

Faculty of Medicine and Health Sciences
Division of Microbiology and Infectious Diseases
QUEEN'S MEDICAL CENTRE

**DEGREE OF MASTER OF SCIENCE IN MOLECULAR MEDICAL
MICROBIOLOGY**

(project dissertation)

**Investigating the Role of Chaperonin 60/GroL
Neisseria meningitidis in Induction of Host
Cytokine Responses**

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B.Sc., M.Sc.**

**A Dissertation Submitted in Partial Fulfilment of the
Requirements of the M.Sc. in Molecular Medical Microbiology**

**Supervised By
Dr. KARL WOOLDRIDGE**

**CARRIED OUT AT NOTTINGHAM UNIVERSITY HOSPITAL/
QUEEN'S MEDICAL CENTRE**

JULY 2005

SUMMARY : 319 WORDS
MAIN TEXT: 5292 WORDS
FIGURES : 9
TABLES : 1
Guide Lines: Infection and Immunity

DECLARATION

I confirm that all work presented in this study is my own, except where otherwise stated .

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AHMED MOHAMMED SALIH

SUMMARY

Meningococcal disease is a world wide problem; it presents various clinical syndromes from benign meningococcaemia to meningitis, fulminant septicaemia with septic shock lead to multi organ failure and death. *Neisseria meningitidis* is a major cause of bacterial meningitis and septicaemia. Lipooligosaccharide (LOS) which is released during the infection, causes activation of the innate immune cells and inducing cytokine production is regarded as a virulence factor. Heat shock proteins have been suggested to play a role in inducing cytokine production by the innate immune cells in the same manner the LOS/lipopolysaccharide (LPS) does. This effect has been suggested to be due to the contamination with LPS/LOS. Work in our laboratory has led to the hypothesis that hsp60/GroEL of *N. meningitidis* binds to LOS. The affinity of GroEL for LOS/LPS may explain the observed LPS/LOS- like induction of cytokine production by GroEL. *N. meningitidis* is unique among gram negative bacteria in that it is possible to construct a mutant that is totally devoid of LOS/LPS. The aim of the current study is to purify the meningococcal GroEL from a mutant *N. meningitidis* strain that does not express LOS. Immobilized metal affinity chromatography was used to purify the product of an N-terminally histidine tagged *groEL* gene, which has been cloned into a *N. meningitidis* wild type strain. Terminally tagged GroEL has been shown to be expressed intracellularly but not in concentrated culture supernatants. This is in contrast to the wild type protein, which is abundant in culture supernatants. Future work will aim to clone and express a C-terminally tagged *groEL* from *N. meningitidis* wild type and the *lpxA* gene, which is essential for LOS biosynthesis, will be cloned into strains expressing both the C-terminally and N-terminally tagged *groEL* genes. Purified GroEL from both backgrounds will be investigated for its potential to induce the cytokine production in cells of the human immune system.

INTRODUCTION

Meningococcal disease was first described in 1805 when an outbreak swept through Switzerland, now meningococcal disease is a worldwide problem. The causative agent is *meningitides* (the meningococcus). Twelve subtypes or serogroups of *N. meningitides* are identified and four (serogroups A, B, C and W135) are recognized to cause epidemics, and for most disease worldwide (1). The pathogenicity, immunogenicity, and epidemic carriage differ according to the serogroup, the bacteria can be carried in the pharynx and some reasons not fully known, overwhelm the body's defences allowing infection to spread through the bloodstream and to the brain. It is estimated that between 10 to 25% of the population carry *meningitides* at any given time, but of course the carriage rate may be much higher in crowded situations (2), Serogroup B is the most common cause of meningococcal disease in Europe, USA and several countries in Latin America and has been of great concern in Norway (3). Systemic meningococcal disease presents various clinical syndromes ranging from meningococcaemia to meningitis, fulminant septicaemia with septic shock and in severe cases lead to multiple organ failure and death (4). The management of fulminant meningococcal septicaemia still presents a great problem and mortality rates of up to 30% are common (5) recent advances in intensive care, the mortality for patients presenting with severe meningococcal sepsis remains between 20% and 50%.

Neisseria meningitides, which is a Gram-negative diplococcus and a strict human pathogen, was first identified in 1887, only infects humans; there is no animal reservoir. During the septicaemic phase, *N. meningitides* interacts with components of the host immune system. In particular, lipoteichoic acid released by the bacterium is a potent inducer of the inflammatory response. (6,7). Meningococcal infections (septicaemia and meningitis) are usually suspected on the basis of clinical signs such as fever, vomiting, neck stiffness, and skin rash (purpura). An etiologic diagnosis is usually made by the isolation of *Neisseria meningitides* from cerebrospinal fluid (CSF), blood, or other body fluids. However, this diagnosis is hindered by the failure to isolate bacteria following antimicrobial chemotherapy (8). Molecular interaction between host mucosal surfaces and membrane components of microbes is crucial in the infection process (9). Like other Gram-negative bacteria, *Neisseria meningitides* is surrounded by two cell membranes separated by an outer periplasm. The outer membrane of pathogenic *Neisseria* contains surface molecules such as PilC, and Opa and a monolayer of lipooligosaccharide (LOS), all of which are involved in interaction with host cells. About 50% of the lipid component of the outer leaflet of the outer membrane is lipooligosaccharide (LOS), a molecule related to lipopolysaccharide of other Gram-negative bacteria but lacking the long polysaccharide side-chains (9).

