Characterization of *Neisseria wadsworthii* 9715

Phenotypic Analyses and Assess of the Natural Competence for Transformation of the *Neisseria* species *Neisseria wadsworthii*

by

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Master program in Biomedicine
Department of Health Sciences

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PREFACE AND ACKNOWLEDGMENT

This work was carried out at the Institute of Molecular Microbiology, Centre for Molecular Biology and Neuroscience (CMBN), at Oslo University Hospital (Rikshospitalet), during the period of August 2012 to May 2013, for the Master's degree in Biomedicine at Oslo and Akershus University College of Applied Sciences.

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TABLE OF CONTENTS

Abstract

The bacterium *Neisseria wadsworthii* (Nw) belong to the genus *Neisseria* in the family *Neisseriaceae.* Two novel strains of Nw, isolated from clinical specimens have been identified and have been only poorly characterized *Neisseria* species so far. *N. meningitidis*, the meningococcus (Mc) on the other hand, is a well-characterized *Neisseria* specie that colonizes the upper respiratory tract, without causing disease. However, for some unknown reasons Mc has the ability to become invasive and cause infection in humans. Mc and the closely related *N. gonorrhoeae*, the gonococcus (Gc) are collectively termed as the pathogenic *Neisseria* species. Mc and Gc are naturally competent for transformation throughout its whole life. The competence for transformation is considered to be essential for genome stability and maintenance and the survival of the bacteria. The ability of the bacteria to transform exogenous DNA plays an important role in their fitness and survival in the human host.

The overall goal of this work was to characterize Nw 9715 in regard to phenotypic traits, natural competence for transformation and a potential DNA uptake sequence (DUS) dependence. Phenotypic identification methods, molecular biological and bioinformatics methods were employed to search for natural competence for transformation in this bacterium. Findings on both phenotypical, molecular biological and bioinformatics level would increase the understanding of Nw 9715 functions.

This study established a rapid procedure to detect natural competence for transformation within Nw. The strain Nw 9715 was used throughout this study and was easily transformed with DNA harbouring a point mutation conferring antibiotic resistance to streptomycin, which was used as a marker. In addition, this strain was transformable with streptomycin-resistant (SR)-PCR fragment and plasmid DNA and found to be naturally competent for transformation in a DNA uptake sequence (DUS)-dependent manner.

The pilus subunit, PilE is one of the major components involved in the Type IV biogenesis in the *Neisseria* pathogens. SDS-PAGE and immunoblot

analysis were performed to observe the expression of PilE in Nw 9715. The analysis revealed that Nw expresses PilE which was detected with cross reactive antibodies directed against Mc pilin.

The Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometer (MS) based Biotyper database was extended with currently poorly or non-represented *Neisseriaceae*. Mass spectra from several *Neisseria*, available in the laboratory, together with Nw 9715 were obtained and edited with the Micro Flex Control setup program and were further calibrated and corrugated by applying the Flex Analysis program. The spectra for the strains were successfully added to the Biotyper 3.1 database and the database was tested for performance.

Sammendrag

Bakterien *Neisseria wadsworthii* (Nw) tilhører slekten *Neisseria* i familien *Neisseriaceae*. To nye stammer av Nw, isolert fra kliniske prøver ble identifisert og har bare vært de dårligst karakteriserte *Neisseria*-arter så langt. *N. meningitidis*, meningokokker (Mc) er en godt karakterisert *Neisseria*-art som koloniserer de øvre luftveier uten å forårsake sykdom. Av ukjente årsaker har Mc evne til å bli invasiv, og dermed forårsake sykdom hos mennesker. Mc og den nært slektede *N. gonorrhoeae*, gonokokker (Gc) er kollektivt betegnet som den patogene *Neisseria*-arter. Mc og Gc er naturlig kompetent for transformasjon gjennom hele sin levetid. Denne evnen er antatt å være viktig for genom vedlikehold, stabilitet og overlevelse av bakterien. Denne muligheten transformasjon gir til å ta opp DNA fra omgivelsene spiller en viktig rolle for overlevelsesevne til bakterien i mennesket.

Det overordnede målet med dette studiet var å karakterisere Nw 9715 i forhold til fenotypiske trekk, naturlig kompetanse for transformasjon og en potensiell DNA opptak sekvens (DUS)-avhengighet Både fenotypiske identifikasjons metoder samt, molekylærbiologisk og bioinformatiske analyser ble benyttet for å studere naturlig kompetanse for transformasjon i denne bakterien. Dermed kan funnene fra både fenotypiske, molekylærbiologisk og bioinformatiske analyser benyttes for å øke forståelsen av Nw 9715 funksjoner.

Dette studiet etablerte en rask prosedyre for å oppdage naturlig kompetanse for transformasjon i Nw. En enkelt stamme av Nw 9715 ble brukt i denne studien og den har vist seg å være naturlig kompetent for transformasjon. Analysene viste at Nw lot seg transformere med DNA med punktmutasjon som resulterte i streptomycin-resistens, som ble brukt som en markør. I tillegg har denne stammen vist seg å være kompetent for transformasjon med streptomycin-resistent (SR)-PCR-fragment og plasmid DNA og transformasjon er DUS-avhengig.

Protein-analyser ble utført på en komponent som er involvert i syntese av Type IV pili i patogene *Neisseria*, subenheten PilE. SDS-PAGE og immunoblot-analyser viste at Nw trolig uttrykket PilE, og ble gjenkjent av antistoffer rettet mot Mc antigener.

Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometer (MS) Biotyper database ble utvidet med dårlig presentert *Neisseriaceae*. Massespektra fra flere *Neisseria* referanse-stammer, tilgjengelig i laboratoriet, sammen med Nw 9715 ble analysert med Mico Flex Control-programmet og ble kalibrert og korrigert ved å bruke et Flex Analysis program. Spektrene fra stammene ble lagt inn i MALDI Biotyper 3.1 database og databasen ble testet.

Abbreviations

APS	Ammonium persulfat
ATCC	American type culture collection
BHI	Brain Heart Infusion
CTAB	Cetyl trimethylammonium bromide
DSMZ	Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)
EDTA	Ethylene diamine tetra-acetic Acid
ermC	Erythromycin gene cassette
Erm^R	Erythromycin resistance
GC	Gonococcal
kDa	Kilo Dalton
LB	Lytic broth by Luria Bertani
LDS	Lithium dodecyl sulfate
LDS-PAGE	Lithium dodecyl sulfate – polyacrylamide gel electrophoresis
NH_4OAc	Ammonium acetate
NaOAc	Sodium actetate
NTPs	Nucleotide Triphosphate
pBSK (+)	Plasmid Bluescript (Cloning vector, ampicillin resistance)
PVDF	Polyvinylidene Difluoride
rpsL	30S ribosomal protein S12 gene
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR	Streptomycin resistance
TEMED	Tetramethylethylenediamine
UV	ultraviolet light
wt	wild type

1 INTRODUCTION

1.1 *Neisseria wadsworthii*

1.1.1 Bacterial characteristics

The bacterial species *Neisseria wadsworthii* (Nw) belongs to the family *Neisseriaceae* in the genus *Neisseria*. Nw is a recently identified neisserial species. Two novel strains of Nw species are isolated from clinical specimens, one from the hand wound and the other from the peritoneal fluid (1). A recent study (1) showed that Nw cells are Gram-negative cocci, 1.3–1.8 μm in diameter, often present in pairs and chains that form moist, yellowish and non-hemolytic colonies. The study also showed that Nw grows at 37°C under an atmosphere containing 5% CO_2, on trypticase soy agar (TSA) plates supplemented with 5% sheep blood. Results from DNA/DNA hybridization and 16S rRNA gene sequence analysis in this study indicate that Nw may be distinguished from the Gc type-species and produce acid only from glucose. The phylogenetic analysis of the 16S rRNA gene sequence alignment also showed that the species; Nw reside in a separate clade as compare to previously described species and genera. Other biochemical features that support inclusion of the Nw isolates in the genus *Neisseria* was the presence of catalase and cytochrome oxidase activity and the absence of motility. Single colony of Nw after 3 days of growth on TSA agar plates is shown in Figure 1. The single colony of Nw is circular, yellowish, smooth and shiny.

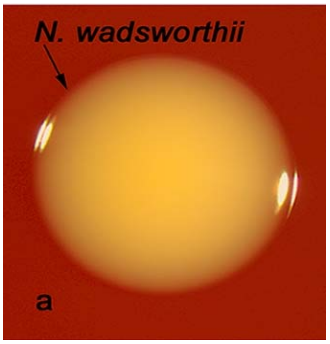


Figure 1: Single colony of *N. wadsworthii* after 3 days of growth on TSA plates with 5% sheep blood. Adapted from (1).

1.2 The Family *Neisseriaceae*

The *Neisseriaceae* belongs to the β subclass of the *Proteobacteria* and represent a diverse collection of Gram-negative cocci, often diplococcic and rods, including the following genera *Neisseria, Eikenella* (rods)*, Kingella* (rods)*, Simonsiella* and *Alysiella* (rods) (2)*.* Most species in the family *Neisseriaceae* are non-pathogenic and part of the normal flora. All genera belonging to the family lack flagella but several are mobile on agar by means of twitching motility, which is only displayed by piliated organisms. Piliation also causes auto-agglutination, a phenotypic trait observable as small aggregates in solution (3).

Bacteria in *Neisseriaceae* are aerobic or facultative in their relationship to oxygen and grow on rich medium, preferably blood agar in an atmosphere containing 5-10% CO_2 (4). They are also sensitive towards external influences as drying, disinfectants and un-physiological temperature. Like the *Moraxella*, the *Neisseriaceae* are all oxidase positive. An overview of biochemical reactions of selected *Neisseria*e species, carbohydrate metabolism is summarized in Table 1-1 which can be used for rapid differentiation of different neisserial species (5).

Table 1-1: Carbohydrate metabolism of selected members of the *Neisseriaceae* family. + = indicator colour change after one day, - = no change of indicator colour after one day.

Specie	Acid production from Carbohydrates			
	Glucose	**Maltose**	**Lactose**	**Sucrose**
N. gonorrhoeae	+	-	-	-
N. meningitidis	+	+	-	-
N. wadsworthii	+	-	-	-
N. sicca	+	+	-	+
N. mucosa	+	+	-	+
N. flavescens	-	-	-	-
N. lactamica	+	+	+	-
N. cinerea	-	-	-	-

1.3 The Genus *Neisseria*

The *Neisseria* is a large genus constituting many species with two important pathogens, Mc and Gc, and many commensal *Neisseria* species. Commensal species defines as an organism that colonizes the host without causing disease. Individual *Neisseria* cells are generally of a coccoid shape, but two species (*N. elongate* and *N. weaveri*) are exceptions in that they are short rods, often arranged in chains. Due to some circumstance, it is possible that cocci are arranged as kidney-shaped diplococci, for example during infections. The taxonomy of the genus *Neisseria* has been challenging to study due to high levels of phenotypic similarity between species and their fastidious growth requirements. However, studies based on phenotypic test, conventional characterization and 16S rDNA sequence analysis, it is possible to divide the different *Neisseria* species into groups. The most clinically important group includes the closely related species Mc and Gc. Mc can occur in carrier state in the pharynx without causing disease, whereas Gc does not constitute as a part of the normal human flora, and is an obligate pathogen that cause disease (gonorrhea) whenever it colonize its human host (3, 6). The second group consists of several, commensal species; such as: *N. subflava, N. sicca* and

N. mucosa. The third group contains *N. elongata, N. weaveri,* and several others (3). These latter species can occasionally be associated with disease but will not be discussed in detail in this paper.

1.4 Horizontal Gene Transfer

Mechanisms for horizontal gene transfer (HGT) in the genus *Neisseria* has been extensively studied, but virtually nothing is known about HGT in Nw. HGT is a potent evolutionary force for creating diversity among prokaryotic microbes (7). Three primary mechanisms of horizontal gene transfer among prokaryotic microbes have been described; phage-mediated transduction, plasmid-mediated conjugation and transformation. The uptake and genome integration of naked DNA from the surrounding environment is transformation (8). Most evidence suggests that transformation provides the predominant means for exchanges, which alter genomic DNA in nature (9). Conjugation is the transfer of genetic material from one cell to another by means of conjugative plasmid (10). Transduction is the third process in which bacterial DNA is moved from one bacterium to another by way of viruses, a bacteriophage.

1.4.1 Transformation

Transformation is binding, uptake and chromosomal integration of exogenous DNA from the environment, in a heritable form, particularly important for genetic exchange and diversity (11). *Griffith* in 1928 discovered the process of transformation by studying the virulence of *Streptococcus pneumonia* in mice (12). Avery and co-workers later demonstrated that the substance of inheritance was DNA by studying transformation (8). Several naturally competent bacteria have been identified and the most studied model organisms is the Gram-positive bacteria *Bacillus subtilis,* and *Streptococcus pneumonia,* and the Gram-negative bacteria *Haemophilus influenza*, Mc and Gc (7).

The Mc and Gc are competent through their whole life cycle whereas competence for transformation may be a tightly regulated process in other species. Today at least 66 bacterial species are identified as naturally

competent (13). The process of transformation differs from conjugation and transduction in both evolutionary and mechanistic characteristics. The transfer of genes occurs by soluble DNA, secreted or liberated spontaneously into the surroundings when bacterial cell die or undergo lysis. DNA is taken up by the recipient cells and integrated into the genome by a recombination process, where it is expressed as a changed phenotype, *e.g.* antibiotic resistance. Wesley Catlin described as early as in 1959 transformation of Mc using DNA isolated from culture slime, today recognized as bio-film, and we know today that *Neisseria* produce complex matrices in the nasopharyngeal mucosa constituted by large amounts of DNA (14). Strings of DNA therefore seem to have dual role; as structural component in biofilms and as allelic reservoir. Bio-film protects the bacteria from host immune defense such as antibodies and neutrophils.

Several studies revealed that transformation is the primary cause of horizontal gene transfer among *Neisseria* spp. Mc and Gc are among the most studied competent human pathogens. Transformation in *Neisseria* is more feasible among closely related species, but also between more distantly related ones, contributing to species diversity and fitness for survival (15).

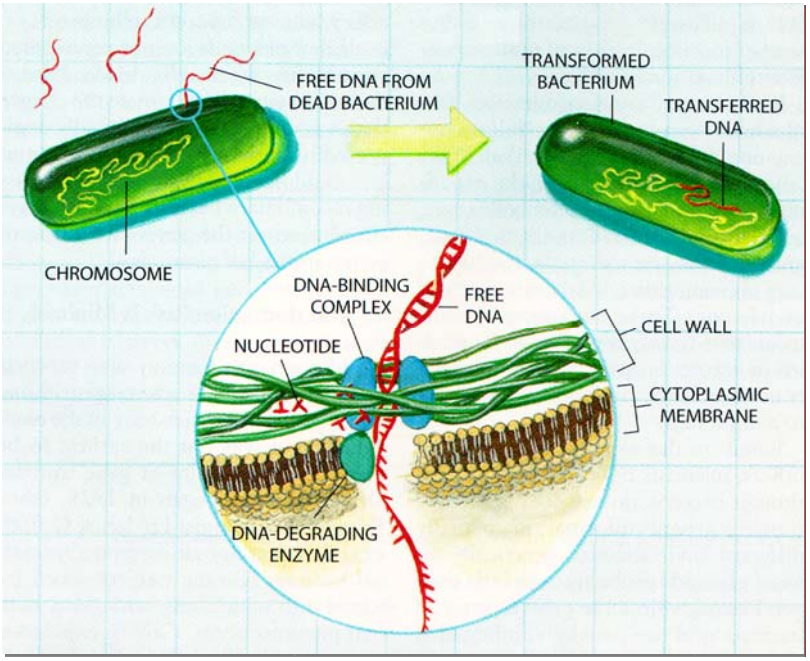


Figure 2: Illustration of transformation of DNA (Miller 1998).

1.4.2 Conjugation

Conjugation is the transfer of genetic material that involves transfer of DNA from one bacterium to another by direct cell-to-cell contact. Bacterial conjugation, transfer genetic material through a mating bridge which requires close contact between the donor and recipient cells. In nature, conjugation most often involves the transfer of plasmid DNA from donor to recipient cell, but chromosomal DNA can be transferred through this process under certain condition. (16). A particular sex-pilus is required for the interaction that precedes DNA transfer between the donor and the recipient cells. This surface structure allows specific contact to occur with a receptor present on the recipient cell and its retraction brings the cells into contact. The cells aggregates as the consequence of being pulled together and the DNA is transferred from one cell to another on a conjugative plasmid (17).

1.4.3 Transduction

Transduction involves movement of bacterial DNA from cell to cell by way of bacterial viruses, bacteriophage. Transduction can either be generalized, where any bacterial DNA can randomly incorporate with viral DNA and genes are transferred. Generalized transduction occurs in a variety of prokaryotes, including *Staphylococcus, Escherichia*, *Salmonella* and *Pseudomonas*. Specialized transduction is another way of genetic exchange, where bacterial phage transfer particular genes, usually those that are adjacent to their integration sites in the genome. Phages are found to be more diffusible and stable in the environment than naked DNA, but not all phages are able to carry out transduction, neither are all bacteria susceptible to this form of horizontal gene transfer (18).

1.5 Competence for Transformation

Competence defines an ability of the bacterium to take up exogenous DNA from the environment and to incorporate it into the genome by way of, homologous recombination (HR) (19). The competent state depends on the expression of several distinct components dedicated to the transformation process. Three basic steps are important during competence process: (1)

binding of the exogenous DNA to a recipient cell, (2) Uptake of the exogenous DNA across the membrane(s), and (3) homologous recombination (HR) involving the integration of the exogenous DNA into the host genome (20). Several physiological factors can affect the competence ability of the recipient cells such as; the growth phase and culture medium. These factors combined with other environmental factors play an important role in the uptake of the exogenous DNA and genome integration (21, 22).

Pathogenic *Neisseria* species; Mc and Gc are the most obligate mucosal pathogens of humans, rarely take up DNA from bacterial species other than their own genus (23). This specificity is similar to that seen in *Hemophilus influenzae* transformation. However, *H. Influenzae* and other *Pasteurellaceae* do not recognize the same or homologous uptake sequence (24). Efficient neisserial transformation depends on the presence of (1) DNA uptake sequence (DUS) in the exogenous DNA, (25), (2) Rec-A dependent HR (24) and (3) expression of type IV pilus (Tfp) (26). Piliated neisserial bacteria are competent for genetic transformation throughout their life cycle, while non-piliated neisserial species had shown low frequencies of transformation (27). The type IV pilus biogenesis apparatus plays an important role in adherence, transformation and motility of the neisserial organism (23).

The widespread occurrence of natural competence for transformation among *Neisseria* species has contributed to an in-depth understanding of genetic exchange and recombination in general and thus enabled the use of transformation process for identification and classification of other bacterial strains. However, tests for natural competence of transformation in the recently identified Nw have not been performed until now.

1.6 DNA Uptake Sequences

The DNA uptake in *Neisseria* species is dependent on the presence of a 10 base pair (bp) specific DNA uptake sequence (DUS) 5´- GCCGTCTGAA-3´ (24). This 10-bp sequence is the hallmark of natural transformation among pathogenic *Neisseria* species (23, 28). Approximately 2000 copies of DUS sequence are accommodated in Mc and Gc genomes, making up about 1 %

of their total genetic sequence (29). The 10 bp DUS 5´- GCCGTCTGAA-3´ is observed to have an effect on transformation, but the extended 12 bp AT-DUS (5´- AT-GCCGTCTGAA-3´) has been observed to elevate the transformation process even further (24). Studies on genome maintenance components suggest that several genes engaged in DNA repair and recombination have higher density DUS within their open reading frame (30).

A recent study (31) reported the presence of eight distinct DUS variants (DUS dialects), in the family *Neisseriaceae*. The DUS sequence variations were observed to have a negative influence on the inter-species transfer of DNA, during transformation and the binding and uptake of DNA. A DUS core of three nucleotides (5'-CTG-3') was found in all DUS dialects, which were essential for the process of transformation. The similarity between the DUS dialect of the recipient species and the donor DNA was observed to be correlated with the level of transformation. The AT-DUS dialect was found in all Mc and Gc genomes. In addition, this DUS dialect was also found in the genomes of other commensal *Neisseria* species; *N. lactamica*, and *N. polysaccharea* (31).

