The function of cysteines and disulfide bridges in LptD of *Neisseria meningitidis.*

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Introduction

Gram-negative bacteria have a very distinct structure of the cell wall. The cell wall consists of an outer membrane (OM) and an inner membrane (IM). These membranes are separated by the periplasm, a hydrophilic compartment that contains a layer of peptidoglycan in the center. The IM of a gram-negative bacterium is a symmetric phospholipid bilayer while the OM is an asymmetric bilayer. The layer of the OM facing the periplasm consists mostly of phospholipids while the layer that forms the cell surface is built up mainly from lipopolysaccharides (LPS) but also from other phospholipids and proteins [1]. The bacterium used in this study is *Neisseria meningitidis*. This is a gram-negative bacterium, which can be found in the nasopharynx of humans.

LPS synthesis

LPS is a glycolipid that is part of the OM layer facing the extracellular environment of a gram-negative bacterium. When LPS is present on the outer leaflet of the OM of gram-negative bacteria the OM is a highly effective permeability barrier. The LPS protects bacteria from many toxic substances such as bile salts, detergents, antibiotics, antimicrobial peptides and other hostile environments [2]. In some gram-negative bacteria LPS is essential for viability [3]. The structure of LPS contributes to the properties of the OM of gram-negative bacteria. LPS is a complex molecule consisting of three parts; the first part is lipid A, which is a hydrophobic moiety ideal to anchor LPS into the outer leaflet of the OM because the hydrophobic moiety of lipid interacts with the hydrophobic part of the membrane. The second part consists of a distinct pattern of oligosaccharides. This pattern is conserved among gram-negative bacteria [4]. Two domains can be recognized in the core oligosaccharides namely an inner core which is composed of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) and L-glycero-D-manno-heptose (HEP) components. The outer core comprises hexoses such as glucose and galactose. Finally there is a polysaccharide moiety also known as the O antigen. The O-antigen is a variable part composed of various saccharides. The O-antigen can differ greatly between gram-negative bacteria strains, and is completely lacking in *N. meningitidis* [5].

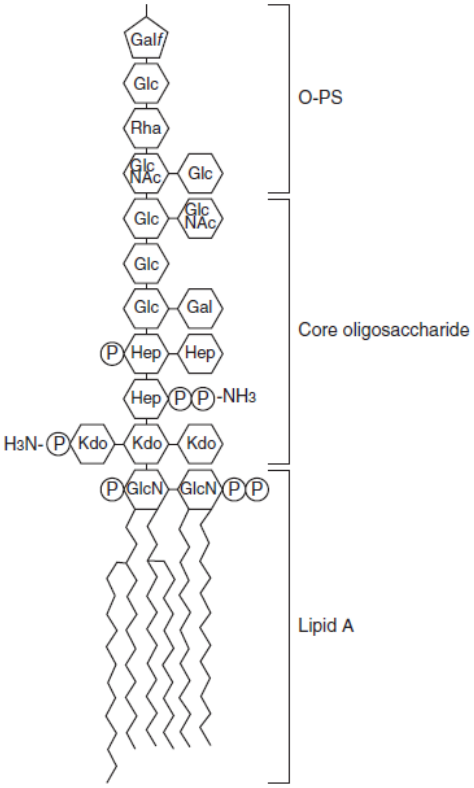

Figure 1 Molecular structure of LPS [3]

The three parts of LPS are synthesized independently from each other in different places in the bacterium but all near the cytoplasmic side of the IM. The O-antigen is synthesized in the cytoplasm at the cytoplasmic side of the IM [6]. After the synthesis it is flipped by to the outer leaflet of IM by a flippase complex composed of two polypeptides Wzm and Wzt [6]. Lipid A and the core oligosaccharide are synthesized separately at the cytoplasmic side of the IM after which core oligosaccharide is attached to Lipid A by specific glycosyl-transferases. Lipid A-core is flipped to the periplasmic side of the IM by an adenosine triphosphate (ATP)-binding cassette transporter called MsbA [6]. At the periplasmic leaflet of the IM the O-antigen and the Lipid A-core are ligated together by the WaaL ligase thereby forming a mature LPS [6].

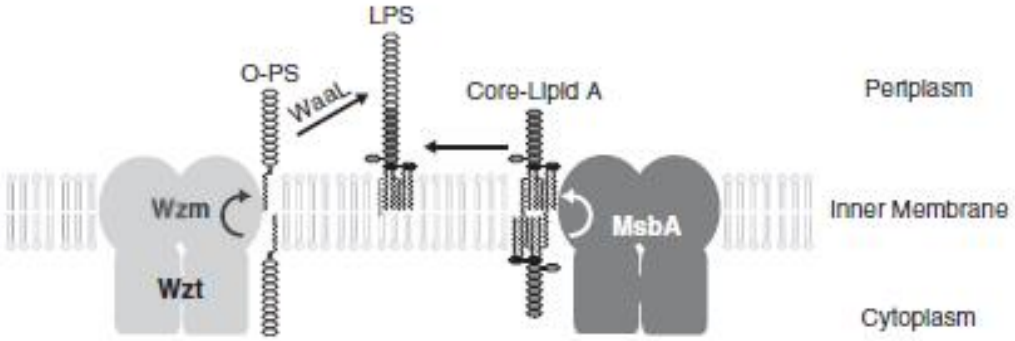

Figure 2 Biogenesis of the LPS parts [6]. O-antigen is flipped by the flippase complex Wzm and Wzt. Lipid A-core is flipped by MsbA. The O-antigen and Lipid A-core are attached to each other by WaaL.

LPS transport

LPS is transported to the cell surface by several proteins that form a complex, these proteins make up the lipopolysaccharide transport (lpt) system. This system is composed of seven different proteins ranging from LptA to LptG each protein has its own function of most enzymes in the lpt system the function has been clear there are still some parts that have to be clarified. There are some differences in the lpt system between gram-negative bacteria, but in *Escherichia coli* the model of the lpt system is the one shown in figure 3. There is a general understanding how the lpt system probably works in *E. coli* and other gram-negative bacteria [7]. When LPS is matured at the periplasmic side of the IM it has to be

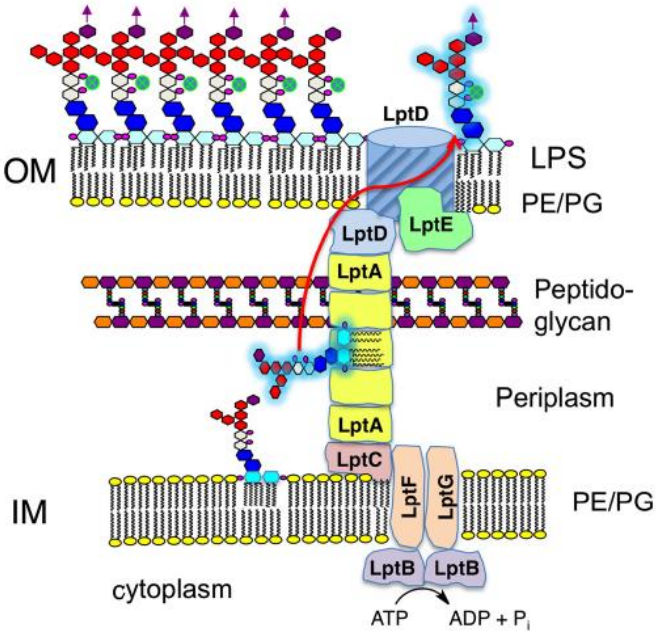

Figure 3 LPS transport pathway in gram-negative bacteria [29]. Schematic representation of LPS transport machinery.

transported through the periplasm, the hydrophilic nature of the periplasm makes it very difficult for a protein with several hydrophobic and hydrophilic regions to be transported towards the outer leaflet of the OM. The energy required for the transport of LPS from the IM to the outer leaflet of the OM is supplied by the protein complex of LptB, LptF and LptG [14]. This complex consist of two copies of LptB, one copy of LptF and one copy of LptG. LptB has an ATP binding cassette (ABC) which binds adenine triphosphate (ATP) necessary for energy. A dimer of LptB forms a LPS ABC transporter with LptF and LptG. LptF and LptG are essential proteins in *E. coli* and are highly conserved among Gram-negative bacteria [13]. This complex probably supplies the other Lpt proteins with energy by hydrolyzing ATP to adenine diphosphate (ADP) with the ATP binding cassette mentioned above [14,15]. Without ATP in the cytoplasm or a defective LptB the mature LPS remains at the leaflet of the IM facing the periplasm [14]. Because the transfer of LPS from the IM to LptC requires energy and the transfer from LptC to LptA requires energy. No energy containing molecules can be found in the periplasm so all the energy needed to transport LPS from the IM to the OM has to come via the ABC transporter complex LptBFG. LPS is first bound to LptC which takes LPS from the IM and passes it to LptA [9]. LptA forms a multimer with other LptA proteins across the periplasm thereby crossing hydrophobic and hydrophilic regions. LPS is bound to LptA and then passed on to another LptA protein thereby moving alongside the LptA chain towards to OM. The LptA transports LPS from the IM to a protein complex composed of LptD and LptE which is located in the OM [10,11,12]. All these proteins except for LptB and LptE share a structurally homologous domain called the Lpt fold [17]. The Lpt fold might be used to build the Lpt transenvelope complex through protein-protein interaction by providing a structural element. Without this Lpt fold LptA and LptC can't interact and there will be no interaction between LptA en LptD [16-19]. LptA interaction with LptC is also an indication of the correct assembly of the lpt system [8].