Most of the host inflammation observed in meningococcal sepsis is generally believed to be induced by lipooligosaccharide (LOS) of *N. meningitidis*, which is an endotoxin structurally similar to lipopolysaccharide (LPS) of enteric Gram negative bacteria (9). The term endotoxin is used to describe the toxic component of the LOS (10). Unlike most enteric LPSs, meningococcal LOS is an antigen and possesses relatively short polysaccharides, only two to five sugar residues, at the meningococcal LOS inner core. LOS is an amphipathic molecule that consists of a hydrophilic carbohydrate portion and a hydrophobic lipid A portion that anchors the LOS to the outer membrane. Endotoxic shock is mediated by the lipid A portion of LOS (9). The carbohydrate portion of the LOS is composed of an inner core which consists of two 3-deoxy-D-glucosamine residues (KDO) and heptose residues (7). There is abundant evidence that the presence of lipid A is important for many of the biological activities of endotoxin, but nevertheless a growing appreciation that endotoxin components other than lipid A also contribute to biological activity. In particular, recent evidence has indicated that the protein component of endotoxin (11). The outer membrane of the meningococcus produces endotoxin-rich vesicles called blebs (5). This occurs during bacterial growth, when the outer membrane separates from the peptidoglycan layer, allowing blebs to form and eventually to be released from the bacterial surface (13). During the course of infection, the growth and division of meningococci results in the release of outer membrane vesicles (OMVs), which disseminate lipooligosaccharide (LOS) throughout the circulation (12). The engagement of meningococcal LOS with the human Toll-like receptor 4 (TLR4) on human macrophages and other host cells is thought to trigger signalling events that ultimately result in the production of proinflammatory cytokines and chemokines. Meningococemia is predicted in large part to be a direct result of stimulation of TLR4 receptors on macrophages and other host cells by circulating meningococcal LOS (14). Over stimulation of this response can lead to meningococcal septic shock (shock), which is characterised by hypotension, organ failure and death (9). LOS has an important role in eliciting pro-inflammatory cytokines and therefore in virulence (15). Tumour Necrosis Factor alpha (TNF- α) and Interleukin 1 (IL-1) are major endogenous mediators of inflammation. Their interactions with target cells (including macrophages) increase their cytotoxic capacity and induce the release of other inflammatory cytokines (4). The morbidity and mortality of meningococcal bacteraemia has been directly correlated with circulating meningococcal LOS (14). LPS-binding protein (LBP) binds to LPS on the surface of gram-negative bacteria. The LPS-LBP complex may then be transferred to CD14 receptors located on the surface of monocytes/macrophages (16). Activation of the Toll receptor results in the stimulation of signalling molecules that are homologous to proteins involved in NF- κ B activation in macrophages (17).

Heat-shock proteins (HSPs) are evolutionarily highly conserved polypeptides with biological functions in protein biogenesis (18). They appear to be produced by prokaryotic and eukaryotic cells to preserve cellular functions under a variety of stress conditions, including heat shock, nutrient deprivation and viral and bacterial infections (19). These proteins are predominantly located in intracellular compartments but recent evidence suggests that some HSPs are also expressed on the cell surfaces and secreted extracellularly (20). Recently, it has been shown that *in vitro* exposure of human monocytes to some HSPs induces the release of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) (18), but little is known about the role of these proteins in the host protective response.

A subset of HSPs, known as the Hsp60/cpn60 family of chaperonins includes GroEL (21) and its homologues may be responsible for tissue injury in infected hosts by autoimmune responses. Antibodies and T cells specific for bacterial Hsp60 can recognize and cross-react with mammalian Hsp60 (23), this, in turn, can result in tissue damage as a result of inflammatory reactions. Many studies have suggested that mammalian and bacterial Hsp60 proteins may have immunomodulatory properties in that they are able to induce cytokine production and dendritic cell activation. Therefore, Hsp60 is reported to induce the production of cytokines such as TNF, IL-1 and IL-6 in monocytes, macrophages and dendritic cells in a manner similar to LPS (26). Recent studies have shown that Hsp60 could induce the production of tumor necrosis factor (TNF) by macrophages and monocytes in a manner similar to lipopolysaccharide (LPS) *e.g.* via CD14 and the Toll-like receptor 4-mediated signal transduction pathway (27). These effects are distinct from the immunomodulatory function of these protein molecules in that they do not require any HSP-associated proteolytic hydrolysis, cofactors, or a protein assembly complex (24). Some studies have reported that Hsp60 homologues of certain bacteria, including *Porphyromonas gingivalis*, have little cytokine-inducing activity (28). Therefore, different Hsp proteins from different species of bacteria may have different cytokine inducing properties (29).

Recent evidence suggests that the reported cytokine effects of HSPs may be due to contaminating LPS and LPS-associated molecules. The reasons for previous failure to identify the contaminant(s) as being responsible for the reported HSP cytokine effects include failure to use highly purified, low-LPS preparations of HSPs; failure to recognize the heat sensitivity of HSPs; failure to consider contaminant(s) other than LPS (24). In low concentrations, LPS is a potent known alarm to the immune system. At higher concentrations, LPS induces an inflammatory response syndrome and the hallmarks of septic shock. In addition, both Hsp60 and Hsp90 have been suggested to be bound to LPS (30). Contamination of experimental preparations with LPS could explain the apparent cytokine-inducing effects of HSPs. It is therefore, likely that HSPs have no cytokine inducing effects (31). Studies reported that LPS-free

when prepared experimentally, retained their normal function as a chaperonins, but lose the ability to induce cytokines. This may not be due to a conformational alteration to the protein when prepared experimentally. (24). The question therefore remains: do HSPs, and in particular, GroEL, have the ability to induce cytokine production? More specifically, does meningococcal GroEL have a direct effect on the immune system which may be important for meningococcal pathogenesis?

The main aim of this project is to purify the recombinant GroEL protein in the absence of LOS and study its effect on cytokine production in human cells. By cloning a histidine-tagged *groEL* gene into a neisserial ectopic expression vector and inserting it into a wild type of *Neisseria meningitidis* (MC58) and a LOS-deficient *Neisseria meningitidis* mutant it will be possible to purify recombinant GroEL expressed in the presence and absence of LOS. This will facilitate a direct comparison of LOS-free meningococcal GroEL with similar preparations which are potentially contaminated with native LOS in assays designed to measure induction of cytokines in human cells.

MATERIALS AND METHODS

Bacterial strains, Media and Growth Conditions :

E. coli strain DH5 α has been used as a host for all recombinant plasmids and in all transfections. *Neisseria meningitidis* strain MC58 is a wild type serogroup B isolate that has been completely sequenced (32), KG115 is a derivative of strain MC58 in which an ectopic *groEL* gene encoding an N-terminally 6-histidine tagged recombinant protein has been inserted and was used previously in the laboratory. H44/76pLAK is a derivative of another serogroup B isolate in which the *lpxA* gene has been disrupted (33). *E. coli* were grown on Luria-Bertani agar at 37°C, or in Luria-Bertani broth (LBB; Fisher Bioreagents), with agitation at 37°C. *Neisseria meningitidis* were grown on Brain-Heart Infusion agar (BHIA) supplemented with Vitox or in BHI broth supplemented with Vitox, at 37°C in an atmosphere of 5% CO₂.

Comment [UoN 1]: Insert appropriate reference here (i.e. Tettelin et al. 2000 Science 287:1809-15.)

Comment [UoN 2]: Insert reference here (Steeghs et al., 1998. Nature 392:449-50)

Antibiotics:

All antibiotics were obtained from Sigma. Ampicillin was added to media at a concentration of 100 μ g/ml for selection of clones containing the pGEM plasmid or its derivatives. Erythromycin was added to media at a concentration of 200 μ g/ml for the selection of *E. coli* clones and 10 μ g/ml for the selection of *Neisseria meningitidis* clones containing plasmid pYHS25 or its derivatives. Kanamycin was used in a concentration of 100 μ g/ml for the selection of the *N. meningitidis* clones containing the *lpxA* gene interrupted with kanamycin resistant gene.

