UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine

Academic Unit of Clinical and Experimental Sciences.

Vaccine potential of Adhesin Complex Protein (ACP) from *Neisseria gonorrhoeae.*

by

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Thesis for the degree of doctor of Philosophy (PhD) Biomedical Sciences

December 2017.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Thesis for the degree of Doctor of Philosophy Biomedical Sciences

VACCINE POTENTIAL OF ADHESIN COMPLEX PROTEIN (ACP) FROM NEISSERIA GONORRHOEAE

Hannia Liliana Almonacid Mendoza

Sexual transmitted disease Gonorrhoea is caused by the organism *Neisseria gonorrhoeae*, infecting ~106 million cases annually. Currently, a lack of an effective vaccine against this pathogen and treatments using last generation of antibiotics are misleading due to emerging antibiotic-resistant superbugs. Recently has been described a small protein in a closed related bacteria, *N. meningitidis (Nm)*, termed as an Adhesin Complex Protein (ACP). *Nm*-ACP is an outer-membrane protein which is a conserved protein in commensal and pathogenic bacteria. Besides Nm-ACP is capable of induce cross-protective bactericidal antibodies. A homologue gene, *ng-acp* (NGO1981) from *Neisseria gonorrhoeae* strain P9-17 is highly conserved among gonococcal isolates reported until the date. The *ng-acp* gene product was cloned into pRSET-A and pET-22b cloning vector systems and expressed as a recombinant protein in *E. coli* BL21pLysS to be used in immunization trials in murine model using a range of adjuvants and delivery formulations. Raised mice serum demonstrated a great reactivity against recombinant rNg-ACP by ELISA and displayed cross-strain reactivity in gonococcal outer-membrane (OMV) and lysate preparations from *N. gonorrhoeae* strains P9-17 and FA1090 by western-blot. Antisera r-Ng-ACP showed high bactericidal properties against homologous and heterologous wild type strains compared to the knockout strains. Furthermore, Ng-ACP plays a role of association on different epithelial cells showing a reduction 75-50% by comparison the wild-type and knockout. Three–dimensional structure of rNg-ACP the overall fold resemble of the lysozyme inhibitors from *Salmonella typhimurium* (PliC family) and recently described *Neisseria meningitidis*.Taken all together, suggest that Ng-ACP from *N. gonorrhoeae* is a potential candidate to develop an anti-gonococcal vaccine.

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Acknowledgements

First and foremost, I would like to thank **Professor Myron Christodoulides** for providing me with guidance and support throughout these last four years. I wish to thank **Dr. Ivo Tews** and **Dr. Moritz Machelett** for providing technical support and expertise in elucidating the three-dimensional structure of the Ng-ACP antigen and for giving me the opportunity to develop my skills in crystal growth and X-ray diffraction technology.

I must thank **Annabel Skarda** and **Zoe Soo**, two medical students who provided the gonococcal variants and worked closely with me to determine the role of this antigen in association with epithelial cells.

I am really grateful to **Dr. Victoria Humbert** and **Dr. Mar Cendra** for providing technical support that improved my research skills and for also providing the best environment and friendship in the laboratory. I would also like to thank my friends outside of the laboratory, namely **Josh**, **Lisa**, **Dan**, **Dominique** and **Matt**, with whom I shared great times with during my PhD, including watching films, sharing food and having a good laugh together.

I would like to acknowledge the Colombian government, **Colciencias**, for giving me the opportunity to study abroad and to develop and improve my research and communication skills in the field of scientific research.

Finally, I am really grateful to **my family**, **my parents**, my lovely sister **Carolina** and all their love, and also my **English partner** and **his family** for giving me great support, affection and love, which helped me to feel really welcomed whilst I pursued my dreams of achieving this PhD in my adopted country.

Definitions and Abbreviations

APS	Amonium persulfate
BCA	Bicinchoninic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GUHCl	Guanidine hydrochloride
HEp-2 Cells	Human epidermoid carcinoma, larynx
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MPLA	Monophosphoryl lipid A (From *Salmonella enterica* serotype Minnesota)
Ni-NTA	Niquel(II)-nitriloacetic acid
Na-Doc OMV	Sodium deoxycholate extracted Outer-membrane vesicles
OMV	Outer-membrane vesicles
ORF	Open reading frame
PBS	Phosphate buffered saline solution
PCR	P:olymerase chain reaction
RNg-ACP	Recombinant gonococcal adhesion complex protein
SBA	Serum bactericidal activity
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyaceylamide gel electrophoresis
TBE buffer	Tris/borate/EDTA buffer
Tfp	Type IV pilus
ZW3-14	Zwitterion detergent 3-14

CHAPTER 1 INTRODUCTION

1.1 *Neisseria gonorrhoeae* (*Ng* gonococcus).

Neisseria gonorrhoeae (Ng, gonococcus) is a Gram-negative diplococcus (Kingdom Bacteria, Phylum Proteobacterium, Class β-Proteobacterium, Order *Neisseriales*, Family *Neisseriaceae* Genus *Neisseria*) and the causative agent of the sexually transmitted disease (STD) gonorrhea. Ng colonises mainly the genitourinary tract, showing a tropism for mucosal epithelial cells, in particular, the columnar epithelial cells of cervix in women and the urethra in men (Ghosh et al., 2004). The World Health Organization (WHO) estimates that there are approximately 106 million new cases of gonococcal infection worldwide each year (WHO), 2012).

Typical features of Ng infection in men include painful urination and urethral discharge, while 30-60% of women with Ng infection are asymptomatic. In women, symptomatic infection is characterised by vaginal and cervical discharge, a 'burning sensation' and itching, bleeding during or after intercourse and an odious discharge. However, interim bleeding and lower abdominal pain are considered the first symptoms of Ng infection in women (van Duynhoven, 1999). A notable difference in gonococcal infection between men and women is the time from which symptoms appear. In men, symptoms occur between two or five days, whereas in women symptoms typically occur after ten days.

If left unthreated, Ng can spread to other tissues, especially into the upper genitourinary tract to cause pelvic inflammatory disease (PID), which encompasses a spectrum of clinical presentations including inflammation of the endometrium (endometritis), the pelvic peritoneum (peritonitis) and the Fallopian tubes (salpingitis). If the infection ascends along the mucosa the gonococcal infection can eventually lead to infertility and ectopic pregnancy. PID is accompanied with pelvic pain, fever, chills and cervical motion tenderness. Disseminated gonococcal infection (DGI) occurs when Ng migrates from the sites of mucosal epithelium colonisation and invades the blood via an incompletely understood multi-step process. Uncontrolled bacterial growth in the bloodstream leads to bacteraemia, whilst systemic infection can occur in the joints, most commonly in the knees, elbows and wrists, leading to development of the dermatitis–arthritis syndrome. Ng can also

colonise and infect the pericardium membrane surrounding the heart, causing pericarditis (Morse, 1997). In addition, infection of the meninges leading to meningitis has also been reported (Mofredj et al., 2000). Finally, infected women can transmit Ng to new-born babies by migrating from the birth canal and infecting the conjunctival mucosa, which can lead to aggressive ophthalmia *neonatorum* and potentially irreversible eye damage (Ghosh et al., 2004).

1.2 Treatment and prevention of gonococcal infection

1.2.1 Current treatment recommendations from the Center for Disease Control and Prevention (CDC)

The current treatment for gonorrhea, as recommended by governmental organisations such as the WHO and Centers for Disease Control and Prevention (CDC), is to combine injectable cephalosporins (one of the latest generation of antibiotics) with an oral antibiotic such as ampicillin, or a sulphonamide, a tetracycline, a quinolone or an macrolide ((CDC), 2013, Tapsall et al., 2009). However, these regimens can result in multiple antibiotic resistance, resulting in gonorrhea infection being potentially untreatable ((WHO). 2011). The current gonorrhoea treatment guidelines suggests for uncomplicated cases of genital, rectal or pharyngeal gonorrhoea suggest combination therapy with an single intramuscular dose of 250 mg of ceftriaxone plus either orally single dose of 1 gram of azithromycin or doxycycline (Bignell et al., 2011, (CDC), 2010). The CDC has reported an increase in the minimum inhibitory concentration (MIC) for the third generation cephalosporins (ceftriaxone and cefixime) during 2006 to 2011, from 0.1% to 1.4% for cefixime and 0% to 0.4% for ceftriaxone. (Centers for Disease and Prevention, 2013, (WHO)). For this reason, there is an urgent need for the development of new antibiotics or new strategies to prevent Ng disease, such as vaccination.

1.2.2 Current treatment recommendations within the European Community.

European reports have outlined in great detail that the preferred treatment differs according to the patient's clinical presentation.The suggested treatment for uncomplicated gonorrhea infections is a single intramuscular (IM) dose of ceftriaxone (500 mg) along with a single oral dose of azithromycin (2 g). Alternative treatments consist of cefixime (400 mg oral dose) in combination

with azithromycin (2 g oral dose), when injectable antimicrobials are not available. A single IM dose of ceftriaxone (500 mg) is recommended when the patient is unable to take oral medication. Alternatively, IM spectromycin (2 g) as a single dose plus azithromycin (2 g) is particularly useful when the patient has penicillin anaphylaxis or cephalosporin allergy, or when *N. gonorrhoea* displays resistance towards broad–spectrum cephalosporins (Unemo and Nicholas, 2012).

1.3 Emerging gonococcal resistance to antibiotics

Over the past seventy years, many antibiotics have been used to treat gonorrhea. However, the bacterium has developed resistance mechanisms that have reduced the efficacy of antibiotics. Antibiotic resistant gonorrhea first appeared in Vietnam in 1967, then spread to the Philippines and finally to the USA in 1990 (Holmes et al., 1967, Rasnake et al., 2005, Schwarcz et al., 1990).Recently, an antibiotic resistant *N. gonorrhoeae* strain was reported on September 2015. An outbreak emerged with 11 cases of azithromycin resistance from the West Midlands and the south of England (BMJ, 2015). This resistance was attributed partly to the continual high rates of antibiotic use in hospitals, which led to increases in the numbers of resistant strains caused by mutation and selection pressure (Laxminarayan et al., 2013). Streptomycin and azithromycin function by interfering with protein synthesis by binding to bacterial ribosomes, but gonococci resistant to these antibiotics carry mutations in 16S and 23S ribosomal RNA genes (Galimand et al., 2000, Ilina et al., 2013, Unemo et al., 2013, Cousin et al., 2003, Douthwaite and Champney, 2001, Roberts et al., 1999). Fluoroquinolone antibiotics function by inhibiting the action of topoisomerase enzymes. Modification of the proteins encoded by the gonococcal genes *gyrA* and *parC*, which are involved in DNA replication, contributes towards Ng resistance to these antibiotics. Meanwhile, three main genes have been identified that result in reduced susceptibility of gonococci to β-lactam antibiotics: *penA*, *mtrR* and *penB* (Ameyama et al., 2002, Zhao et al., 2009). The gonococcal proteins penicillin–binding protein 1 (PBP1) and penicillin-binding protein 2 (PBP2) are involved in cell wall synthesis and especially the formation of peptidoglycan. PBP1 and PBP2 are encoded by the genes *ponA* and *penA*. Mutations in these genes lead to a reduction of the binding efficacy of penicillin.

Another main target for β-lactams and cephalosporins is the major outer membrane porin B (Por B) encoded by the *porB* gene, which was identified as an efflux pump for antimicrobials of various classes. Mutations in PorB have been reported to reduce its permeability and prevent cephalosporin antibiotics entering into the cell. Studies on the role of *penA* gene have been performed with a great variety of gonococcal isolates have revealed resistance against ceftriaxone, with amino acid alterations causing a decreased susceptibility towards cephalosporins, specially cefixime (Unemo and Shafer, 2011, Lindberg et al., 2007).

Another means of transporting antimicrobials out of the gonococcus is the MtrCDE efflux pump, which isencoded by the *mtrCDE* genes (Hagman et al., 1995, Shafer et al., 2001). This type of efflux pump enables export of a wide range of hydrophobic antimicrobials, such as macrolides, β-lactams, ciprofloxacin and tetracyclines. Mutation within the promoter sequence promotes over-expression of the pump proteins and raises the activity of efflux pump complex against antibiotics, in particular ceftriaxone. This phenomenon is crucial for gonococcal survival in experimental infection in the lower genital tract of female mice (Jerse et al., 2003), suggesting that this pump plays a key role in Ng virulence and is relevant for gonococcal fitness in the mouse model (Warner et al., 2007, Warner et al., 2008).Further genetic studies on PorB and MtrCDE have demonstrated that mutations within these proteins confers resistance to cephalosporins, such as ceftriaxone and cefixime (Zhao et al., 2009, Ameyama et al., 2002, Tomberg et al., 2010, Tomberg et al., 2013). The effectiveness of cephalosporins, such as cefixime and ceftriaxone, for treating gonorrhea has been significantly reduced to the extent that the CDC no longer recommends cefixime for treating gonorrhea.

1.4 The pathogenesis of *Neisseria gonorrhoea* (Ng) infection: Role of specific factors.

The interaction of Ng with human cells and tissues involves the interaction of components found within the outer membrane (OM), or the secretome of the organism and specific host receptors. The Ng OM is a phospholipid bilayer membrane containing membrane proteins, surface structures and lipooligosaccharide (LOS) (**Figure 1**). Several of these components function as virulence factors,

adhesins and invasins. Understanding the biological structure of the gonococcus and how it

interacts with the human host is essential for vaccine development.

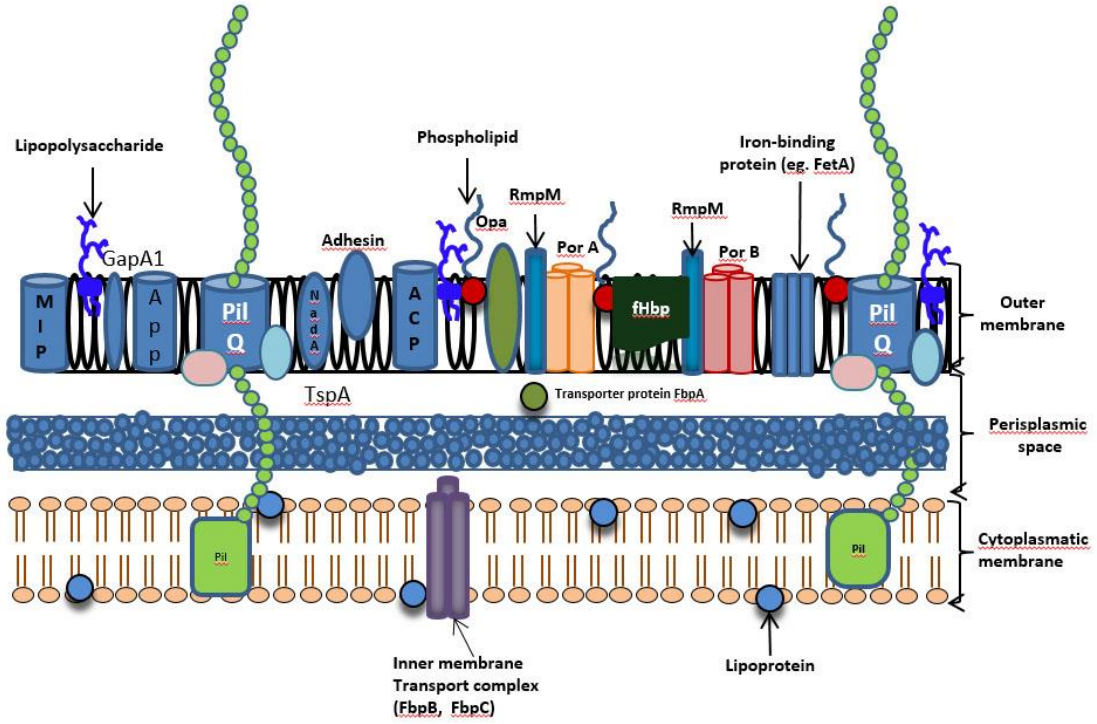


Figure 1 Scheme of *Neisserial* organization of the phospholipid bilayer membrane, outer-membrane (OM) and lipoligosaccharide protein (LOS). The cell wall is composed by two layers separated by perisplasmic membrane. The vast majority of major adhesins and minor adhesins are stablised on the outer-membrane. Major adhesins Pili, Opa, Por A and PorB which are in major composition of the outermembrane. PorB is solely present in *N. gonorrhoeae*. In addition, proteins involved in nutrient aquisition such as iron binding protein. Other molecules such as lipopolysaccharide, marcophage infector potentiator (MIP), and recently described minor adhesin such as Adhesin complex protein (ACP) present in *N. meningitidis* is located present in the outer-membrane.

1.4.1 Type IV Pilus (Tfp).

Type IV pilus (Tfp) mediate a diversity of biological functions and play a vital role in *Neisseria* virulence and host interactions (**Table 1**). Therefore, Tfp have been considered as a target for the development of component vaccines and therapies (Craig and Li, 2008).

Table 1. Main functions of Neisserial Type IV pili Tfp.

Functions of Neisseria Tfp	Reference
Adhesion to host cells	(Mattick, 2002, Virji et al., 1991, Merz and So, 2000, Marceau et al., 1995)
Bacterial motility and gliding	(Mattick, 2002) (O'Toole and Kolter, 1998)
Microcolony formation	(Kirn et al., 2000, Marceau et al., 1995, Pujol et al., 1997)
DNA uptake for natural transformation	(Aas et al., 2002, Rudel et al., 1995b)
Ca^{2+} signalling	Ayala et al., 2005
Protection against immune response? Secretion of proteases and colonization factors immune evasion. modulating host immune response Phage transduction and signal transduction.	(Kallstrom et al., 1997, Boslego et al., 1991)

1.4.1.1 Pilus structure

The type IV pilus is composed of 12 proteins, which are encoded within the same operon. These subunits, display a highly conserved N-methyl phenylalanine, an amino acid at position 32 at the N-terminus. This type of pilus is made of homopolymers of 15-20kDa subunits, and are found on the surfaces of many Gram-negative bacteria (Craig and Li, 2008). Some components that are used to assemble the type IV pilus have homologs in type II pilus and archeal flagellar systems (Craig and Li, 2008, Bardy et al., 2003, Johnson et al., 2006, Craig et al., 2004).

The sequence features of type IV pili are classified according to the N-terminal fragment, which is approximately 25 residues in length, a N-methylated N-terminal residue and a pair of cysteines at the C-terminal region (Strom and Lory, 1993). The structural architecture of the type IV pilin subunit at the N-terminal 54 amino acid residues form an extended α-helix (α1); the N-terminal half of this helix (a1-N), protrudes from the protein and the C-terminal half (a1-C), is embedded in a globular domain and interacts with an anti-parallel four-to five-stranded β-sheet (Hansen and Forest, 2006). A three–dimensional structural study by X-ray of Ng pili has provided insight into the mechanism of gonococcal invasion of epithelial cells (Craig et al., 2006). Pilin is composed of a three-helix spiral that provides flexibility and stability to the structure, as well as enabling interaction with DNA, which is associated with microcolony formation.

In *N. gonorrhoeae,* the Tfp 3-dimensional structure has a modification nearest a β-sheet sequence (Craig et al., 2006). In an X-ray study, at 2,3Å of resolution, unidentified post-transductional modifications were foundbetween Asp 60 and Ser 63 amino acid residues, which confers adherence

to epithelial cells, similar to the bacterial species, *N. meningitidis* (Parge et al., 1995, Virji et al., 1993). Ng expresses a type IV pilus (Parge et al., 1995), which plays a major role in the bacterium-host interaction. Pili are composed of different subunits that play a role in the assembly and disassembly of this organelle, as discussed below.

1.4.1.2. PilE subunit.

PilE is the most abundant pilus subunit, termed pilin, composed of polymerised subunits with a size of 18 kDa and comprised of 160 amino acids (Schoolnik et al., 1984). A high resolution crystallography study and a mass spectrometric analysis revealed a small glycan containing N-acetyl glucosamine (α1-3) GlcNAcN through O-glycosylation (Aas et al., 2007, Banerjee and Ghosh, 2003).

PilE undergoes antigenic variation, mediated by a silent gene PilS, in which antigenic variation of pilus expression, is caused by intra –intergenic non-reciprocal DNA recombination between *pilS* and the pilin gene (Haas and Meyer, 1986, Hagblom et al., 1985, Perry et al., 1988, Segal et al., 1986). Meningococcal and gonococcal pili undergo phase variation resulting in a reversible change between piliated and non-piliated bacteria. Several studies have suggested that pili is involved in receptor recognition (Nassif and So, 1995, Rothbard et al., 1985, Rudel et al., 1992, Virji et al., 1995a). Two binding specificities have been described: (i) binding to epithelial cells, which is dependent on PilC and (ii) binding to human erythrocytes, which suggesting that hemagglutinin is involved in receptor recognition (Rudel et al., 1992).

1.4.1.3. PilC subunit.

The second subunit is **PilC**, with a size of 110kDa and localised at the tip of the type IV pilus structure (Rudel et al., 1995c) within bacterial membrane. In addition, in *N. gonorrhoeae* this subunit is also located on the surface of pili (Rahman et al., 1997, Rudel et al., 1995b). PilC was identified to play a critical role of binding of *N. gonorrhoeae* on human epithelial cells (Rudel et al., 1995c). Purified recombinant PilC has demonstrated that this subunit mediates interaction mostly with epithelial and endothelial cells (Rudel et al., 1992).

There are two homologue variants of PilC (*pilC1* and *pilC2*) showing a role of pilus assembly in *N. gonorrhoeae* and *N. meningitidis*. *pilC1* and *pilC2* enable the adhesion to the host cells in *N. gonorrhoeae* whilst mutation in variant *pilC1* in *N. meningitidis* results in a non–adhesive, piliated transformation competent strain (Jonsson et al., 1994, Rudel et al., 1992, Rahman et al., 1997, Nassif et al., 1994). Furthermore, in *N. gonorrhoeae* strain MS11, PilC expresses small quantities encoding by two variant genes (Jonsson et al., 1991).

The PilC subunit is directly implicated in bacterial adhesion and colony association. PilC mutant did not shows any abnormality in the pilus assembly, but defects on the ability to adhere to human epithelial cells (Rudel et al., 1995a). *In vitro* assays demonstrated the presence of PilC is favoured to bind epithelial cells rather than agglutination on erythrocytes (Rothbard et al., 1985, Schoolnik et al., 1984). PilC pilus adherence on both cell types -erythrocytes or epithelial cells- PilE may influence binding specificity of the PilC receptor. However, the differential binding receptor of PilC may be driven by variant pilus proteins (Scheuerpflug et al., 1999).

The human cofactor protein (Membrane Cofactor Protein, CD46) has been reported as a cellular receptor for gonococcal and meningococcal pili. CD46 is a glycoprotein receptor which serves for several pathogens, it is presented on all nucleated cells and plays the role as an inactivator of complement factors C3b and C4b deposited on self tissue (Riley-Vargas et al., 2004). Phosphorilation on CD46 by Src family tyrosine kinase c-yes upon interaction with piliated *gonococci* suggesting binding of *N. gonorrhoeae* pili with CD46 is a dynamic process that might promote signalling in the host cell that could be important for bacterial virulence (Lee et al., 2002). The N-terminal fragment of PilC also binds to the human complement regulator C4B-binding protein (C4BP). CD46 and C4BP receptors display a competence binding to pili only at high concentrations, indicating that different areas of PilC are involved in these two interactions (Blom et al., 2001).

1.4.1.4. PilF and PilD subunits.

PilF and PilD subunits are related to fiber formation. **PilD** is a pre-pilin peptidase localised to the inner membrane from prepilin. The main role of this subunit is to cleave a short signal peptide from pre-pilin to N-methylate prior to assembly of pilin subunits into pili (Strom and Lory 1987,

Koomey 1991, Reeves 1994) with specific roles in outer membrane translocation. N-methylphenylalanine is the first amino acid in most bacteria expressing type IV pili, including *N. gonorrhoeae* (Fluhrer et al., 2009, Meyer et al., 1984, Strom et al., 1993).

Mutation on PilD demonstrated expression of unprocessed and degraded pilin, to express mature pili (Freitag et al., 1995). A double mutant with PilD/PilT did not show any difference with the strain lacking of PilD, in terms of phenotype. Furthermore, the double mutant strain did not display pili fibre or any growth defect by electron microscopy, with no alteration of degraded pili (Wolfgang et al., 2000).

PilF is a homologue protein of General secretion Pathway protein E (GspE, a type II secretion system protein from *E.coli*) and AAA chaperone/mechanic enzyme family member of cytoplasmatic protein composed of a concensus sequence nucleotide-binding proteins (Morand et al., 2004). Studies on PilF shown that it plays a role in PilE extrusion and helps with the assembling of PilE subunit from the inner membrane, mediated by hexameric ATPase (Craig et al., 2006). A comparison between (PilF/PilT) and PilF mutant, showed pili absence, but not defect in growth (Wolfgang et al., 2000).

1.4.1.5. PilQ subunit.

PilQ is a member of the GspD secretin super family and subunit of the type IV pili, type II and type III secretions systems. PilQ, which is associated with surface localization to gate the chanels which the macromolecules are translocated (Russel et al., 1997, Genin and Boucher, 1994). Transmission electron microscopy (TEM) of PilQ of different bacteria, e.g. *Pseudomonas aeruginosa* (Bitter et al., 1998), *Vibrio cholerae* (Reichow et al., 2010) and *N. meningitidis* (Collins et al., 2001) display a multimeric ring like-structure form that is proposed to act as gate channel through which macromolecules are translocated.

Structural arrangement of PilQ by TEM studies, displayed a double ring structure with 14fold-symmetry of the peripheral ring, demonstrating a multi–component membrane inserted complexes. A PilQ amino acid sequence alignment between *N. meningitidis* and *N. gonorrhoeae* showed 89% of identity, 91% similarity, suggesting that PilQ is highly conserved. A few structural

characteristics show a remarkable differences between these species; a TEM analysis of PilQ from *N. gonorrhoeae* show the symmetry of the inner ring it was not conclusively, likely 14, and was not comparable with the symmetry published in *N. meningitidis* (Collins et al., 2001). The peripheral ring from *N. meningitidis* was bigger than *N. gonorrhoeae*, suggesting the presence of an additional number of copies of the same or smaller protein.

To determine the role of PilT on PilQ, mutants lacking of PilQ or absence of both subunits PilT /PilQ, showed inability to form colonies of normal size and morphology. When expression is re-established, the morphology and appearance of single colonies is restored (Drake et al., 1997). In addition, the viability of the colonies was reduced significantly when both genes were suppressed demonstrating the ability of pili to form aggregates and radiate from cell surface to get the cells interconnected. To determine the influence of this subunit, the knockout of PilT/PilQ were made by recombinantion of pilQ transposon insertion into a strain that carries the pilT gene under the control by an inducible *lac* promoter/operator. The double mutation (PilT /PilQ) demonstrated the absence of diplococcal morphology, characterised by irregular shape and covered with membranous protutions, suggesting that PilQ is crucial as a biogenesis component when PilT expression is suppressed. Besides, mutation of PilT and PilQ showed the formation of prepilin but it is not processed (Jain et al., 2011).

The structure analysis of certain mutants to identify the role of other subunit like PilC, which contains two gene copies *pilC1*and *pilC2,* the lack of those genes present similar structure compared to the wild type strain, demonstrating PilC is not a subunit of the PilQ complex.

Another subunit has been identified known as NgonM_03101. This subunit is a lipoprotein with *Mr*.28kDa containing six tetratricopeptide repeat motifs and an homologue protein in *N. meningitidis* (PilW) and *Pseudomonas aeruginosa* (PilF). A deletion mutant NgonM_03101 has been demonstrated to play a stabilization role of PilQ oligomer generating absence of the PilQ structure. Similar results obtained from PilQ mutant suggesting that NgonM_03101 plays as a chaperone for oligomerisation and part of PilQ complex.

PilP is a lipoprotein of 18kDa that interacts with PilQ. Suppression of the *pilp*-gene shows a phenotype absent of piliation and natural competence (Balasingham et al., 2007). Analysis of the PilQ structures from deletion of *pilP* gene shows a reduction about 30% compared with the wild type suggesting that PilP has an effect of PilQ stabilization.

The evaluation of the changes on the structure of PilQ complex when are suppressed PilE, essential for pili formation , and PilF, crucial for assembly and extrusion of pilin subunits, showed a modified complex structure of PilQ, therefore changes of conformation of secreting complex structure affects the translocation of the pili.

1.4.1.6. PilT subunit.

PilT is an inner membrane-associated ATPase, having a consensus nucleotide –motif that is related with membrane translocation of macromolecular complexes. PilT is involved in pilus retraction and required for force–dependent pilus elongation, enabling the bacteria to modulate interactions with surfaces controlling tension on their pili. This subunit mediates twitching motility (Henrichsen, 1983, Swanson, 1978) and also provides high level competence for DNA transformation in *Neisseria.*

Studies to determine the role of PilT proposed that this subunit is responsible for conditional pili degradation: i) stalling subunit polymerization and extrusion from the inner membrane; ii) localization nascent pili fibres, such that the ends of the filaments are accessible to proteolysis and; iii) disassembly of fibres into subunits, which relocate to the inner membrane and are then degraded (Wolfgang et al., 2000).

Mutation of the *pilT* gene shows expression of prepilin, similar results demonstrated when the strain lack of PilT/PilQ. (Wolfgang et al., 2000) demonstrated that PilT is essential for in twitching motility in an intracellular pathway which stable fiber expression is antagonised to establish a key conditions for organelle retraction (Wolfgang et al., 2000). PilE subunit undegoes degradation, the first 39 amino acids of the PilE subunit are removed to release a mature protein, termed S-pilin. Evaluation of cleavage process by immunoblotting of mutant PilQ/PilT demonstrated that the

absence of pili degradation; in contrast, suppression of PilQ or PilQ/PilU the proteolysis was not perturbed.

A study in *N. gonorrhoeae* has demonstrated that the retraction property mediated by PilT is essential to this process (Merz and So, 2000). The loss of function of PilT by mutation in *pilT* gene improved the biogenesis requirement for PilC, showing the antagonist function in the biogenesis of the organelle expression (Wolfgang et al., 1998a).

The result of PilT lead the expression of pili fibres actings as antagonist and the data showed that the biogenesis comprises three dissociable steps: fiber formation, fiber stabilization and surface localization of the intact organelle. PilT is essential for degradation of pilin subunit in PilQ biogenesis, the presence of fibres but a degraded form (Wolfgang et al., 1998b).

The organelle biogenesis can be described in two main steps: formation of the fiber and translocation to the cell surface (Wolfgang et al., 2000). The twitching motility factor PilT is responsible for the absence of pili fibers and acts as antagonist of stable fiber formation.

When the colonization is mediated by pili to the host cell the bacteria form microcolonies inducing cortical plaques, which are enriched in cortical cytoskeleton and integral membrane proteins. The formation of these components depends not only on the presence of pilus fibre and PilC but also the expression PilT (Wolfgang et al., 1998a).

The pilin subunit is an inner membrane prepilin peptidase which claves the N-terminal of signal peptide, PilD. An ATPase assembly promote the pilus polymerization and the pili recruits the ATPase from the cytoplasm and an outer membrane secretin, known as PilT. PilT inner membrane ATPase associated with retraction of pilus (Merz and So, 2000). Studies lacking of pilin subunit such as pilQ/PilC influence fiber formation by PilT but not seen on mutants with PilD /PilF, suggesting that PilQ and PilC are essential for PilT function.

1.4.1.7. Pil X subunit.

PilX is an 18 kDa pilin-like protein that plays crucial roles in pilus biology. It has been demonstrated that PilX is not required for pilus assembly, but PilX is colocalised with the fiber and

is necessary for aggregate formation and adhesion to the host cells without affecting PilC (Helaine et al., 2005).

PilX is low abundance located on the surface and maintains the aggregation of the other subunits against the retraction (Helaine et al., 2005). Furthermore, other studies encode a pili protein-like, which co-purify with other pili protein with aggregation properties but not adherence (Helaine et al., 2005).

Functional analysis of PilX, has identified that this subunit is relevant for adherence PilX. APilX mutant displays an unaltered fibers and selective loss of Tfp linked properties. The mutant has competent and motile properties but unable to establish interbacterial interactions, being inter-bacterial adhesion fundamental to facilitate adhesion to human cells (Helaine et al., 2005).

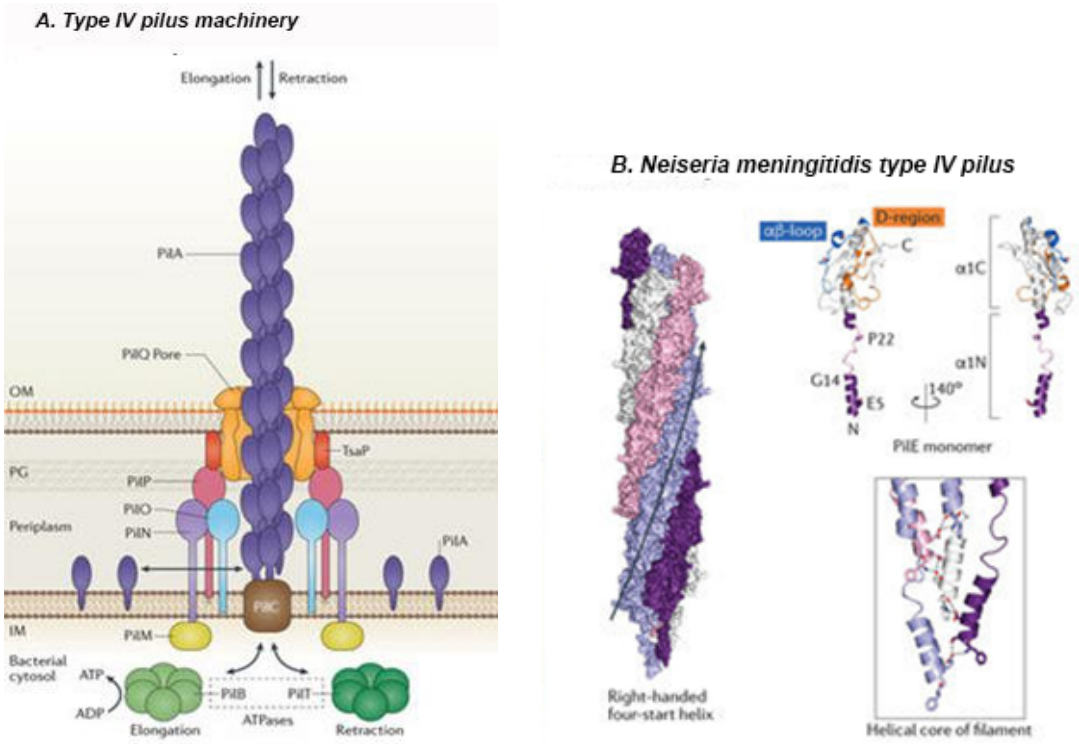


Figure 2. Schematic arrangement of pili type IV and pilus subunits and structure of pili in ***N. meningitidis.*** **A)** Pilus Subunits composing type IV pili machinery.Type iv pilus machinery is composed by outermmebrane secreting subcomplex (PilQ, TsaP and PilF (not shown)), the alignment subcomplex (PiM-PilN-PilO-PilP) and inner membrane submotr complex (PilC-PilB-PilT). ATP hydrolysis of PilT produces energy for piluspilus elongation and retraction (twitching motility), involving rotation of PilC. **B)** A detailed structure of PilE from *N. meningitidis.* Top Right panel ribbon diagraman of PilE monomer. A disulphide bond is represented in orange, αβ region (blue), relevenant for pilus interactions. Residues G14 and P22 are relevant for loss of helical orderat N-terminus. E5 residue is involved for pilus assembly and core stabilization. α1C C-terminus portion of α-helix 1. α1N N-terminal fragment of α-helix 1. Zoom in region helical regions, responsable for stabilization of core pilus. Representation in dotted lines are hydrogen bonds between neighbouring pilins. Images taken from (Hospenthal et al., 2017).

1.4.2 Opacity proteins.

Ng expresses two opacity proteins that are involved in bacterium-host interactions, namely Opa and Opc. There are abundant compared with other antigens binding to cellular receptors; similarly, the cooperative actions of those proteins potentiate the attachment. Opa is major outer-membrane proteins expressed in most of meningocci and gonococci species having similar structure in both species. Opc is exclusively present in *N. meningitidis* (Pujol et al., 2000).

Opa proteins are responsible for the opaque phenotype of the bacterial colony. Opa is expressed in all pathogenic *Neisseria spp.* and some commensal Neisserial strains. However, the differential amount of genes encoding Opa protein between pathogenic *Neisseria* species are significant being

for meningococcal bacteria three or four genes (Aho et al., 1991) whilst for a gonococcal strain 11 to twelve genes (Bhat et al., 1991, Connell et al., 1990).

Three-dimensional structure of Opa is a β-barrel comprised of 8 anti-parallel β- strands embedded into the cell memebrane with four surfaces –exposed loops with a molecular weight of 27-31 kDa (Bhat et al., 1991, Malorny et al., 1998).

Opa expression is variable, due to phase and antigenic variations mediated by intra- or inter-genomic recombination. This phenomenon is a consequence of hyper-variability in two loops, loop 2 and 3, an hyper-variable region, containing HV1 and HV2 regions, loop 1 a semi variable at N-terminal region, and a conserved loop 4 (Hobbs et al., 1998).Those regions have been identified as possible Opa receptor on human cells (Bos et al., 2002, Grant et al., 1999, Virji et al., 1999).

The great diversity of variability loops provide more versatility to binding to cell receptors, analysis of gonococcal Opa variation was estimated 77% diversity, due to recombination within the same isolate, 16% changes for imported genes from other isolates, 7% novo mutation (Bilek et al., 2009).

Phase variation is driven by the variable copies of pentameric repeats (5'-CTCTT-3') at 5' gene region that encodes leader peptide. This repeats recombine into the recipient, Opa gene, and leads frame-shift by mis-pairing of DNA replication generating phase variation (estimated~$1x10^{-3}$ per cell generation). This phenomenon is inducible and regulated by a strength promoter, leading to differential expression levels ranging from lack to multiple Opa (Belland et al., 1997).

The classification of Opa proteins, even though the amino acid variability are based on the binding specificity with human receptors: Group **Opa_{CEA}**-Type specific for carcinoembryonic antigen – related adhesion molecules (CEACAMs) (Virji et al., 1996a, Virji et al., 1996b), being the most extensive group and **Opa_{HS}**-type binding specific with heparin sulphate proteoglycans (HSPGs) (Hauck and Meyer, 2003).

CEACAMs host surface receptor displaying multiple isoforms with different specifities for epithelial and immune cells. The differential expression of these proteins explain the particular tropism displayed with the Opa_{CEA} variants .One consequence of interaction opacity protein-receptor is the rearrangement of the cell wall mediated actin activity skeleton leading to bacterial

internalization in of neutrophil production of cytokines, resulting in opsonin-independent phagocytosis (Billker et al., 2002, Hauck et al., 1998). The bacterial lipooligosacharides (LOS) induces the overexpression of CEACAM producing the binding with Opa protein.

The gonococcal Opa has a certain preference of binding with HSPG (Chen et al., 1995, van Putten and Paul, 1995). Variant opacity proteins with highly cationic loops exposed in the surface presents a highly binding with this receptor causing the invasion in specific cell types. Other molecules that promote interaction between Opa extracellular matrix proteins are vitronectin, and fibronectin. The interaction leading a signalling pathway and the cell undergo a cytoskeletal rearrangement enabling the invasion the gonococcus (Duensing and van Putten, 1997, Gomez-Duarte et al., 1997, van Putten et al., 1998b). Opa is antigenic variant in Neisserial species, in contrast to Opc which is exclusively to *N. meningitidis*. Moreover Opc is homologue with gonococcal Opa_{HS}, high binding with HSPG mediating the attachment with epithelial cells (Virji et al., 1994).

1.4.3. Phase and antigenic variation.

Phase variation refers to a reversible swtich (on/off) expressing phase, as a result, variation of expression levels of one or more proteins between individual cells of a clonal populations. Antigenic variation means to the expression to the functionally of conserved moieties within a clonal population that are antigenically distinct (van der Woude and Baumler, 2004).

In *Neisseria gonorrhoeae* and *N. Meningitidis* expression surface structures are altered by a combination of antigenic variation enabling immune evasion during the recurring infection and generation altered ability to colonise the host (Swanson et al., 1992). The variation of structures is mediated by combination of antigenic and phase variation to alter the composition of type IV pili (Banerjee et al., 2002, Hagblom et al., 1985, Segal et al., 1986) LOS (Banerjee et al., 1998, Danaher et al., 1995) colony opacity proteins (Opa) (Belland et al., 1989, Murphy et al., 1989) and other membrane proteins (Carson et al., 2000, Chen et al., 1998, Cornelissen et al., 2000).

A wide range of genes that undergo phase and antigenic variation have been identified by genome analysis (Snyder et al., 2001). About 72 putative phase variable genes identified in the gonococcal genomes appear to be related on-off switching (Bentley et al., 2007).

Phase variation pilus undergoes phase variation at high frecuencies where piliated (P+) cells produces nonpiliated (P-) variant and viceversa (Kellogg et al., 1963, Swanson et al., 1971). Gonococcal pilin expression experience phase variation. The switch between P^+ or P^- is characterised by deletion of pilE1, pilE2 or both genes (Segal et al., 1985). Phase variation is a consequence of homopolymeric repeats involving different genes in pilus biogenesis and post-translational modification containing homopolymeric strectches within open reading frames (ORFs). Homopolymeric stretch occurs with slipped-strand mispairing during replication (**Figure 3 B.**).

Pilus antigenic variation was observed in two strain *in vitro* and in *vivo* experiments which differ in sequence and that contain new epitopes (Lambden et al., 1979, Virji and Heckels, 1983). Antigenic variation the gene encoding for the major pilin subunit *pilE*, mediates cell-cell interation (Maier and Wong, 2015) which contains semi- and hypervariable regions. Partial recombination from silent pilins pilS containing a variety of sequences and able to recombine into the *pilE* gene recipient represented in **Figure 3A** (Criss et al., 2005, Rotman and Seifert, 2014). Antigenic variation is a genetic recombination of the expression locus of *pilE* with one or more silent pil (*pilS*) gene copy. Recombination between *pilE* and *pilS*, DNA exchange occurs and distinctive *pilS* fragments are transferred through RecA protein (Koomey et al., 1987) and guanine quadruplex motif (G4) upstream of pile shown in **Figure 3A.** (Cahoon and Seifert, 2009).

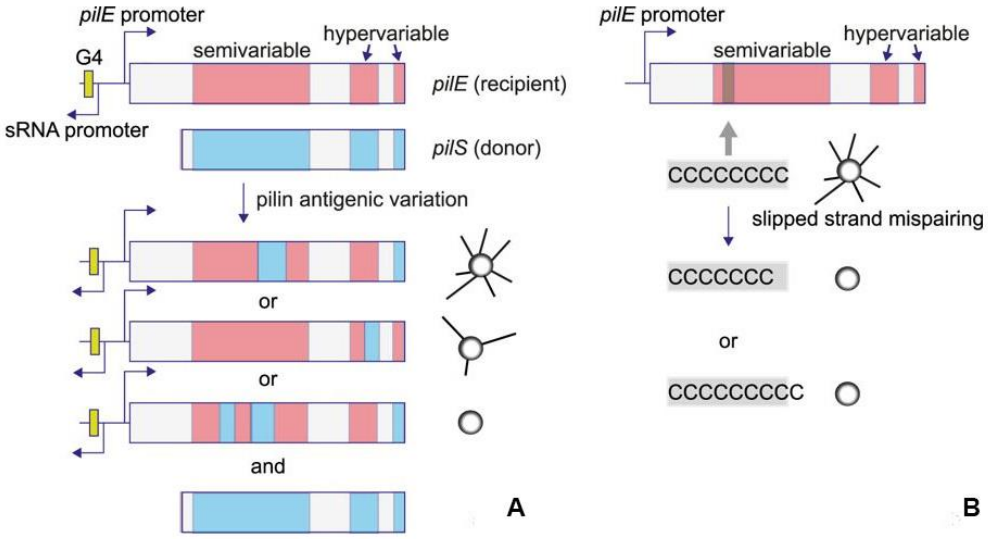


Figure 3. Representation of pilin phase and antigenic variation. **A)** The gene encoding for the major pilin subunit *pilE* contains semi- and hypervariable regions. Silen promoter *pilS* present different sequences and partially recombine *into pilE* gene (recipient), which are dependent of G4 motif and recA. As result, the variant is modified and non-functional, or non-piliated variants. **B)** Phase variation is a consequence of homopolymeric repeats , modifying and often –non functional generating non-piliated post-translational modification containing homopolymeric within open reading frame (ORFs), misparing during replication .The homopolymeric stretch is extended ortruncated by a nucleotide at high probability and the gene is switched off.(Zollner et al., 2017)

1.4.4. Porin.

Porin constitutes about 60% of the total protein of outer-membrane in the *Neisseria spp.* There are two types of Porins, PorA and PorB, with sizes of 41-42 kDa and 34 kDa respectively (Tommassen et al., 1990). Both porins are mostly present in *N. meningitidis,* whereas, PorB is solely present in *N. gonorrhoeae* (Hitchcock, 1989). Porin protein in gonococcus expresses two alleles, PorBIA (35 kDa) and PorB IB (37 kDa) (Gotschlich et al., 1987)**.** In addition *N. gonorrhoeae* has a *porA* pseudo-gene, due to a frame shiftor promoter mutation (Feavers and Maiden, 1998).

The main function of Porin is to control the diffusion and transportation of small metabolites. Pathogenic mechanisms include serum resistance, immune stimulation, host cell survival and invasion (Massari et al., 2003). Studies demonstrated that PorB acts as cation transporter (Kattner et al., 2013). Also, they are capable ofinteracting directly with artificial membranes, planar lipid bilayers (Young et al., 1983) and eukaryotic target cells membranes (Weel and van Putten, 1991).

A three-dimensional structure prediction was published based on a reported structure from *E. coli* porin (Derrick et al., 1999). Despite the lower similarity with the target model, the overall fold

showed 16-strand β-barrel fold distinctive of porins. Meningococal PorB was determined by X-ray crystallography at 2.3Å of resolution, forming a stable trimers, which identified three putative translocation pathways (Tanabe et al., 2010). However, PorA has not been reported completely solved structure but crystal structures of fragments of PorA have been reported such as binding complexes, an interaction of peptide epitopes which are porin subtype variants with monoclonal antibodies (Derrick et al., 1999). Gonococcal PorB1A in the presence of phosphatase and ATP has recently been solved by X-ray crystallography, at 3.3Å of resolution (Zeth et al., 2013).

Studies on porin protein binding receptors have been characterised as heat shock glycoprotein Gp96 and scavenger receptor (SREC) (Rechner et al., 2007). The role of both receptors seems a competitive binding and suggesting Gp96 as an anti-invasion factor whereas SREC play a receptor mediating the host entry in gonococcus (Rechner et al., 2007). Gonococcal porin PorBIA has been associated to increase the bacterial invasines, gonococcal strains carrying PorBIA that are responsible to disseminate the disease and they are difficult to eliminate by human serum and *in vitro* studies demonstrated that PorBIA gonococcus is capable to invade cells more than gonococcal species expressing PorBIB.

The influence on apoptosis of neisserial porins was described by *Muller et.al*, gonococcal porins porIB interacts with mitochondria from Hela cells, inducing calcium efflux and apoptosis (Muller et al., 2000, Muller et al., 2002). Association of PorB with mitochondria suggests mitochondrial modulation, leading to stabilization of the mitochondrial membrane and decreasing cytochrome c release, involved in STS-induced apoptosis.

1.4.5. Lipooligosaccharide (LOS).

LOS is comprised mainly by lipid A, an inner and outer core oligosaccharide and distinctive structurally compared with lipopolysaccharide (LPS) of Gram negative bacteria such as *E. coli*, with out repeating polysaccharide O-side chain (Kahler and Stephens, 1998).The presence of the *ltg* gene, that encodes LOS is present in pathogenic neisserial species, rather than commensal strains, conferring a great diversity. LOS exhibits antigenic variation, due to phase variation of a related gene expression in *N. meningitidis* (Hung et al., 2013).

A LOS characterisation study was performed in 38 *N. gonorrhoeae* strains. LOS from gonococcal strains are composed of by glucose, mannose, and galactose (Burch et al., 1997). In addition, LOS presented glycoses such as N-acetylneuraminic acid, 2- keto-3-deoxyoctulosonic acid (KDO), glucosamine, and galactosamine. LOS comparison between commensal strain, *N. sicca* and *N.lactamica,* with pathogenic gonococcal strains showed a high composition of glycoses (Mannose-KDO, galactose-KDO, and glucose-KDO) in the pathogenic strains compared than commensal species. Furthermore, fatty acid composition analysis showed gonococcal strain is composed by lipid A of 10-, 12-, 14-, 16-, and 18-carbon acids, as well as 3-hydroxytetradecanoic acid, were present are mainly but the fatty acid composition variation is less between pathogenic and commensal strains (Wiseman and Caird, 1977).

1.4.6. Auto transporters.

1.4.6.1 Classical autotransporters.

Neisseria can express monomeric autotransporters, as App and MsA/AusI. In *N. gonorrhoeae,* the common transporter is IgA protease (Pohlner et al., 1987). A modular organization of this type of adhesion i) N-terminal signal peptide, ii) secreted passenger domain and iii) C-terminus translocator domain .The process of transporter exportation starts the N-terminal leader peptide targeting the unfolded passanger domain crossing from inner membrane to periplasmic space via Sec machinery. Follow, c-terminal translocator domain fold into a β-barrel by insertion into the outermembrane (OM) mediated by multi-protein machinery termed Bam protein complex (van Ulsen, 2011). Integral translocator domain posess a hydrophobic channel, like as pore crucial for transportation of passenger domain into the cell surface. The exportation process to the OMV undergo transportation of passenger domain through periplasmic and extra-cellular folding mediated by chaperones.

Another transportater termed as Adhesion and Penetration Protein (APP), with size of 160 kDa. In the App protein contains a serine protease motif in the β-domainwith autoproteolytic activity (Serruto et al., 2003). This transportator is highly conserved in *Neisseria* sps. App is high degree homology *Hap* gene (Haemophilus adhesion and penetration product) from *Haemophilus influenza* (Hadi et al., 2001). Hap protein has been solved by X-ray crystallography (2.2Å of resolution),

intracellular Hap form intracellular complexesthat previously reported plays a role in three processes: i) adhesion to epithelial cells, ii) entry and iii) bacterial aggregation (Spahich and St Geme, 2011).

1.4.7. Other adhesins.

Other specific adhesins have been identified through the complete genome sequences of pathogenic agents *N.meningitidis* and in particular *N. gonorrhoeae* (Chung et al., 2008). Indeed, this playing a role of searching nutrients as well attachment with the host cell through in ligand-receptor interactions involved in pathogenesis in addition auto-transporter pathway. Pohlner and collaborators were the first in describing and characterising an protein auto transporters superfamily known as IgA protease from *N. gonorrhoeae* (Pohlner et al., 1987). The secreted forms of gonococcal porins are able to cleave one region of human secretory IgA1, similar to*Haemophilus* LAMP1, an integral membrane glycoprotein of liposomes, causing the intracellular life of *Neisseria* which displayed a biological function of cytotoxicity, adhesion, enzymatic activity degradation and other virulence factor, being this fragment relevant in biological activity (van Ulsen and Tommassen, 2006).

Another finding from genome sequencing and annotation studies demonstrates one gene associated with adhesion and changing the cellular matrix following the invasion process similarly with *Streptococcal* plasminogen binding proteins. Several studies have characterised adhesive molecules to the cellular matrix, enabling the contact with the endothelial surfaces facilitating bacterial colonization.

1.4.8. Adhesin complex protein (ACP).

An annotated protein was characterised as adhesion complex protein (ACP), identified in proteomic studies (Post et al., 2005, Ferrari et al., 2006) in closely related species of *N. meningitidis.* A bioinformatic analysis of isolates identified with the sequence that encodes ACP in BIGS database (Jolley and Maiden, 2010) showed 31 different *acp* DNA alleles encoding 11 types of ACP proteins. Meningococci express three types of ACP represented by the following *N. meningitidis* strains: MC58 (Type I), MC179 (Type II) and MC90 (Type III). These types of Nm-

ACP displaying a great homology with other related Neisseria species such as *N. lactamica, N. polysaccharea and N. sicca.* Strikingly, *N. gonorrhoeae* does not express these sorts of ACP, but onococcal ACP proteins displayed about 94% of similarity with *N. meningitidis*, suggesting this conserved fragment could be included as a vaccine target (Hung et al., 2013).

Recombinant rNm-ACP elicits bactericidal activity, murine antisera are able to promote complement mediated killing to homologous of type I (*N. meningitidis* MC58) with titres of 512 using murine antisera raised against rNm-ACP with saline or ZW3-14 formulations. Furthermore, mice antisera raised with different formulations showed a high reactivity against the recombinant protein (rNm-ACP) by ELISA, with a great reactivity elicited for mice sera raised with aluminium hydroxide formulation.On the contrary, murine antisera reactivity outer membrane *N. meningitidis* strain MC58 was lower compared the recombinant protein displaying not significant difference among the formulations tested.

Association role of Nm-ACP was evaluated by adherence assays on epithelial cells (Chang and Hep2 cells), endothelial (HUVEC cells) and meningeal cells. A significant difference (~75%) of the amount of recovered associated bacteria between the knockout ACP MC58ΔACP and *N. meningitidis* strain MC58 wild type variant. Similar behaviour was found on endothelial and meningeal cells with a diference of reduction of associated bacteria between 30-50%. (Hung et al., 2013).

Structural analysis of Nm-ACP by X-ray diffraction showed the overall fold composed by eigth-stranded β-barrel stabilized by disulphide bond between Cys38 and Cys 121 (**Figure 4 B**).The structure divided in two four-stranded antiparallel β-sheets (β1-β4) and (β5-β8) (**Figure 4 A**). Analysis of suface electrostatic distribution showed (β1-β4) is mainly basic, in contrast (β5-β8) region is apolar (**Figure 4 C**). Structural analysis of Nm-ACP evidenced shared structural features of lysozyme inhibitors of the MliC/PliC family, but low similarity on the primary sequence (Callewaert et al., 2008). Structural studies for MliC/PliC family lysozyme inhibitors such as *P. aeruginosa* MliC in complex with Hewl (PDB 3F6Z) (Yum et al., 2009), E. coli MliC (Revington et al., 2006) and *S. typhimurium* PliC (PDB 3OE3) (Leysen et al., 2011) have shown overall folding into eight-stranded anti-parallel β-barrels and share two conserved sequence motifs.

The first conserved region is (**SxSGAxY**) located on the fourth loop between β4-β5 strands and the second conserved region is (**YxxxTKG**) placed on the sixth β-strand of proteins distinctive in the MliC/PliC family. Docking simulation studies between human lysozyme (HL) and Nm-ACP involved the loop 4. Comparison loop 4 between amino acid sequence of Nm-ACP or mliC/PliC proteins showed three amino acid longer compared with MliC/PliC loop4 , suggesting that Nm-ACP is a novel lysozyme inhibitor (**Figure 4 D**). In addition, *in vitro* assay showed that at concentration >0.25µg/ml of Nm-ACP or Ng-ACP from *N. gonorrhoeae,* inhibit ~80-100% human lysozyme activity over 24 hours (Humbert et al., 2017).

From bioinformatics findings evidenced a homology of *acp* gene in *N. meningitidis* and other related *Neisseria* species such as *N. gonorrhoeae*. Therefore, Ng-ACP, could display the same biological function described in *N. meningitidis* and these antigen could be categorised as vaccine antigens to develop a vaccine against gonococcal infection.

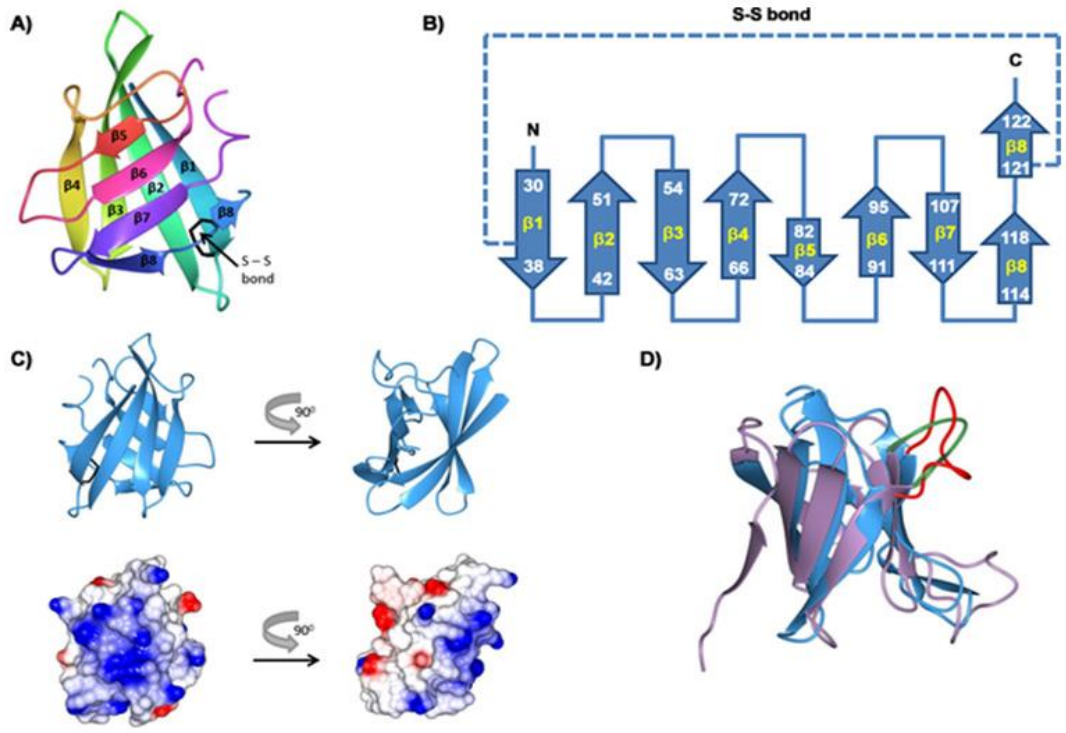


Figure 4. Three-dimensional structure and structural analysis of Adhesin complex protein (ACP) from *N. meningitidis.* A) Ribbon representation of Nm-ACP structure, a β-barrel composed for eight anti-parallel β-sheet, stabilized by disulphide bond. **B)** Topology representation of Ng-ACP amino acid sequence to form β-barrel structure, comprised by disulphide bond between C_{38} and C_{121} **.C)** Stereochemical view of Nm-ACP and electrostatic surface in the different areas of the β-barrel**,** showing region (β-sheets 1-4) display more polar areas compared with the region 2 ((β-sheets 5-8). **D)** Structural comparison by superposition between Nm-ACP (blue) and lysozyme inhibitor MliC-PliC family from *Brucella abortous* (purple). Highlighted loop 4 area in those proteins Nm-ACP (red) and Ba-PliC (green). Images taken from (Humbert et al., 2017).

1.5 Immunology of *Neisseria gonorrhoeae* infection on epithelial cells.

The major adhesins involved in adhesion to the urogenital tract are pili, LOS, Opa and porin. However this process is dependent on the cell histology, gender, and area of infection. One of them, Opa adhesion has a diverse impact on gonococcal invasiveness and symptoms. Opa^+ strains are distributed in all areas of the urogenital tract, causes asymptomatic but invasive disease. In contrast, Opa- strain strain generate a non-invasive disease but more symptomatic (Edwards and Apicella, 2005).

The invasion mechanism by gonococcus was described from infection studies with primary male epithelial uretral cells, whole uretral tissues and analysis of uretral exudates samples (Edwards and Apicella, 2005, Harvey et al., 2001b), gonococcal-positive clinical biopsies and *in vitro* bacterial

infection of human primary cervical epithelial cells (Edwards and Butler, 2011). First, gonococcus infects and colonises exposed mucosal epithelium from the reproductive tract, and there is a slightly differentiation of infection, colonization and bacterial interaction between men and women. In males, in the urethra it is considered that there are two steps involved by interaction of surface ligands to specific host receptors. Firstly, the interaction of the pilus with type I-domain region of α1β1 or α2β1 integrins causing the adherence of gonococcus to the urethral mucosal epithelium (Edwards and Apicella, 2005). Next, a transcient interaction forms between the gonococcus-integrin with sialoglycoprotein receptor, termed ASPG-R leading to a tight contact between the gonococcus and the uretra cell plasmalemma membrane. The ASPG-R receptor also can bind with the galactose present at the terminal of gonococcal LOS.

In females, the infection is influenced by lutheal phase and menses and involves innate immunity such as complement casacade (Edwards et al., 2001). Clinical isolates of gonococcal infections present inactivated C3b (iC3b) on the surface, suggesting the presence of C3b to tackle gonococcal infections (Jarvis, 1994). Human cervix expresses high levels of CR3, a αmβ2 integrin (CD11b/CD18), in gonococcal infection co-localize with CR3 *in vivo* experiments (Edwards et al., 2001). A range of gonococcal molecules interacts with CR3 promoting the internalization and colonization by gonococci, as a result, releases some components such as phospholipase D, suggesting promote colonization. Other examples, glycan moiety of pilus bind with the CR3 domain I leaging the gonococcus association on the epithelial cells. Interaction between iC3b domain I and porin (PorB.1A or PorB.1B) promotes the association with epithelial cells. Moreover, binding interaction of CR3 and factor H facilitates adherence in eukaryotic cells (Edwards et al., 2002, Jennings et al., 2011, van Putten et al., 1998a, Agarwal et al., 2010).

Female urogenital tract presents differential interaction, the Opa adhesion does not mediate association with primary epithelial cells, as well the absence of adhesion of cervical epithelial cells mediated by LOS (Edwards et al., 2002, Harvey et al., 2001a, Swanson et al., 2001).

The transmition between partner to partner has not been described completely. However, there is a finding to confirm the high affinity of piliated gonococci rather than non-piliated ones with the sperm (James-Holmquest et al., 1974). In addition, the interaction between LOS and ASPG-R on

the surface of the sperm can lead the infection. This infection by gonococcus did not cause any changes on motility or viability, but it is still unknown the detaching process of gonococcus and subsequent transmittion from the sperm to the female uro-genital tract (Liu et al., 2002).

1.5.1 Immune evasion mechanisms used by gonococci.

N. gonorrhoeae evade host immune defences using a combination of pathways such as phase-variation, hypervariability of surface antigens andresistance to complement–mediated bacteriolysis. In addition, the lack of generation of mucosal immune response, immunoregulatory and immunosupresive environment in particular female tract, enable the fetus development.

1.5.1 Resistance to complement mediated bacteriolysis.

Antibodies to porin or Opa can mediate complement or phagocyte –dependent killing (Heckels et al., 1989, Virji and Heckels, 1985). In addition, antibodies to reduction-modifiable protein RMP, a closely associated protein that block the bacteriolysis mediated by anti porin antibody (Rice et al., 1986, Virji and Heckels, 1988).

Sialylation of lipooligosaccharide (LOS) inhibits complement–mediated lysis, which was demonstrated from uretral exudates samples. Two mechanism have been characterized in serum resistance such as inhibition of action bactericidal IgM and detrimental of complement. Inhibition of bactericidal IgM was evidendenced by radiolabelling, demonstrating bound IgM showed a reduction of bactericidal activity. Similarly, sialylation increases total factor H which binds with gonococci, as a result reduces the generation of C3 and binding that leads of complement cascade (Wetzler et al., 1992, Smith et al., 1995)

1.5.2 Cell mediated immunity.

In some studies specific antibodies associated with *N. gonorrhoeae* infection were found, but the levels are low and short-lived (Hedges et al., 1999, Price et al., 2004). Weak mucosal immune responses evidenced in gonococcal infection of the rectum. In addition *N. gonorrhoeae* evades the immune response through antigenic and phase variation and resistance to complement –mediated bacteriolysis (Bayliss et al., 2008, Bos et al., 1997).

Interaction between Opa proteins present in *N. gonorrhoeae* and carcinoembryonic antigen related cellular adhesion molecule (CEACAM) , expressed on CD4+ cells, supress the activation and proliferation of T-helper and B cell differenciation (Boulton and Gray-Owen, 2002). CEACAMs host surface receptor displaying multiple isoforms with different specifities for epithelial and immune cells. The differential expression of these proteins explains the particular tropism displayed with the Opa_{CEA} variants. One consequence of interaction opacity protein-receptor is the rearrangement of the cell wall mediated actin skeleton leading to bacterial internalization in neutrophil, cytokine production, resulting in opsonin-independent phagocytosis (Billker et al., 2002, Hauck et al., 1998). The bacterial lipooligosacharides (LOS) induces the overexpression of CEACAM producing the binding with Opa protein.

Studies of gonococcal infection in the murine model observed the expression of Th17. Th17 expression drives an inflamatory immune response, with innate defense mechanism and influx of neutrophils. As a result, Th1 and Th2 adaptative immune response is supressed, including antibody response, which involve TGF-β and regulatory T cells. Supression of TGF-β, using a blocking antibody (anti-TGF-β) allowed the production of Th1 and Th2 response, and evidenced in lymph nodes (Liu et al., 2012).

The effect of neisserial porins on immune cells as human neutrophils inhibits actin polymerization, degranulation, opsonisation and receptor expression for phagocytosis. In addition, the action of porins as an adjuvant an activate B cells and another antigen-presenting cell, this immune response is mediated by upregulation of coestimulatory molecule B7-2 (CD86) (Wetzler et al., 1996).

1.5.3 Cytokine responses.

Murine studies demonstrated the expression of Th17 response which enables the generation of cytokines including IL17, IL6, IL22 and TGF-β. Th17 cells differentiate in presence of TGF-β and a group of inflammatory cytokines, such as IL-6, IL1 or IL23 are needed to maintenance and differentiation of Th17 cells. Epithelial cells and mucosal surfaces respond to stimulation of IL17 and IL22, produced by Th17cells, with upregulation secretion of defensins, S100 proteins and lipocalin-2, which illustrated in **Figure 5**. In addition, patients infected with *N. gonorrhoeae*

presents the same response observed in murine model, which leads to the recruitment of

polymorphonuclear (PMNs) to the site of infection (Liu et al., 2011, Feinen et al., 2010).

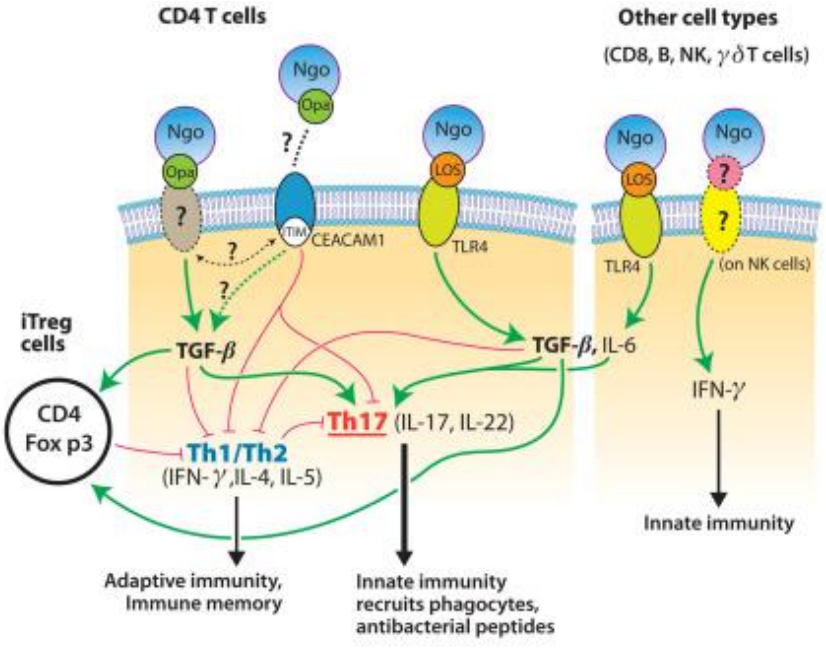


Figure 5. Proposed molecular mechanism for interaction between *N. gonorrhoeae* and the immune system. A Th17 response has been shown in a murine model. High production of IL-17, IL22 interleukins and tumoral growth factor TGF-β which is part of innate immunity and recruits phagocytes for generation of antibacterial peptides . The immune response was triggered in presence of Opa and LOS activated by CEACAM and TLR4 respectively. Blocking TGF-β expression can induce the adaptive immune response characterized by interferon IFN-γ expression and IL-4 and IL-5. (Taken from (Liu et al., 2011).

1.6 Vaccines for *N. gonorrhoea.*

However during the last four decades has developed another prophylactic measurement such as vaccines against *gonorrhoeae*. Vaccine development consist to study of diverse of antigens such as pili, porin, protein II or Opa, lipooligosaccharide, porteins involved in nutrients uptake and minor adhesins that are considered as a potential targets, however, there is still remaining an unknown conserved antigens.

Kellog and coworkers in *in vitro* assays using purified pili to determine the bactericidal effect and the relevance on gonococcal infection (Kellogg et al., 1963). This study confirmed the crucial role in gonococcal infections, discovered its pathogenicity in early 1972. However *in vitro* assays to measure the immunity protection ofpili demonstrated that antibodies against this antigen was not

bactericidal but interfering in bacterial adherence (Punsalang and Sawyer, 1973, Buchanan and Gotschlich, 1973).

Human trials with purified pili vaccine demonstrated that pili does not generates protection from natural infection, even that, produced high levels of antibodies against most common domains in *gonococcal* pili (Johnson et al., 1991). At that time, pili was considered to be responsible to vast antigenic diversity, and a vaccine including this target could be reacted with common areas of the intact organism (Schoolnik et al., 1983). Pili undergoes phase-variation as a mechanism to evade the immune system, which has been demonstrated with the failure of a large pilin vaccine trial in Korea (Boslego et al., 1991).

Serum bactericidal activity (SBA) is considered as a measurement of the efficacy *in vitro* of bactericidal assays and predict vaccine efficacy. Buchanan, *et al* (1978) found bactericidal antibodies recognised lipopolysaccharides (LPS) compared to pili (Buchanan, 1978). Vaccination with LPS provided some protection but less effective than outer membrane protein. The toxicity levels reduce their use in future vaccine formulation (Buchanan, 1978).

Another candidate was evaluated from patients who had gonococcal salpingitis and elimitated *gonococcal* infection without treatment shows high anti-porin antibodies but were reinfected with other porin serotype (Buchanan et al., 1980). *Neisserial* porin's immune-potentiating activity is possible due to interaction with T and B lymphocytes. Measurement of surface expression markers by flow cytometry was identified B7-1 and B7-2 markers. Studies with neisserial porins demonstrated an increase of expression of B7-2. The porin antigen activated B cells and co-stimuli T cells expressing transcriptional markers such as interleukin 2 and interleukin 4 stimulating antibody production (Wetzler et al., 1996). Neisserial porins from outer membrane proteins act as an adjuvant with peptides, when were incorporated in a vaccine one example is outer membrane vesicles with meningococcal porins to boost immune response towards *Haemophilus influenzae* (Liu et al., 1992).

Iron-regulate proteins are expressed by gonococci, in particular, when the environment is limited by free iron, facilitating the interaction with transferrin and lactoferrin. Antibodies against

transferrin binding protein 2 (Tbp2) in murine model was demonstrated protective however there is not any development in human trial. A study performed by Anderson *et. al*. showed Tbp2 is essential for protective response. (Anderson et al., 1994, Mazarin et al., 1995).