The DUS-sequence found in Nw is 5´-CCTGTCTGAA-3'. This DUS-sequence is very similar to the 10-bp canonical DUS and only differs with one T insertion in position three**.** A core genome phylogenetic tree presenting DUS dialects in 23 different members of the family *Neisseriaceae* is presented in Figure 3. The 16S rDNA based cladogram with the connectors on the right (dots) shows the relation to the phylogram on the left (31).

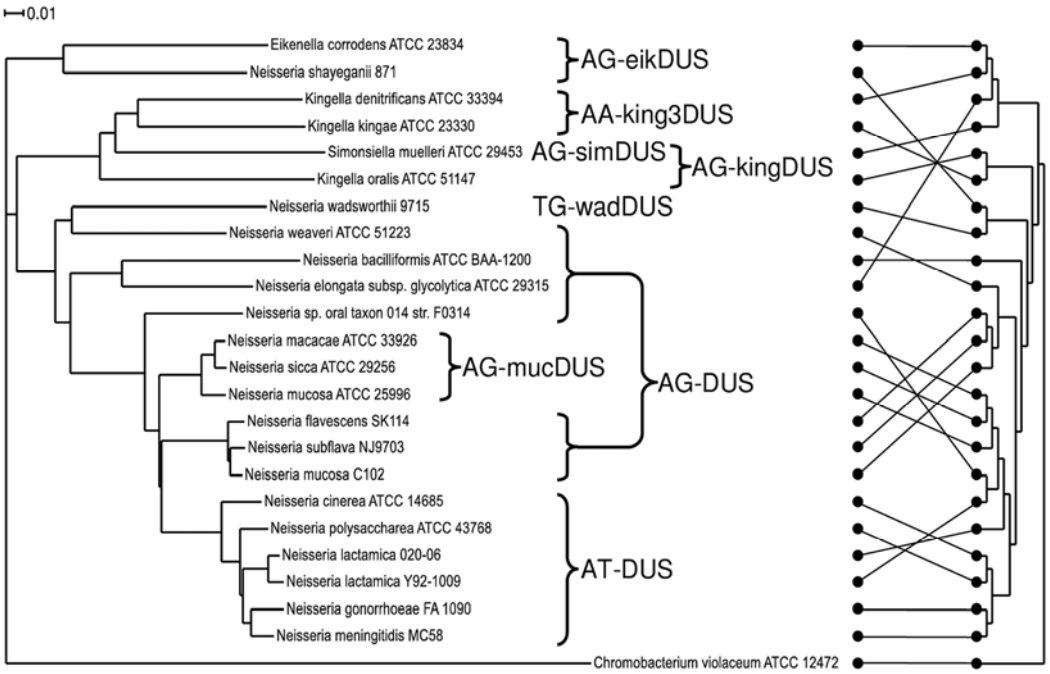


Figure 3: Phylogenetic tree based on the core genome and DUS dialect distribution. Adapted from (31).

1.7 Pili and Pilus Biogenesis

Competence for transformation among pathogenic *Neisseria* correlates with the presence of functional Type IV pili. The ability of pathogenic *Neisseria* to cause infection among humans is associated with the adherence of microbes to host cell surfaces. Fimbriae (pili), termed Type IV pili, are hair-like filamentous appendages, mechanically strong protein fibres extending several micrometers from the bacterial surface. Pili consist of ordered arrays of polymerized subunits called pilin. Fimbriae have been found on the surface of Mc and Gc (32), and non-pathogenic *Neisseria* species (33).

Type IV pili are important cell-associated virulence factors employed by neisserial pathogens, involved in mediating (i) bacterial adherence to, and colonization of mucosal surfaces, (ii) bacterial auto-agglutination (iii) a mode of flagella independent surface translocation known as ‘twitching motility’, (iv) biofilms formation and (v) natural competence for DNA uptake (34, 35). A complex interacting apparatus with several components of the Tfp machinery controls the assembly of pili. This system includes approximately 10 conserved proteins and several additional proteins. Moreover, pilins have a

typical signal sequence (36). Other minor pilins are required for Tfp biogenesis (36, 37). Some components of the Tfp biogenesis machinery are homologous to proteins involved in the general secretory pathway (GSP), part of the Type II secretion system in Gram negative bacteria (35, 38).

The machinery dedicated to Tfp biogenesis in Gram-negative bacteria comprise a conserved proteins including: (i) the major pilin protein (PilE) with an N-terminal domain motif (ii) a specific peptidase (PilD) (iii) a traffic adenosine triphosphate (ATPase) (PilT, PilF and PilP) (iv) an integral outer membrane protein, named secretin (PilQ) and (iv) an integral inner membrane protein (34, 35). The first step of pilus assembly is the insertion of the pilin into the cytoplasmic membrane. After the membrane insertion, the leader peptide is both cleaved at the cytosolic side of the membrane and methylated on the N-terminal region by prepilin peptidase PilD (39). The PilE subunits are assembled and further extruded from the inner membrane by the hexameric ATPase PilF. The ATPase PilT retract the pilus and the pilus passes the outer membrane through PilQ. Several other proteins, called minor pilins, can also be integrated into the growing pilus and were proposed to affect pilus dynamics by influencing the membrane-localization and or polymerization (40). A schematic presentation of the site and possible form of action of many components involved in Tfp biogenesis machinery, extraction/retraction, as well as the transformation process in the meningococcal Tfp biogenesis machinery, is shown in Figure 4 (41).

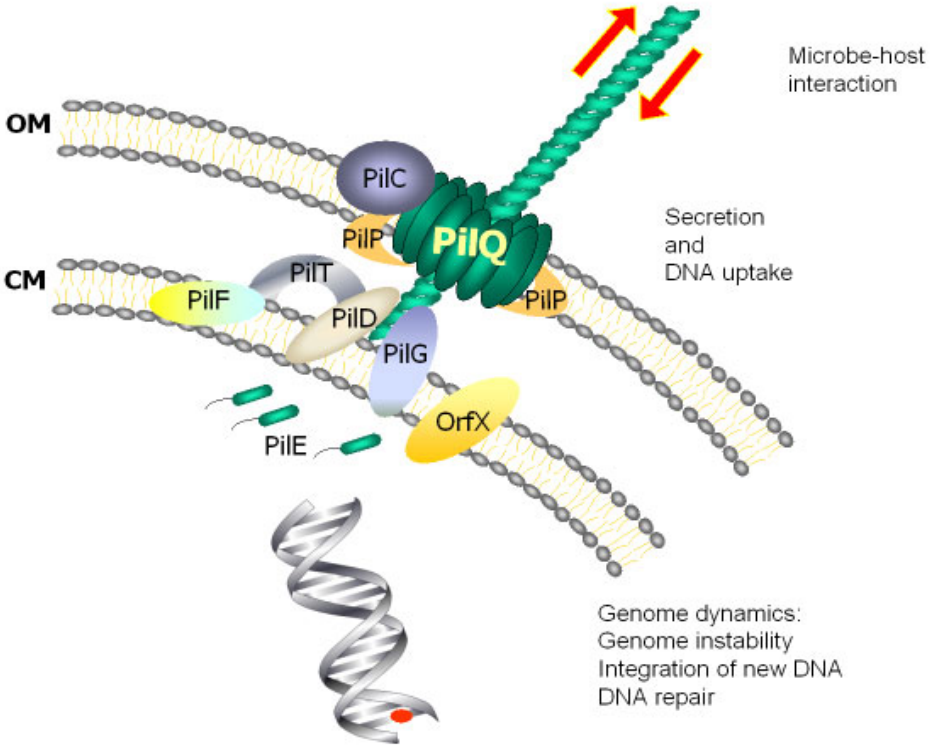


Figure 4: Model of components identified in Type IV pili and their predicted functions as well as the transformation process in the meningococcal Type IV pilus biogenesis machinery. Adapted from Transformation and DNA repair; linkage by DNA recombination(41).

1.8 The Type IV Pilus Biogenesis Components

1.8.1 The pilin subunit, PilE

PilE, 18-22 kDa protein termed pilin, is the major structural component of the pilus filament of Mc and Gc. The pilin subunits make up the majority of the structure of the pilus fiber (42, 43). The Type IV pilin fibers are long, ordered arrays of pilin subunits, PilE. These pilin subunits are synthesized in the cytoplasm as monomer of PilE, harboring a conserved N- terminal leader sequence. PilE is found to be essential for the first step of transformation, during DNA uptake and is also subjected to transformation-mediated phase and antigenic variation (43). The pilin subunit displays features of the Type IV bacterial pilin, including the presence of a highly conserved N-terminal domain and two conserved cysteines residues in the C-terminal domain. The N-terminal sequence is hydrophobic and forms an extended α-helix structure

and the C-terminal region is hypervariable (44, 45) as shown in Figure 5. The conserved N-terminal region of pilin containing a hydrophobic α-helix believed to form the central core of the pilus structure (44).

Figure 5: Schematic presentation of the pilins indicating the relevant regions and residues. The hydrophobic N- terminal with extended α-helix structure. The C-terminal contains two cysteines in a hypervariable domain (D-region). Adapted from (45).

1.8.2 PilQ secretin

The PilQ is a member of the secretin family, identified as an outer membrane protein-macromolecule complex (OMP-MC), especially involved in secretion of Type IV pili among Gc, Mc and *Pseudomonas aeruginosa* (35, 46). The common function served by the secretins appears to involve in the translocation of the large macromolecules complexes across the outer membrane in different species (35, 47). The PilQ protein was named PilQ based on homology with the proteins required for pilus expression in *P. aeruginosa* (48). PilQ found in pathogenic Mc forms a large dodecamer complex of about 900 kDa in size (49), and is proposed to function in the terminal stages of pilus biogenesis by acting as a pilus channel or pore. The *pilP* -gene, located upstream of the *pilQ* -gene, encodes a lipoprotein and the PilP protein is predicted to be located to the outer membrane. PilP is required for stable expression of multimerized form of PilQ. Interestingly, gonococcal mutants with $PilP^-$ and $PilQ^-$ were able to release PilC suggesting that, PilQ and PilC may interact in the terminal steps of pilus biogenesis (47, 48).

1.8.3 Other Type IV pilus biogenesis components

PilC protein is a 70 kDa protein, cooperates with PilE in the DNA uptake process but is not involved in the binding of specie-specific DNA (48, 50). It is therefore still unknown what extend and how PilE and PilC participate during the initial stage of DNA binding. Based on the homologous sequences in *P. aeruginosa;* PilC may be required for optimal stability or membrane

localization of PilD (48, 51). PilD found among neisserial species is homologous to the PilD of *P. aeruginosa*. This protein functions as a prepilin leader sequence peptidase that cleaves off the first five amino acids and adds the α-amino-methylation at the phenylalanine (52). Due to their hydrophobic N-terminal and extended α-helix structure, the prepilins remains in the membrane, with the leader peptide in the cytoplasm and the C-terminal domain in the periplasm (53). These structural properties are required for recognition and processing of prepilin by the prepilin peptidase, an inner membrane protein (35).

The pilus filament assembly also requires energy, which is provided by a conserved traffic ATPase belonging to the core set of proteins (35). PilT is a protein belonging to a large family of molecules sharing a highly conserved nucleotide binding domain motif. PilT protein is homologous to PilF and both function as a putative ATPase, where PilF energize pilus extraction in the Tfp biogenesis system and PilT is involved in the disassembly of the PilE subunits at the cytoplasmic membrane, resulting in retraction of the pilus (54). This mechanism generates through a 'push and pull' mode of action that powers extrusion of the pilin subunits from the inner membrane. ATPase powering pili are required during DNA uptake and translocation across the outer membrane. Furthermore, pili proteins PilC, PilM, PilN, PilO, PilP and PilW are also essential for Tfp biogenesis machinery(35). ComP (competence-associated pilin) is one of the three minor pilins present in pathogenic *Neisseria* species (26). ComP and PilV are dispensable for piliation but found to have an influence on specific binding of DUS-containing DNA. ComA another minor pilin might help the DNA cross the inner membrane into the cytosol. ComE, proposed to bind DNA and aids in transformation, while ComL may allow the DNA to cross the peptidoglycan layer (55).

Despite having similarities in the overall structure, both Gc and Mc pili are known for their variability and antigenic variation. Phase variation, is a genetic mechanisms leading to on and off shifts on pilus expression, from piliated (p^+) to non-piliated (p^-) phenotype and vice versa(56). Furthermore, Mc express

two structurally distinct types of pili termed class I (molecular weight of 17-23 kDa) and class II pili (molecular weight of 15-17 kDa) (57). Class I pili are found to be closely related to gonococcal pili, while pili expressed by commensal *Neisseria* share structural features with the Class II pili of Mc (58). Functional Tfp biogenesis machinery and the set of properties mentioned above are also linked to natural competence for genetic transformation (uptake of free DNA). In an infection-situation, these properties as functional pili, adherence, antigenic variation and adaption through recombination, and DNA uptake could be to the advantage of the bacteria.

1.9 AIMS OF THE STUDY

The overall goal was to characterize the neisserial species, *N. wadsworthii* 9715 in regard to phenotypic traits. As the process of transformation is very essential for the horizontal gene transfer in nature, especially natural competence for transformation and a potential DNA uptake sequence (DUS) dependence of transformation were tested. Methods including phenotypic analyses, molecular biology and bioinformatics, were used to search for competence for transformation in this bacterium. As a side project this study aimed at developing a Biotyper database for the mass spectrometry (MALDI-TOF-MS) based identification of *Neisseriaceae* currently poorly identified by the Biotyper method.

2 MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmid

The bacterial strains and plasmids employed in this study are listed in Table 2-1. The strain Nw 9715 has been used in this study and was purchased from the German collection of microorganisms and cell cultures (59).This type strain (DSMZ number 22247) was first isolated from a hand wound of a 39-year-old-male in New York state, Rensselaer country USA (1) [URL 1].

Escherichia coli (E.coli) strain ER2566, plasmids p0-DUS and pBluescript II SK (+) (pBSK (+)) were already available at the laboratory, the Institute of Medical Microbiology, Department of Molecular Microbiology at the National Hospital, Oslo (Rikshospitalet).

Table 2-1: Bacterial strains and plasmids used in this study.

NAME	CHARACTERISTICS	SOURCE
Plasmid		
p0-DUS	pBSK + harboring *pilG*::Tn3Erm (transposon insertion of *ermC* into *pilG*→Erm^R without DUS)	(29)
pBluescript II SK(+)	General cloning vector, Ampicillin resistance, abbreviated pBSK+	Stratagene
pDV4	Plasmid containing AT-DUS	Provided by Dr. Stephan A. Frye
pSAF62	Plasmid contain ATG-wadDUS	This study
Strain		
Neisseria wadsworthii 9715	Wild-type, clinical isolated, DSMZ 22247	German collection of microorganisms and cell cultures (59)
E.coli strain ER2566	Expression host strain carry with chromosomal copy of the T7 RNA polymerase gene inserted into lacZ gene	New England BioLabs

2.2 Equipment

The equipment used to perform molecular techniques is given in Table 2-2.

Table 2-2: Equipment

EQUIPMENT	DESCRIPTION	MANUFACTURER
PCR machine	Peltier Thermal Cycle PTC-200	Bio-RAD
Spectrophotometer	NanoDrop® ND-1000	Life Science
Incubator	CO_2 water –jacketed incubator	Nuair US autoflow
Microscope	Leica GZ7	Leica
Vortex	Heidolph REAX control	IKA
Tumbler	Reax 2	Heidolph
Safety cabinet	Sterile bench	Holten Laminair
Agarose gel tray	Horizontal Gel Electrophoresis, Horizon 58	Life Technologies
Power supply	Electrophoresis Power supply, EPS 301	Amersham pharmacia biotech.
Micro centrifuge	Micro One	TOMY
SDS PAGE gel tray	BioRad, Mini Protean	BioRad
Protein transfer apparatus	TE series transfer electrophoresis Unit	Hoefer Scientific Instruments
Power supply	Electrophoresis Power supply, EPS 301	Amersham Pharmacia Biotech
Vacuum drier	Hoefer SLAB GEL dryer. GD 2000	Hoefer Scientific Instruments
Transfer membrane	PVDF filterpaper, pore size 0.45 µm	Milipore
Micro Flex LT MALDI-MS	Determine molecular mass of free ions in a vacuum	Bruker Daltonics Germany

2.3 Reagents and Chemical Solutions

The list of reagents and chemical solutions used is given in Appendix 1.

2.4 Recipes for Culture Media, Buffers and Solutions

The recipes for the buffer solutions and culture media used are given in Appendix 2.

2.5 Antibodies

Antibodies used in this study were available at the laboratory, the Institute of Medical Microbiology, Department of Molecular Microbiology at the National Hospital, Oslo (Rikshospitalet).

Table 2-3: Antibodies used in this study.

NAME	SOURCE	REACTIVITY
K849	Rabbit	Pilus fiber from Mc
Goat anti-rabbit IgG-AP	Goat	Whole molecule with heavy chains of rabbit IgG

2.6 Enzymes

Pfu-Turbo DNA polymerase [2.5 Units /µL], Phusion DNA polymerase [2 Units/ µL] and Proteinase K were used and were available at the laboratory, the Institute of Medical Microbiology, Department of Molecular Microbiology at the National Hospital, Oslo (Rikshospitalet).

2.7 Primer Sequences

The list of PCR primers used during this study is given in Appendix 3.

2.8 Oligonucleotides

Primers used for DNA amplification were ordered from Eurofins MWG Operon (Ebersberg, Germany). Some of the primers used in this study were already available at the institute. *Oligocalculator* web server and Primer 3 software [URL 2,3] were used to perform bioinformatics; calculating primer length, molecular weight, GC content and melting temperature (T_M).The 30S Ribosomal protein S12 (*rpsL)* primers were used for DNA amplification by

Polymerase Chain Reaction (PCR). DNA from *N. mucosa* 19696^{SR} and *N. wadsworthii*^{SR} encoding streptomycin resistance were used as a template DNA in the PCR.

Amino acids substitution associated with streptomycin-resistance have been identified in the genes encoding ribosomal protein S12 *(rpsL)* and 16S rRNA *(rrs).* Ribosomal protein S12 stabilizes the highly conserved structure formed by 16S rRNA and mutation in *rpsL* affects the structure of the 16S rRNA, thus confer streptomycin resistance (60). The PCR fragment harboring the *rpsL* starts with the start codon and ends with the stop codon of *rpsL*. The antibiotic prevents the final stage of translation by binding to RpsL and inhibits amino acid synthesis. Streptomycin, combined with other antibiotics is commonly used during the treatment of infection caused by *Neisseria*. A compilation of the *rpsL* primers used for DNA amplification of a fragment encoding streptomycin resistance and erythromycin resistance to construct plasmid DNA (pSAF62) is listed in Appendix 3.

2.9 Bioinformatics Analysis

The software tools and internet services used to design primers and for bioinformatics analysis are given in Table 2-4.

Table 2-4: Software used for bioinformatics analysis and primer design.

SOFTWARE	DESCRIPTION	LINK / SOURCE
Primer 3	Program for designing PCR primers.	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
NCBI Genebank	Database to search microbial gene sequences	http://www.ncbi.nlm.nih.gov/
Genedoc	Multiple sequence alignment editor	http://www.nrbsc.org/
ClustalX	Sequence Alignment software	http://www.clustal.org/clustal2/
Oligonucleotid calculator	Calculate annealing temperature and GC-content in primers.	http://www.basic.northwestern.edu/biotools/OligoCalc.htmL
EDGAR	Efficient Database Framework for Comparative Genome Analysis Using BLAST Score Ratio	http://edgar.cebitec.uni-bielefeld.de (Kindly performed by Dr. Stephan Frye)
Chromas	Sequencing chromatogram viewer	http://technelysium.com.au/?page_id=13

2.10 Bacterial Strain Storage and Growth

Liquid gonococcal (GC) medium with 10 % glycerol medium was used to store Nw 9715 at -80°C. The recipe of the GC-medium is listed in Appendix 2. Standard incubation conditions for *Neisseria* species were 37°C in a humid atmosphere containing 5% CO_2 and were used for the cultivation of Nw 9715. Incubation times varied between 15-18 hours. Bacterial cells from the agar plate were collected with a sterile inoculation loops (Sarstedt) into labeled sterile 1.8 mL screw-cap NUNC CryoTubes and placed directly into -80°C. Nw 9751 was inoculated on Blood agar, Chocolate agar, GC agar, Blood agar with erythromycin, Chocolate agar with streptomycin, or GC agar with kanamycin.

