Investigation of glycosylation in flagellin biosynthesis and LPS O-antigen biosynthesis in *Aeromonas caviae*

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Summary

Aeromonas species are Gram-negative facultative anaerobic bacteria which are widespread in fresh water and salt water. *Aeromonas caviae* and *Aeromonas hydrophila* are two kinds of mesophilic aeromonads which belong to genus *Aeromonas* and are emerging as major pathogens in humans. Flagella are important pathogenic factors of bacteria and there are two kinds of flagella discovered in *A. caviae* and *A. hydrophila* which are polar flagella and lateral flagella (Rabaan et al. 2001; Tabei et al. 2009; Canals et al. 2006a).

The flagella of *Aeromonas* are glycosylated and investigation of glycosylation in flagellin biosynthesis is valuable for the understanding of pathogenicity of *Aeromonas* species. The aim of this project is to investigate the glycosylation during flagellin biosynthesis in *A. caviae*. It has been discovered that the flagella of *A. caviae* are glycosylated 6 to 8 times by Pse5Ac7Ac which is under the control of Maf1 which is known as flagellin glycosyl-transferase (Parker et al. 2012). Theoretically, Maf1 of *A. caviae* Sch3N has the ability to transfer activated pseudaminic acid (CMP-Pse5Ac7Ac) to the hydroxyl group of serine and threonine residues in the central immunogenic D2/D3 domain of flagellin of *A. caviae* Sch3N (Parker et al. 2012; Tabei et al. 2009). The details of how Maf1 interacts with flagellin will be explored in this project.

CMP-Pse5Ac7Ac which is the substrate for glycosylation is generated from UDP-GlcNAc. The biosynthesis of CMP-Pse5Ac7Ac is under the control of *flm* locus including *flmA*, *flmB*, *neuA*, *neuB*, *flmD.* The pseudaminic acid biosynthetic pathway has been confirmed in *Campylobacter jejuni* and related enzymes are homologous proteins of *A. caviae* (Schoenhofen et al. 2006). The proteins encoded by the *flm* genes will be applied in this project to investigate the biosynthesis of CMP-Pse5Ac7Ac. In addition, there is evidence that the LPS O-antigen incorporates with Pse5Ac7Ac. The substrate of O-antigen glycosylation is also CMP-Pse5Ac7Ac (Tomás 2012). The connections between glycosylation in LPS O-antigen biosynthesis and flagellin biosynthesis will be investigated in this project.

Acknowledgment

I am thankful to those who have contributed in assisting me towards the completion of my research project. First of all, I would like to thank my supervisor, Dr. Jonathan G. Shaw, for his dedication in supporting me with this research. All technical advice and valuable feedback from Dr. Jonathan G. Shaw were crucial for the completion of this project and thesis writing. Besides, I would also like to thank Dr. Graham Stafford for his suggestions and advice during my academic career.

Joseph MF Ferner who is my co-worker from Chemistry Department has contributed to my Project and I can not finish my project without his efforts. I would like to preciate the experiments we have done together and the techniques and knowledge we learned from each other. I also want to thank my collegues in the department of IICD. Dr. Yuhang Zhao, Dr. Jennifer L. Parker, Dr. Rebecca C. Lowry, Dr. Sabela Balboa Mendez, Dr. Jamie Hall and, Mr Aaron Savage, Dr. Ruyue Sun and Mr Ben Harvey are all thanked for their help and support in LU 103 and other places. I would also like to thank the PGR group officers and technicians in the department for the autoclaving and materials preparation.

Besides, I also want to thank my parents for their financial support and concern which always support me to finish my research project. Their understanding and support of my study and research career are all essential for my overseas study for 5 years. My cousin Lijie Wang who take care of me with my daily life in the UK would also be appreciated a lot. Thank you all for the time we spent and encouragement that keep me carry on with my study.

At last, I would thank everyone mentioned above again for all the help I received. All the support and help have encouraged me to finish my degrees in the UK.

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Abbreviations

℃: degrees Celsius

µg: micrograms

ACL: sugar-antigen carrier lipid

Amp: ampicillin

APS: ammonium persulphate

BLAST: basic local alignment search tool

bp: base pairs

CBD: chaperone-binding domain

Cm: chloramphenicol

CMP: cytidine monophosphate

CTP: cytidine triphosphate

cm :centimetre

CoA : coenzymeA

D0, D1, D2, D3: domain0, domain1, domain2, domain3 (flagellin)

Da: daltons

dH_2O: sterile MilliQ water

DNA: deoxyribonucleic acid

dNTPs: deoxynucleotide triphosphates

EDTA: ethylenediamine tetraacetic acid

g: grams

GlcNAc: *N*-Acetylglucosamine

HAPs: hook-associated proteins

kb: kilobase

kDa: kilo Daltons

Km: kanamycin

L: litre

LPS: lipopolysaccharide

M: molar

Maf: motility accessory factor

mg: milligrams

ml: millilitre

mM: millimolar

NAc: acetylamino

nm: nanometer

OD: optical density

p(value): probability (value)

PBS: phosphate buffered saline

PEP: phosphoenolpyruvic acid

PPi: pyrophosphate

^{R}: resistant

Sm: streptomycin

STT3: catalytically active subunit of the oligosaccharyltransferase

T3SS: type III secretion system

TEMED: N,N,N,N-tetramethylethylenediamine

Tris: Tris (hydroxymethyl) aminomethane

O-Ag: O antigen

OS: oligosaccharide

OT: oligosaccharyltransferase

UDP: uridine diphosphate

Und-P: undecaprenyl phosphate

UV: ultraviolet

V: volts

xg: times gravity

Chapter 1 Introduction

1.1 *Aeromonas* species

Aeromonas species are Gram-negative facultative anaerobic bacteria which are widespread in fresh and salt water. The genus *Aeromonas* belong to the family of *Aeromonadaceae*. Furthermore，this family also includes the genera *Oceanimonas* and *Tolumonas* (Martin-Carnahan and Joseph 2005).

The understanding of members of the genus *Aeromonas* has been developing for many decades. In 1980, there were only four *Aeromonas* species known (*Aeromonas hydrophila*, *Aeromonas punctata*, *Aeromonas salmonicida*, and *Aeromonas sobria*). As new species have been described, there are now 24 species of *Aeromonas* that are accepted nowadays (Janda and Abbott 2010). At the very beginning, the genus *Aeromonas* were considered as pathogens causing systemic illnesses in poikilothermic animals, but it is acknowledged today that the genus *Aeromonas* is not only responsible for diseases in cold-blooded species but also responsible for various infectious complications in both the immunocompetent and immunocompromised person (Janda and Abbott 2010). It has been discovered that the genus *Aeromonas* can cause primary and secondary septicemia in immunocompromised persons, serious wound infections in healthy individuals and other diseases including peritonitis, meningitis, infections of the joints, bones and eyes and even gastroenteritis (Janda and Abbott 1998).

Generally the genus *Aeromonas* was divided into two principal subgroups based on optimal growth temperature and motility: the mesophilic and motile species including *A. hydrophila*, *A. caviae*, and *A. sobria* as well as psychrophilic and immotile species such as *A. salmonicida* (Krieg and Holt 1984). The mesophilic aeromonads are emerging as major pathogens in humans. Approximately 85% of the aeromonad-related diseases are caused by three mesophilic aeromonads: *A. hydrophila*, *A. caviae*, and *A. sobria* (Janda 1991).

There are many pathogenic factors of *Aeromonas* including surface polysaccharides such as capsule, lipopolysaccharide (LPS), iron-binding systems, exotoxins, S-layers, secretion systems, extracellular enzymes, fimbriae and flagella (Tomás 2012). Furthermore, there are some proteins in *Aeromonas* species that are

glycosylated and glycosylation plays an important role in the survival of both eukaryotes and prokaryotes.

1.1.1 Flagella and flagellins

The prokaryote flagellum is made up of an external part which includes the filament and the hook, a basal body which is the internal part embedded in the cell membrane (Tomás 2012) (Figure 1B). The flagellar filament is formed by flagellins which vary from species to species and filament cap is formed at the end of filament. The assembly of the flagellum starts from the basal body, followed by the hook-associated proteins and the filament (Rabaan et al. 2001).

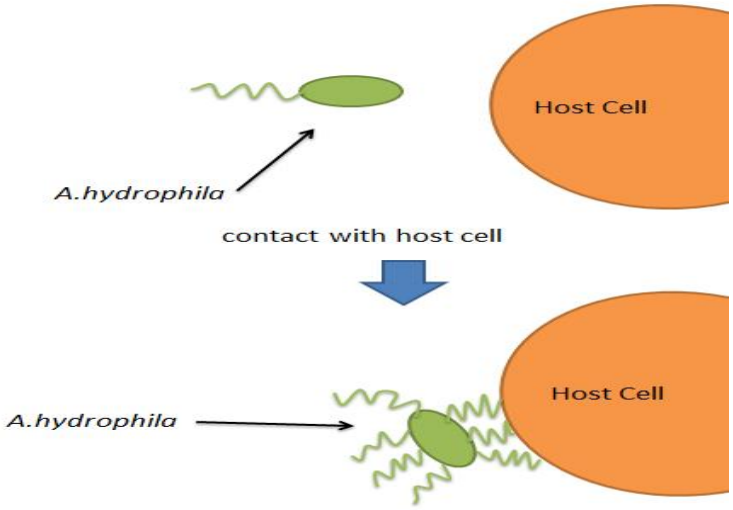
In prokaryotic organisms, flagella are sophisticated structures that exist on the bacterial surface. Flagella allow bacteria to move around and approach optimal environment or avoid harmful factors. When contacting with the host cell, the flagella also play a significant role in adhesion and biofilm formation on the host cell surface. Hence the flagellum is recognized as important bacterial virulence factor in pathogenic bacteria. Such as in *A. hydrophila,* polar flagella are expressed for swimming and lateral flagella are expressed for adhesion (Gavín et al. 2002) (Figure 1A).

A. caviae and *A. hydrophila* are two species of mesophilic aeromonads which have been widely studied. It has been confirmed that there are two kinds of flagella discovered in *Aeromonas* species (Thornley et al. 1997; Shimada et al. 1985; Gavín et al. 2002). The flagella filament is composed of flagellin proteins. There are two polar flagellins (FlaA and FlaB) and two lateral flagellins (LafA1 and LafA2) expressed in *A. caviae* as well as in *A. hydrophila* there are two polar flagellum flagellins (FlaA and FlaB) and one specific lateral flagellum flagellin (LafA) expressed (Rabaan et al. 2001; Tabei et al. 2009; Canals et al. 2006a).

The structure of flagella filament from *Salmonella typhimurium* has been described and the structure of the flagellin is similar in all bacteria. Starting with the N-terminal chain there are four domains including D0, D1, D2 and D3, the C-terminal chain is back to D0 to form a coil. The D0 and D1 are basically buried inside of the structure of filament while the central D2 and D3 are exposed at surface. D2 and D3 are considered to be recognized by glycosyltransferase in bacteria such as *A. caviae,* although they are not glycosylated in *Salmonella* (Tabei et al. 2009; Yonekura et al.