LptA transfers the LPS to the last protein in this transport chain which is called LptD. LptD is a protein with a beta-barrel, so it is able to transport the LPS through the OM and insert it in the outer leaflet of the OM. LPS is transported through the lumen of the beta-barrel part of LptD directly from the periplasm into the outer leaflet of the OM [20]. In this way, the asymmetry of the OM is being maintained by preventing the LPS to be inserted in the inner leaflet of the OM. LptD forms a complex with another protein called LptE, LptE isn't directly involved in the transport of LPS but has probably three functions in the complex with LptD [21]. First LptE seems to help in the folding of LptD and after this it helps to stabilize LptD by keeping it in the correct form. It is also suggested that LptE can form a kind of plug, which could be able to fit inside the beta-barrel of LptD and thereby blocking it [22]. This could prevent access of harmful molecules present in the environment to bacteria.

LptD is essential in the transport of LPS to the cell surface. But it isn't entirely clear how LptD is inserted in the OM and how it is folded. It is known that LptD is folded by a protein called DsbA which is able to form and reshuffle disulfide bonds [27]. This has been found in the *E. coli* bacterium but it is not yet determined if this is also the case for *N. menigitidis*. DsbA is an enzyme that can be found in the periplasm of gram-negative bacteria. This protein is a member of the Disulfide bond (Dsb) family

of proteins that are known as oxidoreductases. DsbA catalyzes the oxidation of a protein via a thioldisulfide transfer mechanism. With this system disulfide bonds are formed between two cysteines in a protein [23-26]. The LptD protein of *E. coli* contains four cysteines and therefore DsbA will form two disulfide bonds creating mature LptD [27]. Several steps can be identified in the folding process of LptD (figure 4). When reduced LptD enters the periplasm DsbA oxidizes the first cysteine creating an LptD with a DsbA adduct. Then an unfolded intermediate of LptD is created in which a disulfide bridge is formed between the first and second cysteine by DsbA [27]. After the folding of the beta-barrel of LptD and insertion of the protein in the OM. Rearrangement of the disulfide bridges is triggered in which the disulfide bond is reshuffled and is between the second and third cysteine [27]. DsbA then creates a new disulfide bond between the first cysteine and the fourth. These disulfide bonds aren't formed between two consecutive cysteines but the disulfide bridges are formed between the first and third cysteine and between the second and fourth cysteine of LptD [27]. It seems that the only one nonconsecutive disulfide bond suffices for proper function of LptD [28]. For the function of LptD two nonconsecutive disulfide bonds aren't necessary. However, it is essential for LptD function that the disulfide bonds are formed nonconsecutively. The disulfide bridges are also necessary to keep LptD in the correct conformation or otherwise it is unable to interact properly with LptA causing problems in forming the lpt bridge in *E. Coli*

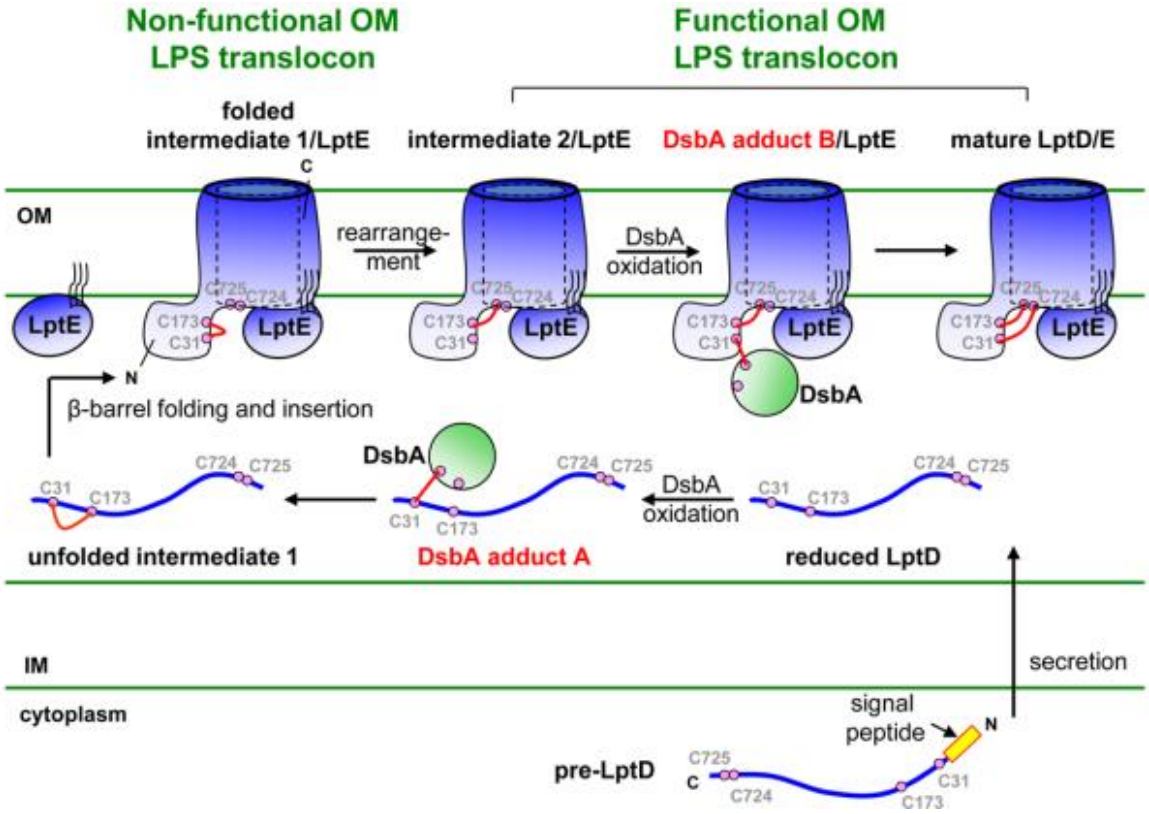

Figure 4 Biogenesis of the transenvelope LPS exporter in *E. coli* [27] DsbA forms disulfide bridges and reshuffles those thereby creating several intermediates of LptD in the end producing a correctly folded LptD.

Interestingly the LptD found in the gram-negative bacterium *N. meningitidis* has five cysteines instead of the four cysteines commonly found in gram-negative bacteria such as *E. coli*. It is unclear why LptD

has five cysteines in *N. meningitidis* because disulfide bonds can only be formed between two cysteines. Therefore it is not entirely clear what the function is of the fifth cysteine. When LptD of *N. meningitidis* is aligned with LptD of *E. coli* (appendix A) the second cysteine of the *Neisserial* LptD seems to be the extra cysteine. The second cysteine is only two amino acids separated from the first cysteine so they are close to each other just like the fourth and fifth cysteine.

LptD is mentioned to be a possible target for antibiotics because it has an important function in the transport of LPS to the OM [29,30]. Also it is possible for antibiotics to enter bacteria through the beta-barrel domain of LptD. Besides this LptD can also be a target for antibiotics itself because when LptD is taken out the transport of LPS to the OM is disrupted. But before this is possible it is necessary first to map the disulfide bonds and function of the cysteines in LptD. This can be done by replacing a cysteine for a serine. Replacing a cysteine has probably effect on the conformation of LptD and thereby on the transport of LPS. *N. meningitidis* is used because this bacterium is able to survive without LPS on the OM unlike *E. coli* [31]. The absence of LPS has a great impact on the growth rate and vulnerability of *N. meningitidis* against hostile environments and toxic substances such as antibiotics and detergents. Thus, changes in LptD can lead to a change in the growth of the bacteria and phenotype, compared to wild-type *N. meningitidis*. This also will be an indication of the impact of mutations in LptD has on the bacterium. In this study several plasmid will be constructed with mutations in the *lptD* present on the plasmid. These plasmids will be introduced in *N. meningitidis* and the effects of the mutations will be examined by several methods such as western blotting and SDS-PAGE.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains are listed in table 1. *E. coli* DH5α was grown in LB at 37˚C. When necessary, antibiotics were added to the growth media for plasmid maintenance: chloramphenicol 25µg/ml, kanamycin 50µg/ml. *N. meningitidis* HB-1 was used for the experiments to harvest the proteins needed. HB-1 is H44/76 with the capsule locus, including the *galE* gene inactivated which results in a strain unable to produce the polysialic acid capsule. Additionally a HB-1 with a chromosomal knock out of *lptD* was used [20]. HB-1 was grown overnight at 37˚C on GC plates supplemented with Vitox (Oxoid). The antibiotics chloramphenicol (10µg/ml) and kanamycin (25µg/ml) were added to the plates when necessary. For liquid cultures HB-1 was grown in sterilized Tryptic Soy Broth (TSB). To induce expression from the plasmids isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the TSB in a final concentration of 1 mM [32]. The optical density (OD) of the bacteria was measured with a spectrophotometer at 600 nm for *E. coli* and 550 nm for *N. meningitidis.*

Strains	**Relevant Characteristics**	**Source/reference**
E. coli DH5α	Cloning strain	Laboratory collection
BL21*LptD*	BL21 with pET26 with *E. coli lptD*	Laboratory collection
N. meningitidis HB-1		Ref. 30
HB-1Δ*lptD*	HB-1 with *lptD* replaced by a kan^R cassette	Ref. 9
HB-1*NlptDS1*	HB-1 with pEN21 *lptD* 1st cysteine replaced by serine	This study
HB-1*NlptDS1k1*	HB-1*NlptDS1* with chromosomal *lptD* replaced by a kan^R cassette	This study
HB-1*NlptDS1k2*	HB-1*NlptDS1* with chromosomal *lptD* replaced by a kan^R cassette	This study
HB-1*NlptDS4*	HB-1 with pEN11 *lptD* 4st cysteine replaced by serine	This study
HB-1*NlptDS4k1*	HB-1*NlptDS4* with chromosomal *lptD* replaced by a kan^R cassette	This study