2.11 Phenotypic Identification of *N.wadsworthii*

The following protocols were adapted from the Department of Microbiology, bacteriology section, Oslo University hospital, Rikshospitalet. These identification methods are being used as a part of the routine diagnosis of *Neisseria* species.

2.11.1 Oxidase test

Oxidase test is a key reaction for the identification of *Neisseria* species and some gram-negative rods like *Pseudomonas*, which are oxidase positive and *Escherichia* which are oxidase negative. Oxidase reduction is based on the ability of the bacteria to produce an oxidase. In the presence of the oxidase enzyme, the reagent N,N Dimethyl-p-phenylenediamine is oxidized by the bacterial cytochrome c, which is the last enzyme in the electron transfer chain ultimately reducing oxygen (61). *Neisseria* species are positive for cytochrome oxidase, and this is visualized by a change of color in the reagent, where a change to blue color indicates oxidase positive cells. Every inoculated growth medium with bacterial growth has been tested in this manner.

2.11.2 Gram staining

The Gram staining procedure is a rapid, day-to-day practice method to detect and identify bacteria in clinical laboratories. Gram staining is based on differentiating bacterial species into two large groups (Gram-positive and

Gram-negative). The reagents used are Crystal violet, Gram iodine, Safranin and alcohol. Crystal violet and Gram iodine interact and produce a complex inside the bacterial cell. The cell wall of the Gram negative bacteria has a different structure as compared to Gram positive bacteria. Gram negative bacteria cells will decolorize when exposed to alcohol and bacteria are afterwards stained Safranin light pink. The cell wall of Gram positive bacteria contains a thick peptidoglycan layer, and does not decolorize by alcohol. The cells of Gram positive bacteria therefore remain dark purple (62).

2.11.3 Carbohydrate metabolism

Neisseria species metabolize different carbohydrates, and a metabolic product is acid. A thick inoculate of bacteria is plated on different agar plates containing GC-agar medium, L-glutamine, Iron (III) nitrate-9-hydrat, phenol red NaOH, distilled water, (Sigma-Aldrich, USA), and only a single carbohydrate (glucose, maltose, sucrose or lactose). When a carbohydrate is metabolized, acids are produced and this is visualized with the help of the pH-indicator, phenol red embedded in the agar (62).

2.11.4 Analysis of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of streptomycin on Nw 9715 growth was measured. MIC is defined as a lowest concentration of antibiotics required to prevent growth of microorganisms and is measured in µg antibiotic/mL (63). It is a semi-quantitative test, applied in almost every diagnostics laboratory. The MIC value for Nw 9715 was used to define the minimum streptomycin concentration to be used in media employed in the transformation assays. An overview of Nw 9715 in Brain heart infusion (BHI)-medium with different streptomycin concentrations is given in Table 2-5, which can be used for the rapid determination of MIC of this strain. The recipe of the BHI-medium is listed in Appendix 2.

For this 2 % of agar was added in 500 mL BHI medium. The agar medium was then dissolved in a microwave oven and cooled in a water bath to reach 60°C. 50 mL of agar medium was then transferred into a new tube containing different amounts of streptomycin. 25 mL of agar medium was poured into petri

dishes. Components used to make the streptomycin antibiotic stock of 5000 µg/mL are given in Appendix 2.

Table 2-5: Mixing table for the MIC determination assay of Nw 9715 tested on BHI-medium containing different streptomycin concentrations.

BHI- medium with streptomycin	BHI-medium (mL)	Streptomycin antibiotic from stock (5000 µg/ mL)
5 µg/mL	50 mL	50 µL
10 µg/mL	50 mL	100 µL
20 µg/mL	50 mL	200 µL
30 µg/mL	50 mL	300 µL
40 µg/mL	50 mL	400 µL
50 µg/mL	50 mL	500 µL
100 µg/mL	50 mL	1 mL
150 µg/mL	50 mL	1,5 mL
200 µg/mL	50 mL	2 mL

2.11.5 Colony morphology

To study colony morphology of Nw 9715; characteristics of piliation, 1% agar plates with GC medium were made (see Appendix 2) and several single colonies were streaked out, incubated under standard conditions (37°C, 5.5% CO_2) overnight and inspected using a dissecting microscope (Leica GZ7).

2.12 Transformation

Single colonies of the Nw 9715 recipient strain were streaked out on blood agar and incubated under standard conditions. After overnight growth colonies were examined in a microscope for colony morphology and for contaminants before they were used in transformation assay. Heavily streaked areas were avoided when colonies were sampled using a sterile cotton swab. BHI broth was prepared and 11.5 µL of 3 M $MgCl_2$ was added to 5 mL of BHI broth. A barely turbid bacterial suspension was made by rotating the loaded cotton swab in the 5 mL of BHI/$MgCl_2$ and aliquots of 0.5 mL was transferred into 15 mL tubes. 5 µL of appropriate donor DNA were added. The DNA exposure

lasted for 30 minutes at 37°C, without agitation in the water incubator. 4.5 mL of BHI broth (prewarmed at room temperature) was added and the tubes were tumbled at 37°C for 4.5 hours to allow growth.

Blood agar, chocolate or GC plates with the appropriate antibiotic in preferred concentration were inoculated with the transformation suspension. The agar plates were incubated overnight under standard incubation conditions. Colonies that are able to grow on these selective medium were scored as competent.

2.12.1 Quantitative transformation

To be able to determine the precise transformation rate of Nw 9715, the transformation assay described above was performed. Appropriate donor DNA with a point mutation resulting in streptomycin resistance (0.5 µg mL^{-1}) and or plasmid DNA containing Erm^R (1 µg mL^{-1}) was added as donor DNA. Chocolate agar plates with streptomycin (50 µg mL^{-1}) or erythromycin (8 µg mL^{-1}) were used to count transformants. To count the number of cells a log 10 dilution was prepared. 100 µL of the each 10^{-0}, 10^{-1}, 10^{-2}, and 10^{-3}, suspension were spread on duplicate or triplicate sets of chocolate agar plates containing streptomycin (50 µg mL^{-1}) or BHI-agar containing erythromycin (8 µg mL^{-1}). A 10^{-6}-suspension was spread on duplicate or triplicate sets of blood or BHI-agar plates without antibiotics to count the total cell number. Transformation frequencies were determined by dividing the number of streptomycin and or erythromycin-resistance colony-forming units (CFU) by the number of total CFU. Each experiment was repeated at least four times.

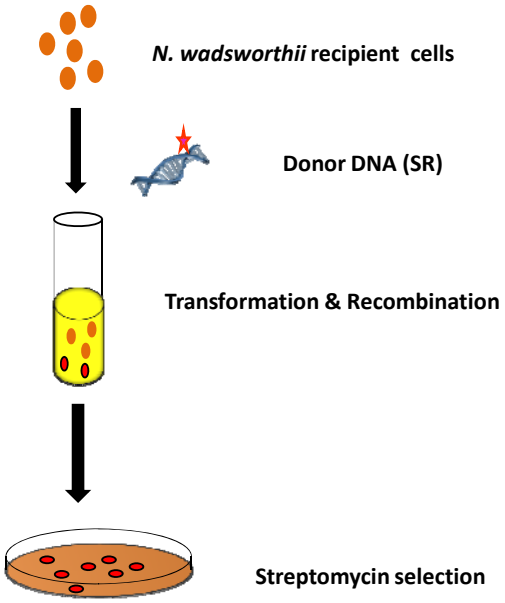


Figure 6: Quantitative genetic transformation test of *N. wadsworthii* 9715. The red star denotes the streptomycin resistance mutation in the donor DNA.

2.13 Nucleic Acid Methods

2.13.1 Isolation and preparation of genomic DNA

Large quantities of genomic DNA are used in a range of applications in the laboratory. Briefly, in the protocol to isolate genomic DNA bacterial cells are collected from agar plates, lysed, and proteins degraded by digestion with proteinase K. After the cell walls are fragmented the remaining proteins, lipids and polysaccharides are removed with CTAB by selective precipitation. Finally, isopropanol precipitation is used to collect genomic DNA.

Several bacterial cells were collected from agar plates and a suspension was made by adding 1.5 mL TE buffer (pH 8). The suspension was centrifuged until a pellet was formed (13.000 × g, 4°C, 5 min). The supernatant was discarded and the pellet was re-suspended in 567 µL TE buffer (pH 8) by repeated pipetting. 15 µL of 20 % SDS and 3 µL of 20 mg/ mL of proteinase K were added. The sample was mixed thoroughly and incubated for 1 hour at 37°C. Then 100 µL of 5 M NaCl was added and mixed thoroughly and 80 µL of 2% CTAB/ NaCl solution was added and mixed thoroughly before the reaction was incubated for 10 minutes at 65°C. 765 µL of chloroform / isoamyl alcohol

(24:1) was added, mixed and the sample centrifuged (13.000 × g 20ºC, 5 min). The supernatant was then transferred to a new reaction tube, leaving the interface behind and an equal amount of phenol / chloroform / isoamyl alcohol (24:24:1) was added followed by centrifugation (13.000 × g 20ºC, 5 min). The supernatant was transferred into a new reaction tube and 0.6 volumes of isopropanol were added to precipitate the nucleic acids. The tube was vigorously shaken until the white stringy DNA clearly became visible and then centrifuged for 5 min at 13.000 × g. To remove the residual CTAB, the DNA was washed in 70 % ethanol. To remove the ethanol, the DNA was dried for 15-20 minutes at room temperature. The DNA was finally dissolved in 100 µL TE buffer (pH 8).

2.13.2 Isolation of streptomycin-resistant mutants of *N.wadsworthii* 9715

Suspensions of the streptomycin sensitive Nw 9715 strain were spread evenly on a blood agar plate and incubated under standard conditions for 4.5 hours until a thin bacterial film was observed. The agar disk was then gently removed from the petri dish using sterile steel spatulas and transferred onto a brain heart infusion (BHI) agar plate containing 5000 µg mL^{-1} streptomycin. After standard incubation overnight and diffusion of streptomycin into the blood agar, streptomycin resistant colonies were picked and streaked onto another blood agar plates containing 5000 µg mL^{-1} streptomycin and incubated to allow growth. Streptomycin resistant strains (MIC ≥ 1000 µg mL^{-1}), were selected and stored for DNA extraction. The genomic DNA of these clones was further used to transform Nw 9715 wild type (wt).

2.13.3 DNA Quantification

A NanoDrop® ND-1000 Spectrophotometer was used to quantify DNA. The instrument can measure both double stranded DNA (dsDNA) and single stranded RNA. The NanoDrop® ND-1000 Spectrophotometer is a cuvette free spectrophotometer, performing absorption in the 220-750 nm spectrums using a sample volume of as little as 1 µL. Two fiber optic cables are connected with the liquid sample and a xenon lamp provides the light source. The spectrophotometer is routinely used for the quantification of extracted genomic

DNA, which in this study is further used during transformation. The maximum absorption of nucleic acid is at a wavelength of 260 nm and the DNA purity is determined by the ratio of sample absorbance measured at 260 nm and 280 nm (260/280 ratio). A ratio of 1.8 is generally accepted as “pure” for DNA. If the ratio is lower, it may indicate the presence of proteins, phenol or other contaminants. The NanoDrop® ND-1000 Spectrophotometer is also used to calculate concentrations of oligonucleotides used in the PCR. Agarose gel electrophoresis was also used as a semi-quantitative approach to measure DNA amounts by using a DNA ladder with a given concentration as a standard.

2.13.4 Agarose gel electrophoresis

Agarose gel electrophoresis is an indispensable tool for DNA analysis and is the standard method of separating DNA-fragments. Agarose is a polysaccharide, extracted from seaweeds and is ideal for the separation of DNA-fragments. The gel electrophoresis technique may be used to quantify DNA and to measure the size of the DNA-fragments. By applying an electric current to an agarose gel, nucleic acids get separated by size. Nucleic acids have a consistent agent negative charge imparted by their phosphate backbone of the DNA helix and thus migrate towards the positive charged electric field. Separation of the nucleic acids (DNA or RNA) is dependable on the size of the molecules. Small molecules migrate faster than larger, due to the impact of obstructive gel density which affects the mobility of larger DNA fragments more severely than smaller DNA fragments (64). After separation, SYBR Safe (Invitrogen) or ethidium bromide were used to strain DNA by intercalation. SYBR Safe is less mutagenic compound as compared to ethidium bromide (65) and is commonly used as a fluorescent strain in gel electrophoresis. Both SYBR Safe and ethidium bromide fluoresce upon exposure to ultraviolet light.

The agarose was melted in 0.5 × TBE-buffer in a microwave oven. The solution was then cooled to 60 °C. 3 µL of SYBR Safe was added to the liquid gel and poured in a gel tray. 0.9%, 1% and 2% (w/v) agarose concentration were used, dependent on the size of the DNA-fragments to be separated. After the gel had solidified, a DNA ladder and individual samples mixed with DNA

loading buffer was applied to individual wells. Electrophoresis conditions were 100 V for 40-60 minutes, and DNA was visualized under UV-light.

2.13.5 Polymerase Chain Reaction (PCR)

The Polymerase chain reaction (PCR) is a rapid and versatile in vitro method for amplification of target DNA sequences with defined oligonucleotides primers. The principle of PCR involves three separate steps: denaturation of double stranded DNA, annealing of primers, and primer extension (66). PCR is an enzymatic reaction that utilizes DNA polymerase that targets and amplifies a defined segment in DNA. The number of DNA molecules in a single PCR may reach up to a million copies in the test tube, yielding large amount of specific DNA for sequencing, cloning or mutagenesis purposes (67).

Primer design

Available sequences in NCBI Gene bank were used to design primers for PCR amplification in this study. Primers for the amplification of the *rpsL* gene encoding the 30S Ribosomal protein S12 were designed to contain the different DUS variations. These primers were designed based on the available sequences of Nw 9715 (HMPREF9370_0706). This sequence was aligned with similar genes from Mc and Gc by using software as ClustalX and Genedoc. Areas that were conserved and matched were highlighted and used as the basis to design primers.

Table 2-6: Set of criteria's was used for designing primers for the PCR amplification.

CHARACTERISTIC	CRITERIA
T_m (melting point)	T_m= 2(AT) +4 Equal melting points in forward and reverse primer
GC-content	A range of 35-65 % of GC content is good to ensure primer binding to the template
Complementarities	To avoid primer self or cross annealing stretches between forward and reverse primer
Primer length	A range of 19-23 nucleotides in length to ensure specificity of binding

PCR reaction

Individual concentrations of the components used in the PCR-reaction were optimized for standard use in the laboratory, at The National Hospital Rikshospitalet in Oslo, and are given in Table 2-7. The Primers were diluted to a final stock concentration of 10 mM in Tris-HCl pH (8) and a solution of 10 µM was made to be used in the setup of the PCR reactions. Genomic DNA from *Neisseria mucosa* harboring the *rpsL* point mutation that encodes streptomycin resistance or the equivalent DNA from Nw were added as template source for the PCR amplification. Genomic DNA from *N. mucosa* with a point mutation resulting in streptomycin resistance was already available in the laboratory, while equivalent Genomic DNA from Nw was prepared during this study. The template was added separately as the last component.

Table 2-7: Components concentration in the PCR-reaction.

REAGENT	AMOUNT [µL]
Template-DNA 100 ng/ µL	2.5
10 x cloned Pfu PCR-Puffer	25
Forward primer(10 µM)	6.25
Reverse primer (10 µ M)	6.25
2,5 mM dNTPs (mix of each NTPs)	10
Pfu Turbo-Polymerase (2,5 U/µL)	5
H_2O	195
Total amount	250

PCR cycling program

The PCR cycling program is summarized in Table 2-8. The DNA template was denaturated for 45 seconds at 95°C prior to temperature cycling. The *rpsL* DNA was amplified by performing 35 cycles of denaturation, annealing and extension at 95°C, 55°C and 72°C respectively. After completion the reaction was kept at 8°C to minimize degradation of DNA. The annealing and extension temperature were kept same for all the different types of primers that were used. The extension time in the PCR plays an important role in adjusting the outcome of the PCR reaction. The Pfu Turbo-polymerase is a highly thermo-stable enzyme, retaining 94-99% of its polymerase activity after 1 hour at 95°C and more time is necessary during the last cycle for the polymerase to complete synthesis of all the products initiated.

Table 2-8: PCR program used for PCR-amplification.

STEP	PROCESS	TEMPERATURE [ºC]	TIME [min/s]
1	Denaturing	95ºC	45 s
START OF CYCLE (35 X)			
2	Denaturing	95 º C	15 s
3	Annealing	55 º C	15 s
4	Extension	72 º C	45 s
END OF CYCLE			
5	Extension	72 º C	10
6	Maintenance	12 º C	∞

2.13.6 Plasmid cloning strategy

In vitro recombination techniques were used to construct the plasmid pSAF62 during this study. *E. coli* strain ER2566 cells were grown and used to propagate plasmid DNA, which was further used to transform Nw 9715. The hybrid DUS-deficient plasmid p0-DUS contain the neisserial pilus biogenesis gene *pilG* harbouring a selective marker encoding erythromycin resistance (29), was used to construct the plasmid pSAF62. Based on p0-DUS, a plasmid was made harbouring a unique ATG-wadDUS, the DUS found in the Nw genome (31).

The plasmid p0-DUS was used as a substrate in a PCR with a single variation of primer SF159 (forward) and 3893OH3_*pilG*3 *SacII* (reverse), together with Pfu turbo-polymerase. The resulting PCR product and the vector pBluescript II SK + were first digest with appropriate restriction enzymes. The amplified DNA was further purified and ligated in the vector pBluescript II SK +. The concentrations of the components used during the PCR-reaction are given in Table 2-9. The PCR program used for DNA amplification is given in Table 2-10.

Table 2-9: Components concentration in the PCR-reaction.

REAGENT	AMOUNT [µL]
Template-DNA 10 ng/ µL	2
Forward primer (10 µM)	5
Reverse primer (10 µM)	5
Pfu Turbo-Polymerase (2,5 U/µL)	4
10 x cloned Pfu PCR-Puffer	20
4 × 5 mM dNTPs (mix of each NTPs)	2
H_2O	162
Total amount	200

Table 2-10: PCR program used for PCR-amplification.

STEP	PROCESS	TEMPERATURE [ºC]	TIME [min]
1	Denaturing	95ºC	45 s
START OF CYCLE (35 X)			
2	Denaturing	95 º C	15 s
3	Annealing	55 º C	15 s
4	Extension	72 º C	45 s
END OF CYCLE			
5	Extension	72 º C	10
6	Maintenance	12 º C	∞

Digestion of the PCR product and the vector pBLUESCRIPT II SK (+)

The plasmid vector and the DNA were digested with the *XhoI* and *SacII* (Cfr421) restriction enzymes. In a reaction tube the following mixture was set up for each digestion reaction at 37°C overnight and further purified with the QiaExII kit. The digestion set-up for PCR product and vector pBSIISK (+) with *XhoI* and *SacII* (Cfr421) is given in Table 2-11.

Table 2-11: Digestion of PCR product and vector pBSIISK (+) with *XhoI* and *SacII* (Cfr421).