Bacterial Transformation:

Competent *E. coli* DH5 α cells (Invitrogen) were transformed by incubation with the bacterial plasmid DNA on ice for 30 minutes, followed by 45 seconds incubation at 42°C and then returned to ice for 2 minutes. 250 μ l of SOC medium (Invitrogen) was added, incubated at 37°C for one hour and then plated out on LB agar overnight. *N. meningitidis* strains MC58 and KG115 were transformed by natural transformation as described previously (34). Briefly, *N. meningitidis* were grown in BHIB to mid-logarithmic phase, 200 μ l of the culture was added to 1.5 ml of LB broth supplemented with Vitox in 50 ml centrifuge tubes (CELLSTAR), incubated for 2 hours for pilus biosynthesis (35). 12 μ l of DNA was added and the culture was incubated overnight. Cells were then harvested and plated on BHIA plates supplemented with Vitox and kanamycin. Cells were incubated for 48 hours in 5% CO₂.

Comment [UoN 3]: Add appropriate reference (e.g. Hadi et al., 2000. Mol. Microbiol. 41:611-23)

PCR Reactions:

A C-terminally 6-histidine tagged *groEL* gene was amplified using MC58 genomic DNA as a template in PCR reactions in which the primers GroEL-F2 and GroEL-R2 were used. The sequences of these primers are shown in table 1. The PCR reaction mixture contained 100 μ l of

DNA, 0.2 μ M of each primer, 200 μ M each of dATP, dCTP, dGTP and dTTP, 10 \times Expa 3.5U Expand DNA polymerase (Roche) in a final volume of 25 μ l. The initial incubation for 3 minutes, followed by 30 cycles of incubation at 50°C for 1 minute, 68°C for 2 minutes for 45 seconds, and a final cycle at 50°C for 1 minute followed by incubation at 68 minutes. The genomic DNA from both MC58 and H44/76pLAK *N. meningitidis* was purified using a DNEasy tissue kit (Qiagen) according to the manufacturer protocol. The *lpxA* mutant was amplified by PCR reaction using *lpxA*-F and *lpxA*-R primers (from MWG- Biotech). The sequences of the primers are shown in table 1.

Comment [UoN 4]: Could you put all PCR reactions together in one section? You need to provide full details of the PCR amplification strategy. Also plasmid and chromosomal DNA preparation could also go together.

DNA Manipulation:

DNA manipulation techniques including digestion with restriction enzymes, DNA ligase extraction of DNA fragments from the agarose gel were done according to the standard protocol (36). The amplified fragment was cleaned up using a PCR purification kit (Qiagen) according to the manufacturer's instructions, then cloned into the pGEM-T Easy vector (Invitrogen) according to the manufacturer's instructions. The cloned insert was excised by digestion with the enzymes *MluI* and *BamHI* (Invitrogen). The 1.6 kb cloned fragment containing the C-terminally tagged *groEL* gene was separated by agarose gel electrophoresis and purified from the QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. DNA concentration in all preparations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies). The extracted and purified C-terminally 6hisitidine tagged *groEL* DNA was ligated into the Neisserial ectopic vector pYHS23 which has previously been digested with the same restriction enzymes. The ligation reaction was used to transform *E. coli* (DH5 α), which was plated on LB supplemented with erythromycin for selection of the plasmid DNA.

Comment [UoN 5]: This should be a new section as it is no longer part of "Bacterial Strains and Growth Conditions"

Comment [UoN 6]: You need to describe this plasmid somewhere. Unfortunately there is no good description in the literature so briefly describe it yourself, indicate that it was a gift from Chris Tang of Imperial college and reference the paper Winzer et al. 2000. Infect Immun. 70:2245-8. Make it clear that it is a suicide vector in Neisseria, which is designed for targeted integration into the chromosome.

Name of primer	DNA sequence
GroEL-F2	5' ACG CGT TGG AGA GCA GCA AAA G 3'
GroEL-R2	5' GGA TCC TAA TGG TGA TGG TGA TGG TGC ATC ATC CCG CCC ATA CC 3'
<i>lpxA</i> -F	5' CCG CCA CCT ACA CCC TG 3'
<i>lpxA</i> -R	5' CAA TGC TGC CGA CTG CC 3'

Table 1: Primers used in PCR reactions.

Protein Isolation, Purification and Immunoblotting:

Bacterial cells from overnight broth cultures of *N. meningitides* strains MC58 and KC harvested by centrifugation at 4000 rpm. The supernatants were cleared of remaining cell filtration using membrane filters (Minisart 0.20µm; Sartorius), then concentrated by osmosis using Vivaspin 20 tubes (Vivasciences) according to the manufacturer's instructions. Bacterial cells from the pellets were lysed by sonication (Soniprep150). The N-terminally tagged GroEL protein was purified by immobilised metal ion affinity chromatography using Ni²⁺-NTA spin columns (Qiagen) using protocols based on the manufacturer's instructions. The procedure was optimised by using a range of different pH values for washing at different buffers.

SDS-PAGE was used for protein separation, 15 µl/lane of each of the washed and eluted samples (from bacterial lysates and cell free supernatant) were loaded onto 10% acrylamide PAGE gels. 10 µl of pre-stained broad range protein marker (from New England Biolabs) was added to a single lane of each gel. Two identical gels were run separately at the same time for each set of protein samples. One of the gels was stained with SimplyBlue SafeStain (BioRad) according to the manufacturer's instructions. Proteins in the other gel were transferred to a nitrocellulose membrane using a Trans-Blot Semi-Dry transfer cell (BioRad) according to the manufacturer's instructions. Membranes were blocked by incubating the membranes in blocking solution (PBS containing 5% dried skimmed milk) overnight and probed with mouse anti-GroEL monoclonal antibodies (Qiagen) diluted 1:2000 in blocking solution for 2 hours at room temperature with gentle agitation. Membranes were then rinsed and washed twice for 5 minutes with PBS containing 0.1% Tween 20 (Sigma), incubated with GAM-HRP (BioRad) diluted 1:1000 in blocking solution for 2 hours at room temperature with gentle agitation, then washed followed by a final wash in 10mM Tris-HCl (pH 7.5). N-terminally 6-his tagged immunoprecipitated GroEL protein was detected using an Enhanced Chemiluminescence ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

Expression and purification of N-terminally 6-his tagged GroEL.