2003). These findings suggest that the D2 and D3 are where the flagellar glycosylation occurred. Recent study about *A. caviae* indicated that D2 and D3 can be recognized by Maf1 and a C-terminal chaperone-binding domain (CBD) can directly interact with FlaJ which is a flagellin-specific chaperone (Parker et al. 2014).

(A)



(B)

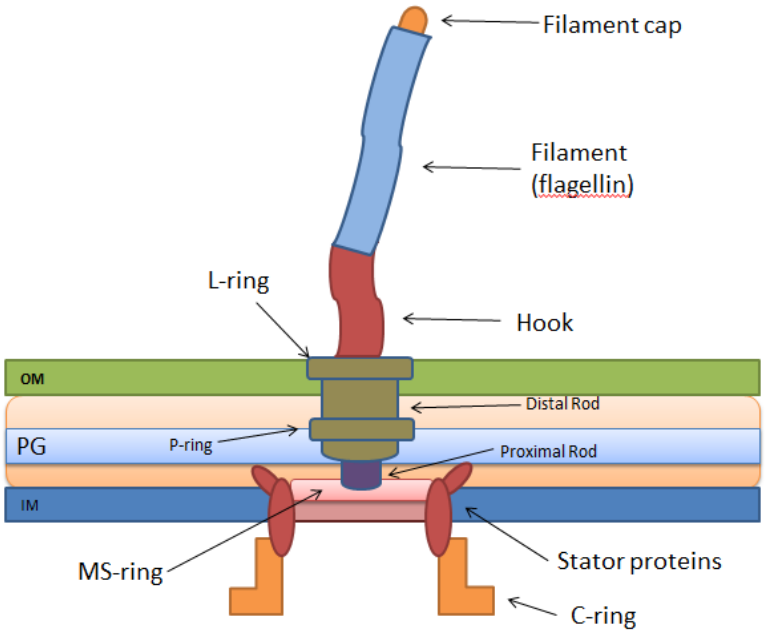


Figure 1.1 The expression of the flagella systems in *A. hydrophila* and Structure of flagellum from bacteria.

(A) Polar flagella system is expressed when the bacteria are living in liquid conditions. Lateral flagella system is expressd when the bacteria are contacting the host cell.

(B) A single flagellum consists of the filament, the hook and a basal body. (OM = Outer membrane PG = peptidoglycan layer CM = cytoplasmic membrane)

1.2 Glycosylation in eukaryotes and prokaryotes

Glycosyl acceptor molecules such as immature proteins attached to a carbohydrate at a hydroxyl or other functional group is known as glycosylation. Protein glycosylation is an extensive post-translational modification that exists in eukaryotes and prokaryotes.

Based on the studies about glycosylation so far, glycosylation can be classified into two pathways: N-glycosylation in which carbohydrates are covalently attached to the amide asparagine residues of proteins as well as O-glycosylation in which carbohydrates are covalently attached to serine or threonine residues instead of asparagine residues (Iwashkiw et al. 2013).

It is acknowledged that both N-glycosylation and O-glycosylation have been identified in eukaryotes and prokaryotes (Iwashkiw et al. 2013). In eukaryotes, N-glycosylation of proteins is the most common post-translational modification (Dwek 1996; Varki 1993). The understanding of N-linked glycosylation and O-linked glycosylation in prokaryotes is developing with time. In prokaryotic organisms, both N-linked glycosylation and O-linked glycosylation pathways have been established and each pathway has been widely investigated in *Campylobacter jejuni*.

In *C. jejuni,* the formation of a heptasaccharide is required for N-linked glycosylation while O-linked glycosylation is involved in flagellum assembly (Kalynych et al. 2014). The glycosylation in prokaryotes will be extensively discussed here, especially related to flagellin glycosylation and LPS O-antigen glycosylation.

1.2.1 N-linked glycosylation and O-linked glycosylation in prokaryotes

Campylobacter species are Gram-negative bacilli which are responsible for most cases of bacterial gastroenteritis worldwide (Allos and Acheson 2001). A well-studied N-glycosylation pathway and O-linked flagellar glycosylation pathway have been established in *C. jejuni.* Therefore, it makes *C. jejuni* a good model for the investigation of prokaryotic glycosylation (Szymanski and Wren 2005).

In *C. jejuni*, biosynthesis of a branched heptasaccharide is involved in the N-linked glycosylation process which is under the control of *pgl* (protein glycosylation locus) genes. PglA, PglB, PglC, PglD, PglE, PglF, PglH, PglI and PglJ are all encoded by the *pgl* locus. Initially, UDP-N-acetyl glucosamine (GlcNAc) is sequentially modified by PglF, PglE and PglD and results in UDP-bacillosamine, and then it is attached to a lipid carrier by PglC and result in UPP-bacillosamine. Eventually, glucose is added by branching enzyme PglI and five N-acetylgalactosamine (GalNAc) moieties are linked by PglA, PglJ and PglH to form the branched heptasaccharide (Merino and Tomas 2014). After the heptasaccharide is produced, it is transferred to the periplasm by PglK which is a flippase and the heptasaccharide is coupled to the asparagine residue of the acceptor polypeptide by PglB. It is remarkable that, PglB of *C. jejuni* shares a similar role to STT3 in eukaryotes which bonds glycan to the acceptor in N-linked glycosylation (Linton et al. 2005b) (Figure 2).

Furthermore, in *C. jejuni* there is a specific O-linked flagellar glycosylation system and a series of nucleotide-activated sugars including activated pseudaminic acid is added to the surface-exposed domains of flagellin monomers separately via an oligosaccharyltransferase.

There is evidence that both polar and lateral flagella are glycosylated in prokaryotes (Merino and Tomas 2014). In *C. jejuni*, flagellin is glycosylated up to 19 times before the protein is completed while the flagellin from *Aeromonas* is glycosylated 6 or 7 times (Thibault et al. 2001; Tabei et al. 2009; Wilhelms et al. 2012). As far as we know, the particular purposes and functions of flagellar glycosylation still remains unknown but Josenhans and the colleagues confirmed that un-glycosylated flagellin cannot be exported from cytoplasm via the flagellar type III secretion system (T3SS) which indicated that glycosylation could be essential for the assembly of flagellins (Josenhans et al. 2002).

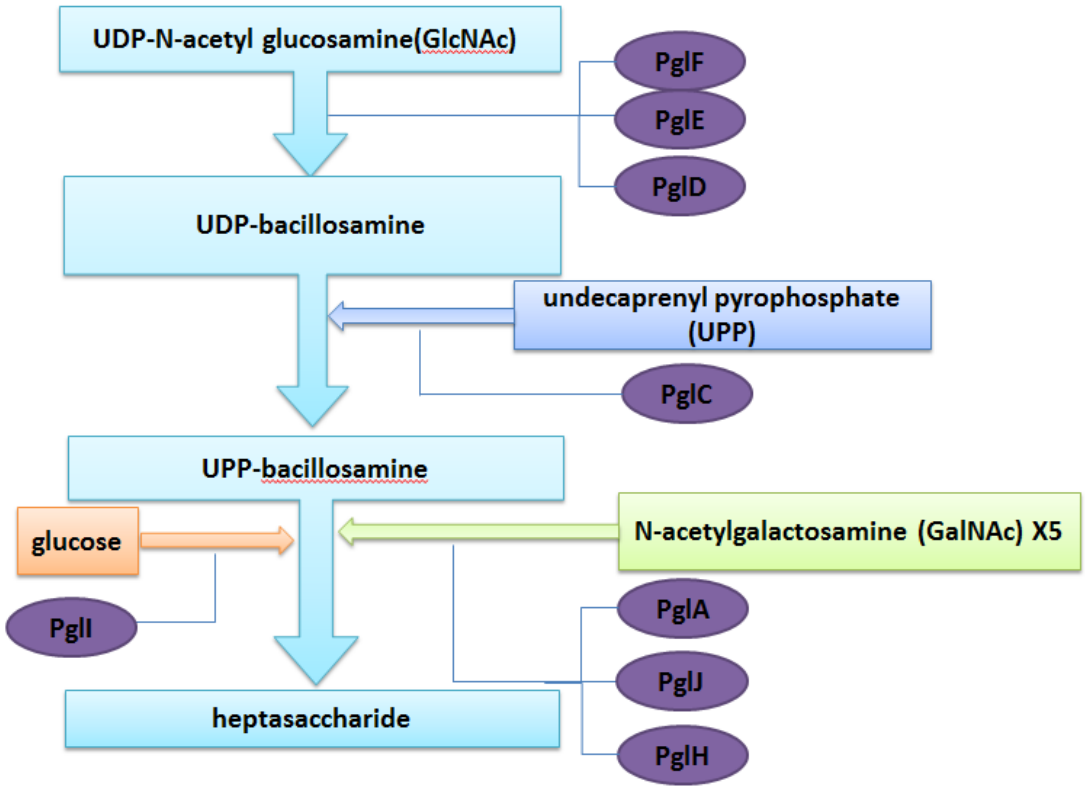


Figure 1.2. Biosynthetic pathway of branched heptasaccharide in *C. jejuni*.

UDP-N-acetyl glucosamine (GlcNAc) is modified by PglF, PglE and PglD and result in UDP-bacillosamine, and then it is tranfered to a lipid carrier (UPP) by PglC and result in UPP-bacillosamine. Eventually, glucose is added by branching enzyme PglI and five N-acetylgalactosamine (GalNAc) residues are all added by PglA, PglJ and PglH to form the branched heptasaccharide (Linton et al. 2005a).

1.3 *Aeromonas* genetic loci of glycosylation

In order to investigate the glycosylation in polar flagellar biosynthesis and other mechanisms, it is necessary to look into the relevant genetic loci including glycosylation genes and flagellum structural genes. The study of these genes has not been completely finished.

Based on the previous research, *flaA*, *flaB*, *flaG*, *flaH* and *flaJ* (*fla* locus) and *flmA*, *flmB*, *flmD neuA*, *neuB* (*flm* locus) were all identified in *A. caviae* Sch3N and *A. hydrophila* AH-3 (Tabei et al. 2009) (Figure 1.3). Such as *flm* genes in *A. caviae*

including *flmA*, *flmB*, *neuA*, *neuB*, *flmD* and polar flagella filament genes including *flaA*, *flaB*, *flaG*, *flaH* and *flaJ* are all interesting targets of this project (Tabei et al. 2009; Rabaan et al. 2001). A deep investigation of the above genes can be very valuable for the understanding of the mechanisms of flagella system and relevant pathogenicity of *Aeromonas* species.