Antibodies raised against reduction modifiable protein (Rmp), similar to OmpA-like proteins found in all Gram-negative bacteria, block the bactericidal activity of PorB or LOS-specific antibodies. This phenomenon is called " blocking antibodies" which is defined as serum that killed the bacteria but immunizacion with this serum reduces significantly the serum bactericidal activity (Harriman et al., 1982)

The current challenge to develop an effective anti-gonococcal vaccine is due to highly antigenically surface. In addition, the animal model, even the best approach it would be BAL/C mice due to the innate response is similar in humans but the limited capacity to mimic human *gonorhoeae* in some way to predict with this model for human vaccines. A lack of human-specific receptors for adherence and invasion such as iron-binding glycoproteins, specific soluble regulators of complement cascade, IgA1 substrate of gonococcal IgA1 protease and FcαR (CD89) a opsonophagocytic receptor for IgA to identify protective response and systematic testing of antigens and immunization routes (Jerse et al., 2014).

1.6.2 Challenges developing a gonococcal vaccine.

The challenge to pursue an effective gonococcal vaccine is an emergency, due to emerging antibiotic resistant gonorrhoea strains and potentially becoming untreatable. In order to curb the sitation, the development involves different aspects from research of potential antigens or subunits which requires use of reverse vaccionology of bioinformatics to identify and characterise more potential antigens, as well as, further studies in clinical trials with the current potential candidates. Furthermore the appropiate animal model to correlate the findings and understanding of immune response in gonococcal infection as well the lack of protection in recurrent infection. Some of the challenges are summarised as below.

1.6.2.1. Adjuvants

The selection of an appropriate adjuvant or delivery systems raises the antibodies with specificity. Recent studies with novel adjuvants as microencapsulated IL-12 administered intravaginally in mice infected with Ng displayed a Ng-specific vaginal serum antibodies, as well as intranasally administration of IL-12 against respiratory diseases demonstrated a potent adjuvant (Metzger, 2009). Oligodeoxynucleotides containing the CpG motif as adjuvants serves as an enhancer to activate TLR9 and induce genital tract responses (Bode et al., 2011).

1.6.2.2. Limited understanding immunity in gonococcal infections and recurrent infection.

The lack of specific immune mechanisms to protect against gonococcal infection. This unknown mechanisms of pathology and host responses, a natural infection induces immunity to reinfection but sometimes limits prospectively defines the types of immune responses for an effective vaccine must induce (Jerse et al., 2014). It is suggested that *N. gonorrhoeae* induce antibodies rather than cell-mediated immunity, however this hypothesis has not been proven. *Gonococcal* infection resides intracellularly thereby escapeing the T cell reponse, for that reason there is a field to take into account. One example when repeat exposure of infection and raised antibodies were associated to decrease the risk of salpingitis (Zhu et al., 2011).

1.6.2.3. Bioinformatics, genomics and structural biology leading reverse vaccionology against N. gonorrhoeae.

The development of genomic and proteomics enables the identification of potential targets that are stable under different conditions and conserved among gonococci. In addition, the successful outcome from reverse vaccinology to develop a vaccine against *N. meningitidis* serogroup B may lead a similar approaches to tackle gonococcal disease (Pizza et al., 2000, Tettelin et al., 2000).

Some of the *N. meningitidis* vaccines contain Outer-membrane vesicles (OMV) and are genetically engineered to down-regulate the expression of phase variation, increasing the range of antigen specifities (O'Hallahan et al., 2005, Oster et al., 2005). Gonococcal outer membrane protein formulation showed protection in mice when is delivered intranasally with CT despite of this

promisory approach, the differences of protein isolation was not successful is this study (Plante et al., 2000).

Hypothesis and Aims

Recent characterised potential vaccine candidate, Adhesin complex protein (ACP) in a closely related bacteria *Neisseria meningitidis* showed a highly conserved candidate among commensal and pathogenic species, which elicits bactericidal activity and plays a role on association of epithelial cells. Based on this evidence, we will test the central hypothesis that the *Neisseria gonorrhoeae-adhesin complex protein (Ng-ACP)* is a potential vaccine candidate and plays a role in mediating bacterial interactions with human cells. Testing this central hypothesis involves characterization of the biological roles of Ng-ACP and examining the immunogenicity of a recombinant protein as a vaccine antigen.

The experimental aims of the work are:

1. To determine *ng-acp* gene expression in gonococcal strains P9 and FA1090 and isolates in the http:/pubMLST.org/Neisseria database. To sequence the genome of *N. gonorrhoeae* strain P9-17.
2. To clone the P9 *ng-acp* gene cloning vector and produce recombinant protein expression in an heterologous host, *E. coli.*
3. To evaluate immunogenicity of this antigen (mature or full-length rNg-ACP) through immunisation trials using diverse adjuvants in different animal models.
4. To assess the immunogenicity and reactivity of raised serum against rNg-ACP with different immunological tecniques.
5. To identify the localisation of gonococci ACP on the outer-membrane.
6. To use a range of epithelial cell lines to determine the potential adhesion and invasion of this pathogen using *in vitro* assays.
7. To determine the serum bactericidal activity (SBA) of raised mice serum.
8. To determine the three-dimensional structure of gonococcal Ng-ACP.

By performing all of these experimental aims it is anticipated that we will be able to understand the biological and immunological role of this antigen, and begin to evaluate whether Ng-ACP represents a good vaccine candidate against *N. gonorrhoeae.*

CHAPTER 2 METHODOLOGY

2.1 Bacteria.

Neisseria gonorrhoeae strains and closely related *Neisseria* species used in this study are summarised in **Table 2**.

Table 2. *Neisseria* species used in this study.

Organism /strain	Origin
Neisseria gonorrhoeae strain P9	1B-26 serovar United Kingdom (UK) isolate from a patient with gonococcal prostatitis at St Mary's Hospital, London (Ward et al., 1970)
N. gonorrhoeae strain P9-1*	*A variant of N. gonorrhoeae* strain P9 Phenotype ($Pili^{-}Opa^{-}$)
N. gonorrhoeae strain P9-2*	*A variant of N. gonorrhoeae* strain P9 Phenotype ($Pili^{+}Opa^{-}$)
N. gonorrhoeae strain P9-16*	*A variant of N. gonorrhoeae* strain P9 Phenotype ($Pili^{-}Opa^{+}$)
N. gonorrhoeae strain P9-17	*A variant of N. gonorrhoeae* strain P9 Phenotype ($Pili^{+}Opa^{+}$)
N. gonorrhoeae strain FA1090	ATCC (ATCC code 700825)
N. gonorrhoeae strain MS11	(ATCC code BA1833)
N. gonorrhoeae strain CEPA-1	Chilean isolate
N. lactamica strain E232	
N. lactamica strain A219	
N. meningitidis strain MC58	Professor Myron Christodoulides laboratory
N. sicca strain C11	
N. sicca strain B521	

Gonococcal P9 variants were picked and purfied by single colony isolation (Lambden, 1979, Lambden et al., 1979)

All bacteria were grown from liquid nitrogen onto Proteose peptone agar (GC) agar plates (Appendix A.8.) at 37˚C with 5% (v/v) CO_2 for 16-18 hours. Then the bacteria were isolated using a stereo-microscope to select the appropriate phenotype. The selection is based on the appearance of opacity and size; small colonies are characterised to express Pili and Opa, which are the major adhesins in the gonococcal OMV. On the contrary, bacteria lacking of these genes are bigger and opaque (Swanson, 1978, Swanson et al., 1971).

Competent cells for cloning as *Escherichia coli* strain DH5α (Invitrogen, UK), and *E.coli* strain GM2163 (Yeastern). Heterologous host for protein expression as *E. coli* strain BL21DE3pLysS (Invitrogen, UK). Both types of bacteria were grown from glycerol stocks onto Luria Bertani (LB) agar and broth (Appendix A.1) with appropriate antibiotics. Super optimal broth (SOB) was used to grow bacteria for protein expression (Appendix A.2) with the appropriate antibiotic agar plate at 37˚C with 5% CO_2 (v/v) for 16-18 hours. Inoculums were made in LB broth or SOB broth with the selective antibiotics, which depends on antibiotic resistance cassette on the plasmid. The inocula were incubated at 37˚C at 200 rpm in an orbital shaker incubator (Sony Gallenkamp, UK).

2.2 Extraction of genomic DNA from *Neisseria* and *E. coli spp*.

Genomic DNA was extracted using the following method, with the main difference for *Neisserial* and *E. coli* being the solvent used to neutralize the DNA. Potassium hydroxide (0.25 M KOH, 10µl) was added to an Eppendorf tube (500 µl), and a 1µl loop (Greiner Bio-one, UK) was used to swab a single colony of the desired bacteria from the agar plate until cell aggregates were sufficient obtained. The bacterial aggregate was suspended in 0.25 M KOH potassium hydroxide, by stirring the mixture and 10 µl of Ultra High Quality water (UHQ) was added. The bacterial suspension was boiled for 5 minutes and 10 µl of neutralization solution then added for *E.coli* samples 0.25 M hydrochloric acid (HCl) was used and 0.25 M Tris-HCl buffer (pH 7.5) for *Neisserial* samples. Finally, 100 µl of UHQ water was used to suspend the genetic material.

2.3 Polymerase chain reaction (PCR).

The *2X Phusion Master mix* (Thermo Scientific, UK) was used to set up a PCR reaction for amplification of fragments with high efficiency and specificity, with the parameters outlined in

Table 3. The selection of annealing temperature (X) was based on the melting temperature (*tm)* of the primers used. In addition, the time for extension (Y) was calculated using the efficiency of the enzyme according to the manufacturer and the amount of base pairs to amplify. All the PCR reactions were done on a thermal cycler T3 thermocycler (Biometra, Denmark).

Table 3. Polymerase chain reaction (PCR) conditions used to amplify a gene.

Step	Temperature (˚C)	Time (sec)	
Denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	X	30	
Extension	72	Y	
Extension	72	300	

Preparation of the reaction master mix was done in PCR tubes (Starlab) on ice and the amount of each reagent depended on the sample amount to amplify. The order of addition of each reagent of the standard mixture preparation was as follows (**Table 4)**.

Table 4. Amount of PCR regents used to prepare master mix per reaction tube.

Reagent	Volume of reagent (µl)	Final concentration
2X Phusion	10	1X
Foward Primer (10mM)	1	0.5µM
Reverse Primer (10mM)	1	0.5µM
UHQ water	7	-
DNA template	1	-

2.4 Amplification of *acp* gene in *Neisseria* spp.

A primer set used to amplify the *nm-acp* gene from *Neisseria meningitidis* (Hung et al., 2013) was used to amplify the fragment of *Neisseria* commensal species (*Neisseria sicca* and *N. lactamica*) and the *Neisseria gonorrhoeae ng-acp* gene (**Table 5**). This region is characterised by a high similarity of the sequences among *Neisseria* commensal and pathogenic species and was therefore

used as a template sequence. In this study, we amplified this gene in different *Neisseria* species for subsequent sequence analysis. All the reactions were done at 63˚C as the annealing temperature, with 40 seconds of extension time using the 2X Phusion Master mix (Thermo Scientific, UK), following the same parameters as shown in **Table 3**.

Table 5. Primer sequences used to amplify the *Neisseria acp* gene fragment.

Primer	Sequence
Forward	5'-GGCTATCTCGAGATGAAACTTCTGACCACCGC-3'
Reverse	5'- GGCTATAAGCTTCTATTAACGTGGGGAACAGTCTT-3'

2.5. Sequencing and bioinformatic analysis of *ng-acp* gene.

2.5.1 Distribucion analysis of *ng-acp* gene in different strains of *N. gonorrhoeae*.

A *Neisseria* database available from the PubMLST **http://pubmlst.org/neisseria/** displays an increasing collection of *Neisserial* commensal and pathogenic isolates, which have been identified and characterised to date (Jolley and Maiden, 2010). This web-server was used to determine the population distribution of *ng-acp* expression amongst gonococcal isolates.

To determine the distribution of *ng-acp* from a collection of *Neisseria* isolates was searched the locus for *acp* gene (**NEIS2075**). From this collection, was selected only the *N. gonorrhoeae* strains. From the gonococcal collection was categorised according to the type of allele and counted the amount of isolates that present the same assigned allele. Then was selected an amino acid sequence from *N. gonorrrhoeae* strain representative of each allele.

All the representative amino acid sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).To evaluate the identity and homology between amino acid sequences Ng-ACP was analysed by a phylogenetic tree generated by JALview (http://www.jalview.org), using average distance and percentages of identityto determine how many redundant alleles are in this collection.

2.5.2 Sequencing and evaluation of *ng-acp* from *N. gonorrhoeae* strain P9-17.

Neisseria gonorrhoeae strain P9-17 was amplified the *acp* gene using a set of primers described on **Table 5** and followed the parameters on **Table 3.** The PCR product was sequenced commercially by BioScience (Nottingham)**.**

2.5.2.1. Amino acid comparison among Ng-ACP protein from N. gonorrhoeae with other homolous and heterologous N. gonorrhoeae strains.

Neisseria gonorrhoeae strain P9-17 *ng-acp* nucleotide sequence was aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) with *ng-acp* nucleotide sequences from *N. gonorrhoeae* strains FA1090 and MS11, which have a complete genome sequences available (Dempsey et al., 1991, Brettin et al., 2005). The EMBOSS-Transeq web–server from EMBL-EBI **(**http://www.ebi.ac.uk/Tools/st/emboss_transeq/) was used to translate the nucleotide sequences into amino acid sequences to interrogate the homology of all types of Ng-ACP proteins using Clustal omega.

2.5.2.2 Amino acid comparison among Ng-ACP preotein N. gonorrrhoeae strain P9-17 and N. meningitidis strains.

From the translated nucleotide sequence of *N. gonorrhoeae* strain P9-17 in EMBOSS-Transeq web–server was compared with other amino acid sequences of the most representative Nm-ACP alleles in *N. meningitidis* strains MC58 (type I) and MC179 (typeII). ACP Amino acid sequences were aligned using Clustal Omega.

2.5.3 Genome sequence of *N. gonorrhoeae* strain P9-17.

From an isolated gonococcal lawn on GC agar plate was swabbed and suspended into 500 µl of PBS. Isolation of genomic DNA was done using Genome Extraction Kit (Promega, USA) and suspended with 30µl of Nuclease free-water. Genomic DNA was evaluated the quality and quantified the amount of DNA by 1% (w/v) agarose electrophoresis gel and Nanodrop (Sections 2.6.6 and 2.6.7).Genome DNA from *N. gonorrhoeae* strain P9-17 sequencing was performed by

Oxford University, Genome sequencing area, Zoology Department) and deposited the information in P PubMLST database (https://pubmlst.org/bigsdb?page=info&db=pubmlst_neisseria_isolates&id=36675).

2.6 Cloning of the *ng-acp* gene.

2.6.1 Preparation of the *ng-acp* gene insert.

A reaction mixture (100 µl) was prepared using the 2X Phusion Master mix (ThermoScientific, UK) and *Neisseria gonorrhoeae* strain P9-17 DNA as a template, with the PCR amplification parameters and conditions for *ng-acp* gene depending on the cloning strategy used (Section 2.7.1.2 and Section 2.7.2.2.1).

2.6.2 Wizard PCR Clean Up.

The Wizard PCR clean up kit (Promega, USA) contains buffers and solvents for removing impurities from genetic material, PCR reactions or bands excised from agarose gels run in Tris-Borate or Tris-EDTA buffers (Appendix A.3). The kit allows purification of material from 100bp to 10kb and uses two steps:

Step i) Binding of DNA to the column (maximum capacity of 40 ng of genomic DNA per column) in the presence of 4.5 M guanidine isothiocyanate and 0.5 M potassium acetate buffer, which enables retention of DNA fragment onto the column for 1 minute, followed by column centrifugation at 20,800 x*g* (Eppendorf Centrifuge 5417R) for 1 minute, followed by removal of the liquid.

Step ii) involves two washes one of 700 µl and then 500 µl with washing solution (10 mM potassium acetate, 80% (v/v) ethanol and 16.7 µM EDTA,pH 8.0), followed by centrifugation at 20,800 x*g* for 1 and 5 minutes respectively between each volume addition to remove impurities. Finally, the purified DNA was suspended in nuclease free water in a minimal volume that provides the most concentrated sample (usually 40-50 µl).

2.6.3 Plasmid extraction.

Plasmid extraction comprised four steps with different buffers from an overnight cell culture:

Step i) *E.coli* cells were centrifuged at 5580 x*g* (Thermo Fisher Megafuge S16 Centrifuge) for 10 minutes and the pellet was suspended with 250 µl of 50 mM tris-HCL pH 7.5, 10 mM EDTA, 100µg /ml RNase A buffer.

Step ii) 250 µl of cell lysis solution (0.2 M NaOH and 1% (w/v) SDS) was added to the pellet follow by treatment with alkaline phosphate, which are 10 µl per lysate tube reaction, twist three times the Eppendorf tube and incubate 5 minutes at room temperature, to improve the quality of the isolated DNA.

Step iii) The addition of 350 µl of 4.09 M guanidine hydrochloride, 0.759 M potassium acetate and 2.12 M glacial acetic acid to the *E. coli* lysate. The tube was then mixed 4 times and the mixture was centrifuged at 14000 rpm (20800 x*g*) for 10 minutes (Eppedorf 5417R).

Step iv) The cleared lysate was placed on mini-columns and incubated for 1 minute. Then, the sample was centrifuged at 20800x*g* for 10 minutes. Serial washing steps followed with wash solution composed of 60% (v/v) ethanol, 60.0 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5 and 0.04 mM EDTA pH 8.0, starting with the addition of 750 µl volume followed by 250 µl volume with centrifugation steps at 14000 rpm (20800x*g*) for 1 and 5 minutes, respectively.

Step v) The purified plasmid was suspended in 30-50 µl nuclease free water. The purity and concentration of isolated plasmid was evaluated by 1% (w/v) agarose gel electrophoresis, as described in Section 2.6.6. and Section 2.6.7.

2.6.4 Double digestion of DNA using restriction enzymes.

The use of one or more restriction enzymes specifically cleaves DNA fragments at specific sequences and enzyme selection is made exclusively from the nature of the nucleotide sequence. In addition, selection of reaction buffer and other additives, *e.g.* Bovine Serum Albumin (BSA), depends on the manufacturer's recommended protocol for a high performance DNA enzyme digestion. The amount of the restriction enzyme added was determined by the amount of DNA

present and the reaction was done on ice, using the reagents in **Table 6** added in the descending order.

Table 6. Reagents used for preparing enzyme digestion mix.

Reagent	Volume of reagent (µl)	Final concentration
DNA fragment	39	~1.5µg
BSA	5	1X
Enzyme 1	1	1U
Enzyme 2	1	1U
Enzyme buffer**	5	1X

** The selection of the buffer largely depended on the high performance (100%-75%), as recomended by the manufacturer.

2.6.5. Ligation of *ng-acp* gene into cloning plasmid.

A T4 ligase enzyme was used to ligate the gonococcal *ng-acp* gene insert into the cloning plasmid through ligation of the overhang ends created by the restriction enzymes. According to the estimated concentrations of both plasmid and insert, and using a molar ratio of insert: vector of 3:1, a reaction mixture was prepared using the following formula:

$$\frac{(10\ ng\ vector * 0.372\ kb\ size\ of\ insert)}{2.98\ kb\ size\ of\ plasmid} * 3 = \text{ng of insert}$$

To increase the efficiency of insertion and ligation, higher amounts of insert to plasmid were used as shown in **Table 7.**

Table 7. Ligation reaction conditions for *ng-acp* gene insert-cloning plasmid**.**

	Positive control (µl)	Negative control (µl)	Final Concentratio
Plasmid	1	1	100-200ng
Insert *ng-acp* gene	1.5	--	10-20 ng
UHQ water	5.5	7	
10X ligase buffer	1	1	1X
T4 ligase	1	1	3 Unit

Each ligation reaction was set up individually in Eppendorf tubes on ice. Each reagent was added follow the order descendent in **Table 7**. Then the samples were kept at 4˚C overnight in the fridge, to maximize the efficiency of the ligation reaction and subsequently the number of transformants.

2.6.6. Agarose gel electrophoresis.

Agarose gel electrophoresis is the most effective way to separate DNA fragments ranging in size from 100 bp to 25 kb. Agarose gel electrophoresis is done in two steps. First, the agarose gel was prepared using agarose powder (Sigma-Aldrich) a 1% (w/v) dissolved in 40 ml of Tri-boric acid-EDTA (TBE) or Tri-EDTA buffer (Appendix A.3). The mixture was boiled for 3 minutes at high power in the microwave and then 5-6 µl of SYBR safe DNA gel stain (Invitrogen) was added as an intercalating agent. The solution was poured into a gel tray (Mini SUB DNA cell Bio-Rad) the gel allowed to set. For samples preparation a mix of 3µl of loading buffer 4X (Bioline) and 2-3µl of sample or 2-5 µl of PCR reaction used with Green Master mix (Promega, USA) was loaded into each well, and finally an exclusive lane for loading 5 µl ladder Hyperladder I (Bioline). Once the samples were loaded, electrophoresis was done at 80 volts for 1 hour, in a Mini SUB DNA Cell (Bio-Rad) chamber filled with 800-900 ml of TBE. The agarose gel was visualised and photographed in a UV-light trans-illuminator (UVP 2VTrans illuminator BiodocitSystem, UK).

2.6.7 Estimation and quantification of DNA.

DNA was estimated relative to the marker Hyperladder I (Bioline), which consists a group of standard DNA fragments with size ranging from 200 bp up to 10 kbp and different concentrations, some of DNA fragments such 1000 bp and 10 kbp have a concentration of 100 ng/µl and enable stimate the concentration of the sample. The volume of hyperladder added to an agarose well was 5 µl, which provided bands of reliable and reproducible brightness for quantifying sample DNA bands, according to the manufacturer's protocol (Bioline). Alternatively, DNA was quantified using a Nanodrop (Nanodrop TI 1000, ThermoScientific) spectrophotometer, with the final concentration expressed in ng/µl, calculated from a combination of two ratio from measurement at

different wavelengths (λ) at λ_{260}nm, which absorbance generated bypresence of organic solvents ($\lambda_{260}/\lambda_{230}$) and at λ_{280}nm , ($\lambda_{260}/\lambda_{280}$) related withpresence of protein in the sample.

2.6.8 Preparation of competent cells and transformation methods.

Chemical transformation of *E. coli* strains such as DH5α, GM2163 or BL21DE3pLysS was required to enable uptake of foreign DNA. Heat shock leads to insertion of foreigner DNA into the cell and enable genetically modified the organism. Calcium and magnesium salts were used to prepare competent cells for transformation, since they perturb the cell membrane to form pores that allow uptake of genetic material from the environment.

2.6.8.1 Preparation of competent cells.

A culture of host *E. coli* bacterium was made overnight in 10 ml LB medium with shaking at 200 rpm at 37˚C in an orbital shaker incubator (Sony Gallekamp, UK). An aliquot of 1ml of this overnight culture was then inoculated into 25 ml of LB medium, which was incubated with shaking at 200 rpm at 37˚C until cell culture growth reached an OD λ_{600nm} of 0.4-0.5. Once this absorbance value was reached, the cell culture flask was placed on ice for 20 minutes. Next, 10 ml of cell culture was poured into an ice-cold 15 ml Falcon tube and centrifuged at 3000 rpm (1690 x*g*) for 10 minutes at 4˚C (ThermoFisher Megafuge S16). The supernatant was discarded and the pellet suspended in 5 ml of ice cold 0.1 M $MgCl_2$ buffer. The cells were centrifuged (1690 x*g* for 10 minutes) and the pellet was suspended again in 1 ml of ice-cold 0.1 M $CaCl_2$. The cell suspension was kept on ice- for 1 hour. To prepare stocks of competent cells, 177 µl of glycerol (100%) was added to the bacterial suspensions and 150 µl of the cell suspension was then dispensed into cryogenic tubes (NNC#375418,NUNC,(ThermoScientific) and stored at -80˚C.

2.6.8.2 Transformation of competent cells.

From an aliquot tube of competent cells (150 µl), equal volumes (50 µl) were distributed into Eppendorf tubes. Three types of transformation conditions were prepared i) a positive ligation control ii) a negative ligation control iii) No DNA control. Depending on the DNA concentration, approximately 5- 10 µl of ligation mixture was added to the cell suspension, which was left on ice for 30 min on ice, with occasional shaking by hand. The cells were heat-shocked by placing in a

water bath at 42-45˚C for 45 seconds and then placing on ice for 5 min. Next, 500 µl of LB medium was added to each tube and the samples were incubated at 37˚C for 1 hour in an incubator. The cell culture was centrifuged at 13000 rpm (15142x*g*) for 2 minutes (Heraeus, Biofuge 13) the supernatant was removed but leaving ~100 µl liquid to suspend the bacteria. This volume was then plated onto an antibiotic-selective agar plates, which was incubated overnight at 37˚C.

2.6.9 Cloning of *ng-acp* gene-plasmid vector construct into *E. coli* DH5α.

In order to clone the *ng-acp* gene into cloning vector, two steps are required, which are i) competent cell uptake of the ligation product. ii) Followed by evaluation of the possible transformants by PCR and nucleotide sequencing to ensure that the candidates contain the appropriate open-reading frame (ORF).

2.6.9.1 Transformation of the ng-acp-cloning vector construct into Escherichia coli DH5α.

Following the protocols described in Sections 2.6.8.1 and Section 2.6.8.2, 1-2µl of ligation reaction was added to the competent *E. coli* DH5α cells in an Eppendorf tube (1.5 µl) and kept on ice for 30 minutes. This was followed by heat –shock activation for 45-50 seconds at 42˚C, and placement of the samples on ice for 2 minutes. LB medium (500-900 µl) was then added and the suspension incubated for 1hours at 37˚C, allowing the bacteria to grow to semi-log phase, (cell suspension turbidity). Once reached, the suspension was centrifuged at 14000 rpm (20800 x*g*) for 1 minute and the supernatant was discarded. The pellet cell was suspended in a minimum volume of LB medium (50-100 µl) and this volume plated onto an antibiotic-selective plate and the plates which was incubated overnight at 37˚C.

2.6.10. Selection of transformants.

2.6.10.1 PCR colony.

Possible transformants that are resistant on antibiotic-selective LB plates were screened by PCR reaction. Single colonies were picked from selective plates and DNA extracted as described in Section 2.2. A PCR reaction mixture containing Go tag Master Mix (Promega, UK) was prepared (**Table 8**) and PCR run with the parameters outlined in **Table 9**.

Table 8. PCR reaction mixture for transformants.

Reagent	Volume (µl)
Go tag master mix (Promega, UK)	10
Forward primer *ng-acp*	1
Reverse primer *ng-acp*	1
UHQ water	7
DNA template	1

Table 9. PCR parameters for transformants.

PCR step	Temperature (˚C)	Time (sec)	
Denaturation	95	30	
Denaturation	95	10	30 cycles
annealing	X*	30	
Extension	72	Y*	
Extension	72	300	

* Being X*annealing temperature from set primers used, Y* time of amplification PCR product depends of the length of the fragment (30sec up to 1min).

2.6.10.2 Sequencing of selected transformants.

Overnight cultures of transformed *E.coli* DH5α containing *ng-acp*-cloning vector constructs were grown and the plasmids were extracted using Wizard Miniprep (Promega) protocol, as described in Section 2.6.3. A random selection of 2 to 5 candidates was made, which were sequenced commercially (SourceBioScience, Nottingham; http://www.lifesciences.sourcebioscience.com/genomic-services/sanger-sequencing-service/) using the Forward primer T7, a T7 promoter sequence included into pRSET-A or pET22b cloning vector, and the appropriate reverse primer of *ng-acp* gene, depending on the cloning strategy. Sequences were analysed by Segman software (Lasergene, DNASTAR).

2.6.11. Transformation into heterologous host *E.coli* strain BL21 DE3pLysS.

E. coli strain BL21(DE3)pLysS was transformed with the *ng-acp*-cloning vector construct for expression of Ng-ACP protein with a N-terminal or C-terminal 6xHis-tag that allows rapid screening of expression levels and protein solubility. The host strain BL21 is deficient in two proteases encoded by the *lon* (cytoplasmic) and *omp*T (periplasmic) genes, thereby eliminating the production of proteases and leading to enhanced accumulation of protein by reducing proteolytic

degradation. Following the protocols described in Sections 2.6.8 *E.coli* BL21 (DE3) pLys bacteria were transformed with the construct selected previously in Section 2.6.10. The selection of the transformants was from selective LB agar plates with 50-100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The transformants were evaluated by then colony PCR. A positive transformant was selected to continue further experiments for optimization of protein level expression and determination of protein solubility and protein stability.

2.7 Cloning strategy for protein expression of rNg-ACP.

A variety of cloning vectors are commercially available for generating recombinant proteins and include features such as terminal His-tags, to enable protein purification by affinity chromatography. Also, an enterokinase site is incorporated to allow removal of the tag to release the protein. In this study two types of cloning vectors were chosen, pRSET-A and pET22b, as well specific modifications of the protein e.g. presence or absence of the leader peptide.

2.7.1 Cloning strategy for *ng-acp* gene into pRSET-A cloning vector.

pRSET-A is a cloning vector for high protein expression in heterologous organisms such as *E. coli*. The high production of recombinant protein is made possible by a T7 promoter (**Figure 6A**) that regulates expression of the gene in the presence of Isopropyl β-D-1-ThioGalactopyranoside (IPTG) (Sorensen and Mortensen, 2005), as well as including a region downstream of the *pRSET-A* DNA sequence that encodes at the N-terminus of the protein a tag fusion sequence of polyhistidine (6xHis) as shown in **Figure 6B**. This enables the recombinant protein be purified by affinity chromatography. This is followed by an enterokinase cleavage recognition site (**Figure 6C**). The size of the cloning vector is 2897 base pairs (bp). This cloning vector in the multiple cloning site contains a restriction sequences for *XhoI* and *Hind III* enzymes was used to insert the *ng-acp* gene (**Figure 6 MCS**). In addition, the cloning vector includes ampicillin (Amp) and chloramphenicol (Chlo) antibiotic resistance marker sequences.

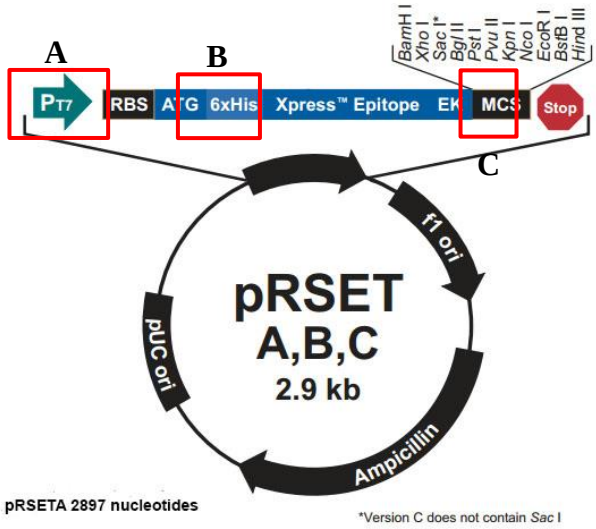


Figure 6. Composition multiple cloning site of pRSET-A cloning vector. A. T7 promoter. B. Polyhistidine (6xHis) at N-terminal region. C. Enterokinase region (EK). MCS Multiple cloning site. This cloning system was used to produce the full-length rNg-ACP recombinant protein. Image taken from PRSET-A manual Life techonologies Invitrogen-Thermo Fisher.

2.7.1.1 Extraction of the pRSET-A cloning vector from Escherichia coli strain DH5α.

The cloning vector pRSET-A was extracted from an overnight culture of *E.coli* DH5α strain grown in 5 ml of LB medium containing ampicillin (50 μg/ml), using the Wizard Miniprep kit (Promega) follow the manufacturer's protocol (Section 2.6.3).

2.7.1.2 Amplification of ng-acp gene from Neisseria gonorrhoeae strain P9-17.

Based on the nucleotide sequence of the gene encoding for *ng-acp* from *N. gonorrhoeae* strain P9-17, a PCR reaction product was performed as described in Section 2.4. A set of primers was designed to amplify with high specificity the region that encodes the Ng-ACP protein. Construction of the primers was done with inclusion of an overhang sequence 5'-GGCTAT-3' and two restriction site sequences for *Xho*I and *Hind*III enzymes, present on MCS or pRSET-A vector (**Figure 6**). The reverse primer contained the CTA stop codon. The PCR was carried out using 2X Phusion Master mix (Thermo Scientific, UK) with an annealing temperature of 62˚C and 11 seconds of extension time, as described in Section 2.4 and using the set of primers shown in **Table 10** and represented in the **Figure 7.**

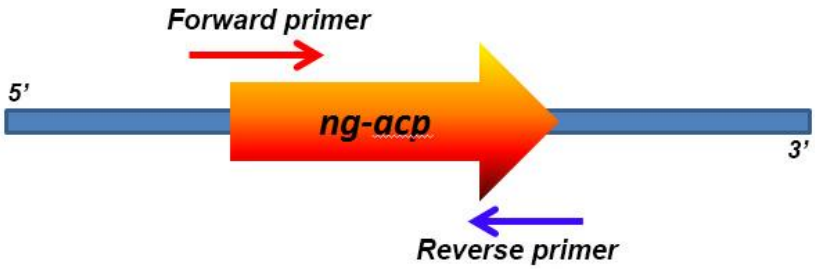


Figure 7. Representation primer localization for *ng-acp* gene amplification from *N. gonorrhoeae* strain P9-17.

Table 10. Sequence primers used to amplify the *ng-acp* gene.

Primer	Sequence
Forward	5'-GGCTAT**CTCGAG**ATGAAACTTCTGACCACTGC-3'
Reverse	5'-GGCTAT**AAGCTT**CTATTAACGTGGGGAACAGTCTT-3'

The sequences in bold represent the restriction sites for *Xho*I (**CTCGAG**) and *Hind*III (**AAGCTT**).

2.7.1.3 Double digestion of gonococcal ng-acp gene.

Based on the estimation of purified gonococcal *ng-acp* gene PCR product, the amount of DNA fragment was calculated as 1.5 µg for optimal digestion with the restriction enzymes. The *ng-acp* fragment was digested with *Xho*I and *Hind*III using a common buffer Buffer B, according to the manufacturer's recommendations, to maintain high efficiency (Promega), using the same reaction conditions described above (Section 2.6.4). The digestion mixture was incubated at 37˚C for 3 hours in an incubator. The sample was then purified using the Wizard PCR Clean Up Protocol (Promega) and DNA fragment quality and concentration was estimated by electrophoresis on an agarose gel 1% (w/v) as described above (Section 2.6.6).

2.7.1.4. Double digestion and dephosphorylation of pRSETA plasmid.

Based on the estimated concentration of the pRSETA plasmid from agarose gel electrophoresis, a digestion mixture reaction was prepared following the standard conditions described in Section

2.6.4. Once the plasmid is linearised by restriction enzymes, this generates an overhang end, which enables re-ligation inside of the vector. To avoid this phenomenon required dephosphorylation of the linearised vectors, which was done by the addition of alkaline phosphatase as described in **Table 11**.

Table 11. Reagents and amount required for plasmid desphosphorylation.

Reagent	Volume added (µl)
Alkaline phosphatase, Calf intestinal (CIAP)	8
Alkaline phosphate buffer (50mM Tris-HCl (pH 9.3 at 25°C), 1mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine)	8
BSA	8
UHQ water	6

Each reagent was added at room temperature and the dephosphorylation mixture was incubated in an incubator at 37˚C for 30 minutes and then inactivated by heating to 56˚C for 15 minutes in a water bath (Grant). The digested-dephosphorylated DNA fragment was purified using the protocol described for PCR Clean Up (Section 2.6.2) and subsequently evaluated by agarose gel 1% (w/v) electrophoresis to confirm quality of the purified plasmid and estimate concentration (Section 2.6.6. and 2.6.7).

2.7.2 Cloning strategy for *ng-acp* gene into pET22b cloning vector.

The pET22b cloning vector was also selected and the *ng-acp* gene without the region encoding the leader peptide of Ng-ACP from *N. gonorrhoeae* strain P9-17. Addition of the 6XHis-Tag at C-terminus was chosen on the pET22b was used to improve protein solubility (**Figure 8).**

pET-22b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
bla coding sequence	4038-4895
f1 origin	5027-5482

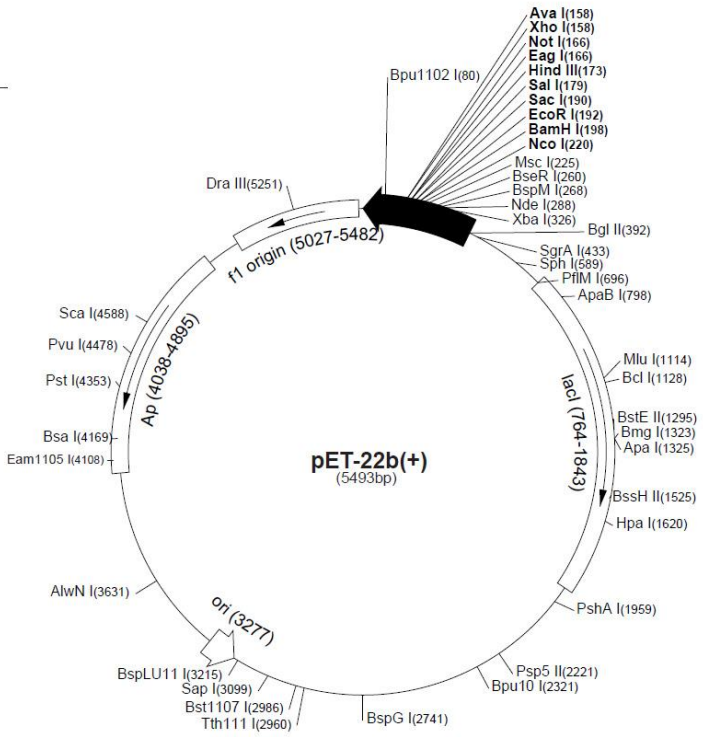


Figure 8. Cloning vector map of pET22b which includes multiple cloning site (MCS), antibiotic resistance (*Amp*),T7 promoter , enhancer of protein expression.

Image taken from manual Novagen (Invitrogen).

2.7.2.1 Extraction of the pET22b cloning vector from Escherichia coli strain DH5α.

The cloning vector pET22b was extracted from an overnight culture of *E.coli* DH5α with the *nm-acp*-pET22b construct grown in 5 ml of LB medium containing ampicillin (50 μg/ml), using the Wizard Miniprep kit (Promega) follow the maufacturer's protocol (Section 2.6.3).

2.7.2.2. Primer design.

A set of primers was designed using the *ng-acp* gene sequence from *Neisseria gonorrhoeae* strain P9-17 in order to construct a soluble protein. Both primers contain an overhang sequence 5'-**GGCTAT-3'** in addition the restriction sites placed in the multiple cloning site (MCS **Figure 8**) of pET22b cloning vector, *Nde*I 5'-CATATG-3' and *Xho*I 5'-CTCGAG -3' which are summarised on **Table 12.**

Table 12. Set of primers sequences for mature (rNg-ACP).

	Sequence
Forward Primer sNg-ACP	5'- GGCTATCATATGAAACTTCTGACCAC -3'
Reverse Primer sNg-ACP	5'- GGCTATCTCGAGACGTGGGGAACAG -3'

The sequences highlighted represent the restriction sites for *Nde*I (**CATATG**) and *Xho*I (**CTCGAG**)

2.7.2.2.1 PCR amplification *ng-acp* protein.

The *ng-acp* gene from genomic DNA from *N. gonorrhoeae* strains P9-17 was amplified using the 2XPhusion enzyme, which an annealing temperature of 62˚C and an extension time was 45 seconds, as described in **Table 13.**

Table 13. PCR protocol to amplify *ng-acp* gene for cloning into pET-22b.

Temperature (˚C)	**Time(seconds)**	
98	30	
98	10	30 cycles
62	30	
72	45	
72	300	

2.7.2.3 Generation of ng-acp-pET-22b construct.

Once each fragment of *ng-acp* PCR product was obtained, the plasmid was double digested with *Nde*I and *Xho*I enzymes as described in Section 2.6.4. Each fragment was then purified using PCR Clean up kit (Promega) and evaluated by agarose gel 1% (w/v) electrophoresis. The purified plasmid was obtained by removing additional fragment of *nm-acp* gene by agarose gel electrophoresis and subsequent purification using PCR clean up kit.

To generate the construct each fragment was ligated using T4 ligase, as described in Section 2.6.5. The ligation product was used to transform *E. coli* strain DH5α as described in Section 2.6.8.2. Possible candidates were selected LB agar plates containing with 0.1 mg/ml ampicillin and tested by PCR as described in Section 2.6.10 using the set of primers in **Table 12**.

Plasmid was extracted from the positive candidates using Wizard Mini prep kit (Promega), and then sequenced and analysed using Segman software (Lasergene, DNASTAR) as described in Section 2.6.10.2.

2.8 Production of recombinant rNg-Gap and full-length/mature rNg-ACP protein.

In order to obtain high levels of expression of the protein in *E. coli* strain BL21(DE3)pLysS, several conditions were tested, including determination of the effect of promoter induction, the time of maximum protein expression and the protein yield.

2.8.1 Pilot expression of rNg-GapC, full-length rNg-ACP and mature rNg-ACP.

For rNg-ACP, an overnight culture (5 ml) of *E. coli* BL21 DE3pLysS was grown in super optimal broth (SOB) supplemented with chloramphenicol (34μg/ml) and ampicillin (50 μg/ml). For rNg-GapC expression, the same conditions were used except the introduction of chloramphenicol (34 μg/ml) and kanamycin (50 μg/ml) or Erlenmeyer flasks (2 x 250 ml) was evaluated with and without add of inducer 1mM IPTG at different time points (**Figure 9**). Protein production was evaluated over 5 hours, with 1 ml of bacterial culture removed every hour for analysis.

2.8.1.1 Extraction of recombinant protein from *E.coli* bacteria.

E.coli bacterial cell pellets were suspended with 100 μl of phosphate buffered saline (PBS, pH7.4) and lysed by repeated cycles of freezing and thawing at -80˚C and 37˚C for 30 minutes, followed by probe sonication with two repeated cycles at 18-22 microns for 6 seconds each (MSE Soniprep 150) on ice. The clarified protein extracts were analysed by SDS-PAGE (Laemmli, 1970) in order to evaluate the presence of protein in the samples.

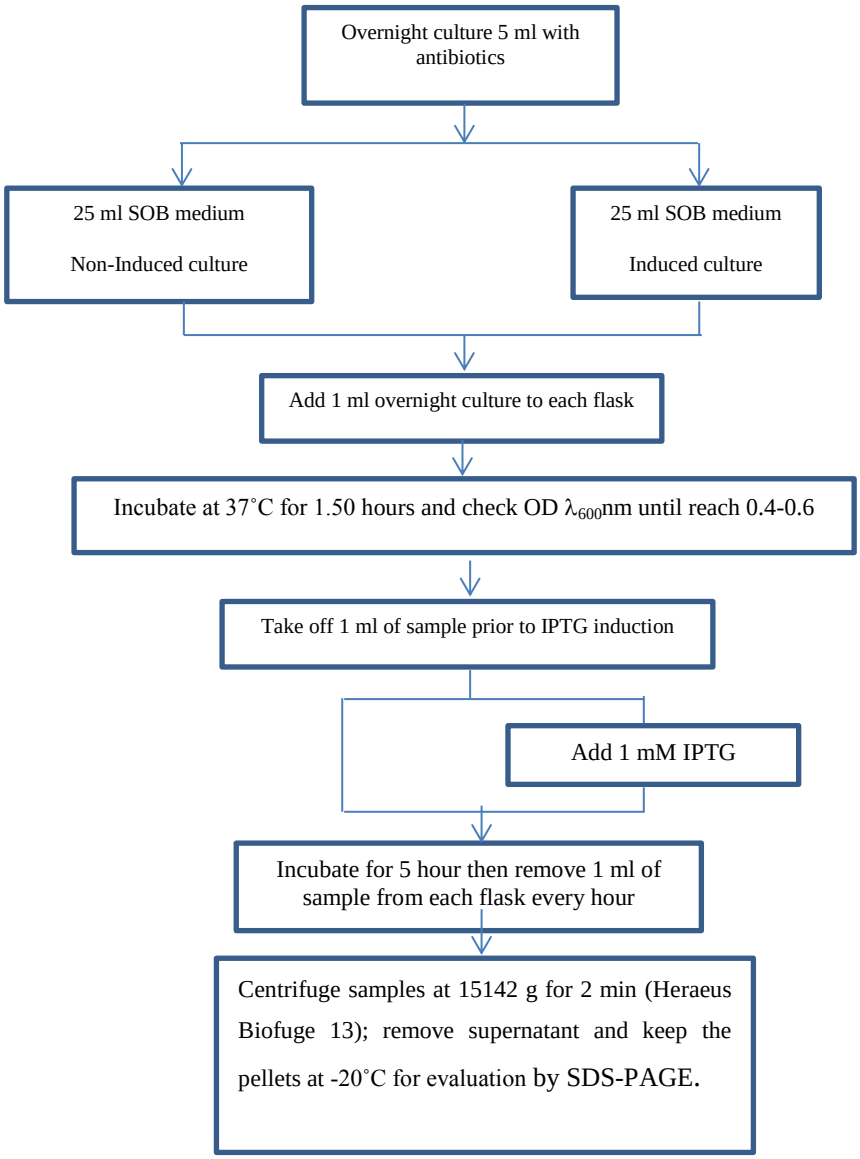


Figure 9. Pilot recombinant protein expression flowchart to evaluate the influence of presence and absence of induction agent and time of incubation to produce the recombinant protein.

2.9. Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE electrophoresis is a rapid method for characterizing and quantifying proteins. This technique separates proteins based on their molecular weight (Laemmli, 1970). SDS binds along the protein polypeptide chain and the length of the reduced SDS–protein is proportional to molecular weight. The SDS-PAGE gel is composed of a lower separating gel layer (prepared using a ratio of acrylamide: bisacrylamide mixture of 37.5:1, with 12% (w/v) used to evaluate the

molecular weight of interest, 15-20 kDa) and an upper stacking gel, which helps to concentrate the protein before separation under current (See Appendix A.4 for composition of reagents).

The polyacrylamide gel was inserted into a Mini-Protean apparatus (Bio-Rad) with 1X running buffer (Appendix A.5.) for electrophoresis. The samples were loaded into the wells with loading buffer (Appendix A.4.3.) at a ratio of sample to buffer of 1:4. The samples were boiled for 5 min and then briefly centrifuged at 20800 x*g* for 1 minute prior to loading (Eppendorf centrifuge 5427R). The gel apparatus was attached to a power pack (Bio-Rad 200/2.00 Power Supply), which was operated at 200 Volts for 1hour.

2.9.1 Staining the polyacrylamide gel with Coomasie Blue.

This method enables protein detection in the range of 0.1-1 μg per band (Diezel et al., 1972, de Moreno et al., 1986). Using a stock solution of Coomassie blue in acetic acid (Appendix A.6.), approximately 20 ml of the solution was poured onto the gel and left for 1h at room temperature. The stained solution was removed and the gel was treated with methanol/acetic acid destaining solution (Appendix A.7) for 1 hour to remove the excess Coomassie blue. Alternatively, the acrylamide gel was placed in a beaker with distilled water and boiled at medium power for 4 minutes in the microwave.

2.10 Pilot purification and evaluation of recombinant protein solubility.

A sterile 250 ml Erlenmeyer flask containing 50 ml of SOB medium with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol for rNg-GapC protein or 50 μg/ml ampicillin and 34 μg/ml chloramphenicol for rNg-ACP protein was inoculated with 1 ml of an overnight culture of *E.coli* strain BL21pLysS *ng-GapC*-pET24 or *E.coli* strain BL21pLysS *ng-acp*-pRSET-A and incubated at 37 ˚C with shaking at 200 rpm (Sony Gallenkamp, UK) until the cell culture growth reached semi-log phase (OD λ_{600}nm of 0.5-0.6). Bacterial suspension (1 ml) was placed in a disposable 10mm cuvette (Fisherbrand) and was measured by UV-Vis spectrophotometryat λ_{600}nm (Vis-spectrum, HITACHI, U-1100) (Section 2.11.1), previously calibrated with SOB broth as the blank. IPTG was added at semi-logaritmic growth to a final concentration of 1 mM and the culture was incubated for the optimal time selected from the pilot expression experiments. Samples (1 ml) were removed

each hour to examine the levels of protein expression by SDS-PAGE. The cell culture was then centrifuged at 5000 rpm (10000 x*g*) at 4˚C (Thermo Megafuge 16R), the supernatant discarded and the pellets stored at -20˚C. On the following day, buffers were prepared for protein purification under denaturing conditions using nickel (II)–nitrilotriacetic (Ni-NTA) (Qiagen) resin affinity chromatography.

Cell pellets were suspended in 2-10 ml of iced Lysis buffer (**Table 14** or **.**

Table 15) and then transferred to Eppendorf tubes. The suspended pellet was centrifuged at 9800 rpm (8000 x*g*) for 30 minutes (Heraeus, biofuge 13), to separate the cell debris and supernatant. The cleared lysate was incubated with 0.5 ml of Ni-NTA (Qiagen) resin for 1 hour at 4˚C on a rotor (Smith Scientific). Then, the lysate suspension was poured into a column dimension of (20 cm ×0.7 mm) and allowed to settle and the flow-through collected under gravity (1-2 fractions of 2 ml each). Washing buffer (**Table 14** or **Table 15**) was added after the flow-through was collected; 10-20 ml of washing buffer was used and 2-3 fractions of 4 ml each were collected under gravity in glass Bijoux bottles and the remaining volume discarded. Finally, elution buffer (**Table 14** or **Table 15**) (10-15 ml) was added and 0.5 ml fractions were collected under gravity into Eppendorf tubes.

The eluted fractions for full-length rNg-ACP under denaturing conditions were precipitated with tri-chloroacetic acid (TCA) as described in Section 2.11.4.2.1 and the precipitates suspended with PBS buffer containing 0.5% (w/v) SDS. Eluted fractions for rGapC and mature rNg-ACP under non-denaturing conditions and samples from denaturing conditions were evaluated on SDS-PAGE (Section 2.9).

2.11 Large scale expression of rNg-GapC and full-length andmature rNg-ACP proteins.

An overnight culture (10 ml) of *E.coli* strain BL21pLysS *ng-GapC*-pET24 or *E.coli* strain BL21pLysS *ng-acp*-pRSETA in SOB medium with antibiotics, (50 µg/ml kanamycin and 34 µg/ml chloramphenicol for rNg-GapC or 50 µg/ml ampicillin and 34 µg/ml chloramphenicol for rNg-ACP) protein was added to 1.5 L of SOB medium in a 2 L sterile Erlenmeyer flask for large scale recombinant protein production. The culture was incubated at 37˚C with shaking at 200 rpm (Sony

Gallenkamp, UK) until an OD λ_{600}nm of (0.4-0.6) was reached. Absorbance was measured with spectrophotometer (HITACHI U-1100) as described in Section 2.14.1. To induce over-expression of the recombinant protein in *E.coli*, which is under control of the T7-lac-operon repressor that regulates protein expression, IPTG was added to a final concentration of 1mM after the bacteria reached semi-log phase growth. Incubation was continued at 37˚C with shaking at 200 rpm for the optimal time of expression. The culture was then centrifuged at 5100 rpm (3879 x*g*) for 30 minutes at room temperature (Optima L-90K Ultracentrifuge, Beckman Coulter, rotor 19) to obtain the bacterial pellet, which was stored at -80˚C.

2.11.1 Measurement of optical density (OD) of bacterial suspension.

To check when the bacterial suspension reached semi-log phase, or reached optical density (OD) of 0.5-0.6, after absorbance 1.5 hours of incubation was measured the cell culture. Bacterial suspension (1ml) was removed and placed into a cuvette (Fisherbrand) and was measured with a spectrophotometer UV-Vis (Hitachi, U-1100), absorbance at wavelength λ_{600} nm previously calibrated with a blank standard (SOB broth supplemented with $MgCl_2$ 0.01 M final concentration).

2.11.2 Suspension and lysis of bacterial cell pellet.

2.11.2.1. Non-denaturing conditions (production r-Ng-GapC and mature rNg-ACP proteins).

The cell pellet was frozen and thawed three times at -80˚C and 37˚C for 30 minutes per cycle. A series of buffers was prepared to suspend the pellet for recombinant protein purification as shown in **Table 14**, using a column with Ni-NTA nickel (II)-nitrilotriacetic acid resin for affinity chromatography.

Table 14. Composition of buffers used to purify and elute recombinant protein under non-denaturing conditions.

Buffer	Composition
Lysis Buffer	50 mM NaH_2PO_4 30 mM NaCl 10 mM Imidazole pH8.0
Washing Buffer	50 mM NaH_2PO_4 30 mM NaCl 20 mM Imidazole pH8.0
Elution Buffer	50 mM NaH_2PO_4 30 mM NaCl 250 mM Imidazole pH8.0

2.11.2.2 Denaturing conditions (production of full-length r-Ng-ACP protein).

A series of buffers was prepared to suspend the pellet for recombinant protein purification as shown in **Table 15**, using the same Ni-NTA resin for affinity chromatography. The raison d'etre for these buffers is to purify insoluble proteins by disrupting hydrogen bonds through the use of agents such as guanidine hydrochloride (GdnHCl) or urea. GdnHCl molecules are believed to bind peptide bonds (Roseman, 1975, Robinson and Jencks, 1965) and as the recombinant protein unfolds, more peptide groups are exposed to denaturing molecules (Mayo and Baldwin, 1993, Pace, 1986).

Table 15. Composition of buffers used to purify and elute recombinant protein under denaturing conditions.

Type of Buffer	Composition
Lysis Buffer	100 mM NaH_2PO_4 10 mM Tris-HCl 6 M Guanidine Chloride, pH8.0
Washing Buffer	100mM NaH_2PO_4 10 mM Tris-HCl 6 M Guanidine Chloride, pH6.3
Elution Buffer	100 mM NaH_2PO_4 10 mM Tris-HCl 6M Guanidine Chloride, pH4.5

The pellet was suspended in denaturing lysis buffer, with the volume used calculated from the net weight of the wet pellet. In general, 25-30 ml of lysis buffer was needed to avoid diluting the protein. The lysate was centrifuged (Beckman Coulter Ultracentrifuge) at 5100 rpm (3879 x*g*) for 20 minutes at room temperature to obtain a clarified supernatant (*Supernatant*) and the remaining

pellet was dissolved in the same amount of denaturing lysis buffer and defined as the cell debris (*Cell Debris*).

The *Supernatant* was centrifuged twice at 10000 rpm (9908 x*g*) for 30 minutes to remove any residual inclusion bodies to generate a clarified *Supernatant* for purification. The clarified *Supernatant* was maintained with 3-5 ml of Ni-NTA resin at 4˚C for 1 hour and then added into a column (dimensions 1.2 cm×30 cm) and the total flow-throw was collected into a Universal tube (20-25 ml). Washing buffer (50 ml) was added when the level of the lysis buffer reached the top of the resin, in order to remove any non-specific protein contamination. The wash fractions (4 ml–volume) were collected into plastic Bijoux tubes. Finally, elution buffer (25 ml) was added to the column and eluted fractions (1 ml–volume) were collected into sterile Eppendorf tubes. Next, all the flow-through, wash and eluted fractions were pooled separately, placed on ice and TCA (10% w/v) added to each sample in order to precipitate the protein, for protein purified under denaturing conditions. Subsequently, protein was recovered with 30 µl of PBS buffer containing 0.5% (w/v) SDS and the samples were evaluated by SDS-PAGE, by loading 5 µl of each fraction with 15 µl of loading buffer onto a 12% (w/v) acrylamide–bis-acrylamide gel as described in Section 2.9. For non-denaturing conditions, all the fractions were evaluated by SDS-PAGE.

2.11.3 Purification of r-NgGapC and full-length and mature rNg-ACP proteins.

2.11.3.1. Non-denaturing conditions.

The *Supernatant* obtained from the clarification process was mixed with Ni (II)-NTA resin (5ml, taking into account the binding capacity of resin) for 1h at 4˚C on a rotary mixer. This mixture was loaded into a column (0.7 cm×20 cm) and a volume of 25-30 ml flow-through was collected. The resin was washed with 40 ml of washing buffer (**Table 14**) to remove any non-specific protein binding. Bound protein was eluted with elution buffer (**Table 14**) and 3.0 ml volume samples were collected in Bijoux tubes, retaining 10 µl of each fraction for SDS-PAGE.

2.11.3.2. Denaturing conditions.

The *Supernatant* obtained from the clarification process was mixed with Ni(II)-NTA resin (3.5 ml) for 1h at room temperature on a rotary mixer. This mixture was loaded into a column (0.7 cm×20

cm) and a volume of 25 ml flow-through was collected. The resin was washed with 50 ml of washing buffer (**Table 15**) and bound protein was eluted with elution buffer (**Table 15)** with 1.0 ml volume samples collected in 1.5ml Eppendorf tubes, retaining 30 µl of each fraction for SDS-PAGE analysis prior to precipitation with TCA 10% (w/v) (Section 2.14.4.2.1).

2.11.4 Dialysis and suspension of rNg-GapC and full-length and mature rNg-ACP proteins.

2.11.4.1 Non-denaturing conditions.

2.11.4.1.1 Dialysis with phosphate buffered saline (PBS) solution.

Dialysis membrane (30 cm in length, 6 mm diameter, 10 mm flat width, *Mr* cut off 14 kDa; (Sigma-Aldrich) was boiled twice for 5 minutes in ultra-high quality water (UHQ), to remove sulphate impurities, and then hydrated (10 times) with distilled water and UHQ.The purified rNg-ACP pooled fraction was placed in the dialysis tube, which was tied and placed into a 2 L glass cylinder containing PBS, pH 7.4. This was left at 4˚C overnight and the buffer replaced twice with fresh buffer during the following day, for a period of 72 hours.

2.11.4.2 Denaturing conditions.

2.11.4.2.1 TCA precipitation of the eluted fractions.

One volume (30 µl) of 10% w/v) TCA was added to the eluted protein samples, which had been diluted previously with six volumes of water, and the samples placed on ice for 30 minutes. Samples were then centrifuged (Eppendorf centrifuge 5417R) at 14000 rpm (20800 x*g*) for 1 minute. The pellets were washed with 80% (v/v) ethanol, followed by centrifugation at 20800 x*g* for 5 minutes. The supernatants were removed and the pellets dried at 37˚C for 30 minutes in an incubator. The final pellets were suspended in PBS, pH7.4 containing 0.5% SDS (w/v) and stored at -20 C.

2.12. Bicinchoninic acid (BCA) protein assay.

The bicinchoninic acid (BCA) assay was used to quantify recombinant protein, and consists of a colorimetric reduction reaction between copper (II) (Cu^{2+}) solution and the protein in an alkaline

medium. BCA reagent reaction with reduced copper (I) (Cu^{1+}), product of reaction, is detected by colorimetry and the intensity of the colour reaction is dependent on the concentration of the protein (Smith et al., 1985, Wiechelman et al., 1988). A standard curve was prepared using known concentrations of Bovine Serum Albumin BSA (25-2000 µg/ml) and 25µl of each concentration of serially diluted BSA was placed into duplicate wells of a 96 well ELISA plate (ThermoFisher Scientific) (Appendix A.17.1). Similarly, 25µl of the protein samples, diluted 1/5, 1/10 and 1/20, in UHQ were added to duplicate wells. Dilutions of test samples were estimated from the intensities of protein bands on Coomassie blue-stained SDS-PAGE gels.

The following solutions were used to prepare the colorimetric reaction: Solution A, sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), bicinchoninic acid ($HO_2CC_9H_5N)_2$, sodium tartrate in 0.1M sodium hydroxide (NaOH) and Solution B containing 4% (w/v) cupric sulphate. The ratio of the Solutions A:B was 1:50 and 200 µl of the mixture was added to each well. Negative controls included UHQ alone and protein suspension buffer alone (25 µl volumes).The plate was incubated at 37˚C for 30 minutes and read on an ELISA reader (Biorad, iMark microplate reader) using a filter of λ_{570}nm. Protein concentration was calculated by interpolation from the BSA standard curve.

2.13 Generation of polyclonal antibodies against full-length and mature rNg-ACP proteins.

Purified recombinant proteins were suspended in PBS buffer (mature rNg-ACP), or with addition of 0.5% (w/v) SDS (full-length rNg-ACP). These recombinant Ng-ACP protein suspensions were used for murine immunization trials using a variety of adjuvants and delivery vehicles i.e- Saline alone, Aluminium hydroxide ($Al(OH)_3$), Zwitterion detergent 3-14 (ZW 3-14), Zwitterion detergent 3-14 with MonoPhosphoryl Lipid A (MPLA), Liposomes, Liposomes+MPLA- in order to prepare polyclonal antisera for functional and biological assays. BALB/c mice ($H\text{-}2^d$ haplotype) were used for these immunological trials where each mouse received 20 µg protein intraperitoneally and volumes of each immunisation mixture were adjusted to reflect this, based on the known concentration of the purified rNg-ACP. This immunization was performed by Dr.

Myron Christodoulides and comsisted in a three dose schedule: dose I (day 0), dose II (day 14) and dose III (day 28) followed by terminal bleeding by cardiac puncture on day 42.

2.13.1 Saline formulation.

A solution of sodium chloride (0.9% w/v) was prepared in UHQ water and sterilised with passage through a 0.22 μm filter (Merck Milipore, UK), in a Class II cabinet to maintain sterility. A saline solution was prepared containing 140 μg full-length or mature rNg-ACP (for n=7 mice) and each mouse was immunised (n=5 mice) with a 100 μl containing 20 μg protein. Control animals (n=5) were immunised with saline alone (100 μl).

2.13.2 Aluminium hydroxide (Al $(OH)_3$) formulation.

A mixture of 1 volume of a solution of 140 μg of rNg-ACP full-length or mature in saline solution (total volume 0.7 ml) was mixed with 1 volume (0.350 ml) of aluminium hydroxide (Al $(OH)_3$); Alhydrogel, Superfos) suspension, which was kept at 4˚C. The mixture was placed on a rotary mixer overnight at 4˚C to allow protein adsorption. A control preparation without protein was produced similarly. Each mouse (n=5 mice) was immunised with 100 μl of Al $(OH)_3$ adsorbed protein (containing 20 μg protein) or adjuvant alone (n=5 mice).

2.13.3 Zwitterion Detergent.

A stock solution was prepared with 80 mg of Zwitterion detergent ZW 3-14 (Calbiochem, USA) in 1ml of sterile saline solution. Simultaneously, a solution of full-length or mature rNg-ACP was prepared to give a final concentration of 500 μg/ml in saline solution. Then, the detergent mixture was prepared by adding 100 μl of ZW 3-14 stock solution (to give a final concentration of 8 mg /ml) with the volume containing 500 μg/ml full-length or mature rNg-ACP, and the final volume adjusted to 1ml with sterile saline. This mixture was kept at room temperature overnight to allow micelle formation; the stock was diluted to a concentration of 140 μg/700 μl with sterile saline and each mouse (n=5) was immunised with 100 μl of ZW 3-14 -protein solution (equivalent to 20 μg protein) or control ZW 3-14 solution alone, prepared without the addition of protein (n=5 mice). Unused solution was stored in aliquots at -20 C for subsequent immunisations.

2.13.4 Zwitterion Detergent with MonoPhophoryl Lipid A (MPLA).

MonoPhosphoryl Lipid A (MPLA) from *Salmonella enterica* serotype Minnesota (1mg, Sigma-Aldrich) was suspended in saline solution to a final concentration of 1 mg/ml. A solution of (8mg/ml) ZW 3-14 (Section 2.13.3) was added 500 µg MPLA (0.5 ml), 500 µg of full-length or mature rNg-ACP and the volume adjusted to 1ml with saline solution. The solution was kept at room temperature overnight. Each mouse was immunised with 20µg full-length or mature rNg-ACP in ZW 3-14 + 20 µg MPLA and control animals (n=5) with the same mixture without protein.

2.13.5 Liposomes.

Liposomes were prepared following the protocol described by (Ward et al., 1996) with the following steps:

2.13.5.1 Formation of the lipid shell.

All glassware used for liposome manufacture (round-bottomed flasks, Bijoux bottles) were soaked in concentrated 70% (v/v) nitric acid overnight followed by exhaustive washing in tap water followed by distilled water and dried in a hot oven (160˚C) to remove all moisture.

The lipid shell is comprised of L-α-phosphatidylcholine, *Mr* 768 (Sigma-Aldrich) and cholesterol, *Mr* 386.6 (Sigma-Aldrich). The ratio of these components in the mixture was 7:2 L-α-phosphatidylcholine: cholesterol for a total of 2 mg. L-α-phosphatidylcholine (87.5 µl of 100 mg/ml stock) was added directly to a round-bottomed flask to which was added cholesterol (125 µl of 10 mg/ml cholesterol solution dissolved in chloroform (BDH, UK). The final volume was adjusted to 3 ml by the addition of chloroform.

A rotary evaporator, with a water bath set at 25˚C (Buchi, Rotavapor, Switzerland), was used to evaporate the solvent and create the dried lipid shell on the inner surface of the glassware. The shell was kept at room temperature until used.

2.13.5.2 Preparation of rNg-ACP-octyl-β-glucopyranoside solution.

A 10 mM HEPES (Sigma-Aldrich) pH7.2 buffer was used to dissolve 50 mg of octyl-β-glucopyranoside (Sigma-Aldrich). The mixture was incubated at room temperature for 3 hours. In

the liposome formulation which contains recombinant protein, full-length or mature rNg-ACP was suspended in PBS with 0.5% (w/v) SDS or PBS respectively to a final concentration of 500 μg/ml, or containing MPLA to a final concentration of 500 μg/ml.

2.13.5.3 Protein-lipid shell.

Prior to addition of protein or control solutions, the lipid shell was examined to confirm that there was no solvent residue and that a multi-layered shell of lipid was produced around the inside of the flask. The protein-MPLA-octyl-β-glucopyranoside solution or control octyl-β-glucopyranoside-MPLA solution was added and used to suspend the lipid shell with constant manual agitation. The mixture was left at room temperature for 1 hour.

2.13.5.4 Dialysis and sonication.

Dialysis was used to remove detergent and SDS; the solution was added to dialysis tubing (0.63 cm diameter, Sigma-Aldrich), which had been boiled previously as described above in 2.11.4.1.1 The samples were dialysed against PBS, pH 7.4 containing 0.0001 % (w/v) thimerosal as a preservative, at 4˚C for 72 hours with two changes of buffer daily. The dialysed samples were transferred to Bijoux tubes and small unilamellar liposomes were prepared by sonication using a MSE Soniprep 150 probe sonicator. Each cloudy liposome mixture was sonicated for 30 seconds, 15-20 times on ice at amplitude of 10-15 micron until the solution was cleared. The final volume of liposome preparations was quantified and the samples stored at -20˚C.

2.13.6. Freund's Adjuvant.

Freund's adjuvant is a water-in-oil emulsion. This formulation differs in composition between the primary and subsequent immunizations -where Freund's Complete Adjuvant (FCA) is used for the former and Freund's Incomplete Adjuvant (FIA) is used for the latter. FCA contains dried *Mycobacterium tuberculosis* in the oil. rNg-ACP protein (100μg of full-length rNgACP) was mixed with an equal volume of FCA/FIA and emulsions produced by repeated passage of the solution through 25 gauge needles until a thick suspension was produced. Rabbits (n=2) were immunised subcutaneously by Davids Biotechnologie, (GmbH, Germany) using a five dose schedule: dose I (day 1), dose II (day 14) and dose III (day 28), dose IV (day 42), dose V (day 56)

followed by terminal bleeding by cardiac puncture under anaesthesia on day 63. A pre-immune bleed was also provided.

2.13.7. Preparation of murine and rabbit sera.

Individual murine sera were collected in sterile 7 ml glass Bijoux (Sarstedt) and labelled with the respective number or code system.

Murine serum were treated with the following procedure: i) An incubation for 1 hour at 37˚C, to allow clotting, followed by ii) An over-night storage at 4˚C. The serum was removed from the Bijoux bottles with aid of 100 µl pipette tip (Alpha Laboratories) avoiding taking any clotted blood. The serum samples were centrifuged at 4000 rpm (1434 x*g*) for 4 minutes to remove any clotted blood remaining, and then the cleared supernatants were transferred to another Eppendorf tubes labelled with numeric system of each animal. Finally sera were aliquot (75-100 µl) into 500 µl Eppendorf tubes for further biological experiments.

For pre-bleed and post–immune rabbit serum was aliquoted 1ml volumes were aliquoted into a 1 ml Eppendorf tubes for biological additional experiments. The volume of pre-immune serum provided provided was one 15 ml falcon tube (~14 ml) per animal and terminal bleed serum with two 50 ml falcon tubes (~96 ml) per rabbit.

2.14. Generation of *N .gonorrhoeae Δng-acp* knockout mutant strain.

In order to supress expression of Ng-ACP (the product of the *ng-acp* gene) the technique of knockout by homologous recombination and insertion of an antibiotic resistance cassette for selection of candidates was used. The knockout construct consisted of different steps of amplification of each fragment and insertion into a plasmid, as shown in **Figure 10**. This construct was used to transform *N. gonorrhoeae* and lead to chromosomal integration by a homologous recombination process.

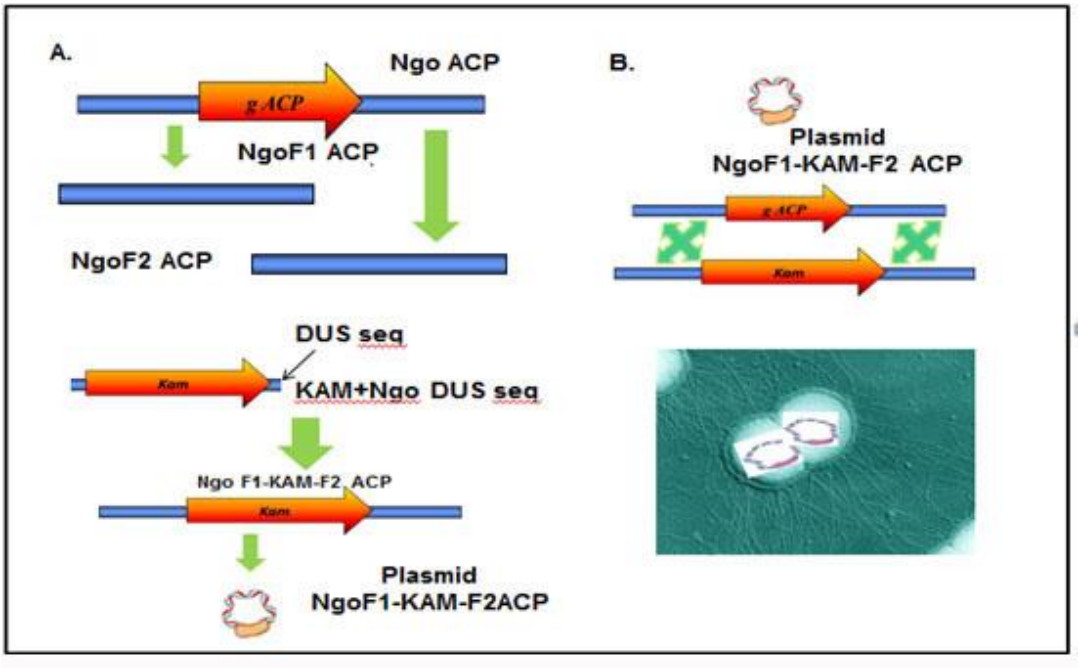


Figure 10. General scheme for knockout construct and insertion of the DNA fragment into *N. gonorrhoeae*. **A)** Generation of PCR products for regions up- (NgoF1 ACP) and down- (NgoF2 ACP) stream of ng-acp gene (Top left panel). Generation of kanamycin (Kam) fragment with addition of DNA uptake sequence (DUs) in both ends (KAM+NgoDUS seq). Ligation PCR fragments up-stream (F1), kanamycin fragment (KAM) and down-stream (F2) of Ng-ACP to generate (NgoF1-KAM-F2 ACP). This construct was inserted into a plasmid (pGEM cloning vector). **B)** Homologous recombination for insertion of foreing DNA into the region encoding and non encoding of ng-acp gene (Top right panel). Insertion of knocokout construct into *Neisseria gonorrhoeae* using spot and liquid transformation (bottom right panel).