Comment [UoN 7]: Need to describe using IMAC.

To confirm the expression of an N-terminally 6-his tagged GroEL in *N. meningitides* strain and to attempt to purify the tagged protein cell lysates were prepared from overnight culture strain and its wild type parent strain MC58. Immobilised metal affinity chromatography was used to purify histidine-tagged proteins. Cell lysates, as well as fractions collected during IMAC procedure, were examined by SDS-PAGE and immunoblotting using anti-penta-histidine antibodies to probe for histidine-tagged proteins. The N-terminally 6-his tagged GroEL was detected in significant amounts in the KG115 bacterial lysate but not in the MC58 lysate (Fig. 1).

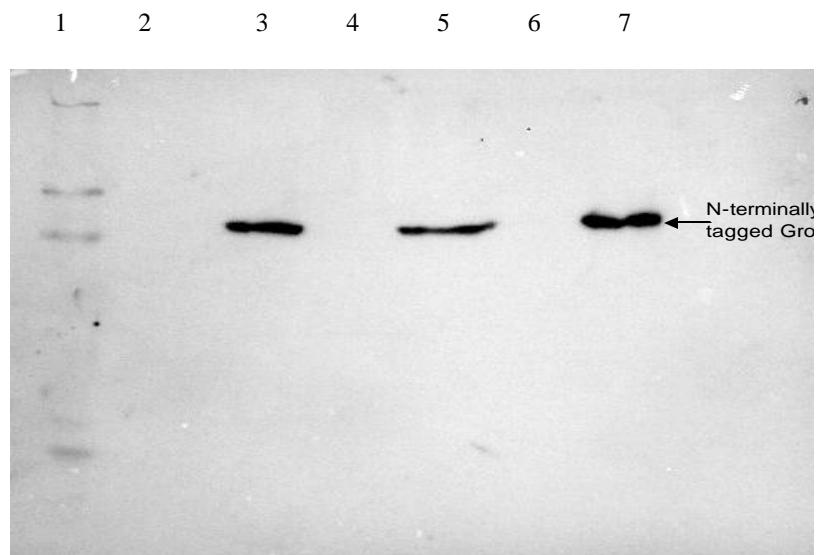


Fig.1. Western blot of the KG115 and MC58 *N. meningitides* cell lysates and fractions during purification. N-terminally 6-his tagged GroEL in KG115 preparations was detected using mouse anti-penta-histidine. Cell lysate, column flow through and elutes of strain KG115 are in lanes 3, 5 and 7, respectively. Similar preparations of strain MC58 are in lanes 2, 4 and 6, respectively. Lane 1 contains pre-stained protein mixture ladder (175-6 kDa).

Cell-free concentrated supernatant fractions of cultures of wild type *N. meningitidis* shown previously to contain large amounts of GroEL (A. Javed, personal communication). To determine whether the recombinant protein containing an N-terminal 6histidine tag exported from the cell during culture a concentrated, cell free supernatant from an overnight culture of strain KG115 *N. meningitidis* was processed using IMAC and fractions were analysed by SDS-PAGE and immunoblotting along side the bacterial lysate as a positive control. Histidine-tagged protein could be detected in the whole cell lysate but no tagged GroEL was detected in the supernatant fraction (Fig.2).

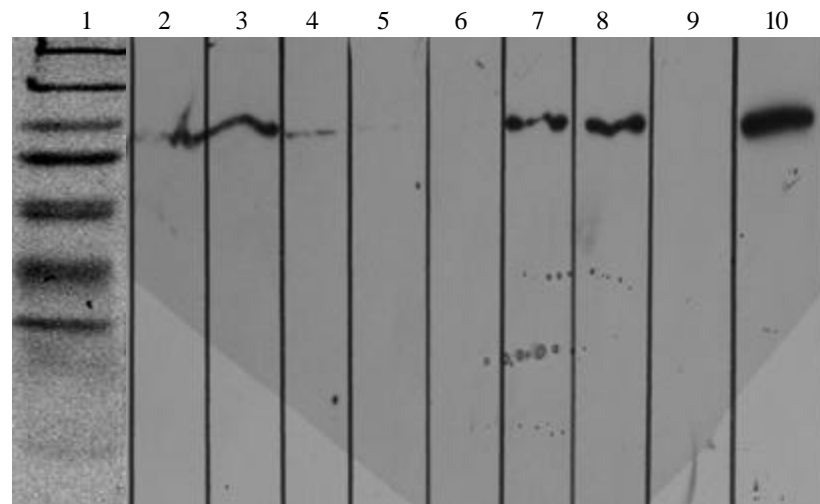
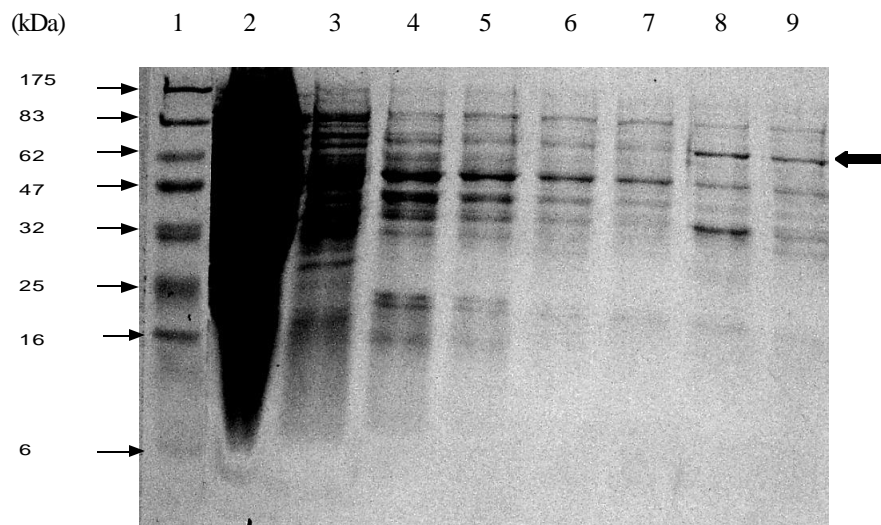


Fig.2. Western blots of cell lysate and cell-free supernatant fractions of KG115 *N. meningitidis*. Histidine tagged protein could be detected in whole cell lysate (lane 2), flow through (lane 3) with pH 5 buffer (lane4) and elute with pH 4.5 buffer (lanes 7,8). No histidine-tagged protein could be detected in the secreted protein preparation (lane9). Lane 10 contains a previously purified histidine-tagged GroEL protein purified from a whole cell lysate added as a positive control (band). Lane 1 containing a pre-stained protein mixture (175-6 kDa) ladder.