Table 1 List of strains and relevant characteristics

Plasmid construction

Plasmids were adapted from the plasmid pCRII-*lptD,* containing the *N. meningitidis lptD*. Single nucleotide mutations were introduced in *lptD* using the Quick-Change site-directed mutagenesis (Agilent) according to the manufacturers protocol using PFU buffer without $MgSO_4$ (Fermentas) instead of the buffer provided in the kit. These mutations were introduced in *lptD* causing cysteine to be replaced by serine in LptD. The list of plasmids can be found in Appendix B. For this PCR 7 sets of reaction primers (Appendix B) were designed to introduce mutations in the *lptD* region of the pCRII-*lptD* plasmid. The DH5α strain was transformed by incubating the cells with the plasmids. The plasmids were sent for sequencing (Macrogen) to confirm that the point-mutations were present. *lptD* was excised from the pCRII vector using NdeI and AatII (Fermentas) and ligated with pEN21 or pEN11. To confirm insertion of *lptD* a PCR was done using the primers lacc cass and pEN rev [32]. A DNA fragment of 2400 base pairs would indicate that the insert was present. The isolated plasmids were then sent for sequencing for confirmation of the insertion and the mutation. HB-1 was transformed with the plasmid containing the insert and the resulting colonies were tested using colony PCR with the primer pair lacc cass and pEN rev to check if the insert was present. HB-1 containing pEN-*lptD* was transformed with a DNA-fragment that replaces the first 1000bp of the chromosomal *lptD* with a Km^R cassette by homologous recombination [10]. This replacement was checked with several primer pairs such as Km-for and *lptD*-dn-rev, *lptD*-up-for and IMP-rev. Primer Km-for is found at the start of the Km^R cassette, *lptD*-dn-rev is located around the 200 bp at the downstream flank of the *lptD* complementary strand. While *lptD*-up-for is located around 250 bp at the upstream flank of *lptD* and the IMP-rev primer is at the end of *lptD*.

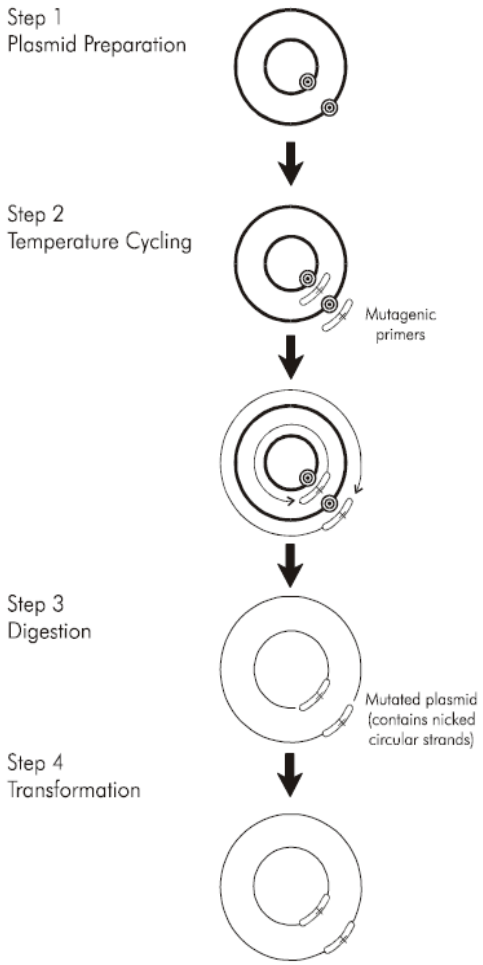

Figure 5 Overview of the QuickChange site-directed mutagenesis method [agilent protocol]

SDS-PAGE and immunoblotting

Proteins were analyzed by native and semi-native sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Whole cells and cells envelope samples were taken up in sample buffer (SDS 1%, Glycerol 10%, 62.5mM Tris/HCL pH 6.8, bromophenol blue 0.01%). For experiments using mPEG-maleimide, sample buffer was supplemented with 4M urea. Native SDS-page was carried out

at 4˚C with a running buffer (25 mM Tris, 192 mM glycine, 0.1 SDS, pH 8.3).Running gel (8% SDS, TEMED, demiwater, RG buffer, Stock 1) and stacking gel (3% SDS, TEMED, demiwater, SG buffer, Stock 2) were poured and the gel was run for 2.5 hours at 12mA per gel. After the SDS-PAGE the gels were heated for 10 minutes to facilitate protein transfer to the membrane. Proteins were transferred to a nitrocellulose transfer membranes which were incubated in block buffer (PBS, Protifar (1 % w/v), Tween 0.1%) over night at 4˚C. The membranes were incubated for 1 hour with primary antibodies (Rabbit anti LptD, Mouse anti LptB, Mouse anti PorA, Mouse anti LptD and Mouse anti His-tag) after washing, 3 times 10 minutes (PBS, Tween 0.1%); the blots were incubated for another hour with the secondary antibody (Goat anti rabbit perodxidase (GARPO) and Goat anti mouse peroxidase (GAMPO)). The excess of secondary antibodies was removed by washing before the antibody was detected using enhanced chemiluminescence (Thermo Scientific).

Silver staining

Whole cell samples in sample buffer were heated to 100°C to denature proteins. Samples were diluted five times before they were incubated with proteinase K (1 hour 60˚C) to remove all proteins. Samples were then analyzed by SDS-PAGE. LPS was fixed in the SDS-PAGE gel with fixative (Isopropanol 40%, Acetic acid 50%) for 1 hour at room temperature. LPS was then oxidized for 5 minutes in periodic acid 0.7%. The oxidizing agent was washed away with MilliQ (4X for 15 minutes). LPS was stained with a silver solution (0,02M NaOH, 1,3%NH_4, 0,04 M $AgNO_3$) for 10 minutes, after which the staining was washed away. LPS was visualized using a citric acid solution (Citric acid 0.05%, Formaldehyde 0.05%). When LPS was visible, the reaction was stopped with acetic acid (7.5%).

Cysteine labeling with mPEG-maleimide 5000

Cells were grown in liquid to a final OD of 1 before being harvested (3300g 3 minutes) and were washed twice with phosphate buffered saline (PBS 1%). The cells were resuspended in AK buffer (80mM Tris/HCl pH6.8, 150mM NaCl). The samples were separated into two groups, one group of samples didn't receive DTT treatment. The control group did receive DTT (200mM) treatment to break all existing disulfide bridges thereby making the cysteines free to bind mPEG-maleimide. The samples were incubated at 4˚C for 30 minutes. Subsequently the samples were incubated at 60˚C for 1 hour. The DTT-treated samples were washed again with PBS. The samples without DTT treatment and the control group were each divided in 2 more groups in which the samples would receive mPEG-maleimide treatment en control samples that didn't receive mPEG-maleimide treatment. mPEG-maleimide (5000 kDa Sigma Aldrich) treatment was performed with 2.5mM mPEG-maleimide for 30 minutes at room temperature.

Proteins were precipitated using trichloroacetic acid (TCA). Briefly, TCA was added to final concentration of 10%. The proteins were collected by centrifugation (14000g for 30 minutes) and washed with ice-cold acetone, after which they were collected by centrifugation again (14000g for 5 minutes). The proteins were resuspended in AK-buffer with SDS 1% and glycerol 5% before sample buffer with urea was added.

Results

Genotype of the mutants

The plasmids are used to determine the function of the cysteines in LptD. The mutations in the *lptD* cause a replacement of cysteine by a serine in the LptD. The plasmids are then introduced in HB-1 which is a *N. meningitidis* strain. The mutants of this strain are used to study several features of LptD such as growth and phenotype. Before the plasmids are introduced in HB-1 the wild-type gene of *lptD* has to be removed or else two types of LptD are produced in the cells. This makes it difficult to study the function of the cysteines in the changed LptD. A DNA-fragment was used to replace the first part of chromosomal *lptD* for a Km^R cassette. The HB-1*NLptDS1* and HB-1*NLptDS4* mutants were transformed with this DNA-fragment. HB-1*NLptDS1* is a strain in which the first cysteine is replaced by a serine and in HB-1*NLptDS4* the fourth cysteine is replaced by a serine.