Digestion of the PCR product	Digestion of the vector pBSIISK (+)
45 µL PCR product	1 µL of 3µg pBSIISK (+)
6 µL NEB4 + BSA buffer	4 µL NEB4 + BSA buffer
3 µL OH3 (reverse primer)	8 µL OH3 (reverse primer)
2 µL *SacII*	4 µL *SacII*
1 µL *XhoI*	4 µL *XhoI*
3 µL H_2O	40 µL H_2O

After digestion the size of the DNA was investigated by agarose gel electrophoresis using 1% agarose gels before continuing with the purification process using QiaExII and the ligation of the insert into the vector.

Ligation of DNA to the vector pBluescript II SK (+)

Ligation is the process in which two or more pieces of DNA are bind together by means of an enzyme, the ligase. The ligation process was carried out using the amplified DNA fragment and the vector pBlueScript II SK (+), with bacteriophage T4 DNA ligase. T4 DNA ligase catalyzes the ligation of blunt-ended fragments of DNA (68). Ligation process was set up in three different reaction tubes with a negative control containing no insert. The ligation mixture was set to incubation overnight at 15 °C. The ligation set-up for DNA and the vector pBSIISK + is given in Table 2-12.

Table 2-12: Ligation of DNA sample into the vector pBSIISK (+).

	1	**2**	**3**
Plasmid vector (pBSIISK (+))	5	5	5
DNA insert	0	3	9
NEB4 + BSA buffer	2	2	2
T4 DNA ligase	1	1	1
NEB4 + BSA buffer	12	9	3
H_2O (final volume)	20	20	20

Transformation of DNA into competent ER2566 cells

Transformation is the method used to introduce exogenous DNA into a competent bacterial cell. *E.coli* is the most common bacterium used in molecular biology to propagate plasmid DNA and *E.coli* strain ER2566 (New England BioLabs) were used as a suitable host cells for the plasmids used in this study. *E.coli* strain ER2566 was transformed with the plasmid pSAF62 by electroporation; a method based on a short pulse of electric charge to facilitate DNA uptake. Chemical transformation by calcium chloride is another method to induce the cells to take up the DNA. This common method was not used during this study. In both cases the purpose is to facilitate the penetration through the cell membranes. The plasmids were transformed into ER2566 cells and selected on Lytic broth by Luria Bertani (LB)-agar plates containing the appropriate antibiotic.

Briefly, electro-transformation was performed using a Gene Zapper 450/2500 apparatus (at 2000 V, 21 µF, 400 Ω). Frozen competent *E.coli* strain ER2566 cells were removed from the – 80 °C freezer and thawed on ice. 3 µL of ligation mixture and 50 µL of competent *E.coli* ER2566 cells were mixed in a sterile reaction tube chilled on ice. The tube was incubated on ice for 30 minutes. The mixture of cells and DNA was transferred to a cold electroporation cuvette and the suspension was gently tapped to allow the sample to reach the bottom of the narrow gap. The cuvette was placed in the chamber slide. The slide was pushed into the chamber until the cuvette was

seated between the electrical contacts in the base of the chamber. The "charge" button was pressed. The cuvette was removed from the chamber and 1 mL of SOC solution (2% tryptone, 0.5 % yeast extract, 10mM NaCl, 10 mM $MgSO_4$, 10 m M $MgCl_2$) was added immediately to the cuvette. The mixture of the cell and DNA was quickly but gently transferred to a new reaction tube and the mixture was incubated at 37 °C with vigorous shaking for 1 hour. The reaction tube was set on quick centrifuge (1 min, 400 × g). Most of the supernatant was poured off and the pellet was re-suspended in the residual. 50 µL and 100 µL were transferred to the center of the LB-agar plates containing appropriate antibiotic concentration (Amp100 + Erm400) and the solution was spread over the entire surface of the plate. The plates were inverted and incubated overnight at 37 °C. After an overnight incubation (37 °C) the number of antibiotic-resistant colonies was counted. The plates were further stored at room temperature.

Colonies were detected on LB-agar plates with (Amp 100 + Erm400) from the ligation with the highest amount of insert (PCR product, Tube 3, Table 2-12). These resistant colonies were further harvested on LB-agar containing (Amp100 + Erm400) and two of the resistant colonies were further used for extraction of plasmid from bacterial cells.

2.13.7 DNA purifications

Purification of DNA from solution and Gel Band

The GFX™ PCR DNA and Gel Band Purification Kit is designed to purify DNA by removing salts, enzymes, primers and unincorporated nucleotides from PCR-products. The DNA is bound to a glass fiber matrix and by using buffer solutions which contains acetate and a chaotropic agent, proteins denature and agarose is dissolved. Once the DNA of interest is captured, the proteins and the remaining contaminants are washed away. The purified DNA is further eluted in water.

Briefly, to purify DNA from the solution, one GFX column was placed in a collection tube, 2000 µL of capture buffer was added to the GFX column. The DNA solution (PCR-product) was transferred to the GFX column, mixed

thoroughly by pipetting, and centrifuged (13.000 rpm, 20 °C, for 30 s). The flow-through was discarded and 500 µL of washing buffer was added to the column, and the column was centrifuged (13.000 rpm, 20 °C, 30 s). The collection tube was discarded and the GFX column was transferred to a 1.5 mL micro-centrifuge tube. 50 µL of elution buffer (10 mM Tris-HCl pH 8.0) was applied directly to the top of the glass fiber matrix in the GFX column. The columns were incubated at room temperature for 1 minute, and centrifuged (13.000 rpm, 20 °C, 1 minute) to recover the purified DNA.

The bands cut from an agarose gel containing the DNA of interest were also purified by using GFX™ PCR DNA and Gel Band Purification Kit. The required DNA fragment was excised from the agarose gel under UV-light using a blade or scalpel and transferred into a reaction tube. The reaction tube containing the agarose slice to the nearest 10 mg was obtained and 500 µL of capture buffer was added. The reaction tube containing gel slice was mixed by vortexing and incubated at 60°C until the gel slice had dissolved completely. To help dissolve the gel, the tube was mixed by vortexing several times during incubation for 10-15 minutes. To bind DNA, the sample was applied to one GFX column in a collection tube, and centrifuged (13.000 rpm, 1 minute) to bind the DNA on the filter. The flow-through was discarded. The column was washed by adding 500 µL of washing buffer to the column and centrifuged for 30 seconds. The flow-through was discarded and the GFX column was placed to a fresh 1.5 mL micro-centrifuge tube. The DNA was eluted by adding 50 µL of elution buffer (10 mM Tris-HCl pH 8.0), incubated at room temperature for 1 minute and centrifuged for 1 minute to recover the purified DNA.

Purification of DNA by QIAGEN Plasmid Mini- and Midi-prep

The QIAGEN Plasmid “Mini and Midi-prep” were used for the purification of plasmid DNA from *E. coli.* Plasmid “Mini-prep” method is a simple, rapid and relatively low cost variant where no column and less amounts of buffers are used. The “QIAGEN Plasmid midi-prep”, is used for larger quantity and quality of DNA. This method yields DNA that is clean enough for transformation assay and for DNA sequencing. Composition of buffers P1, P2, P3 and QBT, QC and

QF used in “Mini-prep” and “QIAGEN plasmid midi-prep” are listed in Appendix 2.

“Mini-prep” method

Bacterial cells were grown overnight, 37 ºC on LB- agar plates and cells were harvested with a 1 µL loop. 250 µL of cold mini-prep re-suspension buffer P1 was added and vortexed thoroughly until all the bacteria cells were re-suspended in the solution. 250 µL of P2 solution (0.2 NaOH, 1 % SDS) was added to lyse the cells, and the tube was mixed by inversion and incubated for 5 min at room temperature. 250 µL of cold 3 M potassium acetate solution (pH 4.8) was added to neutralize the lysate and the tube was mixed by inversion and incubated for 30 min on ice. Potassium acetate precipitate dodecyl sulfate, allowing the removal of protein from DNA. The sample was centrifuged (16.000 × g, 15 min) and the supernatant was transferred to a fresh tube, avoiding the precipitate. 0.7 volumes (630 µL) of 100 % isopropanol were added to precipitate DNA. The tube was vortexed well and incubated for 30 min at 4 ºC prior to a centrifuged step (16.000 × g, 30 min) at 4 ºC. The supernatant was removed and 500 µL of 70 % ethanol was added to the pellet and mixed. A final centrifugation (16.000 × g, 10 min), and residual ethanol was removed. The pellet was dissolved in 100 µL of 10 mM Tris-HCl (pH 8.5).

QIAGEN Plasmid midi-prep

In this study a QIAGEN plasmid midi kits protocol has been used for plasmid DNA purification. Bacteria cells were grown overnight, 37 ºC on LB–agar plates. The bacteria cells were re-suspended in 4 mL re-suspension buffer P1 and vortexed thoroughly. Similar amount of buffer P2 was added, mixed vigorously by inverting 4-6 times and incubated at room temperature for 5 min. 4 mL of buffer P3 was added, mixed by inverting 4-6 times and incubated on ice for 15 min. Centrifuged (≥ 20.000 × g, for 30 min) at 4 ºC. A QIAGEN-tip 100 was equilibrated with 4 mL of buffer QBT, and the column was allowed to empty by gravity flow. The supernatant was further applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with buffer QC (2 × 10 mL). DNA was eluted with 5 mL buffer QF into clean

15 mL tube. Plasmid DNA was further precipitated by adding 0.7 volumes of isopropanol (3.5 mL) and mixed. The sample was centrifuged (≥ 15.000 × g for 30 min) at 4 ºC. Plasmid DNA was washed with 2 m L 70 % ethanol, centrifuged (≥ 15.000 × g for 10 min) and residual ethanol was removed. The pellet was dissolved in 100 µL of 10 mM Tris-HCl (pH 8.5). The yield of plasmid DNA was further determined by electrophoresis on an agarose gel as described and by NanoDrop® ND-1000 Spectrophotometer absorbance at 260 nm.

2.13.8 Sterilization of plasmid DNA

A plasmid DNA preparation of the pSAF62 construct was during this study observed to be contaminated with other microbes. To be able to use this plasmid DNA batch the following sterilization method was used to obtain water-saturated chloroform. Briefly, chloroform (50 mL) was added in 0.5 mL H_2O in a reaction tube and mixed thoroughly.150 µL of water-saturated chloroform was taken from the bottom of the reaction tube and added to about 300 µL plasmid DNA, mixed thoroughly to lyse the contaminating bacteria and centrifuged (13.000 rpm, 20°C, for 10 min). The sterile supernatant was transferred into a new reaction tube and pellet was discarded. The DNA concentration was measured by NanoDrop® ND-1000 Spectrophotometer and gel electrophoresis. The sterilized plasmid DNA was stored in -20°C.

2.13.9 DNA sequencing

Dideoxy DNA sequencing method or chain determination method is an enzymatic sequencing method developed by Fred Sanger (69), based on DNA synthesis in the presence of dideoxynucleotides which contains a hydrogen group on the 3' carbon instead of a hydroxyl group (OH). DNA sequencing is a precise method to determine the order of nucleotides within a DNA molecule. Dideoxynucleotides are chain terminating inhibitors of DNA polymerase and abbreviated as ddNTPs (ddGTP, ddCTP, ddTTP, ddATP), when integrated into a sequence, prevent the addition of the normal nucleotides, deoxynucleotide triphosphate dNTPs (dGTP, dCTP, dTTP, and dATP) found in DNA.

This method allows the synthesis of DNA strands of various lengths that can further be separated by size. Transcript elongation in DNA synthesis depends

on free hydroxyl groups at the deoxynucleotide triphosphate (dNTPs) 3' carbon position and formation of phosphodiester bonds (70). The hydrogen atoms inhibit the formation of phosphodiester bonds and terminate the DNA synthesis. This produces all possible lengths of chains. To determine the base termination of each fragment with the fragment length (which helps determining the order and position of the bases in the DNA molecule), fluorescent labeling of the ddNTPs, by dye molecules can be applied (66).

The DNA sequences of all the constructs; *rpsL* DNA (PCR-product), genomic,-and plasmid DNA were confirmed by DNA sequencing analysis. A list of primers is given in Appendix 3. Genomic- and *rpsL* DNA (PCR products) were sequenced at the Institute of Medical Microbiology, Department of Molecular Microbiology at the National Hospital, Oslo, using ABI Big Dye Terminator v.3.1 DNA sequencing kit (Applied Biosystems). 3.2 pmol reaction mixes of primers, 5-10 ng PCR product (200-500 bp DNA) was used. Plasmid DNA was sequenced at GATC Biotech AG, European custom sequencing center, Germany. 10 pmol reaction mixes of primers, 80-100 ng plasmids DNA product was used.

To retrieve sequence data, the computer program Chromas was used. Chromas display the chromatogram files from the sequencers. Raw data from the sequencing had to be controlled for possible corrections of the base calls. Sequences were aligned using the program ClustalX program, which provides an integrated environment for performing multiple sequence and profile alignments and analyzing of the results. Genedoc was used for viewing and editing the alignment and with the help of the versatile coloring scheme incorporated in the program the conserved areas were highlighted in the alignment.

2.14 Protein Methods

2.14.1 Preparation of protein sample for SDS-PAGE

Whole-Cell Lysate Samples

The neisserial cells were spread on the blood and GC agar and the plates were incubated overnight under standard incubation conditions. The cells were harvested in 1 mL H_2O and vortexed until the cells were totally in suspension. Equal amounts of cells were prepared based on OD measurements (OD_{595}). Cells equivalent to 50 µL with an OD_{595} equals 0.5 were resuspended in 50 µL H_2O. 50 µL of 4 × Lithium dodecyl sulfate (LDS) buffer was added; the solution was mixed thoroughly and boiled at 100 °C for 7 min. The samples were stored at – 20°C until further analysis.

2.14.2 Isolation and Purification of pili

The neisserial cells for pili purification were spread evenly on the GC and blood agar, and the plates were incubated overnight under standard incubation conditions. Briefly, bacteria cells from two 90 mm Petri dish were harvested and dissolved in 1 mL 0.15 M ethanolamine buffer (pH 10.5) and further vortexed in reaction tubes until the cells are totally in suspension and afterwards centrifuged (16.000 × g, at 4 °C, for 5 min). Ethanolamine buffer (0.15 M, pH 10.5) is basic solution and makes the pili shear off from the surface of the cell. The supernatant was aspirated and transferred into a new reaction tube and the cell pellet (cells without pili) was re-suspended in 1 mL H_2O and was saved for total cell protein-determination. 1/10 volume of saturated ammonium sulphate (crystallized the pilus) in 0.15 M ethanolamine buffer was added to the supernatant, the sample was vortexed and incubated at room temperature for 30 minutes. To collect the ammonium sulphate precipitate the reaction tube was centrifuged (16.000 × g, at 4 °C, for 5 min). The supernatant was removed and the pellet was washed twice with 0.05 M Tris-buffered saline (pH 8.0), the sample was vortexed until the pellet was dissolved in the solution and thereafter centrifuged (16.000 × g, at 4 °C, for 5 min). The supernatant was removed carefully. Traces of salt from TBS buffer may prevent solubilization of the pili pellet in H_2O. The pellet was fully

resuspended in 30 µL of H_2O. 30 µL of 4 × Lithium dodecyl sulfate – polyacrylamide gel electrophoresis (LDS-PAGE) sample buffer was added and, the samples were mixed and subsequently stored at -20 °C.

2.14.3 SDS-PAGE

To separate proteins according to their electrophoretic mobility; length and charge of polypeptide chain in an electric field, SDS-PAGE is used. An electric field is used to move the molecules through a viscous gel-like medium. The strongly anionic detergent SDS is used in combination with reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denaturated polypeptides bind SDS and become negatively charged (71).

Tricine gel with 4% stacking gel and 12% separating gel was made and the components were mixed according to the mixture table given in Appendix 2. 4 × LDS-PAGE sample buffers together with ß-mercaptoethanol was added to the proteins and heated to dissociate before they were loaded on the gel. BioRad Mini Protean system was also used for SDS-PAGE and the samples with a protein size ladder was loaded on the gel and separated for 1 hour under constant voltage (200 V). NuPAGE 10% Bis-Tris Gel (1.0 mm × 10 wells, Novex by life technologies) was also used to separate proteins.

2.14.4 Coomassie Blue Staining

The position of the protein-bands in the SDS-PAGE can be revealed by staining with Coomassie Brilliant Blue R-250. The gel was incubated in Coomassie Brilliant Blue R-250 staining solution under agitation for a minimum 30 minutes. The gel was destained in Coomassie blue destaining solution with agitation applied for 3-4 hours. For longer time storage the stained gel was dried in a vacuum drier for 1 hour at 80 ºC.

2.14.5 Western blot and immunoblotting

An alternative method to Coomassie blue staining to detect proteins is by binding of antibodies. This method involved transfer of proteins from the gel to a membrane. An electric field is applied to allow transfer of proteins that were to be examined. Proteins from the gel bind to the Polyvinylidene Difluoride (PVDF)-membrane and thereby gets immobilized due to the membrane

positive charge surface. The membrane is then incubated with the antigen specific primary antibodies directed against the protein. A secondary enzyme coupled antibody was used to detect primary antibody and finally the protein bands were visualized by an enzymatic reaction. Method where antibodies are used to detect proteins is called immunoblotting.

Briefly, proteins sample from SDS-PAGE were transferred to a membrane, Immobiton^{TM}-P Transfer Membrane, filter-type: PVDF, pore size; 0.45 µm. The PVDF-membrane was washed in methanol prior to use. The SDS-PAGE gel was placed directly on the PVDF membrane, packed between two WHATMAN filter papers (3MM) and placed between sponges, in a transfer box filled with Western Blot transfer buffer. The transfer was carried out under constant current 400mA for 1 hour. After the proteins have been transferred to the membrane, the membrane was rinsed with a Western Blot blocking buffer before it was transferred to into 10 mL 2 % transfer blocking buffer with 2 % (w/v) skim milk powder and saturated for 1 hour under agitation. 5 µL of the first antibody (Rabbit antibody-Pilus fiber from MC, see table 2-3) at a dilution of 1:2000 was added in 10 mL Western Blot blocking buffer with 2% (w/v) skim milk and the membrane was incubated overnight (4°C) with agitation. The PVDF membrane was washed with Western Blot blocking buffer 2 × 2 min and 1 × 30 min. 0.4 µL of Secondary antibody (Goat anti-rabbit IgG-AP conjugated) at a dilution of 1: 30 000 was added in 10 mL Western Blot blocking buffer with 2% (w/v) skim milk under agitation and incubated for 1 hour. The membrane was washed with Western Blot blocking buffer 2 × 2 min, 1 × 30 min and further it was washed with Western developing buffer for 2 minutes. 10 mL Western developing buffer containing 20 µL Nitro blue tetrazolium chloride (NBT) (75 mg/mL) in 70% Dimethylformamide (DMF) and 15 µL Bromo-chloro-indolyl phosphate (BCIP) (50 mg/mL in 100 % DMF) was added for the developing of the membrane until distinct bands appeared. The developing step stopped by rinsing the membrane with H_2O. The membrane was dried between to paper sheets in a dark place.

2.14.6 Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometer (MS)

Matrix Assisted Laser Desorption /ionization Time of flight (MALDI-TOF) mass spectrometer (MS) in combination with a dedicated bioinformatics software tool MALDI Biotyper 3.1 was used for the identification of *Neisseria* strains. The technique allows transfer, desorption and ionization of large molecules into the gas phase. For the MALDI Biotyper 3.1 identification approach protein peaks in the mass-to-charge (m/z) ration of 2000-18000 Da were used. The MALDI Biotyper identifies microorganism by analyzing the expression of their intrinsic proteins using MS. An organic acid referred to as the “matrix”, is responsible for ionization, facilitates desorption and prevents analytes from decomposing (72).

For the development of MS database of *Neisseriaceae*, MicroFlex LT MALDI-TOF-MS (Bruker Daltonics, Germany) together with MALDI Biotyper 3.1 software was used. MicroFlex LT uses the same method described above and is designed for automatic protein identification, characterization, detection of biomarkers, quality control of oligonucleotides and single-nucleotide polymorphism (SNP) genotyping, which makes the instrument a rapid and automatic platform for the identification of microorganisms (73). The spectra obtained by MicroFlex LT MALDI-TOF-MS were compared with the database provided by the manufacturer, allowing the comparison and differentiation of bacteria by their protein profiles/ fingerprints (74).

