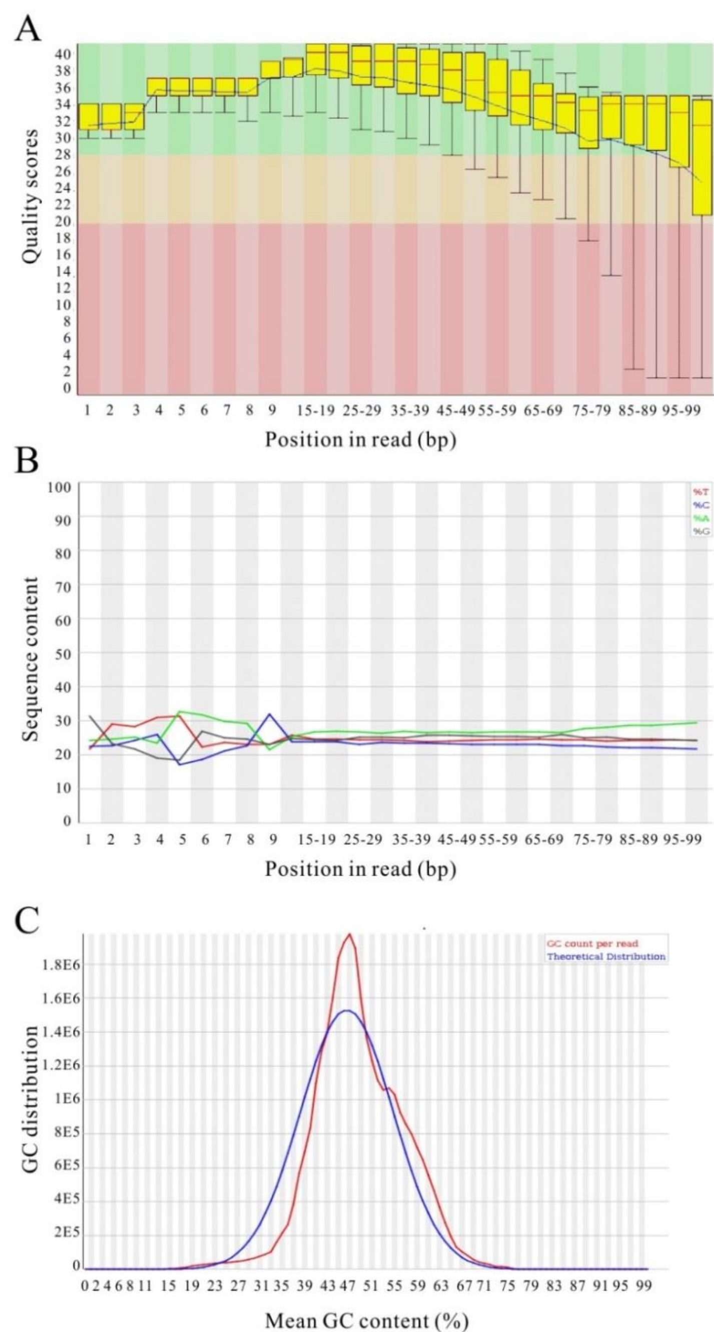

Fig. 3-4 Quality control of the sequencing reads made by Fast QC[2].

(A) Quality scores across all bases. The X-axis represents the position of the base in the sequencing read. The Y-axis represents the quality of the base in −10*lg (p), p: measuring error probability. The green background corresponds to high quality reads, the yellow background to intermediate quality reads and the red background to poor quality reads. Most bases in the reads (93%) were in high quality.

(B) Sequence content across all bases. The X-axis represents the position of the base in the sequencing read. The Y-axis represents the percent of G, A, T and C appeared.

(C) GC distribution over all sequence. The red line is GC count per read and the blue is the theoretical distribution.

[2] Quality of the sequencing reads was analyzed by Dr. Richard Reinhardt and his colleagues.

All these indexes indicated the sequencing were of high quality and thus were used for mapping to the *Neisseria* genome[3]. For mapping, the primer and adaptor sequences were firstly trimmed from the original sequencing reads and all remaining sequences longer than 12 bp (more than 97%) were mapped on the ring chromosome (2,233,640 bp) and the plasmid (4,153 bp) of *N. gonorrhoeae* MS11 version 4 (*Neisseria gonorrhoeae* group Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org/) using Bowtie 2.0.2 [177]. The mapping demonstrated 87,812 unique transposon insertion sites distributed across the whole genome (Table 3.1, Fig. 3-5A). This indicated that almost all the genome sequence contained a very high density of transposon insertions with an average of one insertion every 25 bp. There was no difference in TIS percentages between coding sequences (CDS) and intergenic regions, demonstrating that the TIS distribution was random without a bias for either coding or intergenic regions (Fig. 3-5B). Also there was no bias in the TIS positions within the CDS (Fig. 3-5C). In order to validate the reproducibility of sequencing, genomic DNA was isolated from the same library and two additional sequencing samples were prepared. The subsequent two sequence runs yielded 84,335 (Library 2 or 438_A) and 86,327 (Library 3 or 438_B) transposon insertion sites which were identified on the *N. gonorrhoeae* MS11 ring chromosome. Since reproducibility among the technical replicates was very high (Pearson correlation coefficient p=0.994; p-value < 2.2e-16), library 2 was chosen for further analysis.

Table 3.1 Overview of sequencing results

	Library 1	Library 2 (438_A)	Library 3 (438_B)
Sequencing reads	31,771,224 [a]	54,346,653	30,541,365
Processed [b]	30,888,325 (97%)	40,538,502 (75%)	19,480,906 (64%)
Mapped [c]	11,293,160 (37%)	37,835,517 (93%)	17,708,811 (91%)
Unique TIS [d]	87,812	125,666	129,055
Intragenic TIS	66,509	100,791	102,566

[a] Including internal control sequences from Enterobacteria phage φX174
[b] Passing primer/adaptor trimming (length ≥ 12 bp)
[c] Mapping to new assembly of MS 11 genome sequence and its cryptic plasmid
[d] Unique transposon insertion site (TIS) on the *Neisseria* chromosome and plasmid

[3] Christian Remmele performed raw data processing and mapping.

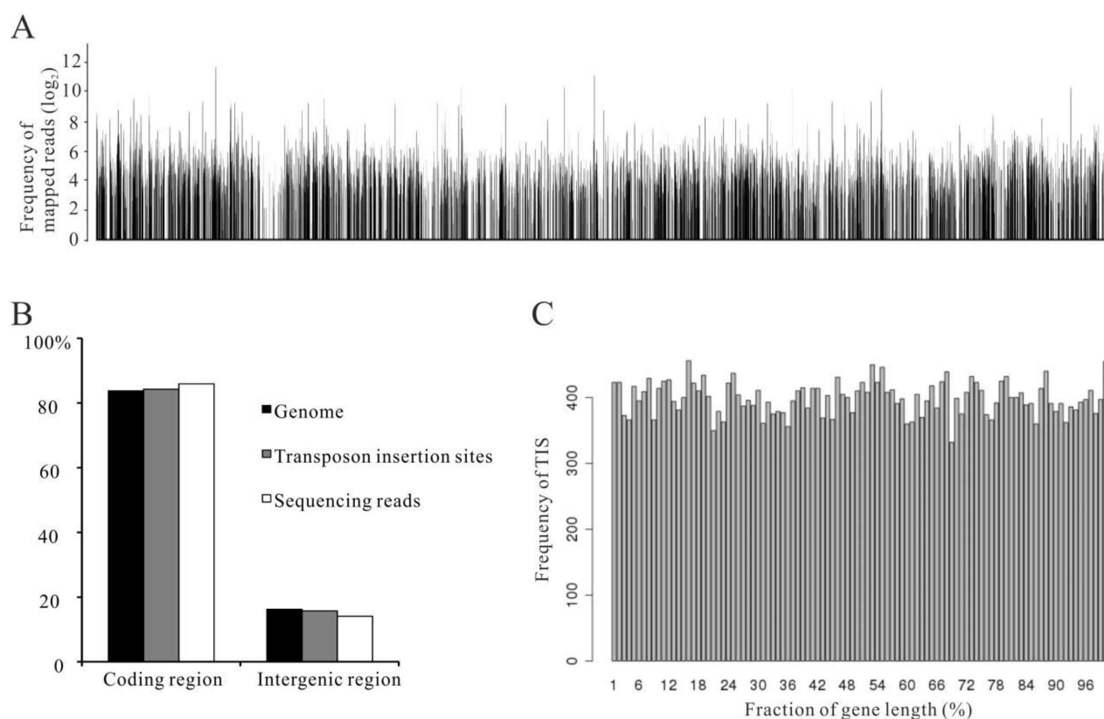

Fig. 3-5 Tn-seq mapping results show that the distribution of Tn5 insertions is random, unbiased, and of high density[4].

(A) Distribution of transposon insertion sites (TIS) across the whole genome of MS11. The X-axis represents the whole genome of MS11 and the Y-axis represents the number of mapped sequencing reads in $\log_2$ scale.

(B) Proportion of the counts of the sequencing reads and the TIS demonstrate that there is no insertional bias between the coding regions or intergenic regions in the *Neisseria* genome.

(C) Positional distribution of TIS in CDS normalized by gene length demonstrates that there is no bias in the position of TIS within coding regions. The full gene length is regarded as 100% (X-axis) whereas the Y-axis represents the number of TIS identified at the relative position within the gene.

3.3 Identification of *Neisseria* essential genes

For the 2526 genes encoded by the *N. gonorrhoeae* MS11 genome (including 350 newly annotated genes)[5], the counts of the sequencing reads and the number of separate transposon insertion sites (TIS) per gene are shown in Table S1. The number of TIS per gene varied between 0 and 506, with a median of 31. Up to 81.9% (2069 genes) of the genes have less than 50 TIS and very few

[4] Tn-seq mapping was done by Christian Remmele.

[5] The preliminary annotation of the *N. gonorrhoeae* MS11 genome (version 4) consists of 2185 CDS (2176 CDS on the chromosome and 9 CDS on the plasmid) (*Neisseria gonorrhoeae* group Sequencing Project, Broad Institute of Harvard and MIT). Christian W. Remmele et al augmented the MS11 genome annotation. 350 CDS on the chromosome and 5 CDS on the plasmid were new annotated (Table S1).

genes contain more than 150 TIS (Fig. 3-6A). The mutants with TIS in essential gene are not viable and missing in transposon mutant libraries, so these TIS located in the essential genes cannot be detected from Tn-seq data. Theoretically, genes without TIS or with strongly depleted TIS were considered to be essential for *Neisseria* survival and growth. However, TIS were detected in nearly all the genes with different frequencies which can be evaluated only with the statistical analysis. P-value was assigned to assess gene essentiality which assume uniform transposon insertion rates across the whole genome and neutral fitness costs of each mutant [152]. The P-value of each gene is shown in Table S1. Occasionally, transposon insertions in a non-essential gene might disrupt the expression of an essential gene downstream in the same operon. This kind of mutants could not survive which might result in false-positive essentiality calls. Therefore, the predicted essential genes were divided into two groups, 480 genes of which are not contained within operon, whereas 301 genes are within an operon structure (Fig. 3-6B). This information is also included in Table S1. Since an unequivocal assignment of sequence reads to duplicated genes is not possible, P-values for duplicated genes were adjusted (Table S1).[6]

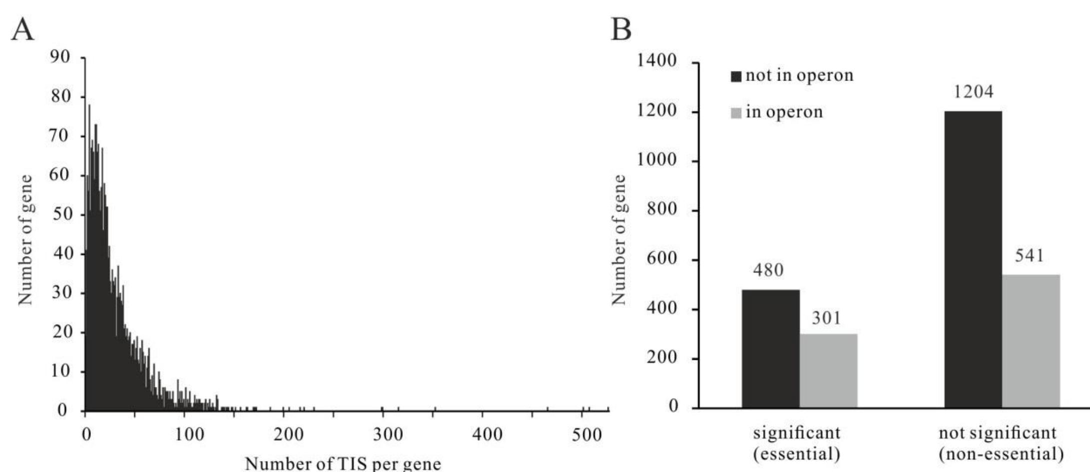

Fig. 3-6 Distribution of TIS in one gene indicated the essentiality of the related gene.
(A) Number of transposon insertion site (TIS) per gene. 19 genes have no TIS, 2069 genes (81.9% of the total genes) have less than 50 TIS, 102 genes (4% of the total genes) have more than 100 TIS, and the average TIS per gene is 31.
(B) Number of the predicted essential genes or non-essential genes belonged to the group of genes in operon or genes not in operon. 480 essential genes are not within operon, whereas 301 essential genes are within an operon structure. 1204 genes are non-essential and not within operon, while 541 non-essential genes are within operon structure.

[6] Bioinformatics analysis of the data was done by Christian Remmele.

In summary, 781 genes with P<0.05 were designated as essential for *Neisseria* survival and growth (Table S2). The list contains some well-known essential genes involved in fundamental biological processes, such as DNA replication, DNA recombination, DNA repair, transcription, ribosomal structure and biogenesis, as well as translation and energy production. For example, all aminoacyl-tRNA synthetase genes and the genes encoding subunits of ATP synthase were identified as essential in this study. In addition, seven out of the eight subunits of the multimeric DNA polymerase III were identified as essential. The remaining gene NGFG_00714, which is annotated as exonuclease and epsilon subunit of DNA polymerase III was unlikely to be essential (P=1), whereas NGFG_00762 (P=0.00073) also encodes an epsilon subunit and thus might functionally replace NGFG_00714.

Exemplary, triosephosphate isomerase (TIM, NGFG_00153) was identified as an essential gene. TIM plays an important role in glycolysis and is essential for efficient energy production [178]. Fig. 3-7 showed the distribution of mapped sequencing reads (black and green lines) in the according chromosomal region. Only very few transposon insertions were detected in the coding region and also in the promoter region of the gene NGFG_00153 encoded TIM (P=0.00073). By contrast, the genes upstream and downstream of TIM, NGFG_00152 and NGFG_00154, displayed high insertion density which resulted in P-values of 0.30367 and 0.99151 respectively, thereby defining NGFG_00152 and NGFG_00154 as non-essential genes (Fig. 3-7). However, NGFG_00152 (encoded preprotein translocase SecG subunit) only shows a lot of reads at the C-terminus and the majority of the ORF is not targeted by Tn5. It is possible that the C-terminal domain of SecG might not be important for the function and the protein without C-terminal domain is still functional, therefore the gene might still be essential.

The putative essential genes list includes 215 of the 307 essential genes in *E. coli* MG1655 [179] that have orthologs in *Neisseria gonorrhoeae* MS11 (70%), suggesting that a high percentage of essential *E. coli* genes have orthologs in *N. gonorrhoeae* that are also essential (Table S3). The rest 566 genes contain genes involved in fundamental cellular processes, such as ribosomal proteins, subunits of ATP synthase and enzymes for DNA metabolism which might be essential in other bacteria just not in *E. coli*. Besides, it shows some species specific essential genes including conjugal transfer pilus assembly proteins, irons binding and transport proteins, ABC transporters

and also 112 hypothetical proteins and 29 phage proteins. Some of them have been previously identified as essential: Omp85 (NGFG_01715, P=0.00073) [180], PorB (NGFG_01725, P=0.00073) [181,182] and the alternative sigma factor RpoH (NGFG_00430, P=0.00073) [169]. The predicted essential genes especially *Neisseria* specific essential genes could be putative targets for vaccine and antibacterial drug development.