The *fla* locus of *A. caviae* Sch3N is linked to the biosynthesis of polar flagella filament. This locus includes *flaA*, *flaB*, *flaG*, *flaH* and *flaJ* which have been listed in genetic order (Rabaan et al. 2001).

The *flaA* and *flaB* genes of *A. caviae* Sch3N encode two flagellin subunits. A strain that lacks *flaA* and *flaB* shows no motility and further study proved that production of flagellins in this strain is absolutely terminated (Rabaan et al. 2001). Motility and adherence drop to about 50% in the strain with single mutation in *flaA* or *flaB* in which flagella are still expressed. This finding indicates that FlaA and FlaB compose flagellin with a 1:1 ratio (Rabaan et al. 2001).

FlaG encoded by *flaG* has been shown not to be essential for polar flagellar biosynthesis and export. Mutation in *flaG* result in decreased of cell adherence while the motility is not affected. The mutant appears to make an elongated filament (Rabaan et al. 2001).

FlaH encoded by *flaH* has been considered as a homologue of hook-associated proteins (HAPs) which is usually acts as flagellum cap. The function of these proteins is to prevent the decomposition of unpolymerized flagellin via modification of the terminal of flagellar filament (Parker et al. 2014). Mutation in *flaH* results in entire loss of motility and flagella and there is no production of flagellins detected (Rabaan et al. 2001). Besides, a study shows that *flaH* mutant of *A. caviae* Sch3N can not produce any fully functional filament while the flagellins that synthesized in cytoplasm can still be exported to the outside of cell membrane (Yonekura et al. 2000). In addition, the glycosylation of flagellin is still functional in this mutant which indicates that the glycosylation of flagellin is irrelevant to the modification of filament (Parker et al. 2014).

The protein encoded by *flaJ* which is downstream of *flaH* is considered as a

homologue of FlaJ of *Vibrio parahaemolyticus*. This protein has been regarded as a chaperone protein that manipulates the flagellin export (Rabaan et al. 2001). Mutation in *flaJ* also results in loss of motility and flagella which is the same as mutation in *flaH* (Rabaan et al. 2001). FlaJ stabilizes the flagellin in an unfolded form which means this chaperone protein prevents unfolded flagellin from polymerization in the cytoplasm via binding to the C-terminal interactive region (Auvray et al. 2001).

Recently, a *flm* locus of *A. caviae* Sch3N was identified. This locus which includes *flm* genes is consists of *flmA*, *flmB*, *neuA*, *flmD*, and *neuB*. The genes are arranged as *flmA-flmB-neuA-flmD-neuB*. Following the *flm* locus, there are *lsg* and *lst* which encode the LPS O-antigen flippase and transferase (Tabei et al. 2009).

In *A. caviae* Sch3N, the *flm* locus is involved in both flagellin glycosylation and lipopolysaccharide (LPS) O-antigen (O-Ag) biosynthesis, the mutants without these genes lost motility, flagella, and their LPS O-antigen (Gryllos et al. 2001). The proposed protein encoded by *flmA* is the homologue of PseB (Cj1293) of *C. jejuni* and the orthologue of FlaA1 of *H. pylori* (Goon et al. 2003; Creuzenet et al. 2000). FlmA is proposed to be essential for the Pse5Ac7Ac biosynthetic pathway (Leclerc et al. 1998; Tabei et al. 2009)

The *flmB* gene is involved in the assembly of flagellar filament which is the same role as *flmA* (Leclerc et al. 1998). PseC (Cj1294) of *C. jejuni* is the homologue of FlmB of *A. caviae* Sch3N and this protein is working as pyridoxal-dependent amino-transferase in general protein glycosylation system (Szymanski et al. 1999)

The *neuA* of *A. caviae* Sch3N encodes a protein that shares similar amino acid sequence to CMP-NeuNAc synthetases (NeuA) of *H. pylori* and these enzymes can synthesis CMP-NeuNAc from N-acetylneuraminic acid (NeuNAc) and CTP (Gryllos et al. 2001). PseF (Cj1311) of *C. jejuni* has been identified as the homologue of NeuA of *A. caviae* Sch3N. In *C. jejuni,* PseF is CMP-sugar synthetase in the biosynthesis of CMP-Pse5Ac7Ac which makes NeuA of *A. caviae* Sch3N the crucial enzyme related to CMP-Pse5Ac7Ac in *A. caviae* Sch3N (Schirm et al. 2003).

FlmD encoded by *flmD* is also involved in assembly of flagellar filament (Gryllos et al. 2001). This protein is a chimera that includes a component that has the same

activity as PseH (Cj1313) of *C. jejuni* and FlmD is considered as the homologue of PseG (Cj1312) and PseH (Cj1313) of *C. jejuni* (Tabei et al. 2009).

PseI (Cj1317) of *C. jejuni* is the homologue of NeuB of *A. caviae* Sch3N and this kind of protein is regarded as sugar synthetase in the biosynthesis of pseudaminic acid (Pse5Ac7Ac) (Thibault et al. 2001; McNally et al. 2006). From previous work, one of three *neuB*-like genes discovered in *C. jejuni* was shown to be required for motility and production of flagellins (Linton et al. 2000).

Based on experimental results, mutants lacking each *flm* gene did not present functional LPS O-antigen and flagella which lack the Pse5Ac7Ac in glycosylation. Therefore, it can be concluded that *flm* locus of *A. caviae* Sch3N is involved in Pse5Ac7Ac biosynthesis (Tabei et al. 2009)

The *fla* locus and *flm* locus are all identified in *A. caviae* Sch3N and *A. hydrophila* AH-3 as well as some *maf*-like genes are also discovered in these strains. However, the glycosylation of polar flagellum in *A. caviae* is different from glycosylation of polar flagellum in *A. hydrophila.*

Based on the recent studies, there are some other genes also involved in flagellin glycosylation in *A. hydrophila.* An enzyme able to link sugars to a lipid carrier in *A. hydrophila* was identified. WecX which is encoded by *wecX* is required for the heptasaccharide glycosylation of the polar flagellin and this enzyme is not involved in the modification of O34-antigen LPS. Additionally, UDP-GalNAc formation is under the control of an enzyme named Gne which is encoded by *gne* and UDP-GalNAc is essential for the biosynthesis of heptasaccharide (Merino et al. 2014).

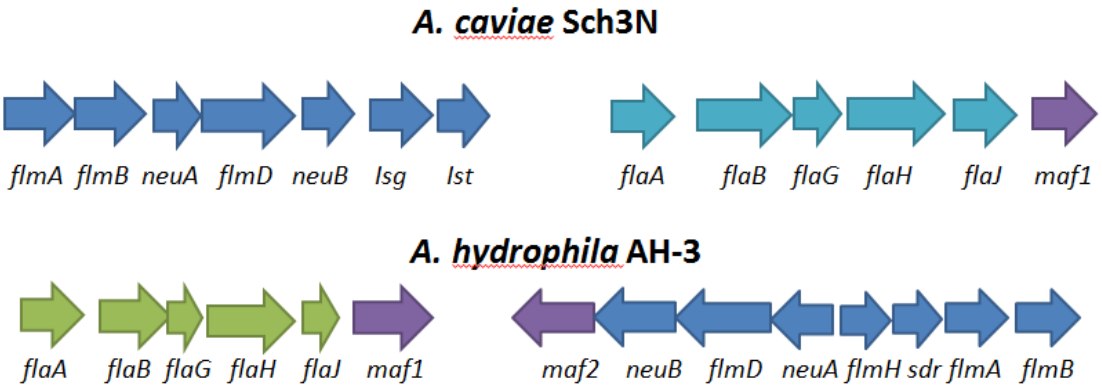


Figure 1.3. Genetic organization of the *A. caviae* Sch3N and *A. hydrophila* AH-3 flagellin and flagellar glycosylation loci.

This diagram shows the *flm*, *fla* and *maf*-like genes identified in *A. caviae* Sch3N and *A. hydrophila* AH-3 (Tabei et al. 2009; Rabaan et al. 2001; Canals et al. 2006a; Canals et al. 2007; Parker et al. 2012).

1.4 Putative flagellin glycosyl transferase

The investigation of flagellin glycosyl transferase has not been finished. There are many homologues encoded by *maf* family in various bacteria species. In most cases, the *maf*-like genes are essential for flagellar biosynthesis (Karlyshev et al. 2002).

In *C. jejuni*, seven putative *maf* genes have been discovered. The genes including *maf1* (*Cj1318*), *maf2* (*Cj1333*), *maf3* (*Cj1334*), *maf4* (*Cj1335/6*), *maf5* (*Cj1337*), *maf6* (*Cj1341*) and *maf7* (*Cj1342*) have been revealed and phase variation was detected in flagellar biosynthesis, but the exact functions of the proteins encoded by these genes still remains unknown (Karlyshev et al. 2002). However, based on the experiment result, *maf5* is considered involved in the formation of the flagellum and it is hypothetical that all of these genes are involved in flagellar biosynthesis and phase variation (Karlyshev et al. 2002).

Some *maf*-like genes have been identified in *A. hydrophila* AH-3. The *maf-1* gene of *A. hydrophila* AH-3 has been proved to be related to glycosylation of polar flagellum filament (Canals et al. 2006b). In addition, *maf-5* gene of *A. hydrophila* AH-3 is

confirmed to be only essential for lateral flagellar biosynthesis and there is evidence that it is not required for polar flagellar biosynthesis (Canals et al. 2006a). Additionally, *maf-2* of *A. hydrophila* AH-3 is identified and it plays a role in both the glycosylation of polar flagella and lateral flagella (Canals et al. 2007) (Figure 1.3).

In *A. caviae* Sch3N, the *maf1* gene named by Parker et al (2012) shared 38% similarity to *maf-1* of *A. hydrophila* AH-3 is considered related to polar flagella glycosylation. The Maf1 encoded by *maf1* in *A. caviae* Sch3N is considered as transferase in the polar flagellar biosynthesis. Based on the experimental results, it is essential for polar flagella-mediated motility and it has been confirmed that the overexpression of *maf1* in cell will result in the generation of overexpressed glycosylated flagellins which leads to the blocking of flagellum synthesis and then it will cause low level of cell motility. This phenomenon indicates that the expression of *maf1* in *A. caviae* Sch3N is precisely controlled and a certain amount of Maf1 is pivotal for the optimal cell motility (Parker et al. 2012).

From the previous studies, there is evidence that FlaA and FlaB of *A. caviae* Sch3N are all modified by Pse5Ac7Ac in the flagellar biosynthesis (Tabei et al. 2009). It has been confirmed that pseudaminic acid is irrelevant to the interaction between Maf1 and flagellins which indicates that Maf1 can directly interact with flagellins (Parker et al. 2014). The experiments confirmed that the Maf1 is not involved in Pse5Ac7Ac and LPS biosynthesis and it is only required for flagellin glycosylation. Therefore, the Maf1 is deemed as a putative flagellin pseudaminyl transferase (Parker et al. 2012). Theoretically, Maf1 of *A. caviae* Sch3N has the ability to transfer activated pseudaminic acid (CMP-Pse5Ac7Ac) to the hydroxyl group of serine and threonine residues in the central immunogenic D2/D3 domain of flagellin of *A. caviae* Sch3N (Parker et al. 2012; Tabei et al. 2009).