Neisserial strains were spread on blood agar and incubated under standard conditions. For sample preparation the formic acid extraction method was applied. For each strain eight spots on the MALDI-target plate were spotted and analyzed three times. The 24 spectra were obtained in the automatic acquisition mode. The identification by MicroFlex LT MALDI-TOF-MS of the strains was performed in duplicates. The identification score cut-off values were applied on each measurement according to the manufacturer’s instructions. Score values ≥ 2.0 is recommended as a probable identification at species level. The obtained spectra (raw data) were calibrated and corrugated by using Flex Analysis program. The modified spectra were added in the

MALDI Biotyper Offline classification (OC) program 3.1 in a separate project folder. To test the performance of the database MALDI-TOF-MS BioTyper 2.0 was used, due to some software problems occurred with the BioTyper database 3.1. A MALDI-TOF-MS profiling protocol was compared with a laboratory strain *E.coli* DH5α, recommended by the manufacturer (Bruker Daltonics Germany). Materials, the matrix solution and the Standard User Bacterial positive control (*E.coli* DH5α 255343, Bruker) used are listed in Appendix 2. The list of *Neisseria* strains used for the establishment for Biotyper database is given in Appendix 3.

Formic Acid Extraction method

300 µL of H_2O was added in reaction tubes. With the help of 1 µL loop, single bacteria colony was picked from the agar-plates and dissolved thoroughly in the H_2O by vortexing. 900 µL of 100% ethanol was added and mixed thoroughly by vortexing and the sample was centrifuged at 13 000 rpm, for 2 min. The supernatant was discarded and the pellet was centrifuged (13 000 rpm, for 2 min). The remaining supernatant was discarded and the reaction tubes were left open for 1-2 min to air dry the pellet. 50 µL of 70 % formic acid was added and the pellet was dissolved by pipetting several times. 50 µL of acetonitrile was added, mixed and centrifuged (13 000 rpm, for 2 min).1 µL of the supernatant was applied on the target plate and let to dry. The sample spot was covered with 1 µL matrix solution and air dried. The samples were directly analyzed by MicroFlex LT MALDI-TOF-MS.

Micro Flex-control setup

The spectra were obtained by using MicroFlex LT MALDI-TOF-MS. The procedure of using Flex-control software is mentioned below.

- Click: AutoXecute tab and select: Run → new → MSP 96 →Next (an overview picture of the plate appears).
- Under AutoXecute Method, choose: MALDI Biotyper (MBT), MBT_AutoX. Other: None.
- Under Data directory; Select the destination folder or create a folder with your initial and under sample name, enter sample numbers or name.

- Select the correct number of points on the plate (= 8), and click the add button (green plus sign) on the top three times.
- Repeat this for all the isolates. Save setup (disk) button in the same folder that you selected in the Data directory. Close the window. You will return to the main page of Flex Control.
- Select Run: Load (your current file that you saved)
- Press → Start Automatic Run.

Flex-Analysis software setup

The spectra obtained by Micro Flex-control software were further calibrated and checked for abnormalities by using the Flex-analysis software.
Following steps were executed:

- Click Open, then select the appropriate folder (similar to Data directory) and open all your spectra.
- Highlight control in the list, press Calibrate→ Internal → Automatic Assign. Right-click the control, select Properties and check the calibration constants. The calibration constant contains three numbers. Check the calibration constants for a range of set of isolate you measures, the numbers should be the same as that of control.
- Choose one set raw specter from the list, right click and select Load.
- Click Method →Open and select MBT_standard. Flex analysis Mass Spectrometry (FAMS) Method → Open
- Click Process →Substract Mass Spectrum Baseline, and then click Smooth Mass Spectrum.
- Zoom in on the peaks and check for any flat lines or other abnormalities (To check the peaks start at about 2-3000 Dalton (first fine top) and check peak at each 1000 D (up to 10,000). The peaks should look the same and have a high enough intensity, not be shifted to the right or left).
- Delete any spectra by selecting them in the list on the left, press Ctrl + F4 (or right click and select close) and select “No” for whether to have been asked to save.
- Go back to the file explorer under Open, right click on the appropriate set of spectra and rename it and then Close.

- Save the current set of modified spectra. You will now have a folder with original raw spectre, and a folder with modified spectra. Repeat the same process for all the spectra.

MALDI Biotyper Offline Classification (OC)

To add the modified spectra to the database the MALDI Biotyper (OC) 3.1 program was used as followed:

- Click Add Spectra; select the appropriate set of modified spectra.
  Select all, click Action →Preprocessing→ Normalize
  Click Action again, MSP Creation → Create
- Give the specter a name (full microorganism name, since this specter is going to be placed in the library), it will now be placed under Unassigned MSPs.
- Right click on it and click Start Taxonomy Tree Editor. Unassigned MSPs is to the left, select the ones you want to move over and click the right arrow (You can also delete the spectra if desired, right click on the appropriate spectrum and select Delete).

3 RESULTS

Natural competence of transformation among pathogenic *Neisseria* species is highly associated with piliation (3). A strain of *Neisseria wadsworthii* (Nw) was examined and studied with regard to phenotypic and biochemical properties and pilus related properties on protein and nucleic acid levels. The reference strain included in this study was Nw 9715 (DSMZ number 22247).

Phenotypic identification methods revealed that Nw bacteria cells in Gram staining are negative, pink colored, coccoid shaped, like coffee beans. Colonies are small and circular, light yellow and non-hemolytic on blood agar. They are oxidase positive, penicillinase sensitive and produce acid only from glucose. The antibiotics MIC on BHI medium with streptomycin was determined to be 50 µg mL^{-1}. Good growth of Nw was obtained on blood and GC agar. No growth was obtained on blood agar containing erythromycin (8 µg mL^{-1}), Chocolate-agar containing (streptomycin 50 µg mL^{-1}) and GC-agar containing (kanamycin 100 µg mL^{-1}).

3.1 Colony Morphology

To study the colony morphology of Nw 9715, several colonies were propagated on solid GC agar plates and incubated under standard incubation conditions. Differences in colony morphologies become readily apparent when studied in a Leica GZ7 dissecting microscope. Earlier studies have reported that the presence of pili among neisserial species is indicated by the bacteria forming colonies with a sharp perimeter defined edge, while colonies formed by non-piliated bacteria grow with a diffuse edge(75).

Nw was observed to have a “high” and rough colony formation on GC agar. The colonies were translucent, grew larger on GC-agar than on blood agar, and grew faster than those of *N. gonorrhoeae* (N400) under the standard cultivation conditions. The colonies of Nw also showed auto-agglutination, indicating the presence of Fimbriae (pili) on their surface of the bacterial cell. Nw colonies showing auto-agglutination on fresh 1% GC- agar observed after 1 day of incubation (15-18 hours) are illustrated in the Figure 7.

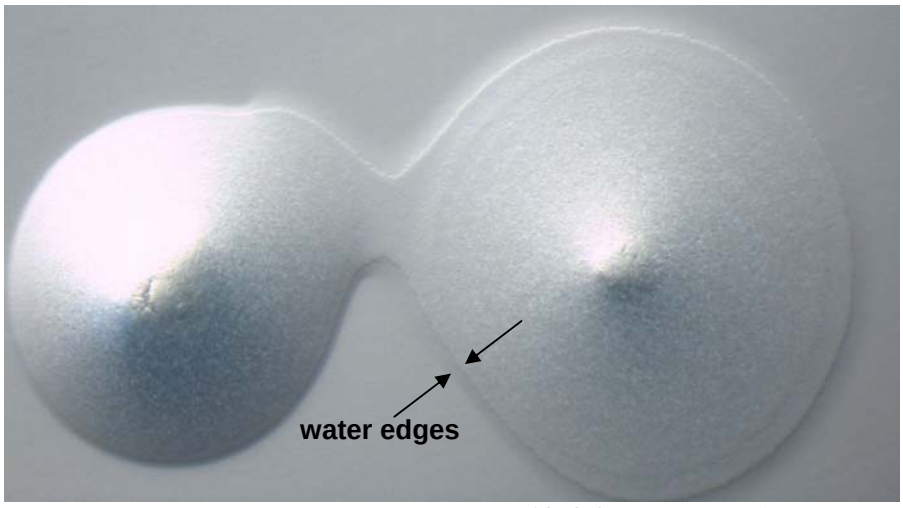


Figure 7: Piliated *N. wadsworthii* colonies on 1% GC-agar. The “water edges” collected around the colonies are visible and indicated by the arrow.

3.2 Homologue genes of the Type IV pilus biogenesis machinery

To identify genes involved in HGT a search in the genome sequence of Nw was performed using BLAST and EDGAR. Several Nw 9715 genes were identified with substantial similarities to genes for Tfp biogenesis components and other genes in *N. meningitidis* (Mc58). Results are given in Table 3-1. This search was kindly performed by Dr. Stephan A. Frye. Of the twenty one genes analyzed almost all homologues were found. Only one copy of *pilC* was found in the genome of Nw 9715.

Table 3-1: Homologue Type IV pilus biogenesis components found in *N. meningitidis* Mc58 and *N. wadsworthii* 9715.

Protein	Involved In	*N. meningitidis* Mc58 gene number	*N. wadsworthii* 9715 gene number
PilM	PB*	NMB1808	HMPREF9370_2326
PilN	PB	NMB1809	HMPREF9370_2325
PilO	PB	NMB1810	HMPREF9370_2324
PilP	PB, HGT**	NMB1811	HMPREF9370_2323
PilQ	PB, HGT	NMB1812	HMPREF9370_2322
PilD	PB	NMB0332	HMPREF9370_2478
PilT-1	PB	NMB0052	HMPREF9370_0153
PilT-2	PB	NMB0768	HMPREF9370_1730
PilF	PB	NMB0329	HMPREF9370_2480
PilG	PB, HGT	NMB0333	HMPREF9370_2479
PilE	PB, HGT	NMB0018	HMPREF9370_0129
ComP	HGT	NMB2016	HMPREF9370_1126
ComL	HGT	NMB0703	HMPREF9370_1267
PilV	PB, HGT	NMB0547	HMPREF9370_1127
PilC1	PB	NMB1847	HMPREF9370_2421
PilC2	PB	NMB0049	
PilW	PB	NMB1309	HMPREF9370_1859
PilZ	PB	NMB0770	HMPREF9370_2487
RecA	HGT	NMB1445	HMPREF9370_1256
DprA	HGT	NMB0116	HMPREF9370_2388
Ssb	HGT	NMB1460	HMPREF9370_1257

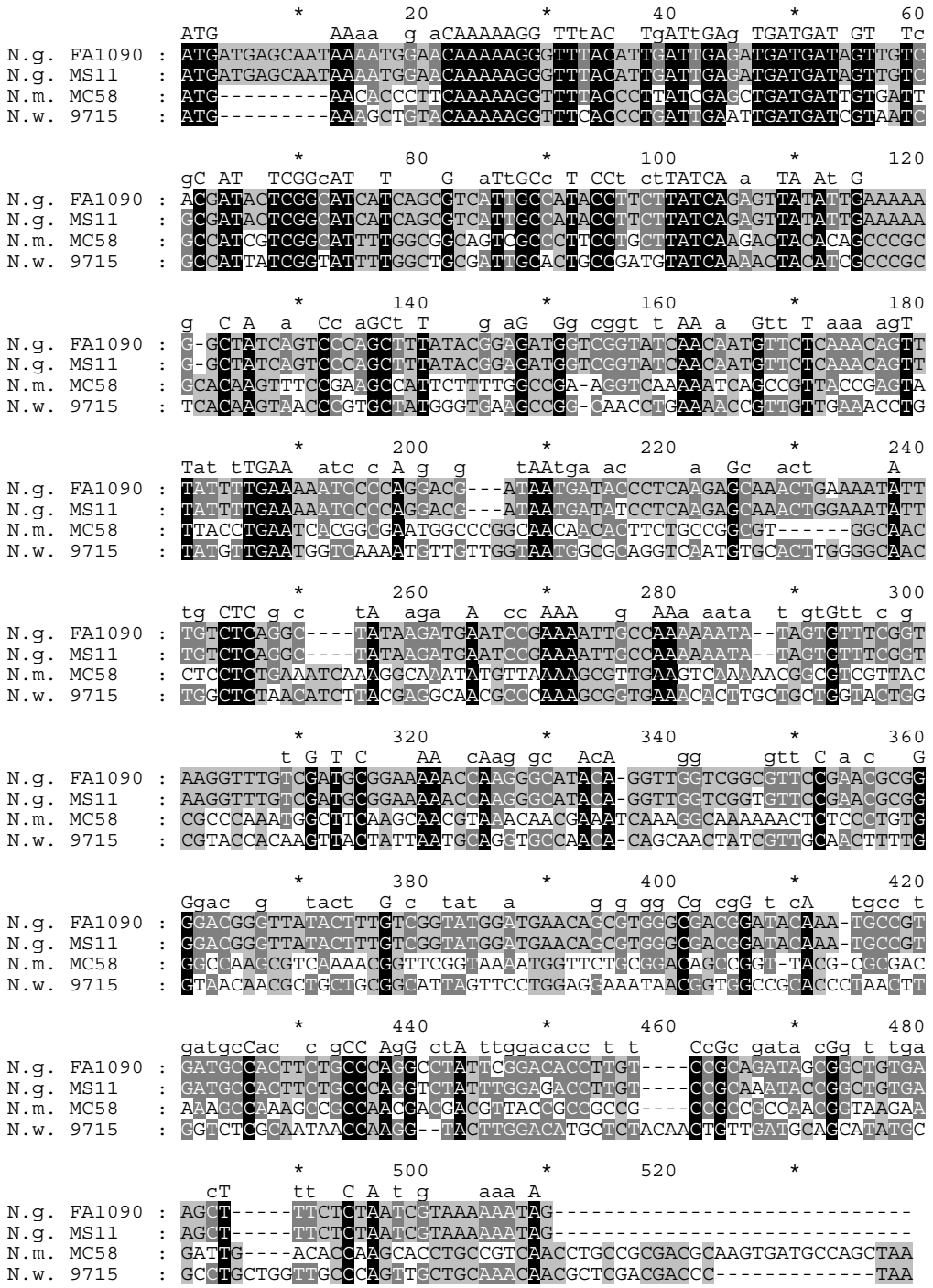
* = pilus biogenesis ** = directly involved in HGT

3.3 DNA Sequence Analysis of the *pilE* gene in *N. wadsworthii*

3.3.1 pilE sequence from N. wadsworthii

The DNA sequence of the *pilE* gene from the *N. wadsworthii* strain 9715 was found as shown above in Table 3-1. The nucleotide sequence was compared to other neisserial *pilE* sequences and the alignment (Figure 8) generated with ClustalX and displayed with Genedoc at standard settings.

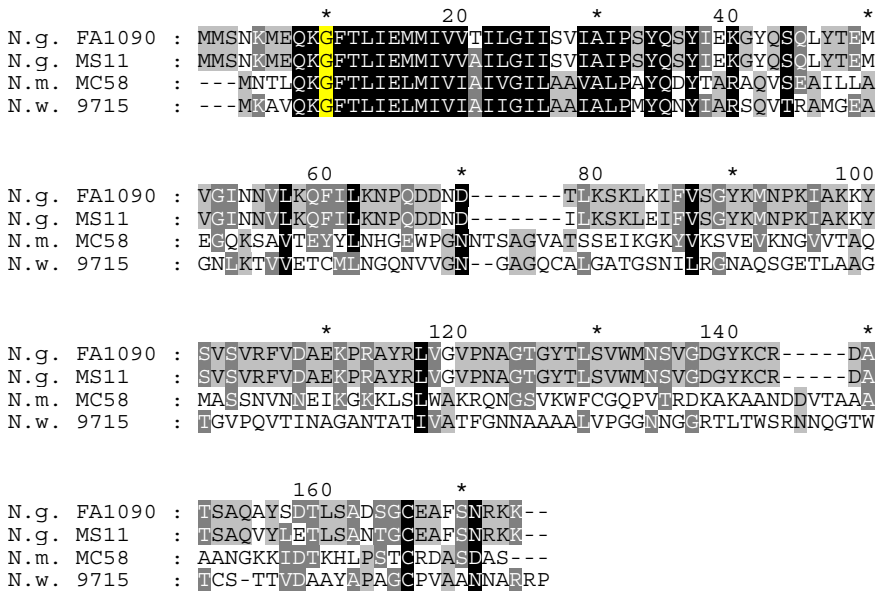
Figure 8: (see next page) *PilE* gene sequences from *N. gonorrhoeae* FA1090 (NGO0456) and MS11 (NGFG00610), *N. meningitidis* MC58 (NMB0018) and *Neisseria wadsworthii* 9715 (HMPREF9370_0129).



The *pilE* gene from *N. wadsworthii* 9715 strain harbour a conserved 5'-end and (hyper)-variable regions towards the 3'-end.

Based on the *pilE* nucleotide sequence the amino acid sequences of the *N. wadsworthii* PilE protein was generated. This sequence was compared to other neisserial PilE sequences (Figure 9). The sequences were aligned using ClustalX and the alignment is displayed by Genedoc at standard settings.

Figure 9: (see next page) Alignment of amino acid sequences of *pilE* genes from selected species. The PilE sequences from *N. gonorrhoeae* FA1090 (YP207607) and MS11 (ZP06132803), *N. meningitidis* MC58 (NP273084) and *Neisseria wadsworthii* 9715 (EGZ51246) are shown. The site processed by the peptidase PilD is marked in yellow.



3.4 Detection of the Pilin Subunit, PilE, by SDS-PAGE and Immunoblotting

Among neisserial species, natural competence for transformation is highly associated with the presence of pili. Analysis of the Nw pilus subunit, PilE was therefore performed. The presence of PilE in Nw was detected by protein analysis, SDS-PAGE and immunoblotting. Proteins from whole-cell lysates were separated according to size on a 12% SDS-PAGE gel. Standard procedure of Western blotting was performed as described in the methods and materials.

Figure 10 shows the detection of the PilE in whole-cell lysates from Nw examined by SDS-PAGE, Western Blotting and immunoblotting analysis, using antiserum generated against the purified pili from Mc M1080 (K849). The antibodies were kindly provided by Dr. Stephan A. Frye. Different amounts of whole-cells lysates were applied onto the gel. Based on the intensity of the bands detected in the immunoblotting, the Nw pili showed weaker reactivity with antibodies raised against class I pili from Mc M1080. The predicted size of the protein bands that reacted with the anti-PilE antibody ranged between 15-16 kDa, indicated with the arrow (A).

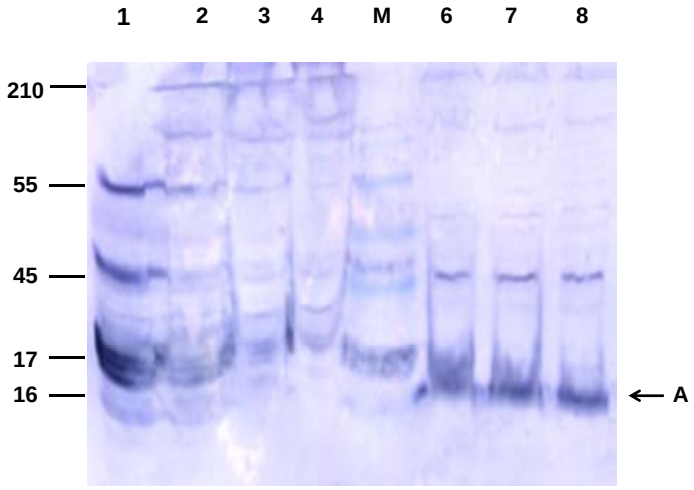


Figure 10: Detection of PilE antigen in whole-cell lysates of *N. wadsworthii* strain and *N. gonorrhoeae* (N400) by immunoblotting. The pili were reacted with antiserum generated against the purified pili from *N. meningitidis* M1080 (K849). Individual lanes were loaded with bacterial lysates as follows: 1and 2; 3 µL of *N. gonorrhoeae* (N400); 3, 6 µL of *N. gonorrhoeae* (N400); 4, 10 µL of *N. gonorrhoeae* (N400); M (5), 4 µL Seeblue plus® Ladder; 6, 10 µL of *N. wadsworthii*; 7, 6 µL of *N. wadsworthii*; 8, 3 µL of *N. wadsworthii*. Lines on the left indicate the positions of the protein size marker and the arrow on the right (A) present the predicted size of the protein from *N. wadsworthii* in kDa.