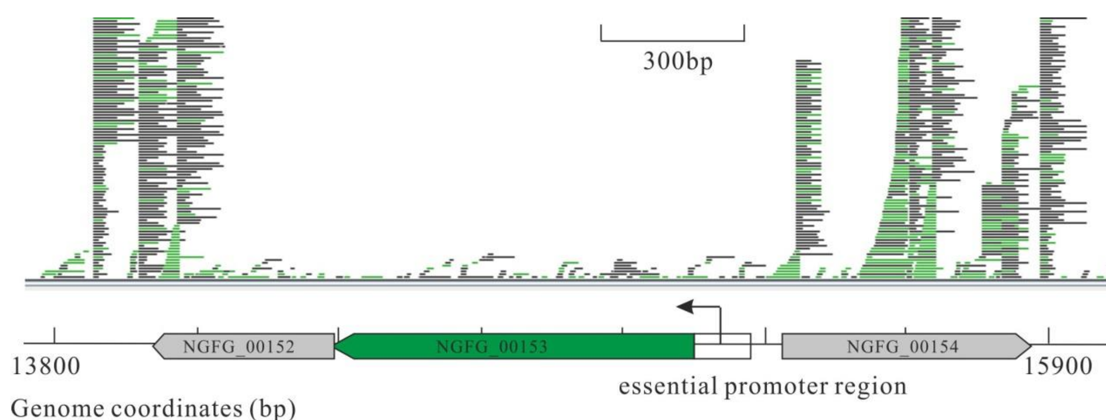

Fig. 3-7 Triosephosphate isomerase (TIM) is an essential gene. Distribution of mapped sequencing reads (black and green lines, merger of three libraries) shows transposon insertion sites on a segment of the *Neisseria gonorrhoeae* MS11 genome encoding TIM. The non-essential genes NGFG_00152 and NGFG_00154 (grey; P=0.30367 and 0.99151, respectively) have a lot of sequencing reads, while TIM encoded by NGFG_00153 (green) does not display such a read density and thus constitutes an essential gene (P=0.00073). The predicted promoter region of TIM (white box upstream of NGFG_00153) is also essential.

3.4 Validation of gene essentiality

Many of the predicted essential genes are required for fundamental biological processes, however, more than 15.4% (120 genes) of the putative essential *N. gonorrhoeae* genes were annotated as hypothetical proteins. To test the essentiality of a subset of these candidate drug and vaccine targets, mutagenesis of the genes, conditional knockout assays and genetic footprinting studies were performed.

3.4.1 Conditional knockout assay

Conditional knockout assay was established to test the essentiality of some candidate essential genes [168-170] which were listed in Table 3.2. The native promoter sequences of the candidate essential genes might be recognized by RpoD (σ70) which represents GC house-keeping sigma

factor, so the promoter sequence were predicted by BPROM (a bacterial sigma 70 promoter recognition program) [183]. Exemplary, in order to test the essentiality of the putative essential genes NGFG_00442 and NGFG_00443 (50S ribosomal protein L35 and L20, with P-values of 0.00073 and 0.00914, respectively), the predicted native promoter was detected 117 bp upstream of the operon. The native promoter was replaced with a kanamycin-P_{trc} cassette containing the IPTG-inducible promoter P_{trc} amplified from Hermes-10 vector [171]. Afterwards, the mutants were conjugated with *N. gonorrhoeae* MS11 N220, a strain containing pTH10a [171] which constitutively expresses the repressor *lac*I^q. By replacement of the promoter region, the expression of the candidate gene was conditional inhibited by omitting IPTG. Accordingly, the conditional knockout of NGFG_00442–00443 grew on GC-plates supplemented with 0.5 mM IPTG but did not survive on GC plates without IPTG. These data indicated that the ribosomal proteins encoded by NGFG_00442 and NGFG_00443 are essential for *N. gonorrhoeae* growth and that the assay was functional. By contrast, for NGFG_01725, NGFG_01315 and NGFG_00686, the P_{trc}-promoter mutants were not obtained after several attempts of transformations (Table 3.3). It may be due to that these genes are so important for *Neisseria* survival and it is not allowed to change their expression pattern. Furthermore, the conditional mutants of NGFG_02103, NGFG_04144 and NGFG_00007 grew even in absence of IPTG (Table 3.3). It is possible that these gene products still exist in the cells even after several passage growths without IPTG. In order to remove the remaining products, the concentration of IPTG was reduced to 0.1 mM to maintain the growth of mutants, and then more subsequent passages (about ten passages) of the mutants on the plates without IPTG were performed. However, no obvious growth defect was found (Fig. 3-8). Further, the expression of these genes in the conditional knockout mutants were tested by RT-PCR using RNA isolated from bacteria grown on the plates with or without IPTG. Fig. 3-9 shows the expression of NGFG_00007 was strongly reduced in the absence of IPTG compared with its expression in the presence of 0.1 mM IPTG, but the detectable expression might be sufficient to support *Neisseria* survival and growth (Lane 1–4 in Fig. 3-9). The same situation was found in conditional mutants of NGFG_04144 (data not shown). This suggested that the P_{trc} promoter is leaky and that a minute amount of protein expression driven by P_{trc} even in absence of IPTG produces enough gene products to support *Neisseria* growth. In conclusion, the conditional knockout assay based on the leaky IPTG-inducible P_{trc} promoter might be suitable for

testing essentiality of the candidate essential genes that require a large amount of protein to sustain *Neisseria* growth such as ribosomal proteins.

Table 3.2 Conditional knockout constructs of candidate essential genes

Gene ID	Annotation	Essentiality (P-value)	CDS_ Start	CDS_ Stop	CDS_ Strand	Promoter region (upstream of ATG)
NGFG_01725	Outer membrane protein P.IB	0.00073	1860158	1861210	+	158 bp [184,185]
NGFG_00442	50S ribosomal protein L35	0.00073	2149030	2151195	+	117 bp
NGFG_00443	50S ribosomal protein L20	0.00914	2151701	2151991	+	within operon of NGFG_00442
NGFG_02103	Hypothetical protein	0.01442	1681299	1681475	-	120 bp
NGFG_01315	Hypothetical protein	0.00699	1662515	1662916	-	100 bp
NGFG_04144	Hypothetical protein	0.00073	1421651	1422010	-	2 bp [a]
NGFG_00007	Hypothetical protein	0.00177	2119773	2120003	-	210 bp
NGFG_00686	Hypothetical protein	0.05433	1078088	1078249	+	57 bp

[a] P_{trc} promoter was inserted into the 2 bp region in front of ATG of the gene NGFG_04144

Table 3.3 Growth phenotypes of conditional knockout constructs

Gene ID	Annotation	Mutant (available)	Growth phenotype	
			With IPTG	Without IPTG
NGFG_01725	Outer membrane protein P.IB	no	ND	ND
NGFG_00442	50S ribosomal protein L35	yes	Growth	Growth defect
NGFG_00443	50S ribosomal protein L20	yes	Growth	Growth defect
NGFG_02103	Hypothetical protein	yes	Growth	Growth
NGFG_01315	Hypothetical protein	no	ND	ND
NGFG_04144	Hypothetical protein	yes	Growth	Growth
NGFG_00007	Hypothetical protein	yes	Growth	Growth
NGFG_00686	Hypothetical protein	no	ND	ND

ND: not determined

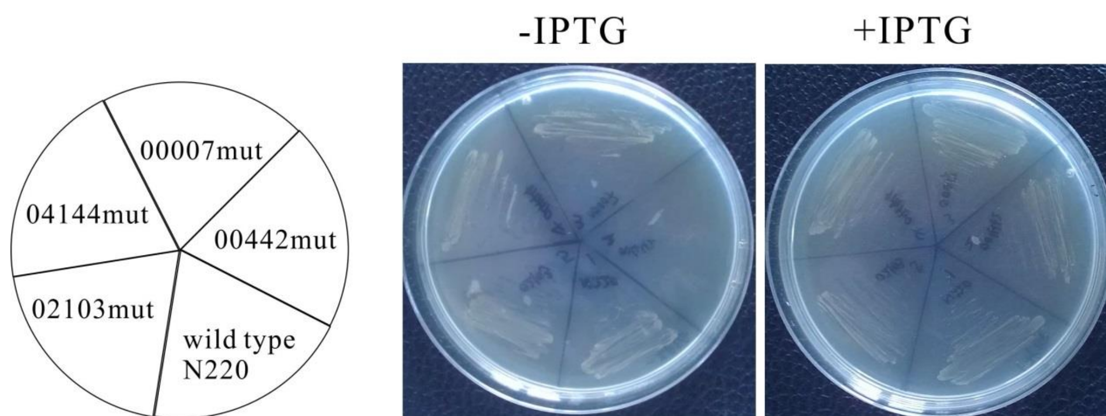

Fig. 3-8 Growth phenotypes of *N. gonorrhoeae* MS11 wild type N220 and conditional knockout mutants on the GC agar plates in the presence (right) or absence (left) of 0.1 mM IPTG. The gonococci were grown on GC agar plate containing 0.1 mM IPTG for 16–20 h at 37 ℃ in 5% CO_2 in a humidified atmosphere and then collected in PPM medium. Approximately 10^7 gonococci were streaked on the GC agar plates with or without IPTG and the phenotypes were recorded after 24 h incubation at 37 ℃ in 5% CO_2. Only NGFG_00442 mutants were not able to grow on the plates without IPTG, which indicated NGFG_00442 is an essential gene.

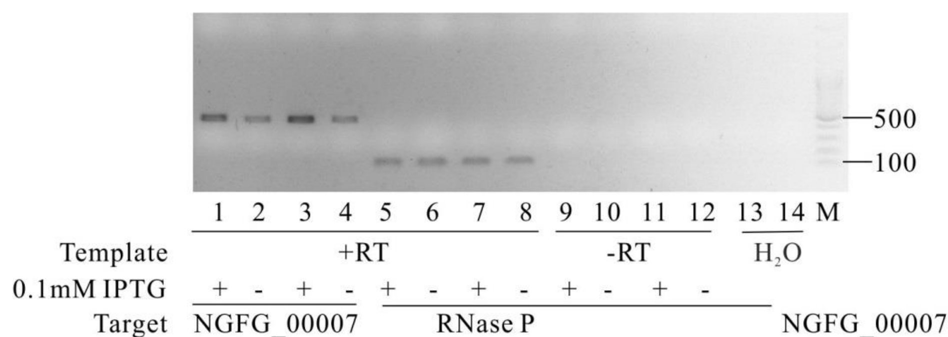

Fig. 3-9 Conditional P_{trc}-driven expression of NGFG_00007. RNA isolated from NGFG_00007 conditional knockout mutants grown on GC agar plates with or without 0.1 mM IPTG (as indicated) was reverse transcribed using random primers. The subsequent PCR was performed with primers specific for NGFG_00007 and RNase P as indicated. Two clones were analyzed. Lanes 1, 2, 5, 6, 9 and 10 are derived from clone 1 whereas the other lanes were from clone 2. The amplification of RNase P from the cDNA samples without Reverse transcriptase (-RT, lanes 9–12) show there are no contaminated genomic DNA in the isolated RNA samples. The amplification with H_2O (lanes 13 and 14) show there are no contamination in primers mix. M: DNA ladder. Results shown are representative of three independent determinations.

3.4.2 Genetic footprinting assay

Gene essentiality was further tested by a genetic footprinting assay [146,172-174]. Here the region of interest containing the putative essential gene as well as upstream and downstream of the open reading frame was amplified by PCR. Subsequently the PCR products were mutagenized *in vitro*

with Tn5 and were transformed in *N. gonorrhoeae* N2009. Resulting mutants were selected on GC agar plates supplemented with kanamycin and were pooled. Afterwards, the bacteria were incubated with DNase I to digest the remaining extracellular transposed DNA. gDNA was re-isolated and footprinting was performed using a chromosome-specific and a transposon-specific primer. As a reference, the PCR was conducted with the mutagenized DNA that had been used for transformation. The size of PCR products indicated where the Tn5 insertions occurred within the region of interest and thereby allows determination of the transposon insertion sites within the locus. Since bacterial mutants with insertions in essential genes are not viable, the Tn5 insertions within the essential genes will be lost during cultivation of the bacteria. Essentiality thus can be determined by comparing the control samples illustrating all Tn5 insertions after *in vitro* mutagenesis with a selective loss of insertions within *in vivo* selected mutants. PCR specificity was assured by carrying out the PCR with chromosome-specific primers derived from both, the 5' and 3' direction of the region. As shown in Fig. 3-10, genetic footprinting was performed in the genomic regions of NGFG_01063-1068 (722834-725537) and NGFG_01048-01053 (736587-732215). The functionally uncharacterized genes NGFG_01066, NGFG_01049 as well as NGFG_01051 encoding ferredoxin-NADP$^+$ reductase were predicted essential genes (P=0.04238, 0.00073 and 0.00129, respectively). *In vitro* transposition of the 4373 bp region NGFG_01048-01053 yielded 226 individual mutants, whereas for the 2704 bp region NGFG_01063-1068, 495 clones were obtained. PCR products corresponding to an insertion in these genes *in vivo* were rarely detected on an agarose gel when compared to the PCR products observed from the *in vitro* template. By contrast, PCR products corresponding to an insertion in the surrounding non-essential genes were detected (Fig. 3-10A). These data are in agreement with the insertion patterns detected in the Tn-seq libraries (Fig. 3-10B) and illustrate that the candidate genes NGFG_01066, NGFG_01049 and NGFG_01051 are indeed essential. Simultaneously these data prove that NGFG_01068, NGFG_01064, NGFG_01063, NGFG_01053, NGFG_01052 and NGFG_01048 are non-essential in *N. gonorrhoeae* under the tested conditions.

In addition to conditional knockouts and genetic footprinting assays, several putative essential genes were validated by inability to produce deletion mutants of whose mutants show strong growth phenotypes. Bacteria with disruption of NGFG_01725, NGFG_01315 and NGFG_00686

(see also conditional knockout assay; Table 3.3), as well as beta-ketoacyl-acyl-carrier-protein synthase II (NGFG_01674, P=0.00385) and uridylate kinase (NGFG_01912, P=0.00177) were not viable (Table 3.4). Similarly, gonococci with knockout in the ABC transporter substrate-binding protein virulence factor Mce (NGFG_00072, P=0.03377), a phospholipase D family protein (NGFG_00827, P=0.00262) and a hypothetical protein (NGFG_01266, P=0.05006) were obtained on GC agar plates, but demonstrated a strong growth defect (Table 3.4). By contrast, gene deletion experiments were performed with several predicted non-essential genes (Table 3.4) and these gene deletions did not influence gonococci survival and growth under the tested conditions.