2.14.1 PCR amplification of up-down-stream fragment of *ng-acp* gene.

To amplify the fragments up- and down-stream of the *ng-acp* gene (**Figure 11**), a pair of primers was designed (**Table 16**) to cover the fragments and include a sequence for the restriction enzyme *XbaI* (restriction site $^{5'}$CTAGA$^{3'}$) for assembly of the construct fragments.

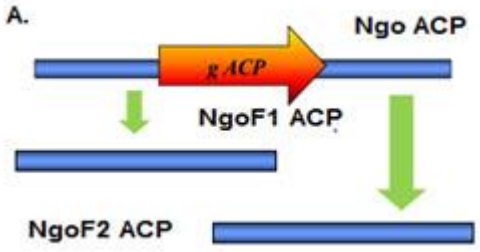


Figure 11. General scheme for amplification of fragments up- (F1) and down-stream (F2) of *ng-acp* gene to generate a *knockout* construct in *Neisseria gonorrhoeae.*

Table 16. Sequence primer design amplify up-stream *ng-acp* gene (Fragment 1, F1).

Primer	Sequence
Forward	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse	5'- GGCTAT**TCTAGA**TTTTATTCCTTTGGATAGATG-3'

The PCR amplification used a 2xPhusion Master mix (Section 2.3) with the parameters of annealing temperature (55˚C) and extension time (14 seconds) to amplify the up-stream fragment of *ng-acp* gene (**Table 17**), using genomic DNA from *N. gonorrhoeae* strain P9-17 as a template.

Table 17. PCR conditions used to amplify *Neisseria* Fragment 1.

Step	Temperature (˚C)	Time (sec)	
Denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	55	30	
Extension	72	14	
Extension	72	300	

Similarly, the down-stream fragment of *ng-acp* gene (**F2**) was amplified from gonococcal genomic DNA using PCR (**Table 18** and **Table 19**).

Table 18. Sequence Primer designed to amplify down-stream *ng-acp* gene (Fragment 2, F2).

	Sequence
Forward Primer	5'-GGCTAT**TCTAGA**TCAGGCAACAAAAAACAGCG -3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC-3'

Table 19. PCR conditions used to amplify gonococcal down-stream *ng-acp* gene fragment F2.

Step	Temperature (˚C)	Time (sec)	
Denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	63	30	
Extension	72	14	
Extension	72	300	

Kanamycin antibiotic resistance cassette from **APCYC177** was kindly provided by New England Biolabs. The antibiotic resistance fragment was amplified in order to insert between the up (**F1**) and down (**F2**)-stream fragments of *ng-acp* gene as a part of selection of transformants. A *Neisseria* DUS sequence (Duffin and Seifert, 2010, Elkins et al., 1991) (**Figure 12**) was also inserted to enable homologous recombination and insertion into the *N. gonorrhoeae* genome (**Table** 20).

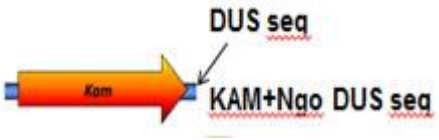


Figure 12. Kanamycin fragment (KAM), including DNA sequence up-take (DUS).

Kanamycin antibiotic resistance cassette was amplified by PCR using the set of primers and conditions as described in

Table 20. The sequence coloured in red is the restriction site *Xba***I** (TCTAGA) and in blu is the DUS sequence for *N. gonorrhoeae* (TTCAGACGGC) and using the PCR parameters from **Table 21**, this fragment is located between up- and down-stream fragments of *ng-acp* gene, would, generate a knockout construct in *N. gonorrhoeae.*

Table 20. Primer sequences designed to amplify the kanamycin cassette gene (*kam*).

	Sequence
Forward Primer	5'-GGT**TCTAGATTCAGACGGC**GTGATCTGATCCTTCAACTC-3'
Reverse Primer	5'- GGT**TCTAGA**TTAGAAAAACTCATCGAGCATC-3'

The sequence couloured in red is the restriction site *Xba*I (**TCTAGA**) and in blue is the DUS sequence for *N. gonorrhoeae* (**TTCAGACGGC)**.

Table 21. PCR conditions used to amplify the kanamycin cassette gene (*kam*).

Step	**Temperature (˚C)**	**Time (sec)**	
Denaturation	98	30	
Denaturation	98	10	30 cycles
Annealing	54	30	
Extension	72	29	
Extension	72	300	

2.14.2. Ligation and amplification Fragment 1 and 2 (F1-F2).

2.14.2.1 Digestion with XbaI of up- and down-stream fragments.

Following amplification, the fragments were purified with a Wizard clean up kit (Promega) (Section 2.6.2). To ligate fragments F1 and F2 they were digested separately using restriction enzyme *Xba*I (Section 2.6.4) using the reagents described in **Table 22**.

Table 22. Digestion reaction with restriction enzyme *Xba*I of fragments F_1 and $F_{2..}$

Reagents	Fragment 1 (µl)	Fragment 2(µl)
Purified PCR product	39	39
Buffer D 10 X	5	5
BSA 10%	5	5
*Xba*I	1	1

The digestion mixtures were incubated at 37˚C for 3 hours to generate single bands, which were separated by agarose gel electrophoresis (Section 2.6.6) and DNA was purified from excised gel bands using a Wizard PCR clean up kit (Promega) (Section 2.6.2).

2.14.2.2. Ligation of up- and down-stream fragments.

The concentration and purity of each purified fragment was examined by agarose gel electrophoresis and Nanodrop spectrophotometry.The ratio between each fragment was estimated for the ligation reaction using a T4 ligase enzyme (Promega) as shown in **Table 23**.

Table 23 Ligation reaction conditions between gonococcal *ng-acp* gene's up- and down-stream fragments.

Reagent	Volume (µl)
Fragment 1	8
Fragment 2	8
T4 Buffer	1
T4 DNA ligase	1

Using the parameters and conditions described in Section 2.6.5, the mixture was kept overnight at 4˚C to obtain maximum efficiency. A PCR reaction with GoTag Green Master mix (Promega) and

primers (**Table 24)** and reaction conditions (**Table 25**) was used to increase the proportion of amplified ligation product F1-F2. This PCR reaction was clone to verify the efficiency of ligation process of gonococcal *ng-acp* gene up- and down- stream fragments.

Table 24. PCR primers used to amplify fragments up- and down-stream (F1 and F2) ligation product.

Primer	Sequence
Forward Primer F1	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer F2	5'-GGTACGGAGATTGTCGCCC -3'

Table 25. PCR conditions for amplifying up- and down-stream fragments F1 and F2 ligation product.

Temperature (˚C)	Time (seconds)	
95	30	
95	10	30 cycles
56	30	
72	60	
72	300	

2.14.2.3. Insertion of up- and down-stream fragments F1-F2 into pGEM cloning vector.

Next, the new insert, ligated F1 -F2, was purified using the PCR Clean Up kit (Promega) and inserted into the *pGEM* cloning vector, which is an open vector with TOPO system enabling the insertion of the insert in different directions and avoiding circularization of plasmid. Activation or induction of the promoter, *lacZ operator*, in the presence of IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) demonstrates β-galactosidase activity, which generates cells of characteristic blue/white colour, and is used to select bacteria containing the insert. In bacteria, the inserted construct interrupts the expression of the *LacZ* operator, generating colonies without colour. The ligation reaction is described in **Table 26**.

Table 26. Ligation conditions for cloning of fragment 1 and 2 (F1-F2) into pGEM vector.

Reagent	Volume (µl)
2X Rapid buffer ligase	5
pGEM cloning vector	1
Insert	3
T4 DNA ligase	1

2.14.2.4 Transformation (pGEM-F1F2) in E.coli strain GM2163.

The new ligation reaction was transformed into *E. coli GM2163* strain, through chemical transformation (Section 2.6.2) and then harvested on selective LB agar plates containing 0.1 mg/ml ampicillin, 80 µg/ml XGal and 0.5 M IPTG. The main characteristic of *E. coli GM2163* strain is that it is a chemically-competent *E. coli* strain that is methyl transferase deficient and suitable for expansion of plasmids free of *dam* and *dcm* methylation. The reason for using this strain is that the restriction enzyme *Xba*I is susceptible to *dam* methylation.

Positive transformants selected, by colour were evaluated by colony PCR using the Go Tag Master Mix (Promega), in order to screen for the correct orientation and insertion of the fragment. The following parameters shown in

Table 27 and Table **28** confirmed the expected single band of ~800bp.

Table 27. Primers used in PCR colony screening of the transformation products.

	Sequence
Forward Primer	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC -3'

Table 28. PCR conditions for colony screening transformation products.

Temperature (˚C)	Time (seconds)	
95	30	
95	10	30 cycles
56	30	
72	60	
72	300	

2.14.2.5. Insertion of kanamycin cassette into pGEM (F_1-F_2) construct

From the selected colonie(s) containing the appropriate open reading frame (ORF) of the insert, the plasmid was extracted and purified by Wizard MiniPrep kit (Promega) (Section 2.6.3) and the product(s) evaluated by 1% (w/v) agarose gel electrophoresis to estimate the concentration of the construct (Section 2.6.6 and Section 2.6.7) . Then, the plasmid was digested with the *XbaI* restriction enzyme using the recipe described in **Table 29** (Section 2.6.4.) and then was treated with alkaline phosphatase to avoid circularization (**Table 30**), in order to insert the kanamycin cassette to obtain the final construct (**Table 31).**

Table 29. Digestion reaction with *Xba*I restriction enzyme of construct pGEM (F_1 and F_2).

Reagents	**Volumen (µl)**
Purified PCR product	39
Buffer D 10 X	5
BSA 10% w/v	5
*Xba*I	1

Table 30. Alkaline phosphate treatment of the construct pGEM (F_1 and F_2).

Reagents	**Volumen (µl)**
Alkaline Phosphate	8
10X Buffer	8
BSA 10% w/v	8
UHQ water	6

Table 31. Digestion reaction with *Xba*I restriction enzyme of kanamycin cassette (*kam*).

Reagents	**Volumen (µl)**
Purified PCR product	39
Buffer D 10 X	5
BSA 10% w/v	5
*Xba*I	1

Similarly, the construct (F1-F2)-pGEM was digested with the same conditions described for the kanamycin cassette (**Table 31**). Both digestion reactions were incubated for 3 hours at 37˚C and were then purified by Wizard CleanUp kit (Promega) (Section 2.6.3) and the quality and concentration were evaluated by 1% (w/v) agarose gel electrophoresis (Section 2.6.6). Ligation of the kanamycin fragment and (F1-F2)-pGEM construct was carried with the following parameters in **Table 32** with the reaction kept overnight at 4˚C, for high efficacy to obtain the maximum number of transformants.

Table 32. Conditions of ligation of fragments pGEM(F_1-F_2) construct with kanamycin cassette.

Reagent	Ligation Reaction	Negative control
pGEM(F_1-F_2) construct	1	1
kanamycin cassette	7	---
T4 Buffer	1	1
T4 DNA ligase	1	1
UHQ water	---	7

The final construct was transformed into *E. coli DH5α,* through chemical transformation as described in Section 2.6.8.2, and finally plated out onto selective LB agar plates containing 0.1 mg/ml ampicillin.Single colonies containing the correct orientation of the insert (F_1-kam-F_2) was done by colony PCR with the reagents and reaction conditions outlined in **Table 33** and **Table 34** The expected band is ~1800 bp in size.

Table 33. Primers used in colony PCR screening of the final transformation product (F_1-Kkam-F_2).

Primer	Sequence
Forward Primer	5’-TAGACTTCTGGGGCAAGGTC-3’
Reverse Primer	5’-GGTACGGAGATTGTCGCCC -3’

Table 34. Colony PCR screening conditions of final transformation product (F_1-Kam-F_2).

Temperature (˚C)	Time (seconds)	
95	30	
95	10	30 cycles
56	30	
72	120	
72	300	

2.14.3 Generating a *Neisseria gonorrhoeae Δng-acp* knockout mutant.

2.14.3.1 Transformation in Neisseria gonorrhoeae.

From an overnight lawn culture of *N. gonorrhoeae* strain P9-17, bacterial growth was removed with a loop and the colonies were suspended in GC broth with Supplements A and B (Appendix A.8.1.1 and A.8.1.2). The appropriate phenotype ($Pili^+Opa^+$) was selected with a stereo-microscope. The bacteria can be suspended in GC broth supplemented with 5 mM $MgCl_2$ or plated out directly onto the surface of an agar plate to contact DNA directly in the presence of 5 mM $MgCl_2$. Homologous recombination occurs, leading to insertion of the construct into the genome along with the gonococcal DUS sequence (Duffin and Seifert, 2010).

2.14.3.1.1 Spot Transformation in *Neisseria gonorrhoeae.*

The method described by (Ilina et al., 2013) was used. *N. gonorrhoeae* bacteria were grown on supplemented GC agar plates as described in Section 2.1. On the bottom of a GC agar plate, two circles of ~1.5 cm in diameter were drawn, and 10-20 µl of 1000 ng/µl of ice-cold PCR product or plasmid in GC Broth with 5 mM $MgCl_2$ were added onto the agar inside the circles and allowed to dry. A few single colonies were then streaked across the circles and the plates incubated at 37˚C with 5% (v/v) CO_2 overnight. Bacterial growth on the plates was swabbed from the surface and suspended in 200 µl of GC Broth (not necessarily supplemented), from which 10-fold serial

dilutions were made in GC Broth (10^0-10^{-4}). Each serial dilution (100-200 µl) was then applied to fresh GC agar and selective GC agar plates (antibiotic resistance) and the plates incubated at 37˚C with 5% (v/v) CO_2 overnight. Single colonies were then selected from these plates and streaked out onto another selective agar plate for evaluation by PCR and western blot to determine presence/absence of the gene/gene product.

2.14.3.1.2 Liquid transformation in *Neisseria gonorrhoeae.*

The protocol described with *N. meningitidis* by Hung *et al.* (2013) was used similarly on *N. gonorrhoeae*. Gonococci were grown overnight on GC agar plates at 37˚C with 5% (v/v) CO_2. A suspension of ~1×10^8 CFU bacteria was made in 1 ml of GC broth containing 5 mM $MgCl_2$. The bacterial suspension (200 µl) was added into the wells of a sterile 24– well tissue culture plate (Greiner Bio-one, UK) followed by ~1000 ng/µl of PCR product or plasmid. The plate was then incubated for ~20-30 min at 37˚C with 5% (v/v) CO_2. At the same time, supplemented GC broth was pre-warmed at 37˚C and 1.8 ml added into each well. The plate was incubated for 4 h at 37˚C with 5% (v/v) CO_2. . From each well, 100-200 µl of bacterial suspension was plated onto selective GC agar plates followed by incubation for 24-48 h. Single colonies growing on these plates were selected onto fresh selective agar media for evaluation by PCR and western blot to determine presence/absence of the gene/gene product.

It should be noted that the rate of growth of each gonococcal strain varied on the selective agar plates. Single colonies could be obtained between 1-3 days of incubation and it was necessary therefore to prepare control (wild-type gonococcal strain) on selective GC agar plates (GC + 0.1 mg/ml of kanamycin).

2.14.3.2 Evaluation of Neisseria gonorrhoeae transformants.

PCR was used to confirm the presence/absence of the *ng-acp* gene and western blot was used to confirm the presence /absence of the protein product, Ng-ACP.

2.14.3.2.1. Colony PCR.

The presence/absence of *ng-acp* gene was determined by using either the primers used for determining *ng-acp* gene or primers for amplifying up- and down-stream of the gene (F1 and F2)

(**Table 35**) and analysing the differences in size compared with the wild type strain following PCR using the conditions outlined in **Table 36.**

Table 35. Primers used in colony PCR screening of the final transformation product (F_1-kam-F_2).

	Sequence
Forward Primer	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC -3'

Table 36. Colony PCR conditions to amplify knockout transformation product (F_1-kam-F_2).

Temperature (˚C)	**Time (seconds)**	
95	30	
95	10	30 cycles
56	30	
72	120	
72	300	

2.14.3.2.2. Western blot (WB).

For each transformant bacteriawere swabbed from lawn growth and suspended in 0.5-1 ml of PBS. Protein concentration of the cell lysate was quantified by BCA (Section 2.12). Each sample was loaded~100-250 µg/ml into a bis-acrylamide gel and separated by SDS-PAGE electrophoresis (Section 2.9). The bis-acrylamide gel was transferred into a nitrocellulose membrane as described in Section 2.18. Ng-ACP protein expression on each sample was detected with mouse serum raised to full-length rNg-ACP-Al $(OH)_3$ (1/100 dilution) and (1/3000 dilution) anti-mouse with alkaline phosphate conjugated (Biorad) as a secondary antibody.

2.15. Complementation *ng-acp* gene in knockout gonococcal transformants (*Δng-acp*).

In order to determine the recovery of biological function of association on epithelial cells mediated by Ng-ACP protein, gonococcal knockout transformants were complemented using pGCC4 cloning vector kindly provided by Hank Seifert's laboratory (Feinberg School of Medicine, Northwestern University) (Mehr and Seifert, 1998). This cloning vector contains a *lac* operator and enables the

expression of *ng-acp gene* with the induction with IPTG. Also, pGCC4 cloning vector has a kanamycin (*kam*) and an erythromycin (*erth)* antibiotic resistance marker for selection (**Figure 13**).

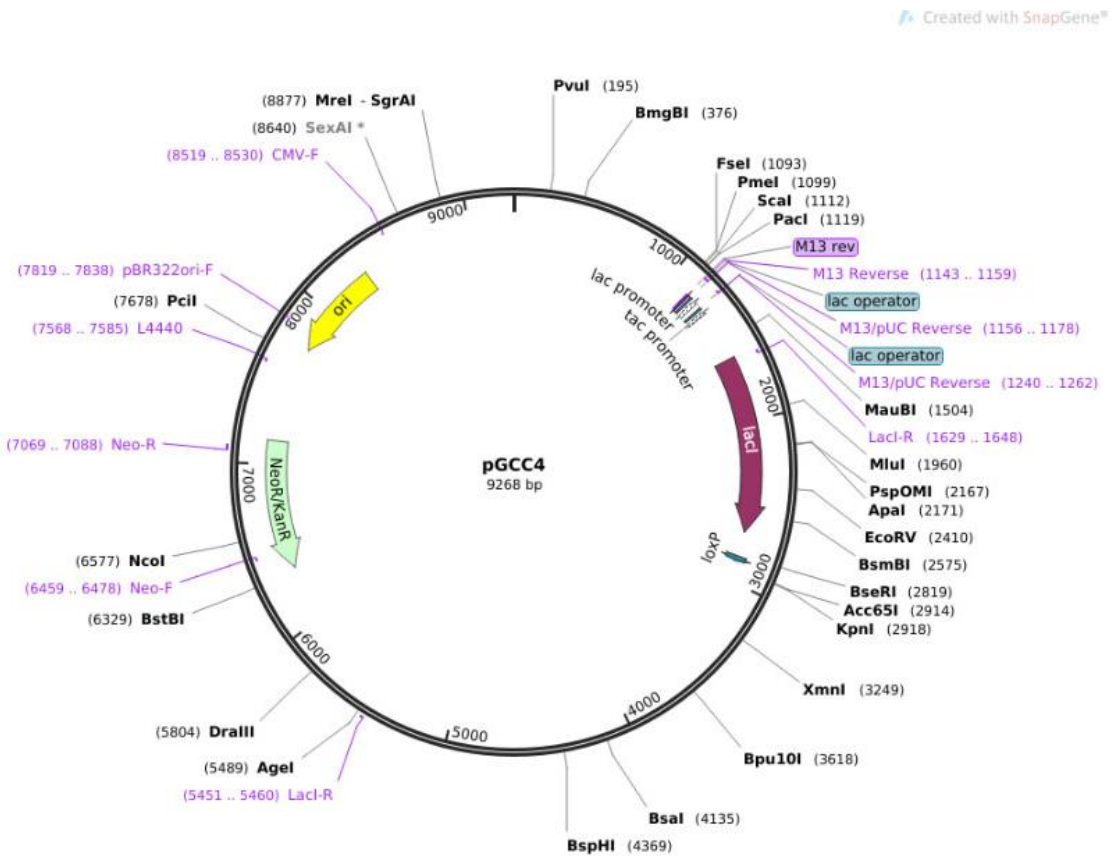


Figure 13. Cloning map pGCC4 plasmid for gonococcal transformation.

Extracted from Addegene website (https://www.addgene.org/37058), Hank Seifert lab group (Feinberg School of Medicine, Northwestern University).

2.15.1. Primer design.

Based on the *ng-acp* gene sequence from *Neisseria gonorrhoeae* strain P9-17 and FA1090, a set of primers was designed to construct a complemented strain to evaluate the recovery of adhesion or other biological properties action of this protein. Both primers contain an overhang sequence 5'-**GGCTA-3'** as well as, restriction site sequences from the multiple cloning site (**Figure 13**) of pGCC4 plasmid for this primers ***PacI*** 5'-TTAATTAA-3' and ***PmeI*** 5'-GTTTAAACT-3' and summarised on **Table 37** which enables the insertion of *ng-acp* gene into the complementation plasmid.

Table 37. Set of primers sequences for complemented strain.

	Sequence
Forward primer Com-gACP	5'-GGCTATTTAATTAAATGAAACTTCTGACCAC-3'
Reverse primer Com –gACP	5'-GGCTATGTTTAAACTTAACGTGGGGAACAG-3'

The underlined sequences represent the restriction sites for *PacI* (**TTAATTAA**) and *PmeI* (**GTTTAAACT**).

2.15.2. PCR amplification for *ng-acp* complementation.

The 2XPhusion mix, with annealing temperature of 62˚C and extension time of 45 seconds, as described in **Table 38** was used to amplify the *ng-acp* gene from genomic DNA from *N. gonorrhoeae* strains P9-17 and FA1090**.**

Table 38. PCR steps to amplify *ng-acp* gene for complemented strain.

Temperature (˚C)	**Time(seconds)**	
98	30	
98	10	30 cycles
62	30	
72	45	
72	300	

2.15.3 Generation of gonococcal complemented (*¢Δng-acp*-pGCC4) construct.

Next the PCR product and cloning vector were double-digested using *Pac*I and *Pme*I restriction enzymes following the protocol described in Section 2.6.4. The digested products were purified and evaluated by 1% (w/v) agarose gel electrophoresis as described in Section 2.6.6 and 2.6.2. The ligation reaction between those fragments was prepared with a 1:2 (vector: insert) ratio to increase the efficiency of ligation (Section 2.6.5). Transformation of the ligation product in *E. coli* strain DH5α was done as described in Section 2.6.8.2. The trasnformants candidates were tested by PCR using the primers used in Section 2.15.1 and with the parameters described in Section 2.15.1.

Plasmid were extracted from potential transformants, as described in Section 2.6.10.2, sequenced and analysed by Segman software (Lasergene, DNASTAR).

2.16 Preparation of *Neisseria gonorrhoeae* lysates and outer membranes (OM).

2.16.1. Lysate.

N. gonorrhoeae homologous strains P9-17 and MS11, heterologous *N. gonorrhoeae* strain FA1090 and the knockout mutants *N. gonorrhoeae* strain P9-17*Δng-acp* and *N. gonorrhoeae* strain FA1090 *Δng-acp*, were grown as lawns on GC agar and growth was removed with a loop and each suspended in 1ml of UHQ. A bicinchoninic assay (BCA) assay was used to quantify protein concentrations as described in Section 2.12.

2.16.2. Preparation of Outer Membranes (OM).

A single plate of P9-17 was grown overnight from liquid nitrogen stock onto a GC agar plate at 37˚C with 5% (v/v) CO_2. Gonococci of the appropriate phenotype (Pili $^+$ Opa$^+$) were selected using a stereo-microscope and plated onto six fresh GC agar plates. Following 18-20 hours incubation at 37˚C with 5% (v/v) CO_2, the bacterial growth from each plate was removed and suspended in GC broth (~1 ml) and 200 µl of this solution distributed onto six large GC agar plates (15 cm diameter). All plates were incubated at 37˚C with 5% (v/v) CO_2 overnight. Each agar plate was then analysed visually for culture purity and the bacterial growth removed and placed into 50 ml of 0.2 M lithium acetate ($LiCH_3COO$), pH 5.8 buffer to which was added ~10 ml of sterile glass beads. The bacterial suspension was then placed in a water bath with a magnetic stirrer plate and incubated at 45 ˚C for 3 hours with continuous stirring.

The supernatant was removed and centrifuged three times (type 52 centrifuge tubes, Beckman coulter) at 10000 rpm (12063 x*g*) for 30 minutes to remove cell debris. The resulting clarified supernatant was centrifuged at 37000 rpm (25000 x*g*) for 2 hours, the supernatant then discarded and the pellet was suspended in 1ml of UHQ water. Protein content was quantified by BCA assay as described in Section 2.12 and the OM preparation stored at -20 °C.

2.16.3 Preparation of sodium deoxycholate extraction Outer-Membrane Vesicles (Na-Doc-OMV).

A buffer was prepared (50 ml) solution composed of 2% (w/v) sodium deoxycholate (Sigma-Aldrich) with 2 mM Tris-HCl (Fisher) and 20mM EDTA (Fisher) with final pH of 8.5. The solution was filtered-sterilised with 0.22 μm filter (Merck-Millipore).

From the OM preparation of *N. gonorrhoeae* strain P9-17 described in Section 2.16.2, ~270 μl was taken and diluted with UHQ to a final concentration of 5mg/ml and afinal volume of 5ml. Next, 0.5 ml of 2% (w/v) sodium deoxycholate buffer was added and incubated at room temperature for 30 minutes. The sample was centrifuged at 37,000 rpm (100,000 x*g*) on a Beckman Coulter Optima Max Ultracentrifuge with TLA-55 rotor at 4˚C for 4 hours. The supernatant was then removed and collected in an Eppendorf tube. The cell pellet was suspended with 1% (w/v) sodium deoxycholate buffer and centrifuged at the same conditions above. Both supernatant and pellet were kept in 1.5 ml sterile Eppenford tubes. The cell pellet was suspended with 0.5 ml of UHQ water for protein quantification by BCA assay (Section 2.12).

2.17. Enzyme-linked immunosorbent assay (ELISA).

The enzyme linked immunosorbent assay (ELISA) is a sensitive technique for quantitative detection of antigens/molecules using specific antibodies and an enzyme-based colorimetric reaction. Primary antibodies are molecule-specific and the secondary antibodies are also specific but labelled with an enzyme, usually horseradish peroxidase that acts on a specific substrate 3,3',5,5- tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H_2O_2) to produce colour that can be quantified on a spectrophotometer. Absorbance is proportional to the amount of the antigen/molecule present. This protocol comprises the following steps: immobilization of protein, incubation with primary (test) antibody, incubation with secondary antibody and colour development.

i) Reagents

Coating buffer (10X): 35 mM sodium bicarbonate ($NaHCO_3$) and 15 mM sodium carbonate (Na_2CO_3), prepared in UHQ, pH 9.6

Wash buffer: 0.15 M sodium chloride with 0.05% (v/v) Tween 20.

Antibody diluent: 50 mM Tris buffer containing 0.15 M sodium chloride, 0.05% (v/v) Tween 20 and 1.0% (w/v) Bovine Serum Albumin (BSA), adjusted to pH 7.4 using acetic acid.

Anti-Mouse horseradish peroxidase (HRP) conjugated antibody (Invitrogen, life technologies)

Anti-rabbit horseradish peroxidase (HRP) (ZYMED, Invitrogen, life technologies)

Color substrate:

0.1 M sodium acetate buffer (100 ml), TMB solution (1ml), hydrogen peroxide (10 µl) mixed before use.

A. **0.1M sodium acetate buffer**, adjusted to pH 6.0 with 1 M citric acid.
B. **3,3',5,5'- tetrametyl benzidine (TMB)** 3.6 mg of TMB was dissolved in 1 ml of dimethyl sulphoxide (DMSO, BDH,UK)
C. **Hydrogen peroxide** (H_2O_2), 30% v/v (Merck)

Stopping solution: 1 M sulphuric acid (H_2SO_4)

ii) Immobilization of protein.

The recombinant protein/antigen/OMV/Na-Doc OMV preparation was prepared in coating buffer (1X) to a concentration of 1 µg/ ml with the addition of sodium azide (0.05% w/v) to prevent contamination. The coating solution (100 µl) was added to each well of a 96-well ELISA microtitre plate (Thermo Scientific), which was incubated at 37˚C overnight in a humid chamber. On the following day, the plate was washed three times with wash buffer and dried by blotting on absorbent paper.

iii) Blocking non-specific binding.

Non-specific protein binding was blocked by the addition to each well of 100µl of Antibody diluent with incubation at 37˚C for 1 hour in a humid chamber. The blocking buffer was removed and the plate dried, without washing.

iv) Interaction with primary (test) antibody.

Serial dilutions of test antibody were made in Antibody diluent buffer and 100 µl of each dilution was added to duplicate wells followed by incubation at 37˚C for 1 hour in a humid chamber. The plate was washed four times and dried.

v) Interaction with secondary antibody.

To each well was added the appropriate anti-species horseradish peroxidase conjugate antibody (100 µl/well, suggested dilution of 1/2000, prepared in Antibody diluent), followed by incubation at 37˚C for 1 hour. The plate was then washed four times and dried.

vi) **Colour reaction development.** Colour substrate **(**100 µl) was added to each well and the plate was incubated at room temperature for 10 min avoiding exposure to direct light. The colour reaction was stopped by the addition of 50 µl/well of stopping solution (1 M H_2SO_4).

vii) Each plate was measured the Absorbance at λ_{450}nm on an ELISA iMark reader plate (Biorad).

viii) Statistical Analysis. Titres was obtained from a lineal regression of titration curve of each sample serum and extrapolated from the absorbance background from the controls. Then was calculated GEOMEAN for each group of animals tested and 95% confidence limits (CL) and represented a bar chart. Comparison of titres obtained from different groups was performed a t-student analysis.

2.18. Western Blot (WB).

Reagents.

SDS-PAGE electrophoresis (Section 2.9): Gonococcal lysate and OM/OMV preparations were loaded at 150-200 μg in a single well, to compare the reactivity of antisera to rNgACP with all range of formulations generated. For determination of expression or suppression of Ng-ACP, gonococcal candidate lysate extract were loaded at 15 μg to 20 μg per well.

Blotting buffer: To prepare 200 ml, mix 160 ml of 1X running buffer (Appendix 9.5) were mixed with 40 ml of 100% methanol (Fisher Scientific).

Washing Buffer, Tris Buffer Solution (TBS): TBS was prepared by dissolving 29.54 g of sodium chloride (NaCl) and 2.42 g of Tris –hydrochloride in 1 litre of distilled water and pH adjusted to 7.5 with NaOH.

Tween 20-Tris Buffer Saline Solution (TTBS):TTBS was prepared by dissolving 29.24 g of sodium chloride (NaCl), 2.42 g of Tris –hydrochloride, adding 0.05% (v/v) Tween 20 in 1 litre of distilled water and pH adjusted to 7.5with NaOH.

Blocking Buffer: To 10 ml of TTBS buffer was added 0.5 g (5% w/v) of skimmed milk powder.

Substrate buffer: Substrate buffer was prepared by dissolving 3.025 g of Tris-hydrochloride, 1.46 g of NaCl and 0.101 g of $MgCl_2$ in 250 ml distilled water, and pH adjusted to 9.5 with HCl.

NBT (nitrobluetetrazolium) stock solution: NBT solution was prepared by weighing 0.06 g NBT and dissolving in 2 ml of 70% (v/v) dimethylformaldehyde (DMF, Sigma Aldrich).

BCIP (4-bromo-3-chloroindoylphosphate) stock solution: BCIP solution was prepared by weighing 0.03 g BCIP (Sigma, Aldrich) in 2 ml DMF (100%) Both NBT and BCIP stock solutions were stored at 4°C in foil-covered glass bijoux to prevent exposure to light.

Preparation of Colour Substrate: Solution was prepared by adding 200 μl NBT and 200 μl BCIP stock solutions into 20 ml Substrate Buffer.

i) **Separation of the protein with SDS-PAGE electrophoresis.** Gonococcal lysate and OM/OMV preparations were separated using a SDS-electrophoresis cell (MINI-PROTEAN Tetra Cell, Bio Rad) with 150-160 mV during the first 15 min to allow separation and penetration of the protein into the stacking gel. Then the gel was run at 200 mV for 45 minutes until the protein ladder was completely separated. Similar pre-treatment was employed with loading buffer to all samples evaluated (Section 2.9).

ii) **Protein transfer to the nitrocellulose membrane.** Proteins separated on the acrylamide gel weres transferred to a nitrocellulose membrane (General Electric) using a SEMIDRY-MINIBLOT apparatus (Bio Rad). Transfer was run with 9-10 V for 30 minutes. An arrangement was prepared as follows: 3x pieces of Whatman paper, 1 piece of nitrocellulose membrane, bis-acrylamide gel and finally 3pieces Whatman the papers were each soacked previously in blotting buffer. The arrangement was rolled on top with a 10 ml pipette to remove the any bubbles, which could prevent efficient transfer.

iii) **Nitrocellulose membrane blocking.** After transfer was completed, the nitrocellulose membrane was placed in a glass dish with blocking buffer for 1h at room temperature in rotatory shaker R100TW (Luckham, UK) to reduce non-specific protein binding during the western blot. The membrane was washed three times for 5 minutes with agitation each in, TTBS buffer with rocking.

iv) **Interaction with primary antibody.** The reactivity of murine and rabbit anti rNgACP sera on gonococcal whole cell lysate and OM /OMV was evaluated. The rabbit and murine polyclonal serum or monoclonal antibodies described in **Table 39** were tested at a 1/100 dilution prepared in blocking buffer. The membrane was stripped in 3mm width and placed each strip into S&S Accutran, Incubation tray and incubated with each serum sample for 1.5 hours with agitation.

Table 39. Murine and rabbit anti sera to rNg-ACP tested in western blot against gonococcal whole cell lysate and OMV/Na-Doc OMV.

rNg-ACP formulation	Sham formulation
Murine sera	
Saline $Al(OH)_3$ Zwitterion detergent ZW 3-14 Zwitterion detergent ZW 3-14+ MPLA Liposomes Liposomes+MPLA	Saline $Al(OH)_3$ Zwitterion detergent ZW 3-14 Zwitterion detergent ZW3-14+ MPLA Liposomes Liposomes+MPLA Normal Mouse Serum (NMS)
Rabbit Sera	
Pre-Bleed of Rabbit 710 (Nm-ACP ,*Neisseria meningitidis*) -	Terminal Bleed of Rabbit 710 (Nm-ACP, *Neisseria meningitidis*) Terminal Bleeding Rabbit 711 (Nm-ACP, *Neisseria meningitidis*)
Pre-Bleed of Rabbit 1 (full-length Ng-ACP ,*Neisseria gonorrhoeae*) - Pre-Bleed of Rabbit 1 (full-length Ng-ACP ,*Neisseria gonorrhoeae*)	Terminal Bleed of Rabbit 1 (full-length Ng-ACP, *Neisseria gonorrhoeae*) Terminal Bleeding Rabbit 2 (full-length Ng-ACP, *Neisseria gonorrhoeae*)

v) **Interaction with secondary antibody:** The blot was washed four times with TTBS (5 minutes each wash with rocking), and then incubated with Anti-Goat species-specific alkaline phosphatase conjugate antibody (Biorad) (500 µl/strip or 10ml/membrane) at a dilution of 1/3000 at room temperature for 1 hour.

vi) **Development colour reaction:** The blot was washed three times with TTB buffer and then twice with TBS, (5 minutes each wash with rocking), and colour substrate (10 ml/membrane or 500 µl/strip) was added. The reaction was incubated for 10-20 minutes, avoiding exposure to direct light, until positive reactivity was observed (visible purple-coloured bands). The reaction was stopped by repeated washing of the blots under running tap water and the blot dried finally between Whatman filter papers.

vii) **Imaging final result:** Each strip from the different formulation evaluated were gathered into a Whatman paper and aligned along with the protein ladder. Strips were scanned with a Scanner HP-Scanjet G2410 photo scanner (Hewlett Packard).

2.19. Flow cytometry (FC).

In order to attempt the presence of determine Ng-ACP on the bacterial surface, FC was used similar as described by (Hung et al., 2013). To determine the appropriate dilution of antisera for FC, initial experiments used mice raised with aluminium hydroxide Al (OH_3) against full-length rNg-ACP. A serial dilution of pooled mice sera (1/20 to 1/100 dilution) was used and a 1/400 dilution of rabbit serum.for rabbit sera.

Neisseria gonorrhoeae strains P9-17 and P9-17*Δng-acp* were grown and isolated on GC agar plates (Section 2.1). Bacteria were suspended in 2ml PBS.and the bacterial suspension was diluted 1/10 or 1/40 in lysis buffer (0.1% (w/v) NaOH and 1.0% (w/v) SDS) to estimate CFU/ml by measuring optical density (λ_{260nm}). A 15 ml PBS with 1% (w/v) BSA with concentration of $2x10^9$CFU were prepared from the master cell suspension. The bacterial suspension was added to in Eppendorf tubes (1 ml aliquots).

Each tube of bacterial suspension was washed twice with PBS containing 1% (w/v) BSA and then the samples were centrifuged at 5000 rpm (2200 x*g*) for 5 minutes and the supernatant was discarded. Each cell pellet was suspended with 100 µl of corresponding dilution of the test sera, and incubated at room temperature for 30 minutes. Next, each tube was centrifuged at 5000 rpm (2200 x*g*) for 5 minutes and the supernatant was removed.

Each cell pellet was washed twice with 1 ml of PBS with 1% (w/v) BSA, followed by vortex and centrifugation at 2200x*g* for 5 minutes for each step. To the cell pellets was added 100 µl ani-Rabbit Antimouse Fluorescein isothiocyanate (FITC) conjugated- (DAKO, UK), which was diluted 1/50 in PBS+ 1% (w/v) BSA and incubated at 4˚C for 30 minutes. Each sample was centrifuged and washed twice with PBS+ 1% (w/v) BSA with the same conditions and reagents used above.

To fix the cells, each cell pellet was kept with 100 µl of paraformaldehyde 0.4% (v/v) for 30 minutes on ice, followed by centrifugation at 5000 rpm (2200 x*g)* for 5 minutes and the supernatant discarded. The cell pellet was washed with PBS+ 1% (w/v) BSA and centrifuged for 5 minutes at 5000 rpm (2200 x*g*). Finally, ~150-200 µl of PBS+ 1% (w/v) BSA was added on each pellet (dependent on the size of the pellet samples), vortexed and kept in the fridge (4˚C).

Each sample was analysed on a FACsAria Flow cytometer (Beckton Dickinson, USA) recording 10,000 events and comparing the signal (Normal distribution bell shape), using FlowJo (Becton Dickinson) with the controls included in the experiment e.g. PBS+1% (w/v) BSA or bacteria with pre-immunization sera (negative control).buffer control and to test the best sera dilution to observe any shift between the wild type and knockout strains.

2.20. Human Serum Bactericidal Activity (hSBA).

This method measures the ability of anti-rNg-ACP serum to promote complement-mediated bactericidal activity, which is one of the hallmark assays for gonococcal vaccine development. This methodology was based on the protocol of described by (Ram et al., 1998).Essentially, the human serum-bactericidal activity (hSBA) assay, contains serum has depleted of innate complement activity byheat inactivation), serial dilutions of test antisera a suspension of gonococci prepared in liquid medium, and the addition of exogenous human serum, which does not have reactivity against gonococcal strains , the survival of gonococci was assessed along a serial dilutions of mice serum tested (Gill et al., 2011, Ngampasutadol et al., 2006, Price et al., 2005, Zhu et al., 2004).

2.20.1 Culture of *Neisseria gonorrhoeae.*

N. gonorrhoeae wild type strains P9-17 ($Pili^+$, Opa^+), *N. gonorrhoeae* FA1090 and knockout variants *N. gonorrhoeae* strain P9-17 *Δng-acp* and *N. gonorrhoeae* strain FA1090 *Δng-acp* were used for hSBA assays and were grown overnight on GC supplemented agar at 37˚C with 5% (v/v) CO_2. Phenotypic variants were isolated using a stereo-microscope and sub-cultured to lawn growth.

2.20.2. Materials.

Phosphate buffered saline (PBS) solution: 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 10 mM disodium phosphate Na_2HPO_4, 1.76 mM potassium dihydrogen phosphate KH_2PO_4 Sterilised by filtration using 0.22 μM filters (Merck, Millipore).

B salts: 0.5 m M magnesium chloride $MgCl_2$, 0.9 mM calcium chloride $CaCl_2$ in UHQ.

PBS +B salts, (PBSB) solution: 100 ml PBS solution with 0.5 ml of B salts.

PBSB +10% (v/v) dFCS (PBSB/dFCS): 100 ml PBSB solution with 2 ml Fetal Calf Serum, which was previously decomplemented by heating at 56°C for 1h in a water bath (dFCS) (GIBCO, Life biosciences, Invitrogen).

Lysis buffer: 0.1% (w/v) sodium hydroxide (NaOH) and 1.0% (w/v) sodium dodecyl phosphate (SDS) in UHQ.

Human serum: Human blood serum, from a donor with no previous contact with *Neisseria gonorrhoeae* was collected in non-heparinised tubes and was allowed to clot at room temperature for 30 min and then the sample was centrifuged at 3000 rpm for 20 minutes at 4˚C. The serum was removed and stored at -80˚C in 500 µl aliquots in 1.5 ml Eppendorf tubes (Sarstedt).

2.20.3 Preparation of bacterial suspension (Master suspension).

Neisseria gonorrhoeae bacteria were removed from lawn growth using a 10 µm loop and suspended in 2 mL PBS, then the samples was pipetted and vortex to obtain an homogenous suspension. The cell suspension was centrifuged at 1000 rpm (200 x*g*) for 1 minute (Thermo Biofuge), to remove any clumps. Finally the supernatant were collected in a new sterile Bijoux tube, to obtain the Master solution. To obtain bacterial colony counts on each agar plate in a range of 200-250 CFU, the working bacterial suspension was estimated at $4x10^4$ CFU/ml. To calculate the number of colony formed units (CFU) units in the gonococci suspension, the master suspension was diluted 1/40 in 1 ml of lysis buffer (Section 2.20.2) and optical density of bacterial suspension was measured on a spectrophotometer (HITACHI U-1100) at λ_{260} nm in UV cuvettes (Sarstedt) using lysis buffer as a blank.

An ODλ_{260} nm of 1 Absorbance (Abs) units is equivalent to~$2x10^9$ CFU/ml for *N. gonorrhoeae* strain P9-17 in the master suspension, previously determined by viable counting. From this was calculated the amount of CFU relative to absorbance or optical density (OD) as follows in the formula:

$$\textbf{Estimated CFU} = 4x2\times10^9 \text{ CFU/ml} \times \text{OD}\lambda_{260}\text{ nm}$$

Serial 10-fold dilutions of the Master suspension were made in PBSB to produce a final working stock of 4×10^4 CFU in PBSB/dFCS.

2.20.4. Viable counting of working stock.

Serial dilutions of the final working stock(s) (4×10^4CFU) were made in PBSB/dFCS to ~2×10^3 CFU, from which four to six aliquots of 15µl were plated onto agar plates.

2.20.5 Bacteridical Assay

The bactericidal assay was done in a sterile 96 well microtitre plate with lid (Greiner Bio-one, UK) and each well contained the components as shown in **Table 40.** The test sera were heated at 56˚C for 30 minutes, to remove any endogenous complement. To evaluate the viability of gonococci in the presence of antibodies and human complement the working gonococcal stock $4x10^4$ CFU was prepared and the sera were serially diluted with PBSB/dFCS including four dilutions from 1/4 to 1/2048.

The bactericidal reactions were done at 100 µl final volume. In order was added PBSB/dFCS, followed by Tgonococcal suspension ($4x10^4$ CFU) to give ~1000 CFU/well, diluted test sera and finally 16.68 µl of normal human serum (**Table 40**). The plate was shaken in a plate shaker (Titertrek) for 5 seconds to mix the contents and was incubated at 37˚C with 5% (v/v) CO_2 for 1 hour.A 10-fold dilution of the working stock of gonococcal suspension to $4x10^3$ CFU/ml was made and 15 µl volume were plated onto 4 to 6 GC agar plates for viable counting.

Table 40. Components of the bactericidal assay.

Component	Amount (µl)
Gonococcal suspension	25
PBSB +dFCS**	52-48.33 (depends on volume of murine serum serial dilution used)
Normal human serum	16.68

**All the test sera were assessed in triplicate over a range of serial dilutions.

The bactericidal assay plate was incubated for 1 hour at 37˚C with 5% (v/v) CO_2 and then from each well, aliquots of 15 µl were plated onto triplicate GC agar plates. Following 2 days incubation at 37˚C with 5% (v/v) CO_2, CFU were counted on the plates using a ProtoCOL automatic colony counter plates (Synoptics Ltd, Cambridge UK) and ProtoCOL software to determine the number of single colonies present on each plate.

Calculation of the serum dilution at which ≥50% of the gonococcal population killed was done using the formula bellow:

$$\%SBA = 100 - \left(\frac{population\ (CFU)\ test\ serum}{population\ (CFU)\ without\ test\ serum}\right) x100$$

The serum bactericidal activity titre was reported as the reciprocal of the highest serum dilution of ≥50% of bactericidal killing, compared with the number of CFU in the absence of serum. Sera that showed bactericidal activity (≥50%) in two or more dilutions were considered positive.

2.21. Cell culture.

2.21.1. Human Chang conjunctival epithelial cells.

Chang conjunctival epithelial cells were obtained from the EuropeanType cultutre collection (ETCC, Proton Down, UK).

2.21.2. Human meningioma cells.

Meningioma cells were obtained from surgically removed tumours as described by (Hardy et al., 2000). Meningeal cells were cultured in tissue culture flask that has been pre-treated with collagen coating solution (50 µg/ml of collagen rat-tail collagen type I (BD biosciences) in 0.02M acetic acid (Fisher, UK) for 1 hour at room temperature. The coated flask was washed thrice with warmed PBS to return to physiological pH before cells added.

2.21.3. Human epidermoid carcinoma, larynx (HEp-2 cells).

HEp-2 cells were obtained from the ETCC (Porton Down, UK).

2.21.4. Human cell culture maintenance, subculture and storage.

2.21.4.1 Maintenance and preservation cell culture media.

Dulbecco's modified eagle's medium (DMEM) with ultraglutamine 1 and 4.5g/L glucose medium (Lonza, UK) with 10% (v/v) decomplemented fetal calf serum (dFCS) (Gibco, Life biosciences) and 2 ml of antibiotics (5000 U streptomycin and 5000 U penicillin, Lonza) (Growth medium) was used for epithelial and meningeal cells culture.

To prepare decomplemented fetal calf serum (dFCS) a 500 ml flask of FCS (Life Biosciences) was placed in a water bath at 57˚C for 1-2 hour(s). Then the serum was stored at -20˚C in 20ml aliquots in universal tubes.

2.21.4.2. Cell resuscitation.

The contents of cryovial of human epithelial cells taken from liquid nitrogen storage, was thawed and suspended into 8 ml of growth medium. Suspended cells were centrifuged at 2000 rpm (1000x*g*) (ThermoScientific Heraeus Megafuge 16) for 3 minutes at room temperature and the supernant was removed. For Chang conjunctival epithelial cells and HEp-2 laryngeal carcinoma cells, the cell pellets were diluted (1/6 dilution) appropriately to culture in a 75 cm^2 tissue culture flask (Greiner Bio-one, UK). The Meningeal cell pellet was suspended in 6 ml of culture media in a 25 cm^2 tissue culture flask (Greiner Bio-one, UK) previously coated with collagen coating solution.

Monolayer of epithelial cells (75cm^2 tissue culture flask) or meningeal cells(25 cm^2 tissues culture flask) were incubated at 37 ˚C with 5% (v/v) CO_2 to confluence and then washed thrice with sterile PBS and treated with 1-2 ml of trypsin-EDTA (Hank's balance salt solution containing 0.5g/L of trypsin and 0.2g/L EDTA Lonza, UK) for 10 min at 37˚C with 5% (v/v) CO_2 or until the monolayer was detached. The detached cells were suspended by the addition of 8 ml of growth medium and centrifuged at 2000 rpm (1000x*g*) for 3 minutes. The pellet was gently detached by tapping and suspended with fresh growth medium to a desired volume dilution in a 75 cm^2 flask or into 1ml of

cell suspension with appropriate dilution to individual wells of a 24 well-plate. Cells were incubated and grown to confluence at 37˚C with 5% (v/v) CO_2.

2.21.4.3. Freezing cell lines for long-term storage.

Cell pellets were individually suspended with 1.8 ml of growth culture medium and with 200 µl of dimethylsulphoxide (DMSO, BDH, Poole, England). The cell suspensions (1 ml) were placed into sterile cryogenic vials (Nunc, Roskilde, Denmark) and frozen gradually from -80˚C overnight before placing into liquid nitrogen for long term storage.

2.22 *In vitro* infection of cultured human cells with *Neisseria gonorrhoeae.*

2.22.1. Bacterial suspension.

Wild–type, knockout (Δ*ng-acp*) and complemented (¢Δ*ng-acp*) strains of homologous *Neisseria gonorrhoeae* strains P9-17, MS11 and FA1090 were grown overnight on GC agar or GC selective agar from liquid nitrogen stocks as described in Section 2.1. Bacterial growth was suspended in 2 ml PBS and diluted appropriately for optical density measurement as described in Section 2.14.1 The CFU concentration of the suspension was calculated as described in Section 2.20.3, based on prior determination of CFU in which OD λ_{260}nm of 1.0 = 2×10^9 CFU and serially diluted to a suspension of 2×10^7 CFU/ml in DMEM medium with 1% (v/v) dFCS without antibiotics (Infection medium).

2.22.2. Viable counting of bacterial inocula.

Serial dilutions of the final bacterial suspension of each strain (2×10^7 CFU/ml) were made in Infection medium down to a dilution of ~2×10^3 CFU, which was then, plated out in aliquots of 15 µl in quadruplicate on GC agar plates. Cultures were grown overnight at 37˚C with 5% (v/v) CO_2 and colonies counted the following day as described above (Section 2.20.4).

2.22.3. Measurement of total bacterial association to epithelial cells.

Confluent cell monolayers in 24 well plates were washed four times gently with pre-warmed (37˚C) PBS solution to remove the antibiotics and any dead cells. Cell bacterial suspensions of (wild-type,

knockout or complemented strains) were added to each well in triplicate (1ml of Infection medium containing $2x10^7$ CFU) and the plate was incubated at 37˚C with 5% (v/v) CO_2 for 3 hours. Next, the bacterial suspensions were removed and the infected monolayers were washed four times with pre-warmed PBS solution. To quantify the numbers of adherent gonococci, to each well was added 250 µl of a Saponin lysis solution 1% (w/v) Saponin from Quillaja Bark (Sigma-Aldrich) prepared in 10 ml sterile PBS and 1% (v/v) dFCS) and the plate was incubated at 37˚C with 5% (v/v) CO_2 for 15 minutes. The lysed wells were scraped with a pipette tip and the triplicate wells were pooled to a final volume of 750 µl. The lysate pools were serially diluted 10 to 1000-fold with PBS in a sterile 96 well plate (Greiner Bio-one, UK) and each serial dilution was assayed on GC agar plates in triplicate (15 µl per plate).

2.22.4 Gentamicin protection assay to measure bacterial internalization.

Epithelial cells monolayers were infected with ~$2x10^4$ CFU/ml using different gonococcal strains and cellular invasion was quantified using a gentamicin protection assay. Gentamicin was used, as this antibiotic agent is unable to penetrate the cells and kill intracellular bacteria. Also, the addition of Cytochalasin D (CD) was used in the invasion process to interrogate whether any bacterial internalization was dependent on actin microfilament activity.

The invasion experiment was performed in two individual 24 well plates. On each plate half of the wells with cell monolayer was pre-treated with 500 µl of (2 µg/ml cytochalasin D (Sigma, Aldrich) in DMEM medium (without antibiotics) +1% (v/v) dFCS) the other half of the cell monolayers was pre-treated with 500 µl DMEM medium (without antibiotics) +1% (v/v) dFCS and incubated for 30 min at 37˚C with 5% (v/v) CO_2, (**Figure 14)**. Next, bacterial suspensions ($2x10^7$ CFU/ml final concentration) of wild-type, knockout (*Δng-acp*) or complemented (¢*Δng-acp*) bacterial strain were added and the plates were incubated at 37˚C at 5% (v/v) CO_2 humid atmosphere for 3 hours.

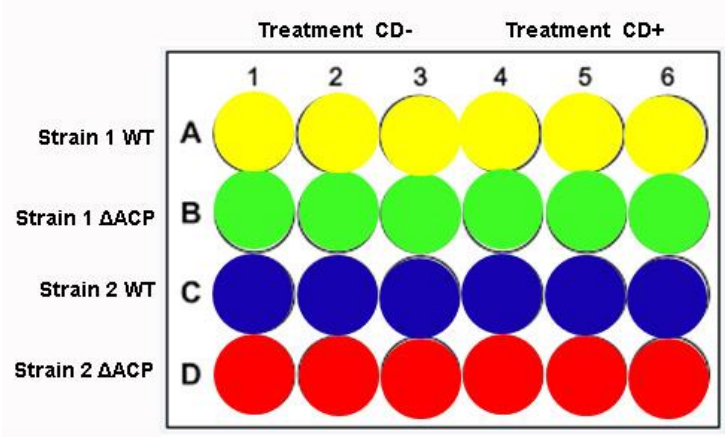


Figure 14. Arrangement to set up cell infection experiments. Evaluation through viable count the infection by different *N. gonorrhoeae* strains on epithelial cells and the influence with or without cytochalasin D (CD) on association and internalization.

After 3 hours of incubation, medium on the internalization plate was replaced with 1 ml per well of Gentamicin solution (DMEM media with 200 μg/ml of Gentamicin final concentration) and incubated at 37˚C at 5% (v/v) CO_2 in a humid atmosphere for 1.5 hours. The total association assay plate was washed thrice with warmed PBS, and lysed to detach the cell monolayer and associated bacteria quantified described in Section 2.22.3.

The gentamicin solution was removed from the internalization assay plate, followed by three gentle washes with warmed PBS. Then, the cell monolayer was detached with the lysis solution, and the triplicate volumes collected into Bijoux tubes. Serial dilutions (1/10) of the cells suspension were plated onto GC agar plates in triplicate and the numbers of recovered bacteria quantified as Section2.20.5.

A student t-test analysis was done from 3 independent experiments to comapre the levels of total association and internalization in presence or absence of cytochalasin D, P values <0.05 were considered significant.

2.23. Binding of Ng-ACP protein to epithelial cells.

FACS was used to determine the binding of recombinant full-length rNg-ACP on Chang epithelial cells was measured by using a method previously described by (Serruto et al., 2003).

Confluent cell monolayers of Chang cell line in a 6 well on a 24 well plate were washed three times with sterile pre-warmed PBS , followed by cell detachment treatment using 250 µl of Versene (0.02% (w/v) EDTA) (Lonza, UK) and incubation at 37˚C for 10 minutes. The cell suspension was collected into an Eppendorf tube and centrifuged at 5000 rpm (2200 x*g*) for 2 minutes. Then the cell pellet was washed twice with cell culture medium without antibiotics (DMEM +1% (v/v) dPBS).The cell pellet was suspended in 1ml of (DMEM +1% (v/v) dPBS) and taken 10 µl to 12 µl cell suspension and mixwith 5 µl of trypan blue stain 0.4% (v/v) (Gibco, Life Technologies). Using a hemocytometer chamber, the number of cells was determined and calculated using the equation below:

$$\textbf{Amount of cells } \frac{\boldsymbol{cells}}{\boldsymbol{ml}} = \boldsymbol{average\ count\ cell\ on\ each\ square} * \boldsymbol{dilution\ factor} * \frac{\boldsymbol{10000\mu l}}{\boldsymbol{1\ ml}}$$

Total cell suspension was diluted with (DMEM media +1% (v/v) dPBS) to a final concentration of $3.7x10^5$cell/ml in a final volume of 1.5-2 ml in order to obtain sufficient cell samples for to FC experiments. The cell suspension was placed in 100 µl aliquots into1.5 ml Eppendorf tubes to which was added a range of concentrations of full-length rNg-ACP from 0- 300 µg and incubated at 37˚C for 1 hour. Then each sample was centrifuged at 5000 rpm (2200 x*g*) for 5 minutes and the cell pellet was washed twice with PBS+5% (v/v) dFCS. Each sample was incubated with a dilution of rabbit anti-full length rNg-ACP (1/400) serum at room temperature for 30 minutes, followed by two of washes of the cell pellet as described above. The cell pellet samples were incubated with FITC-conjugated goat anti-rabbit serum (Dako, UK) 1/50 dilution in PBS+5% (v/v) dFCS on ice for 30 minutes and a series of washes of the cell pellet was done to remove excess antibody. Cell pellet samples were fixed with 0.4% (v/v) paraformaldehyde in PBS+5% (v/v) dFCS for 30 minutes on ice. The cell pellet were washed twice with warmed PBS+5% (v/v) dFCS and suspended in 150-200 µl of PBS+5% (v/v) dFCS.

Each sample was analysed on a FACsAria Flow cytometer (Beckton Dickinson, USA) recording 10,000 events and comparing the signal (Normal distribution bell shape and obtain the medium fluresncence intensity), using FlowJo (Becton Dickinson) with the controls included in the experiment i.e. untreated cells (negative control).buffer control. The analysis of plot Mean

fluorescence intensity (MFI) against concentration of recombinant protein was represented in a graph plot.

2.24 Inhibition of bacterial association on human epithelial cells using rabbit anti- full-length rNg-ACP serum.

To test the presence of rabbit sera anti-full-length rNg-ACP could inhibit the association of *N. gonorrhoeae* strain P9-17 on Chang epithelial cells, was used a confluent cell monolayer in a 96 well plate and was tested with different MOI of *N. gonorrhorreae* strain P9-17, followed by addition of decomplemented rabbit sera in growth medium. To quantify the amount of cell was used per well aTriplicate cell monolayers was washed thrice with warmed with PBS solution and treated with Versene (EDTA) 0.02% (Lonza) and incubation at 37˚C for 10 minutes. Then the cell suspension was diluted with trypan blue 0.4% (Gibco, Life technologies) and quantified the amount of cells using a hemocytometer chamber as described above.

The total association on Chang cells was assessed with a wide range of multiplicity of invasion (MOI), which is taking into account the amount of cells and CFU/ml from cell suspension bacteria obtained from *N. gonorrhoeae* strain P9-17. An amount of bacterial suspension was added, based on the formulas described below, to make a cell suspension with 200 MOI in DMEM media+1% dPBS containing 10% decomplemented pre-bleeding serum (PB) or terminal bleeding (TB) rabbit serum against full-length rNg-ACP. A serial 1/10 dilution of MOI suspension was made from 200-0.02 MOI and 150 µl of each bacterial suspension was placed on each cell monolayer as shown in **Figure 15,** and incubated at 37˚C with 5% (v/v) CO_2 humid atmosphere for 3 hours.

$$MOI = \frac{Amount\ of\ bacteria}{Amount\ of\ cell}$$

$$Amount\ of\ bacteria\ (\frac{CFU}{ml}) = \frac{Amount\ of\ cells\ *\ 200MOI}{150\mu l} * \frac{1000\mu l}{1ml}$$

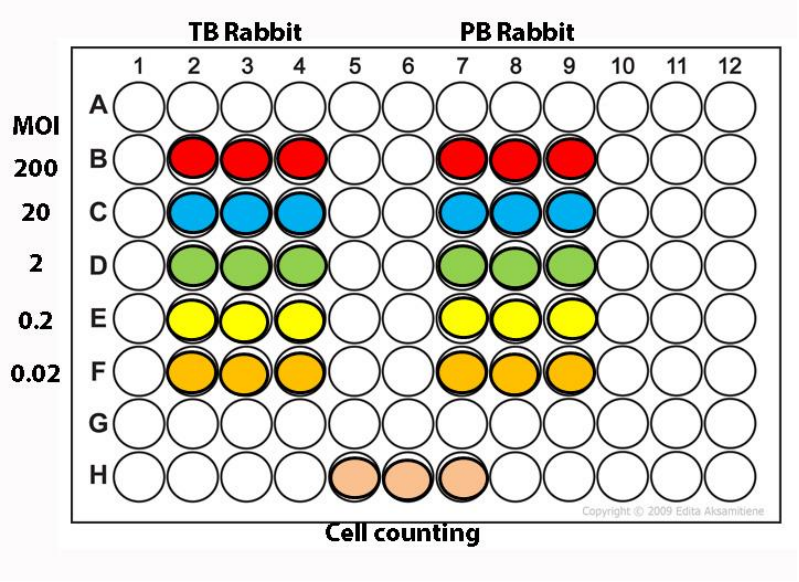


Figure 15. Setting up for inhibition of *N. gonorrhoeae* strain P9-17 association on Chang cells using anti-rNg-ACP rabbit serum. Each color on the same row has the same multiplicity of infection (MOI) 200 MOI denoted in red, 20 MOI (bright blue), 2 MOI (green) , 0.2 MOI (yellow) and 0.02 MOI (mustard). The columns 2-4 were treated with terminal bleeding rabbit sera (TB Rabbit) and columns 7-9 the samples were treated with pre-bleeded rabbit sera (PB Rabbit).

Different Multiplicity of infection (MOI) of *N. gonorrhoeae* strain P9-17 were used to test the adherence on Chang cells in presence of rabbit serum raised against full-length rNg-ACP, terminal bleeding (TB) and pre-bleeding (PB) to determine the amount of recovered bacteria in each case.

Infected cell monolayers were washed with warmed PBS, followed by monolayer detachment using 250 µl lysis solution 1% (w/v) Saponin from *Quillaja* Bark (Sigma-Aldrich) prepared in 10ml sterile PBS and 1% (v/v) dFCS) at 37˚C for 10 minutes. Each individual condition was gathered and diluted (1/100 or 1/1000) for highest MOI. Neat and a 1/10 dilution were used with the lowest MOI (2-0.02) tested and plated onto GC agar plates and the recovered bacteria quantified as described in Section 2.22.3.To calculate the inhibited associated bacteria was used the following equation:

$$\frac{(adherent\ bacteria\ \ pre-immune\ serum)-(adherent\ bacteria\ post-immune\ serum)}{(adherent\ bacteria\ \ pre-immune\ serum)}\times 100$$

2.25 Determination of the three–dimensional structure of Ng-ACP from *N. gonorrhoeae strain P9-17.*

The construct generated for mature-rNg-ACP (pET22b-*ng-acp*) was chosen to produce recombinant protein, in heterologous host *E. coli* strain BL21pLysS. The protein was evaluated with a selection of the appropriate buffers to generate a single crystal unit and analysed by X-ray diffraction. The analysis of recombinant protein diffraction pattern was done by molecular replacement.

2.25.1 Large scale production of soluble mature rNg-ACP.

The selected candidate (Colony 8) was used to inculate 200ml of Luria –Bertani (LB) (containing 0.1 mg/ml ampicillin + 34 μg/ml cloranphenicol)and the cell culture incubated overnight at 37˚C and 200 rpm in a rotary shaker (New Brunswick Innova 43/43R). A large scale cell culture was done in eight 2 L Erlenmeyer flasks, each containing 1 L of LB broth with antibiotics. To each flask was added 10 ml of inoculum and incubated in a rotary shaker at 200 rpm and 37˚C (New Brunswick Innova 43/43R) for 2 hours until OD_{600}nm of 0.4-0.6 was reached. IPTG (100 mM) 10 ml of was then added to each cell culture in semi-logaritmic phase growth, and followed by incubation at 37˚C for 4h to recombinant protein expression.

2.25.2 Purificationof soluble mature rNg-ACP.

From 8L cell culture of mature rNg-ACP the cell pellet was extracted by centrifugation at 4˚C for 30 minutes at 6000 rpm (9000 x*g*) (Avanti J-30I high performance,Beckman Coulter). The cell pellet was stored at -20˚C overnight.

2.25.2.1 Buffer conditions used for non-denaturing protein purification.

A series of buffers was prepared for suspension of the cell pellet extract of the protein and purification of the protein by affinity chromatography and size exclusion chromatography, as summarised in **Table 41**.

Table 41. Buffers used for rNg-ACP protein purification.

Buffer	Composition
Lysis buffer	300 mM NaCl + 50 mM Tris HCl+10% (v/v) glycerol pH 8.5
Affinity chromatography buffer	300 mM NaCl + 50 mM Tris HCl+10%(v/v) glicerol + 500 mM imidazole pH 8.5
Size exclusion chromatography (SEC) buffer	300 mM NaCl + 20 mM Tris HCl pH 8.0

2.25.2.2 Suspension of cell pellet.

The cell pellet was thawed on ice and then lysis buffer was added (**Table 41**) to obtain a homogeneous cell suspension. The cell suspension mixed by vortex and sonicated on a water-ice bath for 5 minutes with the following program (6 micron for 10 SEC ON and 30 sec OFF; Misonic Sonicator XL2020 ultrasonic processor (Fisher Scientific), to disrupt any potential inclusion bodies present in the suspension. The sample was centrifuged at 45000 rpm (208000 x*g*) for 45 minutes (Beckman coulter XPN). The cleared supernatant was collected into 50 ml Falcon tubes and filter-sterilised using 0.22 μm filter (Merck millipore, UK).

2.25.2.3 Protein purification.

A series of purification steps was done on the clarified supernatant including i) affinity chromatography using a Ni(II)-NTA column, to remove any host's proteins and ii) size exclusion protein purification in order to separate oligomers of the rNg-ACP protein.

2.25.2.4. Purification by affinity chromatography–HPLC.

An AKTA *prime* liquid chromatography system (General Electric (GE), which was coupled with 1 ml Ni-His trap HP column (General Electric) placed in parallel was used to purifiy the cell supernatant. The column had been previously washed with 20% (v/v) ethanol (ETOH), and equilibrated with lysis buffer (**Table 41**). The clarified extract was placed on a 50 ml injector during a manual run and fractions were collected at a flow-rate of 1ml/min. A gradient elution buffer containing a range of concentration from 4% (v/v), 8% (v/v) and 60% (v/v) of affinity chromatography buffer (**Table 41**), containing imidazole, was applied in order to remove any

heterologous host proteins and to obtain rNg-ACP of high purity. The eluted fractions were collected (10-15 fractions) during a manual run at flow rate 0.1 ml/min with a collection volume of 2 ml per sample and tracked by UV (λ_{280} nm) absorbance during the process.

2.25.2.5. Preparation of the concentrated protein sample.

The fractions eluted under the chromatography peak were analysed by SDS-PAGE electrophoresis in a Mini-Protean apparatus (Bio-Rad) and run at 160 V for 50 minutes using Powerpac 1000 Power Supply (BioRad). The acrylamide gel was stained using a mixture of Coomasie blue staining for 20 minutes at room temperature, followed by destaining solution–distilled water with boiling for 10 seconds in a microwave set at medium power.

The fractions that contained a single band of *Mr* 12kDa were collected in a Vivaspin -20 5,000 MWCO PES tube (Sartorius). A series of centrifugation steps at 3000 rpm (1932 x*g*) Sorvall legend RT (Thermo Fisher) were done until the samples were concentrated to a final volume of 750 µl to 1ml.

2.25.2.6 Purification by size exclusion chromatography (SEC).

The concentrated sample was collected in a 1.5 ml Eppendorf tube and centrifuged at 13000 rpm (16060 x*g*) for 5 minutes (Heraeus BioFuge Pico Sorvall Centrifuge) to remove any solid particles. Then the sample was injected into a 1ml injector loop coupled AKTA *premier* liquid chromatography system (General Electric) and SEC column high load `6/600 Superdex 75 pg (General electric). An overnight manual run was done at a flow rate of 0.2 ml/min and 62 samples were collected with final volumes of 2 ml per tube and assessed by UV at λ_{280} nm.

The fractions collected under the chromatography peak were pooled and concentrated in a Vivaspin 2 5000MWCO PES tube (Sartorious) and the initial amount of protein quantified using a Nanodrop 2000 c spectrophotometer (Thermo Scientific), expressed in mg/ml and the ratio at λ_{280}nm, revealing the amount of protein and λ_{260}nm, reading the presence of organic solvents in the sample.

A series of centrifugation steps was done for 2-5 minutes at 3000 rpm (1932 x*g*) Sorvall legend RT (Thermo Fisher) until the sample was concentrated to a final volume of 200 µl -100 µl with concentration of 80-100 mg/ml.

2.25.3 Setting up crystallization conditions.

The concentrated protein was tested with a set of screening kits (Molecular Dimensions, UK) of buffers to stablish the optimal crystallization conditions on a 96 well SDC crystallization plate (Molecular Dimensions, UK). The rNg-ACP protein–buffer condition was set up using an IO/IL art Robbins inteliplate 2/3 HP system (Art Robbins Instruments).

A JCSG-plus screening kit set of 96 sterile buffer containing polyethylene glycol (PEG), salts, neutral organic acids or organics reagents with a pH ranging 4 to 10.5, was used to test the crystallization process. In addition, a PAC premier kit (Molecular Dimensions, UK) was used to test the crystallization process in the presence of anions, cations, and different of pH in the presence of polyethylene glycol (PEG) as a precipitant agent.

The crystallization process was assessed at different temperatures (4˚C and 21˚C).and the plates were screened after 3-7 days with a camera on each well (Minstrel Imager UV, Rigaku, California) and visualised using CrystalTrack version 2.27 Software.

2.25.4. Analysis of the crystal by X-ray diffraction.

From screened different crystallization conditions was analysed on a light microscope the size and dimension of a single crystal unit at 4˚C. The crystal unit was taken out from the well with aid of a cryopin, containing a loop needle, and placed into a magnetic cryovial (MiTeGen) immersed in liquid nitrogen bath. A wide range of samples were placed in a SPINE puck transfer (MiTeGen) and stored in liquid nitrogen deward until the date of analysis. The selected crystals were analysed on an ID23 beamline at Grenoble, France.

2.25.5. Analysis of the diffraction pattern.

The analysis of diffraction pattern of the different samples were collected in a database of the center of syncotron at Grenoble, France (ESRF) https://wwws.esfr.fr/ispyb/security/logon.do, which contains all the information i.e. pictures of the crystal, diffraction pattern, all the files to in software specialised in analysis X-ray diffraction pattern. The selection criteria is based on the

completeness or percentage generated from diverse of simulators to generate the molecule, which the crystal condition with high completeness was chosen for further analysis.

All the crystallography information was downloaded and used to run in a Molrep programme as part of CCP4 software. An iterative work was done to determine the possible conformation of the molecule, based on the energy parameters adjust.

Once the three-dimensional structure is determined a PDB file is generated, which contains spatial and conformation of each atom that composed the protein. The molecular file was run into a webserver DALI (http://ekhidna.biocenter.helsinki.fi/dali_server/start) which search homology of structures elucidated and annotated on Protein Data Bank (PDB) database to validate the molecular replacement analysis and interrogate the potential biological function of this antigen, in order to infer a structure –function correlation.

CHAPTER 3– RESULTS

3.1. Bioinformatic analysis to determine the diversity of the *Neisseria gonorrhoeae* Adhesion Complex Protein (Ng-ACP).

A bioinformatic analysis was performed using the *Neisseria* database from PubMLST.org database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_neisseria_isolates) to analyse gonococcal strains reported with the locus of the *acp* gene, **NEIS2075**. The *Neisseria* PubMLST database contains data for a collection of isolates that represent the total known diversity of *Neisseria* species. The database was developed by Keith Jolley and Martin Maiden (Jolley and Maiden, 2010). The results of this analysis showed that within 3822 gonococcal isolates, the *ng-acp* gene was found within 20 alleles (**Table 42)**. The allelic distribution of gonococcal isolates showed that the vast majority of the gonococcal strains presents a protein encoded by allele **10** (3083;80.66%), with a smaller number expressed a protein encoded by allele **60** (440;11.51%) followed by allele **59** (122;3.24%) and allele **93** (133;3.48%).