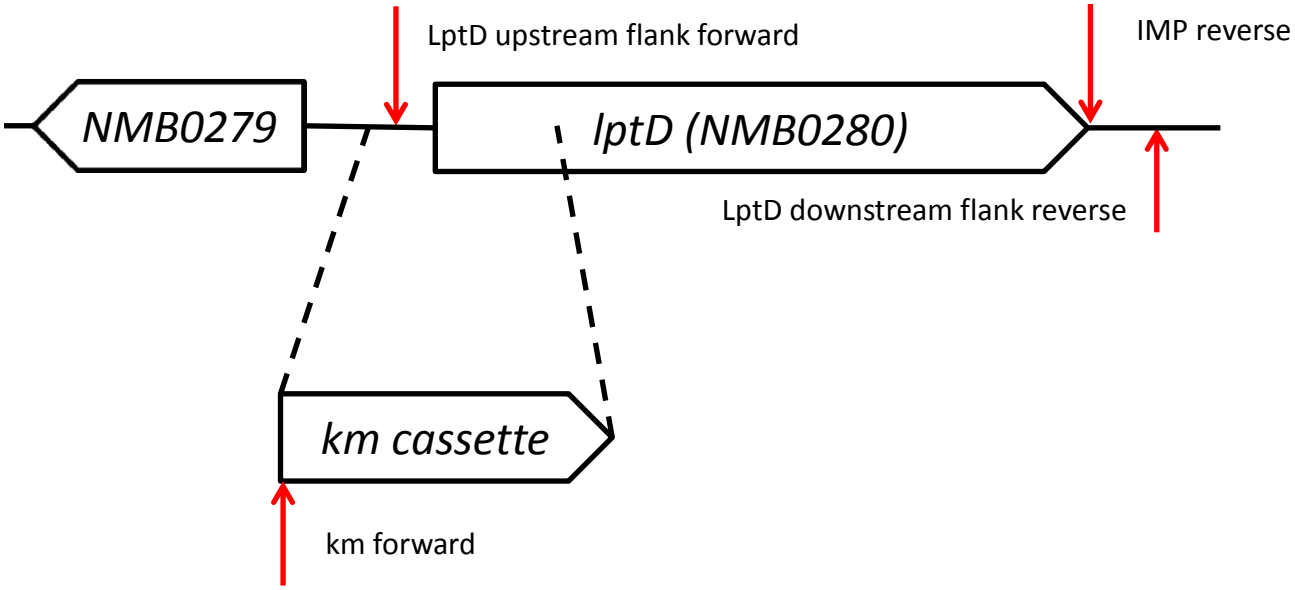

Figure 6 Locus of *lptD* in the chromosome of HB-1. Indicated in red are the places in which the primers bind on the chromosome. The Km cassette inserts in the 5' region of *lptD*.

Transformants were tested by PCR to determine the presence and the correct location of the DNA-fragment in the genome of the clone. As can be seen in figure 6 the Km^R cassette causes a partial removal of the upstream flank of *lptD* including the location at which the primer *LptD*-up-for binds. Therefore, when *lptD* is inactivated by insertion of the kanamycin cassette, a PCR using the primers *LptD*-up-for and IMP-rev will no longer yield a DNA fragment. While the primer pair of Km-for and *LptD*-dn-rev should work and produce a product. However, when a PCR is done with those two primer pairs on the two isolated clones we see conflicting results. If *lptD* has been inactivated, the primer pair of *LptD*-up-for with IMP-rev shouldn't be able to amplify a DNA-fragment. However, we find that this primers pair amplifies a DNA-fragment of 3000 bp. This could mean that the DNA-fragment used to inactivate the wild-type *lptD* didn't recombine correctly (fig. 7B).

Subsequently the primer pair of km-for and *lptD*-dn-rev does give an amplified DNA-fragment of 3000 bp as expected, this means that the DNA-fragment to inactivate the wild-type *lptD* is present in the genome (fig. 7A). Thus, it isn't entirely clear if the recombination of the DNA-fragment with the genome *lptD* works correct or is successful. However, we assume that this is the case because the clones show kanamycin resistance. Besides the genotype we can also verify if *lptD* was removed by analyzing its phenotype.

A **B**

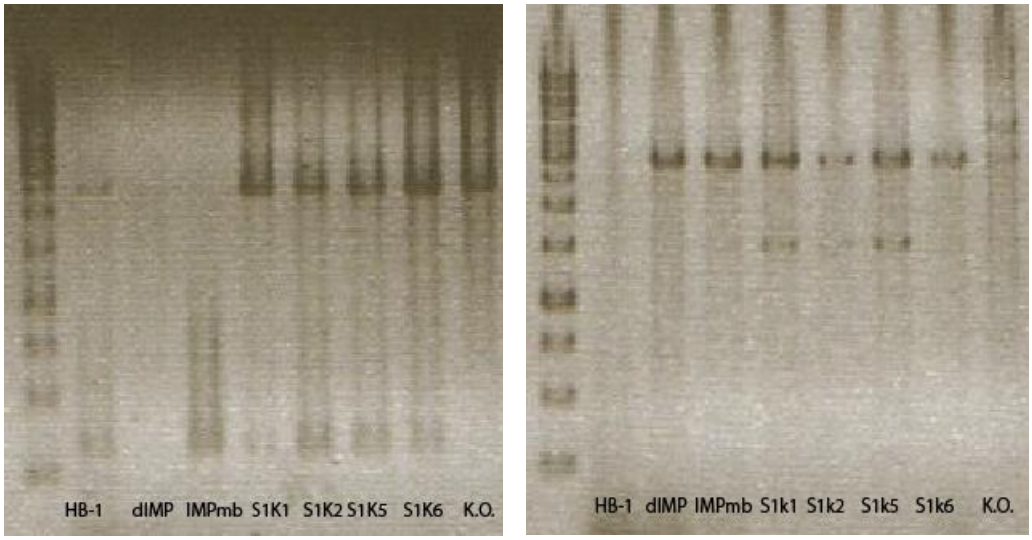

Figure 7A PCR with the primer pair Km-for and *lptD*-dn-rev shows bands at the correct height for the mutants proving that the Km^R cassette is present, 7B shows a PCR with primer pair *lptD*-up-for and IMP rev.

Phenotypes of the mutants

To determine if the mutants with the inactivation construct behaved like a HB-1*ΔlptD* we first look at the colony morphology. When HB-1 is defective in LPS transport there will be no LPS on the outer leaflet of the OM. This causes a coloration of colonies from a "bluish" appearance to an opaque phenotype (fig. 8B) [10].

A **B**

Figure 8A "bluish" phenotype of a HB-1 and B ,an "opaque" phenotype of a LPS deficient mutant.

When the clones of HB-1*NLptDS1* and HB-1*NLptDS4* are transformed with the DNA-fragment to inactivate the chromosomal *lptD,* this should mean that no LPS is transported to the cell surface. If

there is no LPS at the cell surface the colonies should show the phenotype as seen in figure 8B. This means that a color change can be an indication that the DNA-fragment in the HB-1*NLptDS1* and HB-1*NLptDS4* is properly inserted. Therefore, the mutants created from HB-1*NLptDS1* and HB-1*NLptDS4* clones should show the opaque phenotype as shown in figure 8B. However, after 24 hours we see wild-type parts (fig. 9) that looks like the wild-type as seen in figure 8A. Some of colonies remain "opaque" while others exhibit wild-type-parts.

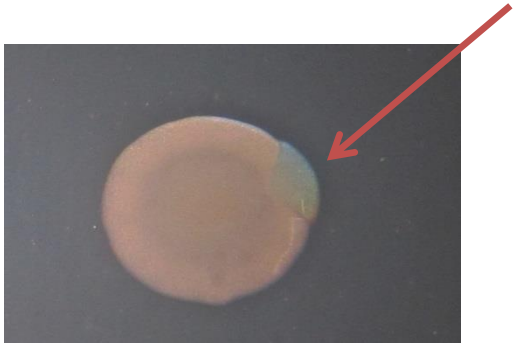

Figure 9 A "opaque" colonies suddenly seems to have developed a "bluish" part, which is indicated with the red arrow.

Nevertheless, we were able to obtain HB-1*NLptDS1* and HB-1*NLptDS4* mutants from the colonies that stayed "opaque".

Normally the growth of HB-1 slows down if the transport or synthesis of LPS is disrupted [10]. This means that if the LptD doesn't fold correctly due to a problem with the cysteines and the formation of disulfide bridges. It will not transport LPS and therefore impair the growth of the mutants. Therefore, we look at the growth of the mutants because this can be an indication if the cysteine disabled in the mutant is essential in the correct folding and thereby the functioning of LptD. Production of mutated LptD from the pEN plasmid was induced by IPTG. In the absence of a functional LptD we expect growth similar to a *ΔlptD* mutant. Therefore, we analyzed the growth of HB-1, HB-1*ΔlptD*, HB-1*NLptDS1* and HB-1*NLptDS4*.

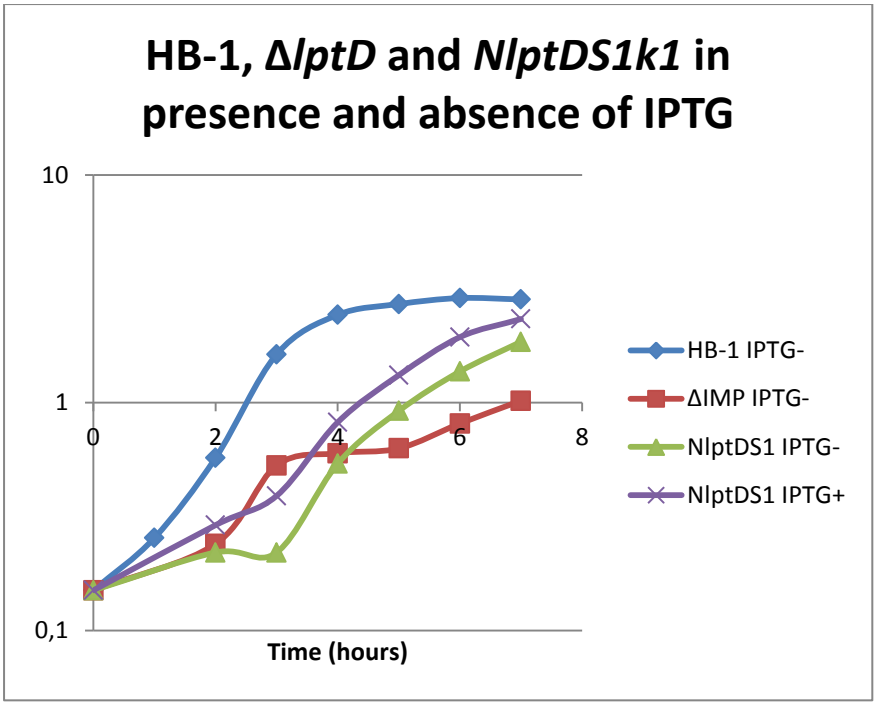

Figure 10 Growth curve of the average OD of HB-1, HB-1*ΔlptD* and HB-1*NLptDS1k1* in presence and absence of IPTG.