1.5 *Aeromonas* flagellin glycosylation system

1.5.1 *Aeromonas* polar-flagella and lateral-flagella

From the previous investigations, it has been revealed that polar flagella systems and lateral flagella systems of the *Aeromonas* species are all correlative in adherence and biofilm formation in both inorganic environments and organic environments such as host cell surface (Kirov et al. 2004). It has been proved that lateral flagella system

of *A. caviae* and lateral flagella system of *A. hydrophila* are essential for swarming motility in solid environment (Kirov et al. 2004; Gavín et al. 2002). Furthermore, polar flagella system is functional when bacteria contacting with liquid environment including water or body fluid (Gavín et al. 2002; Rabaan et al. 2001) (Figure 1A).

1.5.2 Flagellin glycosylation system in *A. caviae* Sch3N

A. caviae which is a typical pathogenic mesophilic species of *Aeromonas* is drawing the attention. In this project, *A. caviae* Sch3N will be researched widely as the model organism. Pseudaminic acid (Pse5Ac7Ac) is a nine carbon sugar similar to sialic acid. The biosynthetic pathway of Pse5Ac7Ac in *A. caviae* Sch3N is based on the predicted functions of proteins encoded by *flm* genes which are the homologues of enzymes of *C. jejuni* and *H. pylori* (McNally et al. 2006; Schoenhofen et al. 2006). With the help of PseF, PseG, PseH, and PseI from *H. pylori* as well as PseG and PseH from *C. jejuni* which are overexpressed in *Escherichia coli* and purified, the pseudaminic acid biosynthetic pathway was reproduced (Schoenhofen et al. 2006).

PseB which is a dehydratase initiates the biosynthesis of pseudaminic acid by catalyzing the conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-2-acetamido-2,6-dideoxy-b-L-arabino-hexos-4-ulose. Subsequently, the product is modified by PseC which is aminotransferase and UDP-4-amino-4,6-dideoxy-b-L-AltNAc is generated. After processing by PseH which is an acetyltransferase, the UDP of UDP-2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose is removed by PseG which is a glycosyltransferase and results in 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose. Eventually, this monosaccharide is converted to pseudaminic acid by PseI which is a sugar synthetase (Schoenhofen et al. 2006; Schirm et al. 2003). PseF acts as a CMP-sugar synthetase in the biosynthesis of CMP-Pse5Ac7Ac (Schirm et al. 2003) (Figure 1.4). Activated pseudaminic acid (CMP-Pse5Ac7Ac) is ready for the glycosylation and it can be transferred onto flagellin by flagellin glycosyl transferase.

In *A. caviae* Sch3N, flagellins of polar flagella are glycosylated 6 to 8 times by CMP-Pse5Ac7Ac which is under the control of Maf1 which is known as flagellin glycosyl transferase (Parker et al. 2012). In a recent study about *A. caviae* Sch3N, the interaction between FlaJ and Maf1 has been basically explained. After flagellins (FlaA/B) were glycosylated by activated pseudaminic acid (CMP-Pse) in a Maf1

dependent manner, the chaperone-binding domain (CBD) of the glycosylated flagellin will be recognized and bound by the flagellin-specific chaperone FlaJ (Parker et al. 2014). Additionally，experimental results show that the affinity between FlaJ and glycosylated flagellin is higher than the affinity between FlaJ and un-glycosylated flagellin. This result is significant for the export of glycosylated flagellin (Parker et al. 2014). Besides, it has been discovered that flagellin glycosylation is irrelevant to FlaJ and CBD of flagellin which are crucial for flagellin export (Parker et al. 2014).

In conclusion, glycosylation and assembly of flagellins of polar flagella in *A. caviae* Sch3N can be described like this: The original flagellin synthesized from FlaA and FlaB has a central D2/D3 domain which can be recognized by Maf1 and a C-terminal chaperone-binding domain (CBD) which can directly interact with FlaJ. First of all, Maf1 is attached to the central D2/D3 domain of original flagellin with CMP-Pse5Ac7Ac and then the original flagellin is glycosylated by Pse5Ac7Ac. After that, Maf1 is released from glycosylated flagellin for next circulation. Secondly, as it was mentioned before, FlaJ is bound to the C-terminal chaperone-binding domain (CBD) of flagellin (FlaA /FlaB+Pse) and this complex is transported to the basal body of flagellum. Finally, the glycosylated flagellin is discharged from intracellular environment via T3SS. The exported flagellin will be polymerized by FlaH at the distal tip and the complete flagellar filament will be produced. Glycans can attach to the exposed central D2/D3 domain after the flagellin is folded (Parker et al. 2012; Parker et al. 2014) (Figure 1.5). Additionally, Parker et al found out that un-glycosylated flagellin is exported via T3SS too. This finding suggests that un-glycosylated flagellin can be recognized by the export apparatus of flagellar T3SS and the glycosylation of pseudaminic acid has no influence on the export of flagellin (Parker et al. 2014). However, the system appears to have a higher affinity for the glycosylated flagellin.

However, there are some evidences about the glycosylation of lateral flagellum in *A. caviae* Sch3N. The glycosyl groups of the lateral flagellum was detected by periodate oxidation and hydrazine biotinylation in a previous study, but the investigation in this field is still insufficient (Gavín et al. 2002).

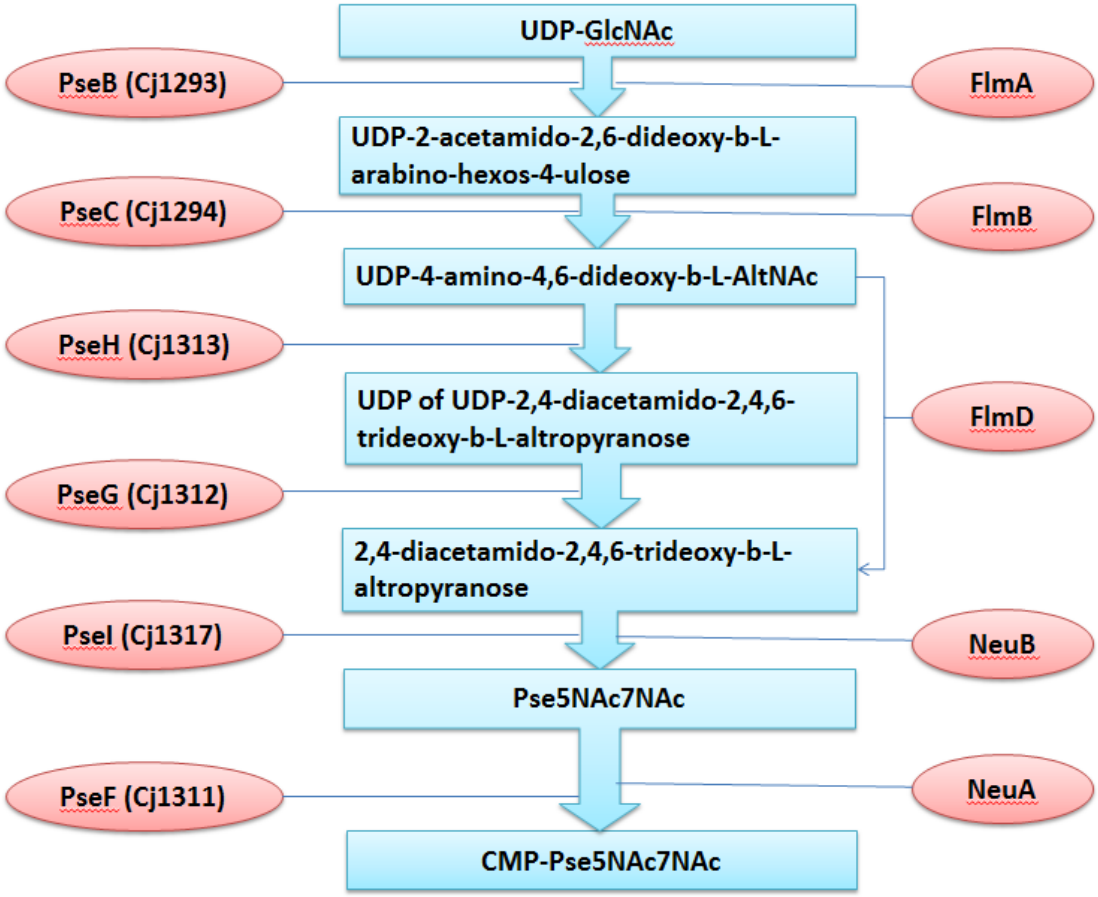


Figure 1.4. The proposed CMP-Pse5Ac7Ac biosynthetic pathway in *C. jejuni* and *A. caviae*.

This diagram shows CMP-Pse5Ac7Ac is synthesized from UDP-N-acetylglucosamine via PseB (Cj1293), PseC (Cj1294), PseH (Cj1313), PseG (Cj1312), PseI (Cj1317) and PseF (Cj1311) or FlmA, FlmB, FlmD, NeuB and NeuA (Schoenhofen et al. 2006).

1.5.3 Flagellin glycosylation system in *A. hydrophila* AH-3

Recently, the glycans involved in glycosylations of flagellins of polar flagella (FlaA/FlaB) and flagellins of lateral flagella (LafA) have been revealed in *A. hydrophila* AH-3. The flagellins of polar flagella are glycosylated 6 times and initially glycosylated by a single monosaccharide which is derivative of pseudaminic acid. The flagellins of lateral flagella are most likely glycosylated by another phosphorylated derivative of pseudaminic acid (Wilhelms et al. 2012).

In a recent study, flagellins of polar flagellum of *A. hydrophila* AH-3 were discovered to be O-linked glycosylated with a heterogeneous glycan. A remarkable finding which is a lipid carrier is involved in flagellin glycosylation has not been reported before. The proposed polar flagellum glycosylation in *A. hydrophila* AH-3 is described: WecX links CMP-Pse-like to undecaprenylphosphate (Und-P) and then two hexoses are added. After that, three N-acetylhexosamines or derivates of N-acetylhexosamine are added and an unknown glycan of 102 Da is added at last. Finally, this heptasaccharide is attached to the threonine/serine amino acid residue of the polar flagellin by a putative OT (Merino et al. 2014).

The lateral flagella of *A. hydrophila* are O-linked glycosylated at least three times with a derivative of pseudaminic acid and there is evidence that this derivative is phosphorylated. It has been confirmed that mutation in *flmA* (*pseB*) or *neuA* (*pseF*) leads to the absence of both flagella which indicates that the biosynthesis of pseudaminic acid is essential for the productions of polar flagella and lateral flagella (Wilhelms et al. 2012).