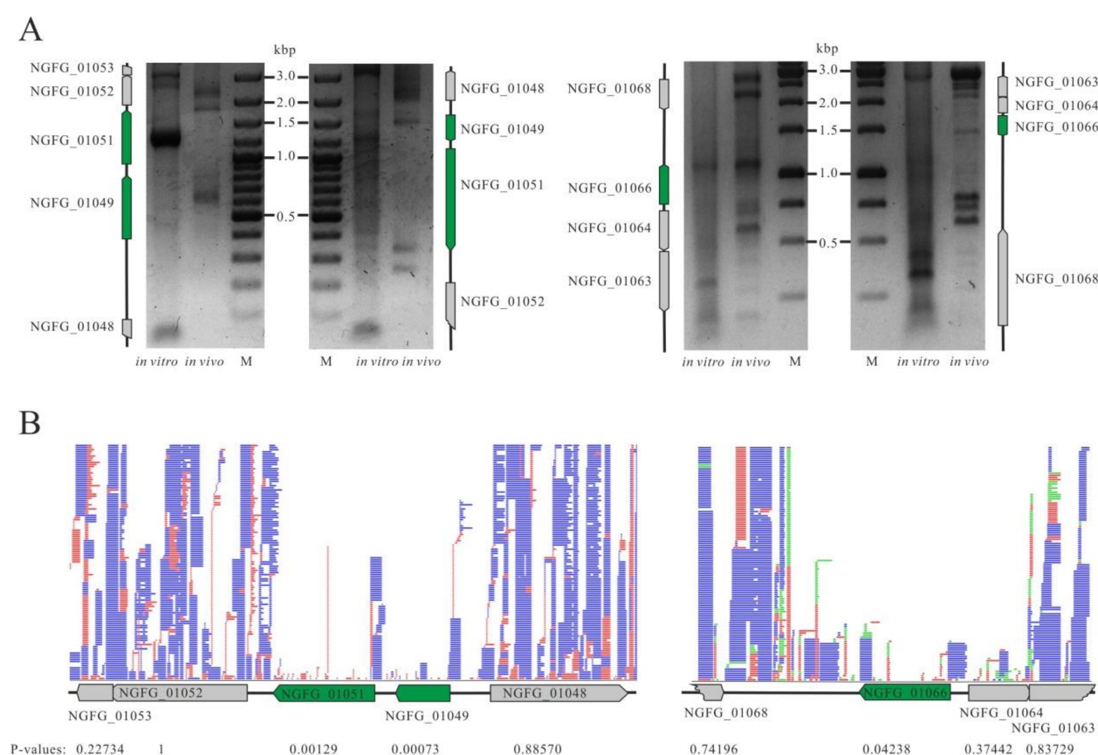

Fig. 3-10 Genetic footprinting and TIS distribution in Tn-seq libraries indicated gene essentiality.
(A) Genetic footprinting demonstrates gene essentiality in *N. gonorrhoeae*. *Neisseria* PCR products are mutagenized with Tn5 *in vitro* and transformed into bacteria. gDNA is recovered from recombinant bacteria and the distribution of Tn5 insertions of *in vivo*-selected mutants is compared to the *in vitro* template by PCR using a transposon-specific primer (Tn ME sequence) and a chromosome-specific primer placed either 5' (left panel) or 3' (right panel) from the locus NGFG_01048-01053 or NGFG_01063-1068. M: DNA ladder.
(B) Distribution of TIS in this region in Tn-seq libraries. The mapped sequencing reads from library 1 (blue line), library 2 (red line) and library 3 (green line) indicate the insertion patterns in this region surrounding the predicted essential genes NGFG_01049, NGFG_01051 and NGFG_01066. Grey bar represents non-essential genes and green bar represents putative essential genes.

Table 3.4 Growth phenotypes of gene knockout mutants in *Neisseria gonorrhoeae*

Gene ID	Gene function	Gene name	P-value	Mutant growth [a]
NGFG_00042	TonB dependent siderophore receptor		0.12712	Normal
NGFG_00506	Hypothetical protein		0.07867	Normal
NGFG_00599	Sulfate ABC transporter	*cys*W	1	Normal
NGFG_00859	DedA family membrane protein		0.58067	Normal
NGFG_00860	Outer membrane protein	*opc*A	1	Normal
NGFG_01393	Hypothetical protein		0.99247	Normal
NGFG_01489	TonB-dependent receptor		1	Normal
NGFG_01605	Predicted protease	*aif*1 [b]	1	Normal
NGFG_01643	ABC transporter ATP-binding/permease protein		1	Normal
NGFG_01836	Membrane-bound lytic murein transglycosylase A	*mlt*A	1	Normal
NGFG_02032	FKBP-type peptidyl-prolyl cis-trans isomerase	*fkp*A	1	Normal
NGFG_00072	ABC transporter substrate binding protein	*mce*	0.03377	Growth defect
NGFG_00827	Phospholipase D family protein	*pld*	0.00262	Growth defect
NGFG_01266	Hypothetical protein		0.05006	Growth defect
NGFG_01674	Beta-ketoacyl-acyl-carrier-protein synthase II	*fab*H	0.00385	Could not obtainable
NGFG_01912	Uridylate kinase	*pyr*H	0.00177	Could not obtainable

[a] Growth curve was performed in *Neisseria* growth medium

[b] aif1 was first named after "**A**dherence and **I**nvasion-associated **F**actor 1" in this study

3.5 Use of Tn-seq to identify *N. gonorrhoeae* virulence factors in DGI

The virulence mechanisms involved in disseminated gonococcal infection (DGI) are not well understood. Therefore the transposon mutant library in *N. gonorrhoeae* strain N2009, an MS11 derivative expressing $PorB_{1A}$ (chapter 3.1) was used to infect the human conjunctiva epithelial cell line Chang (ATCC CCL-20.2) due to the high bacterial invasion efficiency of 0.6 invasive non-piliated bacteria and 0.015 piliated *Neisseria* per host cell after 1h infection at a MOI of 100 (data not shown). Because the library contained about 100,000 mutants and a 100-fold representation of the library was used for screening, approximately 10^7 bacteria from the library were recovered on GC agar plates and were used to infect 4×10^7 Chang cells under low phosphate conditions. After infection for 1h, the cell-associated or invasive bacteria were selected by gentamicin treatment and recovered on agar plates. The recovered bacteria were used in two additional infection rounds. Each time the recovered bacteria were pooled thus yielding three separate “output” libraries. As control, the mutant library was incubated for the same time in infection medium in the absence of host cells and thus constituted the “input” library (Fig. 3-11).

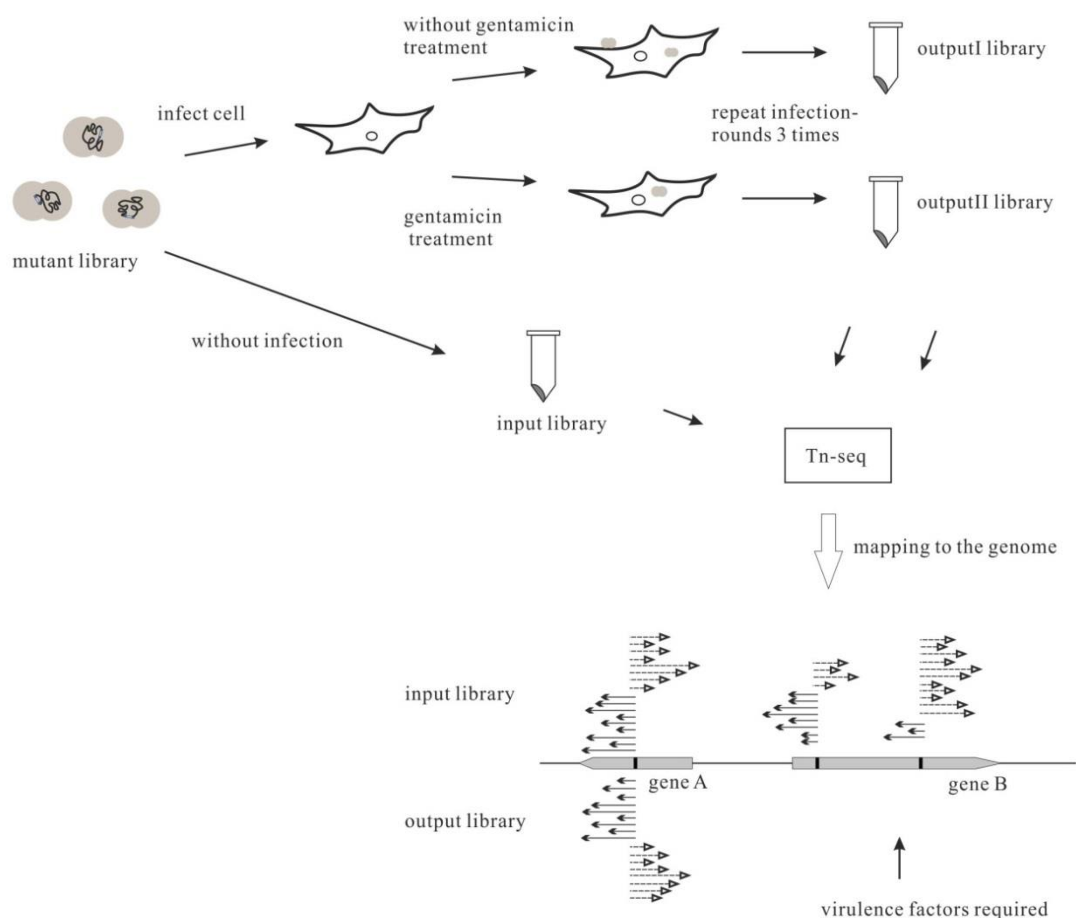

Fig. 3-11 Infection and screening for virulence factors involved in DGI. A high-density Tn5 mutant library was used to infect Chang cells with an MOI 100 for 1 h under low phosphate conditions (HEPES medium). After infection, the unattached bacteria were washed away and the cells were lysed by incubation with 1% saponin for 15 min. The cell-associated bacteria (adherent and invasive bacteria) were recovered on the agar plates for the next round of infection. In order to select for invasive bacteria, 100 µg/mL gentamicin was added after the infection to kill all the extracellular bacteria. Intracellular bacteria were recovered on agar plates as described above. Recovered bacteria were used for two additional subsequent infections thereby increasing stringency of the assay. The mutants without infection constitute input library. Chromosomal DNA from input and output libraries was isolated and TIS were identified as described. A depletion of TIS in the recovered output libraries thus will indicate the factors required for adherence or invasion under the low phosphate conditions.

Chromosomal DNA from input and output libraries was isolated and the sequencing samples were prepared for Tn-seq as described (chapter 3.2)[7].The introduction of different barcodes enabled multiplexing of Tn-seq as well as identification of the source of the sequenced DNA. The

[7] Dr. Richard Reinhardt and his colleagues performed the Illumina sequencing and preliminary quality control of the sequencing reads. Christian Remmele performed raw data processing and bioinformatics analysis.

sequencing reads were separated based on the barcodes and then mapped to MS11 genome (version 4, *Neisseria gonorrhoeae* group Sequencing Project, Broad Institute of Harvard and MIT). More than 50 million raw sequencing reads were obtained for the output I library and nearly 60% were specifically mapped to the genome which indicated 88,625 unique TIS in the annotated CDS regions. For the output II libraries, the samples from the second and the third infection round were sequenced and the results showed 32,198 and 34,147 intragenic TIS, respectively (Table 3.5). The complete data, the distribution of the reads and TIS counts per gene is shown in Table S4. Few sequencing reads were acquired from the input library. It might be due to poor-quality sequencing sample or technical problems during sequencing which can be ruled out by repeating the sequencing of a new prepared sequencing sample of this library. Because the wild-type strain N2009 cannot grow in HEPES medium, it is more likely that most mutants cannot survive or grow in the incubation of HEPES medium which lead to very few mutants in the input library.

Table 3.5 Overview of sequencing results of input and output libraries.

	Input library (438_C) [a]	Output I library (438_D) [b]	Output II library (438_E) [c]	Output II library (438_F) [d]
Sequenced reads	101,178	50,666,104	14,160,012	19,231,784
Mapped reads	69,183 (68.38%)	30,722,996 (60.64%)	8,679,833 (61.30%)	10,593,688 (55.08%)
Uniquely mapped reads	68,302 (67.51%)	30,315,271 (59.83%)	8,569,794 (60.52%)	10,375,019 (53.95%)
Unique TIS	5,980	112,887	42,123	45,321
Intragenic TIS	4,548	88,625	32,198	34,147

[a] non-infection library, sample from the second round selection
[b] output I library, sample from the second round selection
[c] output II library, sample from the second round selection
[d] output II library, sample from the third round selection

3.6 Identification of virulence factors required for DGI

In order to identify the virulence factors required for *Neisseria* adherence or invasion during phosphate sensitive infection, the P-values of each gene in these three different output libraries were calculated based on the TIS counts (Table S4). The depletion of TIS in specific genes during the selection indicated the requirement of the corresponding gene products for *Neisseria*

attachment to or invasion into the host cells. The recovered bacteria resulting in the output I library adhered to or invaded into the host cells. This illustrates that the genes in which significantly reduced numbers of TIS are required for adherence. Conversely, mutants lost in the output II library demonstrated factors required for adherence or invasion. By comparing the data between libraries DGI invasion factors can be identified. Exemplary, the uncharacterized gene NGFG_00506 was predicted as an invasive factor due to a loss of Tn5 insertions lost in the output II library when compared with reads originating from either “input” or “output I” library (Fig. 3-12). In summary, 431 genes with $P< 0.05$ in the output I library (438_D) indicated that the respective mutants were unable to attach to the host cells or were not viable (Table S5). Further analysis showed that the list of candidate genes for *Neisseria* adherence factors contained 333 predicted essential genes (chapter 3.3; Table S5) and 98 non-essential genes (Table 3.6). In this list the genes for the known adhesin type IV pilus as well as its assembly proteins PilP and PilW are found. To analyze gonococcal DGI invasion factors, the two output II libraries 438_E and 438_F were merged and genes with $P< 0.05$ in either library were chosen. Table S6 harbors 184 predicted essential genes and 43 non-essential genes (Table 3.7). The overlap in both libraries consisted of 117 genes, 102 of which comprising essential and 15 non-essential genes (Table 3.7). A subset of candidate genes was chosen for validation and closer characterization in order to learn more about the mechanism of *Neisseria* phosphate sensitive infection.

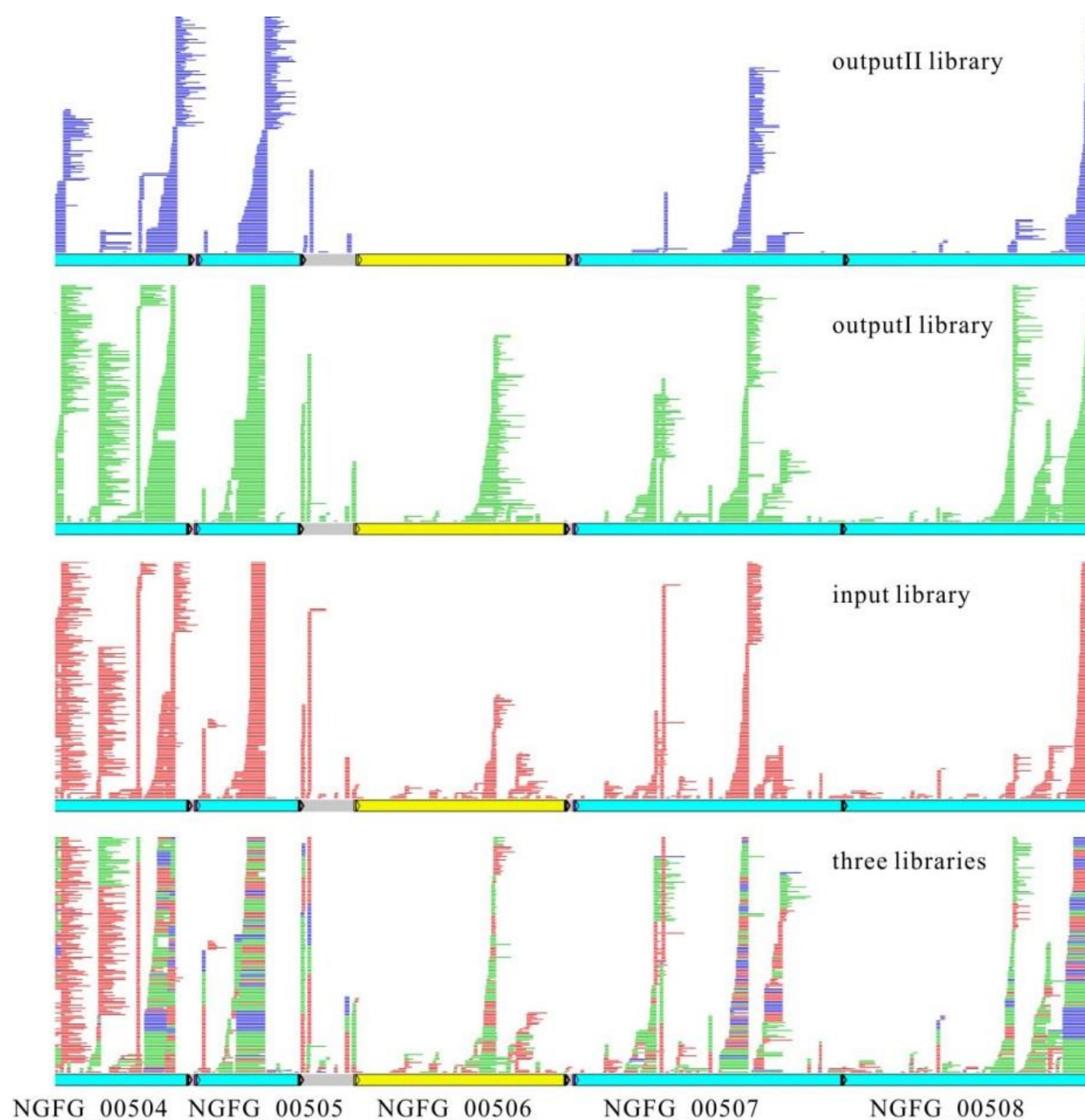

Fig. 3-12 Distribution of mapped sequencing reads on the DNA segment of NGFG_00506 indicates involvement of the ORF in DGI invasion. The different coloration indicates the different libraries from which the sequencing reads were obtained: red reads originate from the "input" library (438_A), green reads from the "output I" library (438_D) and blue reads from the "output II" library (438_E).