An amino acid sequence aligment of the different Ng-ACP proteins encoded by the 20 alleles, demonstated that there were 13 non-redundant alleles. The amino acid alignment of the different gonococcal protein alleles demonstrated that the vast majority showed 93.50% -98% of identity, of the 20 gonococcal proteins, suggesting that all the alleles expressed in the gonococcal population are highly conserved (**Figure 16**). For allele proteins, amino acid substitutions with similar physicochemical properties were found, e.g. in position 19, 22, 96 (Ala toVal), position 29 and 71 (Val to Ile), position 81 (Glu to Asp) or 25 (Asn to Asp). Other substitutions observed was replacement from a charged amino acid to an aliphatic amino acid residue at positions 93 (Ser to Gly) or 80 (Met to Thr). The only deletion was located at position 20 (**Figure 16**).

Table 42. Distribution of *ng-acp* alleles in *Neisseria gonorrhoeae* from PuBMLST.org/*Neisseria* database.

Allele	Number of *N. gonorrhoeae* isolates	Distribution percentage (%)
3	1	0.03
6	1	0.03
10	3083	80.66
13	4	0.11
33	1	0.03
59	122	3.24
60	440	11.51
80	1	0.03
81	3	0.08
82	5	0.13
83	1	0.03
93	133	3.48
94	1	0.03
95	2	0.05
113	11	0.29
146	2	0.05
150	6	0.16
151	2	0.05
152	2	0.05
153	1	0.03
Total	**3822**	**100.00**

*** PuBMLST.org/*Neisseria database was accessed 07/12/2017***

The percentage of identity and homology between the gonococcal Ng-ACP amino acid sequences is represented as a phylogenetic tree/dendrogram generated using Jalview webserver (Waterhouse et al., 2009). This representation showed the relationship among sequences and the differences between alleles, in order to identify the non-redundant alleles. From this analysis, of the 20 gonococcal alleles expressing Ng-ACP, 13 alelles were non- redundant with five major groups identified (Allele 6,59,60); (Alleles 93,94); (Alleles 10,146); (Alleles 3,13,33) and (Alleles 151,152) (**Figure 17**).

```
CLUSTAL O(1.2.4) multiple sequence alignment


allele153       MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVPYGFNKQGLTTYAS  59
allele150       MKLLTTAILSSAIALSSMA-AVGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele113       MKLLTTAILSSAIALSSMA-AADTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele95        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele93        MKLLTTAILSSAIALSSMA-AVGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele94        MKLLTTAILSSAIALSSMA-AVGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele83        MKLLTTAILSSAIALSSMV-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele82        MKLLTTAILSSAIALSSMA-AAGTDNPTIAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele10        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele146       MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele81        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
alelle6         MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele59        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele60        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele151       MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele152       MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele3         MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele13        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele33        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele80        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
                ******************. *..*:***:**************** *************

allele153       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele150       AVINGKRVQTPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele113       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele95        AVINGKRVQMPINLDKSDNMDTFYGKEGSYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele93        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele94        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele83        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele82        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele10        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele146       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
allele81        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAIDSKSYRKQPIMITAPDNQIVFKD  119
alelle6         AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
allele59        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
allele60        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
allele151       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
allele152       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
allele3         AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD  120
allele13        AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD  120
allele33        AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD  120
allele80        AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120
                ********* *:*******::*******.***.**.:*.********************

allele153       CSPR*         123
allele150       CSPR*         123
allele113       CSPR*         123
allele95        CSPR*         123
allele93        CSPR*         123
allele94        CSPR*         123
allele83        CSPR*         123
allele82        CSPR*         123
allele10        CSPR*         123
allele146       CSPR*         123
allele81        CSPR*         123
alelle6         CSPR*         124
allele59        CSPR*         124
allele60        CSPR*         124
allele151       CSPR*         124
allele152       CSPR*         124
allele3         CSPR*         124
allele13        CSPR*         124
allele33        CSPR*         124
allele80        CSPR*         124
                *****
```

Figure 16. Amino acid sequence alignment of different *N. gonorrhoeae* isolates that express Ng-ACP protein (PubMLST.org website).***** represents fully conserved amino acid residue, **: (colon)** represents conservation between groups with similar chemical properties. **.(dot)** denotes conservation between groups with a weak similar properties.

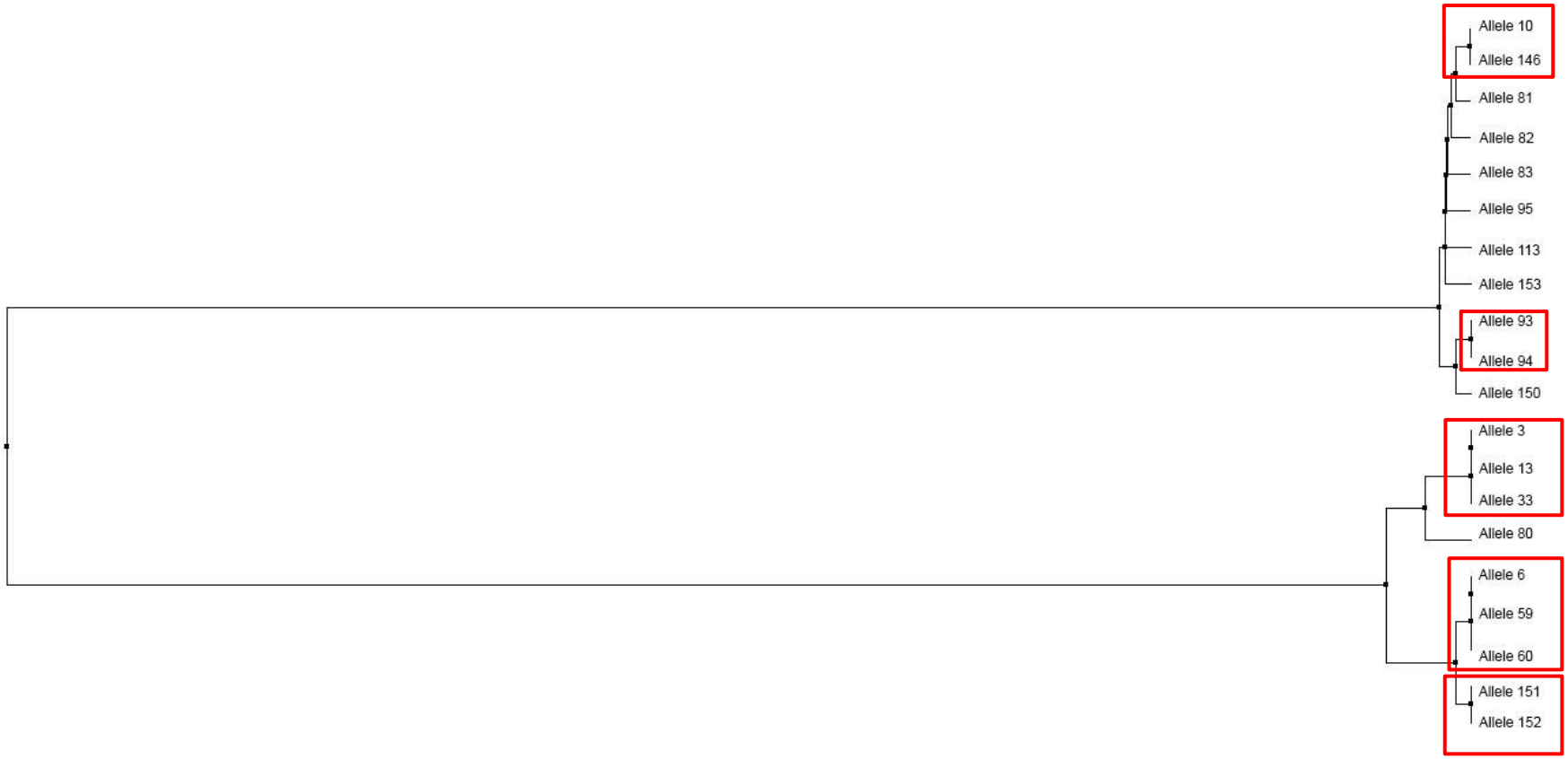


Figure 17. Dendrogram of different gonococcal isolates encoding *ng-acp* gene **(NEIS2075)** in PubMLST.org/*Neisseria* database. The squares denote the redundant isolates are grouped.

Table 43. Amount and distribution of gonococcal isolates sharing non-redundant alleles.

Cluster	Number of isolates	Percentage
6*(+59,60)	563	15
93(+94)	134	3
3(+13,33)	6	0.2
10 (+146)	3085	81
151(+152)	4	0.1
Total	**3792**	**100**

*Lowest number designates the allele cluster.

From the identification of non-redundant alleles from 3792 isolates, the most representative alleles were found to be allele 10 (3085, 81%), followed by allele 6 (563, 15%). The other three remaining clusters presented at low distribution below 3 to 0.1% **(Table 43**).

A comparison of the amino acid sequences of the most representative Ng-ACP proteins (encoded by allele 10 and 6) and the two most common type I and II Nm-ACP proteins from the closely related bacteria, *N. meningitidis* (Hung et al., 2013) showed high homology of 94.3% (**Figure 18**). The difference between the two types of meningococcal Nm-ACP resides in the presence of aspartic acid (D) or asparagine (N) at position 25. The significant changes compared to the meningococcal strains, *N. gonorrhoeae* showed a single deletion at position 20, the substitution with non-polar amino acids of (Val to Ile) at residue 72 (Val to Ala) position 96, on the contrary presence of polar residues (Gly to Ser) at positions 93 and 99, (Val- to Met) on amino acid 80. Amino acid substitution with similar physico-chemical properties (Glu to Asp) at position 81. Among these sequences between closed related bacteria *Neisseria spp*.

```
CLUSTAL O(1.2.4) multiple sequence alignment


P9-17       MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS         59
MC179       MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS         60
MC58        MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS         60
            ****************** ****:**********************************

P9-17       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD         119
MC179       AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD         120
MC58        AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD         120
            **********:*******::***********.**.**.********************

P9-17       CSPR        123
MC179       CSPR        124
MC58        CSPR        124
            ****
```

Figure 18. Amino acid alignment between two types of meningococcal ACP and the most prevalent gonococcal allele of ACP.).***** denotes fully conserved amino acid residue, **: (colon)** represents conservation between groups with similar chemical properties. **.(dot)** denotes conservation between groups with a weak similar properties.

3.2 Identification and amplification of the *acp* gene from *Neisseria* species.

To amplify the *N. gonorrhoeae ng-acp* gene and up and down non-encoding fragments, a set of primers used previously to amplify the *N. meningitidis* (*nm-acp*) gene Appendix A.9.1 was used (Hung et al., 2013). *N. gonorrhoeae* strain P9-17, *N.meningitidis* strain MC58, as well as commensal strains *N. lactamica* and *N. sicca* were grown overnight on GC agar plates at 37˚C in a humid atmosphere with CO_2 (5% v/v). DNA extracted from Neisserial species by basic extraction, PCR reactions were set up and the products were evaluated by agarose gel electrophoresis (**Figure 19**).

A. **B.**

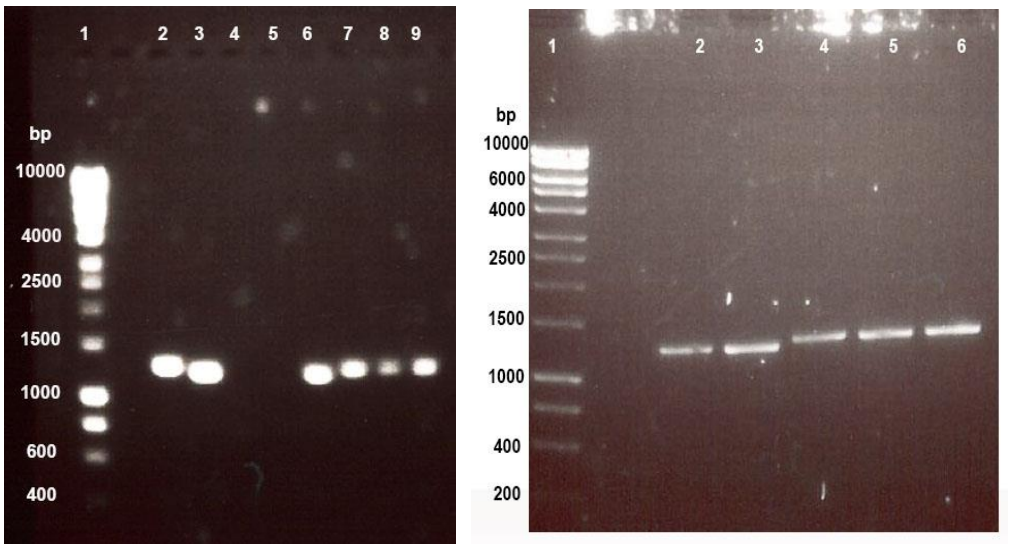


Figure 19. PCR products of *acp* gene of different isolates from pathogenic and commensal *Neisseria* species.

A). Lane 1, ladder. **Lane 2,** *N. meningitidis* MC58. **Lane 3**, *N. lactamica* strain E232.**Lane 4 and 5,** *N. sicca* strains C11 and B521. **Lane 6,** *N. lactamica* strain A219. L**ane 7,** *N. gonorrhoeae* strain CEPA-1. **Lane 8,** *N. gonorrhoeae* strain P9-17. L**ane 9,** *N. gonorrhoeae* strain P9-1. **B. Amplified PCR products of *acp* gene between different strains of *N. gonorrhoeae* and *N. lactamica*. Lane 1,** ladder. **Lane 2,** *N. lactamica* strain E232. L**ane 3,** *N. lactamica* strain A219, **lane 4,** *N. gonorrhoeae* strain CEPA-1 . **Lane 5,** *N. gonorrhoeae* strain P9-17 . L**ane 6,** *N. gonorrhoeae* strain P9-1 .

The majority of the *Neisseria* species displayed a PCR product of ~1,250 bp. There were minor differences in product sizes between species which was likely due to subtitutions or differencies in the number of base pairs encoding the *acp* gene.

The *acp* gene of pathogenic *Neisseria* species (*N. meningitidis and N. gonorrhoeae*) was similar in size, suggesting that they encode closely-related gene products. In contrast, *N. sicc*a strains did not

show any amplification product, because of a significant substitution in the *ns-acp* gene sequence (**Figure 19A**).

Detailed amplification of the *acp* gene of *N. lactamica* and *N. gonorrhoeae* demonstrated the amplification of a single product band~1,250 bp with minor differences in the sizes of the PCR product (**Figure 19 B**).

3.3. Amplification of the *ng-acp* gene in *Neisseria gonorrhoeae.*

A PCR was done using the primers listed in Appendix A.9.2 to amplify specifically the region encoding *ng-acp* gene from *N. gonorrhoeae* strain P9-17,without non-encoding fragments, using a high performance polymerase Phusion 2X kit (New England Biolabs).

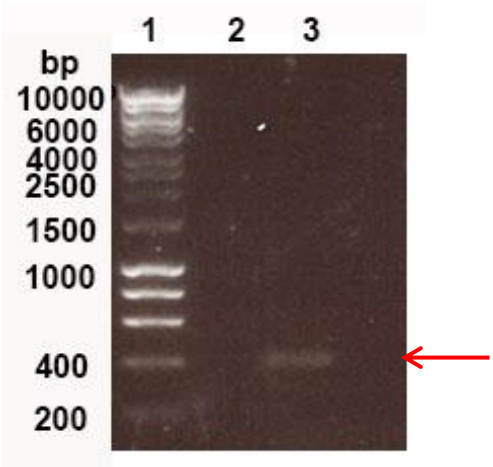


Figure 20 . The amplified PCR product of the *ng-acp* gene of *N. gonorrhoeae* strain P9-17**.**
Lane 1 ladder, **lane 2** negative control (UHQ water) **lane 3** *N. gonorrhoeae* strain P9-17. The arrow denotes the PCR product (size~400 bp).

The product of amplification showed a single band with a size ~400 bp (**Figure 20**). Next the PCR product from *N. gonorrhoeae* strain P9-17 was sequenced and the nucleotide sequence show below (**PubMLST.org ID number 36675**).

The *ng-acp* gene from *N. gonorrhoeae* strain P9-17 was translated to amino acid sequence using the web tool Transeq webserver (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Transeq translates nucleotide sequences into protein sequences and translates to the three forward and three

reverse frames, obtaining an amino acid sequence with multiple frame translations at once as show below (**Figure 21**).

P9-17 nucleotide sequence

5'-
ATGAAACTTCTGACCACTGCAATCCTGTCTTCCGCAATCGCGCTCAGCAGTATGGCCG
CCGCCGGCACGGACAACCCCACCGTTGCCAAAAAAACCGTCAGCTACGTCTGCCAGCA
AGGTAAAAAAGTCAAAGTAACCTACGGCTTCAACAAACAGGGTCTGACCACATACGC
CTCCGCCGTCATCAACGGCAAACGTGTGCAAATGCCCATCAATTTGGATAAATCCGAC
AATATGGACACGTTCTACGGCAAAGAAGGCGGTTATGTGCTGAGCACCGGCGCAATG
GACAGCAAATCCTACCGCAAACAGCCTATTATGATTACCGCACCTGACAACCAAATCG
TCTTCAAAGACTGTTCCCCACGTTAA-3'

P9-17 amino acid sequence

MKLLTTAILSSAIALSSMAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS
AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD
CSPR

Figure 21. *ng-acp* gene nucleotide sequence and Ng-ACP protein amino acid sequence from *N. gonorrhoeae* strain P9-17.

An amino acid sequence comparison between *N. gonorrhoeae* strain P9-17 Ng-ACP and the non-redundant gonococcal alleles (**Figure 22**), showed identity 98.4% and higher homology with the most representative gonococcal allele (Allele 10) shown in **Figure 23.** From a search of the nucleotide sequence that encodes *ng-acp* gene and the genome sequence of the reference *N. gonorrhoeae* strain FA1090, the locus code is **NGO1981**, which is annotated as a conserved hypothetical protein.

```
CLUSTAL O(1.2.4) multiple sequence alignment


allele153       MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVPYGFNKQGLTTYAS  59
allele150       MKLLTTAILSSAIALSSMA-AVGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele113       MKLLTTAILSSAIALSSMA-AADTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele95        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele93        MKLLTTAILSSAIALSSMA-AVGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele83        MKLLTTAILSSAIALSSMV-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele82        MKLLTTAILSSAIALSSMA-AAGTDNPTIAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele81        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
allele10        MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
P9-17           MKLLTTAILSSAIALSSMA-AAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
alelle6         MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele151       MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele3         MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
allele80        MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
                ******************. *..*:***:**************** ************

allele153       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele150       AVINGKRVQTPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele113       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele95        AVINGKRVQMPINLDKSDNMDTFYGKEGSYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele93        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele83        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele82        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
allele81        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAIDSKSYRKQPIMITAPDNQIVFKD 119
allele10        AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
P9-17           AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 119
alelle6         AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 120
allele151       AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 120
allele3         AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD 120
allele80        AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD 120
                ********* *:*******::*******.***.**.:*.********************

allele153       CSPR*     123
allele150       CSPR*     123
allele113       CSPR*     123
allele95        CSPR*     123
allele93        CSPR*     123
allele83        CSPR*     123
allele82        CSPR*     123
allele81        CSPR*     123
allele10        CSPR*     123
P9-17           CSPR-     123
alelle6         CSPR*     124
allele151       CSPR*     124
allele3         CSPR*     124
allele80        CSPR*     124
                ****
```

Figure 22. Sequence alignment among alleles obtained from BIGs database. Allele 3, 80, 81, 82, 83, 93, 95, 113, 150, 151, 153, Allele 6, (*N. gonorrhoeae* strain FA1090) and Allele 10, *N. gonorrhoeae* strain MS11) code P9-17 (*N. gonorrhoeae* P9-17).***** denotes fully conserved amino acid residue, **: (colon)** conservation between groups with similar chemical properties. **.(dot)** denotes conservation between groups with a weak similar properties.

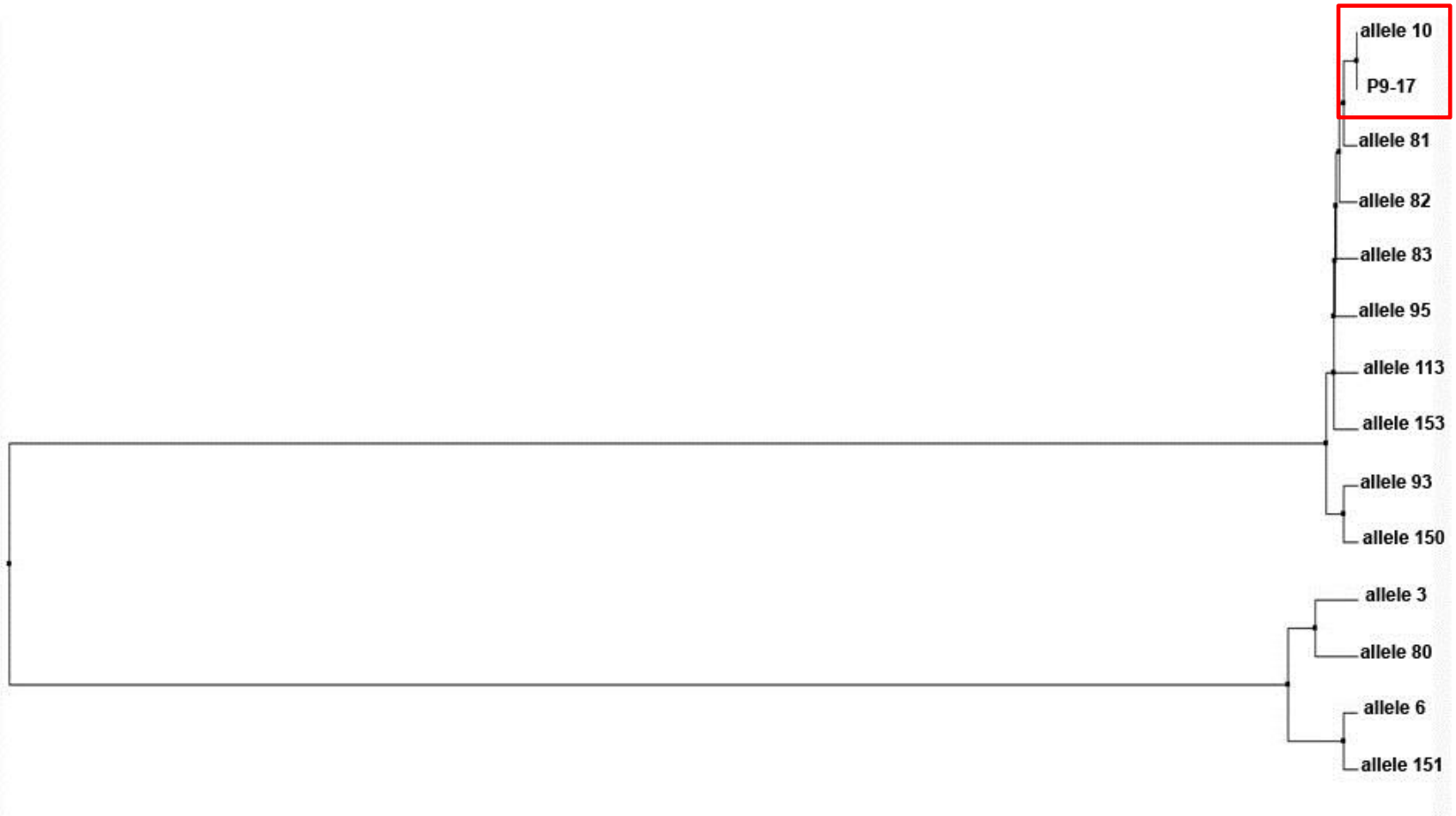


Figure 23. Dendogram to determine the type of allele encoding *N. gonorrhoeae* strain P9-17 Ng-ACP protein.

3.4 Production of recombinant rNg-ACP protein for vacccine studies.

3.4.1 Generation of full-length rNg-ACP protein.

3.4.1.1 Cloning strategy for full-length recombinant protein.

The *ng-acp* gene from *N. gonorrhoeae* strain P9-17 (Allele 10) was inserted into a pRSET-A cloning vector system (size 2897bp), which displays different features such as a 6xHis-tag and enterokinase site located at the N-terminus.The 6xHis tag enables protein purification by Nickel (II)- affinity chromatography and the enterokinase site allows enzimatic cleavage, if necessary **(Figure 24**).

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWMKLLTTAILSSAIALSSMAAAGT DNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYASAVINGKRVQMPINLDKSDNMDT FYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKDCSPR*

Figure 24. Amino acid sequence of *ng-acp*- pRSET-A construct.

The highlighted area in **green** is the vector –derived 6xHis-tag, the **mauve** area is the enterokinase region site, the **turquoise** area is the Ng-ACP leader peptide , and the **yellow** area is the mature Ng-ACP protein.

3.4.1.2. Cloning the ng-acp gene into the pRSET-A vector.

The pRSET-A cloning vector was extracted from competent *E.coli* strain DH5α cells, and visualised by gel electrophoresis as a single band of ~3000 bp at a concentration of ~75 ng/µl s. Subsequently, a double digestion was done using *HindIII* and *Xho*I restriction enzymes, followed by purification by PCR clean up, and evaluation by agarose gel electrphoresis demonstrated a single band of ~3000 bp with a concentration of 40 ng/µl. Similarly, a *ng-acp* gene PCR product was digested with the same set of restriction enzymes and purified. Agarose gel electrophoresis showed a single band of 400 bp of concentration 10 ng/µl **(Figure 73** Appendix .11.1**)**.

The ligation product (*ng-acp*-pRSET-A) was transformed into *E. coli* strain DH5α. Transformants were grown on selective agar (LB+ 50µg/ml Ampicilin (Amp)) plates, from which 12 single colonies were selected to be tested by PCR reaction. From this analysis, four colonies were chosen for sequencing **(Figure 74** Appendix A.11.2.1**)**.

The colonies candidates No.9, 10 and 12 were sequenced and compared by alignment against the sequence of *ng-acp* from *N. gonorrhoeae* strain P9-17. This alignment demonstrated that candidate colonies 9 and 10 did not show any homology with the target sequence **(Figure 75** and **Figure 76** Appendix A.11.2.2). In contrast, candidate colony 12 nucleotide sequence was identical with the gonococcal target sequence **(Figure 77** Appendix A.11.2.2) which was selected for further studies to produce the recombinant protein.

A single colony containing the appropriate open reading frame of *ng-acp* gene (candidate colony 12) was selected and used for further studies. Transformation of the *ng-acp*-pRSET-A construct into *E. coli* strain BL21pLysS was done, followed by a PCR reaction to select potential candidates containing a single band of size ~3400 bp. From these candidates, one was selected for a pilot expression study and a large scale recombinant protein expression study.

3.4.2. Generation of mature rNg-ACP protein.

3.4.2.1 Cloning strategy for mature recombinant protein

To improve the solubility and preserve the protein in native conditions, the *ng-acp* gene from *N. gonorrhoeae* strain P9-17 (Allele10) was inserted into a pET22b cloning system (size 5493bp), which displays a 6xHis-tag located at the C-terminus enable protein purification by Ni-NTA affinity chromatography. Also, a T7 promoter region is present to enhance recombinant protein over-expression **(Figure 25)**. In addition, the rNg-ACP's leader peptide was removed in order to improve the solubility of recombinant rNg-ACP protein.

AGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYASAVINGKRVQMPINLDKSDN

MDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKDCSPRHHHHHH*

Figure 25. Partial amino acid sequence of *ng-acp* and pET22b construct.

The highlighted area in **green** is the vector –derived 6xHistag and the **yellow** area is the mature protein of gonococcal Ng-ACP protein.

3.4.2.2. Cloning the gonococcal *ng-acp* gene into pET22b cloning vector.

The pET22b cloning vector was extracted from competent *E.coli* strain DH5α cells which contain *nm-acp*-pET22b construct demonstrating a single band with size ~6000 bp an a concentration of ~75 ng/µl by gel electrophoresis (**Figure 78A** Appendix A.12.1). A double digestion was done using *Nde*I and *Xho*I restriction enzymes followed by purification and evaluation by agarose gel showed a single band of ~6000 bp of concentration 40 ng/µl, to obtain an opened plasmid. Likewise, a *ng-acp* gene PCR product was digested with the same set of restriction enzymes and purified, to produce a single band 400 bp of concentration 60 ng/µl **(Figure 78B** Appendix A.12.1**)**.

The ligation product (*ng-acp*-pET22b) was transformed into *E. coli* strain DH5α. Transformants were grown on selective agar (LB+ 50µg/ml Ampicillin (Amp)) plates, from which 7 single colonies were selected to be tested by PCR reaction. From this analysis, two colonies were chosen for sequencing. The colonies candidates 10 and 11 were sequenced, and compared with the nucleotide sequence alignment sequence of *ng-acp* from *N. gonorrhoeae* strain P9-17 demonstrated that both colony candidates showed high homology with the target sequence (**Figure 79** and **Figure 80** Appendix A.12.1).

The correct open-reading frame construct was transformed into *E. coli* strain BL21DE3 pLysS and then the transformants were selected on selective agar (LB+ 50µg/ml Amp + 34µg/ml Chloramphenicol (Chl)). Each candidate was screened by PCR reaction which showed a single band of ~400 bp on agarose gel electrophoresis (**Figure 81** Appendix A.12.1). A single candidate was used for a pilot expression study and a large scale recombinant protein expression study.

3.4.3 Pilot expression of full-length andmature rNg-ACP protein.

A pilot protein expression study was done to evaluate the time required and the influence of IPTG induction for optimal recombinant protein expression. A comparison analysis was done of the cell culture in the presence or absence of inducer (IPTG) over 5 time points, up to 5 hours. At each time points, 1 ml aliquots of cell culture (Non-induced or Induced) were collected, centrifuged and the pellets were suspended in PBS. Cell lysate were obtained from the cell pellets by several cycles of freezing-thawing to disrupt the cell membrane.Protein contents were evaluated by SDS-PAGE electrophoresis (Section 2.9).

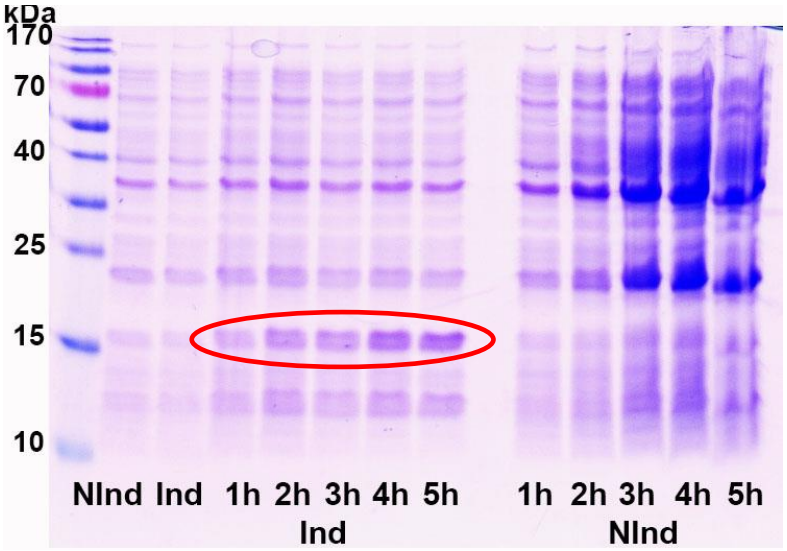


Figure 26. SDS-PAGE of cell lysate samples for pilot expression of full-length rNg-ACP protein.

NInd, Non-induced cell culture (-IPTG). **Ind,** induced cell culture 1mM IPTG (+IPTG). **NInd 1h-5h,** time points from 1 to 5 hours of non-induced samples**. Ind 1h-5h** time points 1 -5 hours of induced cell culture.

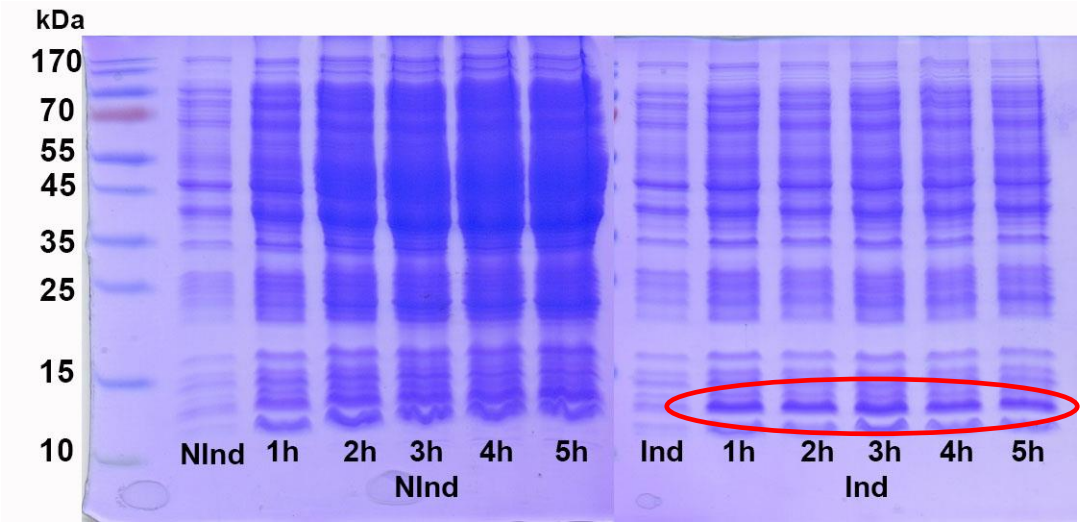


Figure 27. SDS-PAGE of cell lysate samples for pilot expression of mature rNg-ACP protein.

NInd, Non-induced cell culture (-IPTG). **Ind,** induced cell culture 1mM IPTG (+IPTG). **NInd 1h-5h,** time points from 1 to 5 hours of non-induced samples**. Ind 1h-5h** time points 1 -5 hours of induced cell culture.

Comparison between the induced and non-induced cell lysate samples at different time points, showed a selective over-expression of rNgACP (full-length protein of *Mr* 17.5kDa (**Figure 26**) or mature protein of *Mr* 12.5 kDa (**Figure 27**) following induction with IPTG. Through the time course of the experiment, expression appeared to reach a maximum by 4-5 hour of incubation.

Based on this observation, the optimal expression of full-length and mature rNg-ACP was subsequently done with add of IPTG (1mM) and 4 hours of incubation.

3.4.4 Large–scale production of full-length and mature rNg-ACP.

Large scale protein production was done similarly for both full-length and mature rNg-ACP proteins. Started with 1.5L-2.00L of culture medium was prepared and then was added 5-7 ml of An overnight culture of bacterial inoculum in SOB with 50 µl of 1M $MgCl_2$ and antibiotics (50µg/ml Amp and 34µg/ml Chl) 5-7ml was inoculated into 500 ml of SOB and the cultures were incubated on a rotary shaker (Gallenkamp, UK) at 37˚C for 200 rpm until the cell culture reached semi-log phase at an OD λ_{600}nm of 0.4-0.6. Approximately 1.5-2 L of culture medium were provided.Next, 1 ml of cell culture sample was removed (Non-induced (Nind)), centrifuged and stored at -20˚C. IPTG of 100mM stock concentration (5 ml to 500 ml of cell culture) was added to the cell culture, in order to induce over-expression of the protein, with incubation at 37˚C with rotary shaking at 200 rpm for 4 hours.

3.4.4.1. Large scale purification strategy for full-length rNg-ACP.

The cell culture was centrifuged to obtain a cell pellet, which was suspended in denaturing buffer guanidinium chloride 6M GdnHCl (**Table 15** Section 2.11.2.2). Approximately 2-3 ml of lysis buffer was added per gram of wet cell pellet. The lysate suspension was then placed into another centrifugation tube and centrifuged at 9400 rpm (9908*x*g) for 30 minutes at 4˚C. The resulting clarified supernatant was incubated with 3.5 to 5 ml of nickel(II)-nitrilotriacetic (Ni-NTA) resin for 1 hour at room temperature.

The purification process involved three steps using a series of buffers with the denaturing agent 6M GdnHCl **.**

Table 15 Section 2.11.2.2, to gradually remove of hosts proteins, followed washing by finally elution of the protein target from the resin as shown in **Figure 28**. Eluted fracions were precipitated with TriChloroacetic Acid (TCA) 10%(w/v), to obtain the protein, which was then suspended with PBS+0.5% (w/v) SDS and evaluated by SDS-PAGE electrphoresis.

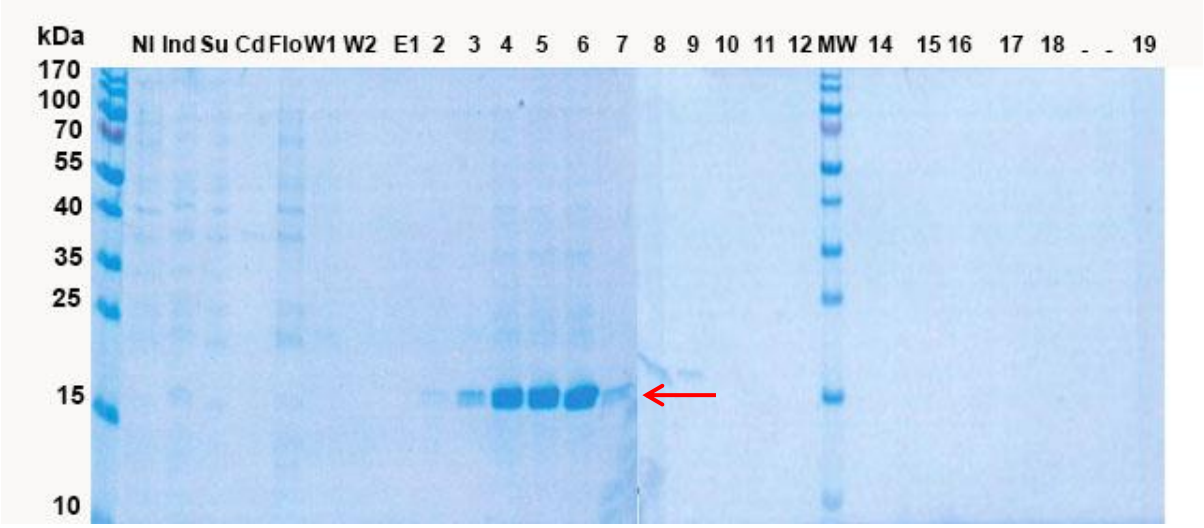


Figure 28. Evaluation of large scale Ng-ACP protein production and purification using Ni-NTA affinity chromatography column.

MW, protein ladder **NI**, Non-induced Ind Induced culture **Su** supernatant cell culture. **Cd**, cell debris. **Flo**, flow through. **W1-W2**, washing fractions. **E1-E19**, eluted fractions from 1 to 19.The arrows denotes a single band of *Mr* 17.5kDa.

SDS-PAGE reveals a gradual removal of the *E. coli* host protein. The arrow denotes the purified full-length rNg-ACP (*Mr*~ 17.5 kDa), which was present in the eluted fractions E3-E7 **Figure 28**. The selectivity of this affinity chromatography process enabled purification of the recombinant protein and the protein concentration was quantified by bicinchoninic acid assay (BCA) using bovine serum albumin (BSA) as a standard (Section 2.12).

From 1.5 L of culture, approximately 1.854 mg/ml of purified recombinant protein was obtained and the total amount of protein was 4.473 mg in an eluate volume of 2.4 ml. Quality assessment by SDS-PAGE of the purified recombinant protein rNg-ACP was done (Section 2.9), and showed a single band of ~17.5kDa (~98% of purity) **Figure 29**.

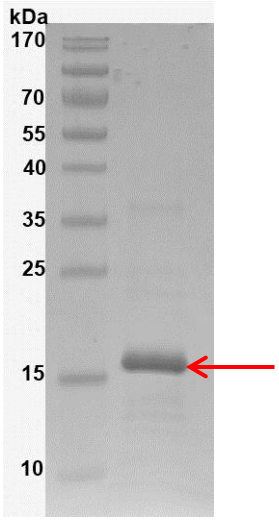


Figure 29. SDS-PAGE of purified rNg-ACP from a large-scale production.The arrow identifies the protein with *Mr*~ 17.5kDa.

3.4.4.2. Large–scale production of mature recombinant protein rNg-ACP.

The cell culture was centrifuged to obtain a cell pellet,which was suspended in non-denaturing buffer conditions (50 mM NaH_2PO_4 +30 mM NaCl+10 mM Imidazole) **Table 14** Section 2.11.2.1. Approximately 2-3 ml of lysis buffer was added per gram net weight,and the lysate suspension was placed into another tube and centrifuged at 9400 rpm (9908*x*g) for 30 minutes at 4˚C to produce a clarified supernatant. The clarified supernatant was incubated with 3.5 to 5 ml of nickel(II)-nitrilotriacetic (Ni-NTA) resin for 1 hour at 4˚C on a rotary shaker.

The purification process involved three steps using a series of buffers with a non-denaturing agent **Table 14** Section 2.11.2.1, to gradually remove host protein, follow by, washing steps and finally elution of ed the protein target from the resin as shown on **Figure 30 A.** Eluted fracions were collected, and dialysed against PBS for 48-72 hours and the dialysate was evaluated by SDS-PAGE electrphoresis.

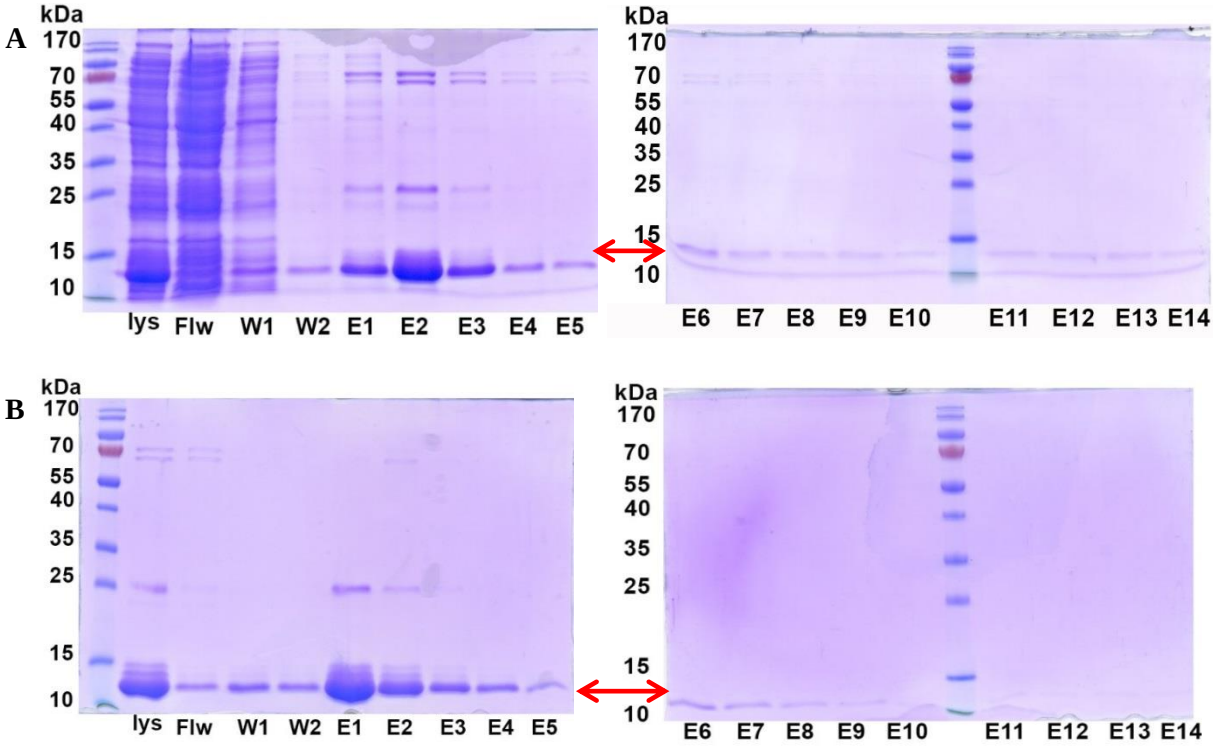


Figure 30. Evaluation of large scale mature Ng-ACP protein production and purification using Ni(II)-NTA affinity chromatography column. **A.** First run purification in affinity chromatography. **B.** Second run purification by affinity chromatography.

Lys, cell lysate. **Flow**, flow through. **W1-W2**, washing fractions **E1-E14**, eluted fractions from 1 to 14. The arrows show a band with *Mr*~12.5kDa.

A gradual removal of *E. coli* host protein until purified protein was obtained with a single band with *Mr*~12.5 kDa in the eluted fractions (E1-E5) **Figure 30 A** To remove further host proteins second purification of the protein, obtaining a similar removal pattern in **Figure 30B**. In the eluted fractions (E1-E4) were observed the presence of the protein in high proportion, making quite selective this methodology to obtain in high proportion of the protein of interest. The purified protein was quantified by bicinchoninic acid (BCA) assay to quantify the protein based on the standard curve quantification of bovine serum albumin (BSA) to calculate the amount of the protein in the mixture or pool of protein obtained Section 2.12.

Approximately 4.0 mg/ml from 2.0 L of cell culture. Quality of the protein was evaluated by SDS-PAGE, which showed a single band of *Mr*~12.5kDa (Section 2.9) **Figure 31**.

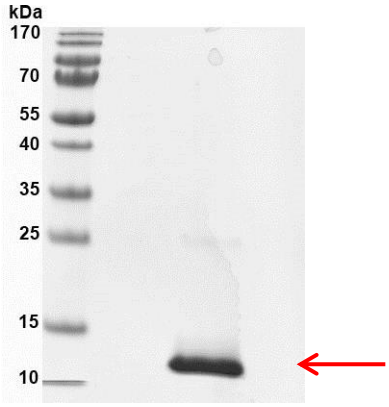


Figure 31. SDS-PAGE electrophoresis of purified fragment from large-scale production of mature rNgACP protein. The arrow displays the protein with *Mr*~ 12.5kDa.

3.5 Characterization of the biological and functional properties of antibodies against full-length rNg-ACP.

To examine the antigenicity of insoluble full-length rNg-ACP, BALB/C mice (H-2^d haplotype) were immunised with the recombinant protein in different adjuvant formulations and delivery systems, using a 3 dose schedule (dose I, day 0; dose II, day 14; dose III, day 28) followed by terminal bleeding by cardiac puncture on day 42. Mice were immunised with rNg-ACP in saline alone and adsorbed to aluminium hydroxide (Al $(OH)_3$) or delivered in liposomes and Zwitterion detergent (ZW 3-14), with and without the addition of exogenous immuno-modulator Monophosphoril Lipid A (MPLA). Antisera were then tested for activity with a variety of *in vitro* assays.

3.5.1 Antigenicity of murine antisera to full-length rNg-ACP.

3.5.1.1 Enzyme-Linked Immunosorbent Assay (ELISA).

Individual murine sera raised against rNg-ACP with the different adjuvant formulations and delivery systems were tested by ELISA. Serial dilutions of test and sham sera were tested initially against immobilised recombinant protein (rNg-ACP) and end point dilution titres were determined from linear regresion analysis and extrapolation of the titration curves for each serum sample. The geometric mean (GM) ELISA end point titre with 95% confidence limits (CL) was calculated for each group of animals per inoculating sample preparation and an independent student-t-test with 95% confidence limits was used to compare the mean values between groups, with $P<0.05$ reporting significance.

All the antisera raised with the different adjuvant and delivery systems showed similar ELISA reactivity against the recombinant protein (**Figure 32**). Antisera raised with Liposome + MPLA and with $Al(OH)_3$ showed the highest mean titres of ~1,825,000 and ~3,660,000 respectively. The lowest mean ELISA tires were shown by the rNg-ACP protein delivered in Liposomes or ZW 3-14 detergent alone, with values of ~17000 and ~12300 respectively. Statistically, there were signficant differences in mean ELISA titres for antisera raised to rNg-ACP in saline, $Al(OH)_3$, ZW 3-14 + MPLA, compared with liposomes + MPLA ($P>0.05$). Addition of MPLA significantly increased mean ELISA titres to rNg-ACP when added to liposomes (from ~17,000 for liposomes to 1,825,000 for liposomes + MPLA) ($P<0.05$), and ZW 3-14 micelles (from ~12,300 for ZW 3-14 to ~160,000 for ZW 3-14+ MPLA) ($P>0.05$).

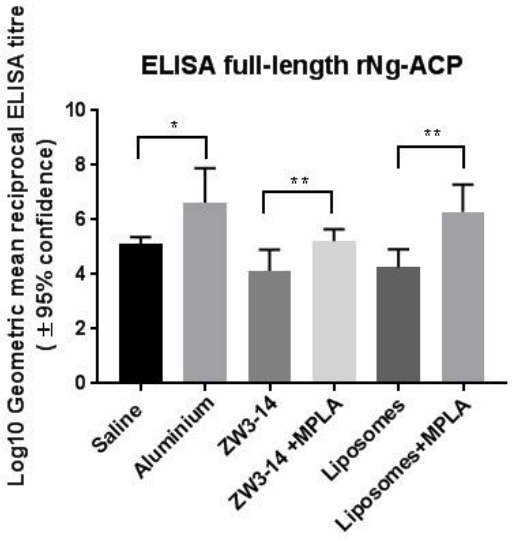


Figure 32. ELISA reactivity of murine antisera raised to full-length rNgACP with different adjuvants and delivery formulations.

Serial dilutions of individual sham and immunised sera were tested against pure full-length rNg-ACP in ELISA. Sham sera do not show any significant reactivity. The columns represents the geometrical mean of reciprocal ELISA titers (n=5 animals/group) and the error bars the 95% confidence limits. * denotes statistical significance ($P<0.05$).

Next, individual sham and anti-rNg-ACP sera were tested in ELISA against both purified P9 outer membranes (OM) and whole P9 bacteria. However, no signficant reactivity was observed for mice immunised with rNg-ACP delivered with any of the adjuvant formulations, compared to sham immunised animals alone ($P>0.05$).

3.5.1.2 Recognition of Ng-ACP protein in Neisseria gonorrhoeae whole cell lysates and OMV by serum reactivity in western blot.

Whole cell lysates and OMV were prepared from *N.gonorrhoeae* strain P9-17 and were separated by SDS-PAGE electrophoresis (Section 2.9) and transfered to a nitrocellulose membrane, following the protocol described in Section 2.18. The membrane was cut into 0.3 cm wide strips and individual stips were reacted in westernblot with pooled antisera to full-length rNg-ACP sera raised with the different formulations and delivery systems or with sham immunised sera. Antisera raised to full-length rNg-ACP in all adjuvant and delivery systems recognised Ng-ACP protein of *Mr*~13kDa in whole cell lysates and (OMV) (**Figure 33A, B**). However, in general, the reactivity with OMV was visually much higher than with the whole cell lysates, probably as a consequence of higher amounts of protein present in the OMV prepared from a larger volume of bacterial growth than was used to prepare the whole cell lysate. Reactivity of these antisera was specific, as shown by the presence of a single band in all of the strips and no reactivity of the corresponding sham-immunised sera (left side of each label), which was similar to that observed with normal mouse serum (**Figure 33 A, B**). In both preparations, a weak reactivity was observed with mice antisera using detergent preparation, whereas sera raised with saline, aluminium hydroxide ($Al(OH)_3$), and liposomes+MPLAshowed stronger reactivity . Rabbit antisera raised to Nm-ACP using Freund's adjuvants was also tested against the gonococcal preparations, but only one rabbit antiserum (R711) showed cross-reactivity with P9-17 OM (**Figure 33B**). Conversely, mice and rabbit antisera to full-length rNg-ACP did not react with whole cell lysate preparations of homologous knockout strains *N. gonorrhoeae* strains P9-17 *Δng-acp* (**Figure 95 A** Appendix A.15**)** and heterologous *N. gonorrhoeae* FA1090 *Δng-acp* (**Figure 95 B** Appendix A.15**)**.

Also mice sera anti- full length rNg-ACP was tested against the recombinant protein full-length rNg-ACP showing similar reactivity in all the formulation tested as shown in **Figure 93 A** Appendix A.15. However, the molar concentration of recombinant protein is high showing some alternative bands (*Mr*~36kDa) due to the inespecificity of recognition. Similarly, murine antisera to full-length rNg-ACP antisera recognised mature rNg-ACP, however a differential reactivity is presented high immuno reactivity with saline, aluminium hydroxide $Al(OH)_3$ and liposomes with

MPLA preparations presents immuno reactive single band of *Mr*~12kDa and additional band at *Mr*~24kDa, suggesting the presence of dimer of rNg-ACP (**Figure 94 A** Appendix A.15).

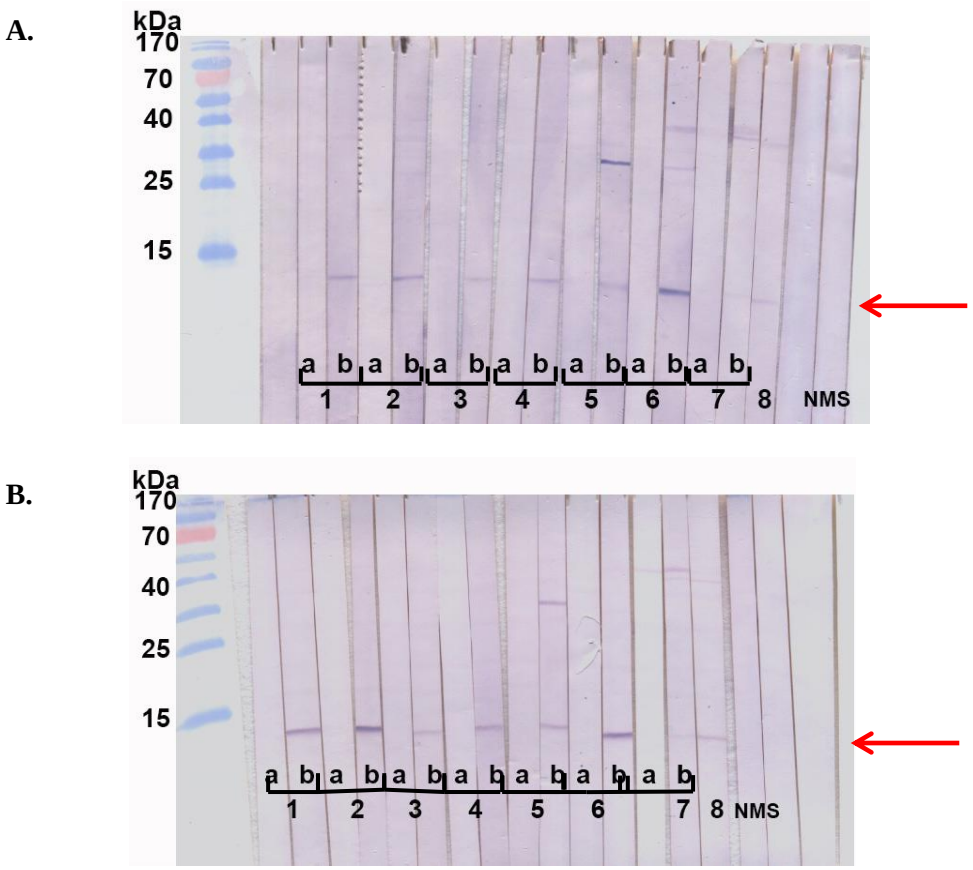


Figure 33. Reactivity of murine antisera to full-length rNg-ACP and sham immunised sera in wester blots of A) whole cell lysate and B) outer membranes from *N. gonorrhoeae* strain P9-17.

Pooled antisera were diluted in TTBS buffer (1/100 dilution) and reacted with the strips for 90 minutes. The arrow denotes the position of Ng-ACP protein (*Mr*~**13kDa)**. Antisera to rNg-ACP raised in saline (Lane 1), $Al(OH)_3$ (Lane 2), ZW 3-14 (Lane 3), ZW 3-14 +MPLA (Lane 4), Liposomes (Lane 5), Liposomes+MPLA (Lane 6), rabbit antiserum (**1/400 dilution**) raised to *Neisseria meningitidis* rNm-ACP (rabbits R710 Lane 7, R711, Lane 8). NMS, denotes reactivity of normal mouse serum, which was also indicative of the reactivity of sham-immunised sera. Stripes labelled with a denotes sham sera of each adjuvant and stripes labelled with b denotes full-length rNg-ACP with each adjuvant.

Anti-rNg-ACP sera were also evaluated for their cross-reactivity with *N. gonorrhoeae* strain FA1090 whole cell lysate and OM (**Figure** 34 **A and B**). *N. gonorrhoeae* strain FA1090 expressed Ng-ACP encoded by Allele 6, whereas the homologous Ng-ACP from P9-17 is encoded by Allele 10.Immunization with full-length rNg-ACP in all of the formulations induced antisera that recognised, with similar reactivity, Ng-ACP of the heterologous strain *N. gonorrhoeae* FA1090

(**Figure 34A, B**). Again, the reactivity with FA1090 OM was visually much higher than with the whole cell lysates.

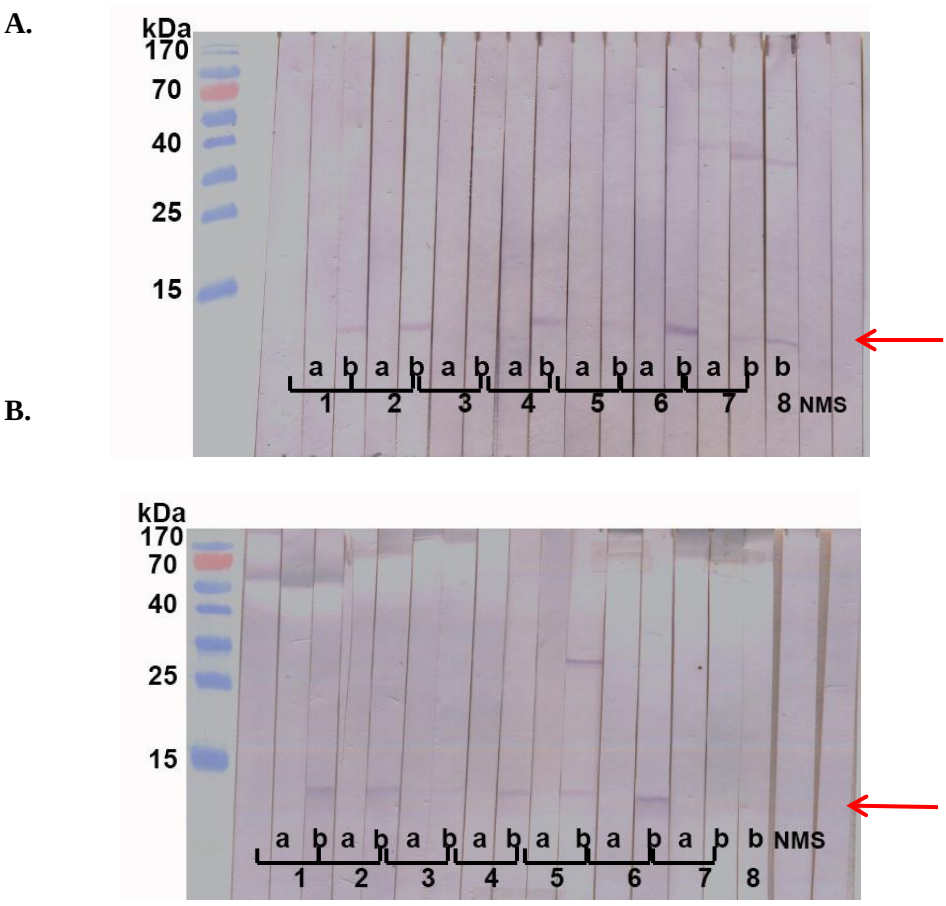


Figure 34. Reactivity of murine antisera to full-length rNg-ACP and sham immunised sera in western blots of A) whole cell lysate and B) outer membranes of *N. gonorrhoeae* strain FA1090.

Pooled antisera were diluted in TTBS buffer (1/100 dilution) and reacted with the strips for 90 minutes. The arrow denotes the position of Ng-ACP protein (*Mr*~12.5kDa). Antisera to rNg-ACP raised in saline (Lane 1), $Al(OH)_3$ (Lane 2), ZW 3-14 (Lane 3), ZW 3-14 +MPLA (Lane 4), Liposomes (Lane 5), Liposomes+MPLA (Lane 6), rabbit antiserum (1/400 dilution) raised to *Neisseria meningitidis* rNm-ACP (rabbits R710 Lane 7, R711, Lane 8). NMS, denotes reactivity of normal mouse serum, which was also indicative of the reactivity of sham-immunised sera.

Reactivity of these antisera was specific, as shown by the presence of a single immuno-reactive band in all of the strips and no reactivity of the corresponding sham-immunised sera (left side on each label), which was similar to that observed with normal mouse serum (**Figure 34A, B**). Rabbit antisera raised to meningococcal rNm-ACP using Freund's adjuvants was also tested against FA1090, and both antisera cross-reacted with FA1090 Ng-ACP present in the whole cell lysate rather than the OM, at the serum dilution tested (1/100). For both gonococcal preparations, there was a lack of reactivity of antisera raised to rNg-ACP with detergent preparation and simililar pattern of reactivity observed in the homologous gonococcal strain.

3.5.1.3 Detection of expression of Ng-ACP on the surface of N.gonorrhoeae P9-17-flow cytometry (FC).

Flow cytometry was used to examine the expression of Ng-ACP on the surface of gonococci, using murine antisera that showed the highest levels of reactivity against full-length rNg-ACP on ELISA. Initially, pilot experiments were done to determine the optimal serum dilution that allowed signficant changes to be observed in fluorescein isothiocyanate (FITC) fluorescence-recorder events between wild type P9-17 and the corresponding knockout strain (P9-17 *⊿ng-acp*) (**Figure 35).** A dilution series of pooled anti-rNg-ACP sera raised with $Al(OH)_3$ and sham-immunised sera was examined: Similar reactivities were observed for most of the dilutions of sham and anti-rNg-ACP sera against both wild-type and knock-out P9-17, with no observable shifts in reactivity curves. However, a serum dilution of 1/100 demonstrated a marginal shift in FITC fluorescence-recorder events **(Figure 35**).

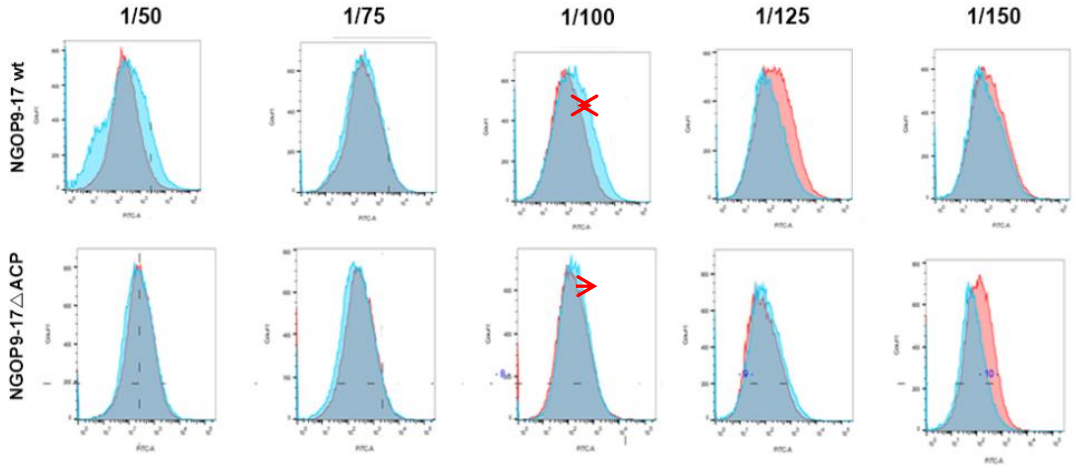


Figure 35. Reactivity of murine anti-full-length rNgACP sera raised with Al $(OH)_3$ and sham immunised sera with P9-17 wild-type and knockout (*Δng-acp*) gonococci.

The areas within the red lines are the reactivity with pooled sham-immunised mice (n=5) and the areas beneath the blue lines denote the reactivity of pooled (n=5) anti- rNg-ACP sera. Data are representative of two experiments using $1x10^6$ CFU/ml of bacteria per flow cytometry sample.

Based on these pilot experiments, recognition of Ng-ACP on the gonococcal surface was weak and only reproducible at a serum dilution of 1/100 tested; higher dilutions did not demonstrate any delineation between rNg-ACP immunised sera and sham sera.

In order to try and increase the signal sensitivity of the flow cytometry (FC) assay, the numbers of bacteria used in the assay were increased to ~1×10^9 CFU/ml and a FC analysis was repeated with a selection of pooled murine antisera raised to full-length rNg-ACP. Significant shifts in FITC fluorescence-recorder events were observed between sham and antisera raised to rNg-ACP with saline, liposomes and liposomes + MPLA, when tested against the wild-type P9-17 strain (**Figure 36**). However, these peak shift events were not significantly different from those observed when the sera were tested on the P9-17 *Δng-acp* variant.

Similarly, this phenomenon was observed on ELISA experiments (data not shown), when was titrated aluminium hydroxide (Al $(OH)_3$) mice sera against outer-membrane (OM) from *N. gonorrhoeae* strain P9-17). Lower antisera titres obtained similar with the control serum. Thus, the studies with OM-ELISA and FACS suggest that surface expression of Ng-ACP is low and the *in*

vitro assays are too insensitive to enable positive reactivity to be observed. By contrast, western

blots are more sensitive and demonstrate the presence of rNg-ACP within the OM preparations.

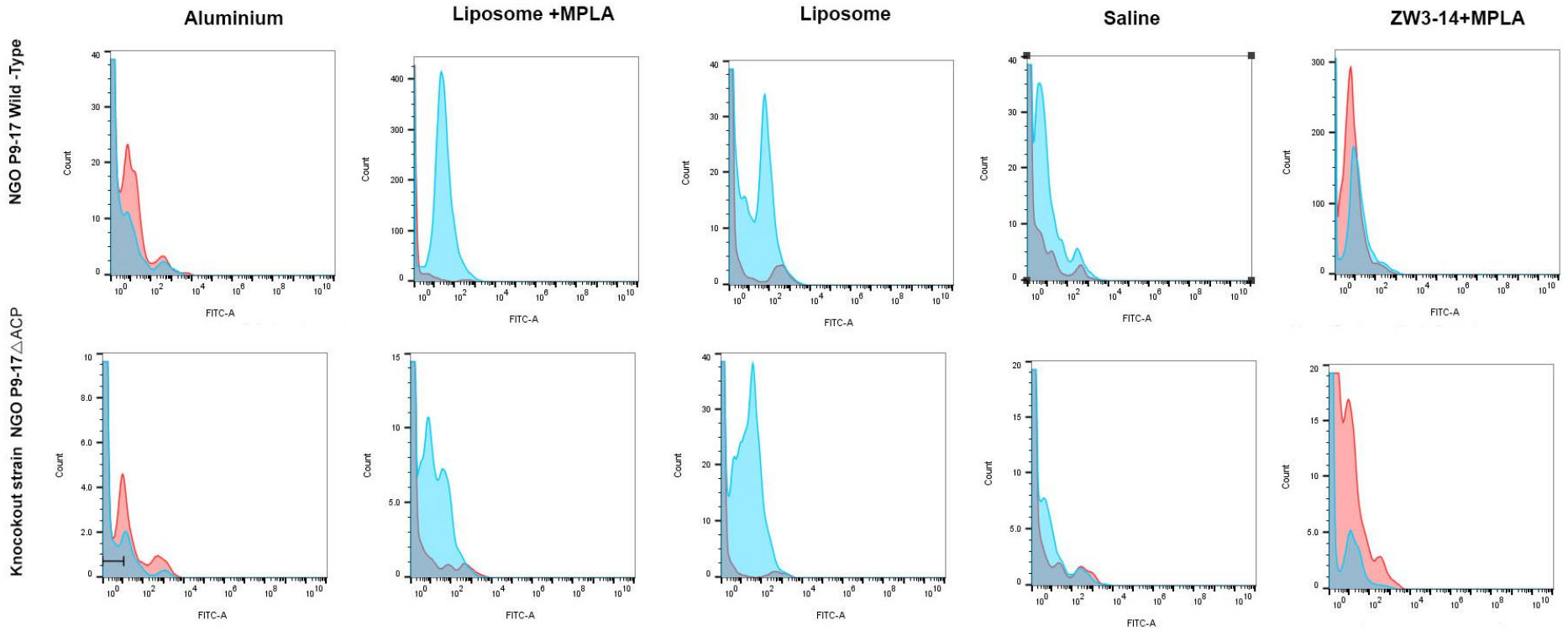


Figure 36. **Reactivity of murine anti-rNgACP sera raised with various formulations and the corresponding sham immunised sera with P9-17 wild-type and knockout (*Δng-acp*) gonococci.**

The areas within the red lines are the reactivity with pooled sham-immunised mice (n=5) and the areas beneath the blue lines denote the reactivity of pooled (n=5) anti-rNg-ACP sera. Data are representative of two experiments using 1×10^9 CFU/ml of bacteria per FC sample.

3.5.1.4 Immunization of mice with full-length rNgACP induces antibodies with serum bactericidal activity (SBA) against wild type Neisseria gonorrhoeae.

Pooled anti-rNg-ACP serum and the corresponding sham-immunised sera were tested for their ability to kill *N. gonorrhoeae* in a complement-dependent manner (Section 2.20). Sera were tested against both wild-type *N. gonorrhoeae* P9-17 (Allele 10) and FA1090 (Allele 6) strains and their corresponding knockout variants (*N. gonorrhoeae* strain P9-17*Δng-acp, N. gonorrhoeae* strain FA1090 *Δng-acp*).

Immunization of mice with rNg-ACP and different adjuvants and delivery systems induced SBA against the homologous P9-17 strains and the heterologous FA1090strain **(Table44)**. The highest SBA titres were observed with rNg-ACP protein in saline, adsorbed to Al $(OH)_3$ and in liposomes and detergent micelles with MPLA (median values of ~256-512 against P9-17 and 64-256 against FA1090). rNg-ACP in liposomes alone and detergent micelles alone induced SBA, with median titres of 64, which were increased significantly with the addition of MPLA to 128 (**Table 44**) All sham-immunised sera did not kill significantly either strain. SBA titres were specific towards Ng-ACP, since the SBA titres for anti-rNg-ACP sera tested against the corresponding knock-out variants P9-17*Δng-acp* and FA1090 *Δng-acp* were significantly lower than those recorded against the wild-type strains (Ng-ACP^+) (**Table 44**).

Table 44. Serum bactericidal activity of murine antisera raised to recombinant full-length *Neisseria gonorrhoeae*-ACP protein delivered with different adjuvants.

rNg-ACP formulation	Serum Bactericidal Activity Titre against strain			
	P9-17 WT	**P9-17 *Δng-acp***	**FA1090 WT**	**FA1090 *Δng-acp***
rNg-ACP-Saline	256 (256, 1024)	16	128 (64, 128)	4
Saline control	64	8 (4,8)	64 (16, 64)	4
rNg-ACP-Al(OH)$_3$	512 (256, 512)	64	256 (256, 512)	64
Al(OH)$_3$ control	16	<4	<8	8
rNg-ACP-Liposomes	64	16 (16,64)	64	4
Liposomes control	4 (4, 16)	8	≤4	4 (4,8)
rNg-ACP-Liposomes + MPLA	128 (16, 256)	32	64 (16, 128)	4
Liposomes +MPLA control	16	4	<4	4
rNg-ACP-ZW3-14	64	<4	64	≤4
ZW3-14 control	<4	<4	<4	4
rNg-ACP-ZW3-14 + MPLA	256 (256, 512)	≤4	256 (256, 1024)	16
ZW3-14+MPLA Control	<4	16	<8	4

Pooled antisera raised to full-length rNg-ACP using different adjuvants and delivery systems and the corresponding sham-immunised control sera were tested for their ability to induce complement-mediated killing of *N. gonorrhoeae* strain P9-17 (homologous Allele 10 encoded Ng-ACP) and FA1090 (heterologous Allele 6 encoded Ng-ACP).The data presented are the reciprocal of the highest serum dilution at which ⩾50% killing was observed. The titers are expressed as the median values from three or more independent experiments. The range of values in parentheses represent the reciprocal SBA titres for the number of experiments done.