To measure pilus expression in Nw 9715, purification of extracellular bacterial pili was also performed during this study. Pilus purification method described above was used to obtain samples of pili from Nw 9715.Purified pili from Nw were analyzed on a Coomassie stained SDS-PAGE gel and by immunoblotting to determine the pili isolated. *N. gonorrhoeae* (N400) was used as a positive control. The purified pilus from Nw 9715 and *N. gonorrhoeae* (N400) detected with the Coomassie Blue staining are shown in Figure 11. The arrows on the right present the predicted size of the protein bands from *N gonorrhoeae* (N400) (A) and from *N. wadsworthii* (B) in kDa.

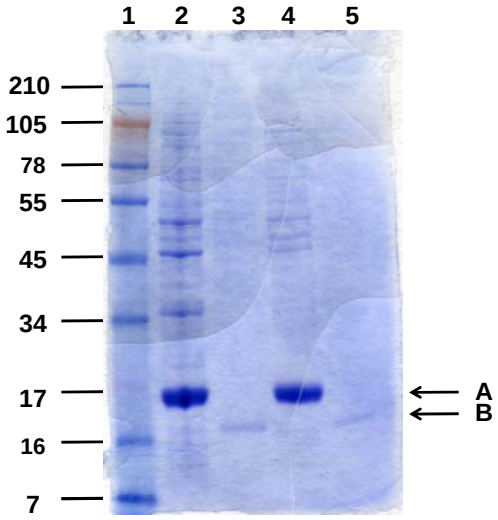


Figure 11: Detection of PilE in the pilus-prep from *N. wadsworthii* and *N. gonorrhoeae* by SDS-PAGE and Coomassie Blue staining**.** Lanes: 1; Seeblue plus® Ladder; 2, *N. gonorrhoeae* (N400); 3, *N. wadsworthii* 9715; 4, *N. gonorrhoeae* (N400); 5, *N. wadsworthii* 9715. 5 µL of sample was loaded on each lane. Lines on the left indicate the positions of the protein size marker and arrows on the right (A) and (B) present the predicted size of the proteins from *N. gonorrhoeae* and *N. wadsworthii* in kDa.

The purified pilus from Nw 9715 and *N. gonorrhoeae* (N400) detected by immunoblotting are shown in Figure 12. The arrows on the right present the predicted size of the protein bands from *N gonorrhoeae* (N400) (A) and from *N. wadsworthii* 9715 (B) in kDa.

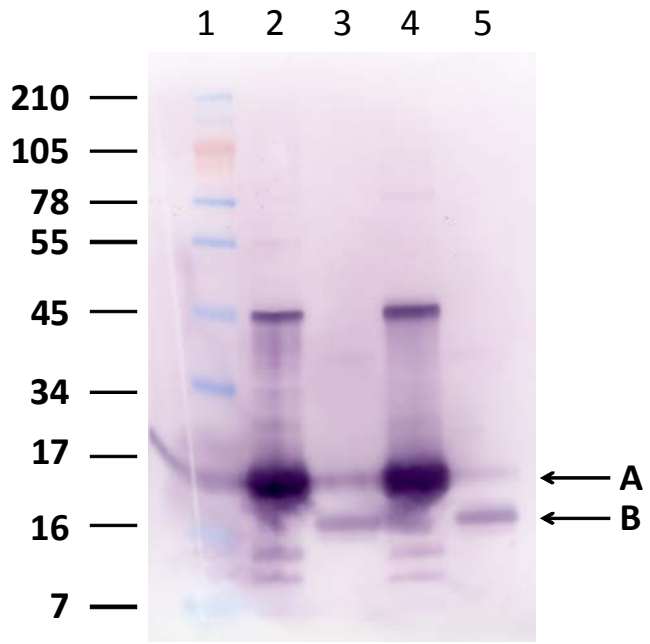


Figure 12: Detection of PilE protein in purified pilus from *N. wadsworthii* and *N. gonorrhoeae* by immunoblotting. The pili reacted with rabbit polyclonal antibodies against purified pili from *N. meningitidis* M1080 (K849). Lanes: 1; Seeblue plus® Ladder; 2, *N. gonorrhoeae* (N400); 3, *N. wadsworthii* 9715; 4, *N. gonorrhoeae* (N400); 5, *N. wadsworthii* 9715. 5 µL of sample was loaded on each lane. Lines on the left indicate the positions of the protein size marker and arrows on the right (A) and (B) present the predicted size of the proteins from *N. gonorrhoeae* and *N. wadsworthii* in kDa.

Results obtained by Coomassie blue staining and immunoblotting showed that Nw 9715 express pili. Pili purified from Nw had a protein size of approximately 16-17 kDa (B), indicating that the Nw strain examined during this study express PilE. The PilE expressed from *N. gonorrhoeae* (N400) had a size of approximately 17 kDa (A). The migration of PilE in immunoblotting indicates a size variation between the PilE subunits of Nw, and Gc (N400).

3.5 Competence for Transformation

Natural competence for transformation is a physiological state which permits the bacteria to take up and integrate exogenous DNA into its genome (19). Nw cells were transformed with DNA encoding different antibiotic resistance. By including different DUS in the DNA, DUS dependency was monitored.

3.5.1 Transformation with genomic DNA conferring streptomycin resistance

Streptomycin resistance may be caused by a single nucleotide variant in the gene encoding the ribosomal protein S12. Spontaneous streptomycin-resistant (SR) mutants of Nw were selected on blood agar containing 1000 µg/mL streptomycin. Genomic DNA from one of the spontaneous streptomycin resistant mutants of Nw was prepared to use in transformation. A pilot-transformation experiment was performed using 0.5 µg DNA per 1 mL cells suspension. Transformants were selected on chocolate agar containing streptomycin at a concentration of 50 µg mL^{-1}. The results obtained by this transformation experiment are given in Table 3-2.

Table 3-2: Pilot-transformation of *N. wadsworthii* 9715 (wt) with genomic DNA conferring streptomycin resistance.

Dilution	Numbers of colonies from 100 µL
10^{-0}	∞
10^{-1}	∞
10^{-2}	361.6
10^{-3}	52.3
10^{-4}	3
10^{-5}	0.7
10^{-6}	0

Donor DNA: *N. wadsworthii* SR mutant. Recipient cells: *N. wadsworthii* 9715 (wt)

Uncountable numbers of transformants appeared on the plates without dilution and with 10^{-1} dilution of the transformed bacterial suspension. The numbers of transformants were countable from the 10^{-2} dilution downwards. It was demonstrated that Nw was highly transformable with genomic DNA and hence is naturally competent for transformation.

3.6 Quantitative Transformation

To determine the transformation frequency of Nw, a quantitative transformation assay was performed. Quantitative genetic transformation made it possible to determine, how many Nw cells were transformed when they are subjected to a given amount of DNA per time unit. The recipient cell numbers were determined by non-selective cultivation on blood agar from the recipient culture. Genomic DNA from *N. mucosa* 19696^{SR} and *N. wadsworthii*^{SR} encoding streptomycin resistance were used as a template DNA in the PCR to generate *rpsL* transforming PCR produced DNA. The primers used in the PCRs contained different DUS-dialects so that sets of transforming DNA were generated, differed only in their DUS dialects. The sizes of the PCR products are given in Table 3-3. Detailed data for primers used in this study are given in Appendix 3.

Table 3-3: PCR-products

PCR products	FORWARD PRIMER	REVERSE PRIMER	SIZE [bp]
0-DUS	OHA2184	OHA2186	400
AT-DUS (a)	OHA2183	OHA2186	400
AT-DUS (b)	OHA2137	OHA2186	400
ATG-wadDUS (a)	SF151	OHA2186	400
ATG-wadDUS (b)	SF153	SF157	400
ATG-wadDUS (c)	SF158	SF157	400

3.6.1 Quantitative transformation with SR conferring DNA PCR produced from *N. mucosa* 19696^{SR}

Nw was transformed with *rpsL* DNA (PCR product) encoding SR containing different DUS-dialects as follows; 0-DUS (negative control without DNA uptake sequence), AT-DUS (positive control with extended 12-mer AT-DUS) and ATG-wadDUS (DUS sequence present in the genome of Nw). Detailed data for *rpsL* primers are given in Appendix 3. The template used to generate PCR products was *N. mucosa* 19696^{SR} encoding SR. The recipient Nw cultures were mixed with 0.5 µg/mL transforming DNA. Quantitative transformation procedure mentioned above was applied (see section 2.12.1).The results given in Table 3-4a are the average transformation frequencies obtained from four independent experiments. The results are also illustrated in Figure 13. Notably, the SR DNA containing ATG-wadDUS produced the highest frequency (2.5×10^{-5}) at statistically significant levels, compared to 6.4×10^{-6} obtained with AT-DUS. The 0-DUS (negative control without DNA uptake sequence) gave only rise to a few transformants, showing a minimal level of DUS-independent DNA uptake. Transformation frequency with 0-DUS was in general very sparse.

Table 3-4a: Result of quantitative transformation with SR conferring DNA (PCR produced) from *N. mucosa* 19696^{SR} Recipient strain: *N. wadsworthii* 9715.

TRANSFORMING *rpsL* DNA (PCR product)	**AVERAGE**
0-DUS	7.6×10^{-7}
AT-DUS (a)	6.4×10^{-6}
ATG-wadDUS (a)	2.5×10^{-5}

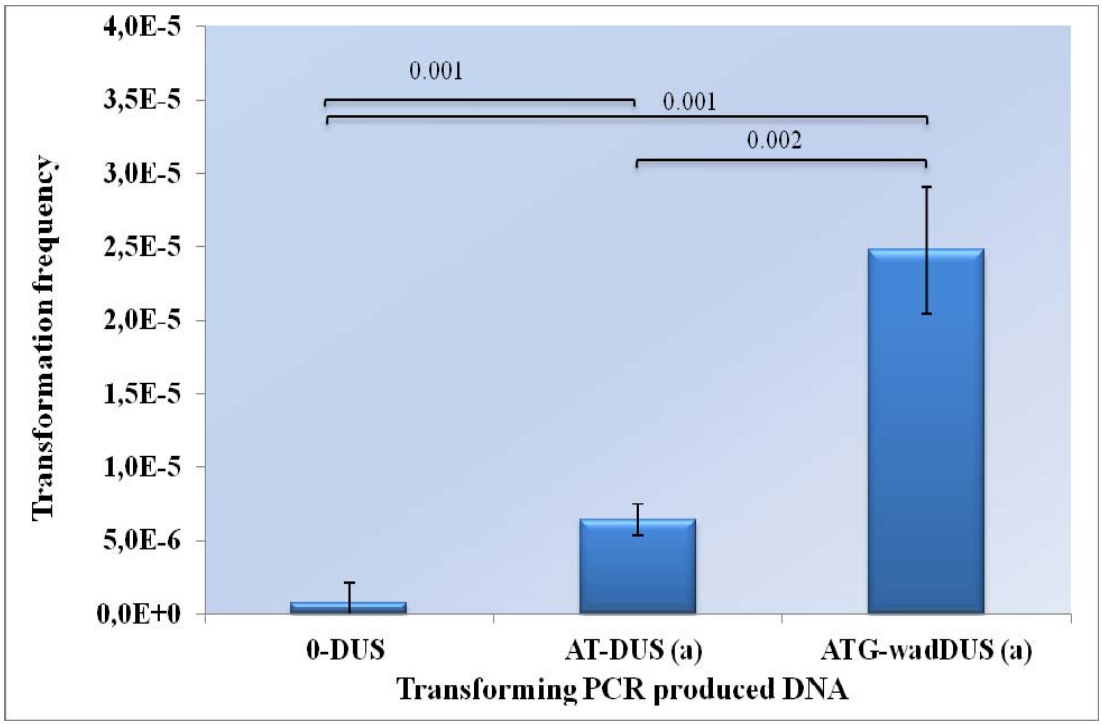


Figure 13: Results from quantitative transformation of *Neisseria wadsworthii* with SR conferring DNA PCR product amplified from *N. mucosa* 19696^{SR}. The X axis shows the three different transforming SR-DNAs used. The Y axis shows the average transformation frequency. Values are the averages of four independent experiments and standard deviations are indicated by bars. Statistically differences calculated by a two tailed paired student's t-test are indicated above the columns.

Once it was determined that Nw is transformable, but yielded low transformation frequency with AT-DUS, which was found in all Mc and Gc genomes and in the genomes of other commensal *Neisseria* species. It was interesting to investigate if transformation efficiency could be affected if the orientation of the DUS were altered. Similar sets of transforming SR-DNA (PCR product) as above were employed (Table 3-4a), with DUS inserted in one of the two possible orientations (forward and reverse complement). Their potential influence of DUS-orientation was investigated in transformation of Nw. A new AT-DUS (b) (see Table 3-3) reverse complement was employed and compared to the other three SR-DNA. The result of the quantitative transformation with SR-DNA (0-DUS, AT-DUS (a), and (b), and ATG-wadDUS (a)) is given in Table 3-4b. The average transformation frequencies are shown in Figure 14. The average

transformation frequency achieved with ATG-wadDUS (a) was 2.5×10^{-5}, compared to 8.0×10^{-6} obtained with AT-DUS (a) (forward) and 6.2×10^{-6} with AT-DUS (b) (rev. comp). No significant transformation was obtained with 0-DUS (negative control without DNA uptake sequence).

Table 3-4b. Result of quantitative transformation with SR DNA (PCR product) from *N. mucosa* 19696^{SR} Recipient strain: *N. wadsworthii* 9715.

TRANSFORMING *rpsL* DNA (PCR product)	**AVERAGE**
0-DUS	2.3×10^{-8}
AT-DUS (a)	8.0×10^{-6}
AT-DUS (b)	6.2×10^{-6}
ATG-wadDUS (a)	2.5×10^{-5}

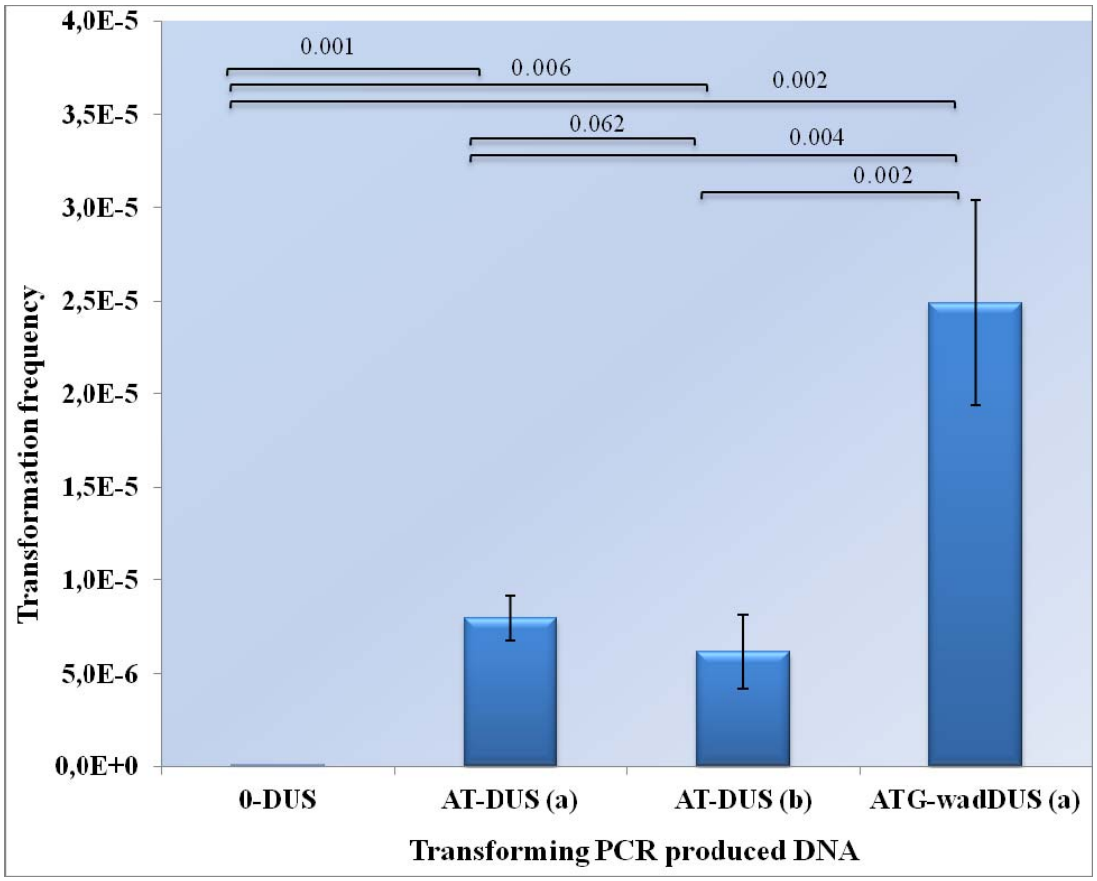


Figure 14: Quantitative transformation of *Neisseria wadsworthii* with SR conferring DNA PCR product amplified from *N. mucosa* 19696^{SR}, AT-DUS in two orientations. The X axis shows the four different transforming SR-DNAs used. The Y axis shows the average transformation frequency. Values are the averages of four independent experiments and standard deviations are indicated by bars. Statistically differences calculated by a two tailed paired student's t-test are indicated above the columns.

As shown in Figure 14, no statistically significant effect of altering the orientation of DUS could be observed by comparing the performances of the transforming *rpsL*-DNA (PCR product) containing AT-DUS (a)(f) and (b)(rev. comp) (p =0.062). Highest transformation frequency was achieved with the SR DNA containing ATG-wadDUS. The transforming SR-DNA AT-DUS (b) appeared to have no effect on transformation than the DNA constructs applied before (Table 3-4a, Figure 13).The differences between the transformation frequencies of SR-DNA (PCR product) are statistically significant (p≤0.05). The SR-DNA containing

ATG-wadDUS construct was considerably higher than the uptake of AT-DUS, confirming the higher efficiency of the ATG-wadDUS, as before.

3.6.2 Transformation of *N. wadsworthii* 9715 with SR conferring DNA PCR produced from *N. wadsworthii* ^{SR}

N. wadsworthii 9715 (wt) was transformed with *rpsL* DNA (PCR product) encoding SR containing different DUS-dialects as follows; ATG-wadDUS (b) (f) and (c) (rev. comp). Genomic DNA from *N. wadsworthii* encoding streptomycin resistance was used as a template DNA in the PCR. The recipient Nw 9715 (wt) cultures were mixed with 0.5 µg/mL transforming DNA. Two independent transformation experiments were performed and the results are given in Table 3-4c. The average transformation frequencies are shown in Figure 15.

Table 3-4c: Quantitative transformation with SR-DNA, ATG-wadDUS in two orientations. Recipient strain: *N. wadsworthii* 9715.