Comment [UoN 8]: I can't see any pre-stained proteins in lane 1 (as it is an ECL image). Can you put the positions of these proteins on the image (using bars)?

Optimisation of IMAC for the purification of N-terminally 6-his tagged GroEL from *N. meningitides* cell lysate

The recombinant 6-histidine-tagged GroEL protein expressed in strain KG115 was retained on a nickel column when the standard method recommended by the manufacturer of the purification was used but it was not pure and the eluted fraction containing the tagged protein also contained many other proteins (Fig. 3). In an attempt to optimise the protocol to facilitate purification, protein cell lysates were passed through a Ni²⁺-NTA spin column, which was then washed with a range of different pH buffers, and proteins eluted at each pH were analysed by SDS-PAGE. The tagged protein was detected in several of the fractions by ECL (Fig. 4).



Comment [UoN 9]: Where is the corresponding immunoblot?
Could you recrop this image to remove the stacking gel and gel plate labels?

Fig.3. SDS-PAGE analysis of KG115 *N. meningitides* bacterial lysates, lane 1 contains protein markers. Proteins were eluted in buffers at pH 6.5, 5.5, 5.0 (lanes 3, 5, 7 respectively) and two final elutions were at pH 4.5 (lanes 8 and 9). The tagged GroEL (black arrow) in each fraction is contaminated with many other proteins.

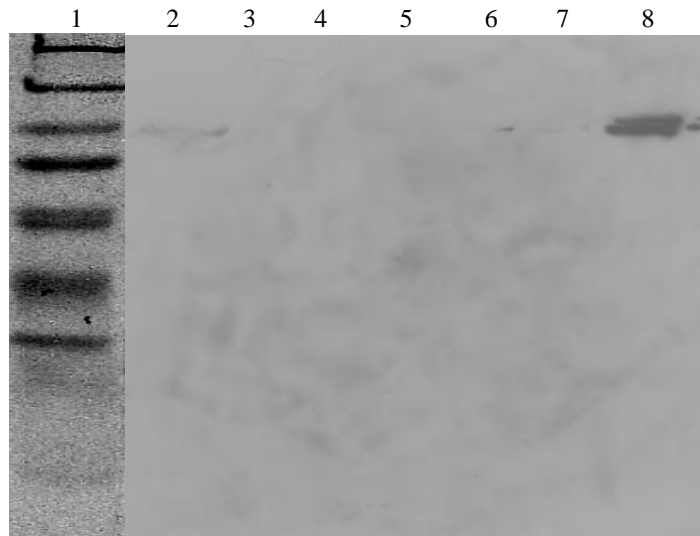


Fig.4. Western blot of the KG115 *N. meningitides* bacterial lysate indicating the presence GroEL (dark bands). Lane 1 is corresponding to the lane SDS-PAGE of the same pre (Fig3) which was containing Pre-stained protein markers 175-6 kDa) . Proteins were buffers at pH 6.5, 5.5, 5.0 (lanes 3 5, 7 respectively). The two final elutions were : containing the tagged GroEL, (darck bands), (lanes 8 and 9).

Cloning of a C-terminally 6-histidine tagged *groEL* gene of *Neisseria meningitides*.

To amplify a C-terminally 6-histidine-tagged *groEL* from *N. meningitides*, the primers, C incorporating MluI site, and GroEL-R2, incorporating *BamHI* site and nucleotides encr terminal 6 histiding extension, were used. PCR amplification of *N. meningitides* str: genomic DNA with these primers yielded a fragment of the expected size of 1.7 kb (Fig5

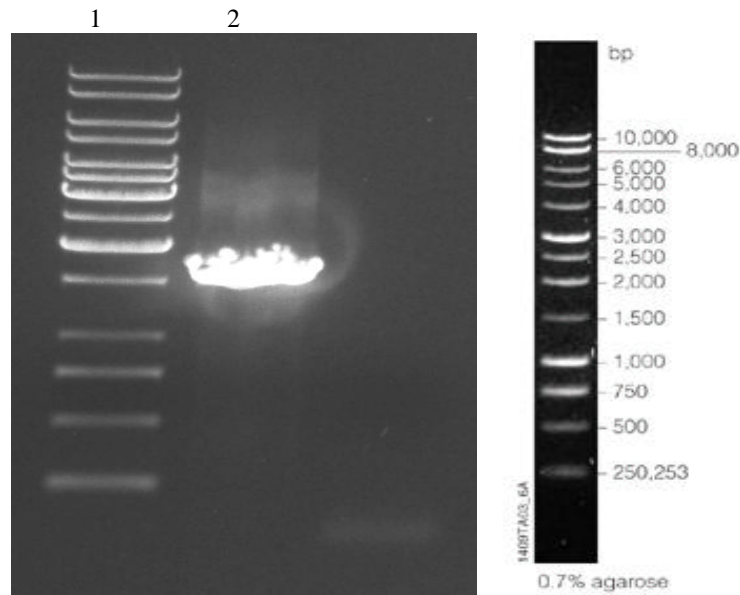


Fig.5. Agarose gel electrophoresis of the PCR amplification product of *N. meningitidis* MC58 genomic DNA amplified with GroEL-F2 and GroEL-R2 (lane 2). Lane 1 contains DNA ladder (Promega). Sizes of the individual ladder bands are indicated in the inset image.

The PCR-amplified histidine-tagged *groEL* gene was purified and ligated to pGEM-T Easy as served as an intermediate cloning vector (Promega). The ligation reaction was used to transform *E. coli* DH5a, resulting in a number of colonies on LB agar supplemented with ampicillin. To confirm the presence of C-terminally 6-his tagged *groEL* in the constructed plasmid, plasmids were isolated from the transformed DH5a *E. coli* clones after growth in broth culture, digested with *Bam*HI and *Mlu*I restriction enzymes and analysed by agarose gel electrophoresis. The gel showed the presence of two DNA fragments: the larger one, of about 3 kb, was the expected pGEM-T Easy vector, the other one, of about 1.7 kb, corresponded to the C-terminally tagged *groEL* gene (Fig6).