3.6 Characterization of the biological and functional properties of antibodies against mature rNg-ACP.

The antigenicity of the mature soluble rNg-ACP was examined in a similar manner to the full-length insoluble rNg-ACP protein (Section 3.5). BALB/C mice (H-2^d haplotype) were immunised with mature rNg-ACP with different adjuvant formulations and delivery systems, using a 3 dose schedule (dose I, day 0; dose II, day 14; dose III, day 28) followed by terminal bleeding by cardiac puncture on day 42. Mice were immunised with mature rNg-ACP in saline alone and adsorbed to aluminium hydroxide (Al $(OH)_3$) or delivered in liposomes and Zwitterion detergent (ZW 3-14), with and without the addition of exogenous immuno-modulator MPLA. Antisera were then tested for antigenicity with a variety of *in vitro* assays.

3.6.1. Antigenicity of murine antisera to mature rNg-ACP

3.6.1.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Individual murine sera raised against soluble mature rNg-ACP with the different adjuvant formulations and delivery systems were tested by ELISA. Serial dilutions of test and sham sera were tested initially against immobilised recombinant protein (rNg-ACP) and end point dilution titres were determined from linear regression analysis and extrapolation of the titration curves for each serum sample, as described above (Section 3.5.1.1). The geometric mean (GM) ELISA endpoint titre with 95% confidence limits (CL) was calculated for each group of animals per inoculating sample preparation and an independent student-t-test with 95% confidence limits was used to compare the mean values between groups, with $P<0.05$ reporting significance.

All the antisera raised with the different adjuvant and delivery systems showed similar reciprocal geometric mean (GM) ELISA titer reactivity against the recombinant protein (**Figure 37**). In general, the levels of ELISA reactivity were lower compared with those observed for the full-length rNg-ACP protein (Section 3.5.1.1). Student t-test- analysis showed no significant differences between the mean ELISA serum titres sera induced by mature rNg-ACP in saline, $Al(OH)_3$, detergent micelles, and liposomes with or without MPLA ($P>0.05$). The highest ELISA titres were observed using saline and liposome + MPLA formulation with mean titres of ~66000 and ~166000 respectively.

Overall reciprocal geometric mean titres ranged from ~7000 for rNg-ACP-ZW 3-14 antisera to ~66,000 for the protein in saline, but with wide confidence limits. By contrast, the geometric mean titre for rNg-ACP in liposomes was increased from ~12,000 to ~ 166,000 with the addition of MPLA (**Figure 37)**.

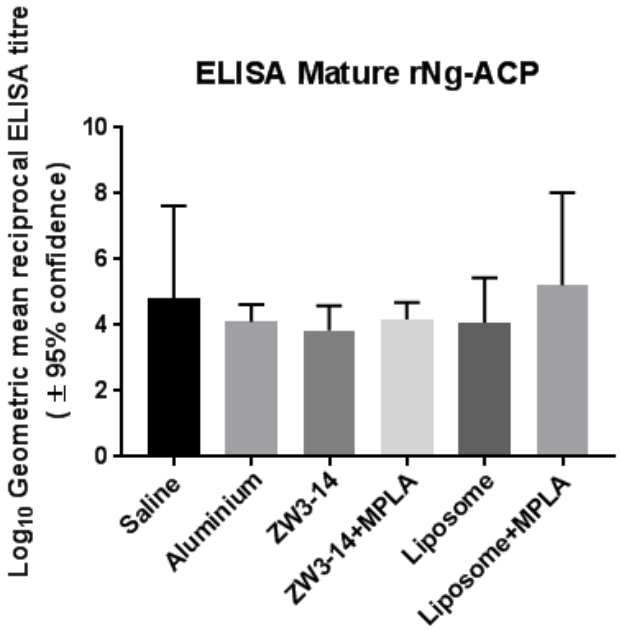


Figure 37. ELISA reactivity of murine antisera raised to soluble mature rNg-ACP with different adjuvants and delivery formulations.

Serial dilutions of individual sham and immunised sera were tested against pure rNg-ACP in ELISA.No reactivity was observed with sham-immunised sera. The columns represent the geometrical mean of reciprocal ELISA titers (n=5 animals/group) and the error bars the 95% confidence limits. * denotes statistical significance (P<0.05).

3.6.1.2 Recognition of Ng-ACP protein in Neisseria gonorrhoeae whole cell lysates and outer membranes by serum reactivity in western blot.

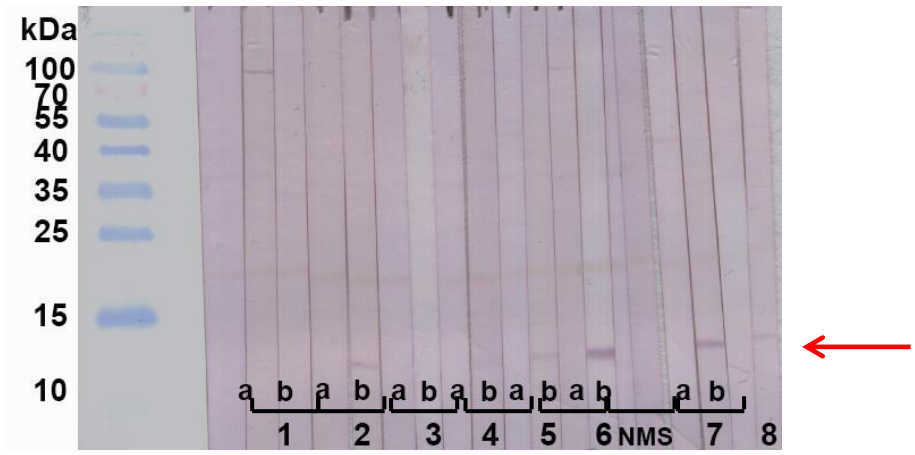
As described above, whole cell lysates and OMV were prepared from *N.gonorrhoeae* strain P9-17 and were separated by SDS-PAGE electrophoresis (Section 2.9) and transfered to a nitrocellulose membranes, following the protocol described in Section 2.18. The membrane was cut into 0.3 cm wide strips and individual stips were reacted in western blot with pooled antisera to soluble mature rNg-ACP sera raised with different formulations and delivery systems or with sham immunised sera.

Antisera raised to soluble mature rNg-ACP recognised Ng-ACP protein of *Mr*~12.5kDa in whole cell lysates and outer membranes (OMV) (**Figure 38A, B**). However, as observed with antisera

raised to full-length protein, in general, the reactivity with OM of antisera to mature protein was visually much higher than with the whole cell lysates. Reactivity of the antisera was specific, as shown by the presence of a single immuno-reactive band in some of the strips and no reactivity of the corresponding sham-immunised sera (left side of each label), which was similar to that observed with normal mouse serum (**Figure 38A, B**). Aluminium hydroxide ($Al(OH)_3$) and liposome+MPLA formulations displayed a high visually immuno-reactive band whereas, detergent (ZW3-14) and detergent+MPLA (ZW3-14+MPLA) preparations displayed a slightly less visually immuno-reactive band. On the contrary, saline and liposome formulation did not show significant immuno-reactivity against Ng-ACP. Rabbit antisera raised to gonococcal rNg-ACP using Freund's adjuvants was also tested against the gonococcal preparations, and sera from both rabbits showed immuno-reactivity with P9-17 OM **Figure 38 B**). In addition, murine and rabbit antisera to mature rNg-ACP did not react with lysate preparation from homologous *N. gonorrhoeae* strains P9-17 *Δng-acp* (**Figure 96 A** Appendix A.15**)** and heterologous knockout strains *N. gonorrhoeae* FA1090 *Δng-acp* **(Figure 96 B** Appendix A.15**).**

Murine anti-mature rNg-ACP sera was tested against the mature recombinant protein, to confirm and display similar reactivity in all the formulations. Additionally, aluminium hydroxide ($Al(OH)_3$), detergent micelles (ZW3-14) and liposomes with MPLA formulations showed an additional band of *Mr*~24kDa and slightly high reactivity compared with the other formulations as liposome and detergent with immunomodulador MPLA (**Figure 94 B** Appendix A.15). Murine antisera to mature rNg-ACP was also reacted against full-length rNg-ACP protein; strong immunoreactivity with aluminium hydroxide ($Al(OH)_3$) and liposome +MPLA preparations, showing a band at *Mr*~17.5 kDa.By contrast, little or not immuno-reactivity was observed with saline and detergent preparations **(Figure 93 B** Appendix A.15).

A.



B.

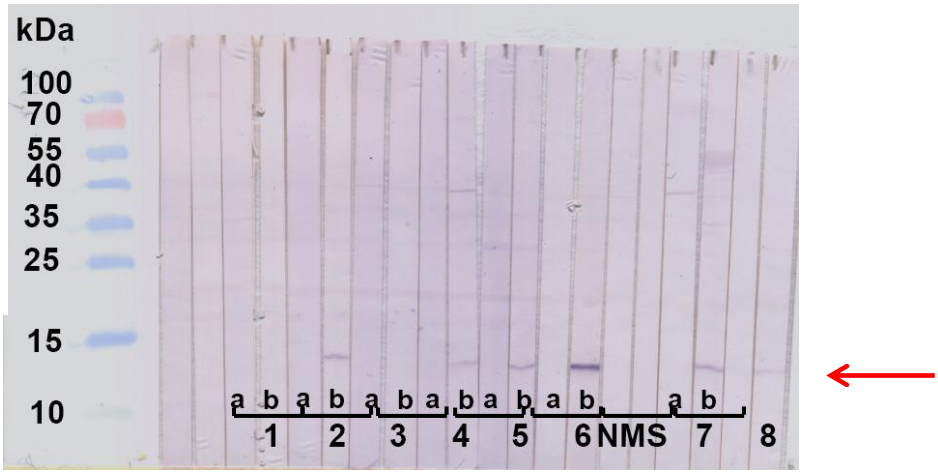


Figure 38. Reactivity of murine antisera to full-length rNg-ACP and sham immunised sera in wester blots of **A)** whole cell lysate and **B)** outer membranes of *N. gonorrhoeae* strain P9-17.

Pooled antisera were diluted in TTBS buffer (1/100 dilution) and reacted with the strips for 90 minutes. The arrow denotes the position of Ng-ACP protein (*Mr*~13kDa). Antisera to rNg-ACP raised in saline (Lane 1), $Al(OH)_3$ (Lane 2), ZW 3-14 (Lane 3), ZW 3-14 +MPLA (Lane 4), Liposomes (Lane 5), Liposomes+MPLA (Lane 6), rabbit antiserum (1/400 dilution) raised to *Neisseria gonorrherrae* rNg-ACP (Rabbit 1 Lane 7, Rabbit 2, Lane 8). NMS, denotes reactivity of normal mouse serum, which was also indicative of the reactivity of sham-immunised (labelled strips with letter a).

Antisera raised to the soluble, mature -rNg-ACP protein were also evaluated for their cross-reactivity with whole cell lysate and OM of the heterologous *N. gonorrhoeae* strain FA1090 (**Figure 39**). Immunization with mature protein induced antisera that recognised, with similar reactivity, Ng-ACP of *N. gonorrhoeae* FA1090 (**Figure 39 A, B**). Again, the reactivity with FA1090 OM was visually much higher than with the whole cell lysates. Reactivity of these antisera was specific, as shown by the presence of a single immuno-reactive band in all of the strips ,especially with aluminium hydroxide ($Al(OH)_3$), detergent+MPLA (ZW3-14+MPLA) and liposome formulations with and without MPLA preparations. No reactivity of the corresponding

sham-immunised sera was observed (left side of each label), which was similar to that of normal mouse serum (**Figure 39 A, B**). Rabbit antisera raised to gonococcal rNg-ACP using Freund's adjuvants was also tested against FA1090, and both antisera reacted with FA1090 Ng-ACP present in the whole cell lysate rather than the OM, at the serum dilution tested (1/100).

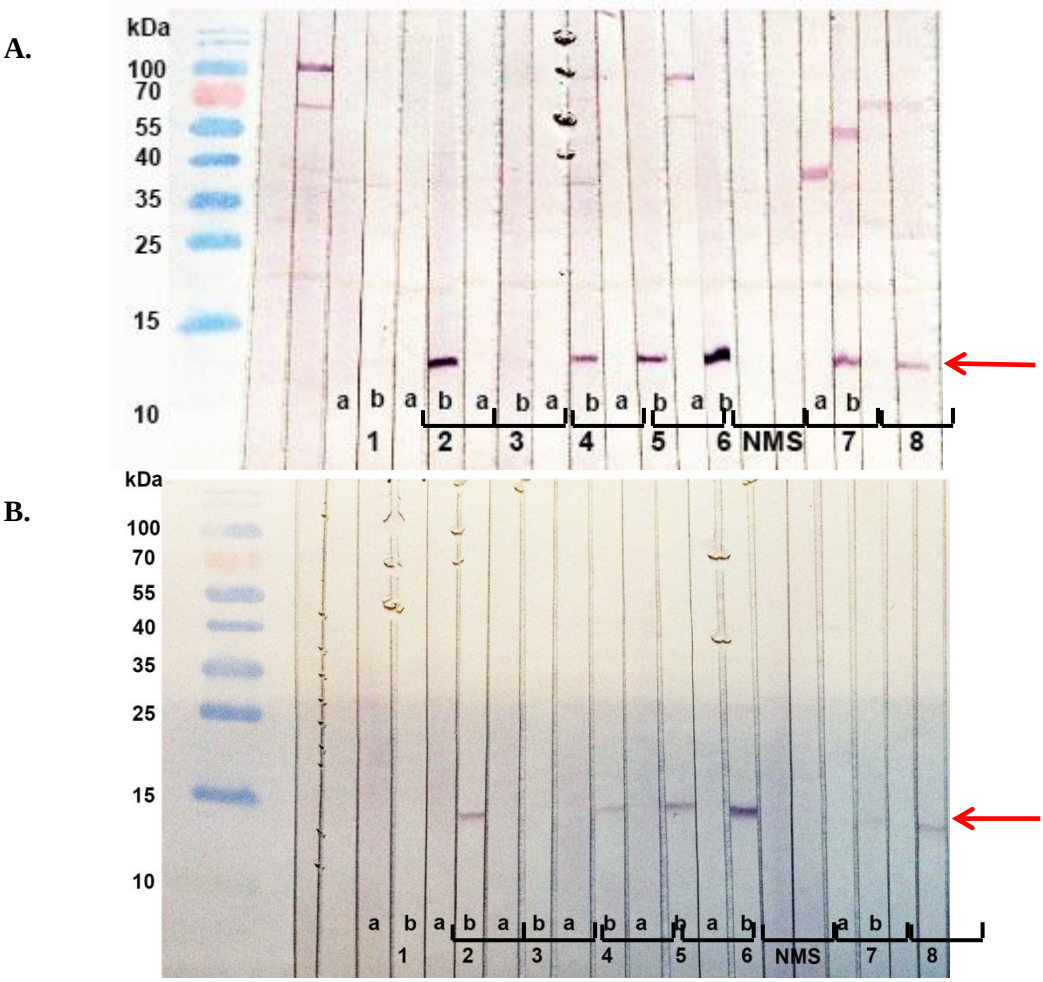


Figure 39. Reactivity of murine antisera to soluble mature rNg-ACP and sham immunised sera in western blots of A) whole cell lysate and B) outer membranes of *N. gonorrhoeae* strain FA1090.

Pooled antisera were diluted in TTBS buffer (1/100 dilution) and reacted with the strips for 90 minutes. The arrow denotes the position of Ng-ACP protein (*Mr*~13kDa). Antisera to rNg-ACP raised in saline (Lane 1), Al(OH)3 (Lane 2), ZW 3-14 (Lane 3), ZW 3-14 +MPLA (Lane 4), Liposomes (Lane 5), Liposomes+MPLA (Lane 6), rabbit antiserum (1/400 dilution) raised to *Neisseria gonorrhoeae* rNg-ACP (rabbits 1 Lane 7, Rabbit 2, Lane 8). NMS, denotes reactivity of normal mouse serum, which was also indicative of the reactivity of sham-immunised sera.

3.6.1.3 Detection of expression of Ng-ACP on the surface of N. gonorrhoeae P9-17 using Flow cytometry (FC).

Flow cytometry was used to examine the expression of Ng-ACP on the surface of P9-17 gonococci, using murine antisera that showed the highest levels of reactivity against the mature rNg-ACP on

ELISA (**Figure 31**). As observed with the full-length protein, antisera raised to the mature rNg-ACP with the different adjuvant and delivery systems, tested at a 1/100 dilution, did not induce signficant (P>0.05) shifts in FITC-recorded events with the wild-type or knock-out P9-17 strain, compared with sham-immunised mice (**Figure40**) or normal mouse serum (not shown).

3.6.1.4 Immunization of mice with soluble mature rNgACP induces antibodies with serum bactericidal activity (SBA) against wild type Neisseria gonorrhoeae.

Pooled anti-rNg-ACP serum and the corresponding sham-immunised sera were tested for their ability to kill *N. gonorrhoeae* in a complement-dependent manner (Section 2.20). Sera were tested against both wild-type *N. gonorrhoeae* P9-17 (Allele 10) and FA1090 (Allele 6) strains and their corresponding knockout variants (*N.gonorrhoeae* strain P9-17*Δng-acp* and *N.gonorrhoeae* strain FA1090*Δng-acp*) (**Table 45**). Immunization of mice with mature rNg-ACP and different adjuvants and delivery systems induced SBA against the homologous strain P9-17 and the heterologous strain FA1090 (**Table 45**). The highest SBA titres were observed with rNg-ACP protein in saline, in liposomes and detergent micelles with MPLA (median values of ~128-256 against P9-17 and 64-256 against FA1090) rNg-ACP in liposomes alone and detergent micelles alone induced SBA, with median titres of 64 (**Table 45**). All sham-immunised sera did not kill significantly either strain. SBA titres were specific towards Ng-ACP, since the SBA titres for anti-rNg-ACP sera tested against the corresponding knock-out variants P9-17*Δng-acp* and FA1090*Δng-acp* (Ng-ACP$^-$) were significantly lower than those recorded against the wild-type strains (Ng-ACP$^+$) (**Table 45**). Antisera raised to mature rNg-ACP adsorbed on $Al(OH)_3$ induced only marginal SBA, when killing was compared between the wild-type P9-17 (median titre 16-64) and corresponding knock-out (median titre 16 (4, 64) strain and the heterologous FA1090 wild-type (median titre 16) and corresponding knock-out (median titre<4) strain (**Table 45**).

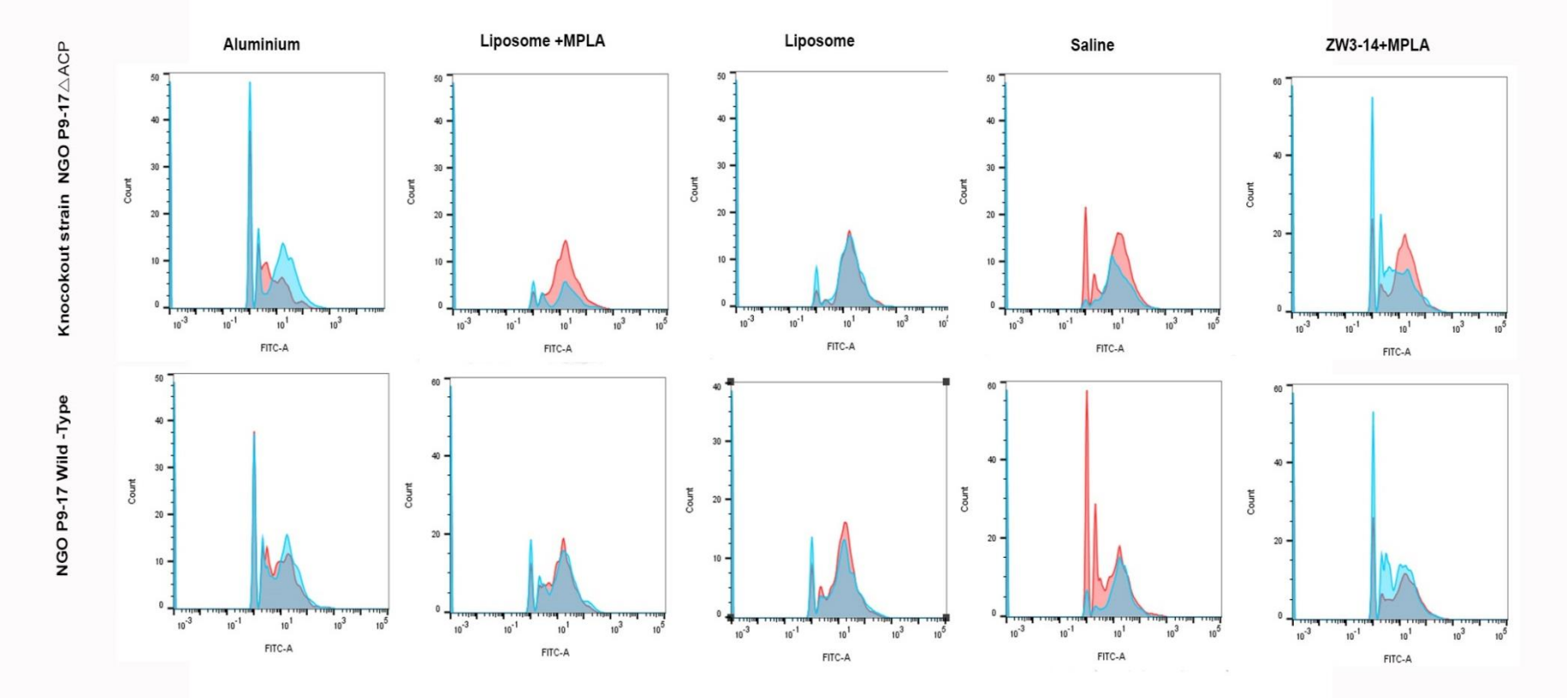


Figure 40. Reactivity of murine anti-rNgACP sera raised against mature rNg-ACP with various formulations, with P9-17 wild-type and knockout (*Δng-acp*) gonococci examined with flow cytometry. The areas within the red lines are the reactivity with pooled sham-immunised mice (n=5) and the areas beneath the blue lines denote the reactivity of pooled (n=5) anti-rNg-ACP sera. Data are representative of two experiments using 1×10^9 CFU/ml of bacteria per flow cytometry sample.

Table 45. Serum bactericidal activity of murine antisera raised to recombinant soluble mature *Neisseria gonorrhoeae*-ACP protein delivered with different adjuvants.

rNg-ACP formulation	Serum Bactericidal Activity Titre against strain			
	P9-17 WT	**P9-17 *Δng-acp***	**FA1090 WT**	**FA1090 *Δng-acp***
rNg-ACP-Saline	256	4 (4, 128)	256	64
Saline control	4	<4	<4	<4
rNg-ACP-Al(OH)$_3$	16-64	16 (4, 64)	16	<4
Al(OH)$_3$ control	<4	8	4	<4
rNg-ACP-Liposomes	128-256	4 (4, 64)	64	<4 (<4, 128)
Liposomes control	4-8	16	4	4
rNg-ACP-Liposomes + MPLA	64	16	64	<4
Liposomes +MPLA control	4	4	<4	<4
rNg-ACP-ZW3-14	64	4	64	<4
ZW3-14 control	4	<4	4	<4
rNg-ACP-ZW3-14 + MPLA	256 (256, 512)	4	256 (256, 512)	<4
ZW3-14+MPLA Control	16	4	16	4

3.7 Antigenicity of rabbit antisera against full-lenth rNg-ACP.

Similar analyses were done to determine the antigenicity of full-length or mature rNg-ACP (Section 3.5 and Section 3.6) in rabbits. An immunization trial was done in rabbits (n=2) with rNg-ACP inFreund's adjuvant by Davids Biotechnologies (GmbH, Germany), using a 5 dose schedule dose I (day1), dose II (day 14), dose III (day 28), dose IV (day 42) dose V (day 56) followed by terminal bleeding by cardiac puncture under anesthesia on day 63.

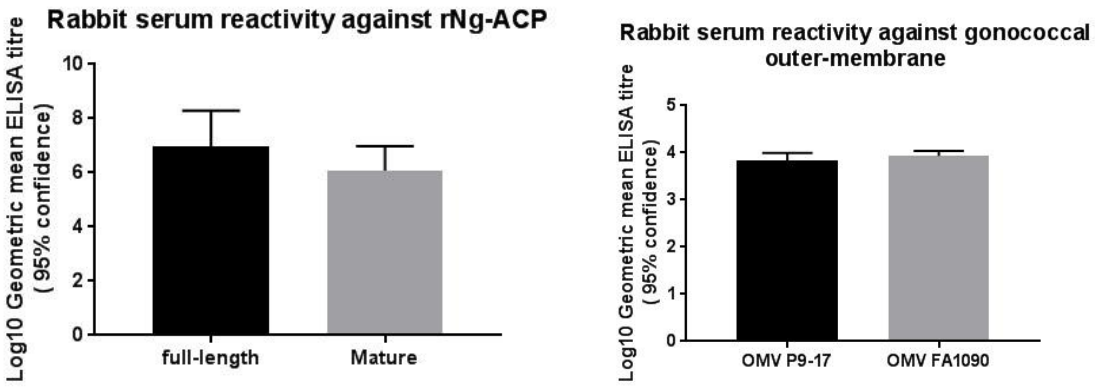
3.7.1. Antigenicity against full and mature rNg-ACP.

Rabbit ELISA titres against the full-length rNg-ACP were similar compared with the titres obtained using murine sera anti full-length rNg-ACP raised with aluminium hydroxide ($Al(OH)_3$) and liposomes+MPLA formulations (**Figure 32**). A small reduction in of titres against the mature rNg-ACP protein was found, but slightly higher compared the murine sera anti mature rNg-ACP (**Figure 41A**). Taken together the rabbit antisera does not show any significant differences between two different antigens ($P>0.05$).

3.7.2. Antigenicity against a diverse outer-membrane preparations from *N. gonorrhoeae.*

ELISA titres against outer-membrane OMV from homologous and heterologous gonococcal strains displayed low reactivity and there was similiar titres found in murine antisera against mature rNg-ACP protein (**Figure 37**). The rabbit antisera reactivity did not show any significant difference ($P>0.05$) between homologous and heterologous gonococcal strains tested (**Figure 41 B**).

Furthermore, analysis of lysate preparation of gonococcal strains panel used in this study (*N. gonorrhoeae* strains P9-17, FA1090 and MS11) display a single band at *Mr*~12.5 kDa on each strain tested (**Figure 42**).



A. **B.**

Figure 41. ELISA reactivity of rabbit antisera raised to recombinant protein full-length rNg-ACP with Freund adjuvant . Serial dilutions of individual sham and immunised sera were tested against **A.**Purified full-length or mature rNg-ACP proteins. **B.** Outer-membrane preparation from homologous and heterologous *N. gonorrhoeae* strains P9-17 and FA1090 respectively. The columns represents the geometrical mean of reciprocal ELISA titers (n=2 animals/group) and the error bars the 95% confidence limits. * denotes statistical significance (P<0.05).

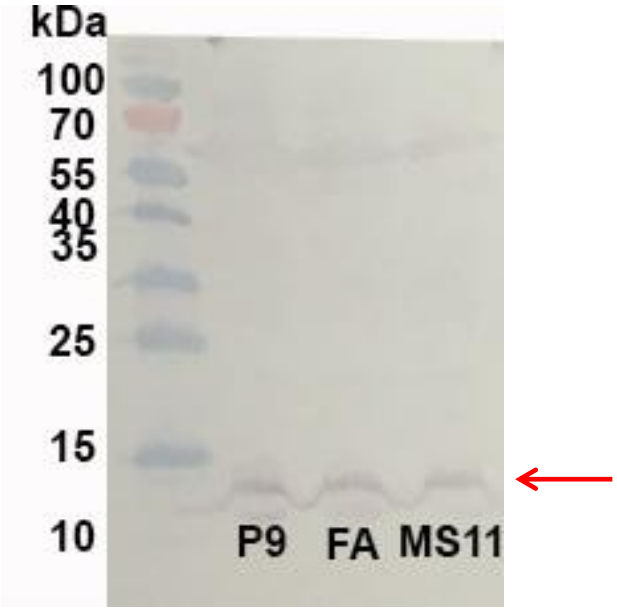


Figure 42. Identification of Ng-ACP protein in the different *N. gonorrhoeae* strains used in this study. **P9** *N. gonorrhoeae* strain P9-17 **FA1090** *N. gonorrhoeae* strain FA1090. **MS11** *N. gonorrhoeae* strain MS11. The arrow denotes a single band with *Mr* ~12.5kDa.

3.7.3 Detection of expression of Ng-ACP on the surface of *N. gonorrhoeae* P9-17 using Flow cytometry (FC).

Reactivity of rabbit antisera against Ng-ACP on the surface of *N. gonorrhoeae* was similar compared with the murine antisera with full-length or mature Ng-ACP. The rabbit sera reactivity did not show any observable shift between wild-type and mutant strains (**Figure 43**). This result confirmed once more the poor reactivity to determine the localization of this antigen on the surface of *N. gonorrhoeae*, with this technique.

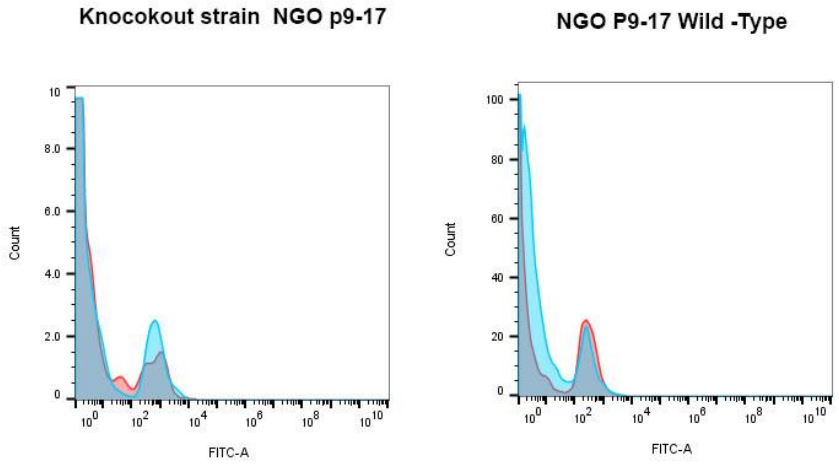


Figure 43. Localization of Ng-ACP on the surface of *N. gonorrhoeae* strain P9-17 by flow cytometry.Reactivity of rabbit antisera against-full-length rNgACP with Freund's adjuvant and the corresponding sham immunised sera with P9-17 wild-type and knockout (*Δng-acp)* gonococci. The areas within the red lines are the reactivity with pooled sham-immunised rabbits (n=2) and the areas beneath the blue lines denote the reactivity of pooled (n=2) anti-rNg-ACP rabbit sera. Data are representative of two experiments using 1×10^9 CFU/ml of bacteria per FACS sample.

3.8. Preparation of native outer membrane vesicles (OMV) from *Neisseria gonorrhoeae* strains P9-17 and FA1090 and detergent-extracted OMV (Na-DOC OMV) from strain P9-17.

An overnight lawn of *N. gonorrhoeae* strain P9-17 and *N. gonorrhoeae* strain FA1090 was prepared from liquid nitrogen stocks onto GC agar. A suspension of bacterial growth was prepared in proteose-peptone broth was prepared and used to inoculate 42 large petri plates. Native outer membranes vesicles (OMV) were prepared by lysis and differential centrifugation and suspended in final volumes of 1ml of sterile UHQ water. Two independent OMV preparations were made for

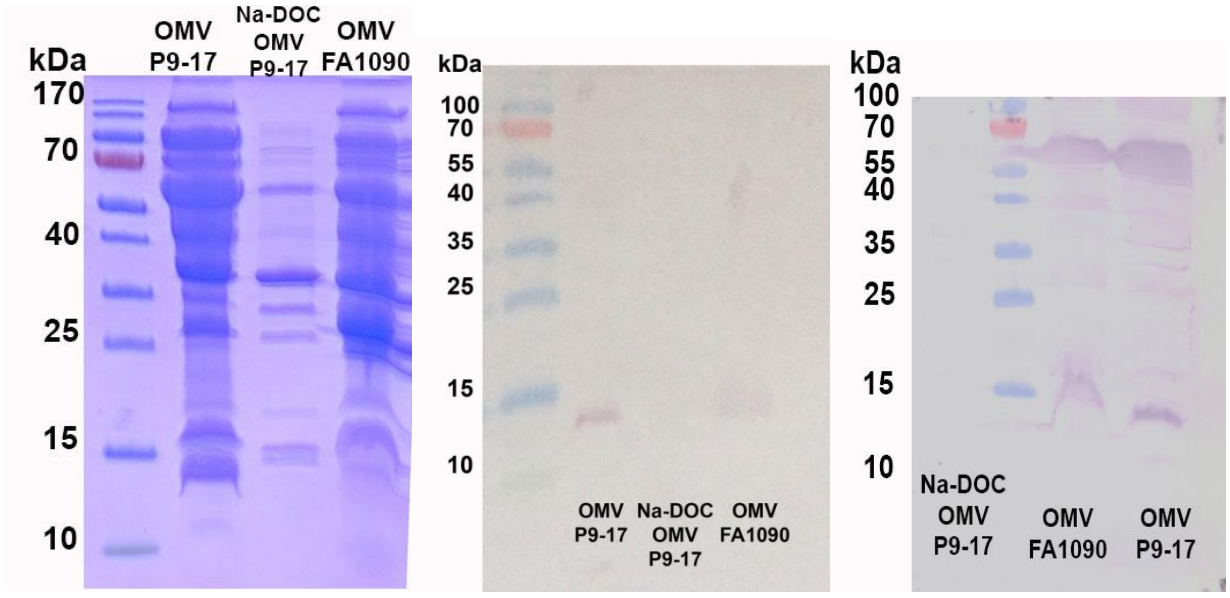
each bacterium. To prepare detergent-extracted Na-DocOMV using sodium deoxycholate (Na-DOC OMV) ~300 µl of *N. gonorrhoeae* strain P9-17 OMV preparation was treated with Na-DOC.Extracted OMV were collected by differential high speed centrifugation and Na-DOC OMV was suspended in a final volume of 500µl of sterile UHQ water.

Protein concentrations of the OMV and Na-DOC OMV preparations was estimated using a bicinchoninic acid (BCA) assay (Thermo, Fisher) using a calibration standard curve of bovine serum albumin (BSA) (**Table 60**, Appendix A.17.1) Protein concentrations were calculated from the calibration curve by linear regression showed in **Figure 98**, **Figure 99** and **Figure 100** (Appendix A.17.2 to A.17.4) and interpolated using the linear ecuation and summarised in **Table 61, Table 62** and **Table 63** (Appendix A.17.2 to A.17.4). Concentrations of the preparations are shown in **Table 46**.

Table 46. Protein concentration of different *N. gonorrhoeae* preparations determined by BCA assay.

Preparation	Protein concentration (BCA asay)
N. gonorrhoeae P9-17 native OMV	9930.12 µg/ml
N. gonorrhoeae FA1090 native OMV	5455.45 µg/ml
N. gonorrhoeae P9-17 Na-DOC OMV	1600 µg/ml

SDS-PAGE and western blot using rabbit antisera raised against full-length rNg-ACP (tested at 1/100 dilution) was done to examine the protein profiles of the OMV and Na-DOC OMV preparations The *N. gonorrhoeae* P9-17 Na-DOC OMV showed a significant reduction in the presence and also the amount of protein, compared with the P9-17 native OMV, particularly with respect to small *Mr* proteins (**Figure 44 A**). Western blot identified a single band of *Mr*~12.5kDa consistent with Ng-ACP protein in both native OMV preparations, with higher reactivity observed with *N. gonorrhoeae* P9-17 compared with *N. gonorrhoeae* FA1090 **Figure 44 B.** and **Figure** 44 **C**). By contrast, no immuno-reactivity was observed against P9-17 Na-DOC OMV at the concentration of antiserum tested (**Figure 44 B.** and **Figure 44C**.). Increased antibody concentration (dilution 1/10) confirmed the presence of Ng-ACP protein exclusively in both OMV preparations, however with a nonspecific bands (**Figure 44 C**).



A. **B.** **C.**

Figure 44. Comparison of native OMV and Na-DOC OMV preparations from ***Neisseria gonorrhoeae*** strains P9-17 and FA1090.

A). SDS-PAGE of native OMV and Na-DOC OMV preparations with Coomassie blue staining. B) Western blot of native OMV and Na-DOC OMV reacted with rabbit antisera against full-length rNg-ACP (1/100 dilution). C) Western blot of native OMV and Na-DOC OMV reacted with rabbit antisera against full-length rNg-ACP (1/10 dilution).

3.8.1 Antigenicity of murine anti-sera raised against outer membrane vesicle (OMV) and sodium-deoxycholate extracted OMV (Na-DOC OMV).

Mice were immunised individually with either 1µg or 10 µg of OMV and Na-DOC OMV preparations of *N. gonorrhoeae* strain P9-17 with saline or aluminium hydroxide (Al $(OH)_3$) adjuvants as described in Materials and Methods (Section 2.13.1 and 2.13.2) and individual sera were tested in ELISA against the OMV homologous and heterologous *N. gonorrhoeae* P9-17 and *N. gonorrhoeae* FA1090 strain immunizing preparations (**Figure 45).**

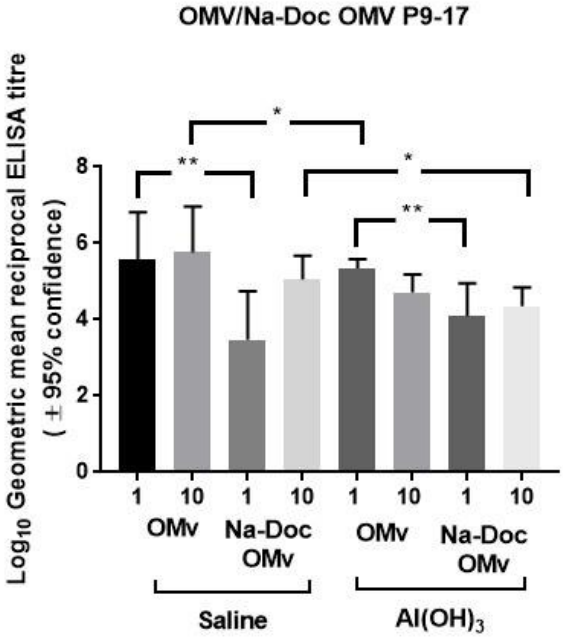
Each group of formulation (n=5) with OMV and Na-DOC OMV against native OMV from *N. gonorrhoeae* P9-17 strain showed similar mean ELISA titres compared anti mice sera raised with recombinant rNg-ACP. Antisera immunised with 1µg/ml and 10µg/ml concentration of OMV in saline formulation showed the highest mean ELISA titres of ~363,000 and ~575,000 respectively (**Figure 45 A**). Similar titres of detergent extracted OMV (Na-Doc OMV) at high concentration ~110,000 (10µg/ml).By contrast, the lowest mean ELISA titres were observed with antiseraraised with (Na-Doc OMV) mean ELISA titres ~2950 with 1µg/ml in saline alone adjuvant. Mean ELISA titres of antisera with (Al$(OH)_3$) adjuvant immunised with OMV were lower compared with OMV

in saline alone adjuvant. Conversely, antibody titres induced (Na-Doc OMV) with with aluminium hydroxide ($Al(OH)_3$) were higher compared with saline formulation, i.e ~12,300 (1µg/ml) and ~22,400 (10µg/ml) respectively (**Figure45A)**.

Antigenicity of the murine antisera against heterologous outer membrane from *N. gonorrhoeae* FA1090 strain slightly lower compared with P9-17 strain. Titres with detergent extracted OMV (Na-Doc OMV) at both concentrations were ~1922600 (1µg/ml) and ~10000000 (10µg/ml). Similarly, titres elicited with OMV preparations with saline at both concentrations tested were ~4933600 (1µg/ml) and ~6492500 (10µg/ml), and in a similar maner only at highest concentration of OMV in saline prerations was observed ~15000000 (10µg/ml). Lowest titres were obtained with detergent extracted OMV (Na-Doc OMV) ~71980 (1µg/ml) and ~997300 (10µg/ml), which was a significant difference showed between the detergent extracted OMV at (10µg/ml) at different adjuvant used ($P<0.05$). For both analysis demonstrated the reactivity is largely depends on the type of strains to be analysed and type of adjuvant used for the analysis.

Immuno reactive bands were observed when the murine antisera reacts with outer membrane prerations of homologous and heterologous strains *N. gonorrhoeae* strain P9-17 and N. gonorrhoeae FA1090. The main difference is the intensity of the immune-reactive bands, which is higher in *N. gonorrhoeae* strain P9-17 compared in the heterologous strain. As well as, murine antisera raise against to detergent extracted Na-Doc-OMV showed the lack of recognition of lower molecular weight bands (**Figure 97**).

A.



B.

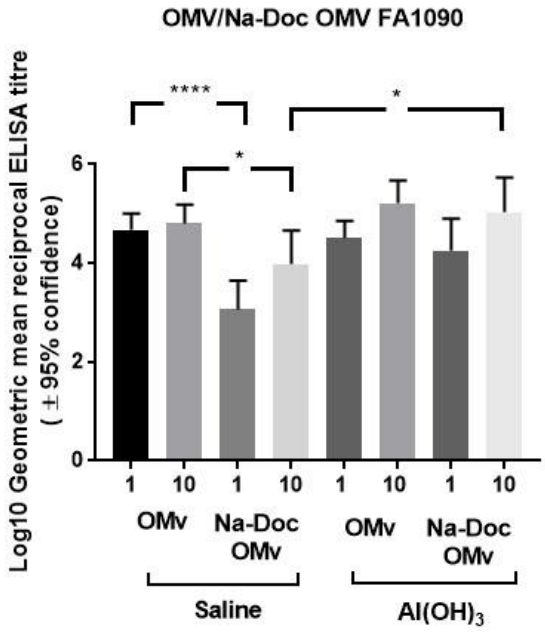


Figure 45. ELISA reactivity of antisera raised with *N.gonorrohoeae* P9-17 OMV and Na-DOC OMV against homologous and heterologous OMV from *N. gonorrhoeae* strains. A) *N. gonorrhoeae* strain P9-17. B) *N. gonorrhoeae* strain FA1090.

Sham sera do not show any significant reactivity. The columns represent the geometric means of reciprocal ELISA titres (n=5 animals /group) and the error bars represent the 95% confidence limits.

3.8.1.1 Reactivity against recombinant protein.

The mean ELISA titres for each group of formulation with different concentration of OMV preparation were similar. There was no significant difference (P>0.05) of reactivity against the recombinant protein full-length rNg-ACP, regardless of different OM or amount of antigen used. The titres observed were lower compared to the reactivity of murine sera agasinst full-length rNg-ACP recombinant protein (**Figure 46).**

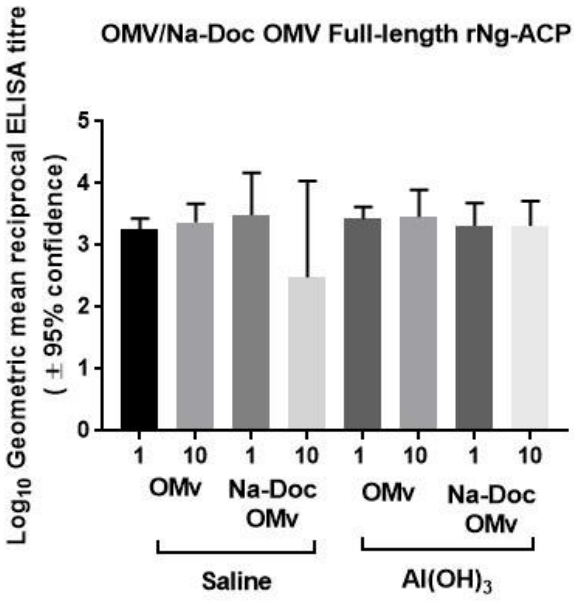


Figure 46. ELISA reactivity of antisera raised with *N.gonorrohoeae* P9-17 OMV and Na-DOC OMV against recombinant full-length rNg-ACP protein.

Sham sera did not showany significant reactivity. The columns represent the geometric means of reciprocal ELISA titres (n=5 animals /group) and the error bars represent the 95% confidence limits.

3.8.2 Bactericidal activity of antisera raised to OMV and Na-DOC OMV from *Neisseria gonorrhoeae* strains P9-17.

Characterization of composition OMV and Na-Doc OMV are comprised a repertoire of molecules, implicated in different mechanisms of host-pathogen. Study of the role of OMV or detergent extracted Na-Doc OMV in the host–pathogen interaction yields new information about the virulence mechanism of pathogenic bacteria (Collins, 2011, Acevedo et al., 2014). Analysis of bactericidal activity of OMV and Na-DocOMV preparations using diverse adjuvants against homologous and heterologous gonococcal strains were compared with the SBA reactivity generated with a single recombinant protein of one of the antigens.

The aim was to compare the serum bactericidal activities of antisera raised to OMV and Na-DOC-OMV from *N. gonorrhoeae* P9-17 preparations, immunised using two different systems (saline and adsorbed to $Al(OH)_3$ at two different doses (1 and 10 μg). Antisera were tested against the wild-type strain P9-17 and the heterologous strain FA1090 (**Table 47**). Antisera raised to 1μg OMV in saline or adsorbed to $Al(OH)_3$ induced SBA titres against P9-17 of 1024, which was increased to 2048-4096 with the higher immunizing dose (10μg). These antisera also killed strain FA1090, with SBA titres just marginally lower at 256-1024 for 1μg OMV in saline and 1024-2048μg for the higher dose (10μg). By contrast, the SBA titres induced by the Na-DOC OMV were lower than those induced by the OMV (**Table 47**).Immunization with Na-DOC OMV (1-10μg) in saline or adsorbed to Al(OH)3 induced SBA titres in the range of 256-1024 against the homologous strain P9-17, and 256 against the heterologous strain FA1090 (**Table 47**).

Table 47. Serum bactericidal activity of murine antisera raised to OMV and Na-DOC OMV from *Neisseria gonorrhoeae* strain P9-17 delivered with two different adjuvants and two different concentrations of the preparations.

Preparation	Dose (μg)	Adjuvant	SBA titre against	
			P9-17 wild-type	FA1090 wild-type
OMV	1	saline	1024	1024
	10	saline	4096	2048
OMV	1	$Al(OH)_3$	1024	256
	10	$Al(OH)_3$	2048	1024
Na-DOC OMV	1	saline	256-512	256
	10	saline	256-1024	256
Na-DOC OMV	1	$Al(OH)_3$	256	256
	10	$Al(OH)_3$	256	256
Control	-	Saline	16-32	16
	-	$Al(OH)_3$	16	4

Pooled antisera raised to P9-17 OMV and Na-DOC OMV preparations (1 and 10 μg/mouse doses) in saline and adsorbed to $Al(OH)_3$ and the corresponding sham-immunised control sera were tested for their ability to induce complement-mediated killing of *N. gonorrhoeae* strain P9-17 (homologous Allele 10 encoded Ng-ACP) and FA1090 (heterologous Allele 6 encoded Ng-ACP).The data presented are the reciprocal of the highest serum dilution at which ⩾50% killing was observed. The titers are expressed as the median values from two or more independent experiments.

3.9 Generation of knockout *ng-acp* gene variants in *Neisseria gonorrhoeae* strains P9-17, FA1090 and MS11 (*N. gonorrhoeae* strain Δ*ng-acp*).

Knockout constructs were generated to determine the effect of suppression of *ng-acp* gene expression (*Δng-acp*) in further biological studies. The gonococcal knockout construction consisted of a series of PCR steps to amplify the regions up-stream fragment *(F1)* and down-stream fragment *(F2)* of the region encoding *ng-acp* gene. An antibiotic resistant marker, Kanamycin (Kam), was inserted to control the process. The generation of this construct was used pGEM cloning vector which contains the knockout fragment (*F1-Kam-F2*). Next pGEM (*F1-Kam-F2*) construct was used to transform different strains of *N. gonorrhoeae* by homologous recombination.

3.9.1 Construction of the knockout construct pGEM (F1-F2).

A couple of PCR reactions were done using a set of primers (Appendix A.9.4.1.1) and (Appendix A.9.4.1.2) with Phusion 2X master mix to generate the regions up-stream *(F1)* and down-stream *(F2)* fragments of *ng-acp gene*. The PCR product showed a single band with size ~ 400 bp for the fragment *F1*. In contrast, the amplification of fragment *F2* showed an additional PCR product above ~1000bp along with the expected band of ~400bp regardless of the annealing temperature used (**Figure 47**).

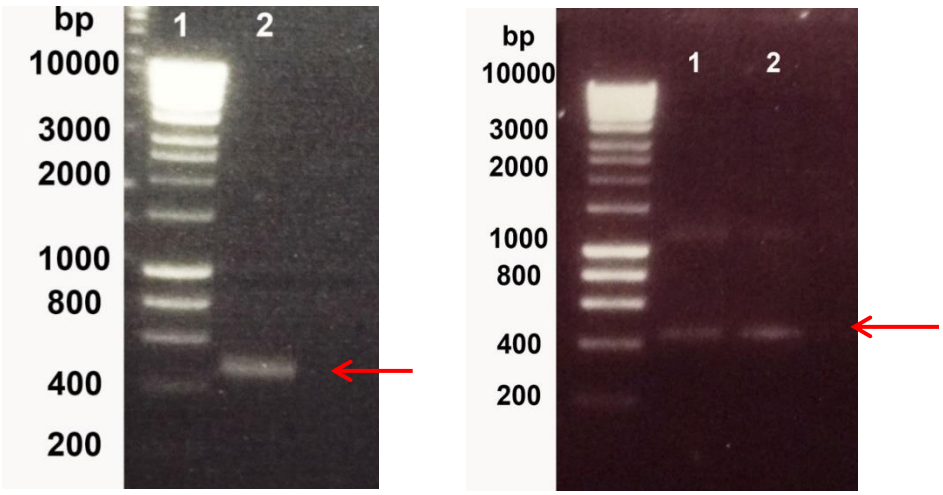
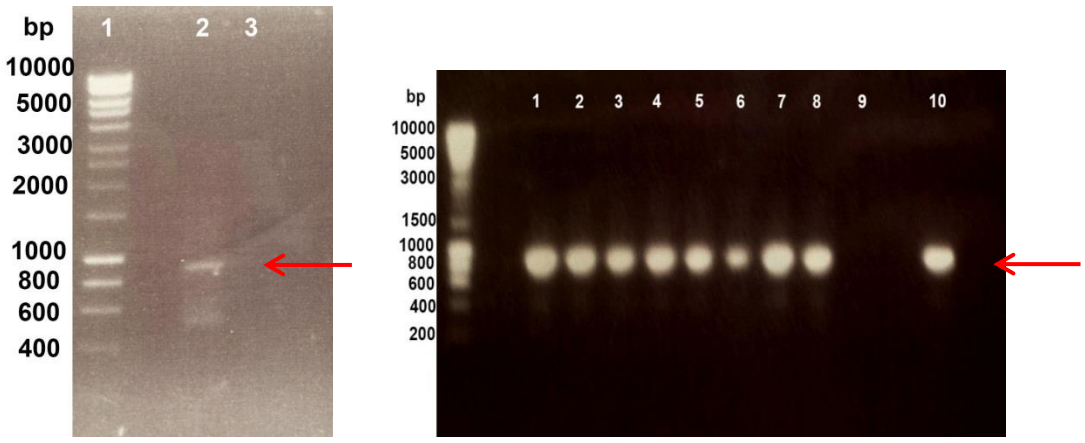


Figure 47. PCR product of region up – and down- stream of *ng-acp* gene.

A. Up-stream fragment *ng-acp (F1)* **Lane 1.** Ladder. **Lane 2.** Fragment F1 PCR product ~400 bp. **B.** Down-stream fragment of *ng-acp (F2)* at different annealing tempratures. **Lane 1.** Tm 63˚C **Lane2.** Tm 65˚C.

Each PCR product was purified by PCR clean up kit (Promega) , followed by digestion using a restriction enzyme *XbaI*. Then, the digested and purified fragments were ligated using T4 ligase. The ligation product was used as a template to generate a single fragment up-down stream fragments (*F1-F2*) by PCR , and the product was cloned into pGEM cloning vector, which generated the first step of knockout construct pGEM (*F1-F2*).

The pGEM(*F1-F2*) vector construct was used to transform *E. coli* GM2163 (Section 2.14.2.4), a genetic modified methyl transferase *Dam* deficient *E. coli* strain, which is suitable for expansion of plasmids free of *Dam* and *Dcm* methylation.This enables use of the restriction site *XbaI*, a restriction enzyme suceptible to methylation. Single colonies were selected on LB+100µg/ml ampicillin agar plates and tested by PCR using a set of primers (*F1-F2*) to determine the appropriate orientation of the insert (**Table 27** and **Table 28**, Section 2.14.2.4). The majority of transformed *E. coli* bacteria displayed a single band of ~900 bp (**Figure 48B**) with same size of the PCR product of the fragment *F1-F2* (**Figure 48A**).



D. **B.**

Figure 48. Amplification, cloning into pGEM and transformation in *E.coli* strain GM2163 of knockout fragment F1-F2. **A**. PCR product *F1-F2*. **B.** PCR colony screening transformation construct (pGEM-*F1-F2*) in *E. coli* strain GM2163

Lane 1. *Ladder* ***Lane2.*** *PCR product F1-F2.* ***Lane 3.*** *UHQ water* ***Lane 1-8****.* Transformants 1-8 *Lane 9.* UHQ water *Lane 10.* PCR product *F1-F2.*

3.9.2 Construction of the knockout construct pGEM (F1-*Kam*-F2).

The second stage of generating the knockout construct was insertion of an antibiotic resistance marker. The aim of the presence of this marker enables control of the knockout process in *N. gonorrhoeae*. The gene for antibiotic marker was extracted from a pACY177 plasmid encoding for kanamycin (*Kam*) resistance.

3.9.2.1 Plasmid extraction of and generation of Kanamycin cassette.

The plasmid was extracted from two single colonies of *E. coli* strain DH5α that contain pACY177 plasmid with the Wizard miniprep (Promega) as described in Section 2.6.3 (**Figure 83** Appendix A.13). A PCR reaction was done to extract the kanamycin cassette from pACY177 plasmid was performed using the parameters described on (Appendix A.9.4.1.3).

The PCR product was evaluated by 1% (w/v) agarose electrophoresis and showed a single band of 1000 bp, (**Figure 85** Appendix A.13). The *Kam* cassette was purified with PCR clean up kit (Promega)(Section 2.6.2) and used in the ligation reaction with pGEM vector (**Table 26**, Section 2.14.2.3).

3.9.2.2 Construction of the knockout construct pGEM (F1-Kam-F2).

One of the *E. coli* transformants containing with the pGEM*(F1-F2*) construct was used to generate the final knockout construct was used. The purified extracted plasmid pGEM(*F1-F2*) and the PCR product of kanamycin (*Kam*) were digested with a restriction enzyme (*XbaI*) following the conditions described in Section 2.14.2.5 (**Table 29** and **Table 31**).

To avoid overhang ends on the plasmid and avoid opening the plasmid, the plasmid was incubated with alkaline phosphatase at 37˚C for 1 hour, and inactivated at 56˚C for 15 minutes, following the protocol suggested by the manufacturer (Section 2.14.2.5).

A 1% (w/v) agarose gel electrophoresis comparison between non-digested plasmid and digested-plasmid a migration difference was observed. The reason for differential migration is due to a different conformation adopted by the uncut (supercoiled conformation) compared with the digested plasmid (linearised conformation) **Figure 86 B**. Appendix A.13. Each digested product

was ligated using T4 ligase with incubation at 4˚C overnight, to generate the **pGEM (*F1-Kam-F2*)** construct.

E. coli strain GM2163 was transformed using the ligation reaction pGEM (*F1-Kam-F2).* The selection of the transformants was performed on selective LB+ 100µg/ml kanamycin agar plates and tested by PCR using a set of primers described in, (Appendix A.9.4.2.2). The result showed that all of the candidates displayed a single band of ~1900 bp, which potentially contains the desired open reading frame construct (**Figure** 87 Appendix A.13).

The plasmid was extracted from one of the selected knockout transformants (Section 2.6.3.). A series of PCR reactions and digestion reactions with restriction enzymes was performed to check the proper orientation of the knockout insert (*F1-Kam-F2*). A digestion reaction was done of the knockout construct using *XbaI*, restriction enzyme whlist showed differential mobility between uncut and digested plasmid with an additional band of~1000 bp, corresponding to the *Kam* fragment, linked in both ends of *XbaI* restriction site inside the knockout construct.

A series of PCR was doneed using a combination of primers that generates a single fragment of knockout construct. One of PCR products in **Figure 49**, Lane 3, was used a set of external primers leading an amplified the entire knockout insert with size of ~1900 bp, indicating that this particular transformant contains the knockout construct. Further studies were performed to interrogate the proper orientation of each fragment that composed the knockout construct by PCR reaction. Lane 4, shows the amplified *F1-Kam* fragment giving a fragment~1400 bp; however, this PCR reaction is not quite specific due to the generation of additional bands (**Figure 49)**. Alternatively another PCR was done to amplify *Kam-F2* and this showed a band with high intensity~1400bp; however, the PCR was non-specific (**Figure 49**, Line 5). The *Kam* fragment was amplified using the internal primers to amplify the antibiotic resistance cassette (**Figure 49,** Line 6). Taken together, the evidence suggests the selection of this transformant enables further studies of transformation in *N. gonorrhoeae* to generate the knockout Δ*ng-acp* strain.

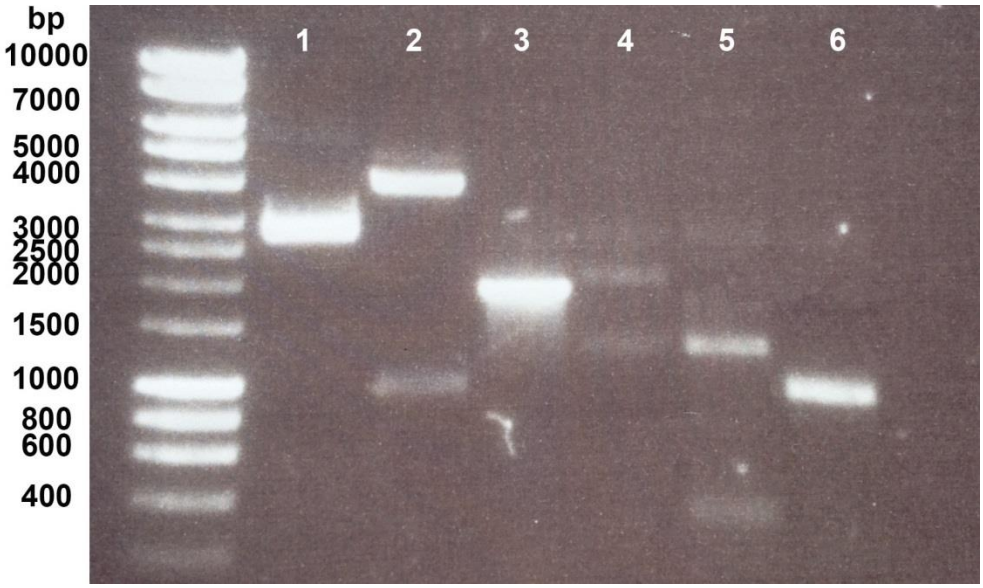


Figure 49. Digestion and PCR reaction to check the appropriate insertion of the knockout insert.

Lane 1. uncut plasmid **Lane 2.** Digested plasmid *(Xba*I) **Lane 3.** PCR using *F1* and *F2* (external primers) **Lane 4.** PCR using *F1-Kam* primers (internal primers) **Lane 5.** PCR with Kam-F2 primers (internal primers) **Lane 6.** PCR reaction with *Kam* primers.

3.10. Transformation and evaluation of *N. gonorrhoeae* knockout mutants P9-17, FA1090 and MS11 strains.

Using the knockout construct previously described in Section 3.9, the plasmid was extracted and a PCR reaction was performed to amplify the knockout insert *F1-Kam-F2* using the primers listed in (Appendix A.9.4.3.1). The PCR product was purified and the DNA concentration was quantified to transform gonococcal strains.

N. gonorrhoeae strains P9-17, FA1090 and MS11 were grown on GC agar, and the appropriate phenotype ($Pili^{+}Opa^{+}$) were then isolated. A liquid transformation was done with a gonococcal lawn as described in Section 2.14.3.1.2 with addition of the knockout construct or PCR product (range from 15 to 30µl of 100-300ng/ml).

The transformants were grown on selective GC agar plates+ 100µg/ml kanamycin and they were evaluated by PCR and western blot to determine the expression or absence of *ng-acp* gene (Section 2.14.3.2.1 and 2.14.3.2.2).

A PCR reaction was done using external primers of knockout construct listed in (Appendix A.9.4.3.1), and this showed a differential PCR product between the wild type strain (~1200bp) and knock strains (~1900bp) (**Figure 50**panel A-C). Expression of the Ng-ACP protein was evaluated by western-blot using murine antisera full-length rNg-ACP with aluminium hydroxide ($Al(OH)_3$) mice antisera (1/100 dilution) and the reactivity was developed with anti-mouse Ig-Alkaline phosphate conjugate. Lack of Ng-ACP protein expression was observed on knockout strains, whereas wild-type strains showing a band (*Mr*~12.5kDa) for gonococcal strains P9-17 and FA1090 (**Figure 50** panel D-E). Similar result was found for gonococcal strain MS11 using rabbit antisera raised against full-length rNg-ACP (1/100 dilution) with the reactivity developed with antisera anti-rabbit Ig- alkaline phosphate conjugate (**Figure 50** panel F).

In addition, knockout gonococcal strains P9-17 and FA1090 were tested with different preparations rasised with full-length rNg-ACP murine antisera (**Figure 95A** and **Figure 95B** Appendix A.15) and murine antisera to mature rNg-ACP preparations (**Figure 96 A** and **Figure 96 B** Appendix A.15), and no reactivity from any of the preparations or sham sera was observed.

Western blot of bacterial lysates of gonococcal wild-type and knockout strains was done using antisera raised to full-length rNg-ACP aluminium hydroxide ($Al(OH)_3$) (**Figure 92** Appendix A.15) and rabbit serum raised with meningococcal Nm-ACP (**Figure 91** Appendix A.15), respectively. This confirmed the immuno-reactivity of wild-type strains tested (*N. gonorrhoeae* strain P9-17 and FA1090) by showing a single band of *Mr*~12.5kDa. A slight cross-reactivity was shown by the wild-type gonococcal (band of *Mr*~12.5 kDa) strains compared with the meningococcal strain MC58, additional bands with high molecular weight displaying potential non-specificity of the amount of dilution used for the first antibody.

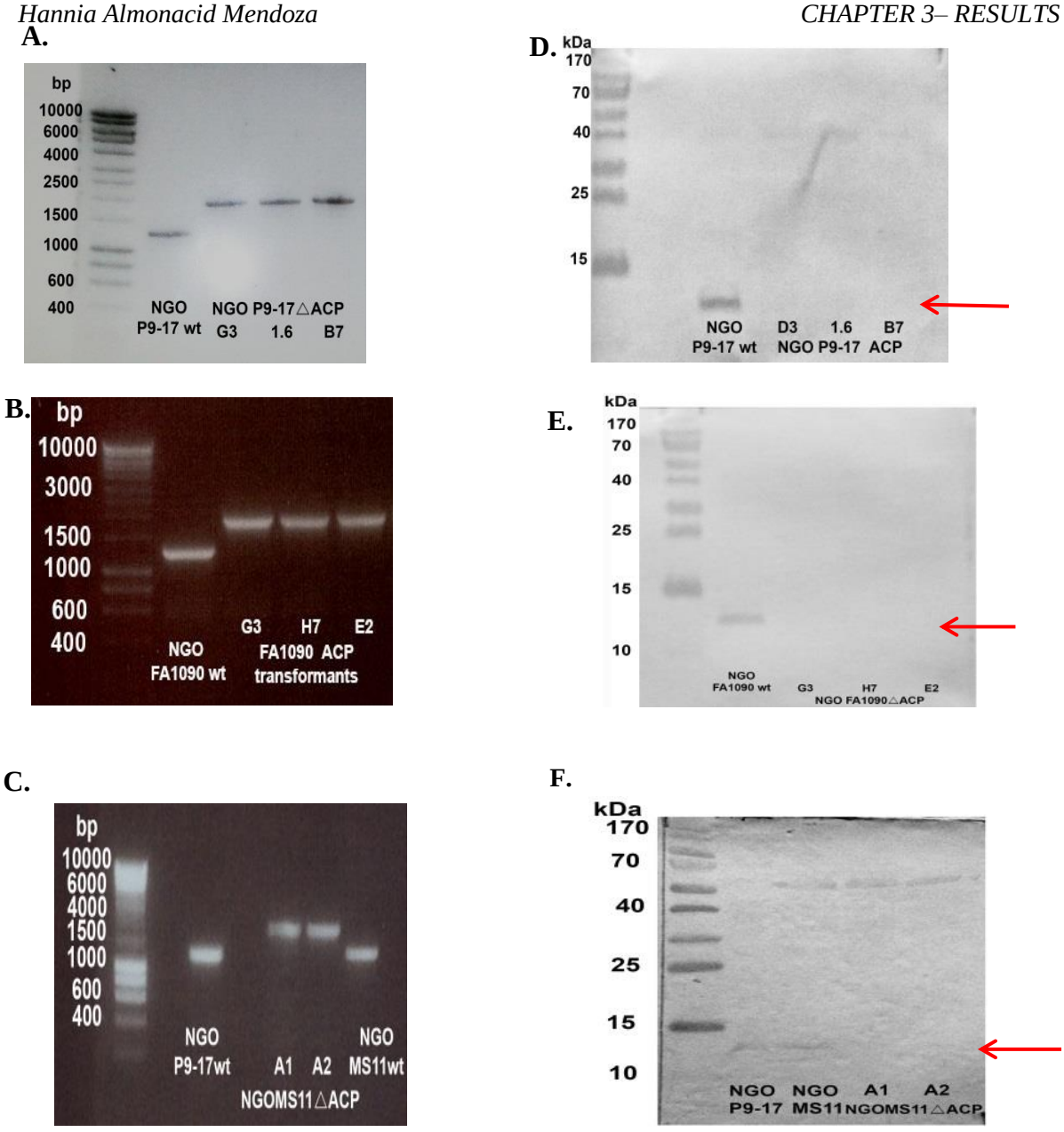


Figure 50. Identification of *ng-acp* gene presence and expression in the different wild-type and knockout mutants from gonococcal strains P9-17, FA1090 AND MS11. **PCR product** *ng-acp* gene .**A**. *N. gonorrhoeae* strain P9-17 **B.** *N. gonorrhoeae* strain FA1090.**C.** *N. gonorrhoeae* strain MS11.**Western-blot** to verify the expression of Ng-ACP protein. **D.** *N. gonorrhoeae* strain P9-17 **E.** *N. gonorrhoeae* strain FA1090.F. *N. gonorrhoeae* strain MS11. The arrow denotes a single band of *Mr* ~13kDa on each sample.

3.11. *Neisseria gonorrhoeae* complemented construct (*ng-acp*-pGCC4).

A complemented construct was generated to determine the recovery of *ng-acp* expression and evaluate the association or internalization on epithelial cells. The cloning process was performed using pGCC4 plasmid, kindly provided by Hank Seifert (Feinberg School of Medicine, Northwestern University). pGCC4 plasmid is a clone of *Neisseria gonorrhoeae* chromosomal DNA, linked with an erythromycin antibiotic resistance cassette. Also, the inserted gene of interest is

under control of the *lac* operator, with size of 9268 bp (**Figure 88 C** Appendix A.14). The pGCC4 plasmid was extracted from *E. coli* strain DH5α host using Wizard miniprep kit (Promega) **Figure 88 A** Appendix A.14.

An individual PCR reaction with genomic DNA from *N. gonorrhoeae* strains P9-17 and FA1090 was performed to amplify *ng-acp* gene using a set of primers **Table 37** Section 2.15.1. Each purified PCR product and complemented plasmid were double-digested using ***PacI*** 5'-TTAATTAA-3' and ***PmeI*** 5'-GTTTAAACT-3' restriction enzymes to clone the *ng-acp* gene into pGCC4 cloning vector. pGCC4 plasmid and digested plasmids were compared by DNA mobility and the concentration estimated by 1% (w/v) agarose electrophoresis gel **(Figure 88 B** Appendix A.14). Digested *ng-acp* fragments were ligated into the cloning vector as described in Section 2.6.5. The ligation reactions were used to transform into *E. coli* strain DH5α (Section 2.6.9.1).

A PCR reaction was done to evaluate the presence of complemented construct *ng-acp*-pGCC4 and this, showed a single band with size of ~400 bp in most the candidates from both gonococcal strains **Figure 51A** and **B**. From these results, the transformant 3 for *N.gonorrhoeae* strain FA1090 and colony 8 for *N. gonorrhoeae* strain P9-17 were selected, in order to analyse the DNA sequence and compare with the sequence of *N. gonorrhoeae* strain P9-17. Both DNA sequence analysis showed high homology with the gene target and were used for further experiments (**Figure 89** and **Figure 90**Appendix A.14).