TRANSFORMING *rpsL* DNA (PCR product)	**AVERAGE**
0-DUS	0
ATG-wadDUS (b)	9.3×10^{-7}
ATG-wadDUS (c)	1.4×10^{-6}

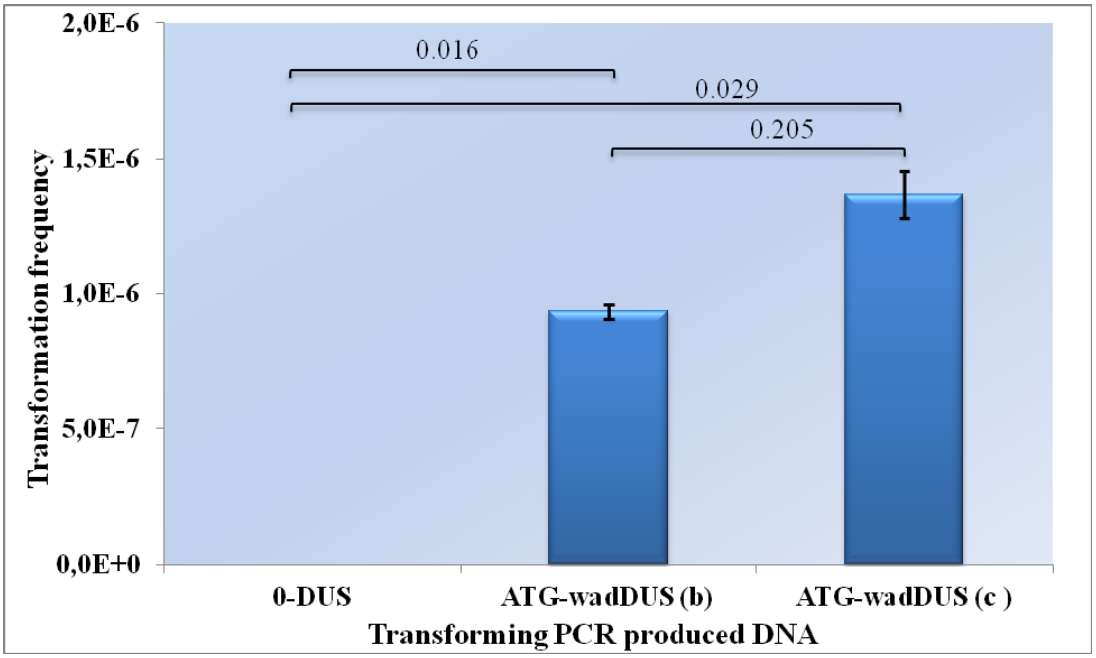


Figure 15: Result from the quantitative transformation with SR-DNA (PCR product) from *N. wadsworthii*^{SR}, ATG-wadDUS in two orientations. The X axis shows the three different transforming SR-DNA used. The Y axis shows the average transformation frequency**.** Values are the averages of two independent experiments and standard deviations are indicated by bars. Statistically differences calculated by a two tailed paired student's t-test are indicated above the columns**.**

No statistically significant effect of altering the orientation of DUS could be observed by comparing the performances of the transforming SR-DNA containing ATG-wadDUS (b) (f) and (c) (rev. comp) constructs. The difference in transformation frequencies between ATG-wadDUS (b) and ATG-wadDUS (c) was found to be not statistically significant (p=0.205). The differences between the transformation frequencies of SR-DNA (PCR product) are statistically significant ($p \leq 0.05$). The average transformation frequency with ATG-wadDUS (b) was 9.3×10^{-7} compared to 1.4×10^{-6} with ATG-wadDUS (c). No growth was obtained with 0-DUS (negative control).

3.6.3 Quantitative transformation with plasmid DNA conferring erythromycin resistance

Nw 9715 was tested to be transformed with plasmid DNA conferring erythromycin resistance. Plasmid pDV4 containing AT-DUS was used as a positive control and plasmid p0-DUS (without DUS) was used as a negative control. The result of the quantitative transformation is given in Table 3-4d. The average transformations frequencies are shown in Figure 16. No growth was obtained with the negative control (p0-DUS). The average transformation frequency with plasmid DNA pSAF62 containing ATG-wadDUS was higher as compare to plasmid DNA pDV4 containing AT-DUS.

Table 3-4d. Quantitative genetic transformation with plasmid DNA Recipient strain: *N. wadsworthii* 9715.

TRANSFORMING PLASMID DNA	AVERAGE
p0-DUS	0
pDV4	6.8×10^{-7}
pSAF62	6.2×10^{-5}

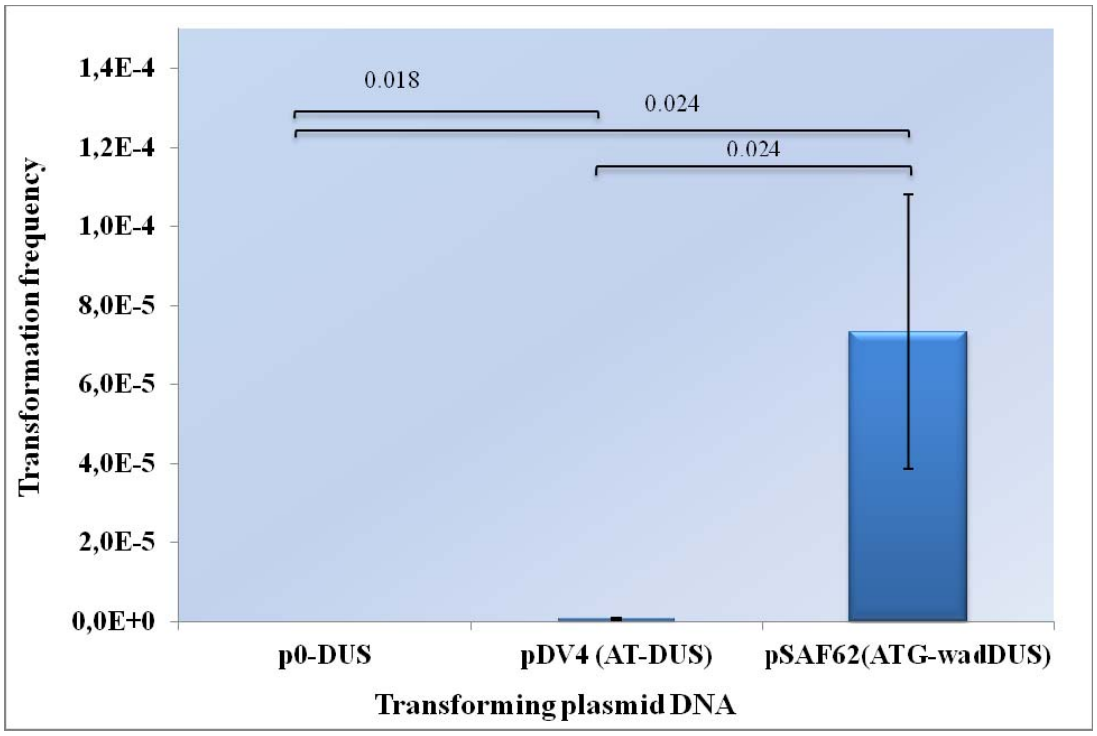


Figure 16: Result from quantitative transformation of *N. wadsworthii* 9715 with plasmid DNA conferring erythromycin resistance. Along the X axis are the different plasmid DNA substrates. The Y axis shows the transformation frequency. Values are the averages of four independent experiments and standard deviations are indicated by bars. Statistically differences calculated by a two tailed paired student's t-test are indicated above the columns.

3.7 Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometer (MS) Biotyper database

The MALDI Biotyper analysis generates a characteristics mass and intensity distribution of mainly ribosomal proteins. Since this mass spectrum is species-specific for a large number of microorganisms, it represents a "molecular fingerprint". Single spectra for thousands of reference strains are stored in the Bruker MALDI Biotyper 3.1 main spectrum (MSP) database. The list of *Neisseria* strains used for the establishment of the Biotyper database is given in Appendix 3.

The spectra produced by the Micro Flex LT MALDI-TOF-MS of each strain were first compared with the MALDI Biotyper Real Time Classification (RTC) program. This program is use to classify unknown samples quickly and easily. An overview of the classification results by RTC program with the two best matches found for each strain is given in Table 3-5a. Reference strains such as, *N. lactamica* (=ATCC 23970, = DSMZ 4691) and *N. mucosa* (=DSMZ 4631, = ATCC 25996), were identified with score values ≥ 2.0, which is the recommended score value for a correct classification. *N. sicca* (=ATCC 9913, =DSMZ 23539) and *N. macacae* (= DSMZ 19175 =ATCC 33926) were identified as the closely related to *N. mucosa* with a score values ≤ 2.0. *N. wadsworthii* (=DSMZ 22247) and *N. mucosa* subsp. *heidelbergensis* (ATCC 25999) were not identified by MALDI-TOF MS Biotyper database 3.1, with the score values 1.65 and 1.55 respectively. *N. weaver*i B3456 (= ATCC 51223 = CTC 8142) identification was not reliable with a score value 1.78.

Table 3-5a: An overview of the classification results of different *Neisseria* strains by MALDI Biotyper RTC.

Strain	Organism (Best Match)	Score Value	Organism (Second best match)	Score Value
N. wadsworthii 9715	Not reliable identification	1.65	Not reliable identification	1.30
N. mucosa 4631	*N. mucosa*	2.17	*N. mucosa*	2.04
N. sicca 23539	*Neisseria mucosa*	1.78	Not reliable identification	1.7
N. weaver B3456	*Moraxella osloensis*	1.78	*Moraxella osloensis*	1.75
N. macacae 19175	*Neisseria macacae*	2.28	*Neisseria mucosa*	2.05
N. mucosa subsp. *heidelbergensis* 25999	Not reliable identification	1.55	Not reliable identification	1.55
N. lactamica 23970	*N. lactamica*	2.16	*N. lactamica*	2.0

The spectrum view between the raw spectra and the modified spectra from Nw 9715 in different ranges were performed and shown in Figure 17. The most difference observed comparing the raw spectra (left) and the modified spectra (right) were in the baseline of the peaks which were further adjusted in the FlexAnalysis program.

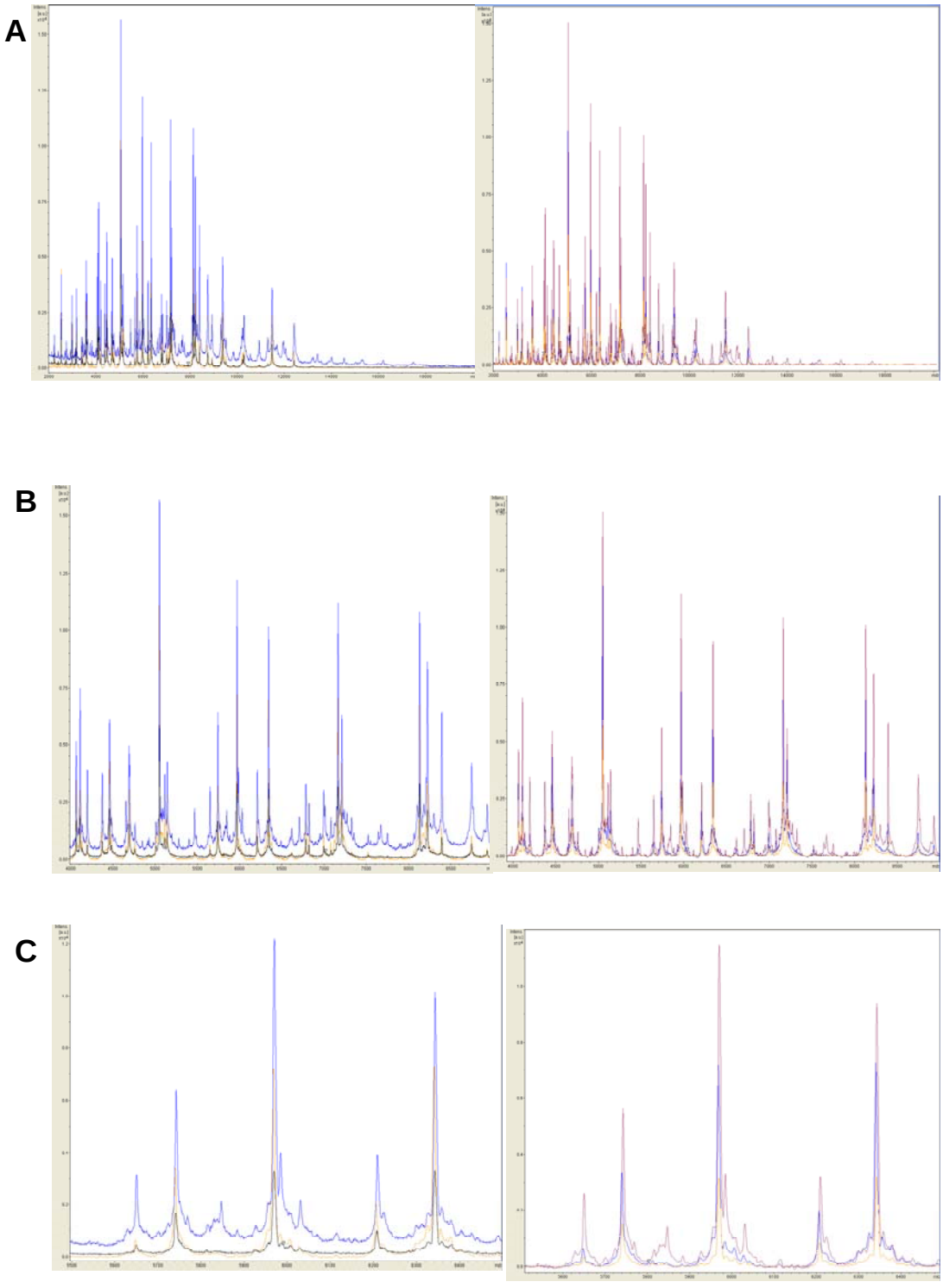


Figure 17 (description see next page)

Figure 17 (see page before): MALDI-TOF-MS of the purified ribosomal fraction of *N. wadsworthii* 9715. The relative intensities of the ions are shown on the Y axis, the mass to charge ratios on the x axis. Variants of peaks corresponding to ribosomal proteins are shown between the raw spectra (left) and modified spectra (right) in different range (A= 2000-18000 Da, B= 4000-9000 Da and C= 550-6500 Da).

Further, sixteen individual spectra for Nw 9715 obtained by Micro Flex LT MALDI-TOF-MS were compared with the MS BioTyper 2.0 (Bruker Daltonics Germany), Bruker Taxonomy database, the Project folder database and a combination of both databases. The score values together with averages and standard deviations obtained for the different databases are given in Table 3-5b. The average score value from the Bruker Taxonomy database was 1.21 (SD = 0.48), as the value from the Project folder database as for the combination of the two databases was 2.19 (SD = 0.61).

Table 3-5b: Score value for sixteen spectra generated from individual Nw 9715 samples analyzed against different databases.

Sample	Database		
	Bruker Taxonomy	**Project Folder**	**Bruker Taxonomy + Project folder**
	Score Value		
A1	1.40	2.36	2.36
A2	1.38	2.46	2.46
B1	1.40	2.38	2.38
B2	1.38	2.48	2.48
C1	1.36	2.51	2.51
C2	1.37	2.24	2.24
D1	1.22	2.41	2.41
D2	1.36	2.37	2.37
E1	1.41	2.49	2.49
E2	1.42	2.30	2.30
F1	Error*	1.79	1.79
F2	Error*	Error*	Error*
G1	1.35	2.31	2.31
G2	1.49	2.34	2.34
H1	1.43	2.32	2.32
H2	1.41	2.35	2.35
Average (SD)	**1.21 (0.48)**	**2.19 (0.61)**	**2.19 (0.61)**

*Error = error reported by MicroFlex software

4 DISCUSSION

Neisseria wadsworthii, N. gonorrhoeae and *N. meningitidis* are species of the genus *Neisseria*. Both, Mc and Gc, are Gram-negative diplococci sharing similar traits, including virulence factors, and share about 95 % of DNA base sequences in their genomes (76). Both bacteria are found to be naturally competent for transformation throughout their life-cycle. Yet they cause diseases that are radically different from each other such as, meningococcal septicemia (blood poisoning) and meningitis by Mc, and gonorrhea (sexual transmitted infection of the urinary tract) caused by Gc (77, 78). Nw is a newly identified species. Since it was observed that Nw constitutes a distinct phylogenetic branch in the *Neisseriaceae* phylogenetic tree (1) and the genome contains the 13-mer ATG-wadDUS 5´-CCTGTCTGAA-3, with an insertion of T in position 3 (31), it was interesting to investigate natural competence for transformation among this *Neisseria* species. Classical methods based on morphological and metabolic characterization of Nw were performed. In addition, several techniques in molecular microbiology were applied to assess natural competence for transformation in this strain.

Recently, Wolfgang and co-workers have characterized Nw cells through several phenotypic and biochemical analyses (1). The observations made during this study are confirmed her as similar results were obtained through Gram-staining, cytochrome oxidase activity testing and carbohydrates metabolism testing in Nw 9715. In addition, the MIC of Nw 9715 was determined (50 µg mL^{-1}) and the bacteria cells have also shown agglutination on solid GC-agar, which occurs due to the presence and interaction between the pili (79), and appears to be essential for reasons such as, competence for DNA transformation (3, 43). *Neisseria* spp., such as Mc and Gc and non-pathogenic *Neisseria* spp., including *N. perflava* and *N. elongate*, also observed to have Fimbriae (pili) on their surface (3). These latter characteristics of Nw was described before.

4.1 *N. wadsworthii* Express Components Necessary for Transformation

The process of natural competence for transformation is tightly associated with the presence of pili in *Neisseria* species (3). The pilus related components were therefore investigated in this study. In Mc and Gc, several of these pilus related components are defined. It was therefore of interest to see if Nw harboured the proteins essential for transformation. Analysis of the Nw pilus subunit, PilE, was therefore warranted.

In this study we showed that Nw 9715 produces PilE, the pilus subunit itself, which plays an important role in the initial part of the transformation process (42). Nw is also capable of expressing several homologue Type IV pilus biogenesis components found in *N. meningitidis* Mc58 (Table 3-1). Only one out of two copies of *pilC* was found in the genome of Nw 9715 by the bioinformatics tools applied. One can speculate that this could be due to the default “cut-off” value used by the software. Lower cut-off values were not tested. The results suggest that *N. wadsworthii* the genome, contains genes for essential elements involved in HGT and pilus biogenesis.

The sequence of the *pilE*-gene in Nw (Figure 8) showed similarities to other *pilE* genes in N-terminal part. This part of the molecule is important for the assembly of the pilus fibre (80). In addition, the conserved Glycin-Phenylalanin (G-F) residues were found in the amino acid sequence of the Nw PilE. These are known to be the substrates for the prepilin peptidase PilD (52), which cleaves and then N methylates the precursor pilin subunits prior to their assembly into pili. The proposed model of the Gc Tfp suggest that the conserved N-terminal region is located in the backbone of the PilE structure(81) and due to little degree of exposure to the environment, could be the reason for the conservation of this part of the PilE sequences. Most of the variations in the sequences of the PilE within Nw were found in the C-terminal part of the amino acid sequence. This part of the protein is exposed at the outer surface of the outer membrane and is in direct contact with the host. Therefore the reason for the high degree of variation in the C-terminal

part of the protein structure, which is predicted to be surface exposed, may be the reason of antigenic variation of the pili (81).

The immunoblotting with whole cell proteins and with pilus preparations showed proteins (16-17 kDa) from Nw 9715, which reacted with antisera generated against class I pili from Mc M1080. The antiserum generated against pili from Mc M1080 showed stronger reactivity against the PilE from Gc (N400), than against PilE from Nw 9715. The weaker reactivity against the PilE of Nw 9715 could be due to several factors. Weaker bands in the blots could be due to methodological reasons such as, relative concentrations of proteins and antisera applied. Also, Mc and Gc are much more closely related to each other than to Nw so that cross-reactivity is expected to be much more prevalent between Gc and Mc. Moreover, antibodies raised against class I pili from Mc M1080 is found to be closely related to Gc pili (58) and our observations by amino acid sequences of PilE indicate that Nw 9715 to some extent differ from the Class I pili of Mc. The non-specific binding and detection of proteins on the membrane observed both in whole-cell lysates and pilus-prep could be due to the polyclonal nature of the antibody used for the detection of, PilE.

4.2 *N. wadsworthii* Is Naturally Competent for Transformation

The transformation assays revealed that the single strain of Nw used in this study was naturally competent for transformation. The Nw strain was transformable with a point mutation conferring antibiotic resistance to streptomycin.