In figure 10 we see that HB-1*ΔlptD* shows decreased growth compared to HB-1. When HB-1 containing the S1 mutation and lacking chromosomal *lptD* is grown, it exhibits decreased growth comparable to that of the *lptD* mutant. However after approximately 3-4 hours it recovers its growth, this is strange because the HB-1 with the S1 mutation should be similar as the *lptD* mutants because the plasmid isn't stimulated to produce LptD. When IPTG is added the S1 mutant grows better compared to the mutant without IPTG. The HB-1 wild-type grows better than the S1 mutant and the *lptD* mutant. In figure 11 we see that the HB-1 containing the S4 mutation and lacking the chromosomal *lptD*, exhibits decreased growth which is also comparable to that of the *ΔlptD*. The growth of the S4 mutant never approaches the level of growth of the *ΔlptD*. When IPTG is added to the S4 mutant to induce the mutated *lptD* the growth is comparable with the S4 mutant without IPTG. Although it is a little higher than when the S4 mutant isn't in the presence of IPTG. When comparison is made between the S1 and S4 mutant it seems that the S4 grows very bad and isn't able to recover its growth as seen in the S1 mutant. Additionally the S4 mutant grows slower than the *ΔlptD* mutant that could mean that this mutation in *lptD* is very severe and has a great impact on the growth of the S4 mutant.

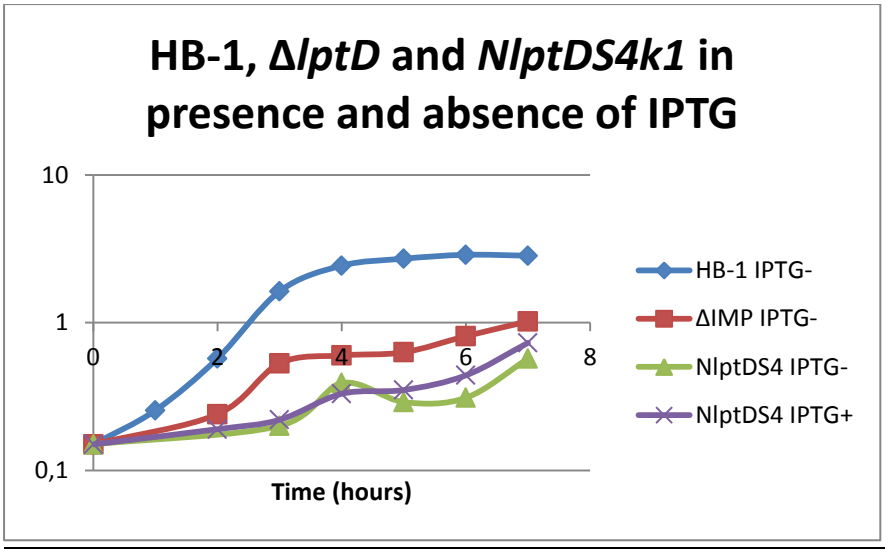

Figure 11 Growth curve of HB-1, HB-1*ΔlptD* and HB-1*NLptDS4k1* in presence and absence of IPTG

Slow growth can mean that there is a problem with the LPS transport to the cell surface [10]. Lack of LPS makes bacteria vulnerable and causes a slow growth rate. With the silver staining method, LPS can be made visible. Any LPS present in bacteria or at the cell surface will be stained. Normally when the transport of LPS towards the cell surface is disrupted, a negative feedback loop causes the synthesis of LPS to halt. We see in figure 12 that the mutant without the mutated LptD is still able to produce LPS. This suggests that there is something wrong with the S1 mutant. Because if there is LPS present, the transport of LPS still works or else the negative feedback loop would have caused the synthesis to stop. There is no difference between the mutant with IPTG or without. This means that the mutated *lptD* is not responsible for the LPS seen in figure 12.

Figure 12 Proteinase K-treated cell lysates were analyzed by SDS-PAGE and silverstaining.

When we look at the LPS production of the S4 mutant we see that there is none in the mutant without and with IPTG. From figure 13 we can say that the mutated LptD is responsible for the lack of LPS. When the mutated LptD is present in this mutant, it could indicate that this LptD is unable to transport LPS that causes the synthesis of LPS to stop. Additionally it could mean that the fourth cysteine is essential in the conformation of LptD and essential for the function of LptD.

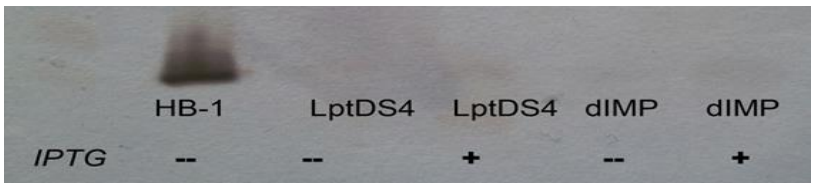

Figure 13 Proteinase K-treated cell lysates were analyzed by SDS-PAGE and silverstaining.

With SDS-PAGE and immunoblotting we can see how much LptD is present. In addition, it would give an insight in the folding and degradation of LptD.

In figure 14 we see the SDS-PAGE and immunodetection of LptD from the HB-1 and the S1 mutant. During the first SDS-PAGE and immunoblotting too much signal caused overlap and made it hard to distinguish the different bands. By making serial dilutions, we hoped to make the signal better and thereby easier to distinguish the different bands. We see that LptD is present in HB-1 and is not present in an *lptD* mutant as indicated with the arrow. LptD can be found around the 88 kDa molecular weight mass. When the S1 mutant is in the absence of IPTG the amount of LptD present is very low compared to the S1 mutant with IPTG. High amount of LptD seen in the S1 mutant with IPTG is probably from the mutated *lptD*. The LptD present in the S1 mutants without IPTG is probably from the wild-type *lptD*. This means that the wild-type copy has not been properly inactivated. There is also the possibility that the plasmid shows some leaky expression. This means that some *lptD* is transcribed from the plasmid although there is not any IPTG present to induce this. Furthermore it is possible that the LptD production is caused by bacteria with the wild-type parts. Subsequently is it difficult to make the distinction, which LptD is responsible for the transport of LPS while the wild-type gene is still present. This means we have to inactivate the wild-type gene.

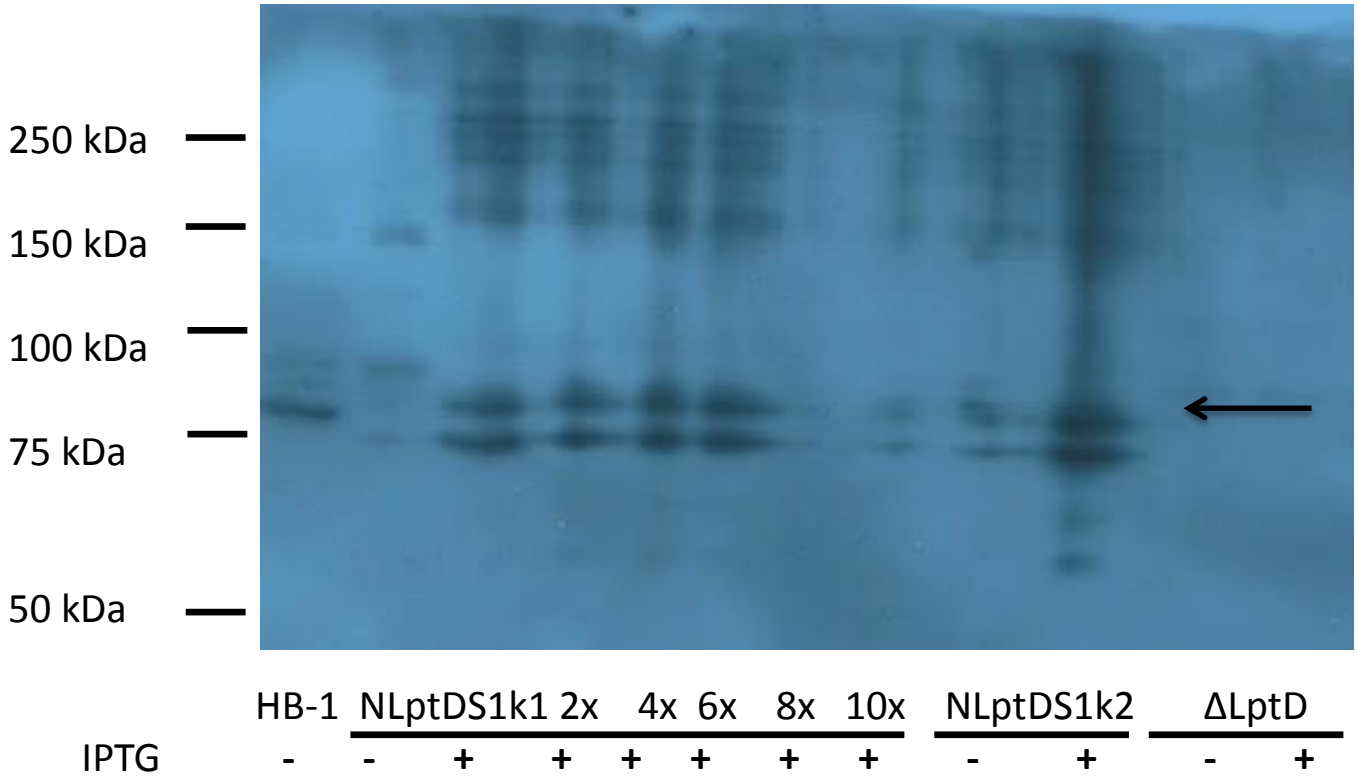

Figure 14 Whole cells samples subjected to SDS-PAGE and western blotting followed by immunodetection with an antibody raised against LptD. Molecular weight markers are shown on the left.