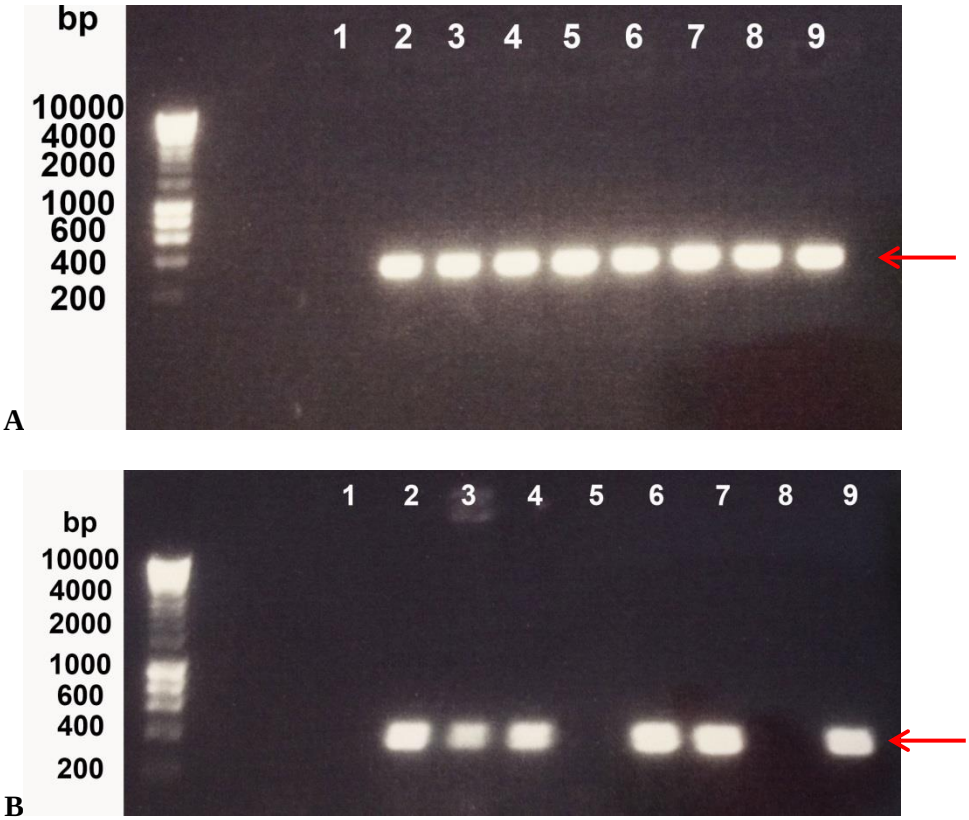


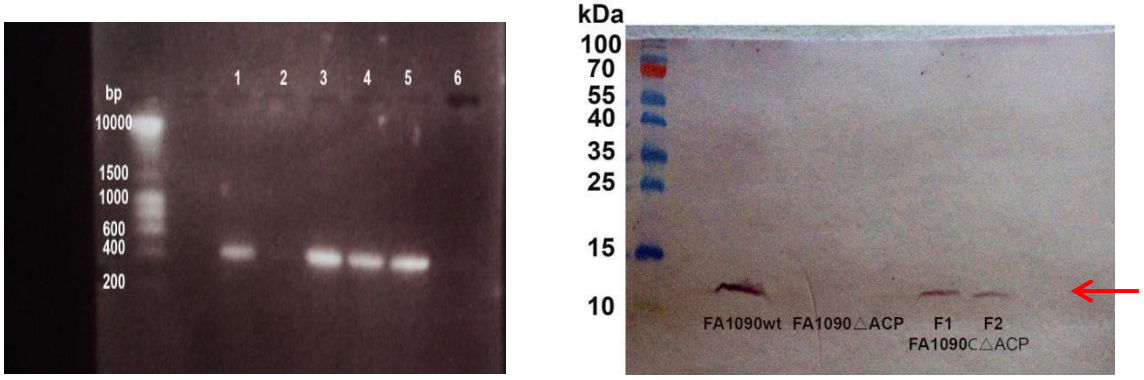
Figure 51. PCR colony to test complementation construct**. A. *ng-acp*-pGCC4 construct from *N. gonorrhoeae* strain FA1090 Lane 1.** UHQ water **lane 2-9.** Colonies 1 to 8 **B. *ng-acp*-pGCC4 construct from *N. gonorrhoeae* strain P9-17 lane 1.** UHQ water **lane 2-9.** Colonies 2-9. The arrows represent a single band of 400 bp on each transformant.

3.12. Generation of complemented variants in *N gonorrhoeae* strains.

The insertion of the complemented construct (*ng-acp*-pGCC4) was done using liquid transformation in gonococci as described previously (Section 2.14.3.1.2). According to the literature, the amount of complemented construct can be increased from 500 to 10000 ng/ml, depending on the gonococcal strain used (Ramsey et al., 2012). Despite, the techniques used to increase the amount of DNA or increase the amount the erythromycin, to improve the selection of the colonies under this type of transformation was successful only in *N. gonorrhoeae* strain FA1090 not result with *N. gonorrhoeae* strains P9-17 and MS11 transformants (data not shown).

Complemented variants from *N.gonorrhoeae strain* FA1090 were grown on GC agar plates + erythromycin (4µg/ml), which is double the amount of *N. gonorrhoeae* strain FA1090 reported in the literature, with 1mM of IPTG, to ensure survival of the potential transformants. PCR evaluation of the potential candidates showed a single band of ~400 bp, which were similar results obtained with the wild-type strain. In addition, the use of knockout variant was used as a negative control, in

order to confirm the complemented strains recovered the protein expression of Ng-ACP by induction of IPTG (**Figure 52 A**). Similarly, evaluation by westen-blot showed a single band with size *Mr*~12.5 kDa, demonstrating recovery Ng-ACP protein expression (**Figure 52B**).



A. **B.**

Figure 52. Verification of complemented strains from *N. gonorrhoeae* strains FA1090. A. PCR reaction of different variants of *N. gonorrhoeae* strain FA1090 **Lane 1** *N.gonorrhoeae* strain FA1090 wild-type **Lane 2** *N.gonorrhoeae* strain FA1090(*Δng-acp*) **Lane 3 to 5** *N.gonorrhoeae* strain FA1090(*¢Δng-acp*) complemented strains F1 to F3. **B.** Western-blot confirmation of the different variants of *N. gonorrhoeae* strain FA1090. **FA1090wt.** *N.gonorrhoeae* strain FA1090 wild-type. **FA1090ΔACP**. *N.gonorrhoeae* strain FA1090 (*Δng-acp*). **FA1090CΔACP** *N.gonorrhoeae* strain FA1090 (*¢Δng-acp*) complemented strains F1and F2.

3.13. Genome sequencing of *N. gonorrhoeae* strain P9-17.

To analyse further *ng-acp* gene in *Neisseria gonorrhoea* strain P9-17 was elucidated its genome (Oxford University, Genome sequencing area, Zoology Department). From an isolated gonococcal lawn on GC agar plate was swabbed and suspended into 500 µl of PBS. Isolation of genomic DNA was done using Genome Extraction Kit (Promega, USA), suspended with 30µl of Nuclease free-water and DNA the quality and amount of DNA was quantified by1% (w/v) agarose electrophoresis gel and Nanodrop (Sections 2.6.6 and 2.6.7).

The amount of DNA sequenced was 100 ng/µl, and the genome sequence was collated into PubMLTS database (**id code 36675**).Some of the characteristics of this genome sequence are summarised in **Table 48.** In addition, the description of the locus that encodes adhesin complex protein *ng-acp* gene in **Table 49**.

Table 48. Description of genome sequence from *N. gonorrhoeae* P9-17 strain.

Description	Amount
Characterization sample	
Disease	Uncomplicated gonorrhoeae
Source	Urethral swab
Sex	Male
Country/Continent	UK, Europe
Year	1973
Genomic characterization	
Contigs	154
Total length	2178201
Minimum length	265
Maximum length	108542
Mean length	14145
σ length	23443
N50 contig	15
N50 length (L50)	47321
N90 contig	50
N90 length (L90)	10591
N95 contig	64
N95 length (L95)	5662

Table 49. Description of locus NEIS2075 encoding for *ng-acp* from *N. gonorrhoeae* P9-17 strain.

Characterization ng-acp						
Sequence	**Sequence method**	**Length**	**Locus**	**Start**	**End**	**Direction**
2294869	Illumina	87612	NEIS2075	7312	7683	←

3.14 Growth curve of *Neisseria gonorrhoeae* strain P9-17 in Eagle's medium DMEM+ decomplemented fetal calf serum (dFCS).

The viability of *N. gonorrhoeae* strain P9-17 wild type and knockout *N. gonorrhoeae* strain P9-17 Δ*ng-acp* were tested in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% (v/v) decomplemented fetal calf serum (dFCS), to perform association and invasion experiments on epithelial cells.

A bacterial suspension of $1x10^6$ CFU/ml of each strain was prepared from 18 to 20 h inoculum on GC agar plates andincubated in 1ml of DMEM+ 1% (v/v) dFCS in a sterile 24 well plate. The

viability of Neisserial strains was evaluated at different time points (0 hours (h) , 1 h, 3 h and 6 h).

At each time point,samples were taken (20µl) and serially diluted in phosphate saline buffer (PBS) and plated on triplicate onto GC agar or GC+Kam 100µg/ml plates, **Figure 53**.

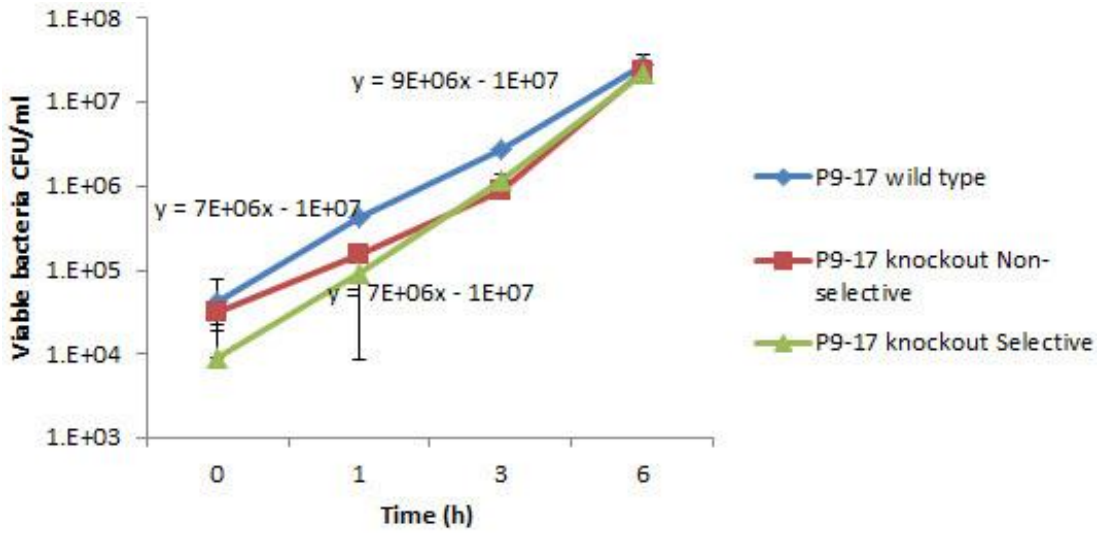


Figure 53. Growth curve of *N. gonorrhoeae* P9-17 in DMEM medium complemented with decomplemented fetal calf serum (δFCS).The representation was taken from 3 individual experiments.

The semi logarithmic relation between the viable bacteria expressed in CFU/ml and time showed both *N. gonorrhoeae* strain P9-17 wild-type and knockout (*N. gonorrhoeae* strain P9-17 *Δng-acp*) gonococi strains had similar rates of growth in this specific medium (rates range 7-9x10^6 CFU/ ml). In addition, there was not a difference between the viability of gonococcal knockout strain when grown on selective or non-selective GC agar, as both linear trends presenting the same rate of growth were observed (7x10^6 CFU/ml).

3.15. Biological role of Adhesin Complex Protein (ACP) on epithelial cells.

3.15.1 Association of *N. gonorrhoeae* strain P9-17 on epithelial cells.

To determine whether Ng-ACP plays a role in *N. gonorrhoeae* adherence to epithelial cells, an association assay was performed with monolayers of conjunctival Chang cells, meningeal cells and HEp-2 laryngeal carcinoma cells in triplicate. The epithelial cells were infected with 2x10^7 CFU/ml bacterial suspensions of gonococcal wild type, knockout and complemented strains for 3 hours at

37˚C. The infected monolayers were lysed with saponin solution and the recovered bacteria were serially diluted (10^{-1} to 10^{-3}) with warmed PBS, plated on GC agar plates and counted using a Protocol cell counter (Cambridge, UK).

Comparison of associated bacteria of different gonococcal strains used on Chang cells of homologous *N. gonorrhoeae* strains P9-17 and MS11 showed a significant reduction of association of knockout (*Δng-acp*) bacteria of ~63% and 38% (P<0.05) respectively. On the contrary, heterologous strain as FA1090 displayed the lowest recovery of associated bacteria (~5x10^5 CFU) with no significant reduction of the knockout bacteria (*Δng-acp*) or recovery of the numbers of associated bacteria of the complemented strain (¢*Δng-acp*) compared with FA1090 wild type strain (**Figure 54**).

The number of associated bacteria recovered from gonococcal infection on HEp-2 laryngeal carcinoma cells was similar on Chang cells. No significant reduction of the number of associated bacteria of gonococcal P9-17. A great number of associated bacteria display a significant reduction (55% reduction; P<0.05) of knockout homologous *N. gonorrhoeae* strain (MS11) on this specific cell line and heterologous strain FA1090 showed a reduction of 30% (P<0.05) **Figure 54**.

The lowest number of associated bacteria was observed with the meningeal cells for all of the strains. No significant reduction of numbers of associated bacteria was found with the homologous strain MS11 on this cell line (**Figure 54**Nevertheless, a significant reduction of the numbers of associated bacteria (~74 %reduction; P <0.05) was found with knockout (*Δng-acp*) *N. gonorrhoeae* strain P9-17 and heterologous strain FA1090 ~40%; (P<0.05).

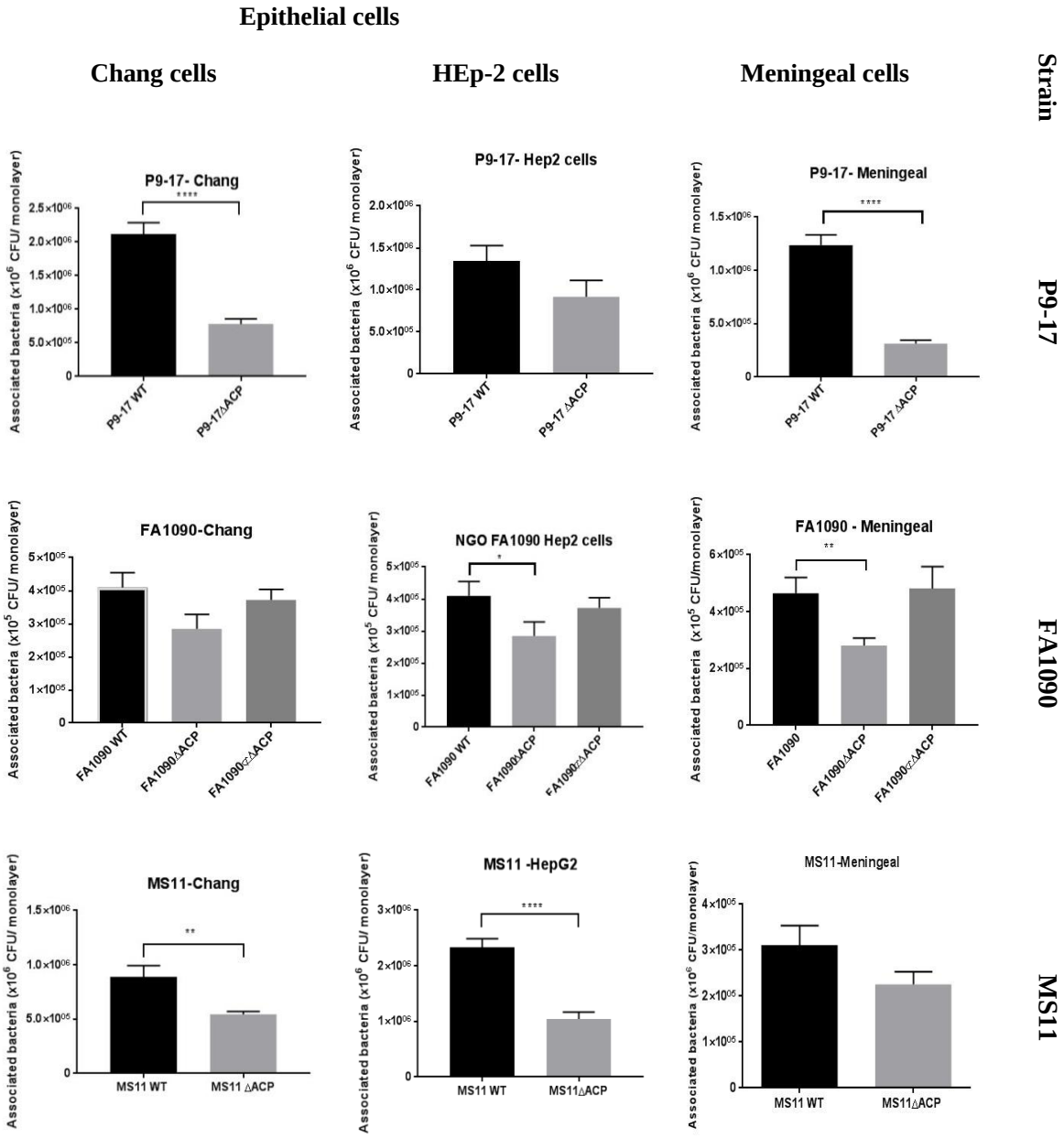


Figure 54. Ng-ACP from *N. gonorrhoeae* mediates adhesion of different gonococcal strains on Chang, HEp-2 laringeal carcinoma and Meningioma cells monolayers. Cell monolayers were infected with $2x10^7$ CFU of gonococcal bacteria. The columns represent the mean values with SEM from 3 individual experiments (n=3).

3.15.1.1. Influence of major adhesins on ACP adherence function.

To investigate whether Ng-ACP protein mediates adherence solely or under influence of the main adhesins (Pili and/or Opa), three gonococcal variants of *N. gonorrhoeae* strain P9-17; **P9-1** ($Pili^-$ Opa^-), **P9-2** ($Pili^+$ Opa^-), **P9-16** ($Pili^-$ Opa^+) and their respective knockout variants (*Δng-acp*) were used in order to perform association assays on Chang cells, as previously described in Section 3.15.1.

Most of the gonococcal variants tested, the knockout variant (*Δng-acp*) did not show a significant reduction of association process ~ 12.5 -27% ($P>0.05$). A reduction in association on epithelial cells was observed one of the gonococcal variants (*N. gonorrhoeae* variant P9-16) showed a reduction about 40% ($P<0.05$). Taken together, these results suggest that gonococcal Ng-ACP has potentially a synergistic function with one of the major adhesins present in the outer-membrane (OMV) of *N. gonorrhoeae,* Opa (**Figure 55**).

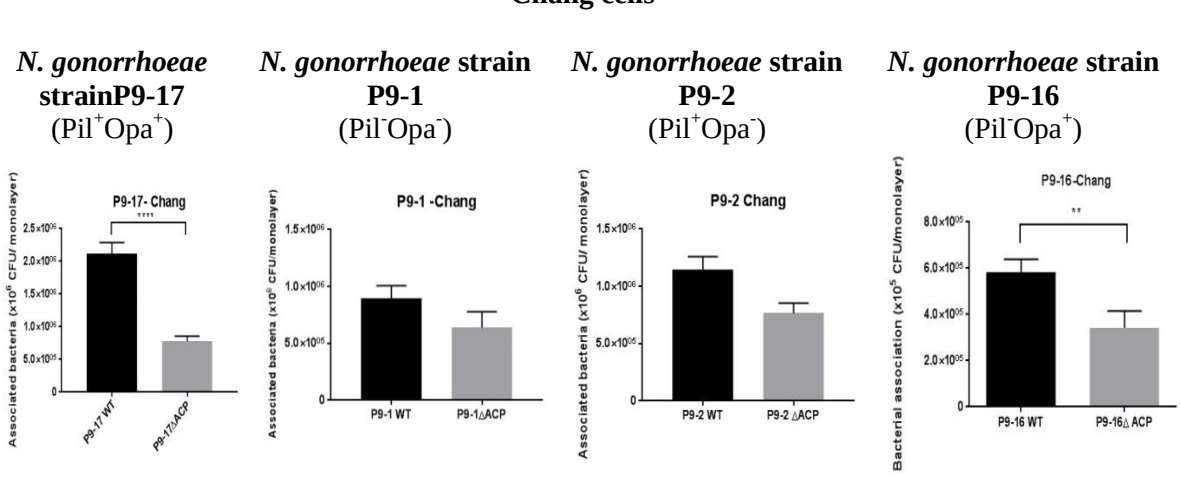


Figure 55. Gonococcal Ng-ACP acts synergistically with the major adhesins to mediate adhesion on Chang epithelial cells. Adherence assays performed on different *N. gonorrhoeae* strain P9-17 variants. The columns represent the mean values with SEM from four individual experiments (n=4).

3.15.1.2. Reduction of Ng-ACP adherence function on epithelial cells.

To further assess the role of Ng-ACP in *N. gonorrhoeae* adherence on epithelial cells, decomplemented anti-rNg-ACP rabbit serum was used to inhibit association at different multiplicities of infection (MOI). MOI is defined by the ratio between amount of *N. gonorrhoeae* strain P9-17 bacteria and number of bacteria on monolayers of epithelial Chang cells. The addition of anti-rNg-ACP serum results a MOI-dependent reduction in adherence. Low MOIs (from 0.02 to 2) caused a reduction of bacterial association by approximately 55%-60 % (**Figure 56**). In contrast, infection of monolayer epithelial cells at highest MOI (from 20 to 200) presentad decreased inhibitory effect by only 20-35%.

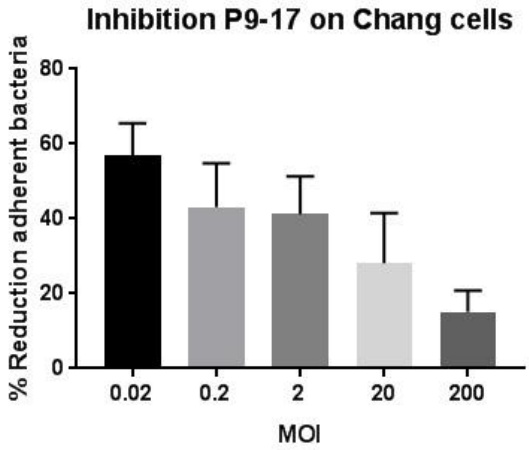


Figure 56. Anti-serum inhibits bacterial association on epithelial cells. Chang epithelial cells were infected with different MOIs of *N. gonorrhoeae* P9-17 strain wild-type in the presence of decomplemented rabbit pre-immune and post-immune serum 10% (v/v).The representative data were determined from four individual experiments (n=4).

3.15.1.3. Binding of Ng-ACP protein on epithelial cells.

To test whether gonococcal Ng-ACP protein binds to cell line cultures, recombinant full-length rNg-ACP was used to treat Chang cell suspension at different concentrations of recombinant rNg-ACP. The cell bound fluorescence was quantified by flow cytometry (FC) binding assay. An anti-rNg-ACP rabbit serum (1/400 dilution) and a secondary swine anti-rabbit Ig- fluorescein isothiocyanate (FITC) labelled antibody.

Preliminary test, Ng-ACP showed a dose –response trend. The binding of Ng-ACP on Chang cells increased in a dose-dependnt manner (**Figure 57**).

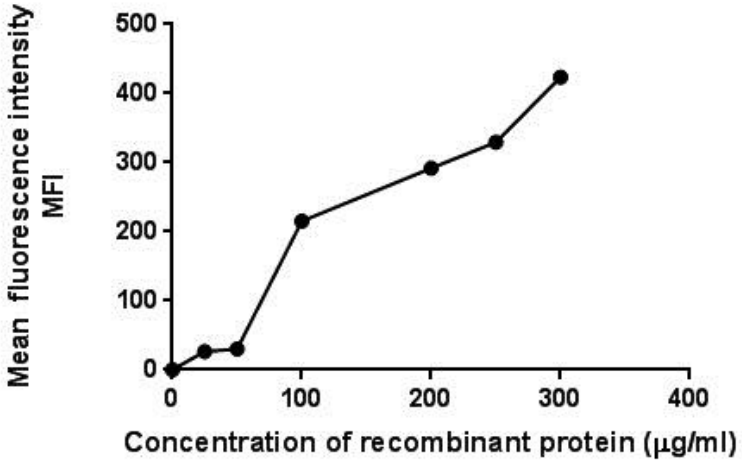


Figure 57. Dose –dependent binding of rNg-ACP on Chang epithelial cell line. Each data point is expressed as net mean fluorescence intensity (MFI). Results are from 3 independent experiments.

3.15.2. Role of Ng-ACP for bacterial invasion of epithelial cells.

A gentamicin assay was used to examine the role of gonococcal Ng-ACP in internalization of epithelial as described by (Virji et al., 1995b, Virji et al., 1993). This assay measures associated bacteria (total bacterial association) and internalised bacteria (gentamicin assay), and these experiments were also performed in the presence or absence of cytochalasin D (CD), to determine whether association or internalisation was mediated by actin microfilament dependent activity.

Total association of the different gonococcal strains on Chang cells demonstated high numbers of associated bacteria for *N. gonorrhoeae* strain P9-17 and homologous strain MS11 and slightly lower recovery of the number of associated bacteria for heterologous *N. gonorrhoeae* strain FA1090. All of the gonococcal strains presented a significant reduction 30-57% ($P<0.05$). The presence of cythochalasin D did not show any significant differences in association without this reagent, suggesting that association is not influenced by actin microfilament dependent activity (**Figure 58**).

Relative high numbers of internalised bacteria in Chang cells were quantified. The highest number was observed with *N. gonorrhoeae* strain MS11 , followed by *N. gonorrhoeae* strain P9-17 and *N.*

gonorrhoeae strain FA1090. A significant reduction of *Δng-acp* knockout strains was found in *N. gonorrhoeae* strains P9-17 and FA1090 ($P<0.05$), with a slightly recovery for complemented strain FA1090. On the contrary, a signficant reduction ($P>0.05$) was not observed for knockout strain for homologous *N. gonorrhoeae* MS11 . For all of the gonococcal strains evaluated there was a significant reduction in the presence of cytochalasin D ($P<0.05$), suggesting that the internalization process was mediated by actin microfilament dependent activity (**Figure 58**).

Similar numbers of recovered bacteria for all of the gonococcal strains were found on HEp-2 larynx carcinoma cells. Likewise, there was sinignificant reduction of the knockout *N. gonorrhoeae* strain P9-17 *Δng-acp* for and heterologous *N. gonorrhoeae* strain FA1090 ($P<0.05$), but not for *N. gonorrhoeae* strain MS11 The presence of CD did not alter the association process (**Figure 59**). For Internalization on HEp-2 cells, a variable recovery amount of internalised bacteria with highest amount for *N. gonorrhoeae* strain P9-17 ($x10^3$ CFU/ monolayer), followed by homologous strain MS11 presented about ($x10^2$CFU/monolayer) and lowest internalised bacteria ($x10^2$CFU/monolayer) for heterologous *N. gonorrhoeae* strain FA1090 (**Figure 59**). There was a significant reduction in internalised bacteria in the presence of CD, suggesting that invasion was mediated by an actin microfilamen dependent activity.

There was a significant reduction of 35-60% in the association of *N. gonorrhoeae* strains P91-7 and MS11 but not for the heterologous strain FA1090 ($P>0.05$) of knockout strain *Δng-acp*. The amount of associated bacteria by strain P9-17 and strain MS11 was similar compared with epithelial Chang and HEp-2 cells lines. Low numbers of recovered bacteria were found for *N. gonorrhoeae* strain FA1090 and also, notably, gonococcal strain FA1090 did not show any significant reduction of the knockout strain (FA1090 *Δng-acp*) (**Figure 60)**.

The numbers of internalised bacteria were lower compared with the numbers of recovered bacteria from the other two epithelial cells tested, (10^1CFU/monolayer**) Figure 60**. The poor or lower recovery indicating that gonococci were not unable to invade meningeal cells and also, is consistent with previous *in vtiro* assays described in the literature with the closely related bacteria *N. meningitidis* (Hardy et al., 2000).

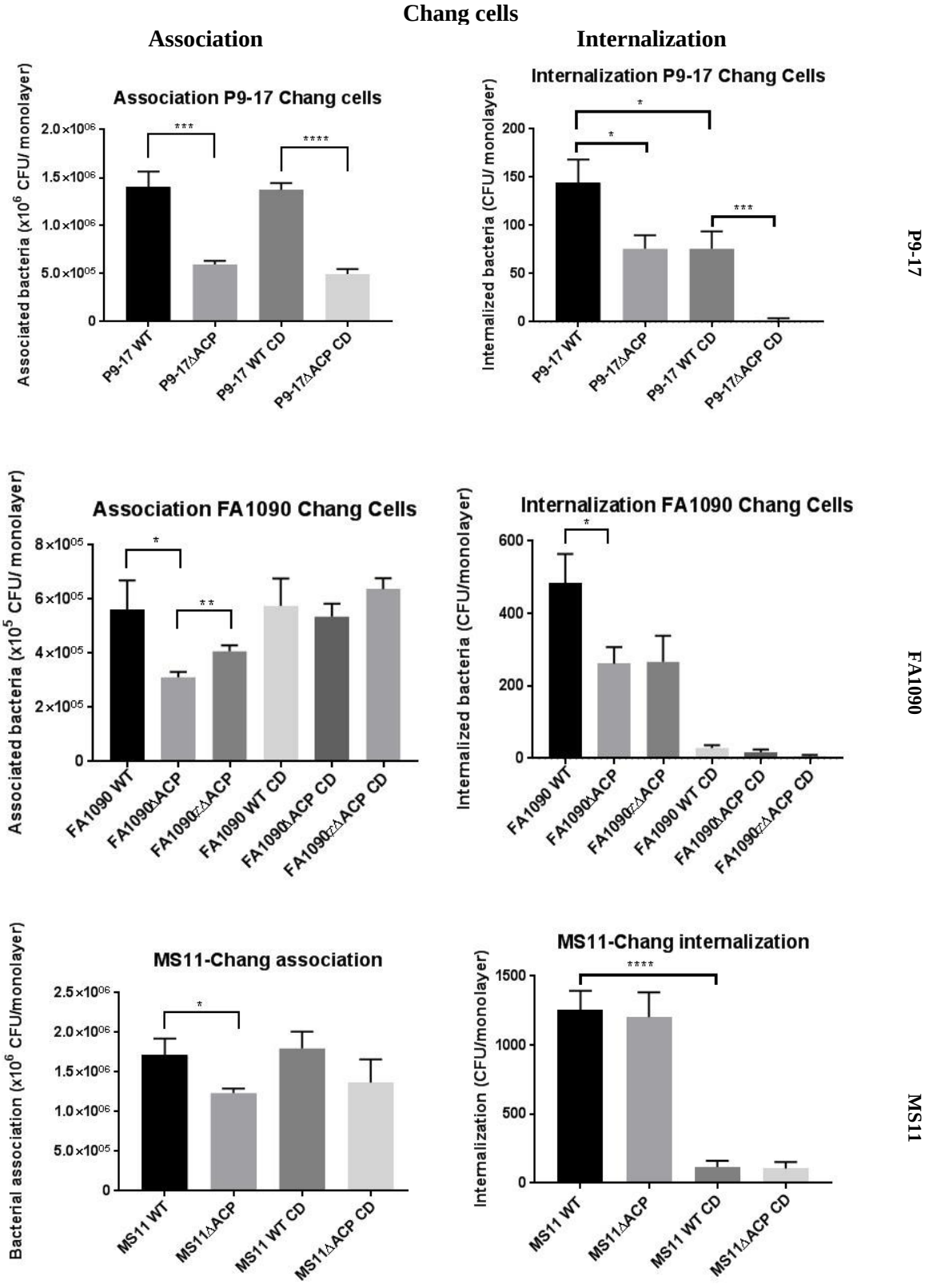


Figure 58. Invassion process on Chang cells for different *N.gonorrhoeae* strains. Left panel representation of total association and the right panel invasion with or without CD. The columns represent the mean values with SEM from 3 individual experiments (n=3).

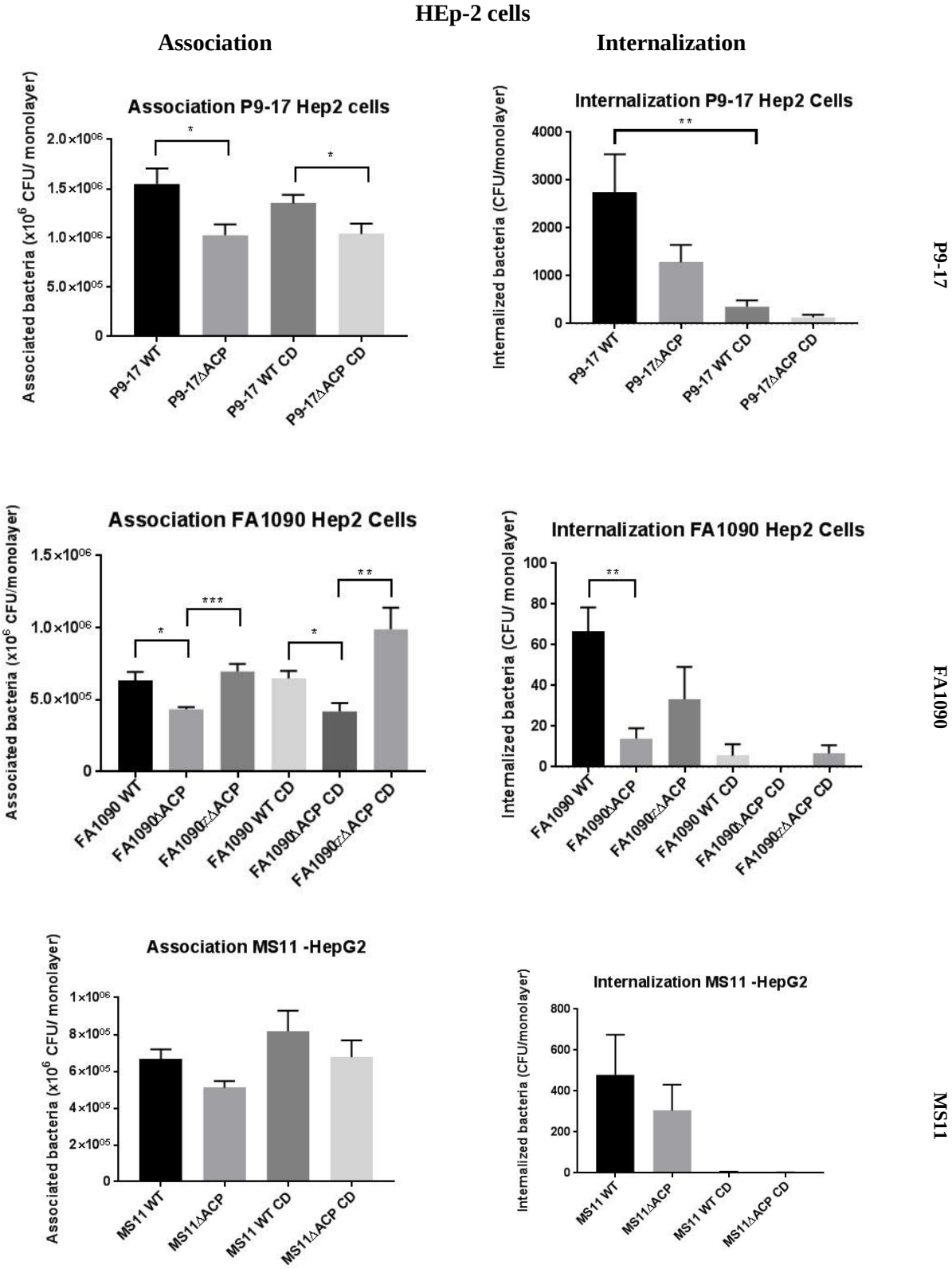


Figure 59. Invasion processon HEp-2 cells for different *N.gonorrhoeae* strains. Left panel representation of total association and the right panel invasion with or without CD. The columns represent the mean values with SEM from 3 individual experiments (n=3).

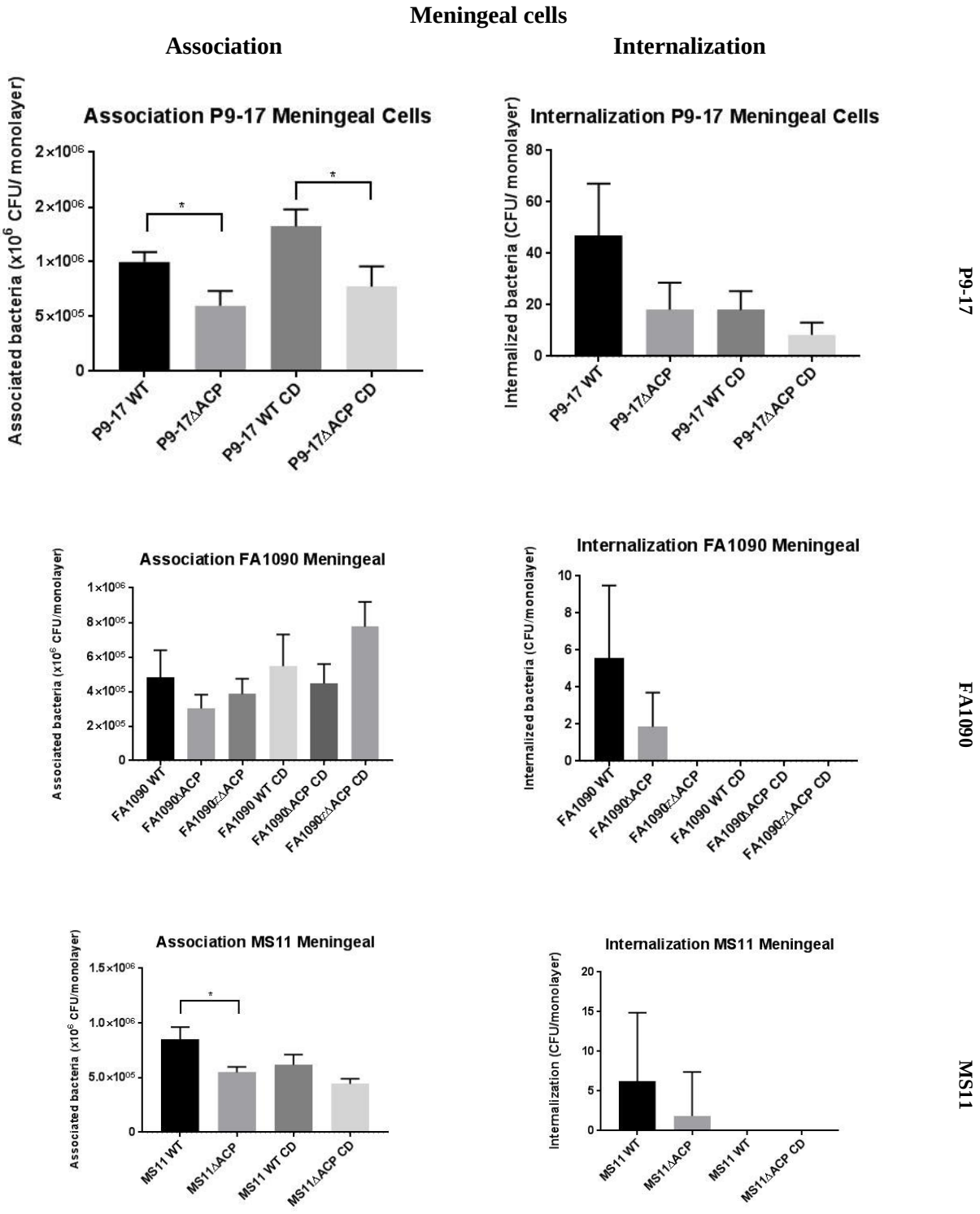


Figure 60. Invasion on Meningeal cells for different *N.gonorrhoeae* strains. Left panel representation of total association and the right panel invasion with or without CD. The columns represent the mean values with SEM from 3 individual experiments (n=3).

CHAPTER 4. Three-dimensional structure of mature rNg-ACP by X-ray diffraction.

This work was conducted in collaboration with the supervision of Dr. Ivo Tews and a PhD student, Moritz Machelet, at the Institute of Biological Sciences (IFLS), University of Southampton. The department also provided the technical support and facilities to obtain the protein. Subsequent analysis of the protein was performed through X-ray crystallography (Sinchroton, Grenoble, France) and elucidation of the structure was performed using molecular replacement.

4.1 Estimation of mature rNg-ACP.

An *E. coli* BL21plysS strain transformed with rNg-ACP-pET22b construct was chosen and used for a large scale production (6-8L) of recombinant mature rNg-ACP protein. To extract periplasmic proteins, a set of buffer was prepared based on the prediction of physicochemical properties of mature rNg-ACP using Protparam (http://web.expasy.org/protparam/). The physicochemical properties of the mature rNg-ACP were molecular weight (*Mr)* of 12 kDa and isoelectric point of 9.2. In order to preserve the native folding of the protein, a low isoelectric point of 8.5 was chosen, and avoiding the reactivity of cysteine (Cys) residues present in the molecule (**Table 50)**.

Table 50. Estimated parameters for mature Ng-ACP from *N. gonorrhoeae* strain P9-17 (Protparam, Expasy webserver).

Parameter	Amount
Number of amino acids	109
Molecular weight	12129.78
Theoretical PI	9.52
Extinction coefficient (all cysteine (Cys) are reduced)	8940

4.2 Large scale production of mature rNg-ACP.

4.2.1. Growth conditions.

Mature recombinant rNg-ACP protein was expressed in *E. coli* strain BL21pLysS grown in LB medium (1 L per flask) in eight 2 L flasks, on a rotary shaker (New Brunswick Innova 43/43R, Eppendorf) for 200 rpm at 37˚C. After 2 hours of incubation, the cell culture was analysed by optical density at λ_{600} nm. When the cell culture reached an optical density at λ_{600}nm between 0.4-0.6 absorbance, 10 ml of IPTG 100 mM stock solution was added per flask, and each flask was incubated for another 4 hours at 37˚C, 200 rpm. To obtain the cell pellet from 8 L of cell culture, the medium was centrifuged at 6000 rpm (9000x*g*) for 30 minutes at 4˚C (Avanti J-30I high performance centrifuge, Beckman Coulter) and the cell pellet was collected in a 50 ml Falcon tube and stored at -20˚C.

4.2.2. Extraction of recombinant protein.

The cell pellet was thawed on ice, and a set of buffers was simultaneously prepared (**Table 51**). The cell pellet was then suspended in ~125 ml of lysis buffer by pipetting and vortexing to obtain an homogeneous suspension. The bacterial suspension was sonicated on ice (Sonicator brand Misonix Sonicator Xl2020 ultrasonic processor) with amplitude of 10,000 microns for 10 seconds ON, 30 seconds OFF for 5 minutes, in order to disrupt the bacterial cells and release the protein. In order to remove the inclusion bodies, the sample was centrifuged at 45000 rpm (208000 x*g*) on a Beckman Coulter XPN for 45 minutes at 4˚C. The supernatant was kept on ice for subsequent purification by affinity chromatography.

4.2.3. Purification of mature rNg-ACP by immobilised metal ion affinity chromatography.

Metal ion chelate affinity chromatography (IMAC) is a highly efficient method for protein purification based on the interaction of metal ion chelating agent present on the surface of a resin support binding with proteins containing (x6) histidine (His) tag (Porath et al., 1975).

The sample was filtered-sterilised with a 0.22 μm syringe filter (Merck-Millipore) to be purified using IMAC on a of 1 ml of Ni-His trap HP column (General Electric GE) coupled in parallel, in a AKTA *prime* (General Electric) liquid chromatography system, which had been previously washed

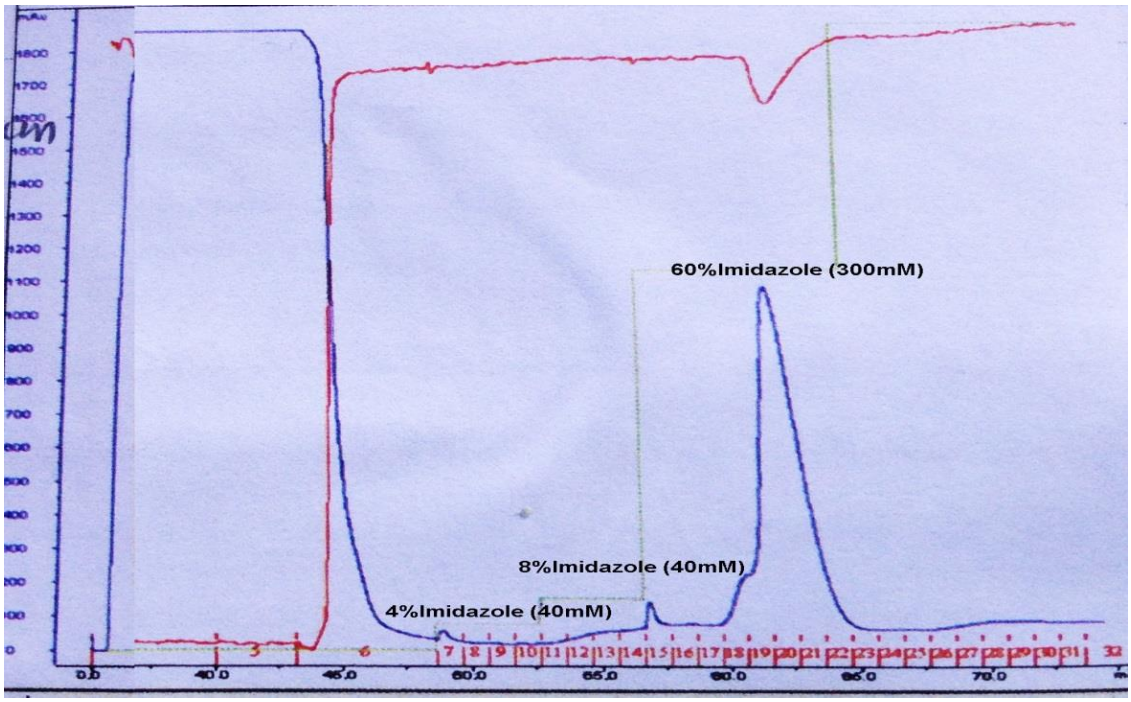
with ethanol 20% (v/v) ETOH and equilibrated with lysis buffer (**Table 51**). The clarified protein extract (~120 ml) was placed in a 50 ml injector and during a manual run with lysis buffer; ten fractions of 10 ml were collected at a flow-rate of 1 ml/minute. A gradient of elution buffer containing a range of concentrations of imidazole from 4% ~20 mM first peak (retention time ~50 minutes), (**Figure 61 A**), 8% ~40mM second peak (retention time ~55 minutes) for elution, (**Figure 61A)** and 60% ~300mM of imidazole third peak (retention time ~60 minutes for elution, **Figure 61A**). Each chromatography peak was fractioned thoroughly at a flow-rate of 0.1 ml/minutes, and 30 fractions of 2 ml of eluent were collected.

Table 51. Buffers used to purify mature rNg-ACP.

Buffer	Composition
Lysis buffer	300 mM NaCl + 50 mM Tris HCl+10% (v/v) glycerol, pH8.5
Affinity chromatography buffer	300 mM NaCl + 50 mM Tris HCl+10%(v/v) glycerol + 500 mM imidazole, pH8.5
Size exclusion chromatography (SEC) buffer	300 mM NaCl + 20 mM Tris HCl, pH8.0

SDS-PAGE electrophoresis was used to evaluate the eluted fractions of the different peaks collected. Host *E. coli* proteins were found mainly in the flowthrough and early eluted fractions did not contain any rNg-ACP bands. Eluted fractions collected from the main peak (**Figure 61A**) contained the protein in high concentrations with a band of *Mr*~12 kDa along with a slight smear of low concentrations across the fractions collected (P19-P23) (**Figure 61B**).

A. Ni(II)-NTA affinity chromatography for rNg-ACP purification.



B.

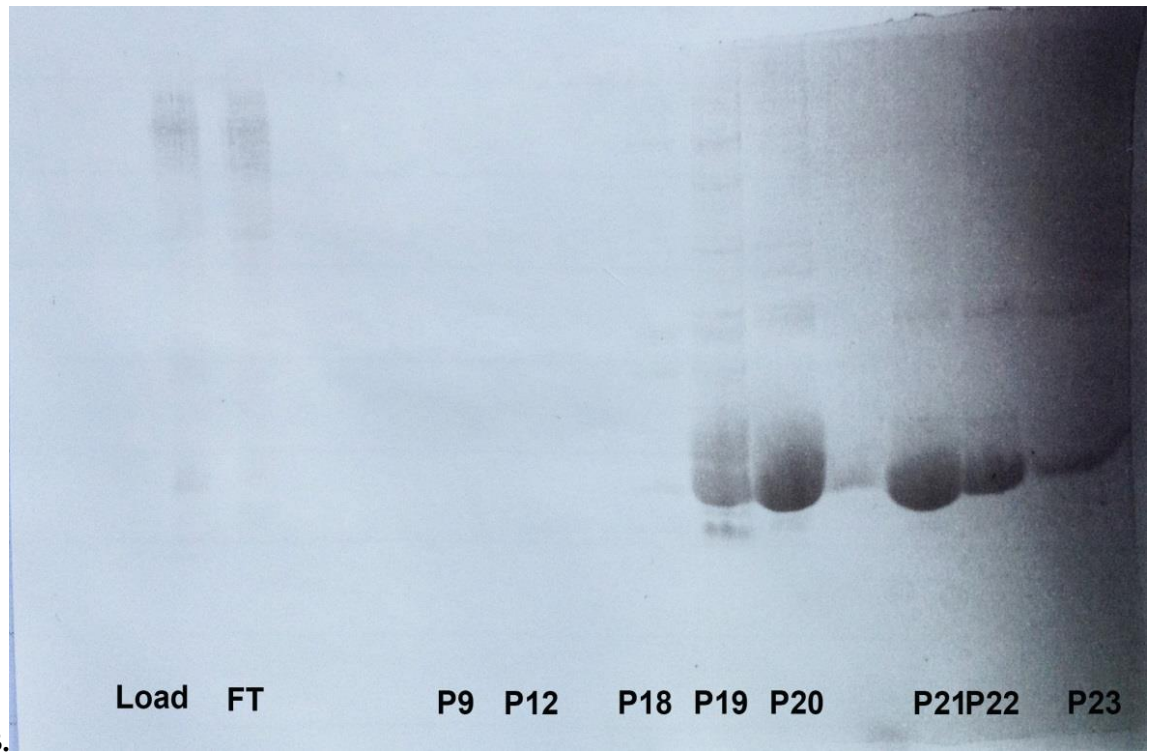


Figure 61. Purification of mature rNg-ACP by immobilised metal ion affinity chromatography (IMAC). A) Chromatogram purification process of recombinant protein was assessed by UV λ_{280}nm and conductivity. B) SDS-PAGE assessment of collected fractions from protein purification. **Load** supernatant cell culture, **FT** flowthrough, **P9-12** and **P18-P23** are eluted fractions 9-12 and 18-23, showing a band with *Mr*~ 12 kDa.

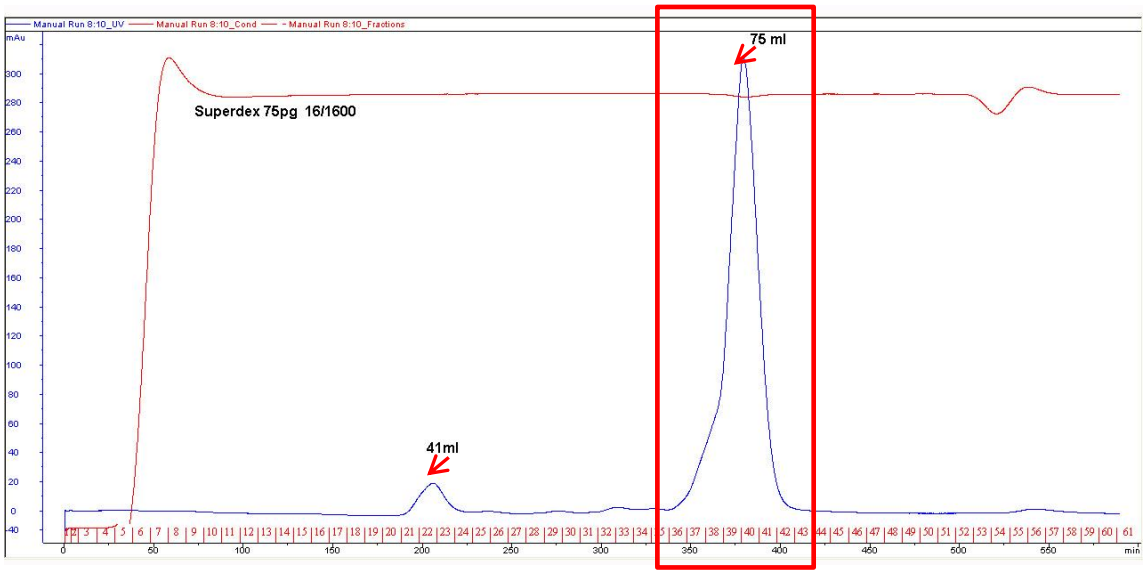
4.2.4. Purification of mature rNg-ACP by Size Exclusion Chromatography (SEC).

Eluted fractions containing the protein (P19-P23, **Figure 61 A and B**) were pooled in a Vivaspin-20, 5,000 MWCO PES (Sartorius) tube. To concentrate the protein, the pooled fractions were centrifuged repeatedly at 3000 rpm, (1865*x*g) (Sorvall Legend RT), at 4˚C for 3-10 minutes, until ~1 ml of concentrated protein was obtained. Next, the sample was injected into the 1 ml loop coupled with the AKTA *premier* chromatography system and separated on a SEC column (High load 16/600 Superdex 75 pg (GE)). An overnight run at a low flow-rate of 0.2 ml/minutes was done and ~62 samples, with 2 ml per sample (**Figure 62 A)**.

SEC chromatogram of pooled fractions showed two peaks at fractions (P34-P37) and (P38-P43), eluted at volumes of 41 ml and 75 ml, respectively. SDS-PAGE analysis of the collected fractions displayed a single band at a high concentration with *Mr*~12 kDa from the samples comprised from P38 to P43 (Second peak, **Figure 62 B)**.

The protein fractions were pooled in a Vivaspin 2 5000 MWCO PES tube (Sartorious) and centrifuged repeatedly for 3-5 minutes, and the protein concentration of the sample was estimated using the molecular weight and extinction coefficient with the aid of the a Nanodrop (Thermo Scientific), until a solution of 100 µl with 50-100 mg/ml was reached.

A.SEC chromatography of collected eluted samples from Ni(II)-NTA column (P19 to P23).



B.

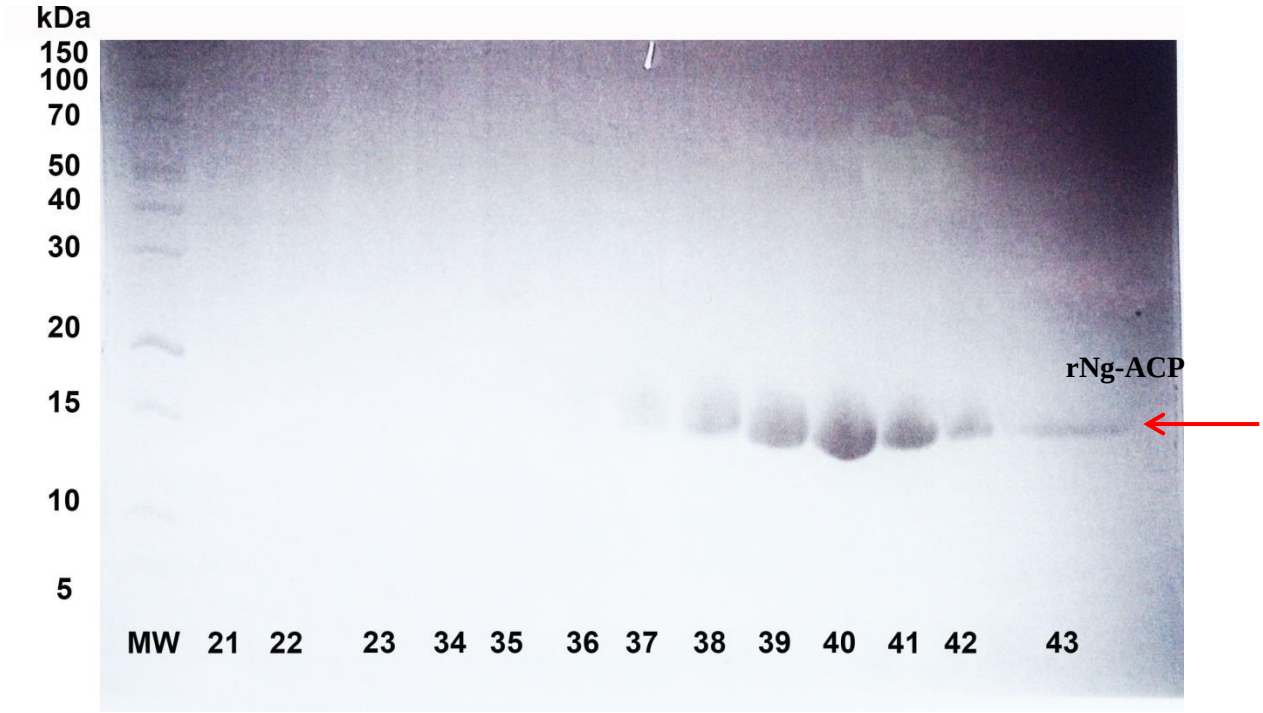


Figure 62. Mature rNg-ACP purified by size exclusion chromatography (SEC). **A)** Chromatogram purification process of recombinant protein assessed by UV λ_{280}nm and conductivity. **B)** SDS-PAGE assessment of the collected elution fractions of the peaks (**P21-P23** eluted fractions peak one) (**P35-P43** eluted fractions 35 to 43 second peak), showing a single band with *Mr*~12 kDa.

Approximately, 80 mg/ml of rNg-ACP was obtained from 8 L of *E. coli* cell culture, and the purified protein showed a single band of *Mr*~12 kDa by SDS-PAGE analysis **(Figure 63).** Bands at a lower concentration were observed at *Mr*~24 kDa and *Mr*~70k Da, which suggested the generation of rNg-ACP oligomers, which required further analysis by mass spectrometry.

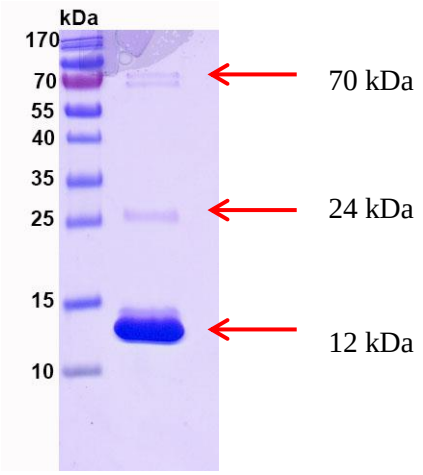


Figure 63. SDS-PAGE of purified mature rNg-ACP (*Mr*~12 kDa) used for crystallography studies**.** The arrows display the purified protein Mr~12 kDa and possible oligomers of rNg-ACP *Mr*~ 24 kDa and *Mr*~70 kDa.

4.3 Crystallography conditions to obtain rNg-ACP crystal.

The purified rNg-ACP protein was used to set up a 96 well SDC crystallisation plate using an IO/IL Art Robbins inteliplate 2/3 drop HP (Art Robins Instruments). A different range of buffers was used at different ratios to optimise the conditions of crystallisation, which is dependend on protein solubility and the presence of salts or precipitant agents. A JCSG-plus screening kit (Molecular Dimensions, UK) was used for crystallisation, containing 96 sterile filtered reagents incorporating polyethylene glycol (PEG), a range of salt additives and neutralised organic acids or organic precipitants across a pH range from 4.0 to 10.5.

The rNg-ACP protein preparation was also tested for crystal growth using a PAC premier screening kit (Molecular Dimensions, UK). This system was designed to test the effect of pH, and

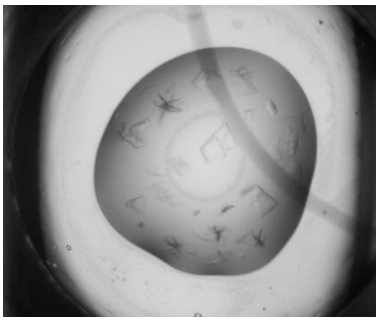
the influence of anions and cations in the presence of polyethylene glycol (PEG) used as precipitant agent.

Some of the conditions tested in the aforementioned screening kits revealed rNg-ACP crystal growth as summarised in **Table 52** as well as the crystal arrangement growth with buffers as shown in **Figure 64.**

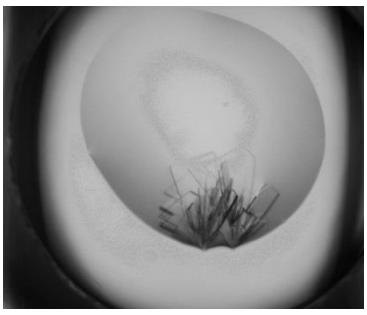
Table 52. Chemical conditions suitable for crystal growth for mature rNg-ACP.

Kit selected	Spot id	Chemical conditions
JCSG+	A12	0.2 M Potassium nitrate, 20% (w/v) Polyethylene glycol 3350
	B3	0.1 M Bicine pH 9, 20% (w/v) Polyethylene glycol 6000
Pact Premier	D6	10% (v/v) malate-MES-tris pH 9, 25% (w/v) Polyethylene glycol 1500

JCSG+ A12



JCSG+ B3



Pact premier D6

Figure 64. Optimal crystallisation conditions for rNg-ACP from different screening kits used. **A12** JCSG screening condition. **B3** JCSG screening growth condition. **D6** Pact premier crystal growth condition.

Generation of the rNg-ACP crystal was favoured by the presence of PEG precipitant agent with a wide range of molecular weights from 1500 to 6000 at pH 9. More organied crystal was obtained with a low density PEG (Pact premier condition D6, **Figure 64)**. In contrast, in the presence of a high molecular weight PEG, the rNg-ACP crystal was more dispersed (JCSG condition B3, **Figure 64)**. Another condition that influenced crystal growth was temperature. The screening plates were tested at 4˚C and 21˚C. 4˚C was found to be optimal for rNg-ACP crystal growth. The rNg-ACP crystals obtained from these selected panels were tested by small angle X-ray scattering (SAXS). The crystallography data was collected in a X-ray beam ID23 beamline at Synchrotron Grenoble, France. Test conditions and properties of the rNg-ACP are summarised in **Table 53**.

Table 53. Crystal structure of Ng-ACP data collection.

DATA COLLECTION			
Space group	C2		
Unit cell parameters	***a***	***b***	***c***
	49.1 Å	31.1 Å	67.5 Å
	α	**β**	**γ**
	(90˚)	(103.255˚)	(90˚)
x-ray source and wavelength	ID2316 to 20 keV energy (0.966 Å)		
Resolution range(Å)	26 - 1.5 (1.53- 1.5)		
multiplicity			
Total refletion	51584 (2530)		
N0. Unique reflections	15962 (781)		
Completenes	99.3 (97.3)		
Rmerge(%)	0.048 (0.151)		
Rpim(%)	0.031 (0.10)		

4.4 Molecular replacement for determining the structure of rNg-ACP.

Molecular replacement is a method for elucidating the three-dimensional structure of proteins, and numerous structures have been generated and deposited onto Protein Data Bank (PDB), in the last decade. The method consists of elucidating of an unknown structure based on an homologous structure as a template (Scapin, 2013), which is selected from sequence alignments using BLASTP, search database Protein data bank proteins (PDB). In this project, a PDB file from the BLAST analysis (3OE3) was used, and the reflection pattern obtained from rNg-ACP X-ray analysis was

run in the programme package MOLREP, which is part of the CCP4 suite. A series of refinement calculations were performed to obtain the molecule. The Dali webserver was used to identify the homologue structures of the solved protein target.

4.4.1 Generation of three-dimensional structure of Ng-ACP using MOLREP from the CCP4 suite.

MOLREP is a package programme included in the CCP4 suite (Collaborative Computational Project, 1994), which is part of a large pipeline of diverse programmes for full crystallographic structure determination. The programme is user-friendly and requires only X-ray data and the atomic model selected from the BLAST analysis of the protein of interest. MOLREP performs a rotation and translation search of a homologous structure (in this case 3OE3), against the data collected for rNg-ACP. The model was then iteratively corrected to modifiy the sequence of the search model to the target sequence. The final outcome is summarised in **Table 54**, using the programmes Coot and REFMAC, which are part of the CCP4 suite (Vagin and Teplyakov, 2010).

Table 54. Refinement statistics of Ng-ACP crystal structure.

Refinement statistics	
Protein residues	98
Rfree (5.0% data)	0.1951
Non-hydrogen atoms	997
Mean overall B (Å)	
RMSD from overall values	
Bond distance (Å)	0.030
Bond angle (degrees)	2.957

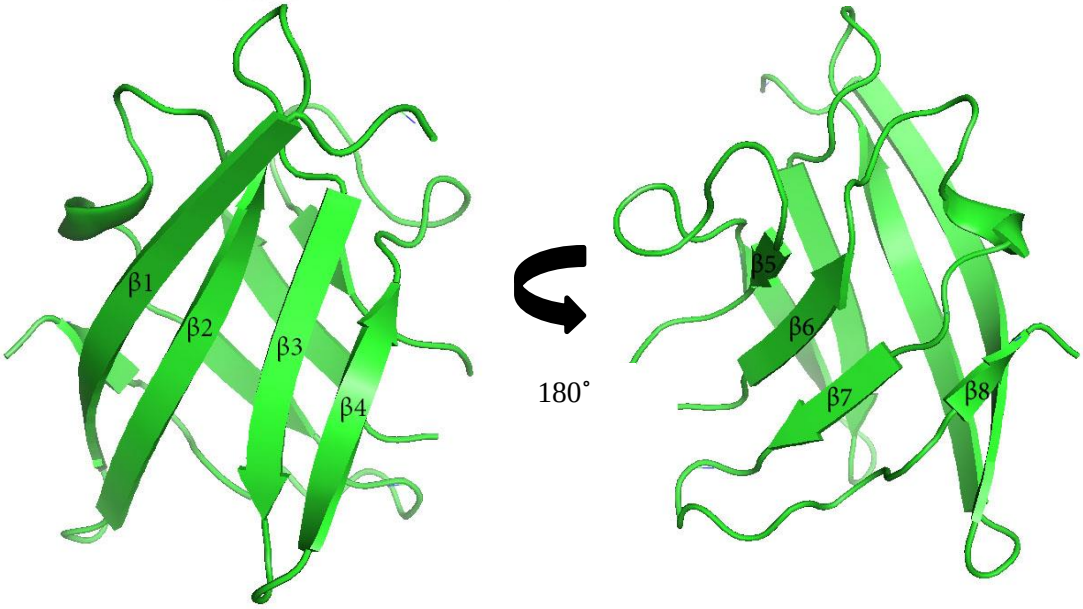


Figure 65. The three–dimensional structure of rNg-ACP using Pymol. A 180˚ degree view of the molecule shows the anti-parallel β-sheet arrangement.

The rNg-ACP structure resembles a β-barrel comprised of 8 stranded β-sheets. The structure divides into two 4–stranded anti-parallel β-sheets (β1-β4 and β5-β8) (**Figure 65**). The structure contains a disulphide bond between Cys_{37}-Cys_{120}, which stabilises the overall fold of the protein (**Figure 66)**.

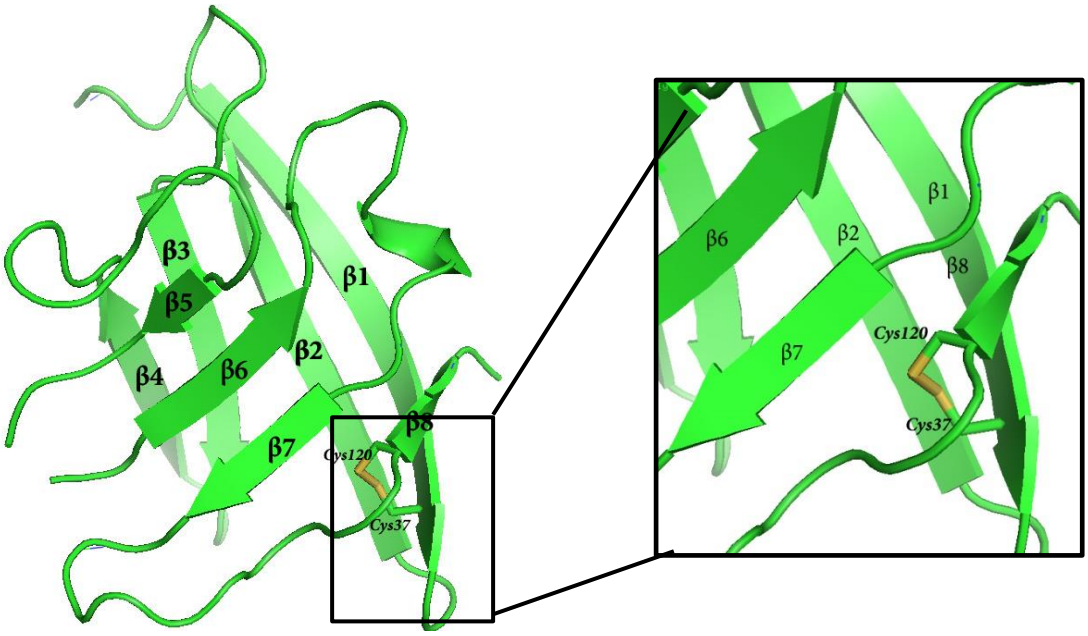


Figure 66. (Cys-Cys) disulphide bond stabilisation of β-barrel fold of rNg-ACP wider magnification of cysteine residues involved in disulphide bond (Cys_{37}-Cys_{120}).

4.4.2. Conservation mapping using Dali webserver.

rNg-ACP homologue proteins were interrogated in Dali webserver to predict the potential biological function (Holm and Rosenstrom, 2010). Dali makes comparative analysis of protein sequences and structures collected on the PDB database; based on the assumption of evolutionary continuity of the structure and function, the biological function of newly discovered proteins can be determined.

Residue conservation is a particularly powerful means of highlighting the key residues involved in interactions, as well as for identifying the location of protein functional sites, and is also one of the tools used in molecular replacement. In this project, the list in rank order for rNg-ACP were molecules recently characterised as Adhesin Complex Protein from *N. meningitidis* (Nm-ACP), lysozyme inhibitor proteins, such as PliC from *Salmonella typhimurium*, MliC from *Pseudomonas aeruginosa* and PliC from *Brucella abortus*, all of which belong to the MLiC/PliC family of lysozyme inhibitors (**Table 55**).

Table 55. Dali research results for homologue proteins to *Neisseria gonorrhoeae* Ng-ACP.

Rmsd*	**% identity**	**Number of residues**	**Protein**	**Organism**	**PDB access code**
0.7	92	93	Adhesin complex protein	*N. meningitidis*	5my7-A
1.8-2.3	18-19	84-88	lysozyme inhibitor of C-type lysozyme Putative periplasmic protein	*Salmonella typhimurium*	3oe3-D
2.5	*98*	*16*	Crystal structure of Brucella abortus PliC in complex with human lysozyme	*Brucella abortus*	4ml7-D
2.4	98	18	Hydrolase inhibitor PliC (orthorhombic crystal form)	*Brucella abortus*	4mis-A
2.4	96-98	18	Hydrolase inhibitor PliC (hexagonal crystal form)	*Brucella abortus*	4mir C-B
2.2-2.4	93	17-19	MliC in complex with hen egg white lysozyme	*Pseudomonas aeruginosa*	3f6z
2.6	82	18	*ydhA unknow function*	*Escherichia coli*	2f09-A

Rmsd *is the average distance (Å) between atoms of superimposed proteins.

4.5 Comparison of rNg-ACP with Adhesin Complex Protein from *N. meningitidis* (Nm-ACP).

Comparison of Ng-ACP structure with the recently described structure of the closely related bacterium *N. meningitidis* (Humbert et al., 2017) displays structural homology (92%, from Dali webserver). The overall structures are similar (Humbert et al., 2017). For Nm-ACP, the structure is stabilised by a disulphide bond between Cys_{38} and Cys_{121} and, in *N. gonorrhoeae* the disulphide bond involves the residues Cys_{37} and $Cys_{120,}$ as shown in **Figure 67**.