The transformation rate for Nw strain transforming genomic DNA harboring a single point mutation in the *rpsL* gene conferring antibiotic resistance to streptomycin was determined to 10^{-7} – 10^{-5}. The transformation rate for Mc was 10^{-4} (79). Nw 9715 was also transformed with plasmid DNA conferring Erm^R, confirming that the strain is also transformable with plasmid DNA. The transformation rates also suggest that Nw binds DNA in a DUS-specific manner with preference for the most similar DUS sequence ATG-wadDUS as compared to the AT-DUS. One can speculate that this 13-mer

(ATG-wadDUS) recognition sequence increases the specificity of DNA uptake and as a consequence, facilitates the discrimination of foreign DNA entering into the Nw recipient cell. Such DUS preference was also observed for *N. meningitidis* Mc58, which displayed an extreme transformation enhancement when exposed to the 12-mer (AT-DUS) and a near lack of affinity for the 10-mer DUS 5´- GCCGTCTGAA-3´(29).

For successful transformation of DNA into *Neisseria* spp., particular features must be in place; DUS in the transforming DNA, a multi-component competence machinery and RecA-mediated HR (11, 28). Also the absence of efficient restriction modification systems (RMS) and the presence of DNA homology between the transforming DNA and the recipient chromosome are affecting the transformation process. RMS imposes a negative influence on the movement of foreign DNA in transformation by making double-stranded DNA breaks. This causes discontinuity and the disruption of transforming DNA so that certain sequences cannot successfully be integrated into the chromosome. Sufficient homology in the DNA sequence is also a prerequisite for transformation and has an impact in the transformation assay.

During this study, it was observed that both transforming SR-DNA (PCR-product) containing AT-DUS and plasmid pDV4 (AT-DUS), yielded relatively low transformation frequency in Nw 9715 as compare to transforming DNA containing ATG-wadDUS. This could be due to several factors. Firstly, Nw 9715 harbours ATG-wadDUS (31) in its genome and not AT-DUS. It is therefore expected that Nw 9715 will better recognize ATG-wadDUS than AT-DUS. Recently, Cehovin and co-workers demonstrated that the Mc Type IV pili bind DNA through the protein ComP and that ComP displays an exquisite binding preference for DNA uptake sequence (55). One can speculate that the ComP of Nw 9715, likely has higher affinity for ATG-wadDUS than for AT-DUS. Another factor of low transformation frequency obtained with plasmid DNA containing AT-DUS could be due to low homology between the plasmid employed and the *N. wadsworthii* genome. If homologous regions are dissociated from the *ermC* resistance marker in pDV4 by restriction, transformation is greatly affected (28). In this recent study (28) it was shown

that only a single restriction site makes a substantial difference in transformation assay. It was also demonstrated that DUS orientation did not significantly affect transformation in Mc by comparing the transforming potential of different plasmids harbouring DUS in different orientation (28). This observation was confirmed here as, no effect on transformation frequency could be observed using SR-DNA PCR-products containing DUS in opposite orientations. Both orientations of DUS performed equally well in transformation, suggesting that DUS is not a starting point for directional DNA processing during transformation. Instead, DUS could possibly influenced several steps during transformation (20).

Variability in transformation assays were observed when different DNA was used in transformation experiments. One can speculate that this variability could be caused by nuclease activities present in the culture medium and in the cells that has affected DNA differentially. However, despite the variability in transformation frequency seen, Nw 9715 was confirmed competent for transformation for the first time. Furthermore, competence in Nw 9715 was confirmed DUS-dependent with a preference for its own DUS, the ATG-wadDUS. Finally, the results obtained will help to understand and execute any experiments involving transformation in this strain.

4.3 Development of a MALDI-TOF-MS Biotyper Database

For the identification of *Neisseria* sp., such as Nw, both phenotypic, biochemical and methods on molecular level was used during this study. Instead of applying these conventional phenotypic or molecular biological techniques, MALDI-TOF-MS technology can be used as a rapid and precise method for the identification of intact bacteria (74). Identification of whole cell bacteria by MALDI-TOF MS has also been demonstrated earlier (82). In addition, sample preparation and analysis with MALDI-TOF-MS is much easier and less time consuming than with genotypic methods.

A MALID-TOF-MS based fingerprint analysis using the Biotyper 3.1 program was applied in this study for the identification of Nw 9715. Other

Neisseria sp., were also included for the expansion of the existed *Neisseriaceae* MALDI-TOF-MS database.

The existing MALDI-TOF-MS Biotyper 3.1 database has 60 reference strains of the genus *Neisseria*. Two strains were correctly identified by the MALDI-TOF-MS technique out of seven strains in total. Discrepant results regarding species identification from Biotyper RTC were obtained with *N. sicca, N. macacae* and *N. mucosa,* where MALDI-TOF-MS was unable to distinguish between these species. Likewise, the lack of MALDI-TOF-MS identification was observed for *N. wadsworthii.* The reason for this could be due to the absence of sufficient spectra from the reference database.

The average score values obtained from 16 spectra (Table 3-5b) showed that the identification and characterization of the *Neisseria wadsworthii* 9715 was much more reliable when using of the spectra in the Project folder database. Therefore, an even further expansion of the Biotyper database for non-pathogenic *Neisseria* species could improve the performance of the Biotyper system.

4.4 The Impact of This Study

Neisseria wadsworthii 9715 was here for the first time found to be naturally competent for transformation in a DUS-dependent manner. Permanent natural competence and high transformability among *Neisseria* species makes the organism interesting for genetic manipulation and the study of transformation as the DUS has an unusual insertion in its sequence. The study of genetic transformation in Nw is based on observations that this species is readily transformable with genomic,-PCR and plasmid-DNA. This novel finding makes Nw 9715 available for genetic manipulation. This feature is also important for Nw antigenic variation and fitness in its natural environment in the human host. Competence may allow for swift changes in gene content and may allow for effective adaption to new environments. This life style can be compared to Mc, which is one of the large numbers of mucosal pathogens of human. Mc, has been widely used as a model species for several studies on DNA uptake and natural genetic transformation. These

features for rapid genome variability, adaptability and maintenance are clearly essential for these bacteria to ensure their survival, and virulence in their natural environment in the human host.

Genome modification, recombination, horizontal gene transfer, as well as a continuously changing environment will constantly challenge the genomes of Mc and related human associated microbes. These genome variations will increase the diversity in the bacterial populations and might ultimately influence the survival of the bacteria. The mechanism of natural competence for transformation has therefore provided a unique tool for measuring genetic distances for classification and identification of new or poorly characterized bacterial species such as, *N. wadsworthii*. Antigenic variability and sequence variations have a great input on this perspective.

N. wadsworthii 9715 has at least one important surface pilus component which is common both in Mc and Gc, PilE. The limitation is that *N. wadsworthii* seems to be restricted towards integration of DNA containing different DUS dialects into its genome. Gene-manipulations in this species therefore require a more in-depth characterization of the transformation system. Yet an aspect of natural competence in *Neisseria* species*;* *N. wadsworthii*, has been described. It is also a possibility to use *N. wadsworthii* as a model in laboratory experiments.

5 Conclusion and Further Perspectives

In this study it is demonstrated for the first time that *N. wadsworthii* is competent for transformation. This competence was further shown to be DUS-dependent similar to competence in Mc and other well-characterized *Neisseria*. Importantly, *N. wadsworthii* was able to distinguish between the two DUS-dialects ATG-wadDUS and AT-DUS; with preference for the former which is the isogenic DUS found in the *N. wadsworthii* genome. A single strain of *N. wadsworthii* was characterized during this study and with the help of phenotypic, biochemical and molecular biological methods; it was found that *N. wadsworthii* 9715 was competent for transformation. *N. wadsworthii* 9715 strain was easily transformable with genomic and PCR-generated DNA containing a point mutation conferring SR and with, plasmid DNA conferring erythromycin resistance. DUS orientation has found to have no effect on transformation frequency in this strain, confirming observations made in a previous study using Mc.

N. wadsworthii colonies were shown to express pili; and a candidate for PilE was shown to be expressed. MALDI-TOF-MS associated with MALDI Biotyper database 3.1 appears to be a reliable and accurate tool for the identification of poorly characterized *Neisseria* sp. For further development of the Biotyper database, large collection of *Neisseria* sp., of diverse origins are needed.

The most important experiments to perform now is the sequencing of the other genes of interest thought to be involved in transformation, in *N. wadsworthii*, and secondly further development of the MALDI-TOF-MS Biotyper database 3.1 with currently poorly or non-represented *Neisseriaceae*.

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URL LIST

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2. Oligocalculator web server: http://www.basic.northwestern.edu/biotools/OligoCalc.htmL
3. Program for designing PCR pimers: http://www.ncbi.nlm.nih.gov/tools/primer-blast/

APPENDIX LISTS

APPENDIX 1: List of the Reagents and Chemicals

Chemicals

- Acetonitrile (CH_3CN) 50%
- Acrylamide/Bis Solution (40%, 29:1)
- Agarose
- Ammoniumpersulphate
- Ammoniumsulphate
- Brain heart infusion agar
- Bromo-chloro-indolyl phosphate (BCIP)
- Coomassie Brilliant Blue R-250 (CB Blue)
- CTAB (cetyl-trimetyl-ammonium-bromic)
- Ethidium bromide (EtBr)
- Ethylene-diamine-tetra-acetic acid (EDTA)
- Fat free dry milk powder
- Formic acid (HCO_2H) 100%
- Glycine
- IsoVitaleX
- KCl
- $MgCl_2$
- N,N,N tetrametyl p-phenylenediamine
- NaCl
- NaN_3 (NatriumAzid)
- NaOH
- Nitro blue tetrazolium chloride (NBT)
- Sodium dodecyl sulfate (SDS)
- Starch
- Tricine

Liquid Chemicals

- β-2-mercaptoethanol
- Ethanol (96 % , 100% EtOH)
- Hydrochloric acid (HCl)
- Isoamylalcohol
- Isopropanol
- Phenol/chloroform/isoamylalcohol (24:24:1)
- Tetra-methyl-ethylene-diamine (TEMED)
- Trifluoroacetic acid 100%
- Tween 20

Nucleotides and Proteins size standards (Marker)

- GeneRuler DNA ladder mix 50 ng/µL
- LC 5925 invitrogen Life technology pre-stained protein standards SeeBlue Plus2

Antibiotics

- Streptomycin
- Erythromycin
- Kanamycin

Components of PCR

- PCR 2.5Mm dNTP´s

APPENDIX 2: Recipes for Culture Media, Buffers and Solutions

Streptomycin antibiotic "stock"

5000 µg/mL streptomycin "stock" was made by adding 10.45 mL of H_2O to 50.0mg streptomycin.

GC broth (4X concentrate)

- 20g NaCl
- 16g K_2HPO_4
- 4g KH_2PO_4

Chemicals above were dissolved in 500 mL H_2O.

- 15g trypticase peptone
- 30g thiotone peptone

The chemicals above were added with H_2O to a final volume of 1 liter. The solution was autoclaved, and stored at 4°C. Before use, the 4X concentrate was diluted to 1X, and added 10 mL 100X IsoVitaleX pr liter GC broth.

1% GC-agar for colony morphology

GC-agar powder mix contents:

- 20g NaCl
- 16g K_2HPO_4
- 4g Monobase K_2PO_4
- 15g Tryptone, BD Bacto Tryptone
- 30g peptone
- 2g soluble starch
- 68.04g agar

4g of GC-agar powder mix with 200mg of starch was dissolved in 100 mL 4 × GC-medium and 400 mL water. Solution was autoclaved for 20 minutes at 121°C, and cooled down in a water bath at 60 °C. 5 mL of IsoVitalex was added in the agar medium. 25 mL of agar medium was poured into petri dishes and the agar was allowed to solidify. Petri dishes were placed at room

temperature for 2 days to allow evaporation of residual moisture from the plates. For selective GC agar antibiotics were added to the liquid agar-solution. The antibiotic selection used is given in the table below.

Antibiotic	Concentration [µg/mL]
Erythromycin	8

Brain Heart Infusion Agar

- 37g Brain Heart Infusion Agar
- 1000 mL water

LB-agar with Amp100 + Erm400

25 g of LB-agar with 20 g agar mixed in 1 L water. Autoclaved for 20 min in 120 ºC, cooled down to 60 ºC in water bath. 1mL of Ampicillin 100 mg/µL and 8 mL of Erythromycin 50 mg/µL from the stock was added to the agar. Mixed well and poured in petri dishes.

Transformation Assay

- PBS-buffer
- 8.0g NaCl
- 0.2g KCl
- 1.4g Na_2HPO_4
- 0.2g KH_2PO_4

Added H_2O to a final concentration of 1 L. pH is adjusted to 7.4

Nucleic Acid Methods

TE-buffer

- 10 mM Tris-HCl, pH 8.0
- 1 mM EDTA, p H 8.0

DNA loading-buffer

- 30 % glycerol
- 20 mM EDTA
- 0.01 % Bromphenol blue

Agarose gel

- 0,9%, 1% and 2% (w/v) Ultra prep-agarose
- 0.5X TBE-buffer
- Solution was mixed and heated in microwave until the Ultra prep-agarose was completely dissolved.

Composition of buffers used in both “Miniprep” and “QIAGEN Plasmid midiprep”

Buffer	Composition	Storage
Buffer P1 (re-suspension buffer)	50 mM Tris-HCl (pH 8.0) 10 mM EDTA 100 µg/mL RNase A	2-8 ºC, after addition of RNase A
Buffer P2 (lysis buffer)	0.2 N NaOH 1% SDS (w /v)	15-25 ºC
Buffer P3	3 M potassium acetate solution (pH 5.5)	15-25 ºC or 2-8 ºC
Buffer QBT (equilibration buffer)	750 Mm NaCl; 50 m M MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)	15-25 ºC
Buffer QC (wash buffer)	M NaCl 50 m M MOPS, pH 7.0 15% isopropanol (v/v)	15-25 ºC
Buffer QF (elution buffer)	1.25 M Na Cl 50 m M Tris-cl, pH 8.5 15% isopropanol (v/v)	15-25 ºC

Protein Methods

Ethanolamine buffer

- 9.15 mL Ethanolamine stock (16.4 M)
- Add 1 liter water to a final volume and pH is adjusted to 10.5 by adding several drops of concentrated HCl.

Ammoniumsulphate solution

- $(NH_3)_2SO_4$
- 0.15 M Ethanolamin buffer
- 4 × PAGE-LDS sample-buffer
- 0.05 M TBS (Tris buffered saline) buffer
- 8g NaCl
- 0.2g KCl
- 50 mL 1M Tris-HCl, pH 8.0
- H_2O was added to a final volume of 1 liter.

SDS-PAGE gel mixture

REAGENT	4%	12%
H_2O	3.145 mL	1.841 mL
Gelbuffer	1.25 mL	3.333 mL
60% Glycerin	25.0 µL	1.666 mL
20% SDS	25 µL	50 µL
40% Acrylamide/Bis (29/1)	500 µL	3 mL
10% (w/v) APS	75 µL	100 µL
TEMED	5 µL	10 µL
Total volume	5 mL	10mL

Gel buffer

- 3.0 M Tris-HCl, (pH 8.45)

Anode buffer

- 200 mM Tris Base (pH 8.9)

Katode buffer

- 100 mM Tris Base
- 100 mM Tricine
- 0.05-0.1% SDS

Coomassie Blue staining solution

- 0.1% Coomassie Brilliant Blue R-250
- 10% acetic acid
- 40% ethanol

Coomassie destaining solution

- 40% methanol
- 4% glycerol
- 10% acetic acid

Western blot transfer buffer

- 5.8g Tris Base
- 0.37g SDS
- 200 mL methanol
- 29.9g Glycine
- H_2O was added to the final volume of 1 liter.

Western blot blocking buffer

- 10 mL 1M Tris-HCl, pH 7.8
- 100 mL 5M NaCl

- 5 mL Tween 20
- 20g of fat free dry milk powder
- 1 mL 1M NaN_3
- H_2O was added to the final volume of 1 liter.

Western blot developing buffer

- 6, 05g Tris HCl, pH 9.6
- 3 mL 1 M $MgCl_2$
- 1 mL 1M NaN_3
- H_2O was added to the final volume of 1 liter.

Western blot blocking alkaline phosphatase developing solution

- 10 mL developing buffer
- 20 µL NBT (75 mg/mL) in 70% DMF
- 15 µL BCIP (50 mg/mL) in 100% DMF

Matrix Assisted Laser Desorption /Ionization Time of Flight (MALDI-TOF) Mass Spectrometer (MS)

Reagent

- Matrix: α-cyano-4-hydroxycinnamic acid (2, 5mg)
- Acetonitrile 50%
- Trifluoroacetic acid 100%
- Ethanol absolute
- Formic acid 100%
- Bruker Bacterial Standard Positive control
- H_2O

Organic solution (1 mL of Organic solution, 2.5 % TFA, 50 % acetonitrile)

- 475 µL H_2O
- 500 µL acetonitrile
- 25 µL 100% TFA

Matrix solution:

250 µL Organic solvent added to matrix solution containing α-cyano-4-hydroxycinnamic acid from Bruker. Vortex suspension for 1-2 min and mix thoroughly. Solution should be clear; there may be some sediment, but no more than barely visible amount. Matrix solution should store in dark place and can stand in room temperature.

70 % Formic Acid:

7 mL of 100% formic acid added in 3 mL H_2O.

Bacterial Standard solution

Add 50 µL OL and dissolve pellet by pipetting several times, minimum 20 times at room temperature. Avoid bubbles and let the solution stand for 5 min at room temperature. Repeat pipetting step minimum 20 times. The solution can afterwards dilute in OL (1:10) and divide in small portion 20 µL. BTS solution is stored at – 18 ºC. Bruker Bacterial Standard (Bruker # 255343; E.coli DH5α) is used as a calibrator and positive control.

Appendix 3 Lists of Primer Sequences

Table 1. The list of primers used during this study.

Primer Name	Sequence (5'-3')	*ORI
SF151 (ATG-wadDUS)	gtgaGctc**atGcctgtctgaaATGC**CAACTATCAACCAATTGGTACGCA	for
OHA2184 (0-DUS)	gtgaGctcATGCCAACTATCAACCAATTGGTACGCA	for
OHA2183 (AT-DUS)	gtgaGctcat**GccgtctgaaATGC**CAACTATCAACCAATTGGTACGCA	for
OHA2186 (0-DUS)	gtctcgagTTATTTAGGACGCTTAGCACCGTATT	rev
SF153 (ATG-wadDUS)	gtgaGctc**atGcctgtctgaaATGC**CTACTATTAATCAATTAGTACGCA	for
SF158 (ATG-wadDUS)	gtgaGctc**TTCAGACAGGCATATGC**CTACTATTAATCAATTAGTACGCA	for
SF157 (0-DUS)	gtctcgagTTACTTAGGACGTTTTGCACCGTATT	rev
OHA2137 (AT-DUS)	gtgaGctc**TTCAGACGGCATATGC**CAACTATCAACCAATTGGTACGCA	for
3893OH3	tagaccGcggtcagGcgaccacgttGcc	rev
SF159 (ATG-wadDUS)	acgactcgagATGCCTGTCTGAAatgGctaaaaacggaggat	for

*ORI: orientation; Forward (for) and Reverse (rev).

Table 2. Donor plasmid used in this study.

Donor plasmid	Sequence	Referance
pSAF62 ATG-wadDUS	acgactcgagATGCCTGTCTGAAatgGctaaaaacggaggat	This study

Table 3: The list of *Neisseria* strains used for the establishment of Biotyper database.

STRAINS	SOURCE / REFERENCE
N.wadsworthii 9715	DSMZ 22247
N. mucosa 4631	DSMZ 4631/ ATCC 25996
N. sicca 23539	DSMZ 23539/ ATCC 9913
N. weaveri B3456	B3456/ATCC 51223
N. macacae 33926	DSMZ 19175/ ATCC 33926
N. mucosa subsp. *heidelbergensis* 25999	ATCC 25999
N. lactamica 23970	ATCC 23970/ DSMZ 4691
E.coli DH5α 255343	Bruker Daltonics