1.6 Connection between glycosylation in flagellin biosynthesis and LPS O-antigen biosynthesis

Besides the flagellar systems, lipopolysaccharide (LPS) O-antigen which is an important pathogenic factor is also investigated in this project.

LPS molecule which embedded in the outer leaflet of the outer membrane of many Gram-negative bacteria is an important part of bacterial cell envelope and this molecule also play a significant role in innate immune responses. LPS is consists of a lipid portion, a core oligosaccharide (OS) and an O-specific polysaccharide which is known as the O-antigen (Nikaido 1996; Whitfield and Valvano 1993). The surface-exposed LPS O-antigen which is required for the subsistence of bacteria prevents the cell from elimination by immune system (Tomás 2012). An understanding of the molecular mechanisms involved in production and modification of O-antigen can be very helpful for the development of new drugs and vaccines.

The surface-exposed O-antigen consists of repeating sequences of three to six sugar residues and the O-antigen is irreplaceable for the survival of bacteria because it protects bacteria from immune responses. The O-antigen need to be glycosylated before it is added to the LPS. The substrate of O-antigen glycosylation is CMP-Pse5Ac7Ac which is activated pseudaminic acid (Tomás 2012). In cytoplasm, Pse5Ac7Ac needs to be bound to CMP which is under the control of CMP-sugar synthetases including PseF (Cj1311) of *C. jejuni* and NeuA of *A. caviae* Sch3N to form CMP-Pse5Ac7Ac. Additionally, in *A. caviae* Sch3N, the CMP-Pse5Ac7Ac is the substrate of Lst and Lsg which were involved in LPS O-antigen formation (Tabei et al. 2009). In *A. caviae* Sch3N, *lsg* and *lst* are located at the downstream of *flm* locus (Tabei et al. 2009). CMP-Pse5Ac7Ac is transfered onto a sugar-antigen carrier lipid (ACL) by Lst which is transferase and then the Pse5Ac7Ac-sugar-ACL complex is transported to the outer membrane by Lsg which is LPS-specific transporter (Kalynych et al. 2014). Eventually, Pse5Ac7Ac-sugar-ACL complex passes the cytoplasmic membrane with the help of Lsg and it is added to the inner-core of oligosaccharide for the purpose of generation of full functional LPS (Tabei et al. 2009) (Figure 1.4).

Based on the papers published before, the glycosylations in LPS O-antigen biosynthesis and flagellin biosynthesis are cross correlated. In *A. caviae* Sch3N, the flagellin is O-glycosylated with Pse5Ac7Ac while LPS is glycosylated with the same sugar (Tabei et al. 2009; Parker et al. 2012). Based on the two mechanisms summarized above, it is clear that Pse5Ac7Ac is acts as a pivotal factor which connects the two mechanisms with glycosylation. Because of that, the investigation of the proteins related to Pse5Ac7Ac becomes very valuable. NeuA which directly interacts with Pse5Ac7Ac is a crucial target has been applied in this project.

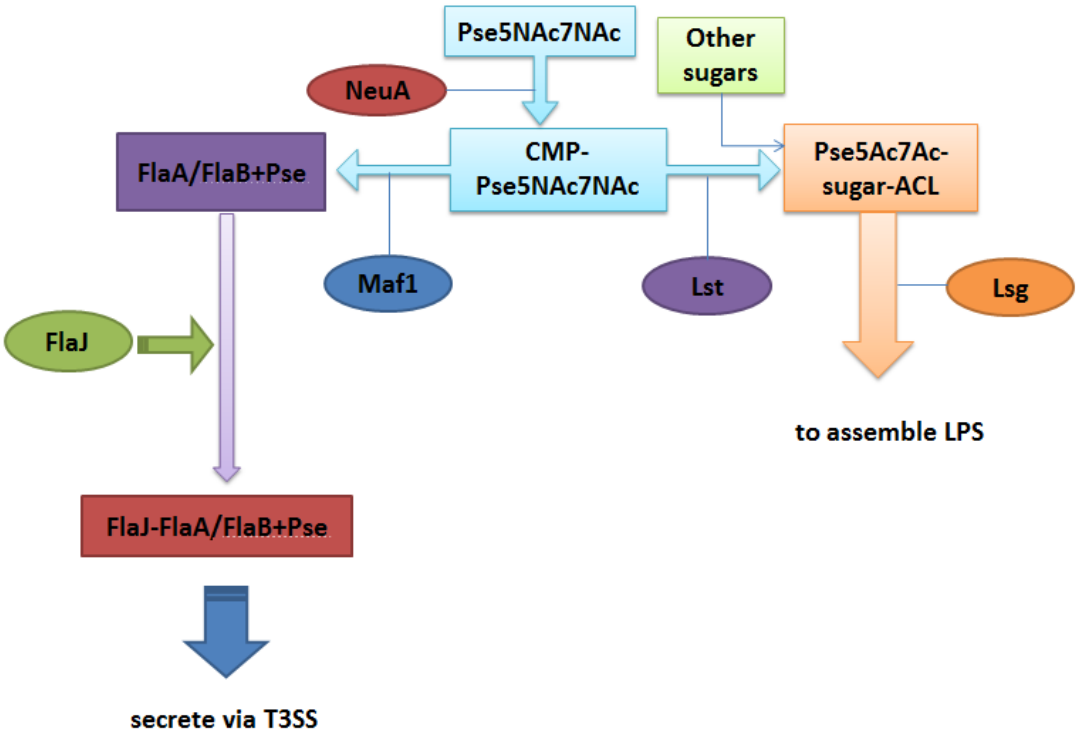


Figure 1.5. The assumed flagellin glycosylation pathway and O-antigen formation in *A. caviae* Sch3N.

This diagram shows NeuA and Maf1 are all involved in the proposed pathway of flagellin glycosylation and CMP-Pse5Ac7Ac is the key substrate connects the two mechanisms in *A. caviae* Sch3N (Tabei et al. 2009; Parker et al. 2012).

1.7 Project aims and design

The aim of this project is to investigate proteins associated with flagellar glycosylation in *Aeromonas caviae*. Maf1 the motility-associated factor is involved in polar flagellar biosynthesis and it is thought to be the glycosyl-transferase that directly transfer of pseudaminic acid onto the polar flagellin proteins in *A. caviae* Sch3N (Tabei et al. 2009; Parker et al. 2012). Hence the detail of how the Maf1 interacts with flagellin and which protein domains or amino residues that are directly related to these interactions are all interesting targets of this project. Furthermore, to prove that Maf1 is indeed the glycosyl-transferase the substrate for the reaction need to be isolated or created, namely flagellin and the activated form of pseudaminic acid (CMP-Pse5Ac7Ac). Therefore, in order to create CMP-Pse5Ac7Ac, the Flm proteins of the pseudaminic biosynthesis pathway will be overexpressed and purified. Hence the biosynthesis of Pse5Ac7Ac and CMP- Pse5Ac7Ac can be looked into via these reactions. Flagellin proteins, are usually insoluble, previous work has shown that the

C-terminal chaperone binding domain of the *A. caviae* flagellin FlaA is not involved in the glycosylation process. Attempts will be made to create deletion derivatives of flagellin that are still glycosylated and are more soluble to provide more suitable substrate for future glycosylation assays.

Chapter 2 Methods and Materials

2.1 Media

All media are autoclaved to sterilize before use.

2.1.1 Luria Bertani broth (LB broth)

1% tryptone (Oxoid)

0.5% yeast extract (Oxoid)

1% NaCl (Melford)

In dH_2O

2.1.2 Luria Bertani agar (LB agar)

1% tryptone (Oxoid)

0.5% yeast extract (Oxoid)

1% NaCl (Melford)

1.5% Bacteriological Agar

In dH_2O

2.1.3 Brain heart infusion broth (BHIB)

3.7% Brain Heart Infusion powder (Oxoid)

In dH_2O

2.1.4 Tryptone soya agar (TSA)

3.7% Tryptone Soya Agar powder (Oxoid)

In dH_2O

2.1.5 Motility agar

0.5% tryptone (Oxoid)

0.5% NaCl (Melford)

0.25% agar (Oxoid)

In dH_2O

2.1.6 X-gal agar

1% Typtone

0.5% Yeast Extract

1% NaCl

1.5% Agar

In dH_2O

400µl of 2mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) in 200ml

200µl of 1M IPTG (isopropyl β-D-1-thiogalactopyranoside) in 200ml

2.1.7 Blood agar

horse blood agar plates (Oxoid)

1.5% Proteose peptone

0.25% Liver digest

0.5% Yeast extract

0.5% Sodium chloride

1.2% Agar

2.2 Bacterial strains used in the study

Table 2.1 Bacterial strains used in this study

Name of strains	Properties	Reference
Aeromonas caviae strains		
Sch3N	Wildtype, Nal^R	(Gryllos et al. 2001)
Sch3N *neuA*::Km	Km^R and Nal^R，*neuA* knockout	This study
Sch3N *flmA*::Km	Km^R and Nal^R，*flmA* knockout	This study
Sch3N *flaA*::Km::*flaB*	Km^R and Nal^R，*flaA* and *flaB* knockout	This study
Escherichia coli strains		
DH5α	competent strain used in plasmid transformation	Invitrogen Life Technologies™
S17-1 λ*pir*	Sm^R, Km::Tn7 (Tc::Mu) λ*pir,* donor plasmid in conjugation	(Miller 1972)
BL21(DE3)	T7 expression strain, used to express target genes	Novagen
NEB	Versatile non-T7 expression strain, Protease deficient	New England Biolabs (NEB)

A. caviae Sch3N strains were incubated at 37℃ on LB agar and TSA or in LB broth and BHIB. Appropriate antibiotics were added when required and there is no antibiotics present in swarming agar. *E. coli* strains were grown at 37℃ on LB agar or in LB broth, in which antibiotics were added when required.

2.3. Plasmids used in the study

Table 2.2 Plasmids used in this study.

Name of plasmids	Characteristics	Reference
pBBR1MCS	Broad-host-range plasmid, Cm^R	(Kovach et al. 1994)
pBBR1MCS-5	Broad-host-range plasmid, Gm^R	(Michael E. Kovach a and a 1995)
pKNG101	RK6 derived suicide plasmid, Sm^R	(Kaniga et al. 1991)
pET28a	Containing T7 promoter, N-terminal His tag, used for protein expression, Km^R	Novagen
pMAL-c5x	designed to produce maltose-binding protein (MBP) fusions, Amp^R	New England Biolabs (NEB)

2.4. Antibiotics used in the study

Table 2.3 Antibiotics used in this study

Antibiotics	Working concentration	Concentration made
Kanamycin (Km)	50μg/ml	50mg/ml (dH_2O)
Streptomycin (Sm)	50μg/ml	50mg/ml (dH_2O)
Gentamicin (Gm)	25μg/ml	50mg/ml (DMSO)
Chloramphenicol (Cm)	50μg/ml	50mg/ml (Ethanol)
Ampcilin (Amp)	100 μg/ml	100 mg/ml (dH_2O)
Nalidixic acid (Nal)	30 μg/ml	30 mg/ml (dH_2O)

2.5 – Growth conditions

2.5.1 – Standard growth conditions

The certain bacterial strain was incubated in 10 ml of the required growth media with appropriate antibiotics in a 25 ml sterile universal tube overnight with shaking. The optimal temperature for both *E. coli* and *A. caviae* cultures is 37^0C.