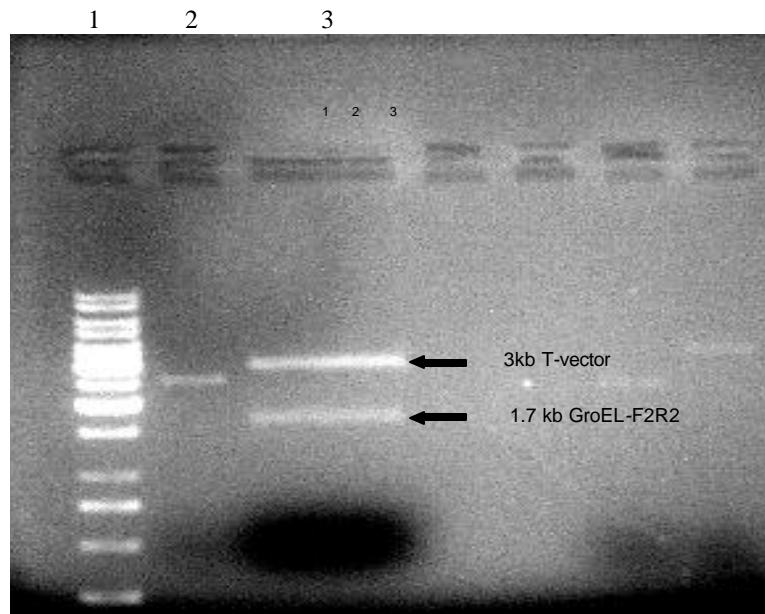


Fig.6. Agarose gel electrophoresis of the putative 6histidine-tagged groEL gene clone pGEM-T Easy digested with *Bam*HI and *Mlu*I (lane 2). Lane 1 contains a 1kb DNA ladder (Promega). Lane 2 contains undigested plasmid.

Sub-cloning of the GroEL-F2R2 gene in to Neisseria ectopic expression vector pYHS

In order to introduce the tagged groEL gene into the *N. meningitidis* chromosome it was to first clone it into a targeting vector. Plasmid pYHS25, (a gift from Chris Tang of college), is a neisseria ectopic vector, which does not replicate in meningococcal cells, but flanks two genes, which are in a head-to-head orientation in meningococcal genome. In the vector, two genes flank a strong promoter, responsible for the driving of the expression of the cloned gene. erythromycin resistance gene to facilitate selection of the successfully transformed meningococci. Transformation of meningococci with this vector results in insertion of the cloned gene into the meningococcal chromosome at an ectopic site that does not affect the transcription of meningococcal genes (37). The relevant features of plasmid pYHS25 are depicted in Figure

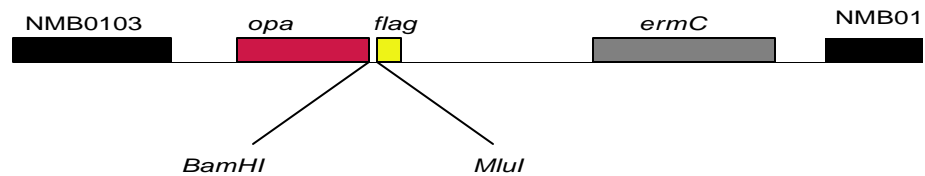


Fig.7. The structure of *Neisseria* ectopic expression vector pYHS25. NMB0102 and NMB01 normally found in a head-to-head configuration in the meningococcal chromosome and undergo homologous recombination after DNA uptake by natural transformation. The *opa* promoter controls expression of genes cloned using the enzyme sites *Bam*HI and *Mlu*I. The *ermC* gene confers resistance to erythromycin.

The C-terminally-tagged *groEL* gene was released from the intermediate vector by digestion with *Bam*HI and *Mlu*I and ligated to plasmid pYHS25 digested with the same enzymes. The ligation reaction was used to transform *E. coli* strain DH5a, which were plated on erythromycin supplemented LB agar. Despite repeated attempts to subclone the tagged gene, no erythromycin resistant colonies were observed.

In order to troubleshoot the transformation reaction a previously constructed plasmid containing an N-terminally histidine tagged *groEL* gene in the same vector was used to transform a sample of competent cells. This transformation resulted in many erythromycin resistant colonies, indicating that the transformation reaction itself was not the reason for the failure of transformation with the ligation products. To determine if the ligation reaction itself was the cause, samples of the ligation mixture before adding the T4-DNA ligase after addition of the ligase and overnight ligation were compared by agarose gel electrophoresis. This experiment showed the presence of multiple fragments of DNA of different sizes, indicating that the ligation of the tagged *groEL* gene to the digested pYHS25 vector was successful (Fig.8).

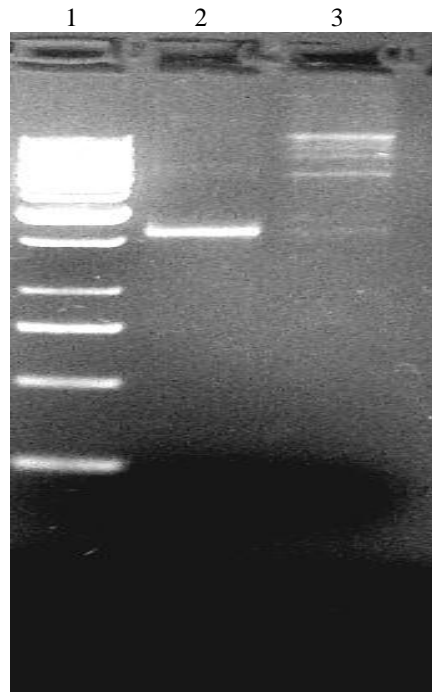
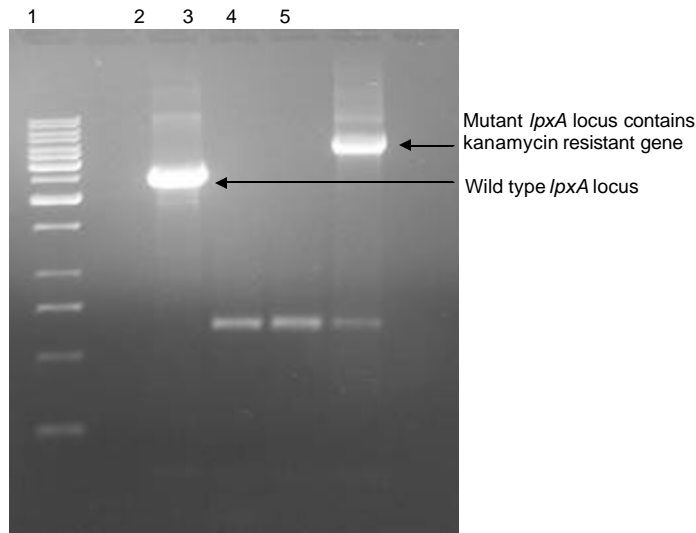


Fig.8. Agarose gel electrophoresis of the GroEL-F2R2 fragment and pYHS25 ectopic vector digested with *Bam*HI and *Mlu*I, before and after ligation with T4 DNA ligase. The upper band represents the vector and the lower band represents the *groEL* insert (lane2). Ligation product is apparent after the ligation reaction (lane3). Lane 1 contains a 1kb DNA ladder (Prom

Mutagenesis of the *lpxA* gene in the N-terminally 6-histidine-tagged GroEL-expressing *N. meningitidis* strain KG115.