Table 3.6 Non-essential candidate genes for *Neisseria* adherence

Gene ID	Gene function	Gene name	P-value (438_D)
Adhesion			
NGFG_00608	type IV pilus assembly protein	*pil*W	0.00795
NGFG_00233	type IV pilus assembly protein	*pil*P	0.00643
NGFG_01202	type IV pilus biogenesis/stability protein	*pil*W (silent copy)	0.02190

Transporters			
NGFG_00598	sulfate/thiosulfate import ATP-binding protein	*cys*A	0.00302
NGFG_00071	ABC transporter permease		0.04406
NGFG_02085	histidine-binding protein	*his*J	0.02218
NGFG_04085	cell division ABC transporter ATP-binding protein	*fts*E	0.00207
Nucleic acid metabolism			
NGFG_01045	protein RecA	*rec*A	0.00384
NGFG_00705	single-stranded DNA-binding protein	*ssb*	0.04562
NGFG_01851	DNA recombination protein RmuC	*rmu*C	0.04681
NGFG_01096	recombination factor protein R		0.04056
NGFG_01641	Holliday junction ATP-dependent DNA helicase	*ruv*A	0.03411
NGFG_01413	Rrf2 family protein		0.02336
NGFG_02201	transposase		0.00384
NGFG_04232	transposase		0.01588
NGFG_01885	transferase		0.01863
NGFG_02198	replication initiation factor		0.03540
NGFG_00391	transcription antitermination factor	*nus*B	0.02038
Protein metabolism			
NGFG_01644	ribosome small subunit-dependent GTPase A		0.02190
NGFG_00413	ribosomal RNA small subunit methyltransferase A	*rsm*A	0.02190
NGFG_01129	ribosomal RNA small subunit methyltransferase B	*rsm*B	0.01985
NGFG_00566	ribosome-associated protein		0.00207
NGFG_01786	methionyl-tRNA formyltransferase		0.04416
NGFG_00172	(Dimethylallyl) adenosine tRNA methylthiotransferase	*mia*B	0.00207
NGFG_00439	queuine tRNA-ribosyltransferase		0.00207
NGFG_02117	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase		0.00384
NGFG_01618	deoxyribodipyrimidine photo-lyase	*phr*B	0.03823
NGFG_01692	dihydrodipicolinate reductase		0.01079
NGFG_02065	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gpm*A	0.00207
NGFG_01787	peptide deformylase		0.00207
NGFG_02048	Imidazole glycerol-phosphate dehydratase		0.00570
Metabolic enzymes			
NGFG_02228	lacto-N-neotetraose biosynthesis glycosyltransferase	*lgt*E	0.03091
NGFG_00164	orotate phosphoribosyltransferase, OPRTase		0.01531
NGFG_00193	4-hydroxyphenylacetate 3-monooxygenase, reductase component		0.00207
NGFG_00187	carbamoyl-phosphate synthase small chain		0.01014
NGFG_00654	isocitrate dehydrogenase, NADP-dependent		0.00207
NGFG_00713	2-nitropropane dioxygenase		0.00207
NGFG_00758	acetate kinase 1	*ack*A1	0.00570

NGFG_00764	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*isp*F	0.02218
NGFG_00888	HAD hydrolase, family IB		0.00643
NGFG_00895	short chain dehydrogenase		0.02564
NGFG_00918	dihydroxy-acid dehydratase		0.00207
NGFG_01074	glucokinase		0.01985
NGFG_00247	oxidoreductase		0.02872
NGFG_00338	phosphoglycolate phosphatase		0.02816
NGFG_00350	phosphate acetyltransferase		0.00207
NGFG_00390	dihydroorotase		0.03055
NGFG_01648	NADH-quinone oxidoreductase subunit N	*nuo*N	0.03152
NGFG_01654	NADH-quinone oxidoreductase subunit J	*nuo*J	0.04222
NGFG_01656	NADH-quinone oxidoreductase subunit H	*nuo*H	0.00486
NGFG_01663	NADH-quinone oxidoreductase subunit B	*nuo*B	0.00725
NGFG_01329	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, YrbI family	*yrb*I	0.03270
NGFG_01335	shikimate dehydrogenase		0.04790
NGFG_01499	thiamine biosynthesis lipoprotein	*apb*E	0.01478
NGFG_01501	Na(+)-translocating NADH-quinone reductase subunit F	*nqr*F	0.00643
NGFG_01505	Na(+)-translocating NADH-quinone reductase subunit B	*nqr*B	0.04633
NGFG_01542	biopolymer transporter	*exb*D	0.04710
NGFG_01574	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	*ubi*D	0.02614
NGFG_01605	protease		0.00795
NGFG_02134	NADPH-dependent 7-cyano-7-deazaguanine reductase		0.02162
NGFG_01608	guanylate kinase		0.00384
NGFG_01791	aspartate carbamoyltransferase regulatory chain	*pyr*l	0.00930
Others			
NGFG_00049	lipoprotein		0.04847
NGFG_00141	Lipoprotein Mlp	*mlp*	0.02218
NGFG_01832	UPF0409 lipoprotein		0.04255
NGFG_00197	GTP-binding protein	*ych*F	0.00302
NGFG_00448	restriction endonuclease		0.04633
NGFG_00560	type I restriction enzyme, S subunit		0.02237
NGFG_00901	[2Fe-2S] ferredoxin, ISC system protein		0.04807
NGFG_04139	bacteriocin resistance protein		0.00795
NGFG_01630	integral membrane protein, virulence factor MviN	*mvi*N	0.00302
NGFG_01826	mechanosensitive ion channel protein		0.04178
NGFG_02173	UPF0210 protein		0.00207
NGFG_00461	UPF0042 nucleotide-binding protein		0.00384
Phage proteins			
NGFG_00623	phage protein		0.03951

NGFG_00632	phage protein		0.02663
NGFG_00642	phage protein		0.04178
NGFG_01054	phage protein		0.03750
NGFG_01283	phage protein		0.02190
NGFG_01285	phage protein		0.01820
NGFG_02190	phage protein		0.02283
Hypothetical proteins			
NGFG_00183	hypothetical protein		0.01768
NGFG_01157	hypothetical protein		0.00486
NGFG_04145	hypothetical protein		0.03540
NGFG_04198	hypothetical protein		0.01820
NGFG_04225	hypothetical protein		0.03906
NGFG_01266	hypothetical protein		0.04663
NGFG_00979	hypothetical protein		0.02564
NGFG_01031	hypothetical protein		0.03712
NGFG_01650	hypothetical protein		0.03152
NGFG_00264	hypothetical protein		0.04489
NGFG_00591	hypothetical protein		0.03500
NGFG_00295	hypothetical protein		0.04681
NGFG_04237	hypothetical protein		0.04790
NGFG_02177	hypothetical protein		0.00207
NGFG_02058	hypothetical protein		0.01223
NGFG_02204	hypothetical protein		0.02190
NGFG_02108	hypothetical protein		0.02336

Table 3.7 Non-essential candidate genes for *Neisseria* adherence or invasion

Gene ID	Gene function	Gene name	P value (438_E)	P value (438_F)
Transporters				
NGFG_01643	ABC transporter ATP-binding/permease protein		**0.02700**	**0.04892**
NGFG_00598	sulfate/thiosulfate import ATP-binding protein CysA	*cys*A	**0.02377**	**0.04892**
NGFG_04085	cell division ABC transporter ATP-binding protein	*fts*E	**0.01419**	0.08746
NGFG_00159	iron chelate ABC transporter, periplasmic iron chelate-binding protein	*afe*A	**0.01508**	0.09711
NGFG_00152	preprotein translocase, SecG subunit	*sec*G	**0.01943**	0.35033
Protein metabolism				
NGFG_01787	peptide deformylase		**0.00544**	**0.03385**

NGFG_00439	queuine tRNA-ribosyltransferase		**0.00544**	**0.01451**
NGFG_00172	(Dimethylallyl) adenosine tRNA methylthiotransferase	*mia*B	**0.00738**	**0.02543**
NGFG_01692	dihydrodipicolinate reductase		**0.01508**	**0.01978**
NGFG_02117	2,3,4,5-tetrahydropyridine-2,6-dicarboxylat e N-succinyltransferase		**0.03229**	**0.04067**
NGFG_02065	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gpm*A	**0.01508**	**0.01036**
NGFG_00566	ribosome-associated protein		**0.04197**	0.07569
NGFG_01129	ribosomal RNA small subunit methyltransferase B	*rsm*B	**0.02798**	0.08402
NGFG_02048	Imidazole glycerol-phosphate dehydratase		0.0570595 56	**0.01036**
Metabolic enzymes				
NGFG_00350	phosphate acetyltransferase		**0.00738**	**0.01036**
NGFG_00654	isocitrate dehydrogenase, NADP-dependent		**0.00544**	**0.03072**
NGFG_00913	UDP-N-acetylmuramate:L-alanyl-gamma-D -glutamyl-meso-diaminopimelate ligase		**0.03563**	**0.04314**
NGFG_01568	3-oxoacyl-[acyl-carrier-protein] reductase	*fab*G	**0.02100**	**0.04983**
NGFG_01605	protease		**0.01327**	**0.01813**
NGFG_01656	NADH-quinone oxidoreductase subunit H	*nuo*H	**0.01145**	0.13267
NGFG_01648	NADH-quinone oxidoreductase subunit N	*nuo*N	**0.03229**	0.18745
NGFG_01501	Na(+)-translocating NADH-quinone reductase subunit F	*nqr*F	**0.0141913 04**	0.10362
NGFG_00193	4-hydroxyphenylacetate 3-monooxygenase, reductase component		**0.04172**	0.10362
NGFG_01574	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	*ubi*D	0.08188	**0.04387**
Phage proteins				
NGFG_04194	phage protein		**0.04904**	0.05973
NGFG_00623	phage protein		**0.03563**	0.09597
NGFG_00642	phage protein		**0.02477**	0.09711
Hypothetical proteins				
NGFG_00506	hypothetical protein		**0.00544**	**0.03510**
NGFG_00574	hypothetical protein		**0.03563**	**0.02919**
NGFG_01157	hypothetical protein		**0.01419**	0.08063
NGFG_02058	hypothetical protein		**0.03709**	0.11198
NGFG_01266	hypothetical protein		**0.00544**	0.10312

NGFG_00295	hypothetical protein		**0.01145**	0.18208
Others				
NGFG_00062	hemoglobin-haptoglobin utilization protein B	*hpu*B	**0.04870**	0.13321
NGFG_01045	protein RecA	*rec*A	**0.00544**	0.06963
NGFG_01893	Ngo I restriction endonuclease		**0.04904**	0.10090
NGFG_00682	5'-nucleotidase surE	*sur*E	**0.01419**	0.12138
NGFG_02198	replication initiation factor		**0.03563**	0.19394
NGFG_04172	cell-surface protein		**0.04730**	0.25084
NGFG_00822	toxin component of toxin-antitoxin system		**0.03285**	0.31050
NGFG_01630	integral membrane virulence factor MviN	*mvi*N	0.06695	**0.01978**

P-values < 0.05 in bold.

3.7 Validation of candidate invasive genes

In order to validate the involvement of identified candidate genes in *Neisseria* disseminated infection, the respective genes knockout mutants were generated and analyzed for their ability to adhere to and invade into the host cells under low phosphate condition. To construct gene knockout mutants, about 500 bp upstream and downstream of the target gene were amplified from *Neisseria* gDNA and combined with a kanamycin cassette (chapter 2.2.13). The resulting PCR fragments were transformed into *N. gonorrhoeae* N2009 and the recombinant bacteria were selected on GC plates supplemented with kanamycin. The mutants were checked by colony PCR and subsequently the correct location of the kanamycin cassette within the genome was confirmed by sequencing the PCR-amplified region from 1000 bp upstream to 500 bp downstream of the target gene. Furthermore, the lack of expression of the target gene in the gene knockout mutants was checked by reverse transcription-PCR (RT-PCR) with cDNA derived from mutants.

Here, three candidate genes were tested, the hypothetical protein NGFG_00506, NGFG_01605, a predicted protease, and NGFG_01643, an ABC transporter ATP-binding/permease protein. The gene knockout mutants for each gene were prepared in triplicate. Growth curves of the mutants in rich medium showed that the mutants grew similar to the wild type which indicated the loss of the genes did not influence the viability of the mutants (Fig. 3-13). However, the mutants within NGFG_01605 or NGFG_01643 showed a significantly decreased adherence and invasion in

infections under low phosphate conditions (Fig. 3-13B and C) and suggested a function in gonococcal adherence and invasion. Mutants within NGFG_00506 showed similar adherence when compared to the wild type, but were significantly decreased in their invasion rates (Fig. 3-13A) which indicated that NGFG_00506 might be an invasion factor rather than an adhesin in this process. It is in agreement with TIS patterns described in Tn-seq data (Fig. 3-12). To confirm this result, a differential immunofluorescence assay was performed with mutants lacking either NGFG_01605 or NGFG_00506. Invasive bacteria in 50 randomly selected cells were counted. Whereas in the wild-type about one invasive bacterium per cell was detected, the mutants' infection rates decreased to less than 0.5 bacteria per cell (Fig. 3-15, [163]). This confirmed that a loss of either NGFG_01605 or NGFG_00506 was required for efficient host cell invasion.

With this technique other genes were tested. For example, mutants within the phospholipase D family protein NGFG_00827 were defective in adherence and invasion but also showed growth defects in rich medium. This was not surprising since NGFG_00827 was predicted to be essential in the input library (P=0.00262 in 438_A library, Table S1; Fig. 3-14B). Mutants lacking NGFG_01266 (hypothetical protein; P=0.05007 in 438_A library, Table S1) grew very slowly in PPM medium and the adherence as well as invasion of the recombinant bacteria were strongly reduced when compared to the wild-type (Fig. 3-14A).

In the MS11 genome there are several duplicated genes. One of the repeated genes, the hypothetical protein NGFG_004218 was analyzed since a sequence alignment demonstrates that, NGFG_004218 has identical sequences as *mafI* gene of *N. gonorrhoeae* strain FA1090. The respective gene knockout mutants grew very well under the tested condition but adherence to and invasion into the host cells were decreased (Fig. 3-14C).

In addition, some genes with $P > 0.05$ in the output libraries (P_{output}) were tested as described above (Table 3.8). The mutants within these genes grew normally in rich medium and did not show any defect in adherence and invasion when compared to the wild type (data not shown).