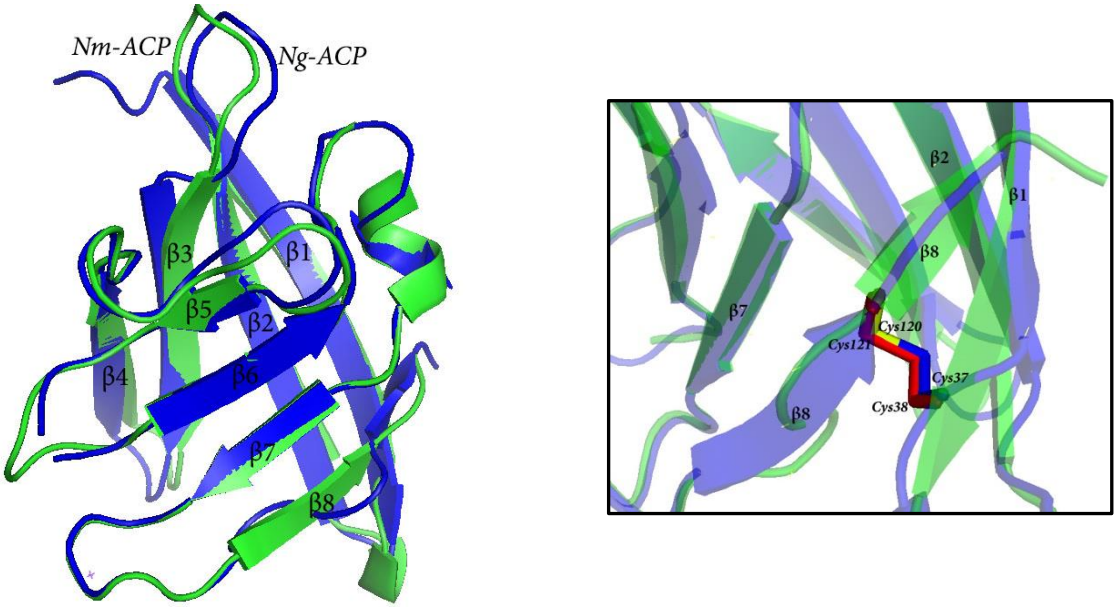


Figure 67. Structural comparison of *Neisseria* species (Adhesin Complex Protein ACP). Ng-ACP from *N. gonorrhoeae* is shown in blue and Nm-ACP from *N. meningitidis* is shown in green. Visualization of molecules was done with PyMOL.Disulphide bond represented in red is between **Cy38-Cys121** from *N. meningitidis* Nm-ACP. Amino acid residue in yellow is **Cys120** and blue colour **Cys37** to form the disulphide bond in ACP from *N. gonorrhoeae* Ng-ACP.

4.6 Comparison of Ng-ACP with proteins of the MliC/PliC family.

The bacterial cell wall is composed mainly of peptidoglycan (PG). The main role of PG is to provide cell structures integrity and resistance against PG hydrolases or other hydrolytic agents of the innate immune system. PG hydrolases target the β1-4 glycosidic bond between N-acetylmuramic and N-acetylglucosamine on the bacterial cell wall (Leysen et al., 2011). Bacteria have developed diverse mechanisms to evade the innate immune system such a PG modification, a mechanism to avoid the action of PG hydrolases, and the generation of lysozyme inhibitors. Lysozyme inhibitors are found in most Gram-negative and some Gram-positive bacteria, and served to protect the bacterial cell membrane from lysozymal lytic activity. (Vollmer, 2008,

Bernard et al., 2011). There are a number of different lysozyme inhibitors, and they have been characterised and classified into several families.

The most similar to *Neisseria* ACP proteins is the MliC/PliC family (membrane bound /periplasmic lysozyme inhibitors of C-type-lysozyme). Chicken type lysozyme inhibitors have mainly been characterised in Gram-negative bacteria (Leysen et al., 2011). Alignment of amino acid sequences of some MliC/PliC inhibitors has shown that they share between 24% and 39% sequence identity only (**Figure 68**). A sequence motif is shared with MliC/PliC family with PliC (bacterial lysozyme inhibitor from *Escherichia coli*) and PliI (bacterial lysozyme inhibitor from *Aeromonas hydrophila*) families (Callewaert et al., 2012).

```
CLUSTAL O(1.2.4) multiple sequence alignment

3od9                            ------mkallmtlglltlplasqaadgffkqltlpsgqvvtvsegrge-----pastgs   49
4G9S:B|PDBID|CHAIN|SEQUENCE     mkiksirkav-lllalltstsfa--agk-----------nvnvefrkghssaqysgeikg   46
3OE3:A|PDBID|CHAIN|SEQUENCE     ----mmkrkl------ipftlflaalsa-----------stts----------i--aa-s   26
4ML7:B|PDBID|CHAIN|SEQUENCE     ----------------------------------------GAMGEITIKLPDSV-K   15
3F6Z:D|PDBID|CHAIN|SEQUENCE     -----mkkalwlllaavpvvlv--acgg-----------sdddkqtaqvdylalpgda-k   41

3od9                            y--dvrly-sganpqfpldqfidgkvlprdgsikelklldlngdkqpelivvvesagsgs   106
4G9S:B|PDBID|CHAIN|SEQUENCE     ydydtytfyakkgqkvhvsisnega-----dtylf-----gpgidds-vdlsr-------   88
3OE3:A|PDBID|CHAIN|SEQUENCE     qeisksiytcndnqvmeviyvntea-----gnaya-----iisqvnemipmrlmkmasga   76
4ML7:B|PDBID|CHAIN|SEQUENCE     VSTNSILYKCGA-KDLSVTYYNAGD-----ISLAK-----LELED-ETVVASNVISGSGA   63
3F6Z:D|PDBID|CHAIN|SEQUENCE     ldtrsvdykcengrkftvqylnkgd-----nslav-----vpvsdnstlvfsnvisasga   91
                                       : .     . :             .               :

3od9                            ylsa----daftlnpqegldsfnhvegla------pnedviqalktprd      145
4G9S:B|PDBID|CHAIN|SEQUENCE     yspeldshgqyslpa-sgkyel-rvlqtrndarknktkkynvdiqik--      133
3OE3:A|PDBID|CHAIN|SEQUENCE     nyeaidknytyklyt-kgktae-lvegddk------pv--lsncslan-      114
4ML7:B|PDBID|CHAIN|SEQUENCE     KYAG----SVYIWWT-KGKTAS--------------------------      80
3F6Z:D|PDBID|CHAIN|SEQUENCE     kyaa----gqyiwwt-kgeeat-lygdwkgge---ptd--gvacker--      127
                                         :     .*
```

Figure 68. Amino acid alignment of different lysozyme inhibitors including MliC/PliC family. **PliI** *Aeromonas hydrophila* **(**PDB acess code **3OD9), PliG from** *E. coli* ***(***PDB access code **4G9S*)*, *PliC*** from *Salmonella typhimurium* ***(***PDB acess code **3OE3*)*,*PliC*** from *Brucella abortous* ***(***PDB access code **4ML7*)*, *MliC from Pseudomonas aeruginosa(***PDB access code **3F6Z*)*.**

Elucidation of the interaction of MliC with hen-egg-white lysozyme (HEWL) provided an insight into the nature of the MliC/PliC motifs **SGxxY** (located in loop4) and **YxxxTKG** located in (loop5) that bind with HEWL by a 'double key-lock' mechanism, which involves the hydrogen bond betweenSer (**S**) and Tyr (**Y**) residues within the binding site MliC (Yum et al., 2009).

In the PliC and MliC family proteins, the overall fold displays an 8 stranded anti-parallel β-barrel. By contrast, PliI family proteins contain the same number of strands in different arrangements such

as a β-sandwich with an α-helix at the C-terminus (Callewaert et al., 2012). Superposition of MliC from *P. aureginosa* and PliC from *Salmonella typhimurium* showed homology with the functional motif in those conserved regions in the loop 4 (**SGxxY**) and loop 5 (**YxxxTKG**), suggesting that they display similar ways of interacting with C-type lysozyme (Van Herreweghe et al., 2010).

Structural comparison of PliC from *Salmonella typhimurium*, PliC from *Brucella abortous* against with the three-dimensional structure of rNg-ACP, demonstrates the similarity in terms of the overall fold. However there is a slight variation in the length of loop 4, which is involved with the binding motif with lysozyme **(Figure 69).** This finding is also observed in the structural analysis of Nm-ACP with some lysozyme inhibitors showing differences within the loop critical for interaction with HEWL (loop 4) (Humbert et al., 2017). Sequence analysis of the most relevant lysozyme inhibitors with rNg-ACP showed an insertion of amino acids around the lysozyme binding site (loop 4) and the lack of functional motifs (**Figure 70**), which suggests another binding mechanism for Ng-ACP. This mechanism should be interrogated with molecular docking analysis.

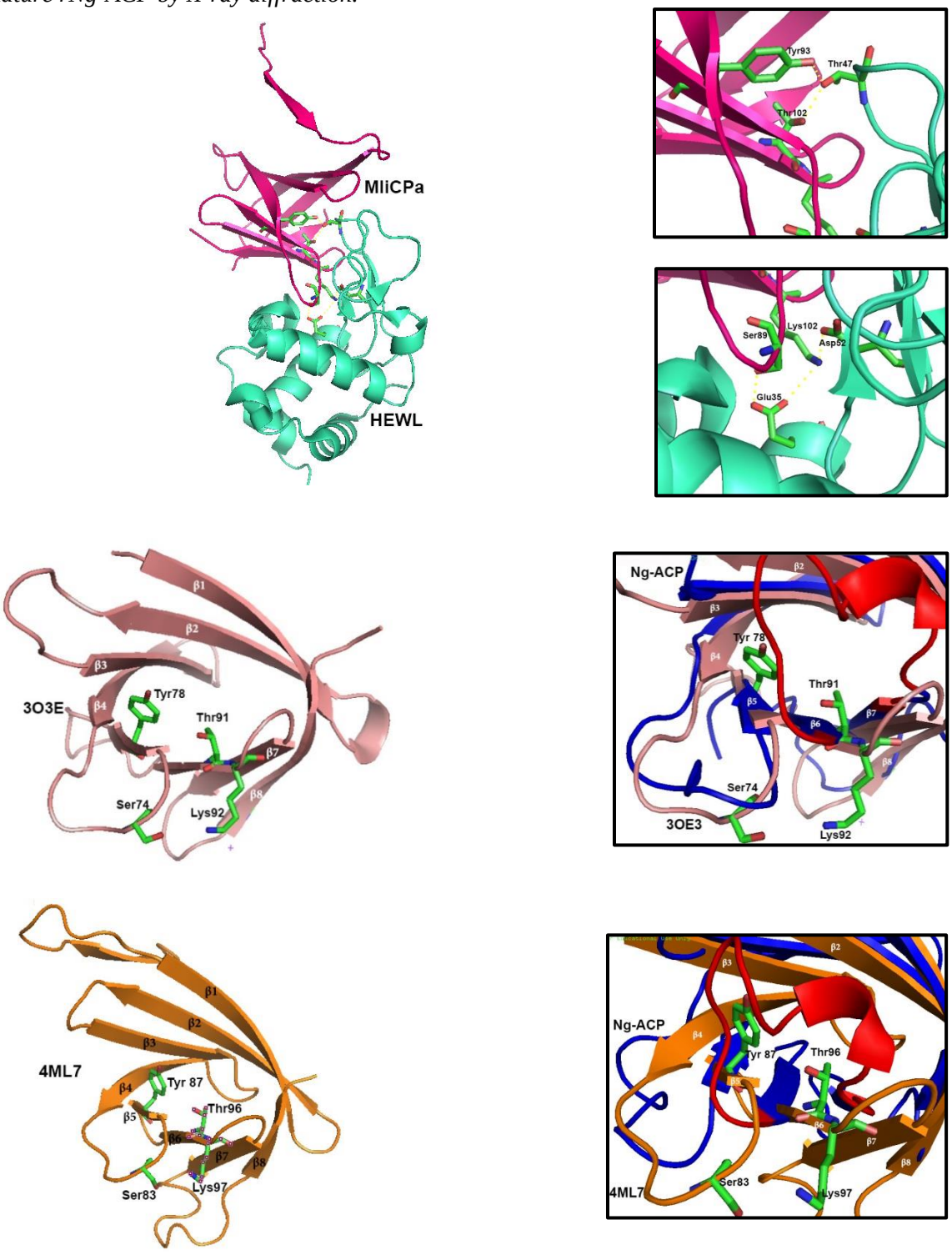


Figure 69. Structural comparison between lysozyme inhibitors **from MliC/PliC protein family with *N. gonorrhoeaeae* Ng-ACP. Three-dimensional structures of common lysozyme inhibitor**s from different species (left panel) and **structural comparison** of inside the loop involved in inhibition mechanism with Ng-ACP (right panel)**. Organisms as *Pseudomonas aeruginosa* MliC in complex with hen egg white lysozyme (PDB accession code 3F6Z), *Salmonella typhimurium* (PDB accession code 3OE3) and *Brucella abortus PliC* (PDB accession code 4ML7). PyMOL was used to visualise the proteins.**

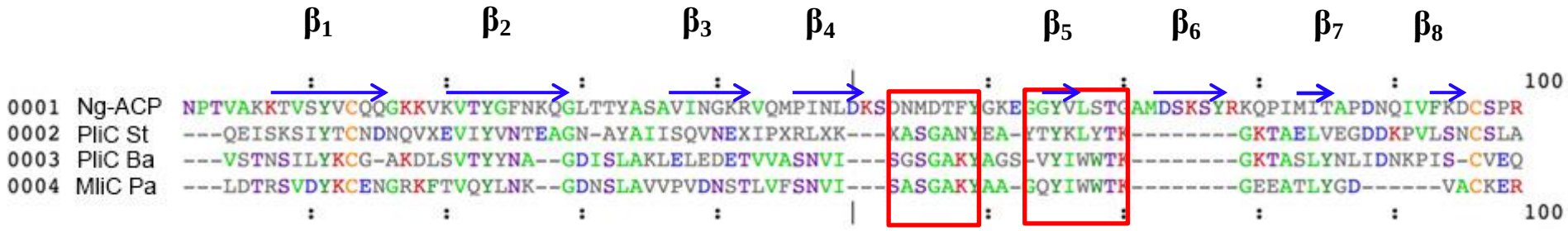


Figure 70. Sequence alignment analysis between the most representative lysozyme inhibitors and Ng-ACP (mol1A), displaying the secondary motifs and differences included inside the conserved binding site with lysozyme.

CHAPTER 5– DISCUSSION

5.1. Pursuing vaccine candidates against *N. gonorrhoeae.*

To date, only few antigens have been evaluated in human clinical trials to develop a gonococcal vaccine (**Table 56**)**.** However, none of these were successful, due to difficulties in producing blocking antibodies and the lack of protect immunity, despite the expectation of antibodies generated against *N. gonorrhoeae* could overcome infection (Eyre, 1909), or that antibodies from genital secretions could block *in vitro* adherence to buccal epithelial cells (Tramont et al., 1980). Most of the antigens tested undergo antigen variation and phase variation, which is characteristic of pathogenic *Neisseria* immunogenic proteins. For this reason the challenge for gonococcal vaccine development is to identify more conserved gonococcal antigens (Rice et al., 1986).

From these examples, gonococcal vaccine development raised some challenges as to find a robust infection model, expand the limited understanding of immunity and the immunity required for protection against recurrent infection (Edwards et al., 2016, Wetzler et al., 2016). Pursuing gonococcal vaccine development requires the identification of conserved antigens, to tackle the conventional pathways of invasion by the host-pathogen. In the last thirty years, a diverse collection of antigens has been identified with promising properties to design a gonococcal vaccine, but many are still in the discovery phase (**Table 57**).

Some antigens are characterised to be immunogenic proteins, despite undergoing variation which expressing multiple variants of the major appendages pili, lipopolysaccharide (LPS) and opacity-associated outer membrane proteins, which are the major components in the outer membrane. In contrast, several immunogenic proteins could potentially be vaccine candidates distinguished by high levels of conservation and stable expression within and between strains (**Table 57**). These conserved antigens may target important physiological functions and compromise the survival of *N. gonorrhoeae*. Some of them involve colonization and invasion, nutrient adquisition, and immune evasion.

Table 56. Antigens used on clinical trials for gonococcal vaccine development.

Antigen	***Outcome***	***Doses***	***Trial***	***References***
Whole cells	Non-protective			(Eyre, 1909)
Partially autolysed bacteria	Non-protective			(Greenberg et al., 1974)
Pilus vaccines	Poor and short lived immune response Non-protective Genital secretion and anti-pilus antibodies could block *in vitro* adherence to human buccal epithelial cells	100 µg vaccine /placebo intradermally on day 1 and day 14.	3123 men and 127 women volunteers. ELISA antibody response to homologous and heterologous pili 40% as high as the homologous pilus antibody rises	(Boslego et al., 1991)
Protein I based vaccine				(Tramont, 1989)

Table 57. Potential vaccines candidates and progress of analysis.

				Immunogenicity			
Antigen	***Function***	***Expression***	***Variability***	***In vitro***	***In vivo***	***Species of immunization***	***References***
PilC	Pilus adhesin , phase variable expression	Phase variable	Variable		Poor and short lived immune response Non-protective		(Tramont et al., 1981)
PilQ	Outer-membrane channel which pili are extruded	Stable	Conserved at C-terminus	Bactericidal properties recombinant $PilQ_{406-770}$ *N. meningitidis* Serogroup A (400) Serogroup B (800)			(Haghi et al., 2012)
PorB	Major porin, involved in invasion of cervical cells. Binds negative regulators of the complement cascade	Stable	Variable	Bactericidal activity (cyclic loop peptides)			(Edwards and Apicella, 2002, Rechner et al., 2007)
Opa proteins	Mediates adherence to immune cells. Phase variable, antigenically distintitve per strain.	Phase variable	Variable	Bactericidal activity Protective in mouse model			(Callaghan et al., 2011, Cole and Jerse, 2009)
OmpA	Mediates invasion of malignant cervical endometrial cell lines	Stable	Highly Conserved	Localization on the surface by FACS Binding protein into cells ME-180	Bactericidal activity		(Serino et al., 2007)

Phospholipase D (PLD)	Regulator of gonococcal invasion and survival in cervical epithelia		Highly conserved	Inhibitory antibodies reduction of adherence and invasion of primary cervical cells		(Edwards et al., 2003, Apicella, 2007)
			Nutrient acquisition and metabolism			
LbpA, LpbB	Lactoferrin receptor	Induced under iron-limited condition	Semiconserved not present in all of gonococcal strains	Bactericidal activity		(Pettersson et al., 2006, Adamiak et al., 2012)
TbpA TbpB	Transferrin receptor Semiconserved,	Induced under iron-limited condition		Bactericidal antibodies against FA19 (200) FA1090 (50-100) MS11 (400)	From recombinant proteins presents Bactericidal antibodies in mice	(Price et al., 2007, Hobbs et al., 2011)
AniA Nitrite reductase	Surface exposed Important for growth and biofilm formation Plays a role serum resistance	Induced under anaerobic conditions.	Highly conserved	A trunctcated AniA without glycosylated C-terminus induced bactericidal (203000) activity and reduce nitrite reductase activity.		(Shewell et al., 2013, Shewell et al., 2017, Falsetta et al., 2009)

Immne system evasion process					
Rmp Outer-membrane Reduction Modifiable Protein	Role in immunosuppresion Almost highly conserved protein	Stable	Highly conserved	Bactericidal activity with knockout Δ*Rmp* (625)	(Li et al., 2014)
2C7 epitope (LOS epitope)	Inner glycose core of LOS	Phase variable gene *lgtG* Expressed by 95%	Highly conserved	Bactericidal activity Whole serum and purified IgG (1/5) but not purified IgM +IgA and opsonphagocytic from Active and passive protection in mice	(Gulati et al., 2013, Ngampasutadol et al., 2006)
MtrE	Surface –exposed channel Mtr efflux pump complex	Stable	Highly conserved	Bactericidal activity	(Jerse and Deal, 2013)
Lst	α2,3 sialyltransferase Addition of sialic acid to the moiety of LOS, protect from complement	Variation expression between strains	Highly conserved	Inhibiting antibodies to reduce sialylation.	(Packiam et al., 2006)
NspA	Factor H binding and serum resistance	Stable	Highly conserved	Bactericidal (NGOWHO-A, china, (200)) and opsonic activity	(Li et al., 2011, Martin et al., 1997)

Continuing to pursue the identification and characterization of novel antigens, a homologue of the *acp* gene in a closely related bacteria *N. meningitidis* (Hung et al., 2013) was identified in *Neisseria gonorrhoeae* with the size of 372 bp. Bioinformatic analysis of gene encoding the *ng-acp* (NGO1981, NEIS2075) for gonococcal isolates in the PubMLST.org/Neisseria database, showed that from 3822 isolates there were 20 redundant alleles encoding Ng-ACP protein, which share from 93% to 98% homology. Amino acid alignment and a phylogenetic tree based on identity percentage showed 13 out of the 20 alleles are non-redundant. The remaining gonococcal alleles isolates are clustered into five groups having high similarity comprised by alleles 6, 59 and 60, which are represented with isolate *N. gonorrhoeae* strain FA1090 and alleles 3, 93, 151. The most predominant allele was 10, represented by *N. gonorrhoeae* strain MS11, which the vast majority of gonococcal isolates (81%) characterised to date expresses this type of allele.

Amino acid alignment comparison between gonococcal alleles (alleles 6 and 10) and two types of meningococcal ACP isolates shows high homology of ~93% and also displayed different substitution and deletion at residue 20 exclusively in the prevalent gonococcal allele (allele 10). First, the gonococcal diversity of Ng-ACP protein is less when it is compared with meningococci that display two types of Nm-ACP protein (Hung et al., 2013). Gonococcal Allele 10 has high homology with Type II meningococcal Nm-ACP, allele 6 has high homology with type I Nm-ACP protein, based on the residue at position 25 (aspartic acid (D) or aspartate (N)). Along the protein, there were a few substitutions with similar physicochemical properties (Valine (V) to Isoleucine (I)) at position 72 (aspartic acid (D) to glutamic acid (E)) at position 81.

PCR reaction confirmed the high similarity between different *acp genes* from other *Neisserial* commensal strains. The *ng-acp* gene from *N. gonorrhoeae* strain P9-17, an UK clinical isolate, was amplified and sequenced, the PCR product with size ~400bp. Comparison of amino acid sequence between the *N. gonorrhoeae* strain P9-17 with non-redundant gonococcal alleles expressing Ng-ACP, displayed high homology with the most prevalent isolate 10, which demonstrates once more that the vast majority of gonococcal isolates characterised express the same allele. This suggests that *ng-acp* gene is a promising vaccine candidate due to *ng-acp* gene is highly conserved and potentially could be expressed in most strains (Edwards et al., 2016).

5.2. Expression of recombinant *N. gonorrhoeae* adhesin complex proteins.

One of strategies used in vaccine development is the generation of recombinant protein, comprised of highly purified recombinant proteins or subunits from pathogens. Potential vaccine candidates are cloned, expressed and purified and using heterologous host to express proteins such as bacteria, yeast and mammalian cells. Some of the factors that might have an effect on efficacy or antigenicity of recombinant protein as vaccine candidates are the specific expression vector and promoter, selection marker and presence or absence of post-translational modification (Hansson et al., 2000). The challenge of this vaccine strategy is to reach the desirable antigenicity and effectiveness as a vaccine, which invariably requires the presence of adjuvant prefered, route of administration and serial doses of immunization. Despite those challenges, many vaccines have been developed as different examples to tackle a wide range of infectious diseases, e.g a vaccine against hepatitis B, a chronic liver disease affecting worldwide. A surface antigen (HBAgs) is produced in yeast, which involves post-translational modification, and then the surface antigen is assembled into virus like particles (VLP), which are extremely immunogenic, and therefore potentiates the efficacy of the vaccine (Adkins and Wagstaff, 1998). Another example is Human PapillomaViruses (HPV), a sexually transmitted disease that can lead to cervical, vulval and vaginal cancers and genital warts. The vaccine is comprised of virus like particles derived from the main prevalent virus subtypes (HPV 11, 16, 6 and 18) assembled with most representative surface protein (VL1) produced in yeast and insect-cell system and the use of aluminium potassium sulphate adjuvant, induces high titres of virus-neutralizing serum antibodies (Govan, 2008). Recently a vaccine has been developed against *N. meningitidis* serogroup B, as result of reverse vaccinology and proteomics, composed by multi-component recombinant proteins (Neisseria heparin-binding antigen NHBA fusion protein, Neisserial adhesin A NadA protein, fHbp fusion protein with Outer membrane vesicles (OMV), New Zealand) from *Neisseria meningitidis* serogroup B, which are the most antigenic tested alone which elicits bactericidal antibodies and could confer protective immune response (Donnelly et al., 2010).

Two cloning strategies and different purification strategies were performed for rNg-ACP. The main differences were on the length of the protein. **Full-length Ng-ACP** was comprised of the Ng-ACP

protein plus the leader peptide and additional 39 amino acids including the poly 6xHis tag at the N-terminus. On the other hand, the **mature Ng-ACP protein**, the leader peptide was removed and added a poly 6xHis tag was presented at the C-terminus. This strategy of placing the 6xHis tag at either the N- or C-terminus could improve significantly the solubility of recombinant proteins (Bornhorst and Falke, 2000, Crowe et al., 1994). For both recombinant proteins, *E.coli* BL21De3pLysS strain was used as a heterologous host to produce the recombinant proteins. The Full-length rNg-ACP was purified using guanidine hydrochloride (GuHCl), as a denaturant agent. GuHCl is a hydrogen bond disruptor to handle insoluble proteins, which the guanidinium ion can transiently stack in a flattened conformation and coat the protein hydrophobic surfaces. GuHCl also interacts with amino acid side chains and with aliphatic side chain, to reduce unfavourable exposure with water (Wingfield, 2001, England and Haran, 2011). On the contrary, mature Ng-ACP, which did not contain the leader peptide was purified under native conditions in the presence of imidazole and was used for further structural analysis.

The ideal recombinant protein for vaccine inclusion must be soluble and invariably does not contain any leader sequence. This is shown by the proteins used in Bexsero® meningococcal vaccine for example (Bai et al., 2011, Gorringe and Pajon, 2012).

5.3. Gonococcal full-length and mature rNg-ACP proteins are antigenic.

The antigenicity of full-length and mature rNgACP was evaluated generating murine antisera and analysed by ELISA. This showed that similar titres were observed with all of the adjuvant and delivery systems used. Lower ELISA tires against full-length rNg-ACP were observed when the protein is delivered in detergent and liposome preparations.On the contrary, the addition of immunomodulators such as MPLA induced the highest titres comparable to the aluminium hydroxide ($Al(OH)_3$) preparations. These results are also correlated in the same way to identify the native Ng-ACP gonococcal protein (*Mr*~12.5kDa) on lysate and outer-membrane preparations from *N. gonorrhoeae* strain P9-17 and heterologous cross-strain reactivity (*N. gonorrhoeae* strain FA1090) by western blot. In both cases, the highest response were obtained with mice sera with aluminium hydroxide ($Al(OH)_3$) and liposome with MPLA.

For mature rNg-ACP protein similar ELISA titres reactivity was presented between the delivery systems tested but lower titres compared with the full-length rNg-ACP. The highest titres were induced using saline or liposomes with MPLA preparations. Conversely, titres of mature rNg-ACP with aluminium hydroxide ($Al(OH)_3$) and detergent preparations were slightly lower.

Likewise, the immuno-reactivite band of (*Mr* ~12.5kDa) in homologous and heterologous gonococcal strains lysate and outer-membrane preparations displayed the same pattern of reactivity as observed in ELISA. In this case, despite the highest titres in Liposomes with MPLA does not have any implication on the native conformation of the antigen. On the contrary , absorption of mature protein rNg-ACP with aluminium hydroxide ($Al(OH)_3$) may have an effect on the native conformation of the protein, to expose the immunogenic epitopes of Ng-ACP (Jones et al., 2005).

Vaccine development involves designing combinations of antigens and adjuvants may reveal unique properties of immune potency or efficacy. Adjuvants are molecules, compounds or macromolecules that boost the potency quality and longevity of specific immune response to antigens causing minimal toxicity. Some of them in the last fifty years have been introduce in vaccine development for prevention of human diseases.

Alum preparations are the most well know to vaccine development. Alum is typically used one of these three salts, such as aluminium hydroxide, aluminium phosphate or potassium aluminium sulfate. The application of these best stabliched salts are included in vaccine formulations for prevention of Hepatitis B, Diphtheria, Pertussis and Tetanus and human papillomavirus (HPV),a licensed vaccine Cervarix (GlaxoSimthKline)(Brito et al., 2013). The interaction between antigen and alumn resides on the surface and associated by surface adsorption which involves electrostatic, hydrophobic interactions and ligant exchange (Hem and Hogenesch, 2007).

Immune potentiators enhace immune response by activating signalling pathways, which trigger innate immunity. Immune potentiators are comprised a wide range of molecules including oligonucleotides, phospholipids, and small imidazoquinolones (O'Hagan, 2007). Lipopolysaccharides such as MPLA a non-toxic version of LPS with beneficial immunomodulatory activities .An examples with introduction of immune modulators in licensed

vaccine producs as Cervarix and Fendrix for prevention of HPV and Hepataitis B, which contain MPL, a MPLA manufactured by GlaxosmithKline) located on the surface alum particles (Didierlaurent et al., 2009, Casella and Mitchell, 2008)

Liposomes are vesicles composed by phospholipids bilayers, fistly decribed in 1964. The antigen can be associated with lipososmes in numerous ways including encapsulation of the antigen within aqueous core, association through the lipid bilayer and transmembrane regions of the antigen, or by surface adsorption. Two licensed vaccines included this type of formulation, such as Inflexal V an influenza vaccine which contains influenza virosomes with flu hemagglutinin (HA) embedded in the liposomal structure. Epaxal is a hepatitis A which contains a formalin inactivated hepatitis virus, electrostatically bound withinfluenza virosome containing HA and viral lipids from influenza virus (Gluck et al., 1992, D'Acremont et al., 2006).

5.4. Full-length and mature rNg-ACP proteins elicited serum bactericidal activity against homologous and heterologous gonococcal strains.

Correlation between protection against gonococcal infection with increased levels of complement bactericidal activity is believed to be one of the key parameters for vaccine development against *N. gonorrhoeae* (Jerse et al., 2014, Zhu et al., 2011). The major finding of this study was that this antigen induced bactericidal activity for both types of rNgACP proteins and elicited the highest titres against wild-type homologous and heterologous gonococcal strains, but not against knockout mutant strains (*N.gonrrhoeae* strain P9-17 *Δng-acp* and *N. gonorrhoeae* strain FA1090 *Δng-acp*). For both cases, murine antisera with delivery systems such as detergent micelles ZW3-14 plus immunomodulator as MPLA elicited highest titres in the range from 256 to 1024 compared with other adjuvants tested. The reason that this particular adjuvant generates immune response is due to MPLA is derived from a lipopolysaccharide fraction of the cell walls from *Salmonella minesota*; which boost adaptative immunity via TLR4 (Casella and Mitchell, 2008). Similar observation of high bactericidal activity were found for meningococcal proteins PorB (Wright et al., 2002) and Opc (Jolley et al., 2001), suggesting that for outer membrane (OMV) proteins the generation of bactericidal titres is largely dependent on native conformation of the epitope (Hung et al., 2011). Similar titres were observed in most of the formulations using full-length rNg-ACP. However, the

lowest titres were observed with detergent or liposomes alone formulations, suggesting that the reactivity of bactericidal antibodies is influenced by the physicochemical properties of the adjuvant.

Murine antisera with mature Ng-ACP showed similar bactericidal titres but lower titres compared with full-length rNg-ACP. However, high antigenicity with aluminium hydroxide ($Al(OH)_3$) and Liposomes with MPLA preparations did not produce higher bactericidal titres, suggesting that the presence of these adjuvant possibly affects the native conformation of the protein, likewise the exposure of the epitopes involved in bactericidal activity (Jones et al., 2005) or induce another pathway of adaptive or innate immunity route (Moon et al., 2011, Casella and Mitchell, 2008).

A comparison of bactericidal titres between full-length and mature rNg-ACP and vaccine candidates discovered for vaccine development against *N. gonorrhoeae* showed the amount of titres are similar with most of them, indicating that Ng-ACP (NGO1981) could potentially be included as a vaccine candidate and deserve more investigation of this antigen and evaluation for further clinical trials.

OMV vaccine strategy for gonococcal vaccines is considered due to the capacity to elicit immune response against different components on the bacterial surface (Zhu et al., 2011). In this study, the bactericidal activity elicited with outer membrane and detergent extracted outer membrane vesicles (Na-Doc OMV) at different concentrations (1μg and 10μg) with adjuvants (saline andaluminium hydroxide ($Al(OH)_3$)) was also examined. High bactericidal titres were observed in both outer membrane preparations and slightly difference of the amount of titres elicited when compared between the concentrations of antigen used. A remarkable difference of elicited bactericidal titres was observed between with outer-membrane preparations and rNg-ACP, demonstrating the great repertoire of molecules present in the outer-membrane, in particular molecules highly immunogenic (presence of LPS, PorB, Pili) can potentiate the response against *N. gonorrhoeae*. Detergent extraction of OMV (Na-Doc OMV) preparation demonstrates the removal of small molecular weight molecules showed a slightly reduction of bactericidal titres but higher compared with rNg-ACP, suggesting the presence of remaining highly immunogenic antigens. Taken together Ng-ACP generates highest bactericidal titres comparable with some of vaccine candidates with deserve more research on this potential target against *N. gonorrhoeae*. In addition, the use of a

repertoire of components on the surface from the outer membrane from *N. gonorrhoeae* could boost the bactericidal titres suggesting that inclusion a group of proteins for vaccine development in a similar manner as the Bexsero®, *N. meningitidis* serogroup B vaccine recently introduced in UK (Donnelly et al., 2010, Carter, 2013, Gorringe and Pajon, 2012).

5.5 Ng-ACP expression is variable expressed on the surface.

To further characterize Ng-ACP as a vaccine candidate, we studied the expression by immunoblotting and binding of antibodies by ELISA against outer membrane preparations. These data provided evidence that this protein was expressed and available evidenced by immune-reactivity by murine and rabbit antisera. However, the antigenicity by ELISA detection was lower. Surface exposure also was examined by flow cytometry analysis, measuring binding of antibodies to bacterial strains. The localization of Ng-ACP on the surface of gonococci by flow cytometry analysis were very poor and the reactivity of murine sera raised with full-length and mature rNg-ACP against outer-membrane preparation from homologous and heterologous gonococcal strains (*N. gonorrhoeae* strains P9-17 and FA1090) appear to display a lower detection of recognition, suggesting potentially a reduced amount of expression of this antigen on the surface or possible differentiation of regulation in cell envelope and membrane vesicles. A transcriptome analysis performed on *N. gonorrhoeae* determined that about 198 genes were differentially expressed (~10% of the genome) in response to anaerobic conditions (Isabella and Clark, 2011). Some genes involved in binding were anaerobically induced under this conditions, and one of them was NGO1981 (*ng-acp* gene), suggesting the expression of Ng-ACP is variable under aerobic conditions (Isabella and Clark, 2011). Ng-ACP is a homolog in another member of *Neisseria* family *Eikenella corrodens*, has been shown to be a part of an adhesin complex key on adherence to host tissues (Azakami et al., 1996). In addition, a proteomic analysis from four gonococcal strains showed that the abundant proteins were identified in the cell envelope (305), the membrane vesicles (46), and associated proteins (34). In both cell envelopes and MVs were found 11 of these proteins presented a differential regulation (Baarda and Sikora, 2015, Zielke e t al., 2014). The regulated proteins were confirmed also in an independent study of MVs (Perez-Cruz et al., 2015).

Furthermore, a qualitative analysis of strength of immune-reactive bands of ACP from protein lysate preparations of gonococcal and *N. meningitidis* strain MC58 detected with rabbit antisera rNm-ACP, displayed cross-reactivity and differential reactivity between these closed related bacteria. In addition, a strong immuno-reactivity band (*Mr* ~12.5kDa) was evidenced for meningococcal strain compared with gonococcal strains, suggesting and evidence once more the potentially lower expression of this antigen in *N. gonorrhoeae*. Taken together, more investigation is required on the expression of *ng-acp* gene under anaerobic conditions and the presence of this gene in more gonococcal isolates. It is possible, that transcription differences are not translated to differences in protein expression.

The challenges observed for the localisation of Ng-ACP on the surface *N. gonorrhoeae* may be due to a low amount of the protein present on the outer membrane which was discussed above. Further studies of Ng-ACP from *N. gonorrhoeae* demonstrated that Ng-ACP is released and then the protein interacts with the membrane through electrostatic interactions (Ragland S.A. , 2018). The Ng-ACP phenomenon from *in vitro* experiments suggests difficulties or unresponsive detection observed using flow cytometry (FC). The potential use of Ng-ACP as a vaccine candidate will be need to be in consideration, however the fact that it may have variable expression and release to the surface of the outermembrane may work against it as an effective vaccine against *N. gonorrhoeae*. Due to a variability of the antigents present in *N. gonorrhoeae* requires a multi-component vaccine which includes other immunogenic antigens or a repertoire such as modified outer-membrane to cover the vast majority of the protein, which was included in the successful vaccine against *N. meningitidis* (Bexsero) (Cartwright et al., 1999).

5.6 Ng-ACP acts as a minor adhesin for gonococcal attachment, but is not significantly involved in invasion to epithelial cells.

Adherence assays demonstrated that Ng-ACP protein plays a role in association on epithelial cells. *In vitro* association assay was performed using conjunctival Chang cells, HEp-2 laryngeal carcinoma cells and meningeal cells and showed a significant difference of associated bacteria numbers between wild-type and mutant strains. In addition, comparison of association between homologous and heterologous gonococcal strains was found similar recovered bacteria on

epithelial cells (Chang and HEp-2 laringeal carcinoma cells). For association of gonococcal strains on meningeal cells, the amount of associated bacteria was slightly lower. Taken together, those data suggest that Ng-ACP plays a role in association on epithelial cells. In addition, Ng-ACP function was evidenced by homology with other adhesin from *Eikenella corrodens*, has been shown to be a part of an adhesin complex and key for adherence to a variety of host tissues (Azakami et al., 1996) in previous transcriptomic studies (Isabella and Clark, 2011). Previous studies of Nm-ACP on *N. meningitidis* demonstrated that Nm-ACP plays a role in association on epithelial cells but not significant on endothelial cells (Hung et al., 2013). Furthermore, preliminary studies of binding of full-length rNg-ACP with conjunctival Chang cells by flow cytometry, demonstrated a dose response trend, in a similar way as another characterised adhesin OmpA like protein in *N. gonorrhoeae* (Serino et al., 2007), suggesting once more the potential role inassociation on epithelial cells. However, the potential receptor used by *N. gonorrhoeae* is still unknown which enables association on epithelial cells. Inhibition assays with addition of rabbit serum raised with full-length Ng-ACP inhibited the association process at different multiplicities of infection (MOI) of *N. gonorrhoeae* P9-17 strain, showing a dependent reduction in total association which reduces 60% of total association at MOI on the range between 0.02 to 2. Whether this represents an interruption of bacterial-ligand and host cell receptor interaction or disruption of an electrostatic interaction is unclear.

Mayor adhesins such as Pili mediate interactions between gonococci and host epithelial cells (McGee et al., 1981, Mosleh et al., 1997, Rudel et al., 1995a). Pili is composed of PilE, the major pilus subunit; PilC, an adherence-associated protein; and other components (Ryll et al., 1997, Rudel et al., 1992). Opa proteins have an important role in gonococcal adherence to host cells. Adherence of P2 Opa1 gonococci to epithelial cells in vitro involves heparan sulfate (Chen et al., 1995). Opa proteins confer on gonococci a cell tropism, with some Opa proteins promoting adherence to polymorphonuclear leukocyte cells and others promoting adherence to specific of epithelial cell lines or other cells *in vitro* (Kupsch et al., 1993). To investigate the influence ofthese main adhesins (Pili and Opa) on Ng-ACP association with epithelial cells different gonococcal variants were assessed with their respective knocokout mutant (*Δng-acp*). Variants P9-1 ($Pili^{-}Opa^{-}$) and P9-2 ($Pili^{+}Opa^{-}$) did not show a significant variation between the wild-type and knockout

strains (*Δng-acp*). On the contrary, a significant 40% reduction of associatiated bacteria on Chang cells between knockout and wild-type mutant strain using gonococcal variant P9-16 *(*Pili^{-}Opa^{+}) was observed suggesting synergic action between Ng-ACP and Opa. Based on this evidence, it is suggested that on the surface of epithelial cells the presence of specific binding receptors for Opa, similarly as *N. meningitidis* association with neutrophils and epithelial cells human colonic (HT29) and lung (A549) which binds (CD66) receptors (Virji et al., 1996b, Virji et al., 1996a). Also this *N. gonorrhoeae* variant can bind with heparin suphate proteoglycans (HSPG) (Chen et al., 1995, van Putten and Paul, 1995, Freissler et al., 2000). A significant reduction on association (75%) was observed when a synergic action of (Pili, Opa and Ng-ACP). Similar studies of adherence and invasion on another epithelial cell line, HEC-1-B, confirmed the synergic action of Pili and Opa (Griffiss et al., 1999).

A study of contact interactions between epithelial cells and *N. gonorrhoeae* showed rearrangement of actin filaments in host epithelial cells reported in Chang epithelial cells through microscopy (Grassme et al., 1996). Cytochalasin D (CD) was used to study the effect of Ng-ACP protein on invasion by gonococci, an actin–filament disrupting agent. Similar results obtained a significant reduction of the numbers of internalised bacteria in the presence of CD when evaluating the influence of Opa on the invasion process (Grassme et al., 1996). Despite the difference found on Ng-ACP, the amount of internalised bacteria in all epithelial cells evaluated was small. On the contrary, the action of CD on adherence on epithelial cells does not affect the process, which was not a significant variation between the numbers of associated bacteria with and without the presence of this actin filament disrupting agent. Similar studies demonstrate gonococci required an actin-based microfilament-dependent activity to enter the host cell but not to adhere to the surface of the epithelial cell (Richardson and Sadoff, 1988).

5.7. Structural vaccininology of Ng-ACP.

Structural biology is progresively being introduced to vaccine development to determine and understand the basics of the antigen of interest and enable the rational design of peptides from specific antigenic epitopes (Serruto and Rappuoli, 2006, Dormitzer et al., 2008).

Recent research on adhesin complex protein (ACP) from the closely related bacterium *N. meningitidis* showed similar overall folding found in gonoccoccal rNg-ACP. Meningococcal, Nm-ACP three-dimentional structure resemble a β-barrel fold comprised with 8 alternated β-sheet and stabilised with a single disulphide bridge (Humbert et al., 2017).Those molecules has been elucidated by molecular replacement, which enable predict the three–dimensional structure based on homology with closed related molecule and potentially predict the potential biological function, which was found in lysozyme inhibitors from *Salmonella typhimurium* (Leysen et al., 2011) and *Brucella abortus* (Um et al., 2013). These types of lysozyme inhibitors have been characterised with the binding site of interaction with hen egg-white lyzozyme within the loop 4 and 5. Amino acid sequence comparison of the loop involved in interaction with lysozyme evidenced two types of motifs SxSGAxY and YxxxTKG, conserved in the MliC/PliC families proteins (Leysen et al., 2011, Callewaert et al., 2012). However the amino acid comparison within these regions demonstrated that *Neisseria* ACP proteins did not share any conserved motif binding regions from the MliC/PliC families proteins, suggesting another alternative of binding with lysozyme, which it will be deserved investigate by simulation using molecular docking or generation of crystal complex Ng-ACP–human lysozyme. Humbert *et al*. also perfomed *in vitro* lysozyme inhibition assay using human lysozyme demonstrated rNg-ACP gonococcal protein elicited ~80-100% inhibition, similar observation as for *N. meningitidis* (Humbert et al., 2017).

5.8 Current state of gonococcal vaccine development.

Efforts continue for identification of novel antigens to develop a gonococcal vaccine. Different approaches have been used lately to identify some potential candidates, involved in a diverse biological process, that are still ongoing **(Table 58).** Recently vaccine candidates discovered from proteomics or bioinformatics analysis, targeted different essential and immune evasion processes. Remarkably, bactericidal titres against heterologous *N. gonorrhoeae* strain FA1090, homologous *N. gonorrhoeae* strain MS11, or other *N. gonorrhoeae* strains displaying similar titres foundwith two types of Ng-ACP recombinant proteins using different formations and delivery systems (**Table 59**) but significant slower compared with the outer memebrane preparations (**Table 59**).

Highest bactericidal titres (~512) against homologous *N. gonorrhoeae* strain MS11 was observed with different vaccine candidates determined from proteomic analysis (**Table 58**) and similarly this bactericidal titres was found using murine sera raised against full-length rNg-ACP with aluminium hydroxide ($Al(OH)_3$) against homologus *N. gonorrhoeae* strain P9-17 (**Table 59**). This titre observed suggesting that can be potentially strain–dependent however requires further analysis. Lower bactericidal activities (~16) were observed using mature rNg-ACP and a few preparations with full-length rNg-ACP (e.g. Liposomes) nevertheless these titres are two or four times higher compared titres observed using NGO1801 and NGO2054 (**Table 58**). However, the progress of characterization of the biological role and structure of these potential antigents is ongoing compared with Ng-ACP to pursue an effective vaccine against *N.gonorrhoeae.*

Table 58. Newly gonocccal antigens recently characterised from proteomics and bioinformatic analysis as a potential gonococcal vaccine candidate.

Antigen	Function	Expression	Variability	Bactericidal activity (SBA titre)	Biological function	3D structure	Ref
α-BamA (NGO1801)	Outer membrane protein assembly factor Essential protein	Stable	Highly conserved	**FA1090** (32) **MS11** (512)			(Zielke et al., 2016)
NGO2054	Unknown subcellular location	Stable	Highly conserved	**FA1090** (16) **MS11** (512)			(Zielke et al., 2016)
MetQ (NGO2139) (homolog from *E. coli* and *N. meningitidis*)	Methionine transport adhesin, component of an ATP binding cassette ABC trasnport system.	Stable	Highly conserved	**FA1090** (24) **NGO1291** (320) **MS11** (512)	• Adherence human cervical cells. • Binding.		(Zielke et al., 2016, Semchenko et al., 2017)
Ng-ACP (NGO1981)	Adhesin Complex Protein	Unknown	Highly conserved	**FA1090** (128) **P9-17** (512)	• Adherence and Invasion on epithelial cells. • Binding.	Characterised by X-ray diffraction	

Table 59. Summary bactetricidal titres against homologous and heterologous different recombinant rNg-ACP proteins, outer-membrane (OMV) from N. gonorrhoeae P9-17 and sodium deoxycholate outer membrane (Na-Doc OMV) with a varity of adjuvants and delivery system.

	Full-length rNg-ACP				Mature rNg-ACP				OMV				Na-Doc OMV			
Strain	**NGOP9-17 WT**	**NGOP9-17Δ ACP**	**NGOFA1090WT**	**NGOFA1090Δ ACP**	**NGOP9-17 WT**	**NGOP9-17Δ ACP**	**NGOFA1090 WT**	**NGOFA1090Δ ACP**	**1µg NGOP9-17**	**10µg NGOP9-17**	**1µg FA1090**	**10µg FA1090**	**1µg NGOP9-17**	**10µg NGOP9-17**	**1µg FA1090**	**10µg FA1090**
Saline	256(256,1024)	16	128 (64,128)	4	256	4(4,128)	256	64	1024	4096	1024	2048	256, 512	256, 1024	256	256
Aluminium hydroxide	512(256,512)	64	256(256,512)	64	(16,64)	16(4,64)	16	<4	1024	2048	256	1024	256	256	256	256
Detergent	64	<4	64	≤4	64	4	64	<4								
Detergent +MPLA	256(256,512)	≤4	256(256,1024)	4	256(256,512)	4	256(256,512)	<4								
Liposomes	64	16(16,64)	64	<4	128-256	4(4,64)	64	<4(<4,128)								
Liposomes +MPLA	128(16,256)	32	64(16,128)	16	64	16	64	<4								

CHAPTER 6– CONCLUSION AND FUTURE EXPERIMENTS

In the pursuit of discovering novel antigens for developing a gonococcal vaccine, this study identified a conserved novel antigen expressed by 81% of the gonococcal strains characterised,which demonstrated high bactericidal activity against homologous and heterologous gonococcal strains with similar titres of other vaccine candidates characterised in the literature to date. Additionally in this study, murine antisera was raised with different rNg-ACP proteins and evaluated through a range of adjuvants and delivery systems. Similar bactericidal activity against wild-type homologous and heterologous strains was observed. However, the bactericidal titres where dependent on the effect of the native conformation of the protein and adjuvant used.

In this project it was discovered that Ng-ACP plays a role in mediating adherence on epithelial cells. Adhence assays demonstrated that there was a significant 50-75 % reduction in associated bacteria from a knockout mutant (*Δng-acp*) compared with the wild type strain. Moreover, Ng-ACP association on epithelial cells was shown from a preliminary binding assay using flow cytometry. Structural analysis of rNg-ACP revealed the same overall fold as found in *N. meningitidis*.

Recently, an adhesin complex protein has beeen characterised from a closely related bacteria *N. meningitidis*. Similarly, *N. gonorrhoeae* Ng-ACP acts as a lysozyme inhibitor with *N. gonorrhoeae* strain FA1090 (Humbert et al., 2017). Although, the mechanism of the binding interaction with lysozyme remains unknown.

In this study, it was not possible to identify the surface localisation Ng-ACP on *N. gonorrhoeae* by flow cytometry or to determine the reactivity against the whole cells or against the outer membrane, possibly due to the low presence of Ng-ACP in aerobic conditions. These results suggested that this antigen may be upregulated in the presence of iron (III) Fe^{3+} or under anaerobic conditions.

For further characterisation of the Ng-ACP antigen to determine whether it represents a good gonococcal vaccine candidate, the following studies should be performed.

Biological studies.

- **Further characterisation of types of alleles and levels of expression of Ng.ACP.**

Determine if all gonococcal strains have the same level of expression of Ng-ACP. This will be carried out by characterisation of allele type, RT-PCR and western blot analysis using a panel of antibiotic resistant *N. gonorrhoeae* strains from CDC (https://www.cdc.gov/drugresistance/resistance-bank/currently-available.html).

- **Determining the serum bactericidal activity of other gonococcal strains.**

Test bactericidal titres using a range of gonococcal multi-resistant strains from a panel from the CDC organisation, as well as samples collected from Professor Sanjay Ram (University of Massachusetts, Medical School, USA).

- **Measure Ng-ACP expression levels in the presence of Fe^{3+} or anaerobic conditions.**

Transcriptome studies have shown that gonococci survive under anaerobic conditions, suggesting some genes may play an important role during the course of infection. One of these genes is the *ng-acp* gene (NGO1981) (Isabella and Clark, 2011). Ng-ACP expression levels can be assessed with RT-PCR or western-blot analysis to investigate the differential expression of Ng-ACP in response to anaerobic conditions or presence of iron using different strains, in order to determine the stability of expression of this gene.

- **Characterise the binding receptor of Ng-ACP.**

  In this project it was demonstrated through flow cytometry binding assays that the full-length Ng-ACP protein binds on epithelial cells (Chang cells). However, the detailed mechanism of this binding interaction between Ng-ACP and epithelial cells deserves to be interrogated, which can be achieved through surface plasmon resonance studies.

- **Further characterisation of Ng-ACP adherence with alternative cell lines.**

  Adherence assays can be performed on human cervical epithelial cells with other characterised adhesins, such as OmpA or MetQ from *N. gonorrhoeae* (Serino et al., 2007, Semchenko et al., 2017). Human cervical carcinoma ME-180 and endometrial cells HEC-

1-B and human cervical epithelial cells (tCX) can be used to determine whether *N. gonorrhoeae* adheres and invades during colonisation of mucosal epithelial cells. Additionally, whether *N. gonorrhoeae* can survive inside these cell types as a means to evade the innate immune response.

- To demonstrate the reduction of Ng-ACP association on Chang cells or epithelial cells using GFP-expressing *N.gonorrhoeae* strain P9-17*Δng-acp* by confocal microscopy.

- **Animal model studies.**

- An animal model has been used in the literature to study the adaptation of *N. gonorrhoeae* in the urogenital mucosa. In this model, female BALB/c mice were treated with 17-β-estradiol and antibiotics for prolonged gonococcal infections. For single infections, groups of 7 to 8 mice were inoculated intravaginally with 10^6 CFUs of wild-type or mutant *N. gonorrhoeae*. The bacteria were typically recovered from vaginal swabs 12 to 13 days following inoculation (Jerse, 1999). This methodology has since been applied to test the efficacy of numerous potential vaccine targets, such as 2C7 lipooligosaccharide epitope with MPLA (Gulati et al., 2013), outer membrane vesicle OMV intranasally with QuilA adjuvant (Plante et al., 2000), and also with a recombinant refolded porin protein with virus replication particles subcutaneously (Zhu et al., 2011). In each study, a significant reduction in the colonisation load and/or duration of colonisation compared with unimmunised mice was observed (Wetzler et al., 2016). Therefore, the Ng-ACP protein could be used to further examine its potential role as a vaccine candidate using this animal model.
- Investigate whether murine or rabbit antisera raised against NgACP with human complement can inhibit or reduce the association of *N. gonorrhoeae* on epithelial cells. This assay can be performed through a bactericidal assay with human epithelial cells.

Structural studies.

- **Further characterisation of the binding interaction between human lysozyme and Ng-ACP and key epitopes with high serum bactericidal activity (SBA).**

In this project, structural biology was used to elucidate the three-dimensional structure of rNg-ACP and in a closely related bacteria *N. meningitidis* by using X-ray crystallography. Studies in the literature performed demonstrated the ability of a meningococcal protein to inhibit the action of lysozyme, as well as showing that mature rNg-ACP exhibited the same inhibition activity with the *N. gonorrhoeae* strain FA1090 (Humbert et al., 2017). Taken together, further studies with rNg-ACP need to be investigated.

- Generate the crystal of rNg-ACP human lysozyme (HL) complex and determine the possible interactions of the primary (amino acid sequence) and secondary structural motifs involved in the binding interaction. Alternatively, a different approach could use molecular docking to determine the residues involved in the binding interaction. The elucidation of these important epitopes will improve the knowledge on the the structural basis behind this binding interaction (Rinaudo et al., 2009). Consequently, it is anticipated that the characterisation of these epitopes with serum bacteridal activity against gonorrhoeae will lead to the generation of subunit, peptide or DNA epitope-based vaccines.

Appendices.

A.1 Preparation Luria Bertani (LB) broth and agar plates.

		Liquid	Plates
	Brand	**Amount (grams) per 1 Liter**	**Amount (grams) per 1 Liter**
Tryptone	Becton Dickinson	10	10
Yeast Extract	Becton Dickinson	5	5
Sodium chloride (NaCl)	Fisher	5	5
Bacteriological Agar	Oxoid	--	15

Reagents are dissolved in water and autoclaved at 2.68 Kg/cm^2 and 121˚C for 15 min.The solution is allowed to cool (50˚C) prior to the addition of the antibiotics.

A.2 Super optimum broth (SOB).

		Liquid
Reagent	**Brand**	**Amount (grams) per 1 Liter**
Tryptone	Becton Dickinson	20
Yeast Extract	Becton Dickinson	5
Sodium chloride (NaCl)	Fisher	0.5
Potasion Chloride (KCl)	Oxoid	0.186

Reagents are dissolved in water and autoclaved 2.68 Kg/cm^2 and 121˚C for 15 min. The solution is allowed to cool (50˚C) prior to the addition of the antibiotics.

A.3 Composition of Tris-EDTA (TE buffer 10X).

		Liquid
Reagent	**Brand**	**Amount (grams) per 1 Liter**
Tris-Hidrochloride	Fisher	30.3
Ethylenediaminetetraacetic acid (EDTA)	Fisher	144.0
Sodium dodecyl sulphate (SDS)	Fisher	0.010

Disolve each component in 800 ml of water, then adjust to pH 8.3 and make up with water to final volume 1 liter. For long term storage at 4˚C.

A.4 Composition ofpolyacrylamide gel (Mini-Protean).

		Separating gel	**Stacking gel**
Reagent	**Brand**	**Amount**	**Amount**
37.5:1Acrylamide:Bis-Acrylamide	Fisher	2 ml	300 µl
Separating or Stacking Buffer		1.9 ml	1 ml
UHQ water	--	1	600 µl
10% (w/v) Sodium dodecyl sulphate SDS	Fisher	50 µl	20 µl
10% (w/v)Amonium persulphate (APS)	--	20 µl	10 µl
TEMED	Fisher	5 µl	2 µl

Separating was prepared in a universal tube and poured into glass miniprotean (Biorad) and added 40 µl isopropanol, to obtain an uniform surface, and let to set up for 45 minutes. Then was extracted the remaining liquid onto the surface. Stacking mixture was prepared in a bijoux bottle respectively and poured in to the glass Miniprotean (biorad) and finally added on the top a plastic comb (0.75 mm) to allowed to form each sample well for 30 minutes. Once the gel was set , was stored in a humid paper and wrapped in cling film at 4˚C.

A.4.1Composition Separating buffer.

Reagent	**Brand**	**Amount**	**Amount**
1.5 M Tris-HCl	Fisher	18.5 g/100ml	pH 8.8

The reagent was dissolved in 75 ml of water and adjusted to pH 8.8 (with HCl 1M) then was make up to a final volume of 100 ml. The solution was stored at 4˚C.

A.4.2 Composition Stacking buffer.

Reagent	Brand	Amount	Amount
0.5 M Tris-HCl	Fisher	6.0 g/100ml	pH 6.8

The reagent was dissolved in 75 ml of water and adjusted to pH 6.8 (with HCl 1 M) then was make up to a final volume of 100 ml. Then, the solution was stored at 4˚C.

A.4.3 Composition Loading buffer (4X).

Reagent	Brand	Amount
Coomasie blue	Merck	4 mg
Glicerol	Fisher	4 ml
UHQ water	--	0.7 ml
SDS		0.8 mg
0.5M Tris/HCl pH6.8	--	4.8 ml

Mix every component to make a solution up to 9.5 ml, then aliquot 950 µl in Eppendorfs tubes and add to each tube 50 µl of β-mercaptoethanol.For storage was kept at -20˚C.

A.5 Composition Running buffer (10X).

Reagent	Brand	Amount
Tris-HCl	Fisher	30.3 g
Glycine	Fisher	144 g
SDS	Fisher	10 g

Dissolve all the reagents in 900 ml of distilled water, then when is dissolved all the reagents, adjust pH to 8.3 and add water to a final volume of 1L.the solution was stored at 4˚C.

A.6. Composition Staining Solution.

Ingredient	Brand	Amount
Acetic Acid (CH3COOH)	Fisher	10% (v/v)
Isopropanol	Fisher	20% (v/v)
PAGE-Blue-83	Merck	0.5 mg/ml

The component are dissolved in 500 ml of water with stirring until obtain a homogenous solution. Then the bottle was covered with foil to avoid any contact with direct light. The solution was stored at room temperature.

A.7. Composition Destaining solution.

Reagent	Brand	Amount
Acetic Acid (CH3COOH)	Fisher	10% (v/v)
Isopropanol	Fisher	10% (v/v)

The solution was prepared to a final volume of 1 L then , was kept at room temperature and placed in a dark area to avoid direct sunlight.

A.8. Recipe for GC- Proteose peptone agar.

Ingredient	Brand	Amount (grams) per 500 ml
Agar bacteriological	Oxoid	6.0
monopotassium phosphate (KH_2PO_4)	Biochemical	0.5
Potassium acid phosphate ($K_2HPO_4.3H_2O$)	Fisher	2.62
Proteose peptone	Becton Dickinson	5.0
Sodium Chloride	Fisher	2.50
Starch	Fisher	0.5

All components are dissolved in distilled water and autoclaved at 2.68 Kg/cm^2 at 121˚C for 15 min .Supplement A (4 ml) and B (1 ml) are added when the mixture is cool (~50˚C) after autoclaving.

A.8.1 Composition of GC-proteose peptone agar supplements.

A.8.1.1. Supplement A.

Component	Amount (mg/g) per 800 ml
Co-carboxylase	100 mg
Cyanocobalamin	10 mg
Ferric nitrate	20 mg
Glucose	100 g
L-glutamine	10 g
Para-amino-benzoic acid	13 mg
Thiamine hydrochloride	3 mg
β-nicotinamide adenine dinucleotide	250 mg

All the components are dissolved in distilled water, filtered and sterilised with 0.22 μm filter (Merk-Millipore) then aliquot in universal tubes (20 ml) and stored at -20 ˚C.

A.8.1.2. Supplement B.

Component	Amount (mg/g) per 200 ml
L-cysteine hydrochloride	26 g
Adenine	1 g
Guanidine hydrochloride	30 mg
Uracil	800 mg
Hypoxanthine	320 mg

The above compounds were dissolved in 100 ml of boiling hydrochloric acid (0.1 M), which was prepared with 800 μl of concentrated hydrochloric acid (37%, 11 M) in 100 ml. Cysteine is added when the solution is cool (45-50˚C) and the volume made up withdistilled water to 200 ml.Aliquots of 20 ml in universal tubes were stored at -20˚C.

A.9. Set of primers used to amplify different DNA sequence.

A.9.1 Amplification neisserial *n-acp* gene.

Primer	Sequence
Forward	5’-CGGGCTGAACCAGATAGACT-3’
Reverse	5’-GCTCCAGTTTGGTACGGAGA-3’

A.9.2 Amplification *ng-acp* gene from *Neisseria gonorrhoeae*.

Primer	Sequence
Forward	5'-GGCTAT**CTCGAG**ATGAAACTTCTGACCACTGC-3'
Reverse	5'-GGCTAT**AAGCTT**CTATTAACGTGGGGAACAGTCTT-3'

Sequences in bold represent the restriction sites for *XhoI* (**CTCGAG**) and *HindIII* (**AAGCTT**)

A.9.3 Primer used for sequencing *ng-acp*-pRSETA construct.

Primer	Sequence
Forward	5'-TAATACGACTCACTATAGGG-3'
Reverse	5'-GGCTAT**AAGCTT**CTATTAACGTGGGGAACAGTCTT-3'

A.9.4. Generation knockout construct.

A.9.4.1 Generation fragments included in knockout construct.

A.9.4.1.1 Generation fragment one (F1) in knockout construct.

Primer	Sequence
Forward	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse	5'- GGCTATTCTAGATTTTATTCCTTTGGATAGATG-3'

A.9.4.1.2 Generation fragment two (F2) included in knockout construct.

	Sequence
Forward Primer	5'-GGCTATTCTAGATCAGGCAACAAAAAACAGCG -3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC-3'

A.9.4.1.3 Generation kanamycin fragment (Kam) included in knockout construct.

	Sequence
Forward Primer	5'-GGTTCTAGATTCAGACGGCGTGATCTGATCCTTCAACTC-3'
Reverse Primer	5'- GGTTCTAGATTAGAAAAACTCATCGAGCATC-3'

A.9.4.2 Set of primers of first fragment knockout construct.

A.9.4.2.1.PCR primers used to amplify fragments up- and down-stream (F1 and F2) ligation product.

Primer	**Sequence**
Forward Primer F1	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer F2	5'-GGTACGGAGATTGTCGCCC -3'

A.9.4.2.2.Primers used in PCR colony screening of the transformation products.

	Sequence
Forward Primer	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC -3'

7.9.4.3 Set of primers of second and final fragment knockout construct.

A.9.4.3.1 Primers used in PCR colony screening of the final transformation product (F_1-Kam-F_2).

Primer	**Sequence**
Forward Primer	5'-TAGACTTCTGGGGCAAGGTC-3'
Reverse Primer	5'-GGTACGGAGATTGTCGCCC -3'

A.10. Pilot expression of *Neisseria gonorrhoeae* rNg-GapC (Glyceraldehyde-3-phosphate dehydrogenase).

In order to develop the technique for gonococcal recombinant protein, initially we focused on the rNg-GapC protein, a collaborative work with Dr. Paula Rodas (University of Andres Bello, Santiago, Chile) to produce the recombinant protein and prepare immunization in murine model using a wide range of adjuvant and delivery formulations.

The gonococcal *ng-GapC* gene was cloned into the pET24b cloning vector. The construct was tranformed into *E.coli* DH5α and selection of single colonies was done on LB medium with chloramphenicol (34 μg/ml) and kanamycin (50μg/ml). The appropiate construct (Ng-GapC-pET24b) was transformed into *E.coli* Bl21DE3pLysS to express the recombinant protein.

For pilot expresion of gonococcal rNg-GapC, *E. coli* BL21DE3pLysS –(pET24b-Ng-GapC) bacteria were grown overnight in 2-3 ml of SOB medium with kanamycin (50μg/ml) and chloramphenicol (34μg/ml). From this cell culture, 1 ml of the inoculum was added into two flasks with 25 ml of fresh medium,and then were incubated in a orbital shaker at 37˚C for 200 rpm (Gallenamp, UK) until appropiate optical density of 0.5-0.6 ($OD\lambda_{260nm}$) was reached. Then, one of the flasks was induced with 1mM IPTG and 1ml of cell culture of both conditions were taken every hour to estimate the amount of protein expressed for up to 5 hours of incubation. Each sample was centrifuged at 13000 rpm (15142*x*g) for 2 minutes to obtain the cell pellet (Heraus, Biofuge). All the samples were suspended in 100μl of PBS buffer and subsequently frozen (-80˚C) and thawed (37˚C) three times for periods of 30 minutes per step, to obtain a cell lysate. Each time point sample was evaluated by SDS-PAGE (Section 2.9).

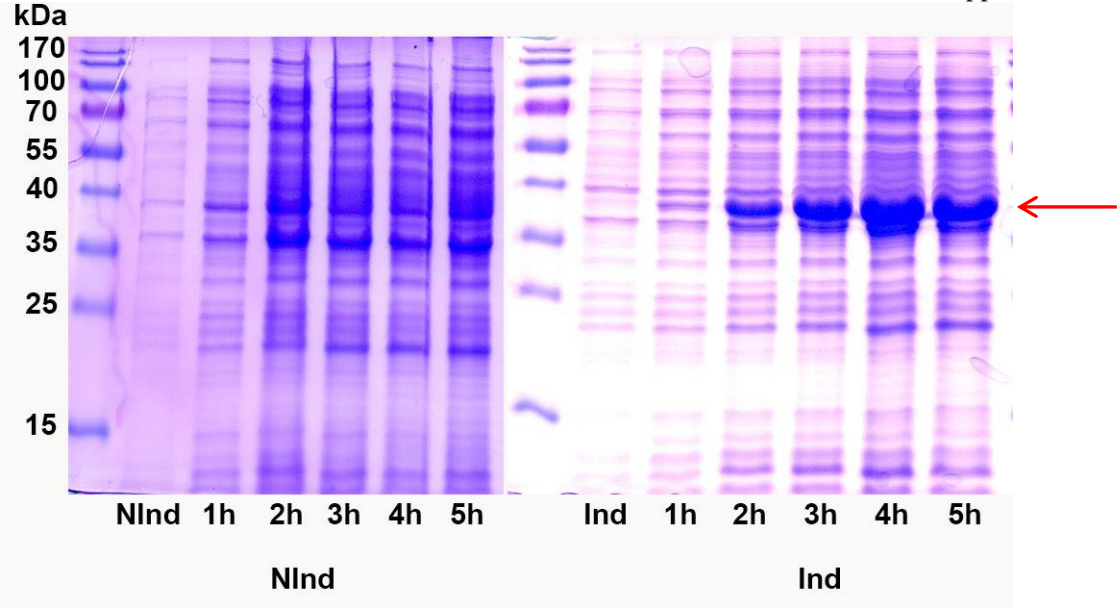


Figure 71. Pilot expression of gonococcal recombinant rNg-GapC protein, evaluating the effect of the inducer (IPTG) and optimal time of expression of the protein**.**

NInd(Non-Induced cell culture (-IPTG) **Ind**. Induced samples (+IPTG). **NInd 1h-5h** Time points from 1-5 hours of Non-induced samples (-IPTG). **Ind 1h-5h**. Time points from 1-5 hours, Induced samples (+IPTG).

This method allows a rapid screening of both protein expression levels and protein solubility. Initially, the optimal parameters of protein expression, i.e. time of maximal expression and effect of the inducer Isopropyl β-D-1-thiogalactopyranoside (IPTG) were determined.

A comparative effect between induced and non-induced sample showed that addition of the inducer IPTG enhanced the expression of rNg-GapC, which is reflected in the thickness and intensity of the band (*Mr*~40 kDa) **Figure 71**, right panel. In contrast, the samples obtained from non-induced cell culture showed an expression of protein, significally lower in concentration; known as basal expression (**Figure 71**, left panel). The time estimation of rNg-GapC protein expression displayed a gradual increase of the amount of protein at each time point. However, in the last two induced samples there were no significant changes of the amount of protein produced (hours 4 and 5).

A.10.1. Purification of rNg-GapC protein.

Purification of rNg-GapC was done using nickel-nitrilotriacetic resin (Ni-NTA) affinity chromatography under native conditions (Section 2.11.3.1). Protein solubility was confirmed by the presence of high amount of protein on supernatant extract compared with the cell debris. Freeze and thaw of the cell pellet and subsequent suspension of the cell pellet with Lysis buffer containing

a small amount of imidazole (20mM) was done, to avoid non-specific binding on the resin. Incubation of the cell lysate with the resin enabled the recombinant protein to bind specifically to the resin through affinity between 6XHis-tag and nickel reagent on the resin.

After incubation, the cell lysate (27 ml) was eluted from Ni-NTA resin. A washing buffer (54 ml) was added to the resin-bound protein, to remove the heterologous host proteins in the cell extract and protein with no affinity with the resin. An elution buffer containing high amount of imidazole (up to 250 mM) was added to the resin, to purify the protein **(Figure 72**).

Eluted fractions that contained the recombinant protein were combined into a dialysis tube and dialysed against PBS buffer to remove imidazole and leave the protein in native conditions.

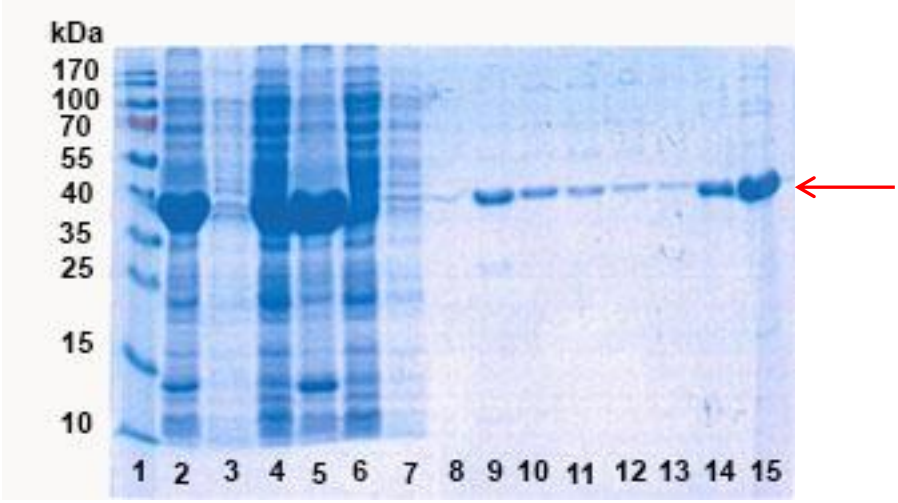


Figure 72. Evaluation of different fractions collected during induction, purification and dialysis of Ng- GapC from *Neisseria gonorrhoeae.*

Lane 1. Ladder **Lane 2.** Induced culture **Lane 3.** Cell debrix **Lane 4.** Supernantant **Lane 5.** Flowthough **Lane 6 and 7.** W1-W2 washing step **Lane 8- 13** E1-7 Eluted fractions **Lane 14** WD Washed dialysis **Lane 15** PBS protein in PBS in SDS 1.0%.

The figure above, shows the different stages from the cell lysate to purified protein using Ni-NTA Ni(II) nitrilotriacetic column. Over-expression of the recombinant protein was observedwhen the inducer IPTG was added (lane 2 **Figure 72)**. Host proteins was gradually removed by washing until the purified protein was obtained (lane 5-7 **Figure 72**). A single band with *Mr*~40 kDa, representing rNg-GapC is shown on lane 8 to 13. Dialysis against PBS to removed residual imidazole from the eluted protein fractions and protein was suspended on PBS with SDS 1.0% w/v,

displaying a high concentrated protein lane 14-15. This pilot expression and purification provided training in recombinant protein expression for the studies with rNg-ACP that followed.