2.5.2 – Glycerol stock

Glycerol stock was made by pipetting 500ul of the overnight culture of the selected strain which was prepared before into a 1.5 ml microcentrifuge tube containing 500ul of 50% glycerol. The stock was stored at -80^0C.

2.5.3 – Swimming motility assays

Swimming motility assays were carried out by setting up an overnight culture of the selected strain. Motility agar was prepared following 2.15 motility agar protocol. The motility agar was prepared before using. The assays were carried out on motility agar plates. 1 ml of the overnight culture was centrifuged (13,000xg) in a 1.5 ml microcentrifuge tube (Sigma) for 1 minute and the supernatant was discarded. About 10µl of the pellet was directly injected into the center of the motility agar by pipette. Swimming motility plates were incubated overnight at room temperature and swimming motility was analysed by measuring the radius of any motility halos appear.

2.6 Chromosomal DNA extraction

Bacteria growth was gained from overnight culture in 10ml LB broth with appropriate antibiotics. The culture was centrifuged at 1,600xg for 15 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 500µl of lysozyme. The sample was incubated in water bath at 37℃ for 60 minutes. Then 50µl of 10%SDS was added and mixed with the sample and the sample was vortexed until it was viscous or creamy. After that, the sample was incubated in water bath for 15 minutes at 37℃.Then the sample was vortexed again until viscosity was decreased.

After that, 300ul phenol/chloroform was added and the sample was centrifuged at 1600xg for 10 min. aqueous layer (top) was transferred into a fresh Eppendorf tube and 200µl dH_2o and 300µl phenol/chloroform were mixed with the aqueous layer. This procedure was repeated twice with phenol-chloroform and dH_2o. A 1/10 volume of 3M sodium acetate and 1 volume of ice-cold isopropanol were added and mixed by inversion. Then the sample was left on ice for 10-20 minutes and centrifuged at 1,600xg for 15 minutes. The supernatant was discarded and the sample was mixed with 0.5ml of 70% ethanol and centrifuged at 13,000xg for 1 minute and the supernatant was discarded. This procedure was repeated once with 0.5ml of 70% ethanol. The DNA pellet was left to dry for at least 2 hours and resuspend in 50µl of dH_2O.

2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was applied in 50µl mix containing reagents of PCR in thin-walled PCR tubes using an Invitrogen T100^{TM} Thermal Cycler (PCR machine). The PCR reaction mixtures used in this project were listed as follows:

2.7.1 Normal PCR and SOE PCR condition

10X Pfx Amplification Buffer (invitrogen)	5µl
2mM dNTPs	5µl
50mM $MgCl_2$	2µl
Primers	1µl +1µl
Polymerase (Platinum Pfx)	0.5µl
Template DNA	1µl

dH_2O was added up to 50µl of total volume

2.7.2 Colony PCR screen condition

5X GoTaq Buffer (PROMEGA)	10µl
2mM dNTPs	5µl

50mM $MgSO_4$ 2µl

DMSO 2µl

Primers 1µl +1µl

Polymerase (GoTaq DNA Polymerase) .5µl

dH_2O was added up to 50µl of total volume.

Bacterial colonies were taken out by clean tips as templete DNA and these colonies were transferred into each tube separately.

2.7.3 PCR cycles

PCR conditions for Normal PCR (Pfx), SOE PCR (Pfx) and Colony PCR (Taq) are listed in table 2.4. Annealing temperature was adjusted according to different primers and Extension time was determined according to the length of the PCR fragment following the manufacturer's instructions.

Table 2.4 PCR conditions for different polymerase.

Name of step		Taq polymerase	Pfx polymerase
Heated lid		105℃	105℃
Denaturing	1 cycle	95℃ 3min	94℃ 3min
Denaturing	30 cycles	95℃ 40sec	94℃ 40sec
Annealing	30 cycles	55℃ 30sec	56℃ 30sec
Extension	30 cycles	72℃	68℃
Final extension	1 cycle	72℃ 2min	68℃ 2min
Infinite Hold		4℃	5℃

2.7.4 – DNA sequencing

DNA was sent to be sequenced by the Genomic Medicine Service at the Medical School of Sheffield University. FinchTV, SnapGene and BLAST (online resource)

were applied for sequencing and checking DNA products.

2.8 Isothermal Assembly (ISA)

The ISA was designed to join several DNA fragments into one plasmid in a single reaction and each fragment applied in ISA contained an approximately 40bp adapter sequence overlapping each other. The concentrations of DNA fragments were measured by using nanodrop. Then an appropriate amount of each DNA fragment was added into an Eppendorf tube and all DNA fragments were mixed with ISA Buffer and three enzymes including T5 exonuclease, Phusion polymerase and Taq ligase. After incubation for 1 hour at 50°C, the mixture was transformed into *E. coli* DH5α and cells were spread on agar containing the appropriate antibiotic to recover bacteria containing the plasmid.

2.8.1 5X ISA Buffer (1ml)

1M Tris-HCl pH 7.5	500μl
2M $MgCl_2 \cdot 6H_2O$	25μl
100mM dATP	10μl
100mM dTTP	10μl
100mM dCTP	10μl
100mM dGTP	10μl
1M DTT	50μl
PEG-8000	0.25μl
100mM NAD	50μl
dH_2O	335μl

2.8.2 ISA reaction (5 tubes)

5X ISA Buffer	20μl
Diluted T5 exonuclease (10 units)	0.5μl
Phusion Polymerase (1 unit)	1.25μl

Taq Ligase (80 units)	10µl
dH_2O	43.3µl

The reagent was aliquot into 5x 15µl in each reaction

2.9 Agarose gel electrophoresis

2.9.1 Preparation of 1X TAE (pH 8.0)

40 mM Tris (Fisher)

1.142% Acetic acid (Melford)

1 mM EDTA (Melford)

2.9.2 Preparation and electrophoresis of agarose gels

100ml 1X TAE Buffer was mixed with 1g of agarose powder to make 1% agarose gel and the mixture was heated in a microwave for 3-5 minutes until the agarose powder was dissolved. Then the liquid agarose gel was poured into a gel tray which was sealed at both ends with rubber caps and a comb was placed in the agarose gel to make wells. The agarose gel was left to solidify for 15-25 minutes. After the gel was solid, the tray was put into a gel tank filled up with 1X TAE buffer. The DNA samples with loading dye (5:2) and corresponding DNA ladder (Appendix 1) were loaded into the gel wells. The agarose gel was run at 100V for 60 minutes and then stained with ethidium bromide (0.5µg/ml) for 15 minutes. After that, the gel photo was taken on an ultraviolet trans-illuminator.

2.9.3 Gel extraction using QIAgen agarose gel extraction kit

The DNA fragment was excised from the ethidium bromide stained agarose gel with a specific tip under ultraviolet light. The gel slice was weighed in a colourless tube. 3 volumes of Buffer QG to 1 volume of the agarose gel (100mg~100µl) was added and the mixture was incubated at 50°C for 10 minutes and mixed by inverting for several times until the gel slice was completely dissolved. The colour of the

mixture should be yellow. If the colour of mixture was orange or violet, 10µl of 3M sodium acetate (pH 5.0) was added. Then 1 volume of isopropanol to the mixture was added and mixed. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1min at 13000xg and the flow-through was discarded. To wash the DNA, 750µl of Buffer PE wash buffer was added and the sample was centrifuged for 1 min at 13,000xg and the flow-through was discarded too. Then the sample was centrifuged again for 1min at 13,000xg to remove residual wash buffer. To elute the DNA fragment, 30µl of Elution Buffer was added to the centre of the spin column and the desired DNA fragment was obtained after centrifugation for 1 min at 13000xg. Buffer QG, PE wash buffer and Elution Buffer were provided by QIAgen agarose gel extraction kit.

2.10 Restriction enzyme digestion

The digestion was set up with the required restriction enzyme and corresponding buffer. All reagents were mixed together following the reaction conditions in 2.10.1. The mixture was incubated at 37℃ for 3-4 hours. After the incubation, the sample was purified following the PCR purification protocol in 2.11.

2.10.1 Reaction conditions:

DNA samples	5µl-16µl
Restriction enzyme (10u/µl)	1µl
10X Buffer	2µl

dH_2O was added up to 20µl of total volume.

2.11 PCR purification using the QIAgen PCR purification kit

The PCR product was transferred into an Eppendorf tube and mixed with 500µl of Buffer PB. The mixture was then transferred into a QIAquick spin column which was placed in a provided 2ml collection tube and the tube was centrifuged at 13,000xg for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. To wash the sample, 600µl of Buffer PE was added to the

column followed by centrifugation at 13,000xg for 1 minute. The flow-through was discarded and the column was placed back again. Then the collection tube was centrifuged at 13,000xg for 1 minute again to remove residual wash buffer and the column was transferred into a new Eppendorf tube. To elute the sample, 20-50μl of Buffer EB was added to the centre of the column membrane and the Eppendorf tube was centrifuged at 13,000xg for 1 minute. Buffer PB, Buffer PE and Buffer EB were provided by QIAgen PCR purification kit.

2.12 Mini-preparation of plasmid DNA (using QIAgen plasmid extraction kit)

The cells were cultured in 10ml LB broth with appropriate antibiotics at the 37℃ with shaking for 12 hours before plasmid extraction. To harvest the cells, the sample was centrifuged at 1,600xg for 15 minutes at room temperature to pellet the cells. The supernatant was discarded and the cell pellet was resuspended in 250μl of Solution 1/RNase A solution. The sample was then transferred into an Eppendorf tube and 250μl of Solution 2 was added and mixed by gently inversion and rotation. Then the sample was incubated for 2 minutes at room temperature. After that, 350μl of Solution 3 was added and immediately mixed with the sample by inversion.

The sample was centrifuged at 13,000xg for 10 minutes at room temperature and the supernatant was transferred to the QIAprep spin column. The sample was then centrifuged at 13,000xg for 1 minute and the flow-through was discarded. A volume of 500μl of Buffer PB was added followed by centrifugation at 13,000xg for 1 minute and the flow-through was discarded. A volume of 600μl of Buffer PE was added followed by centrifugation at 13,000xg for 1 minute and the flow-through was discarded. The sample was centrifuged at 13,000xg again for 1 minute to remove residual wash buffer. The QIAprep spin column was placed into a new Eppendorf tube, to elute the sample, 50μl of Buffer EB was added to the centre of the column membrane and the Eppendorf tube was centrifuged at 13,000xg for 1 minute. Solution 1/RNase A solution, Solution 2, Solution 3, Buffer PB, Buffer PE and Buffer EB were provided by QIAgen plasmid extraction kit.