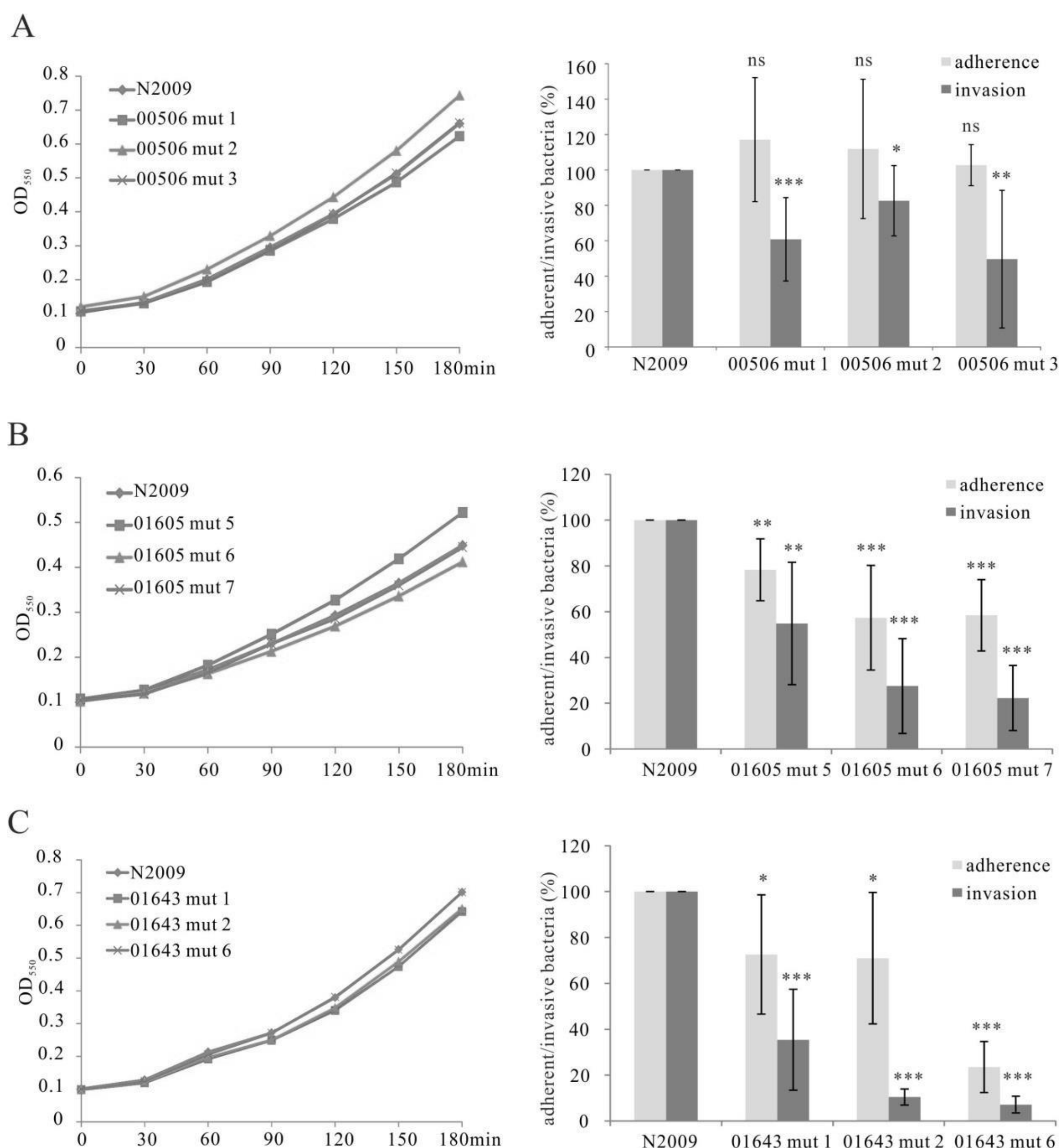

Fig. 3-13 Growth, adherence and invasion phenotypes of mutants within NGFG_00506 (A), NGFG_01605 (B) and NGFG_01643 (C).

Left, growth curves were performed in PPM using strains that were non-piliated and Opa-negative. The experiments were repeated two or three times independently with similar results and one representative data was shown.

Right, adherence and invasion of the bacteria were determined by gentamicin protection assays under low phosphate conditions with a MOI of 50 for 30 min. The numbers of adherent or invasive bacteria were determined with rates of the wild type strain set to 100%. Data represent the mean ± SD of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

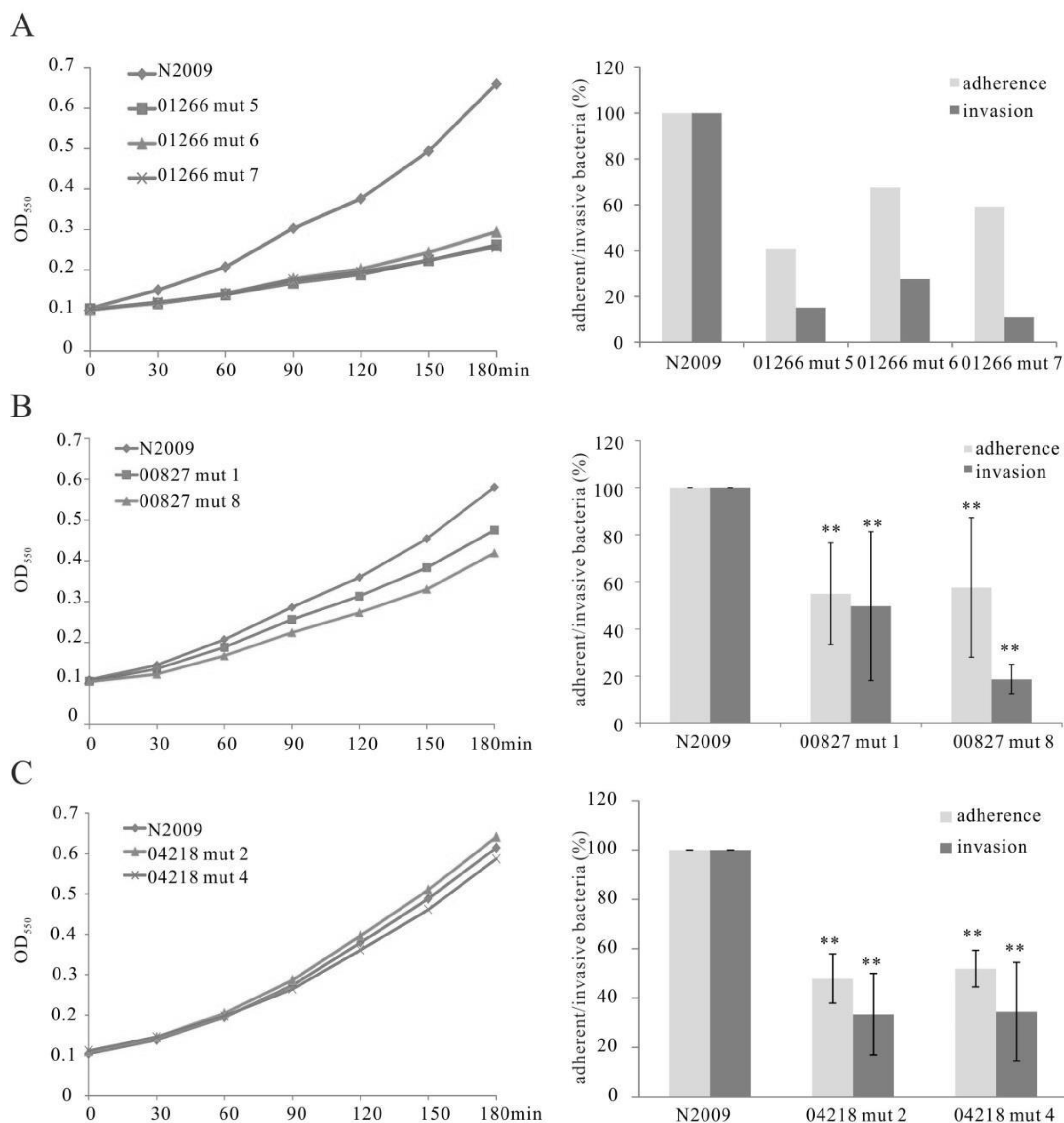

Fig. 3-14 Growth, adherence and invasion phenotypes of mutants within NGFG_01266 (A), NGFG_00827 (B) and NGFG_04218 (C).

Left images show growth curves of the mutants performed in PPM medium using strains with colony morphology of non-pili and non-Opa expression. The experiments were repeated two or three times independently with similar results and one representative data was shown.

Right images show adherent and invasive bacteria were determined by gentamicin assays under low phosphate conditions with a MOI of 50 for 30 min. The number of adherent or invasive bacteria with infecting of wild type strain was set as 100%. Data represent the mean ± SD of three independent experiments. ** $p < 0.01$. The experiment of NGFG_01266 mutants were repeated twice and one representative data was shown.

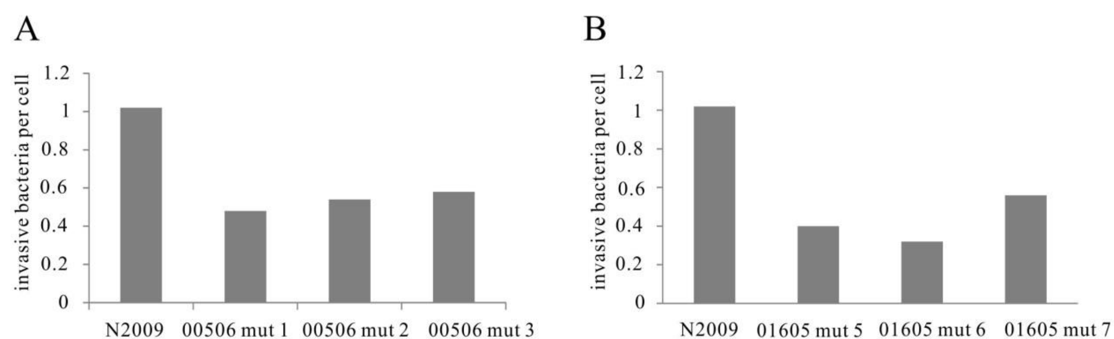

Fig. 3-15 Differential immunostaining demonstrates invasion deficiency of mutants within NGFG_00506 (A) and NGFG_01605 (B). Number of invasive bacteria was counted from 50 randomly chosen cells using differential immunostaining and confocal microscopy [163].

Table 3.8 Genes tested for adherence or invasion defects, which are not in the candidate list

Gene ID	Gene function	P-value (438_D)[a]	P-value (438_E)[b]	Growth [c]	Adherence and invasion phenotype [d]
NGFG_00042	TonB dependent siderophore receptor	0.68657	0.76619	Normal	ns
NGFG_01393	Hypothetical protein	0.83074	0.56661	Normal	ns
NGFG_01489	TonB-dependent receptor	0.96054	0.95802	Normal	ns
NGFG_01836	Membrane-bound lytic murein transglycosylase A	0.13808	0.40787	Normal	ns
NGFG_02032	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	0.94414	0.93860	Normal	ns

[a] 438_D, output I library
[b] 438_E, output II library
[c] Growth curves were determined in PPM medium
[d] Gentamicin assays were performed to test *N. gonorrhoeae* adherence and invasion
ns: not significant, means the mutants did not show significantly defects in the adherence to or invasion into the host cells compared to the wild type strain

3.8 NGFG_01605 is required for gonococcal internalization

The growth in PPM medium of NGFG_01605 knockout mutants was similar to the wild type strain (Fig. 3-13B), however, under low phosphate conditions, the mutants showed a significant decrease in the adherence to and invasion into human epithelial cells as evidenced by gentamicin assays (Fig. 3-13B). Further, the ratio of invasive to adhesive bacteria was determined which was

smaller for the mutants when compared with the wild-type (Fig. 3-16A). This indicated that NGFG_01605 not only influences *N. gonorrhoeae* adhesion but also invasion into the host cells. This was further confirmed by differential immunofluorescence assay (Fig. 3-15B; [163]). In order to test if NGFG_01605 was required for the initial attachment to host cell, gentamicin assays were performed with piliated mutants. Compared to the wild type strain N2009, the mutants showed similar number of adherent bacteria, which demonstrated that NGFG_01605 did not affect pili-mediated initial attachment of *N. gonorrhoeae* to the host cells (Fig. 3-16B).

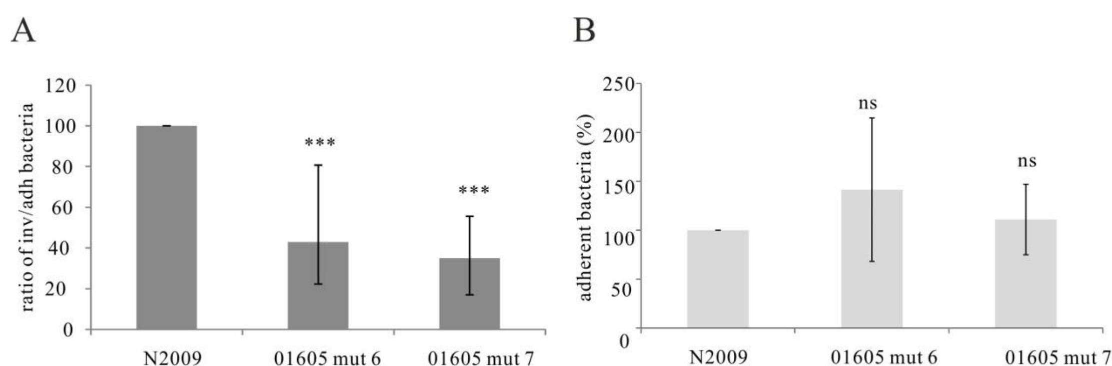

Fig. 3-16 Ratio of invasive/adherent bacteria demonstrates involvement of NGFG_01605 in neisserial host cell invasion under low phosphate conditions but is dispensable for pili-mediated attachment.
(A) Ratio of invasive/adherent bacteria was determined by a gentamicin assay. The ratio of wild type strain N2009 was normalized to 100%.
(B) Chang cells were infected with piliated NGFG_01605 knockout mutants and the wild type strain N2009 at a MOI 10 for 1 h in HEPES medium.
The adherent bacteria were quantified by gentamicin assay. The number of adherent wild type was set to 100%. The data depict the mean values ± SD of three independent experiments. ns: not significant. *** $p < 0.001$.

Aside from $PorB_{IA}$-mediated internalization, *N. gonorrhoeae* efficiently enters host cells through Opa proteins [64,65,69,74,76]. Two distinct Opa groups recognize different receptors on the surface of host cells. Opa_{50} binds to HSPGs and Opa_{51-60} interacts with CEACAMs (chapter 1.1.3). In order to test the involvement of NGFG_01605 in Opa-triggered invasion NGFG_01605 deletion mutants were constructed that stably expressed Opa proteins. NGFG_01605 knockout mutants were conjugated with either N931 harboring pTH6a with an opa_{50} expression cassette or N313 containing an opa_{57} expression cassette on pTH6a, yielding strains N2020 and N2021, respectively (Table 2.1). To test the Opa_{50}-triggered pathway, Chang cells were infected with Opa_{50} expressing NGFG_01605 mutants (N2020) or wild type strain. The numbers of adherent

and invasive bacteria were greatly decreased for N2020 (Fig. 3-17A). A similar result was found when CEACAM1-expressing HeLa cells were infected with Opa_{57}-expressing N2021 (Fig. 3-17B). These results demonstrated that NGFG_01605 is also involved in Opa-triggered neisserial invasion of host cells.