The *lpxA* locus of the *lpxA* mutant strain H44/76pLAK33 has been disrupted by insertion of a kanamycin resistance gene (33). The aim of this part of the project was to amplify the *lpxA* locus of this strain, including flanking DNA and neisserial DNA uptake sequences, to enable mutagenesis of the tagged GroEL-expressing strain KG115. To amplify the *lpxA* mutant locus, specific primers were used in the PCR reaction, *lpxA*-F (forward) and *lpxA*-R (reverse). The pLAK33 *N. meningitidis* genomic DNA has been used as a template for the amplification. *lpxA* not mutated (not mutated) has been amplified from MC58 *N. meningitidis* using the same primers to serve as a control. The PCR reaction resulted in a specific amplification when confirmed by agarose gel electrophoresis (Fig. 9).

Comment [UoN 10]: Insert reference
()



Comment [UoN 11]: I can't edit the annotation on your figure as I don't know what program you used to annotate it. You should change the top annotation to "mutant *lpxA* locus containing a kanamycin gene" and "wild type *lpxA* locus"

Fig.9. PCR-amplification of the mutant *lpxA* locus. mutant and *lpxA* normal gene, negative control (lane 2), MC58 *N. meningitidis* genomic DNA (lane 3), DNA template not added (lanes 4 and 5), *N. meningitidis* genomic DNA (lane 6). ; 1kb DNA ladder (lane 1)

The amplified *lpxA* mutant locus was used as a source of mutagenic DNA for natural transformation of *N. meningitidis* strain KG115 and its wild type parent strain MC58. The amplified wild type locus was used as a negative control. After selection with kanamycin, no clones of transformants were obtained. Due to time constraints the experiment has not been repeated but this will be repeated at a future date.

DISCUSSION

Heat shock proteins (HSPs), including the chaperones GroEL (also known as cpn60) and its homologues have been claimed to be “one of the most potent stimulators of the immune response” (38). It has been noted that the cpn60 molecules of some bacteria are potent immunogens, elicit high titres of serum antibodies (39), in addition, they have been shown to be activators of the innate immune system (40). A study demonstrated that among HSPs, cpn60 was able to induce the release of TNF- α , IL-1 α , IL-6 and sICAM-1 from keratinocytes. HSPs (including cpn60/GroEL)-induced cytokine effects appear to be mediated via the CD14-like receptor (both TLR2 and TLR4) complex, which is a known LPS receptor, signal transduction pathways leading to the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs), thus the reported GroEL homologue-induced effects on cytokine production are similar to those of LPS and bacterial lipoproteins. GroEL homologue preparations are often contaminated with LPS (42), and it has been known that purified natural and recombinant GroEL preparations frequently harbour other contaminating proteins/peptides that have been detected by the presence of tryptophan-fluorescing material in GroEL preparations, since the GroEL contains no tryptophan residues (28). Recent studies, in which HSPs preparations free of LPS were used, suggest that previously reported cytokine function of HSPs may be due to the contamination (42), and Wallin *et al* (40) suggested that hsp70 (another member of the HSP family) should behave as a direct activator of the innate immune system, it should be a poor inducer of an immune response on its own, however associated with other molecules capable of presenting cell activation. Investigators have attempted to exclude the possibility of contamination being responsible for the observed HSP cytokine effects. Some studies have shown the fact that LPS is heat resistant as a base in these investigations (43,44) and others have used HSP antibodies to attempt to neutralise the effects of HSPs (45,46).

Comment [UbN 12]: Can you clarify this sentence – I’m not sure what you are trying to say.

The major objective of this study was to rule out the effect of contaminating LPS on the cytokine-inducing effects of meningococcal GroEL. The strategy was to express an N-terminally and C-terminally histidine-tagged recombinant GroEL molecules from meningococcal cells, and from meningococcal cells unable to produce lipooligosaccharide, the meningococcal equivalent of the LPS molecules of other Gram-negative bacteria. Unlike enteric LPSs, meningococcal LOS lacks O-antigen and possesses relatively short polysaccharide chains, only two to five sugar residues, attached to the meningococcal LOS inner core. Lipooligosaccharide is a amphipathic molecule that consists of a hydrophilic carbohydrate portion and a hydrophobic portion that anchors the LOS to the outer membrane. The LpxA protein, is responsible for the transfer of the 0-linked 3-OH fatty acid to UDP-*N*-acetylglucosamine which is the first committed

lipid A biosynthesis, a part of the LPS molecule essential for bacterial growth. In some *E. coli* inhibition of this step by mutating the *lpxA* gene may be lethal (17), but *lpxA* *Neisseria meningitidis* is viable (46,47).

Comment [UoN 13]: Put the highlighted material further up and amend according to my earlier comment regarding LOS/LPS.

Comment [UoN 14]: Put some information in here on the nature of LOS (how it compares with LPS) and on how, uniquely, it has been possible to construct a mutant in *N. meningitidis* that is completely void of LOS/LPS – a mutation that was previously thought to be non-viable since it has never before been possible to construct a mutant gram negative bacterium that does not produce any LPS. Include appropriate references. – OK I just noticed that you deal with this further down but this would probably be the best place to introduce this.

The N-terminal 6-his tagged *groEL* gene has been constructed in a previous part of this s recombinant gene encoding a C-terminally 6-histagged GroEL was initially amplified by cloned into an intermediated vector: pGEM T Easy. During the PCR reaction, DNA polymerase adds a single deoxyadenosine in a template-independent fashion to the 3'-end of PCR-amplified fragments (48). The single 3'-T of the T-vector overhangs at the insertion site greatly improves the efficiency of ligation of Taq-amplified PCR products (37). Subcloning of C-terminally 6his tagged *groEL* into the Neisserial ectopic vector pYHS25 was unsuccessful. Successful ligation of the tagged *groEL* gene with ectopic vector was confirmed by agarose gel electrophoresis, but many attempts to subclone the construct into the ectopic vector failed. The reason for this is not known but it is possible that the C-terminally-tagged molecule is toxic to the *E. coli* host used for cloning. However, identical ectopic vectors have been used successfully to clone the N-terminally tagged GroEL gene.