2.13 Ligation

Ligation reaction was set up with T4 DNA ligase (50 units) (Promega) and Ligase

Buffer. Purified linear plasmids (30-50μg/μl) and purified PCR fragments (80-100μg/μl) with corresponding restriction sites on both terminals were added to be ligated. The ligation reaction conditions are shown in the table below. All samples and controls were incubated for 12 hours at 15℃.

Table 2.5 Ligation reaction conditions.

Name of reagant	Ligation Reaction	+Ligase Control	-Ligase Control
Linear Plasmid (30-50μg/μl)	2μl	2μl	2μl
Target DNA fragment (80-100μg/μl)	6μl	0μl	0μl
Ligase Buffer	1.5μl	1.5μl	1.5μl
Ligase (10-20μg/μl)	1μl	1μl	0μl
dH_2O	Added up to 15ul of total volume		

2.14 Preparation of competent cells

E. coli (DH5α, BL21 and S17-1 λ*pir*) were cultured overnight in 10ml LB broth with appropriate antibiotics at 37℃. 1ml of bacterial cells in LB broth was transferred into 100ml of sterile LB broth to make a 1:100 dilution. Then the culture was shaken at 37℃ for 2-4 hours until the OD_{600nm} of the culture reached 0.3 (approx. 4-7 x 10^7 cfu/ml). Then the culture was collected into two 50ml universal tubes and kept on ice for 15 minutes followed by centrifugation at 1,600xg for 20 minutes at 4℃. Then the cell pellets were resuspended in 33ml (1/3 of the original volume) of RF1 solution and the cells were incubated on ice for 15 minutes followed by centrifugation at 1,600xg for 20 minutes at 4℃. The cell pellet was then resuspended in 8ml (1/12.5 of the original volume) of RF2 solution and the cells were incubated on ice for 15 minutes. After that, the cells were aliquoted into chilled sterile Eppendorf tubes (200μl) and stored at -80℃.

RF1 solution

100mM KCl

RF2 solution

10mM MOPS buffer pH 6.8

50mM $MnCl_2$	10mM KCl
30mM Potassium acetate	75mM $CaCl_2 \cdot 2H_2O$
10mM $CaCl_2 \cdot 2H_2O$	15% glycerol (w/v)

2.15 Transformation

A volume of 100µl of competent cells was added into a sterile Eppendorf tube and 5-20ul of plasmid DNA was added to the Eppendorf tube and the cells were mixed with plasmid DNA. The Eppendorf tube was left on ice for 30 minutes. Then the cells were heat shocked in a 42℃ water bath for 90 seconds. After that, 1ml of LB broth was added into the Eppendorf tube and the sample was incubated at 37℃ for 1 hour. The cell culture (150µl) was then spread on LB agar plates with appropriate antibiotics. The cells were incubated overnight at 37℃.

2.16 Bacterial conjugation

Bacterial strains were cultured overnight in 10ml LB broth with appropriate antibiotics at 37℃ with shaking. The cell cultures were centrifuged at 1,600xg for 15 minutes to collect the cells. The supernatants were discarded and the pellets were resuspended in 5ml of steriled PBS Buffer. To wash the cells, the suspensions were centrifuged at 1,600xg for 15 minutes and the supernatants were discarded. The cell pellet of conjugal bacterial strain (donor) was resuspended in 1ml of sterile PBS Buffer. 0.5ml of the suspension of the conjugal bacterial strain (donor) was mixed with the pellet of the conjugal bacterial strain (recipient). 200µl of the mixed strains were plated onto blood agar plate. The plate was incubated at 37°C for 8 hours. Then the cells were scraped off the blood agar with 1ml of PBS Buffer. Serial dilutions (10^{-3} and 10^{-4}) were made and 200ul of each strain was spread onto LB agar plates with appropriate antibiotics. The plates were incubated overnight at 37°C to select colonies with the desired resistance.

Phosphate Buffered Saline (PBS) Buffer

1 tablet of PBS (SIGMA™) in 200ml dH_2O

200ml of PBS containing:

10mM Phosphate buffer

2.7mM KCl

137mM NaCl

2.17 Lipopolysaccharide (LPS) extraction

Bacterial cells were obtained from overnight LB broth culture grown at 37°*C*. 3ml of the culture was washed once in PBS Buffer and then centrifuged at 10,000xg for 3 minutes. The supernatant was discarded. The pellet was mixed with 1ml of PBS Buffer and vortexed. After that, the suspension was centrifuged at 13,000xg for 1 minute and the PBS solution was discarded. The pellet was resuspended in PBS Buffer until the A_{600} of suspension reached 1.0. The bacterial suspension was then centrifuged at 10,000xg for 3 minutes and resuspended in 200µl dH_2O. 200µl of 90% (w/v) phenol solution was added into the bacteria suspension and the mixture was vortexed and incubated for 10 minutes at 37°C. The mixture was kept on ice for 2 minutes followed by centrifugation at 2,300xg for 5 minutes. The upper liquid phase was collected and mixed with 1 volume of Lysis buffer and boiled for 5 minutes. The extracts were stored at -20°C. The sample was usually loaded in an SDS-PA gel and stained following the silver stain protocol (section 2.18).

2.18 Silver stain (Silver stain kit)

The SDS-PAGE gel was washed twice for 5 min with ultrapure water and fixed twice for 15 min in 30% ethanol and 10% acetic acid solution. Then the gel was washed twice for 5mins in10% ethanol and washed twice with ultrapure water for 5 min. After that, the gel was sensitized in Sensitizer Working solution for 1 min and washed twice with ultrapure water for 1 min. Then the gel was stained in Stain Working solution for 30 min. Then the gel was washed twice with ultrapure water for 20 sec and developed in Developer Working solution for 2-3 min until the bands were observed. Then the gel was stopped with 5% acetic acid for 10 min. Sensitizer Working solution, Stain Working solution and Developer Working solution were provided by Silver Stain kit.

2.19 Protein overexpression

The *E. coli* protein expression strain (BL21(DE3)) containing the plasmid of interest was incubated overnight at 37°C with shaking in 10ml LB broth with appropriate antibiotics. 5ml of the overnight culture was transferred into 500ml of LB broth with appropriate antibiotics on second day. The culture was incubated at 37°C for 2 - 4 hours until the OD_{600} reached 0.6-1.0 and 500µl of 1M IPTG was added to induce the protein expression. After overnight incubation at 37°C with shaking, the cells were harvested by centrifugation at 5,000xg for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 25ml Binding Buffer or Column Buffer and stored at -20°C. Then the sample was sonicated 5 - 6 times using Jencons Vibracell at 20 kHz (20% amplitude) for 30 seconds with 1 min intervals on ice. The sonicated sample was then centrifuged at 12,000xg for 15 minutes at 4°C to separate soluble and insoluble proteins. The supernatant which contained soluble proteins was stored in a new universal tube at -20°C and the centrifuge tube which contained the pellet was also stored at -20°C.

2.19.1 Binding Buffer preparation

Binding Buffer (50ml)	
25mM Tris-Cl pH 7.4	1.25 ml of 1M Tris-Cl stock solution
10mM Imidazole pH7.4	0.25ml of 2M imidazole stock solution
400mM NaCl pH7.0	4ml of 5M NaCl stock solution

2.20 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.20.1 Preparation and electrophoresis of polyacrylamide gel

Polyacrylamide gel consisted of resolving gel and stacking gel. The resolving gel was prepared firstly and poured into the electrophoresis device. It takes about 5

minutes to become solid. Then the stacking gel was prepared and poured into the electrophoresis device and a comb was inserted into the gel to make wells. The stacking gel takes about 5 min to become solid. The components of these gels are listed in the Table 2.7

The protein samples were prepared by boiling with an equal volume of Laemmli Buffer at 100°C for 5-10 minutes. To each well of SDS-PA gel, 20µl of the sample was loaded and corresponding protein ladder is shown in Appendix 1. The gel was run at 160V for 90-120 minutes in 1X SDS Running Buffer. After that, the gel was stained with Coomassie Blue Stain Solution for 12 hours and then destained with Destain Solution until the bands were clear.

Table 2.6 SDS-PAGE Resolving Gel components (Pour first).

%Gel	Acrylamide (30% w/v)	Tris (1.5M pH 8.8)	H_2O	TEMED		APS (10% w/v)	SDS (10% w/v)
5	1.65ml	2.5ml	5.65ml	7µl		200µl	100µl
10	3.3ml	2.5ml	4.0ml	7µl		200µl	100µl
12	4.0ml	2.5ml	3.3ml	7µl		200µl	100µl
14	4.66ml	2.5ml	2.64ml	7µl		200µl	100µl
15	5.0ml	2.5ml	2.3ml	7µl		200µl	100µl

Table 2.7 SDS-PAGE Stacking Gel components (Pour last).

Acrylamide (30% w/v)	Stacking buffer	H_2O	TEMED	APS (10% w/v)
0.68ml	1.25ml	3ml	5µl	50µl

The percentage of resolving gel used was dependent upon the expected size of the protein sample. 12% of resolving gel was used in this project to check the products of protein overexpression and protein purification.

2.20.2 Buffer preparation

Tris (1.5M pH 8.8)	45.43g in 250ml of dH_2O

Laemmli Buffer

62.5mM Tris (pH 6.8)

2% (w/v) SDS

5% (v/v) β-Mercaptoethanol

10% (v/v) Glycerol

0.02% (w/v) Bromophenol Blue

10X SDS Running Buffer (pH 8.3)	Amount for 1 litre dH_2O
250mM Tris-Base	30.2g
1.92M Glycine	144g
1% (w/v) SDS	10g

Coomassie Blue Stain Solution (1L)

500ml Methanol

2.5g Coomassie Blue

100ml Acetic acid

dH_2O added up to 1L

Destain Solution (1L)

400ml Methanol

100ml Acetic acid

dH_2O added up to 1L

2.21 Histidine-tagged protein purification protocol

2.21.1 Column preparation

The HisTrap™ HP Column was washed with 5ml distilled H_2O and equilibrated with 10ml binding buffer.

2.21.2 Purification protocol

The supernatant which contained soluble proteins (Load) was taken out from the -20°C freezer and the sample was prepared by filtration through a 0.45µm filter to remove any debris and the flow-through (FT) was collected. Then the protein sample was applied to the HisTrap™ HP Column using a flow rate of 1-3 ml/min and the flow-through (Wash) was collected. The proteins with His-tag were all bound to the nickel ions in the column. The column was washed with 10ml binding buffer. The proteins with His-tag were then eluted with 5ml Binding Buffer with increasing imidazole concentration (50mM to 500mM) in 1ml fractions. All fractions were checked by SDS-PAGE including the sample Load, sample FT and sample Wash. From each sample, 20ul was taken and mixed with 20ul of Laemmli buffer. Then all the mixtures were boiled for 10 minutes and checked by SDS-PAGE.