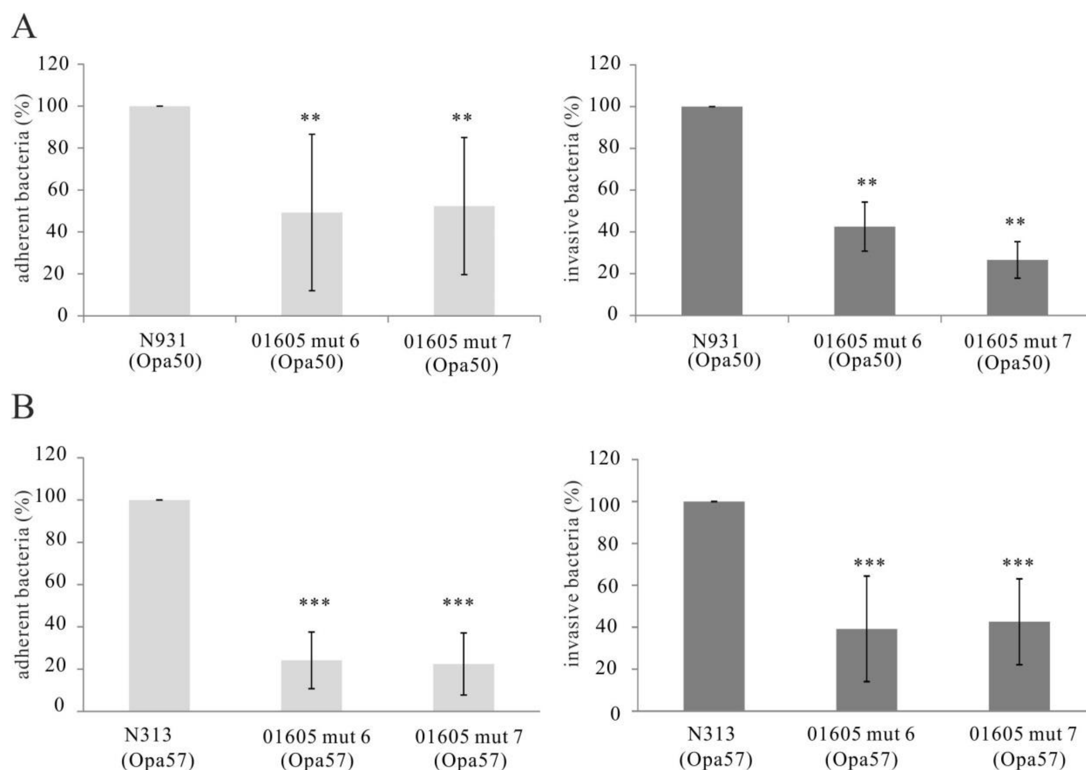

Fig. 3-17 NGFG_01605 functions in Opa-triggered *N. gonorrhoeae* internalization.
(A) Chang cells were infected with Opa_{50}-expressing strains in the 1640 medium at MOI 5 for 2 h for adherence and MOI 50 for 4 h for invasion.
(B) HeLa CEA cells were infected with Opa_{57}-expressing strains at an MOI of 5 for 2 h for adherence and MOI 50 for 2 h for invasion.
The number of adherent or invasive wild type bacteria was set to 100%. The mean ± SD of three independent experiments is shown. ** $p < 0.01$, *** $p < 0.001$.

NGFG_01605 encodes a protein of 451 aa (amino acid), here named AIF1 (**A**dherence and **I**nvasion-associated **F**actor 1). Further research on the function of the AIF1 necessitated the generation of a specific antibody. An antigenicity prediction of AIF1 was performed (ImmunoGlobe GmbH) and showed that the C-terminal part of the protein staring from aa185 ($AIF1_{185\text{-}451aa}$) was optimal for protein expression and subsequent immunization of rabbits. The corresponding DNA sequence was cloned into the vector pET28b at BamHI and HindIII restriction sites, which fused an N-terminal His-tag to the protein. The vector was transformed in

E. coli soluBL21, however, the recombinantly expressed protein was insoluble even after mild induction by 0.25 mM IPTG at 16 ℃ overnight. So the inclusion bodies of recombinant protein were purified (chapter 2.2.6.6, Fig. 3-18A) and dissolved in 7 M urea, which was removed prior to immunization by electro-elution. The resulting anti-AIF1 serum specifically detected the NGFG_01605 protein in the wild type strain N2009 and no protein was detected in the gene knockout mutant (Fig. 3-18C). In order to improve the specificity, anti-AIF1 serum was affinity purified (Fig. 3-18D). Besides, the whole AIF1 protein fused with His-tag on the N-terminus was successfully purified for further study (Fig. 3-18B).

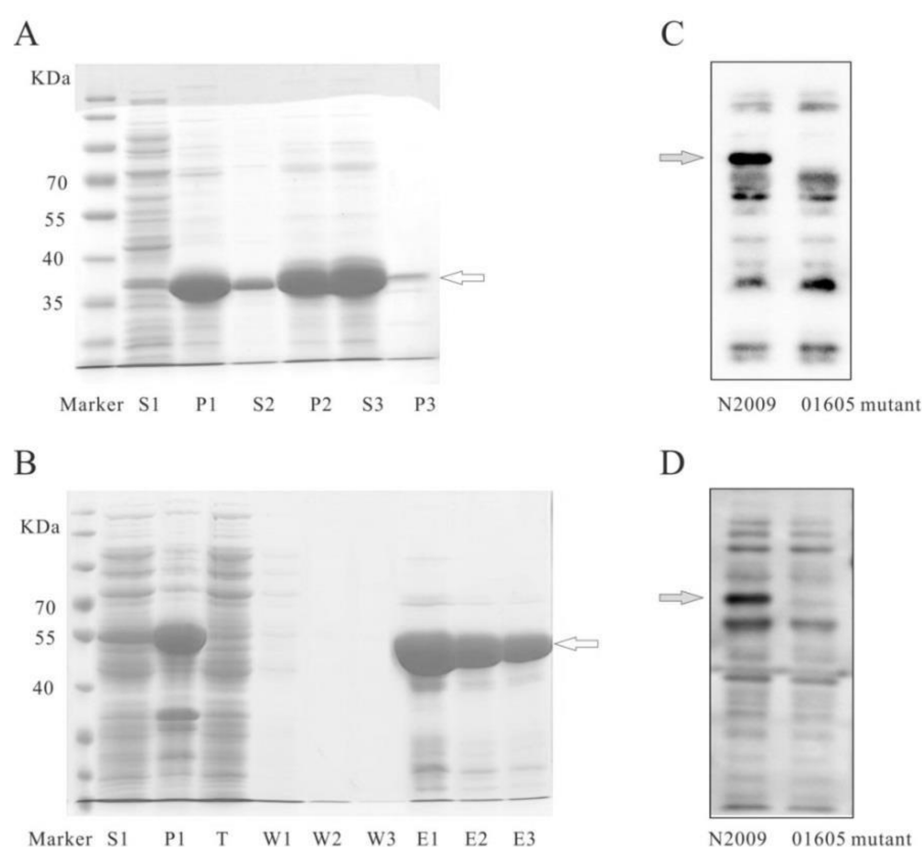

Fig. 3-18 Purification of AIF1$_{185\text{-}451aa}$ inclusion bodies (A), recombinant AIF1 protein (B) and specificity of anti-AIF1 antibody before (C) and after (D) affinity purification.

(A) Isolation and purification of AIF1$_{185\text{-}451aa}$ inclusion bodies. After induction with 0.25 mM IPTG at 16 ℃ overnight, the recombinant *E. coli* were lysed by sonication. Most of the protein was detected in inclusion bodies in the pellet (P1). P1 was suspended in buffer containing 1 M urea, sonicated and centrifuged. A fraction of the protein was soluble in the supernatant (S2), whereas the majority was still unsoluble (P2). P2 was resuspended in buffer containing 7 M urea. After sonication and centrifugation, most of protein was found in the supernatant (S3). All the samples were separated by10% SDS-PAGE and stained with Coomassie blue. S: supernatant; P: pellet.

(B) Purification of recombinant AIF1 protein from *E. coli* soluBL21. The samples were separated by 10% SDS-PAGE and stained with Coomassie blue. S1: supernatant after sonication; P1: pellet after sonication; T: flow-through; W1-3: washing samples; E1-3: elution samples.
(C and D) Specificity of anti-AIF1 antibody before (C) and after (D) affinity purification. Bacterial lysates of N2009 (wt) and N2009 ΔNGFG_01605 were separated by 10% SDS-PAGE and analyzed by western blotting using anti-AIF1 antibody (C: 1:500; D: 1:1000 dilutions).

AIF1 functions in gonococcal adherence to and invasion into host cells (chapter 3.8), hence the expression dynamics of AIF1 during the infection was analyzed. The wild type strain N2009 was used to infect Chang cells at an MOI of 100. After 15, 30, 60 and 120 min, the cell-associated bacteria were collected and AIF1 expression was analyzed by Western blotting using affinity-purified anti-AIF1 antibody. With increasing infection time, AIF1 expression increased about three-fold (Fig. 3-19A). The transcription of NGFG_01605 during the infection was analyzed by real-time PCR with NGFG_01605-specific primers (L-01605-rt and R-01605-rt, Table 2.9). Contrasting the protein data, the qPCR demonstrated that the transcription of NGFG_01605 was nearly unaltered during the course of infection (Fig. 3-19B).

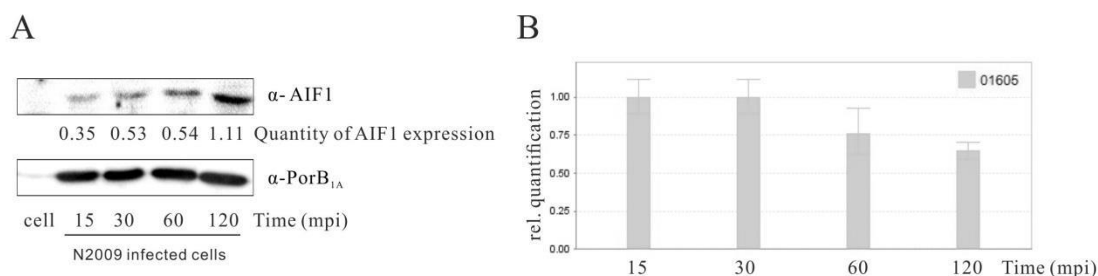

Fig. 3-19 AIF1 expression increases at protein level during the course of infection, whereas transcript levels are unaltered.
(A) Chang cells were infected with N2009 at a MOI of 100 and samples were collected at different time points. AIF1 expression was detected by anti-AIF1 antibody and quantified by ImageJ. The expression of PorB$_{IA}$ was used as loading control.
(B) NGFG_01605 transcription during infection was analyzed by real-time PCR. N2009 was used to infect Chang cells at MOI 100. Total RNA from the samples was isolated and cDNA was synthesized and qPCR was used to determine transcript abundance. *5sRNA* was used as internal standard. Experiments were repeated twice.

The sequence analysis indicates no signal peptides in AIF1 (predicted by SignalP 4.1 Server, http://www.cbs.dtu.dk/services/SignalP/) [186] and the putative localization predicted by PSORTb (http://www.psort.org/psortb/) [187] is cytoplasmic. The amino acid sequence alignment indicated NGFG_01605 encoded protein AIF1 is highly conserved in *Neisseria spp.* and more than 96 %

sequence identity are found in most homologues (Fig. 3-20). The database searching using AIF1 sequence shows its homologues occur in other bacteria of the family *Neisseriaceae* besides the genus *Neisseria*, such as the genera *Kingella* (e.g. U32 family peptidase in *Kingella kingae* ATCC 23330 with 86%/93% sequence identity/similarity), *Simonsiella* (e.g. HMPREF9021_00333 in *Simonsiella muelleri* ATCC 29453 with 86%/93%) and *Eikenella* (e.g. HMPREF1177_00155 in *Eikenella corrodens* CC92I with 86%/92%). Besides the family *Neisseriaceae*, AIF1 homologues are identified in the bacteria belonged to other family in the order *Neisseriales*, even in other order of the class β–proteobacteria the identity sequence up to 60%-80% [188,189]. It seems AIF1 is highly conserved in the evolution and may have important roles besides of virulence factor in gonococcal engulfment into host cells.

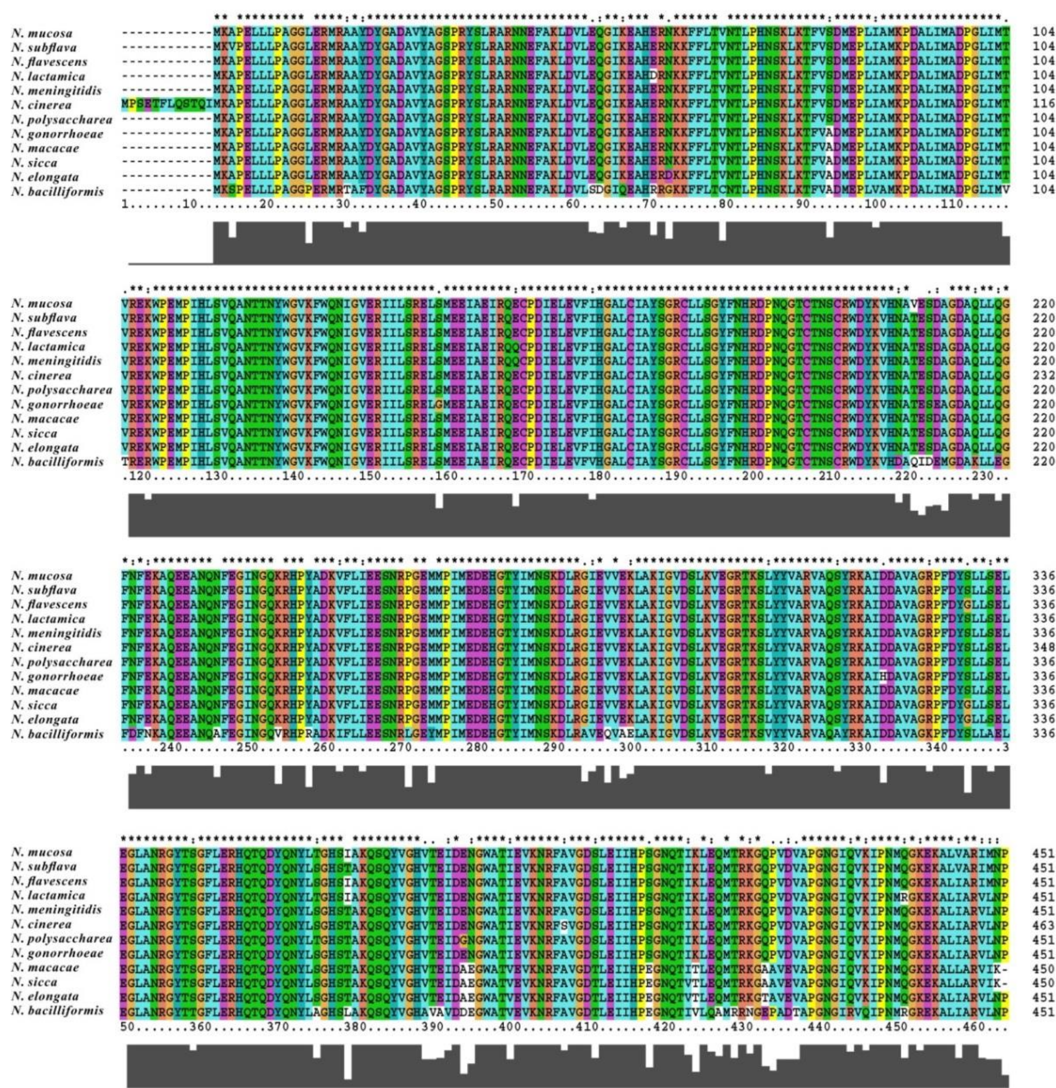

Fig. 3-20 Multiple alignments of amino acid sequence of AIF1 with its homologues in *Neisseria spp* by ClustalX2 (Accession numbers of these sequences are in Supplementary Table 6.2).

4 Discussion

4.1 Transposon mutagenesis in *N. gonorrhoeae*

DNA transposons are mobile DNA sequences in the genome and frequently found in many organisms including *Neisseria*. In *Neisseria gonorrhoeae* and *Neisseria meningitidis,* a small repetitive element, often called "Correia elements", has transposon-like properties of 25 bp inverted repeats, a TA duplication at the target site and a functional integration host factor binding site [190-192]. DNA transposons take up a high percentage in their host genome and are considered to play important roles in genetic plasticity during evolution. Besides, the transposition achieves the translocation of linear DNA fragment to a new position by recombination process which is independent of homologous sequence or any other host factors. These properties enable DNA transposons to serve as useful and powerful genetic tool which have been successfully used for transgenesis and insertion mutagenesis in a wide variety of organisms in order to identify associated genes in pathogenesis of pathogens, analyze the functional and regulatory genome and even be suggested novel methods for gene therapy.