In figure 15 an SDS-PAGE and western blot is seen for the S4 mutant. Again, LptD is present in HB-1 and absent in the *lptD* mutant. In the S4 mutant without IPTG no LptD is visible. In the S4 mutant in the presence of IPTG there is LptD present. This means that the LptD present is probably the LptD derived from the plasmid in the S4 mutant. When we look at figure 13 it is seen that almost no LPS is present. This suggests that the LptD present in the induced S4 mutant is unable to transport LPS. This could mean that the mutation in this LptD is so important that LptD loses its function.

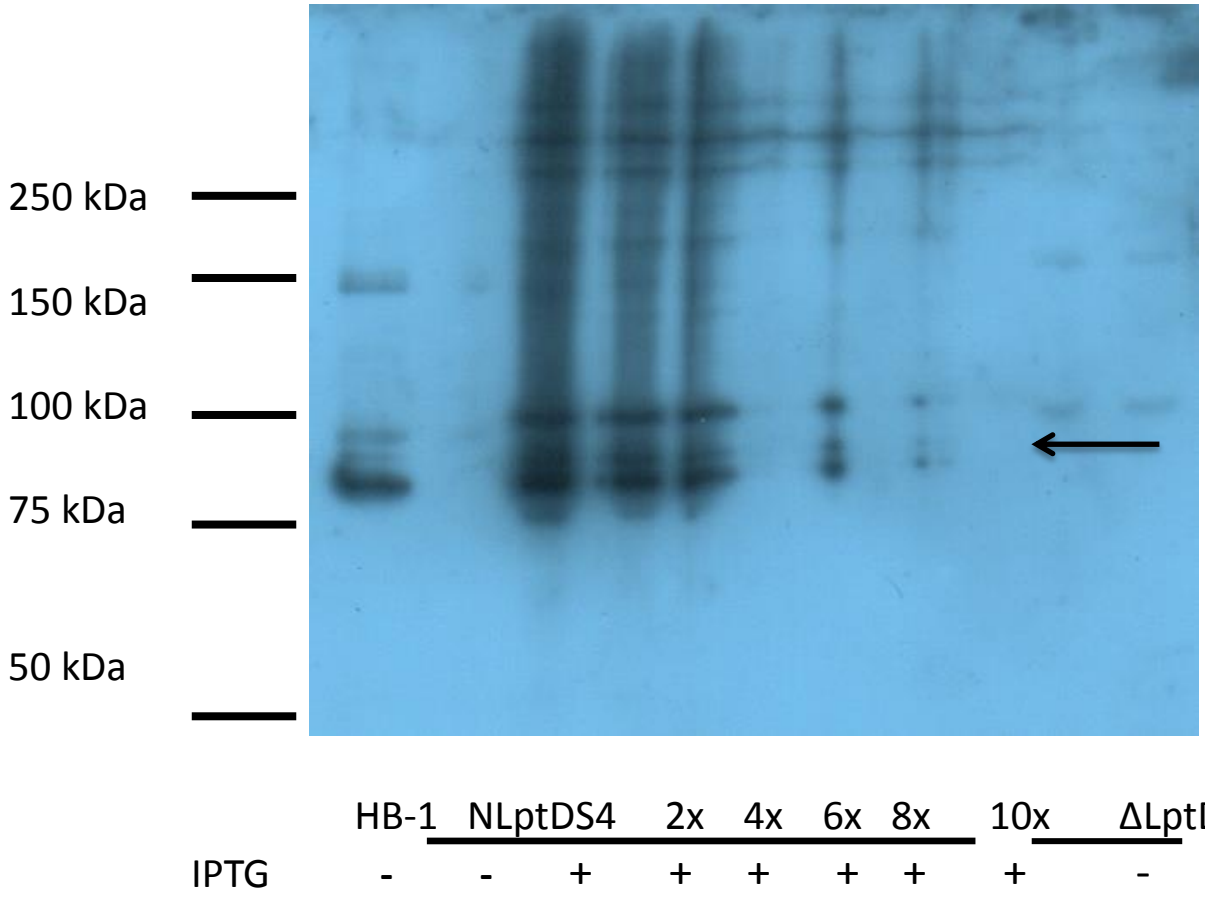

Figure 15 Whole cells samples subjected to SDS-PAGE and western blotting followed by immunodetection with an antibody raised against LptD. Molecular weight markers are shown on the left.

The influence of cysteine 1 and 4 on the conformation of LptD.

LptD from *N. meningitidis* probably contains at least one free cysteine. A disulfide bridge can only be formed between two cysteines. LptD has five cysteines so we can presume that two disulfide bridges are formed making this fifth cysteine free. It is also possible that only one disulfide bridge is formed which means that there are three free cysteines however this seems highly unlikely. In *E.coli* the LptD can function with only one disulfide bridge but only if this disulfide bridge is formed between the two correct cysteines as seen in figure 4.

With a mPEG-maleimide treatment the amount of free cysteines can be made visible after SDS-page and immunoblotting. mPEG-maleimide is a substance composed of maleimide and a mPEG tail. Maleimide is able to bind free cysteines while the mPEG tail adds a molecular weight of 5 kDa to it. Before this treatment could be used it had to be optimized for gram-negative bacteria and proteins used in this study. To optimize the protocol we used the LptD from *E.coli* because this LptD has four cysteines and two disulfide bridges. We used this mutant because it works properly and this LptD can function with only one disulfide bridge formed [27]. Before adding mPEG-maleimide to the proteins some samples were treated with DTT. DTT is used to reduce all the disulfide bridges present in the protein. This means that all cysteines become free cysteines and are therefore able to bind mPEG-maleimide. The arrows in figure 16 indicate the amount of bound cysteines of LptD. With the bottom arrow indicates that one cysteine is bound while the top arrow indicates four bound cysteines.

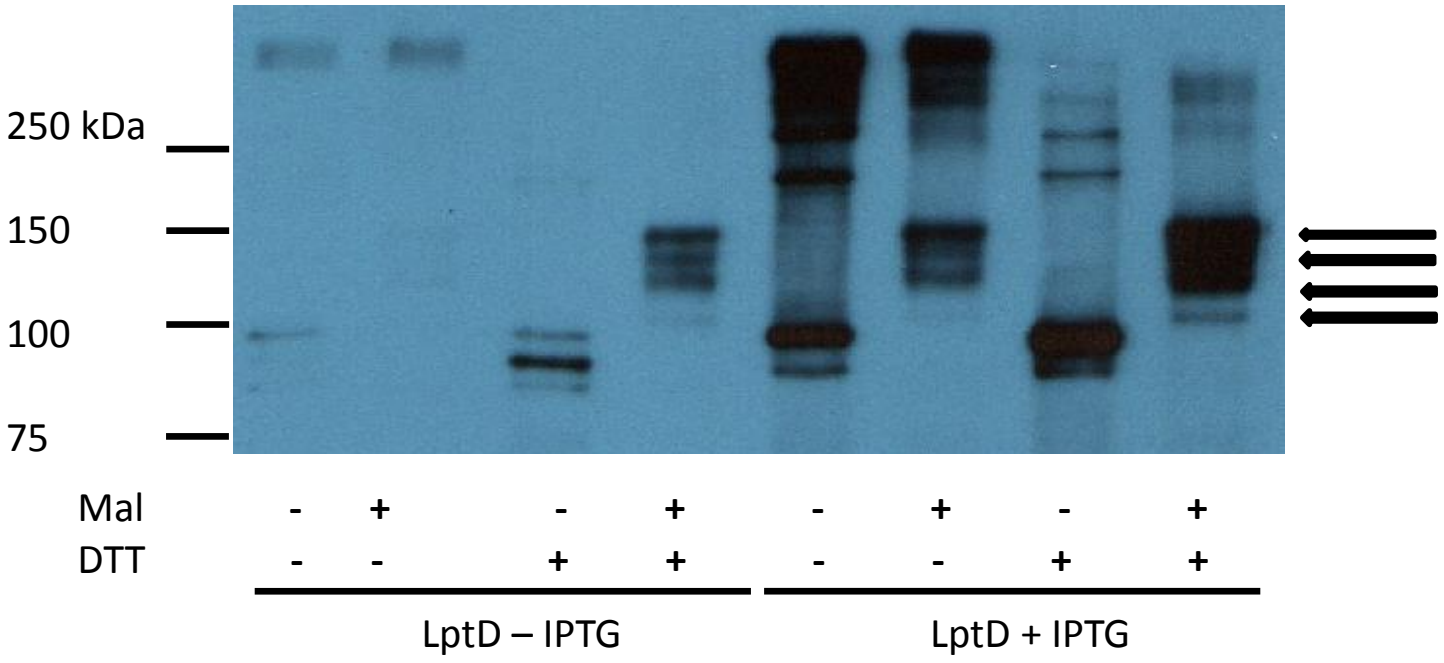

Figure 16 Whole cell samples treated with DTTand mPEG-maleimide subjected to semi-native SDS-PAGEand western blotting followed by immunodetection with an antibody raised against LptD. Molecular weight markers are shown on the left.