A.11. Amplification and Purification fragment construct for Full-length rNg-ACP protein.

A.11.1. Generation of *ng-acp* gene and pRSET-A plasmid extraction.

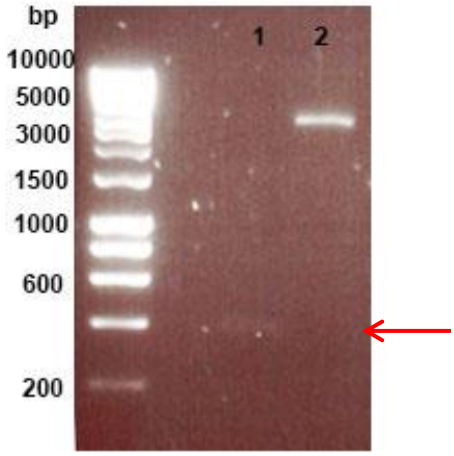


Figure 73. Qualitative estimation by 1%(w/v) agarose gel electrophoresis fragments construct (*ng-acp* –pRSETA)

Lane 1. Gonococcal *ng-acp* gene from *N. gonorrhoeae* strain P9-17. **Lane 2.** pRSETA cloning

A.11.2. Screening of transformants containing *ng-acp*-pRSET-A construct.

A.11.2.1 Screening transformants by PCR reaction.

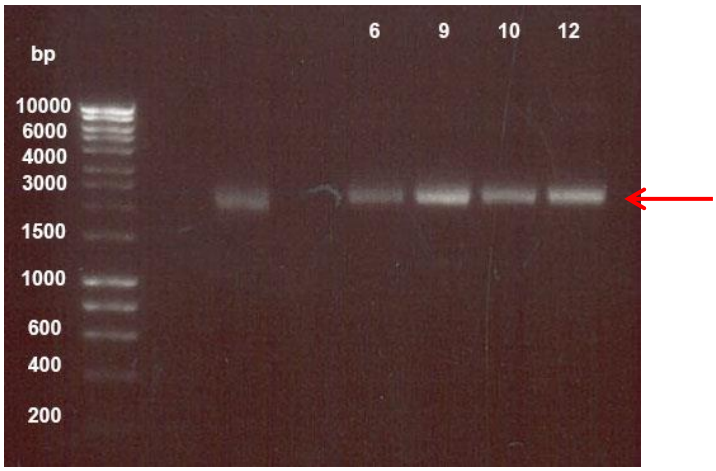


Figure 74. Purified plasmid extracted from selected colonies to sequencing.Lane 6,9,10,12 candidates 6,9,10,12 were tested for subsequent sequencing. The arrow displays a single band ~3200 bp.

A.11.2.2. Screening sequenced transformants by nucleotide sequencing alignment comparison.

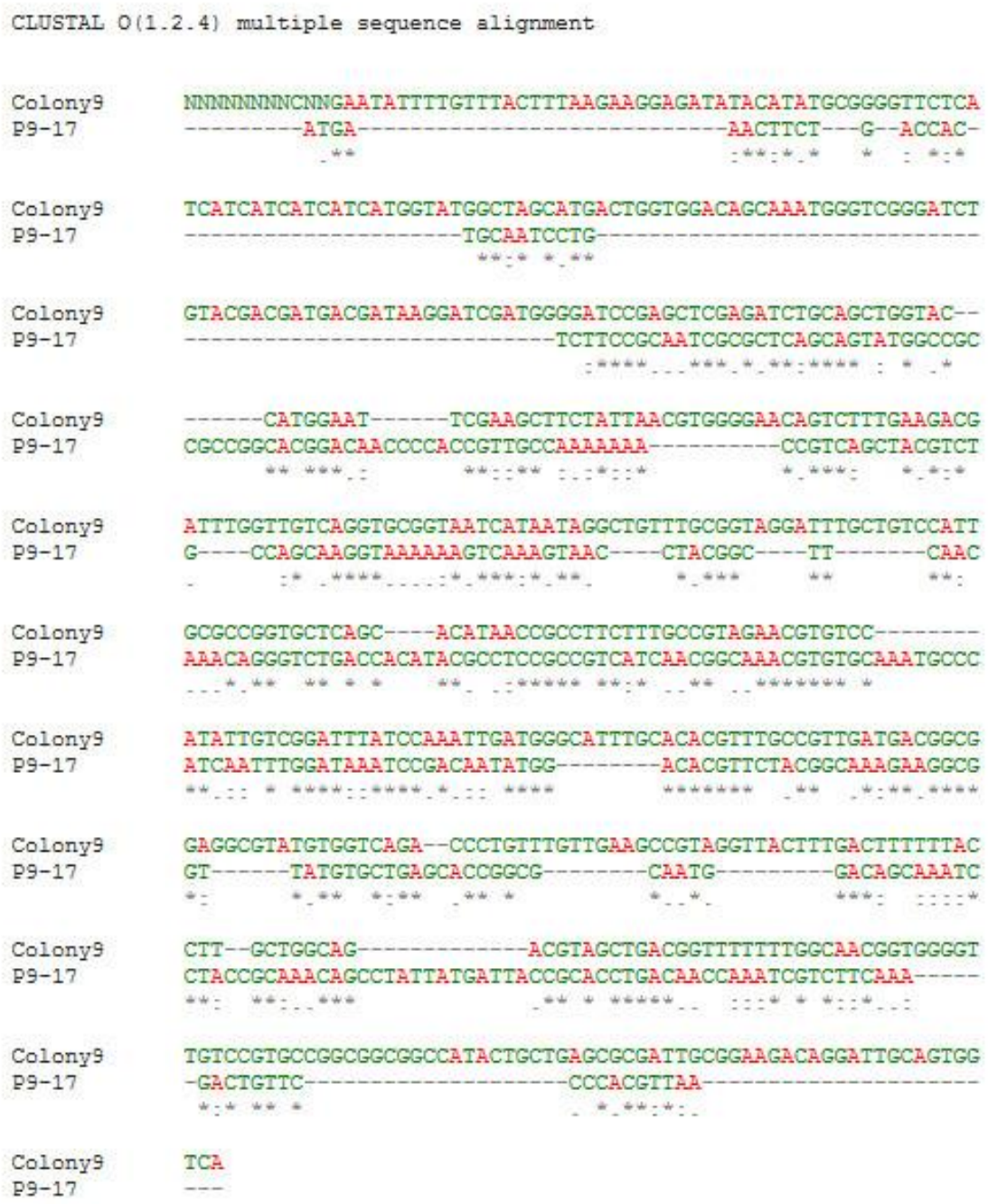


Figure 75. DNA sequences alignment candidate 9 (*ng-acp*-pRSET-A) compared with *N .gonorrhoeae* strain P9-17 *ng-acp* gene.

Figure 76. DNA sequences alignment candidate 10 (*ng-acp*-pRSET-A) compared with *N .gonorrhoeae* strain P9-17 *ng-acp* gene.

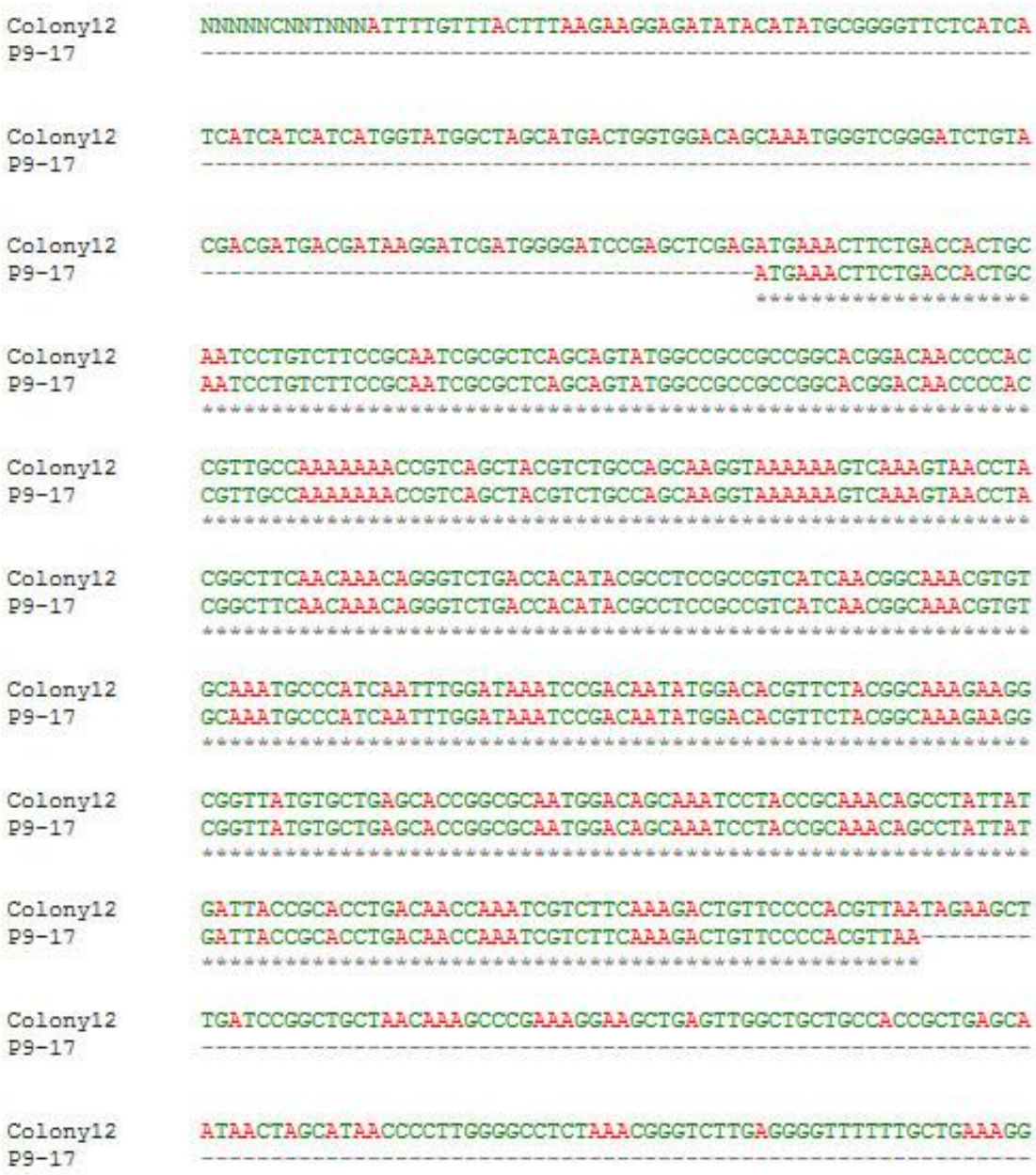
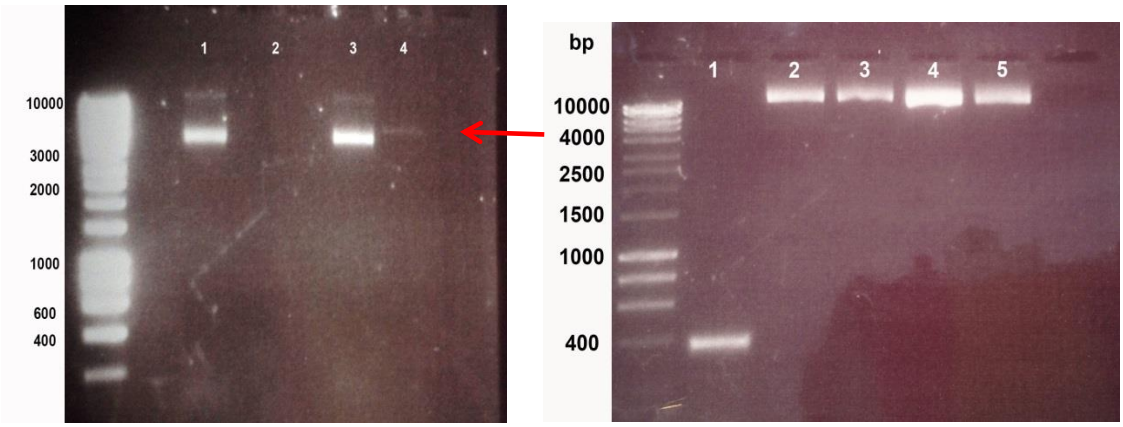


Figure 77. DNA sequence alignment between candidate 12 (*ng-acp*-pRSETA) and *N .gonorrhoeae* strain P9-17 *ng-acp* gene.

A.12. Generation of mature rNg-ACP protein pEt22b construct.

A.12.1 Construction of mature r-Ng-ACP protein on pET22b cloning vector.

Expression of the recombinant protein with high yield and without any refolding process was done for further studies of the three-dimensional structure of this antigen. The cloning strategy was changed to using the pET22b cloning vector, a bacterial expression vector containing a 6xHis-tag located at the C-terminal of the protein, with ampicillin antibiotic resistance, and a size of 5493 bp (**Figure 78**). The plasmid was obtained from a construct made in our research group and *nm-acp* gene was cloned into pET22 cloning vector.



A. **B.**

Figure 78. A. Double digestion of pET22b-*nm-acp* construct, for subcloning with gonococcal *ng-acp*. ***B.*** Fragments to generate construct *ng-acp*-pET22b.

lane 1. PCR product soluble *ng-acp*. **Lane 2-4** Plasmid extraction *nm-acp*-pET22b construct from *Neisseria meningitidis.*The arrow indicates the size of the plasmid ~**5493 bp.**

To make possible subcloning *ng-acp* gene into pET22b, the meningococci construct was digested, and there was showing a difference of mobility when the plasmid was digested (**Figure 78**). In the same way the fragment encoding *ng-acp* gene was double digested with NdeI and *XhoI* Section 2.7.5. Each fragment insert and vector was purified by PCR clean up (Section 2.6.2) and ligated with T4 ligase using the ratio 1:2 (vector:insert). The ligation product was used to transform *E. coli* strain DH5α described in Section 2.6.9, the selection of the colonies was evaluated by PCR. The positive candidates were sequenced sequences were aligned and analysed using Clustal omega. Candidates showing high homology with the gene target (**Figure 79** and **Figure 80**), were chosen for further experiments.

Figure 79. DNA sequence alignment between Colony 10 transformant and *N. gonorrhoeae* strain P9-17 *ng-acp* gene.

```
CLUSTAL O(1.2.4) multiple sequence alignment

colony11sNgACP      NNNNNNNNNNNNNNNNNNNNNNNCCNNCTAGANATAATTTTGTTTAACTTTAAGAAGGAGA
P9-17               ------------------------------------------------------------

colony11sNgACP      TATACATATGAAACTTCTGACCACTGCAATCCTGTCTTCCGCAATCGCGCTCAGCAGTAT
P9-17               -------ATGAAACTTCTGACCACTGCAATCCTGTCTTCCGCAATCGCGCTCAGCAGTAT
                           ************************************************************

colony11sNgACP      GGCCGCCGCCGGCACGGACAACCCCACCGTTGCCAAAAAAACCGTCAGCTACGTCTGCCA
P9-17               GGCCGCCGCCGGCACGGACAACCCCACCGTTGCCAAAAAAACCGTCAGCTACGTCTGCCA
                    ************************************************************

colony11sNgACP      GCAAGGTAAAAAAGTCAAAGTAACCTACGGCTTCAACAAACAGGGTCTGACCACATACGC
P9-17               GCAAGGTAAAAAAGTCAAAGTAACCTACGGCTTCAACAAACAGGGTCTGACCACATACGC
                    ************************************************************

colony11sNgACP      CTCCGCCGTCATCAACGGCAAACGTGTGCAAATGCCCATCAATTTGGATAAATCCGACAA
P9-17               CTCCGCCGTCATCAACGGCAAACGTGTGCAAATGCCCATCAATTTGGATAAATCCGACAA
                    ************************************************************

colony11sNgACP      TATGGACACGTTCTACGGCAAAGAAGGCGGTTATGTGCTGAGCACCGGCGCAATGGACAG
P9-17               TATGGACACGTTCTACGGCAAAGAAGGCGGTTATGTGCTGAGCACCGGCGCAATGGACAG
                    ************************************************************

colony11sNgACP      CAAATCCTACCGCAAACAGCCTATTATGATTACCGCACCTGACAACCAAATCGTCTTCAA
P9-17               CAAATCCTACCGCAAACAGCCTATTATGATTACCGCACCTGACAACCAAATCGTCTTCAA
                    ************************************************************

colony11sNgACP      AGACTGTTCCCCACGTCTCGAGCACCACCACCACCACCACCACTGAGATCCGGCTGCTAACAA
P9-17               AGACTGTTCCCCACGTTAA-------------------------------------------
                    ***************** :.

colony11sNgACP      AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCT
P9-17               ------------------------------------------------------------

colony11sNgACP      TGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATT
P9-17               ------------------------------------------------------------

colony11sNgACP      GGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGC
P9-17               ------------------------------------------------------------

colony11sNgACP      AGCGTGACCGCTACACTTTGCCANCNCCNTANCGTCCGCTCCTTTTCGCTTTTCTTCCCT
P9-17               ------------------------------------------------------------

colony11sNgACP      TCTWNNTCNTCACGTTNGCCGGCTTTTCCCGTCAAGATCTAAATCGGGGGCCTCCCTTTA
P9-17               ------------------------------------------------------------

colony11sNgACP      AGGTTTCCAATTTCTTGGCTTTCCCGCN
P9-17               ----------------------------
```

Figure 80. DNA sequence alignment between Colony 11 transformant and *N. gonorrhoeae* strain P9-17 *ng-acp* gene.

Transformation of *ng-acp*-pET22b into *E.coli* strain DE3pLysS using, followed by PCR evaluation, demonstrated that most of the candidates had incorporated the plasmid.

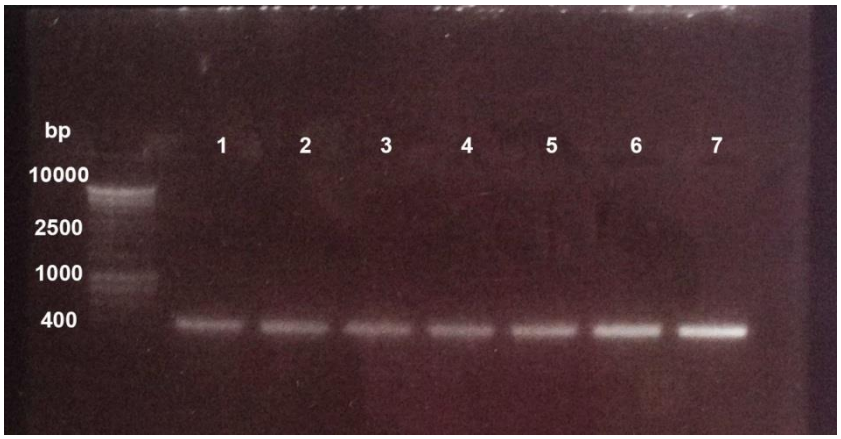


Figure 81. Colony PCR screening transformant in *E. coli* BL21pLysS containing construct (pET22b-matureNg-ACP).
Lane 1-7 transformats 1 to 7. On each sample shows a single band with size of 400bp.

A.12.2. Pilot expression and large–scale protein expression of mature rNg-ACP

To check the quality of expression of this exogenous protein in *E. coli* one of the trasnformants was chosen for a pilot expression. Analysis of each fraction diplayed that most of the protein was in the supernatant, indicating that the protein was soluble. In addition, analysis from previous protein preparations showed that maximum expression was reached at 4-5 hour of expression, due to increase the thickness and brightness of the band. Using the same conditions of purification under non-denaturing condition, showed a gradual removal containing host's proteins leading by the end of the elution process to a single band with *Mr*~13 kDa) **Figure 82.**

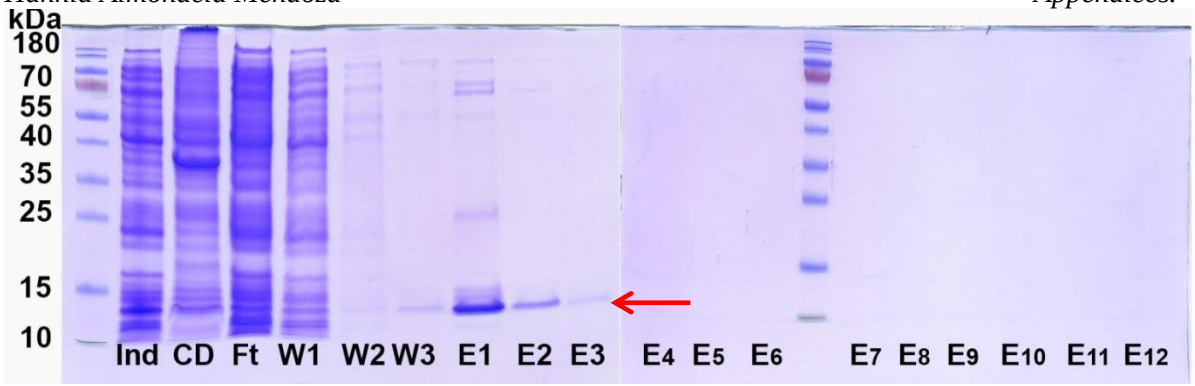


Figure 82. Pilot expression and purification of mature rNg-ACP protein.

Ind induced cell culture **CD** cell debrix **FT** flow-through **W1-3** wash 1 to 3 **E1-12** eluted fractions 1 to 12. The arrow indicates the band of *Mr*~12.5kDa in high concentration on the eluted fractions. The arrow indicates a band of *Mr* 12.5kDa.

A.13 Generation of knockout (*Δng-acp*) construct for *N. gonorrhoeae.*

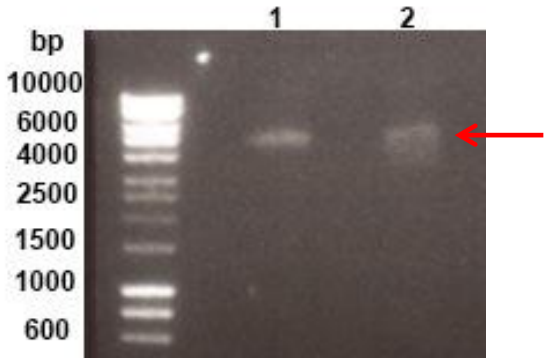


Figure 83. Extraction of plasmid pACYC177 (Kanamycin cassette) from different single colonies. **Lane 1.** colony **2' Lane 2.** colony 3'. The arrow show the size of the pACYC177 plasmid ~3941

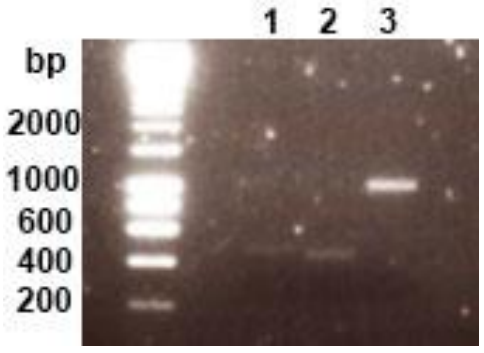


Figure 84. Purified samples of different fragments of knockout construct.

Lane 1. Fragment 1 gonococcal acp gene (10 µg). **Lane 2.** Fragment 2 gonococcal acp gene (10 µg) **Lane 3.** *Kam* cassette.

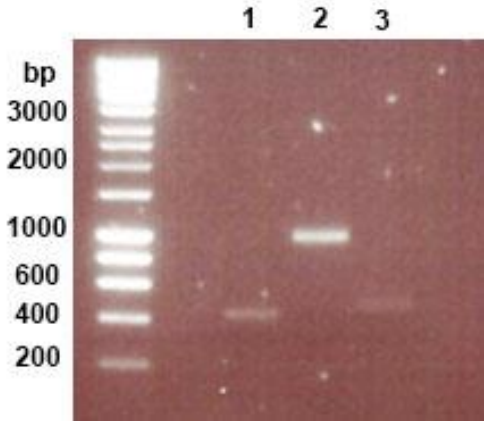
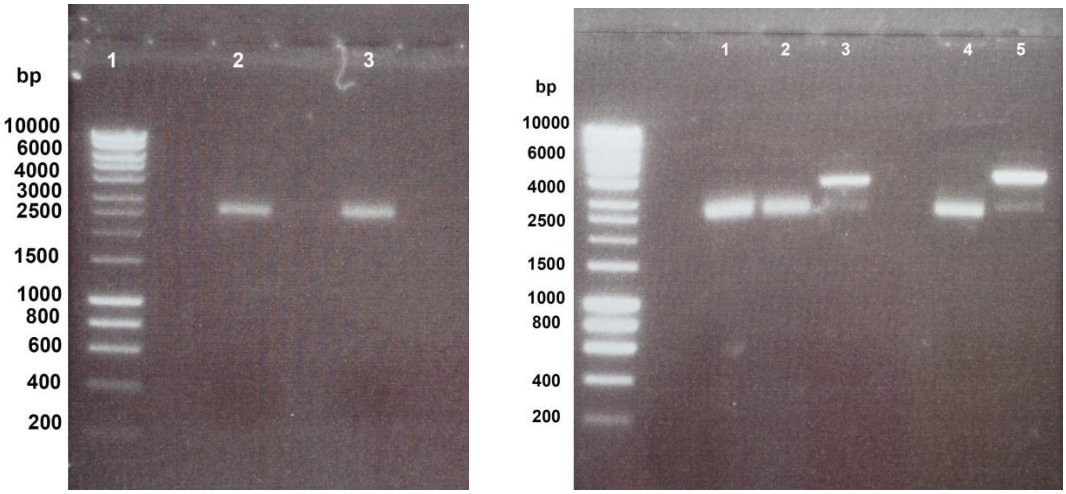


Figure 85. Purified PCR products of up-down stream fragments of *ng-acp* gene and antibiotic resistance kanamycin cassette (*Kam*)

Lane 1. Fragment 2 **Lane 2.** Kanamycin cassette **Lane 3.** Fragment 1



A. **B.**

Figure 86. Preparation construct pGEM-F1-F2 to insert antibiotic resistance cassette. **A**. plasmid extraction of selected candidates containing pGEM-F1-F2 construct .

Lane 1. Ladder **Lane 2.** Colony 8.3 **Lane 3.** Colony 8.8 .**B.** digestion plasmids with *XbaI* **Lane 1 and 2**. undigested colony 8.3 **Lane 3.** digested colony 8.3 **Lane 4.** undigested colony 8.8 **Lane 5.** Digested colony 8.8.

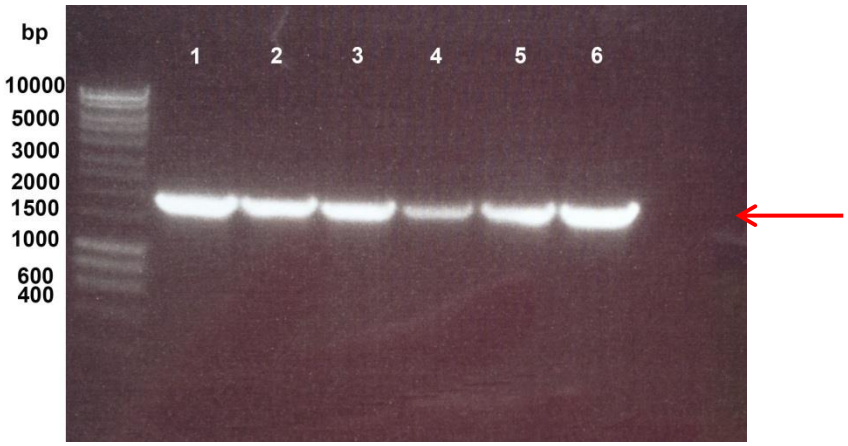


Figure 87. Colony PCR screening of candidates including knockout constructs (pGEM *F1-Kam-F2).*

Lane 1-6 *E. coli* transformants 1 to 6 including pGEM *F1-Kam-F2*construct. The arrow shows the amplified fragment 1800 bp.

A.14 Generation of gonococcal complemented (¢Δ*ng-acp*) construct.

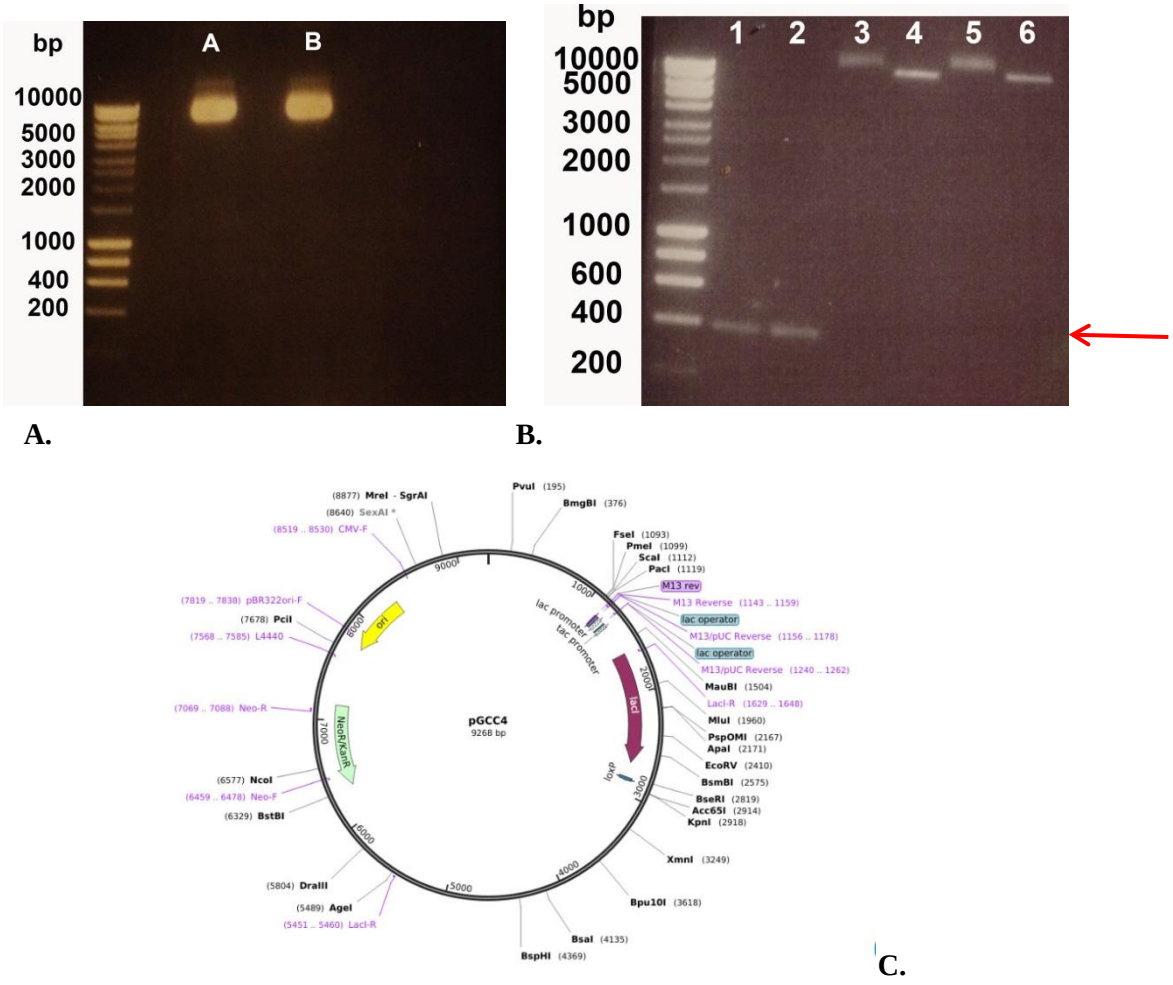


Figure 88. Generation complemented strains for *N. gonorrhoeae* .A. Plasmid extraction of pGCC4 for Neisseria complementation**.**

Lane A. colony 1 **Lane B.** colony 2**. B. Purified fragment of *ng-acp* gene and pGCC4 plasmid for complementation construct. Lane 1.** *N.gonorrhoeae* strain P9-17 *ng-acp* gene **lane2.** *N.gonorrhoeae* strain FA1090 *ng-acp* gene **lane 3.** undigested plasmid colony 1 **lane 4.** Digested plasmid colony 1 **lane 5.** Undigested plasmid colony 2 **lane 6.** Digested plasmid colony 2**. C.** map vector for complementation in *Neisseria gonorrhoeae*. The arrow denotes *ng-acp* gene with size of 400 bp.

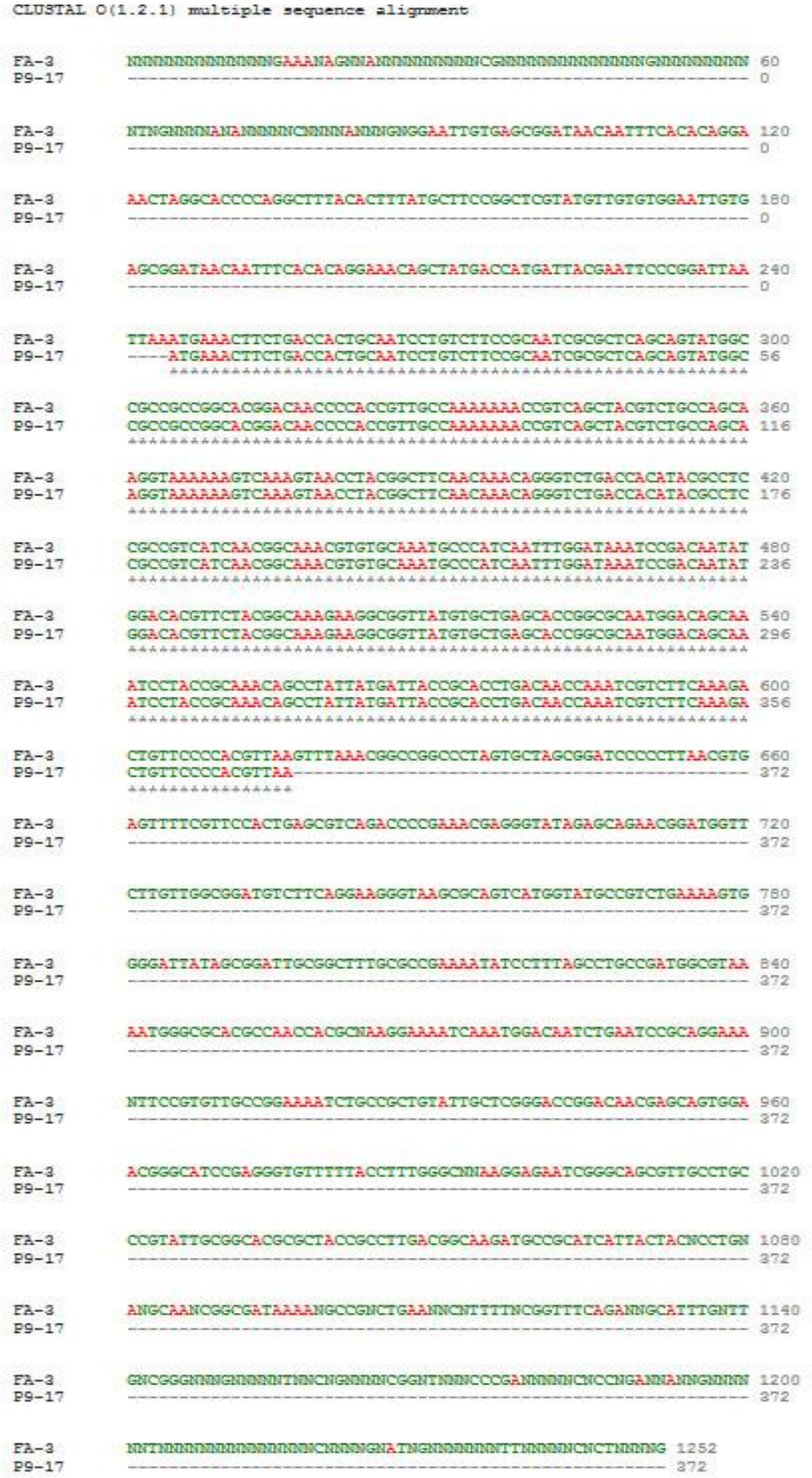


Figure 89. DNA sequence alignment between complemented transformant Colony 3 (FA-3) with *N. gonorrhoeae* strain FA1090 *ng-acp* gene.

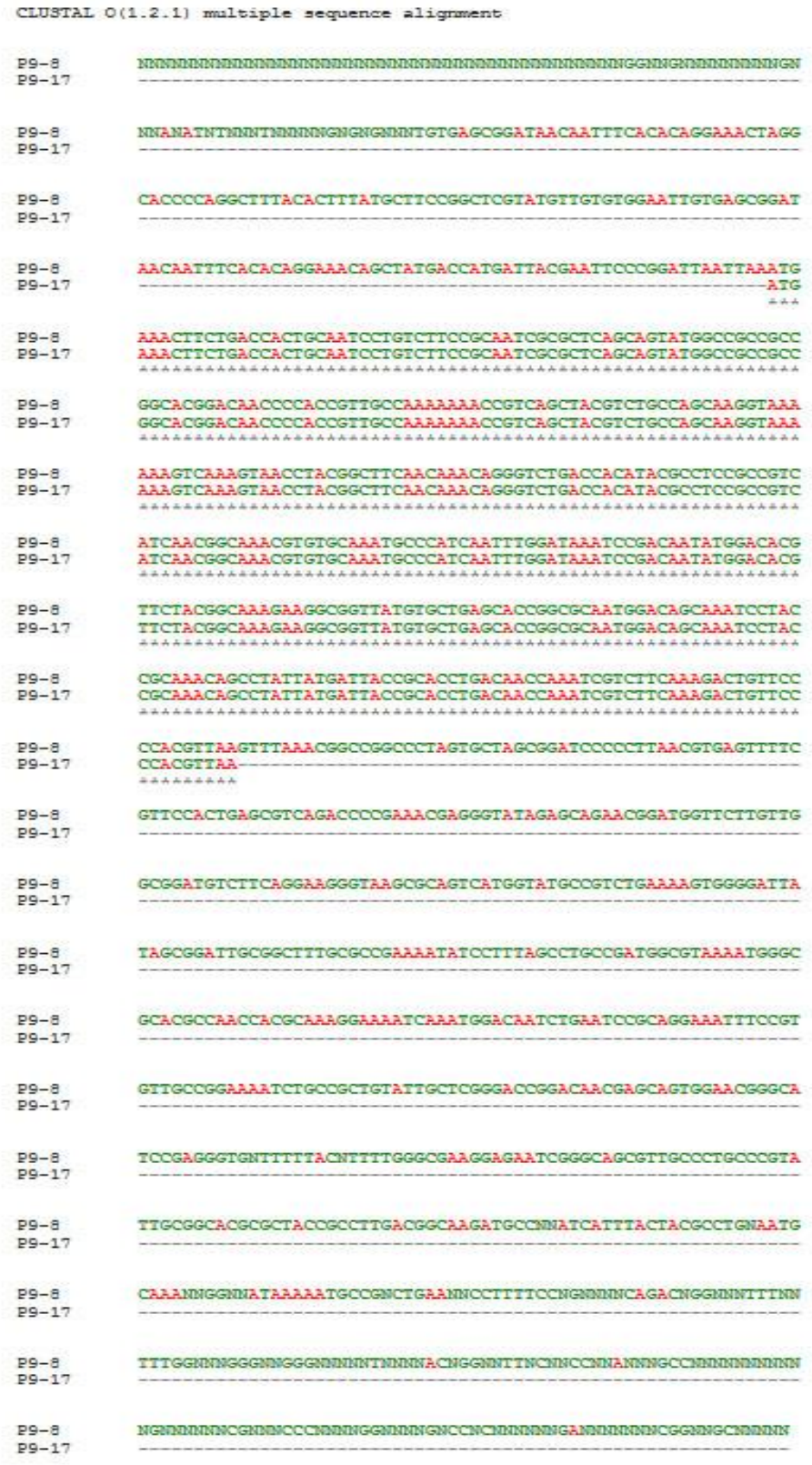


Figure 90. DNA sequence alignment between complemented transformant Colony 8 (P9-8) with *N. gonorrhoeae* P9-17 *ng-acp* gene.

A.15 Recognition of gonococcal variants with rabbit and murine sera to full-length rNg-ACP protein.

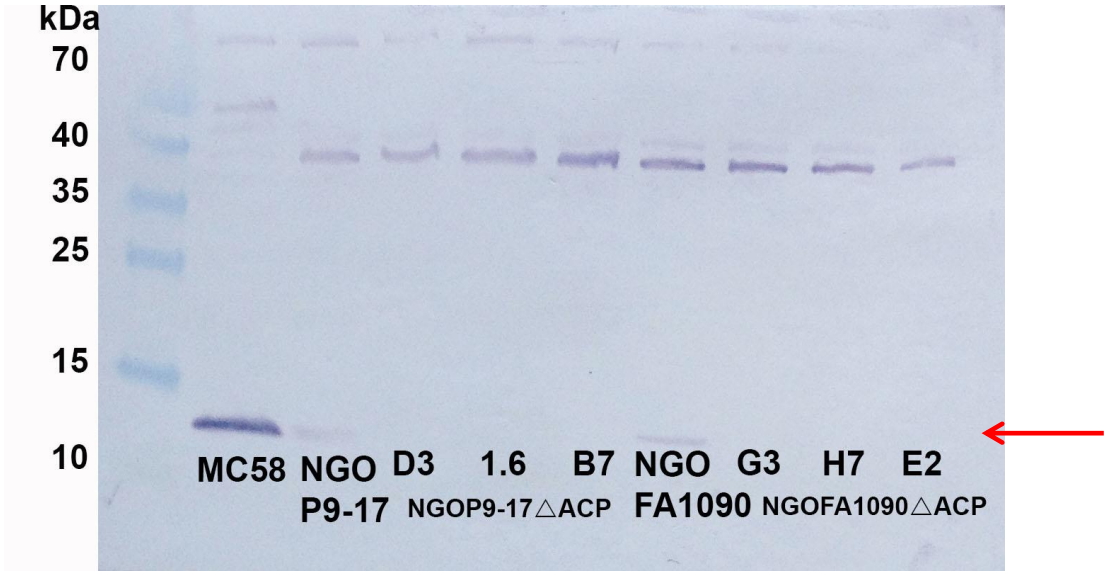


Figure 91. Recognition of gonoccal variants by rabbit antisera to meningococcal rNm-ACP (Rabbit 711) with Freund's adjuvant**.**

NGOP9-17 wild type *N. gonorrhoeae* strain P9-17..**NGOFA1090** wild type *N. gonorrhoeae* strain FA1090. **(D3, 1.6, B7**) knockout variants of *N. gonorrhoeae* strain P9-17 Δ*ng-acp.* (**G3,H7,E2**) knockout variants of *N. gonorrhoeae* strain FA1090 Δ*ng-acp.*The arrow denotes a band at *Mr* 12kDa.

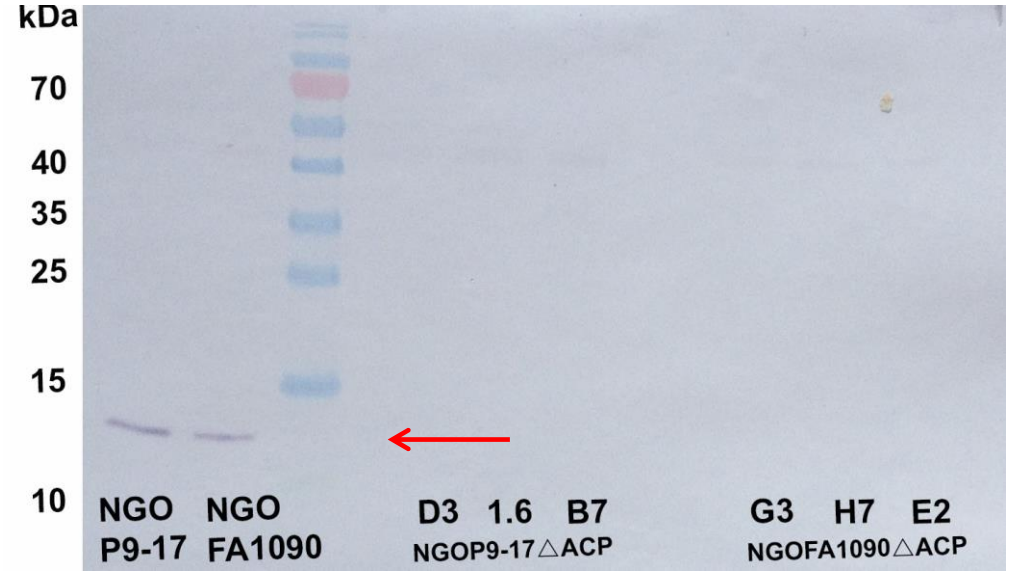
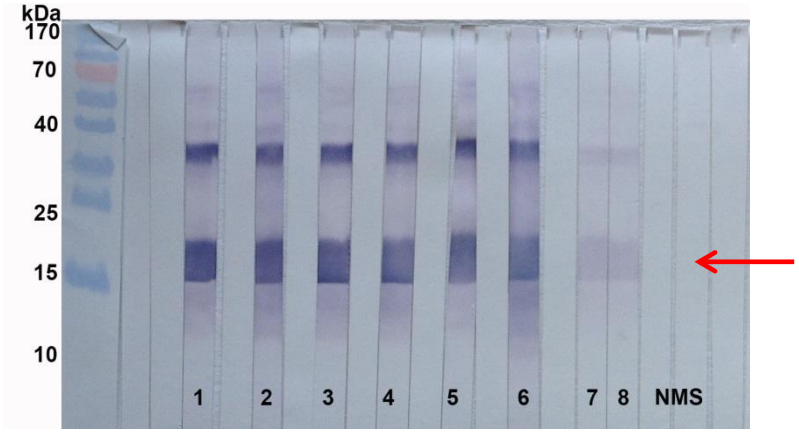


Figure 92. Recognition of gonococcal variants by mice anti-sera to full length rNg-ACP with aluminium hydroxide ($Al(OH)_3$) preparation.

NGOP9-17 wild type *N. gonorrhoeae* strain P9-17. **NGOFA1090** wild type *N. gonorrhoeae* strain FA1090. (**D3, 1.6, B7**) knockout variants of *N. gonorrhoeae* strain P9-17 Δ*ng-acp* (**G3,H7, E2**) knockout variants of *N. gonorrhoeae* strain FA1090 Δ*ng-acp*. The arrow denotes a band at *Mr*. 12.5kDa.

A.



B.

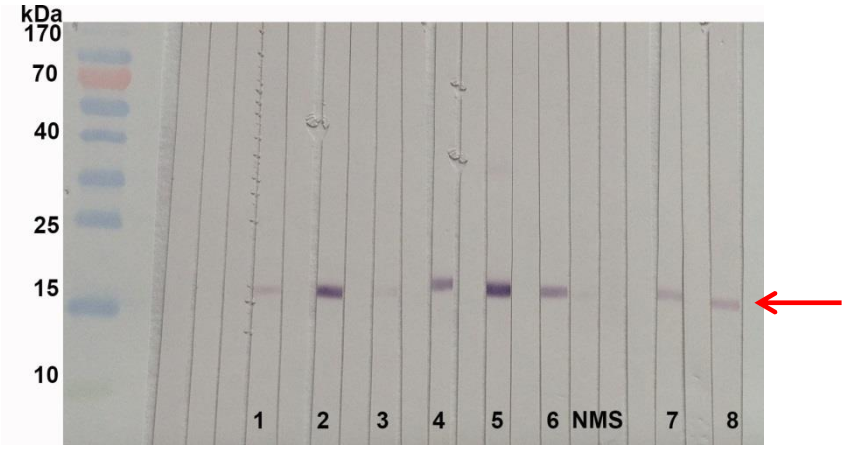
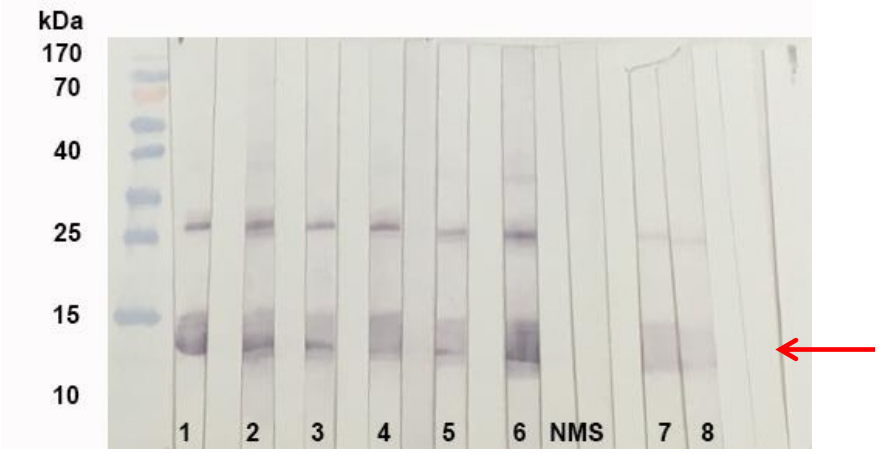


Figure 93. Recognition of the full-length rNg-ACP (70µg protein) using different sets of mice anti-sera **A.** Mice antisera to full-length rNg-ACP with different formulation and delivery systems. Mice and rabbit sera (1/100 dilution). **B.** Mice antisera to mature rNg-ACP with different formulation and delivery systems.

Mice (1:100 dilution) and rabbit sera (1/100 dilution). Antisera to rNg-ACP raised in saline (**Lane 1**), Al (**Lane 2**), ZW 3-14 (**Lane 3**), ZW 3-14 +MPLA (**Lane 4**), Liposomes (**Lane 5**), Liposomes+MPLA (**La** rabbit antiserum (**1/100 dilution**) raised to *Neisseria gonorrhoeae* rNg-ACP (Rabbit 1 **Lane 7**, Rabbit 2, **La** **NMS**, denotes reactivity of normal mouse serum, which was also indicative of the reactivity of sham-immu sera. Sham sera for each formulation is located on the left of each label. The arrows represent a molecular w *Mr*. 17.5kDa .

A.



B.

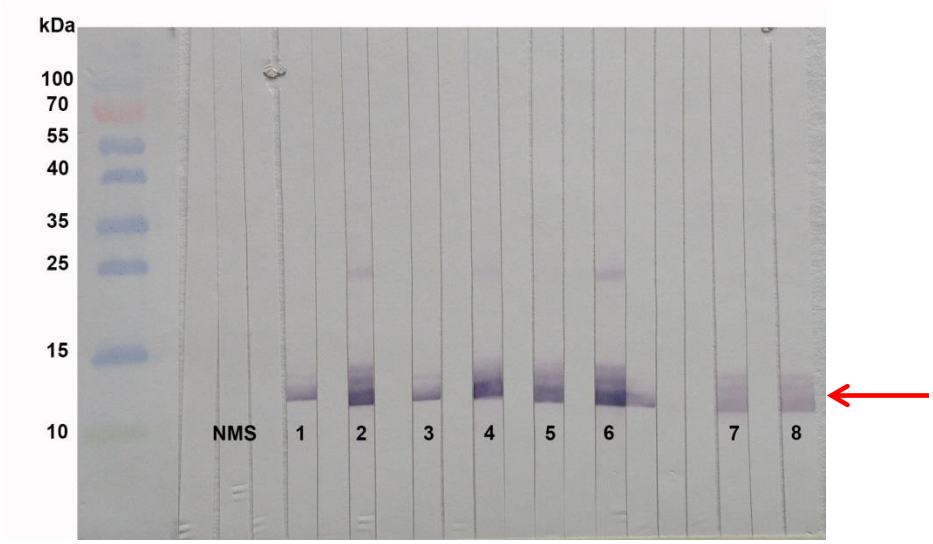


Figure 94. Recognition mature rNg-ACP using a set of mice anti-sera **A.** Mice antisera to full-length rNg-ACP with different formulation and delivery systems. Mice (1:100 dilution) Rabbit (1/100dilution). **B.** Mice antisera to mature rNg-ACP with different formulation and delivery systems.

Mice (1:100 dilution)Antisera to rNg-ACP raised in saline (**Lane 1**), $Al(OH)_3$ (**Lane 2**), ZW 3-14 (**Lane 3**), ZW 3-14 +MPLA (**Lane 4**), Liposomes (**Lane 5**), Liposomes+MPLA (**Lane 6**), rabbit antiserum (**1/100 dilution**) raised to *Neisseria gonorrhoeae* rNm-ACP (Rabbit 1 **Lane 7**, Rabbit 2, **Lane 8**). **NMS**, denotes reactivity of normal mouse serum, which was also indicative os the reactivity of sham-immunised sera. Sham sera for each formulation is located on the left of each label. The arrows denote a molecular weight Mr 17.5kDa and 12.5 kDa respectively.

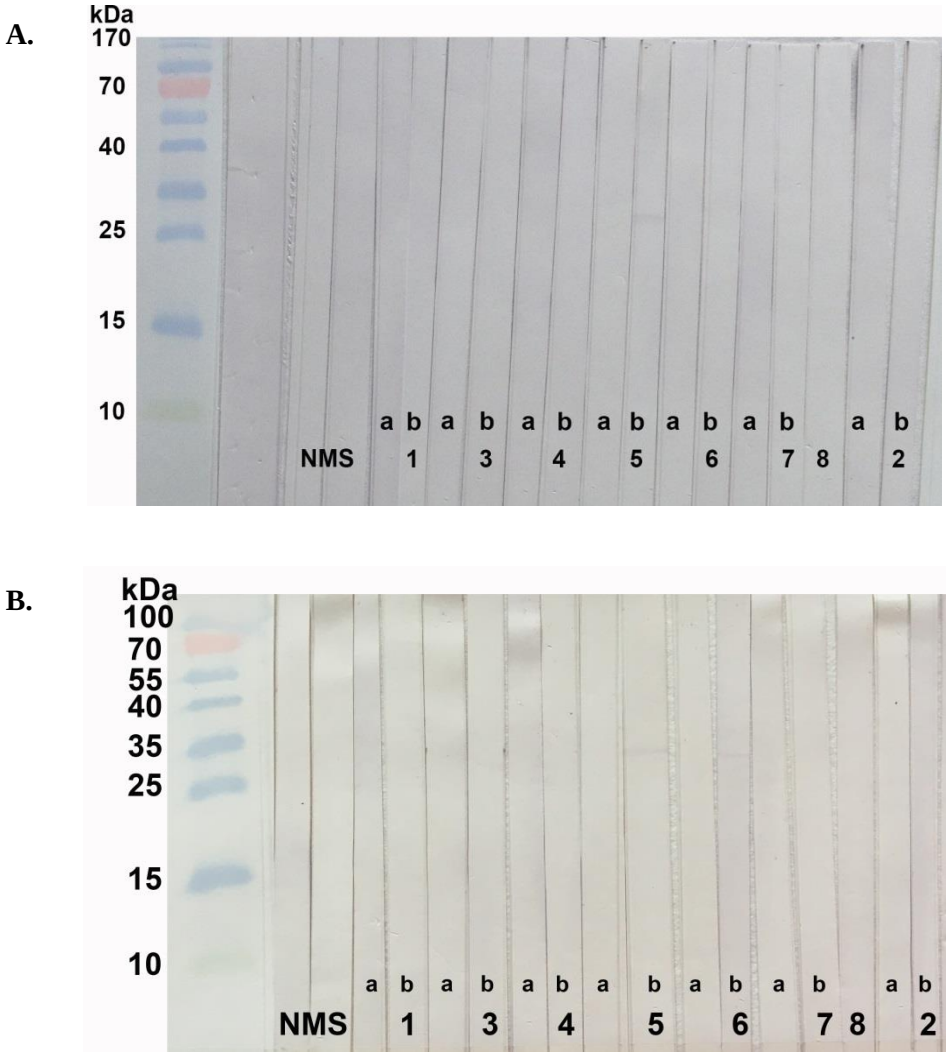
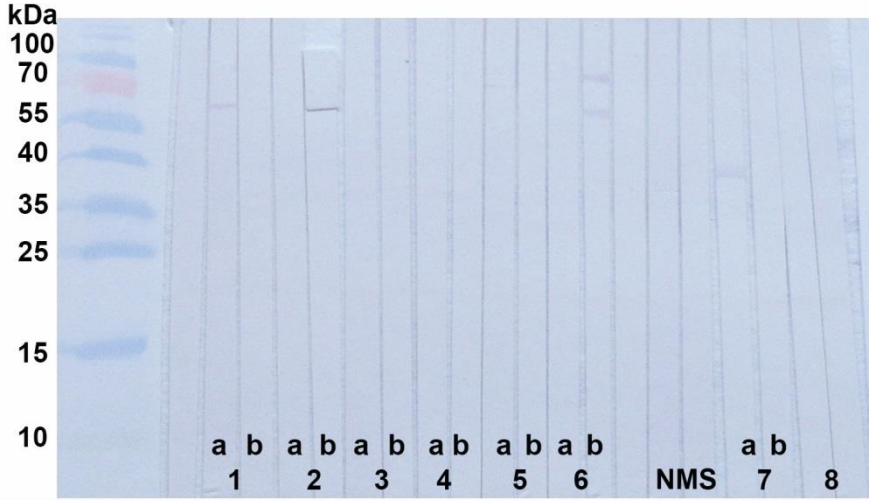


Figure 95. Recognition *N. gonorrhoeae* knockout variants lysate with the set of mice anti-sera full-length rNg-ACP with different formulations and delivery systems. Mice and rabbit sera (1/100 dilution) **A.** *N. gonorrhoeae* strain P9-17 Δ*ng-acp*. **B.** *N. gonorrhoeae* strain FA1090 *Δng-acp*.

Antisera to rNg-ACP raised in saline (**Lane 1**), $Al(OH)_3$ (**Lane 2**), ZW 3-14 (**Lane 3**), ZW 3-14 +MPLA (**Lane 4**), Liposomes (**Lane 5**), Liposomes+MPLA (**Lane 6**), rabbit antiserum (**1/100 dilution**) raised to *Neisseria gonorrhoeae* rNm-ACP (Rabbit 1 **Lane 7**, Rabbit 2, **Lane 8**). **NMS**, denotes reactivity of normal mouse serum, which was also indicative os the reactivity of sham-immunised sera. Sham sera for each formulation is located on the left of each label.

A.



B.

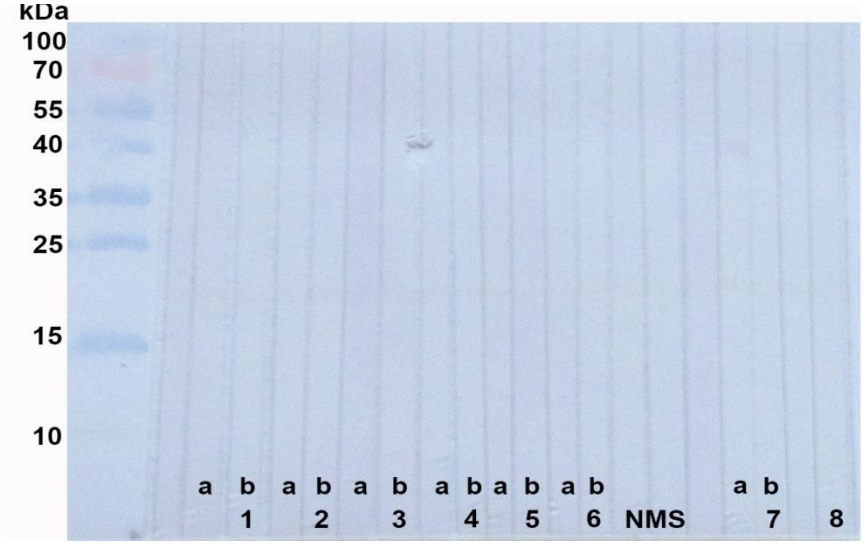


Figure 96. Recognition of *N. gonorrhoeae* knockout variants using murine antisera to mature rNg-ACP and rabbit antisera with full-length rNg-ACP. **A.** *N. gonorrhoeae* strain P9-17 Δ*ng-acp*. **B.** *N. gonorrhoeae* strain FA1090 Δ*ng-acp*.

Antisera to rNg-ACP raised in saline (**Lane 1**), $Al(OH)_3$ (**Lane 2**), ZW 3-14 (**Lane 3**), ZW 3-14 +MPLA (**Lane 4**), Liposomes (**Lane 5**), Liposomes+MPLA (**Lane 6**), rabbit antiserum (**1/100 dilution**) raised to *Neisseria gonorrhoeae* rNm-ACP (Rabbit 1 **Lane 7**, Rabbit 2, **Lane 8**). **NMS**, denotes reactivity of normal mouse serum, which was also indicative os the reactivity of sham-immunised sera. Sham sera for each formulation is located on the left of each label (letter a).

A.16. Reactivity murine antisera raised against OMV and Na-Doc-OMV against outermembranes from *N. Gonorrhoeae* strains and recombinant full-length and mature rNg-ACP.

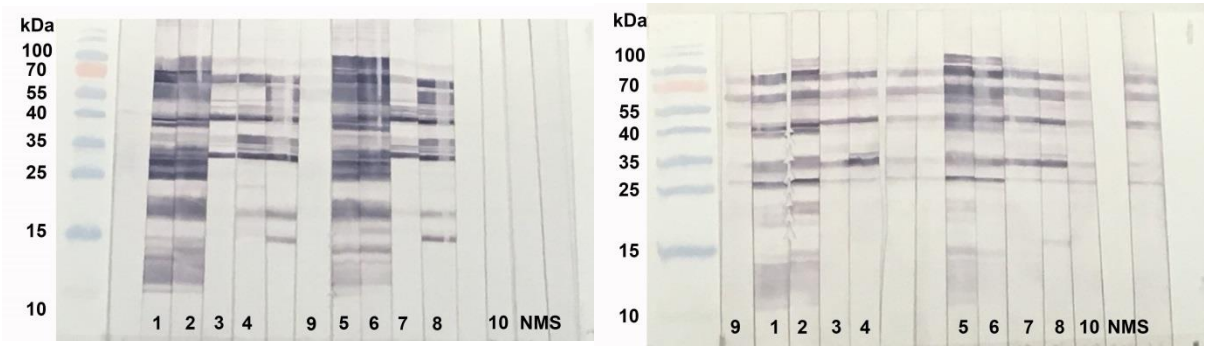


Figure 97. Recognition of *N. gonorrhoeae* outermembrane and recombinant proteins (full-length and mature rNg-ACP) using murine antisera to OMV and Na-Doc-OMV. **A.** outer membrane OMV from *N. gonorrhoeae* strain P9-17. **B.** outer membrane OMV *N. gonorrhoeae* strain FA1090.

Antisera to OMV 1 μg raised in saline (**Lane 1**), Antisera to OMV 10 μg raised in saline (**Lane 2**), Antisera to Na-Doc-OMV 1 μg raised in saline (**Lane 3**), Antisera to Na-Doc-OMV 10 μg raised in saline (**Lane 4**), Antisera to OMV 1 μg raised in $Al(OH)_3$ (**Lane 5**), Antisera to OMV 10 μg raised in $Al(OH)_3$ (**Lane 6**), Antisera to Na-Doc-OMV 1 μg raised in $Al(OH)_3$ (**Lane 7**), Antisera to Na-Doc-OMV 10 μg raised in $Al(OH)_3$ (**Lane 8**) Saline control (**Lane 9**) $Al(OH)_3$ control (**Lane 10**) (**1/100 dilution**). **NMS**, denotes reactivity of normal mouse serum, which was also indicative os the reactivity of sham-immunised sera.

A.17. Protein quantification of *N. gonorrhoeae* outer-membrane (OMV) preparations.

A.17.1 Serial dilution used with bovine serum albumin (BSA) as a standard curve of BCA assay.

Table 60. Serial dilution bovine serum albumin (BSA) (Range concentration 20-2000mg/ml), taken from Thermo Fisher BSA aasay.

Label	Volumen Diluent (µl)	Volumen BSA (µl)	Final Concentration BSA (µg/ml)
A	**0**	**300 (Stock)**	**2000**
B	**125**	**375 (Stock)**	**1500**
C	**325**	**325 (Stock)**	**1000**
D	**175**	**175 (B solution)**	**750**
E	**325**	**325 (C Solution)**	**500**
F	**325**	**325 (E Solution)**	**250**
G	**325**	**325 (F Solution)**	**125**
H	**400**	**100 (G Solution)**	**25**
I	**400**	**0**	**0**

A.17.2. Outer-membrane preparations from *N. gonorrhoeae* strain P9-17.

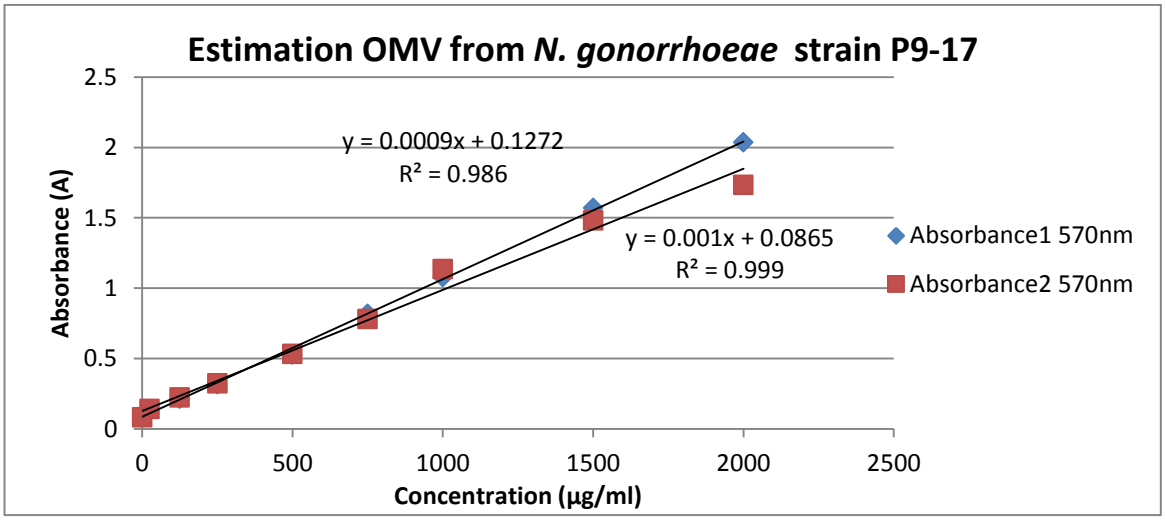


Figure 98. Calibration curve bovine serum for protein quantification of outer-membrane (OMV) from *N. gonorrhoeae* strain P9-17.

Table 61. Determination of protein concentration of outer-membrane from *N. gonorrhoeae* strain P9-17 using BSA as standard.

	Abs λ_{570}**nm**	**Concentration (µg/ml) x dilution factor**	**Concentration (µg/ml)**
		dilution 1/10	
Sample 3	1.322	1235.35	12353.5
Sample 4	1.062	975.35	9753.5
		dilution 1/5	
Sample 5	1.836	1749.35	8746.75
Sample 6	1.86	1773.35	8866.75

A.17.3. Outer-membrane preparations from *N. gonorrhoeae* strain FA1090.

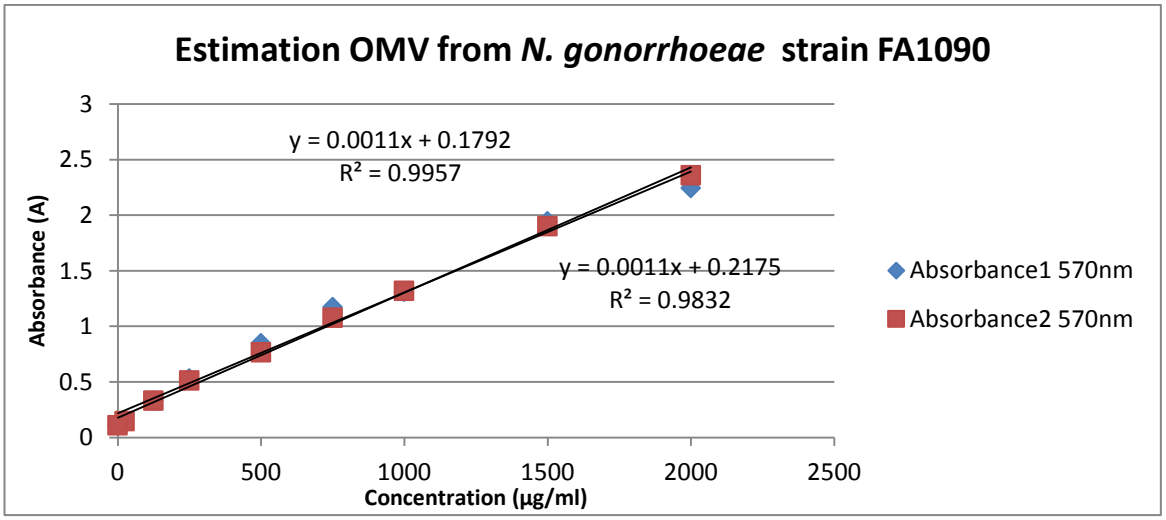


Figure 99. Standard curve of protein quantification of outer-membrane from *N.gonorrhoeae* strain FA1090.

Table 62. Determination of protein concentration of outer-membrane from *N. gonorrrhoeae* strain FA1090 using BSA as standard.

	Abs λ_{570}nm	**Concentration (µg/ml) x dilution factor**	**Concentration (µg/ml)**
		dilution 1/10	
Sample 3	0.6565	433.90909	4339.0909
Sample 4	0.7095	482.09091	4820.9091
		dilution 1/5	
Sample 5	1.346	1060.7273	5303.6364
Sample 6	1.798	1471.6364	7358.1818

A.17.4. Sodium-deoxycholate extracted Outer-membrane preparation from *N. gonorrhoeae* strain P9-17.

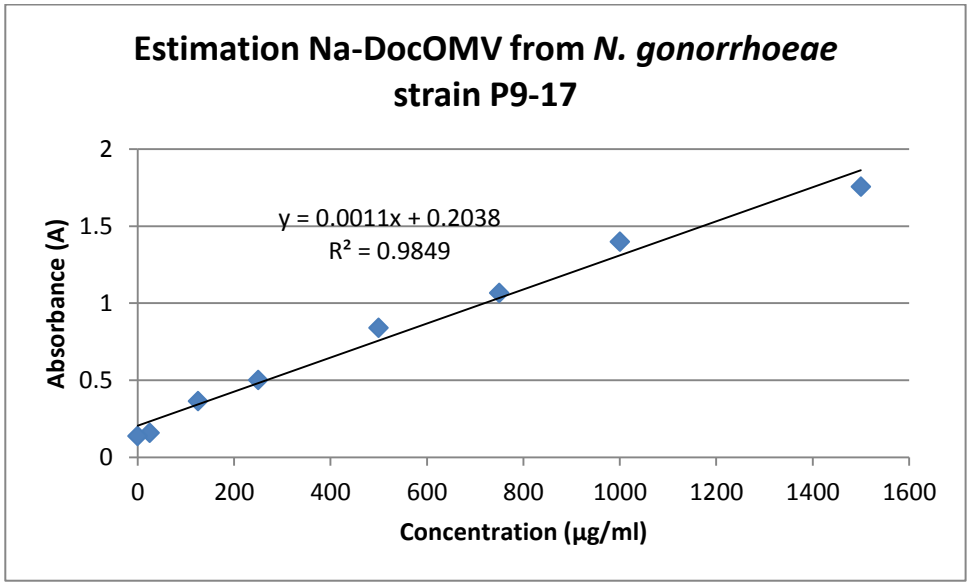


Figure 100. Standard curve of protein quantification of detergent extracted outer-membrane from *N. gonorrhoeae* P9-17.

Table 63. Determination of protein concentration of detergent extracted outer-membrane from *N. gonorrrhoeae* strain P9-17 using BSA standard curve at different dilutions.

	Abs λ_{570}**nm**	**Concentration (µg/ml) x dilution factor**	**Concentration (µg/ml)**
		dilution 1/5	
Sample 1	0.56	323.8182	1619.091
Sample 2	0.568	331.0909	1655.455

CHAPTER 7– REFERENCES

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