The conjugative transposons Tn916 [193,194] and Tn1545 [195] from Gram-positive bacteria were adapted for mutagenesis in *N. meningitidis*. One study indicated that the conjugative transposon Tn916 can be introduced into different sites on the chromosome of recipient meningococci [193], but further research showed that in some cases only the tetM determinant was inserted [194]. Moreover, Thomas et al. constructed a Tn5 derivative containing a functional kanamycin resistance marker to perform *in vivo* transposon mutagenesis of *N. gonorrhoeae* [196]. The modified Tn5 is successfully integrated into the chromosome randomly but most insertions contained only incomplete transposons. Further it was found that the transposition is independent of transposase but requires RecA instead. This event was suggested to represent the outcome of some type of illegitimate recombination system in *Neisseria* rather than of transposition [196]. Another transposition system is shuttle mutagenesis with mini-transposon (mTn), mini-Tn3 [197]. There, neisserial chromosomal DNA is partially digested and cloned into plasmids which constitute a gene bank of *Neisseria* in *E. coli*. In *E. coli*, the neisserial DNA is mutated by mini-transposons containing an antibiotic marker for selection. Subsequently, the transposed

plasmids are isolated from *E. coli* and transformed into *Neisseria* where it inactivates the corresponding genes via allelic exchange [198]. This technique has been broadly applied in *Neisseria* with construction of a series of mini-transposons derivatives for different purposes, such as the creation of lacZ transcriptional fusions [199] in order to understand gene regulation mechanisms, or the production of phoA fusions [200] for identification of exported pathogenicity factors [201].

Although complete genome sequences for several gonococcal strains are available [175,176], our understanding of gene function remains limited. The lack of a suitable method for saturation mutagenesis remains a major obstacle to the unraveling of the pathogenicity of *N. gonorrhoeae*. The methods described above are not suitable, because *i)* transposition by conjugative transposons is not perfectly stable [194] and displays only low transposition frequency, *ii)* transposition of GC with a Tn5 derivative *in vivo* does not work properly [196], and *iii)* although shuttle mutagenesis can be used for generation of a large pool of mutants, some neisserial genes are difficult to clone in or are even lethal for *E. coli*.

In vitro transposition systems using DNA transposons like Tn7, Tn5 and Himar1 mariner have been successfully applied for the mutagenesis of naturally competent bacteria, such as *Haemophilus influenza* [174,202], *Streptococcus pneumoniae* [174] and also *Neisseria meningitidis* [203,204]. Thereby a PCR-amplified DNA segment or isolated chromosomal DNA is mutated *in vitro* by transposons and a purified transposase. After mutagenesis, the DNA is transformed back to the target bacteria. In the bacteria genes are inactivated via allelic exchange with their mutagenized counterpart. This technique makes the transposition stable due to the omission of transposase *in vivo* and does not require the cloning of target DNA in *E. coli*. Therefore it has developed into a standard for the generation of genome-wide mutagenesis libraries in many microorganisms [174,202-204]. Combined with PCR-based amplification methods and microarrays, such as transposon site hybridization (TraSH) [204] and transposon-based genetic footprinting [174,205], or using the NGS-based technologies, such as Tn-seq [148] or TraDIS [147], high-through transposon mutant libraries were applied to rapidly identify mutants with fitness deficits in various conditions to predict gene function and genetic interaction.

In this study, Tn5 was used to create a saturated transposon insertion library in *N. gonorrhoeae*. *Neisseria* chromosomal DNA was isolated and incubated with purified transposase and equal molarity of a Tn5 derivative containing a kanamycin resistance selection marker. Transposons were randomly inserted into chromosomal DNA. Subsequently, the transposed DNA was reintroduced into *N. gonorrhoeae* by natural transformation and the bacteria were selected for kanamycin resistance (Fig. 3-1A). In order to increase the efficiency of mutagenesis in *Neisseria*, the *in vitro* transposition and natural transformation to *Neisseria* are critical steps. Attempts with fragmentation of genomic DNA for *in vitro* transposition and/or introduction of *Neisseria* DNA uptake sequence (DUS) in Tn5 element did not improve the efficiency of mutagenesis in *Neisseria* (data not shown). Further it was found that the quality and purity of isolated genomic DNA is critical for the efficiency of mutagenesis since purification of genomic DNA with affinity columns led to a higher insertion frequency when compared to phenol-chloroform-based DNA extraction most likely because of the inhibition of transposition by remaining phenol or ethanol. Another important factor is the recover efficiency of the mutated DNA in the steps after transposition *in vitro* as well as after gaps filling up steps. Here, we adopt phenol-chloroform extraction and ethanol precipitation instead of column-based method in order to increase the recover efficiency (chapter 2.2.7). One round transformation of mutated DNA into *N. gonorrhoeae* yielded about 20,000 kanamycin-resistant transformants. By pooling of mutants from six independent mutagenesis rounds the final library comprising about 100,000 colonies was obtained. Southern blot analysis confirmed that Tn5 transposition in *N. gonorrhoeae* is random and only one insertion site occurs in each bacterial colony [159]. Transposon insertion sites were then identified by Illumina sequencing (Fig. 3-5 and Table 3.1) and the data demonstrate that the library is the result of saturated mutagenesis in *N. gonorrhoeae*. Compared with the previous similar works in *N. meningitidi*s, 14,000 Tn5 insertional knockouts [204] or 10,000 single colonies with Himar1 mutagenesis [203] was obtained across the whole genome. In *N. gonorrhoeae*, Kline, K. A et al performed transposon mutagenesis on upstream of *pilE* gene to identify DNA sequences that facilitate pilin antigenic variation [206]. Chen, A et al performed *in vitro* saturating mutagenesis on *porB* gene to identify essential versus mutable residues in gonococcal porin [207]. However, this study is the first time to perform a genomic-scale mutational analysis of this important human pathogen *N. gonorrhoeae.*

Further, quality control of the sequencing reads made by Fast QC indicated that the sequence quality was sufficient for further analysis (Fig. 3-4). Then the bias of transposon insertions was tested by analysis of the distribution of TIS, for example, the distribution across the whole genome of MS11 (Fig. 3-5A), on the non-coding region and coding region (Fig. 3-5B), and the distribution in the single gene (Fig. 3-5C). All the indexes showed there was no bias of transposon insertions in our *Neisseria* genome library. Therefore, this saturate mutagenesis library combined with high-throughput sequencing system will be a powerful tool for the system-level understanding of gonococcal physiology and pathogenesis.

4.2781 essential genes in *N. gonorrhoeae*

The emergence and spread of multidrug-resistant *gonococci* became a major public health challenge as the loss of treatment options will significantly increase morbidity and mortality in the future (chapter 1.1.2). A straight forward approach for the identification of potential new drug targets is the identification of essential gonococcal proteins. In order to screen the genes which are critical to survival of *N. gonorrhoeae*, the genome-wide transposon mutagenesis library in *N. gonorrhoeae* strain MS11 was screened for genes which demonstrated a relative depletion of TIS indicative of important functions for bacterial growth.

As is shown in Fig. 3-7, almost no insertions were detected in the CDS and promoter of gene NGFG_00153, but in contrast the adjacent genes NGFG_00152 and NGFG_00154 displayed high insertion density. NGFG_00153 encodes triosephosphate isomerase, which is required for glycolysis, the main pathway of energy production [178]. The absence of TIS is the direct result of the inability of mutants within the locus to grow on selective agar plates. Hence these mutants are lost from the library leading to the absence of Tn-seq reads in this genomic region, and therefore demonstrate that NGFG_00153 is essential for *Neisseria* viability. Genes with strongly reduced TIS thus might be necessary for *Neisseria* growth or survival and therefore comprise candidate essential genes. Due to a very high coverage of TIS in most genes, a statistical analysis can clearly assess the essentiality of genes, by calculation of a P-value [152]. The algorithm, developed by Christian Remmele (Bioinformatics, University of Würzburg) assumes that the transposon is randomly and uniformly inserted across the whole genome and every mutant has the same fitness

under the selective condition. However, this evaluation does not consider polar effects of the transposon insertion on downstream essential genes in operons. Because the transcriptional terminator of Tn5 might terminate transcription of the downstream gene, the mutagenized gene might be false-positively identified as essential, although the absence of the gene product of the essential downstream gene led to the phenotype. To avoid this misclassification, the location of the genes in operons was taken into account. Only genes with $P < 0.05$ not within operon structures were analyzed which resulted in a candidate list of 480 genes (Fig. 3-6B; Table S1). Besides, the sequencing reads of duplicated genes are randomly and equally mapped to repeated DNA sequences on the genome, so the original and true insertion sites on single gene cannot be identified by this technology and individual validation is needed.

Besides of the well-known essential genes required for bacterial fundamental biological processes, *Neisseria*-specific genes which have been previously identified as essential also were identified in our dataset, such as outer membrane proteins PorB [181,182], Omp85 [180], and the alternative σ factor RpoH [169]. Notably, 120 predicted essential genes are of as of yet unknown function, which might be candidates for targets of anti-gonococcal drugs. For testing the essentiality of these genes, conditional knockouts were performed. The native promoter of essential gene was exchanged with the IPTG-inducible promoter P_{trc} and gene expression was conditionally inhibited by omission of IPTG in the growth medium. The assay confirmed essentiality of the ribosomal protein encoded by NGFG_00442-00443 for *Neisseria* survival (Fig. 3-8). By contrast, promoter replacement failed for the genes NGFG_01725 (PorB), NGFG_01315 and NGFG_00686. Possibly the promoter regions themselves could be essential or fulfill other important functions and thus do not allow to be exchanged (Table 3.3). Moreover, the mutants in the hypothetical proteins NGFG_02103, NGFG_04144 and NGFG_00007 still grew on agar plates lacking IPTG (Fig. 3-8). The gene expression of the mutants with or without IPTG induction was determined by RT-PCR (Fig. 3-9). The results indicate that the P_{trc} promoter is leaky leading to a base level transcription of the P_{trc}-controlled gene even in absence of IPTG, which in turn might provide be enough gene product to support *Neisseria* survival and growth. In other studies, the conditional knockout of *Neisseria relA* [168] and *rpoH* [169], employed two tandem *lac* operator sequences in order to enhance the repression of an uninduced promoter [208], but the leakage problem still existed.

Thus conditional knockouts can only be used for validation of essential genes whose products are needed in a large amount for bacteria survival, such as ribosomal proteins. Since the limitation of conditional knockout assay, genetic footprinting (Fig. 3-10) as well as gene knockout trials (Table 3.4) were performed to experimentally verify essentiality of candidates genes identified by the Tn-seq screen. The experimental data were highly coincident with statistic predictions based on p-value of the genes for 11 essential genes and 17 non-essential genes with the exception of a single non-essential gene, NGFG_01266 (hypothetical protein; P=0.05007). NGFG_01266 knockout mutants grew well on agar plates. However the growth curve measured in PPM medium indicated that the mutants are deficient in growing in liquid medium (Fig. 3-14A). Interestingly, this difference was reflected in the p-value, the calculation of which is based on the mutants' growth on agar rather than growth in liquid medium.

Some predicted essential genes might not only affect gonococcal fitness, but may lead to direct killing of the mutagenized bacteria. One example is the antitoxin gene NGFG_00971 (P=0.0011). The co-transcribed genes NGFG_00971 (hypothetical protein) and NGFG_00972 (hypothetical protein, P=0.13597) were identified as a toxin-antitoxin (TA) pair with the RASTA-Bacteria prediction tool (Rapid Automated Scan for Toxins and Antitoxins in Bacteria, http://genoweb1.irisa.fr/duals/RASTA-Bacteria) [209]. Inactivation of antitoxin gene NGFG_00971 may release the toxic activity of NGFG_00972 which will cause the death of NGFG_00971 mutants whereas mutants within the toxin gene NGFG_00972 will grow well. Other interesting examples are the predicted essential phage associated proteins, NGFG_00630 (homologous to NGO0479 located on the prophage island NgoΦ1 of strain FA1090 [210]) and NGFG_02188 (homologous to NGO1116 on the prophage island of NgoΦ2 in strain FA1090 [210]). NGO0479 and NGO1116 are homologues of the lambda repressor cI which reactivates a lysogenic phage. When NGO0479 and NGO1116 were expressed in *E. coli,* the expression inhibited the growth of *E. coli* and the propagation of phage lambda [210]. Besides, it was reported that phage repressors can be regulate host genes expression in the lysogenic cells [211]. It was found that NGO1116 was able to inhibit transcription of *N. gonorrhoeae* genes and *Haemophilus influenzae* HP1 phage promoters [210]. NGFG_01287 (homologous to NGO0509 of NgoΦ1 in FA1090 [210]) and NGFG_02185 (homologous to NGO1119 of NgoΦ2 in FA1090

[210]) belong to the transcriptional regulator family which is critical in the lysogenic stage of neisserial phages. Therefore these phage associated genes might regulate lysogenic phages or other neisserial genes which lead to death of mutants.

4.3 Screening for DGI virulence factors

To screen for *N. gonorrhoeae* adhesins and invasins involved in gonococcal internalization into human epithelial cell during low phosphate-dependent invasion (LPDI), a genome-scale ‘‘negative selection’’ technology was applied [146] (Fig. 3-11). A saturated Tn5 mutagenesis library was successfully established in *N. gonorrhoeae* N2009, a MS11 derivative strain expressing $PorB_{IA}$, the hitherto only confirmed factor required for *N. gonorrhoeae* LPDI. A 100-fold representation of each mutant within the library was used for infection of Chang cells at an MOI 100 for 1 h. Adherent or invasive bacteria were recovered on agar and the recovered bacteria were used in subsequent repetitions of the infection assays in order to deplete mutants from the library that were unable to adhere to or invade into Chang cells. The chromosome-transposon junctions from these libraries were PCR-enriched, barcoded and sequenced by massively parallel sequencing. The sequencing reads were separated according to the barcode and mapped to the genome to identify the TIS (Table 3.4). Again, P-values were determined for each gene to evaluate the gene’s fitness/essentiality in the adherence or invasion process. Genes with $P < 0.05$ were hypothesized to be functional in LPDI. Among these the candidate essential genes (chapter 3.3) are excluded from further analysis, because the essentiality would cause depletion in the output libraries obtained in the LPDI invasion screen. Of the remaining 1745 non-essential genes 98 may function in gonococcal attachment to host cell under low phosphate condition (Table 3.6). These include type IV pilus-associated proteins, lipoproteins, integral membrane protein, diverse enzymes, 8 phage proteins and 15 hypothetical proteins. The 43 candidate invasion factors include various enzymes, 6 unknown proteins, 3 phage proteins and 3 ABC transporter associated proteins (Table 3.7). LPDI is a complicated process starting with bacterial attachment to the host cell surface, engulfment by the cells and intracellular survival. The identified factors may participate in one of these three steps. This hypothesis was confirmed by validating a selection of candidates. Therein the candidate gene was deleted via allelic exchange and the resulting mutants were used within

gentamicin protection assay to test their adherence and their host cell invasion rates. The results indicated the hypothetical protein encoded by NGFG_00506 functions in the invasive process not adherent stage or intracellular survival. By contrast another hypothetical protein NGFG_01266 suggests a more important role in gonococcal survival rather than in infection, whereas genes NGFG_01605, a predicted protease, and NGFG_01643, an ABC transporter ATP-binding protein/permease, participate in both adherence and invasion (Fig. 3-13 and 3-14).

It is interesting that phospholipase D (PLD, encoded by gene NGFG_00827) mutants were impaired in their ability to adhere to and invade into epithelial cells in LPDI (Fig. 3-14B). A previous study showed that *N. gonorrhoeae* secretes PLD to augment complement receptor 3 (CR3)-mediated endocytosis of primary cervical epithelial cells [212] by interacting with Akt kinase in a PI3 kinase-independent manner [213]. It remains to be elucidated, however, if PLD functions in gonococcal LPDI with a similar signaling pathway.