During the mPEG-maleimide treatment four conditions are created. LptD that is not treated with mPEG-maleimide nor with DTT, this is a control for the next condition. LptD that is only treated with

mPEG-maleimide so the cysteines aren't reduced so only free unbound cysteine can be targeted with mPEG-maleimide. To some samples only DTT is added which reduced all the cysteines but there aren't treated with mPEG-maleimide. Which means that the free cysteines aren't bound with mPEG-maleimide this is a control to check what the exact effects are of DTT on the proteins. At last, we have samples treated with DTT and mPEG-maleimide. This means that all disulfide bridges will then be reduced and all cysteines will be free to bind mPEG-maleimide. When we look at figure 16 we see that there is a shift when all reduced cysteines are treated with mPEG-maleimide. The arrows at the right indicate shifts seen from bound cysteines. These shifts are a result from the decreased electrophoretic mobility of LptD caused by the mPEG tail that adds a weight of 5 kDa when it is bound to a free cysteine. This means that each arrow corresponds with the amount of cysteines bound to mPEG-maleimide. LptD without any cysteines bound to mPEG-maleimide cannot be seen in figure 16.

The lowest arrow for instance corresponds to one bound cysteine increasing the molecular weight of LptD with 5 kDa. The second lowest arrow thus indicates two bound cysteines with an extra molecular weight of 10 kDa. The second top arrow indicates three bound cysteines with an extra molecular weight of 15 kDa and the top arrow indicates four bound cysteines with an extra molecular weight of 20 kDa. We expected a to see only one spot indicated with the top arrow but probably the amount of DTT or mPEG-maleimide used in the treatment wasn't high enough. This caused the several forms of LptD in which not all four cysteines are bound or reduced.

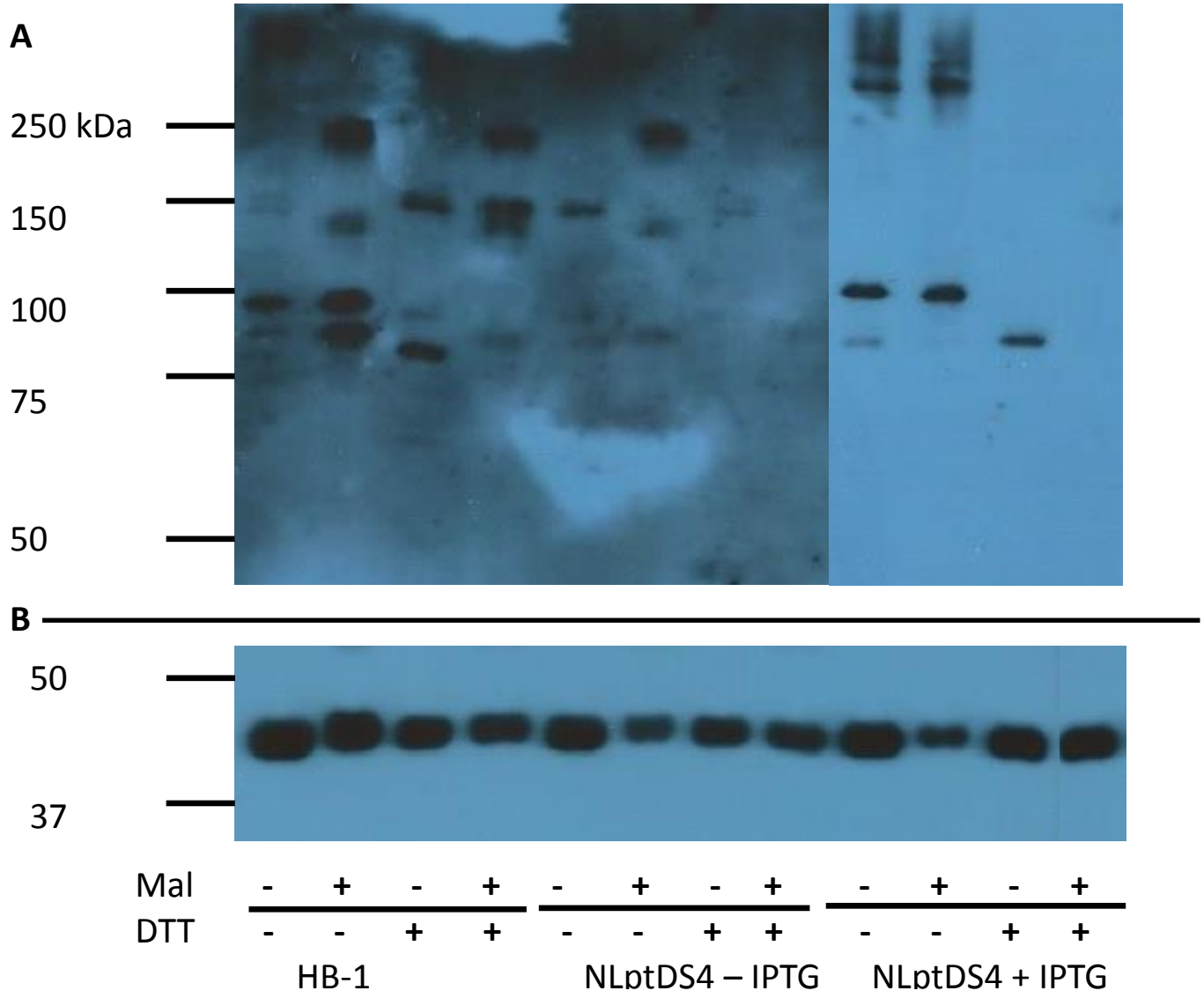

Figure 17 Whole cell samples treated with DTTand mPEG-maleimide subjected to semi-native SDS-PAGEand western blotting followed by immunodetection with an antibody raised against LptD and PorA. Molecular weight markers are shown on the left.

The treatment with mPEG-maleimide seems to work so then we moved on and started to treat LptD of *N.meningitidis*.

During this experiment we used PorA as a negative control. PorA is a protein found in *N. meningitidis* and it doesn't have any cysteines. PorA clearly shows (fig. 17B) that there is no decrease or increase of electrophoretic mobility visible. This means that the treatment with mPEG-maleimide doesn't work for proteins that don't have any cysteines. There are some slight changes visible but these are probably caused by the samples no running straight through the SDS-PAGE gel. When we look at figure 17A some differences are seen between HB-1 and the S4 mutant. We were unable to use the S1 mutant in this experiment because of all the problems with the wild-type gene still present in the strain. When the S4 mutant wasn't induced with IPTG this gave a weak signal which indicates that almost no LptD is present. This can also be seen in figure 15. In the sample of HB-1 we see that

when LptD is reduced with DTT the LptD migrates faster through the SDS-page gel which result in a lower band. It seems that there is some variation in the amount of LptD in the samples. For instance there is more LptD in the second sample compared to the first sample. The LptD in sample four shows some decreased electrophoretic mobility compared to the LptD of sample three. This could indicate that there is one bound cysteine present but unfortunately, this is hard to tell. The S4 mutant, in the presence of IPTG shows in its untreated condition the presence of LptD that disappears when mPEG-maleimide is added. The LptD seen in sample nine can't be seen in sample ten but it reappears in sample eleven. It is also disappointing that sample twelve shows no LptD. We expected to see four forms of LptD as seen in the last sample of figure 16.
Nevertheless, this experiment with mPEG-maleimide seems to work so far with LptD of *E.coli* as shown in figure 16. However, the experiment still needs some adjustments before it can be used for LptD from *E.coli* and *N. meningitidis*.

Discussion

During this study we tried to get some more insight into the function of cysteines and disulfide bridges in LptD of *N. meningitidis*. LptD is an important part of the lpt system and essential in the transport of LPS to the cell surface of bacteria [19]. To determine which cysteines are essential and which cysteines are important in the formation of disulfide bridges we tried to make point-mutations for all the cysteines in LptD so far all the single, double and triple cysteine mutants are present in the pCRII plasmid as shown in appendix B. Only a few of these mutants are also in the pEN11 or pEN21 plasmid. We were able to get the S1 and S4 mutant in HB-1. However, there is a strange phenotype occurring when chromosomal *lptD* is tried to knock out. The DNA-fragment used to inactivate chromosomal *lptD* with homologous recombination gives some contradicting results when a control PCR is done (fig. 7A/B). Occasionally transformed colonies with the opaque phenotypes show wild-type parts. We don't know why this is happening and how it works.

It is possible that there is something wrong with the DNA-fragment. This fragment could recombine with the *lptD* in the plasmid. Maybe the remaining parts of *lptD* on the chromosome recombine with the *lptD* in the plasmid. In either case it could be smart to look deeper into the DNA-fragment used for inactivation of chromosomal *lptD*. This could lead to the production of a new DNA-fragment that entirely replaces the chromosomal lptD rather than pieces of it. Thereby would it also be interesting to send some DNA of the colonies with the wild-type parts for sequencing to see what is happening with *lptD* at both the chromosomal and plasmid level.