Comment [UoN 15]: There is no need to invoke this reference as you only need to point to the N-terminally tagged *groEL* gene, which has been cloned using the identical vector in this laboratory.

Recent work in our laboratory, focusing on secreted meningococcal proteins (SMPs), found that these proteins are important in inducing host pro-inflammatory genes and they are the subject of an extensive investigation (49). One of the major proteins found in SMP preparations was found to be GroEL (A. Javed, personal communication). There are three major pathways for protein secretion in Gram-negative bacteria, the general secretory pathway (GSP), the ATP-binding cassette (ABC) pathway and the type III pathway (50), meningococcus is known to secrete protein

Comment [UoN 16]: Say something here about the known protein secretion pathways of Gram neg. bacterial (just a sentence or two), that the meningococcus is known to secrete proteins by Type I and Type V pathways (in addition to secretion of Type IV pilus by a Type II-like pathway). Indicate that GroEL does not have the characteristics of a protein that would likely be secreted by these, or any, of the known secretion pathways but that it has been reported to be secreted (or at least found extracellularly) by a number of pathogens.

I and Type IV pathways. It has been found that removal of LOS from secreted protein preparations by incubation with Polymyxin B agarose beads resulted in qualitative alterations in the preparation profile, boiling the Polymyxin B agarose beads in SDS-PAGE sample buffer for 5 minutes. These investigations showed that many proteins had bound specifically to the beads, one of which was GroEL (51). In this study the N-terminally 6-histidine-tagged recombinant GroEL protein was not detected in the concentrated supernatant of cultures of *N. meningitidis* strain KG11. This finding suggests that the N-terminally tagged GroEL is not secreted extra-cellularly. In the GSP pathway, the protein, via its N-terminal signal sequence, enters the Sec machinery to cross the inner membrane and reach the periplasmic compartment (52). Although GroEL does not have a classical N-terminal signal peptide, it is possible that the terminal tag interferes with the normal processing of wild type GroEL that leads to its secretion. The mechanism of such a hypothetical secretion pathway, however, is unknown. This finding, however, might ultimately lead to an understanding of the mechanism by which

Comment [UoN 17]: Add here something on why this finding was important – suggests that contaminating LOS might be bound to GroEL tightly enough for the protein to be removed along with LOS.

Comment [UoN 18]: Indicate that, while this suggests that the tag might be interfering with secretion (by whatever unknown mechanism) it is not possible to directly compare with secretion of the native protein as the antibody only recognises the tag. Furthermore, we do not have a mutant that does not express wildtype GroEL.

GroEL is apparently secreted. could. This could be further investigated by comparing the of both N-terminally and C-terminally tagged GroEL.

Purification of the 6-his tagged GroEL from the KG115 meningococcal background, de metal affinity chromatography, which is based on the affinity of histidine residues in a tag for certain metal ions immobilized on a resin column. Nitrilotriacetic acid (NTA) is of choice for immobilizing nickel ions for this purpose. This is a tetradentate chelating l forms a complex with four of six sites in the nickel coordination sphere. To opt purification and reduce the associated contaminant proteins, reducing the buffer pH, or a the imidazole (10-20mM) or β - mercaptoethanol (20mM) may be helpful (53), however the buffer pH, it was found that the tagged GroEL remained associated with significant qu contaminating proteins.

In order to express LOS-free meningococcal GroEL it will be necessary to express the rec proteins in a strain that does not express LOS. An *lpxA* mutant of *N. meningitides*, H44/76 available (33). However, this mutant is in a different background to the strain used for t studies so far and, furthermore, is resistant to transformation. It will, therefore, be d introduce recombinant genes into this mutant The *lpxA* gene in strain H44/76pLAK disrupting with a kanamycin resistance gene. The H44/76pLAK strain is resistant to transf which means that it was not possible to introduce the recombinant GroEL genes into thi For this we attempted to transform the N-terminally tagged *groEL* cloned *N. meningitid* with the *lpxA* mutant gene. For this purpose the mutant *lpxA* locus of strain H44/76p amplified from H44/76 pLAK chromosomal DNA using specific primers upstream and dc of the mutated locus. The amplified region contained flanking DNA to facilitate hor recombination and neisserial DNA uptake sequences to facilitate natural transformation. the first attempt to transform *N. meningitides* strain KG115 and its wild type parent str with the amplified gene was unsuccessful.

Comment [UoN 19]: Add a new section entitled "future directions" or similar

The next steps in the current work will be to attempt to clone the C-terminally 6histidi *groEL* gene into *N. meningitides* and to mutate the *lpxA* locus in *N. meningitides* strains c recombinant N- and C-terminal GroEL. Once both N- and C-terminally tagged GroEL-e strains are available it will be possible to further investigate the apparent secretion of Gro meningococcus, which could provide clues to the mechanism of the GroEL secretion ir cells. Transformation of both N-terminally and C-terminally his tagged *groEL* c *meningitides* with the *lpxA* mutant gene will be repeated and the expression of the tagg from both backgrounds will be examined. The next step will be to purify the tagged Gr both *lpxA* mutant and the wild type *N. meningitid es* backgrounds using meta chromatography. The two preparations then will compared for the cytokine product

Enzyme Linked Immunosorbant Assay (ELISA) (28) or the cytokine produced can be intracellularly using the Fluorescence Activated Cell Sorting (FACS) technique for the analysis (54). The effect of tagged GroEL preparations on TNF α , IL-1 α , IL-1 β and IL-6 will be investigated first, because it has been demonstrated in previous studies that homologues have the effect on the production of such cytokines (24, 28).

CONCLUSION

The work presented in this study, demonstrated that the N-terminally tagged GroEL expressed by *N. meningitidis* does not appear to be secreted into the extra cellular environment, unlike the native GroEL molecule, which is found in the extracellular compartment in large amounts. This result could make a base for further investigations about the mechanisms of GroEL secretion from bacterial cells.

Comparing the effect of GroEL from both *lpxA* mutant and wild type *N. meningitidis* based on enhancing the cytokine production, may give a clear answer on the exact role of the free GroEL homologues in activation of the innate immune system.

ACKNOWLEDGEMENTS

I would like to thank deeply my direct project supervisor, Dr. Karl Wooldridge, for his continuous support, encouragement and advice through the work, also for his time and critically evaluating and editing the final manuscript.

Special thanks to professor Ala'addin and all the members of the MBIG for their assistance in the work.

Thanks to Naumi Bullin, who has constructed a Neisseria clone used in this study.

Thanks to Afzal Javid, who provided us, through personal communications, some information about the meningococcal secreted proteins.

Deep thanks to all the staff of the division of Microbiology and Infectious Diseases.

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