Another interesting finding is that infection of the host cells with mutants in NGFG_04218 (hypothetical protein) showed significant decrease in the number of the cell-associated bacteria (adherent and invasive bacteria, Fig. 3-14C). NGFG_04218 is a newly annotated gene (our unpublished observations) that has many copies in the MS11 genome. Notably, the reverse-complementary sequence of NGFG_04218 is homologous to *maf*I (NGO1066) in FA1090 and the upstream region of NGFG_04218 is the coding regions of NGFG_04217 (MafA adhesin, homologous to NGO1067 in FA1090) and NGFG_00672 (MafB family adhesion protein, homologous to NGO1068 in FA1090). The deletion of NGFG_04218 may influence the expression of NGFG_04217 and NGFG_00672, but it is difficult to quantify the transcription of NGFG_04217 and NGFG_00672 within NGFG_04218 mutants because both genes have many copies in the MS11 genome. Multiple sequences homologous to both, mafB and the adjacent gene mafA, are present in the pathogenic species *N. gonorrhoeae* and *N. meningitidis,* and also in the commensal species *N. lactamica*. A previous study indicates that gonococci bind to gangliotetraosylceramide [GgO4, Gal(β1-3) GalNAc (β1-4) Gal(β1-4) Glc(β1-1)Cer], isoglobotriaosylceramide [Gal(α1-3) Gal(β1-4) Glc(β1-1) Cer], gangliotriaosylceramide [GgO3, GalNAc (β1-4) Gal(β1-4) Glc(β1-1) Cer] and lactosylceramide [LacCer, Gal(β1-4) Glc(β1-1) Cer]. The latter two glycolipids are found in glycolipid preparations from ME180 cells, an epithelial cell

line derived from a human cervical carcinoma and the glycolipid-binding proteins on the surface of GC are distinct from pili and Opa proteins [214,215]. The glycolipids, LacCer, GgO3 and GgO4 share lactose as the core sugar moiety. The GC gene encoding the GgO4-binding adhesin is identified with a size of 36 kDa [216] which might be MafA according to the molecular weight. Research within the laboratory of Thomas F Meyer revealed that the glycolipid adhesin is part of a multiple adhesin family (Maf) exhibiting different binding specificities [217]. Further, mafB2 (NGO1587) was found up-regulated upon adherence to the endocervix-derived cell line A431 which suggests requirement of mafB2 for infection [169]. A recent study further showed repression of MafA1 and MafA2 (encoded by NMB0375 and NMB0652 in MC58) by the regulator NadR in *N. meningitidis* was in response to signals present in human saliva thus enabled *N. meningitidis* to adapt to the relevant host niche [218].

4.4 Characterization of NGFG_01605

NGFG_01605 mutants showed strongly diminished adherence to and invasion into human epithelial cells in the $PorB_{IA}$-triggered pathway under low phosphate conditions (Fig. 3-13B) as well as in Opa-dependent pathways (Fig. 3-17). In order to check whether the decreased number of recovered bacteria in infections was due to a decreased fitness of the mutants, the bacterial growth was monitored under different conditions. However, NGFG_01605 mutants did not show a growth phenotype in rich media such as GC agar plates (P=1 in the input library; Table S1) or PPM liquid medium (Fig. 3-13B). Further, the differential immunostaining assay confirmed that the decrease of recovered bacteria was indeed due to the deficiency in invasion rather than intracellular survival (Fig. 3-15B). Furthermore, the ratio of invasive to adherent bacteria as determined by gentamicin assays was less than half of the wild type strain, which demonstrated that NGFG_01605 was involved in gonococcal invasion as well as adherence (Fig. 3-16A). Pili-dependent initial attachment to host cells was not disturbed in NGFG_01605 mutants (Fig. 3-16B), so that the influence of the knockout on neisserial adherence was due to other unknown reasons. Further, the transcription of NGFG_01605 at 15, 30, 60 and 120 min post infection was analyzed by real-time PCR and was found to remain unaltered at a similar level during the course of infection (Fig. 3-19B). By contrast the amount of AIF1 protein encoded by NGFG_01605 was

detectably increased (Fig. 3-19A) which suggested an involvement of AIF1 in gonococcal infection. The regulation of AIF1 is more likely at post-transcription level.

AIF1 is annotated as a putative protease or U32 family peptidase with a hitherto unknown catalytic type. It is characterized by the consensus sequence E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S containing two active site cysteine residues [219]. The prototype of this family is PrtC from *Porphyromonas gingivalis*. It has been characterized biochemically as a Ca^{2+}-dependent collagenase and degrades type I collagen leading to periodontal tissue destruction [220]. Another interesting member of this group is a secreted collagenase, encoded by *hp0169* in *Helicobacter pylori.* It has been identified and functionally verified as a new essential virulence factor for *H. pylori* stomach colonization. However, the alignment between these two proteins and AIF1 reveals only moderate sequence identity and similarity with PrtC (25%, 45%) and with HP0169 (37%, 54%). The predicted protease or collagenase activity of AIF1 should be confirmed by experimental verification. The known virulence factor with protease activity in *Neisseria* is IgA1 protease which is a secreted serine protease found in all pathogenic *Neisseriae*. IgA1 protease specifically cleaves mucosal immunoglobulin A1 to escape host immune response [100] and degrades LAMP1 to promote neisserial intracellular survival within epithelial cells [102,103]. However, a detailed sequence analysis indicates the absence of signal peptides in AIF1 and the putative localization predicted by PSORTb is cytoplasmic. Therefore, AIF1 is likely involved in gonococcal infection via regulation of bacterial intracellular factors.

4.5 Prospects

The identified essential genes from this work provide a large list of potential targets for the development of vaccines or anti-gonococcal drugs. For example, the candidate essential genes with enzymatic functions could be used to develop new drugs to inhibit their activity. Further, it will significantly reduce the occurrence of new resistance if the new drugs can target more than one essential gene products. In addition, the list of essential genes contains 120 genes with hitherto unknown function. Because these genes as of yet have no homologous genes in any organisms, their functions can not be predicted from homologues but it can be speculated via

structural analysis of purified protein products and also the structural analysis might give some hits for new drug design.

The data sets obtained within the present study revealed gonococcal factors involved in adherence to and invasion into host epithelial cells in a phosphate sensitive condition. The candidate genes have been validated and NGFG_01605 encoding a predicted protease was confirmed to participate in gonococcal engulfment to host epithelia cells not only in $PorB_{IA}$-triggered pathway, but also in Opa_{50} and Opa_{57} mediated *Neisseria* internalization. Therefore, it might be a common and important factor involved in various routes of neisserial infections. The putative protease activity of AIF1, the NGFG_01605 gene product, should be tested with protease activity assays, such as azocasein assays, and gelatin or casein zymography. The localization of AIF1 can be investigated through immunofluorescence staining of reporter gene fusion protein or testing AIF1 expression in separated cell components. The interaction partner of AIF1 could provide critical information to elucidate the molecular mechanism works in the infection process, which may be investigated by co-immunoprecipitation.

Further, the established method of Tn-seq in *N. gonorrhoeae* can be easily applied to identify other novel gonococcal factors in a variety of environments, such as different growth conditions, host cell death or some available infection models. The disadvantage of Tn-seq is supposed to be the PCR amplification step in template preparation which may introduce amplification bias or create mutations. Newly developed sequencing technologies, so called “third generation sequencing”, can perform single molecule sequencing to circumvent any amplification step [221]. Additionally, a set of improvements to the standard Illumina protocols may reduce bias and reliably obtain high yields of data [222]. Finally, the Tn5 transposon and *in vitro* transposition can be used in many other microorganisms, which are refractory to *in vivo* mutagenesis.

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6 Appendix

6.1 Abbreviations

A	ampere
aa	amino acid
Ac	activator element
AHU	Arg-Hyx-Ura
AIF1	adherence and invasion-associated factor 1
APS	ammonium persulfate
Arg	Arginine
ASM	acidic sphingomyelinase
ATP	adenosine triphosphate
ble	bleomycin
bp	base pair
BSA	bovine serum albumin
C4bp	C4b-binding protein
cDNA	complementary DNA
CDS	coding sequence
CEACAM	carcinoembryonic antigen cellular adhesion molecule
CFU	colony forming unit
CHO	Chinese hamster ovary cell
CNBr	cyanogen bromide
cPCR	colony polymerase chain reaction
CR3	complement receptor 3
C-terminal	carboxy-terminal
dA	deoxyadenosine
Dam	deoxyadenosine methylase
Dc	dissociation element
ddNTPs	dideoxynucleotides
DGI	disseminated gonococcal infections
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTPs	deoxynucleotides
DOC	sodium deoxycholate
DTT	dithiothreitol
DUS	*Neisseria* DNA Uptake Sequence
E. coli	*Escherichia coli*
e.g.	Example gratia, for example

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ES	end sequences
FCS	fetal calf serum
FI-dNTPs	fluorescently-labeled nucleotides
FU	fluorescence unit
GC	*Neisseria gonorrhoeae*, gonococcus
GC-kan	GC agar plates supplemented with kanamycin
gDNA	genomic DNA
Gp96	glycoprotein 96
h	hour
H. pylori	*Helicobacter pylori*
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	human foreskin fibroblast cells
His	histone
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSP	heat shock protein
HSPG	heparan sulfate proteoglycan
IE	inside end sequences
IF	immunofluorescence staining
IgA1	immunoglobulin A1
IGB	Integrated Genome Browser
Inh	transposition inhibitor
IP	immunoprecipitation
IPTG	isopropyl-D-thiogalactopyranoside
kan	kanamycin
kb	kilobase
kDa	kilodalton
L	liter
LAMP1	lysosomal-associated membrane protein 1
LB	lysogeny broth
LINEs	long-interspersed nuclear elements
LOS	lipooligosaccharide
LPDI	Low phosphate-dependent invasion
LPS	lipopolysaccharide
LTRs	long terminal repeats
M	mol/L
maf	multiple adhesin family
ME	mosaic end sequences
min	minute(s)
MOI	multiplicity of infection
MPS	massively parallel sequencing

mTn	mini-transposon
MWCO	molecular weight cut-off
N. gonorrhoeae	*Neisseria gonorrhoeae*
N. meningitidis	*Neisseria meningitidis*
N.lactamica	*Neisseria lactamica*
NADH	nicotinamide adenine dinucleotide
NGS	next generation sequencing
NIH 3T3	mouse embryonic fibroblast cells
N-terminal	amino-terminal
OD	optical density
OE	outside end sequences
Omp85	outer membrane protein 85
OMV	outer membrane vesicle
Opa	opacity-associated proteins
P^-	non-piliated phenotype
P^+	piliated phenotype
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC-PLC	phosphatidylcholine-specific phospholipase C
PFA	paraformaldehyd
PI(3,4,5)P	phosphatidylinositol 3, 4, 5-phosphate
PI3K	phosphatidylinositol-3' kinase
PID	pelvic inflammatory disease
pilE	pilin expression locus
pilS	silent pilin loci
PKD1	PKCμ, protein kinase C μ
PLC ɣ1	phospholipase Cɣ1
PLD	phospholipase D
PMN	polymorphonuclear leukocyte
PorB	outer membrane porin protein B
$PorB_{IA}$	PorB serotype A
$PorB_{IB}$	PorB serotype B
Porin	pore-forming proteins
PPM	proteose peptone medium
P^s	S-pilin, a soluble form of pilin
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative real-time PCR
Rac1	ras-related C3 botulinum toxin substrate
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
s	second
SD	standard deviation

SDS	sodium dodecyl sulphate
SINEs	short-interspersed nuclear elements
siRNA	small interfering RNA
SREC-I	scavenger receptor expressed on endothelial cells I
sRNA	small non-coding RNA
STI	sexually transmitted infection
str	streptomycin
TbpB	transferrin receptor protein
TCA	trichloroacetic acid
TE	transposable element or transposon
TEMED	tetramethylethylenediamine
Tfp	type IV pili
TIM	triosephosphate isomerase
TIRs	terminal inverted repeats
TIS	transposon insertion site
Tn	transposon
Tnp	transposase
Tn-seq	transposon sequencing
TraDIS	transposon directed insertion-site sequencing
TraSH	transposon site hybridization
TSD	target site duplication
U	enzyme unit
UEC	urethral epithelial cell
UV	ultra violet
V	volt
v/v	volume per volume
VDAC	mitochondrial voltage-dependent anion channels
VRPs	viral replicon particle
w/v	weight per volume
WB	western blotting
WHO	World Health Organization

6.2 Supplementary materials

Table 6.1 Available *N. gonorrhoeae* genome sequences

Strain	GenBank ID
N. gonorrhoeae FA1090	AE004969.1 [a]
N. gonorrhoeae NCCP11945	CP001050.1 [175]
N. gonorrhoeae NCCP11945 plasmid pNGK	CP001051.1
N. gonorrhoeae TCDC-NG08107	CP002440.1 [176]
N. gonorrhoeae TCDC-NG08107 plasmid pNGTCDC08107	CP002441.1
N. gonorrhoeae MS11	CP003909.1 [b]
N. gonorrhoeae MS11 plasmid pMS11	CP003910.1

[a] submitted by University of Oklahoma, unpublished.

[b] submitted by Broad Institute, release date 10/19/2012.

Table 6.2 AIF1 homologues from different *Neisseria spp* used for multiple alignments

Strain	Accession number
Neisseria mucosa	WP_003748589.1
Neisseria subflava	WP_004519683.1
Neisseria flavescens	WP_003684307.1
Neisseria lactamica	WP_004048244.1
Neisseria meningitidis	WP_0022236041.1
Neisseria cinerea	WP_003677710.1
Neisseria polysaccharea	WP_003753715.1
Neisseria gonorrhoeae	EEZ48438.1
Neisseria macacae	WP_003777098.1
Neisseria sicca	WP_003768744.1
Neisseria elongata	WP_003771571.1
Neisseria bacilliformis	WP_007342950.1

6.3 Publications and presentations

Publications

Christian W. Remmele*, **Yibo Xian***, Marco Albrecht*, Michaela Faulstich, Martin Fraunholz, Elisabeth Heinrichs, Marcus T Dittrich, Tobias Muller, Richard Reinhardt and Thomas Rudel (2014). Transcriptional landscape and essential genes of *Neisseria gonorrhoeae*. * authors contributed equally, Nucleic Acids Research, in revision

Michaela Faulstich, Franziska Hagen, Elita Avota, Ann-Cathrin Winkler, **Yibo Xian**, Sibylle Schneider-Schaulies and Thomas Rudel (2014). Neutral sphingomyelinase 2 is a key factor for invasion of *N. gonorrhoeae* associated with disseminated infection. Cellular Microbiology, in revision

Patent applications

Yibo Xian, Christian W. Remmele, Marco Albrecht, Michaela Faulstich, Martin Fraunholz and Thomas Rudel (2014). Essential genes of *Neisseria gonorrhoeae* as candidates for drug or vaccine development. patent pending

Poster Presentations

Yibo Xian, Christian Remmele, Michaela Faulstich, Martin Fraunholz, Richard Reinhardt and Thomas Rudel. A high density transposon library identifies essential genes in *Neisseria gonorrhoeae.* 3rd Mol Micro Meeting 2014, Wuerzburg

Yibo Xian, Michaela Faulstich, Marco Albrecht, Christian Remmele, Martin Fraunholz and Thomas Rudel. Pool screen of a gonococcal high density transposon library to identify novel virulence factors. XVIIIth International Pathogenic Neisseria Conference (IPNC) 2012, Wuerzburg

Yibo Xian, Michaela Faulstich, Marco Albrecht, Christian Remmele, Martin Fraunholz and Thomas Rudel. Pool screen of a gonococcal high density transposon library to identify novel virulence factors. EPOS-Everything's Part Of Science, 7th International Symposium organized by the students of the Graduate School of Life Sciences (GSLS), 2012, Wuerzburg

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6.5 Declaration of independence

I hereby declare that my thesis entitled:

Identification of essential genes and novel virulence factors of *Neisseria gonorrhoeae* by transposon mutagenesis

is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 27.6.2014

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