The normal growth and the presence of LPS in the S1 mutant shows that the LptD derived from the plasmid isn't responsible for the LPS transport. This could mean that there is another copy of *lptD* present somewhere in the S1 mutant. That is still facilitating the transport of LPS. The SDS-PAGE and immunoblotting show a low amount of LptD when the S1 mutant is in the absence of IPTG compared to the wild-type. We expect a higher amount of LptD because the plasmid is induced with IPTG. However, this mutant is still able to produce as much LPS as when the S1 mutant is in the presence of IPTG. This makes it impossible to conclude if the first cysteine is essential in the conformation of LptD. When we look at the S4 mutant we see that when the plasmid is induced with IPTG it makes LptD. However, this mutant doesn't produce any LPS while LptD is present. This means that its LptD is unable to transport LPS to the cell surface indicating that there is something wrong in the folding of LptD. This could mean that the fourth cysteine has an important role in the conformation of LptD from this can be concluded that the fourth cysteine is essential. The growth curve of this mutant also shows that this mutant has a severely decreased growth compared to the *lptD* mutant.

With the mPEG-maleimide treatment, we tried to get some insight in the function and arrangement of the disulfide bridges present in LptD. Before we could start with this treatment, we had to optimize the

protocol. Figure 16 clearly show the decrease of electrophoretic mobility of LptD caused by the extra 5 kDa molecular weight of the mPEG tail. Four forms of LptD can be seen in this figure each form has a different amount of bound cysteines. However, we expected to see only one LptD form with an extra molecular weight of 20 kDa if all the cysteines were bound to mPEG-maleimide and all disulfide bridges reduced. This could mean that not all disulfide bridges are reduced which indicates that the amount of DTT wasn't high enough. It is also possible that the amount of mPEG-maleimide wasn't high enough causing not all cysteines to be occupied. Nevertheless, these results shown in figure 16 prove that the treatment with mPEG-maleimide works. It only needs more adjustment to work perfect because in figure 17 we see that the treatment doesn't work that great for the LptD from *N.meningitidis*.

In summary, it seems that the fourth cysteine is essential in the correct folding and thereby the function of LptD. However, more research has to be done to determine the exact change in the conformation of LptD. In some colonies we see that they have wild-type parts. This could be caused by the incorrect recombination of DNA-fragment or due to recombination between the lptD of the plasmid and the DNA-fragment. Nevertheless, the cause of this strange phenotype is not entirely clear but could be interesting. The mPEG-maleimide treatment seems to work for the LptD of *E. coli* it only requires some fine-tuning. The protocol for the LptD of *N. meningitidis* requires some major adjustments.

For future research it would be necessary to look at the other mutants with the double and triple point mutations in *lptD* causing the replacement of cysteines by serines in LptD. In addition, it is important to look at the arrangement of the disulfide bridges in which the mPEG-maleimide experiment could play an important role. Above all, we can conclude that still a lot of research has to be done before we fully understand LptD of *N. meningitidis.*

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Appendix A

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Query  50   QPTSLSLGSTCLFCSNESGSPERTEAAVQGSGEASIPEDYTRIVADRMEGQSQVQVRAEG
            Q  +  L S C+      G P      VQG     +P     I AD  +G         G
Sbjct  21   QGLAADLASQCML-----GVPSYDRPLVQGDTN-DLP---VTINADHAKGDYPDDAVFTG

Query  110  NVVVERNRTTLNTDWADYDQSG--------DTVTAGDRFALQQDGTLIRGETLTYNLEQQ
            +V + +  + L  D     Q            TV A        +  +++G     NL
Sbjct  72   SVDIMQGNSRLQADEVQLHQKEAPGQPEPVRTVDALGNVHYDDNQVILKGPKGWANL--N

Query  162  TGEAHNVRMEIEQGGRRLQSVSRTAEMLGEGHYKLTET-QFNTCSAGDAGWYVKAASVEA
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Query  221  DREKGIGVAKHAAFVFGGVPIFYTPWADFPLDGNRKSGLLVPSLS-AGSDGVSLSVPYYF
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            R+   W H   +    +  VD+ +VSD  Y+ DF      + +    ++  + Y    A
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Query  392  GSLNAGLSVLKYQTLANQSGYKDKPYALMPRLSVEWRKN-TGRAQIGVSAQFTRF--SHD
             + NA +S  ++Q  + Q+      Y+  P+L V + +N  G     +  Q   F  + D
Sbjct  366  QNFNATVSTKQFQVFSEQNT---SSYSAEPQLDVNYYQNDVGPFDTRIYGQAVHFVNTRD

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Query  677  GRNEKIYLK-----SDGSYFYDKLSQLDLSAQWPLTRNLSAVVRYNYGFEAKKPIEVLAG
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Query  732  AEYKSSCGCWGAGVYAQRYVTGEN------TYKNAVFFSLQLKDLSS  772
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```

Appendix B

Plasmids	Cysteine in LptD	Characteristics	Source
pCRII *lptD*	CCCCC	pCRII containing *lptD*	Laboratory collection
pEN21	CCCCC	pEN21 containing *taq*-promotor	Laboratory collection
pEN11	CCCCC	pEN11 containing dual *laq-taq* promotor	Laboratory collection
pEN21 *NlptDS1*	SCCCC	pEN21 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 1 in LptD	This study
pEN11 *NlptDS2*	CSCCC	pEN11 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 2 in LptD	This study
pEN11 *NlptDS3*	CCSCC	pEN11 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 3 in LptD	This study
pEN11 *NlptDS4*	CCCSC	pEN11 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 4 in LptD	This study
pEN11 *NlptDS5*	CCCCS	pEN11 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 5 in LptD	This study
pCRII *NlptDS12*	SSCCC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 12 in LptD	This study
pCRII *NlptDS13*	SCSCC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 13 in LptD	This study
pEN11*NlptDS14*	SCCSC	pEN11 containing *lptD* with point mutation causing a replacement of cysteine for serine at position 14 in LptD	This study
pCRII *NlptDS15*	SCCCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 15 in LptD	This study
pCRII *NlptDS23*	CSSCC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 23 in LptD	This study
pCRII *NlptDS24*	CSCSC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 24 in LptD	This study
pCRII *NlptDS25*	CSCCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 25 in LptD	This study
pCRII *NlptDS34*	CCSSC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 34 in LptD	This study
pCRII *NlptDS35*	CCSCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 35 in LptD	This study

pCRII *NlptDS45*	CCCSS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 45 in LptD	This study
pCRII *NlptDS123*	SSSCC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 123 in LptD	This study
pCRII *NlptDS124*	SSCSC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 124 in LptD	This study
pCRII *NlptDS125*	SSCCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 125 in LptD	This study
pCRII *NlptDS134*	SCSSC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 134 in LptD	This study
pCRII *NlptDS135*	SCSCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 135 in LptD	This study
pCRII *NlptDS145*	SCCSS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 145 in LptD	This study
pCRII *NlptDS234*	CSSSC	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 234 in LptD	This study
pCRII *NlptDS235*	CSSCS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 235 in LptD	This study
pCRII *NlptDS245*	CSCSS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 245 in LptD	This study
pCRII *NlptDS345*	CCSSS	pCRII containing *lptD* with point mutation causing a replacement of cysteine for serine at position 345 in LptD	This study
Cysteine codon	**Serine codon**		
TGC	TCC		
TGT	TCT		

Restriction enzyme	**Restriction site**
AatII	GACGT^C
NdeI	CA^TATG

Name primer	Sequence 5’ to 3’
LptDS1 for	AGCCTCGGTTCGACCTCCCTGTTTTGCAGTAAC
LptDS1 rev	GTTACTGCAAAACAGGGAGGTCGAACCGAGGC
LptDS2 for	TCGACCTGCCTGTTTTCCAGTAACGAAAGCGGC
LptDS2 rev	GCCGCTTTCGTTACTGGAAAACAGGCAGGTCGA
LptDS3 for	ACCCAATTCAACACCTCTTCCGCCGGCGATGCC
LptDS3 rev	GGCATCGCCGGCGGAAGAGGTGTTGAATTGGGT
LptDS4 for	GAATACAAAAGCAGTTCCGGCTGCTGGGGCGCG
LptDS4 rev	CGCGCCCCAGCAGCCGGAACTGCTTTTGTATTC
LptDS5 for	GCAGTTGCGGCTCCTGGGGCGCGGG
LptDS5 rev	CCCGCGCCCCAGGAGCCGCAACTGC
LptDS12 for	CTCGGTTCGACCTCCCTGTTTTCCAGTAACGAAAGC
LptDS12 rev	GCTTTCGTTACTGGAAAACAGGGAGGTCGAACCGAG
LptDS45 for	GAATACAAAAGCAGTTCCGGCTCCTGGGGCGCGGGCGTG
LptDS45 rev	CACGCCCGCGCCCCAGGAGCCGGAACTGCTTTTGTATTC
Lacc cass	TCTGGATAATGTTTTTTGCGCCGAC
pEN rev	AAGGGAATAAGGGCGACACG
Km for	TCGTCACTCATGGTGATTTCTCAC
LptD flank up for	GCTGGCATTGGGCCTC
LptD flank down rev	CTGCCTTTGATGGCGGG
0279 for	ATGCCTGCAACCTTCAAGTG
IMP rev	GAACGTGGCATCGAGATTG
lptD 1800 rev	GCGTTTGTCGTTTTGGTTG
lptD 1300 for	CGGACTGCACGCCACC
lptD 600 for	CCCTTGGGCGGACTTC
lptD 1100 rev	GAGGGCATACGGTTTGTC
lptD 600 rev	GCGGACAGTGAGGGAAC