Table 2.8 Binding Buffer with increasing concentration of imidazole[a].

Imidazole concentration	2M imidazole[b] (ml)	1M Tris-Cl (ml)	5M NaCl (ml)	dH_2O (ml)
50mM	0.5	0.5	1.6	17.4
100mM	1	0.5	1.6	16.9
150mM	1.5	0.5	1.6	16.4
200mM	2	0.5	1.6	15.9
250mM	2.5	0.5	1.6	15.4
300mM	3	0.5	1.6	14.9
400mM	4	0.5	1.6	13.9
500mM	5	0.5	1.6	12.9

(a) Binding Buffer was prepared according to protocol 2.19.1.

(b) 2M Imidazole stock was prepared by dissolving 6.8g imidazole in 50ml dH_2O and adjusted to pH 6.8.

2.22 MBP-tag protein purification protocol

2.22.1 Column preparation

The MBPTrap™ HP Column was washed with 5ml distilled H_2O and equilibrated with 10ml column cuffer.

2.22.2. Purification protocol

The supernatant which contained soluble proteins (Load) was taken out from the -20°C freezer and the sample was prepared by filtration through a 0.45μm filter to remove any debris and the flow-through (FT) was collected. Then the protein sample was applied to the MBPTrap™ HP Column using a flow rate of 1-3 ml/min and the flow-through (Wash) was collected. The proteins with MBP-tag were all bound to the beads in the column. The column was washed with 10ml binding buffer. The proteins

with His-tag were then eluted with 5ml column binding buffer with increasing maltose concentration (20mM to 300mM) in 1ml fractions. All fractions were checked by SDS-PAGE including the sample Load, sample FT and sample Wash. From each sample, 20ul was taken and mixed with 20ul of Laemmli buffer. Then all the mixtures were boiled for 10 minutes and checked by SDS-PAGE.

2.22.1 Column Binding Buffer preparation

Column buffer:	Amount per litre dH_2O
20 mM Tris-HCl	20 ml 1 M Tris-HCl (pH 7.4)
200 mM NaCl	11.7 g NaCl
1 mM EDTA	2.0 ml 0.5 M EDTA

2.23 Western Blotting

After SDS-PAGE, one of the two gels was applied to be analysed by Western Blot.

The gel was placed in a western blotting cassette (BIO-RAD™) along with a nitrocellulose membrane (Whatman™ 0.45μm pore size) for proteins to be transferred onto and 3 filter papers of similar shape with the gel. The blotting cassette was assembled and placed in a tank filled up with 1X Transfer Buffer and run at 10V overnight. The blotting cassette was set up to direct the proteins passed from the gel towards the membrane. Then the membrane was collected and soaked in 20ml of 5% (w/v) skimmed milk solution (Marvel™) for 1hour to block all non-specific binding sites. Then the membrane was washed in 20ml PBS Buffer for 10 min. After that, the membrane was soaked in 20ml of 5% (w/v) skimmed milk solution in which 1-5μl (1:2000) of primary antibody was added for 1 hour. Then the membrane was washed 3 times in 10ml of PBS solution for 5 minutes. To bonded by the secondary antibody, the membrane was soaked in 20ml of 5% (w/v) skimmed milk solution in which 1-5μl (1:4000) of secondary antibody was added for 1 hour. Then the membrane was washed 3 times in 10ml of PBS Buffer for 5 minutes again.

To develop the membrane, Pierce™ ECL Western Blotting Substrate (Thermo Scientific™) was applied. 1ml of Detection Reagent 1 (Peroxide Solution) was mixed with 1ml of Detection Reagent 2 (Luminol Enhancer Solution) before using and the mixture was poured on the membrane. The western blot photo was taken using Chemidoc™ XRS+ System (Bio-Rad™).

2.23.1 Buffer preparation

10X Transfer Buffer (1L) (pH 9.0)

Glycine	144g
Tris-Base	30.2g

dH_2O added up to 1L

Chapter 3 Investigation of glycosylation in flagellin biosynthesis

3.1 Introduction

The flagellum consists of a basal body, the hook-associated proteins and the filament and they are essential for bacterial motility. The flagella filament is composed of a polymer of flagellin proteins. There are two polar flagellins (FlaA and FlaB) and two lateral flagellins (LafA1 and LafA2) expressed in *A. caviae.* In *A. caviae* Sch3N, flagellins of the polar flagella are glycosylated 6 to 8 times by Pse5Ac7Ac using the enzyme Maf1 which is known to be a flagellin glycosyl transferase (Parker et al. 2012).

The structure of flagella filament from *Salmonella typhimurium* has been described and the structure of the flagellin is similar in all bacteria. Starting from the N-terminus there are four domains including D0, D1, D2 and D3. The C-terminal chain folds back to D0 to form a coiled coil (Figure 3.1). The D0 and D1 are basically buried inside of the structure of the filament while the central D2 and D3 domains are exposed at the surface when the filament is polymerised. The D2 and D3 domains are considered to be recognized by glycosyltransferase in bacteria such as *A. caviae,* although they are not glycosylated in *Salmonella* (Tabei et al. 2009; Yonekura et al. 2003). These findings suggest that the D2 and D3 are where the flagellar glycosylation occurs. A recent study in *A. caviae* suggests that Maf directly binds the flagellin and is responsible for glycosylation, it does this in a chaperone independent manner, as deletion of the chaperone or removal of the chaperone binding domain on the flagellin still results in glycosylated flagellin. Although the flagellin is not secreted.

The *fla* locus of *A. caviae* Sch3N is linked to the biosynthesis of polar flagella filament. This locus includes the genes *flaA*, *flaB*, *flaG*, *flaH* and *flaJ* which have been listed in genetic order (Rabaan et al. 2001). The *flaA* and *flaB* genes of *A. caviae* Sch3N encode two flagellin subunits. Strains that lack *flaA* and *flaB* show no motility and further study proved that production of polar flagellins in this strain is absolutely terminated (Rabaan et al. 2001). Motility and adherence drop to about 50% in the strain with single mutation in either *flaA* or *flaB* in which flagella are still expressed. This finding indicates that *flaA* and *flaB* compose flagellin with a 1:1 ratio (Rabaan et al. 2001).

In conclusion, glycosylation and assembly of flagellins of polar flagella in *A. caviae* Sch3N can be described like this: The original flagellin synthesized from FlaA and FlaB has a central D2/D3 domain that is glycosylated by Maf and a C-terminal chaperone-binding domain (CBD) which can directly interact with the chaperone FlaJ. It is not known where Maf binds the flagellin, but it does not appear to be the CBD as the removal of the CBD still results in a glycosylated flagellin. However, Maf glycosylates the central D2/D3 domain of the flagellin with Pse5Ac7Ac using the activated form of CMP-Pse5Ac7Ac as a substrate (Tabei et al. 2009; Yonekura et al. 2003).

Maf1 is thought to be bound to the central D2/D3 domain of the flagellin and uses CMP-Pse5Ac7Ac to glycosylate the flagellin with Pse5Ac7Ac. After that, Maf1 is released from the glycosylated flagellin for the next circulation (Parker et al. 2012). Secondly, as it was mentioned before, FlaJ is bound to the C-terminal chaperone-binding domain (CBD) of glycosylated flagellin (FlaA /FlaB+Pse) and this complex is transported to the basal body of flagellum. Finally, the glycosylated flagellin is discharged from intracellular environment via flagellar specific T3SS. The exported flagellin will be polymerized by FlaH the cap protein at the distal tip and the complete flagellar filament will be produced. Glycans can attach to the surface of the filament after flagellin is folded (Parker et al. 2012; Parker et al. 2014) (Figure 3.1).

Therefore, in this chapter we wished to determine, how much of the C-terminal domain of the flagellin could we remove before glycosylation stopped, this would allow further mapping of the flagellin for glycosylation purposes.

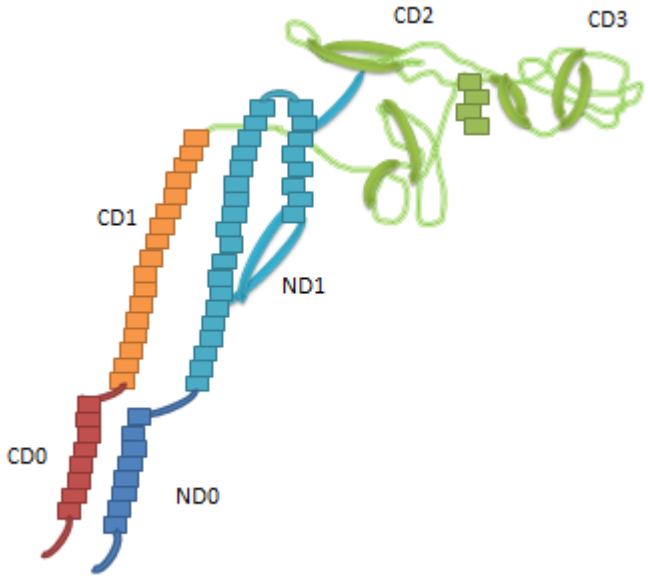


Figure 3.1 Cartoon of the structure of the flagellin subunit.

The flagellin subunit is composed of several domains, the D0 domain is composed of two alpha helices at both the N and C-terminal end of the polypeptide, as is the D1 domain. These two domains are internalized in the polymerized filament. The D2 and D3 domains make up the surface exposed areas of the filament and are the ones that are glycosylated.

3.2 Construction of *A. caviae flaA-flaB* mutant

In order to investigate the properties of a series of flagellin deletion constructs in *A. caviae* from the plasmid pBBR1-MCS (Cm^R), an *Aeromonas caviae* flagellin negative background strain needed to be created. The previous one described by Rabaan 2001 had insertions in both FlaA and FlaB, carrying chloramphenicol resistance and kanamycin resistance respectively. Therefore, it was decided to create an insertional deletion mutant strain in which part of both *flaA* and *flaB* were deleted and replaced with the selectable kanamycin resistance marker.

In order to create this mutant, 2 DNA fragments including *flaA1* and *flaB2* were designed and obtained by PCR first and these DNA fragments represented the 5' portion of *flaA* and 3' portion of *flaB* respectively as shown in Figure 3.3. A kanamycin resistance cassette which is a PCR amplified Tn5-derived kanamycin resistance cartridge (*nptII*)) was also isolated. Subsequently the mobilizable suicide plasmid carrying streptomycin

resistance pKNG101 was digested using the restriction enzyme BamHI (Figure 3.2). All DNA fragments were substrates for isothermal assembly.

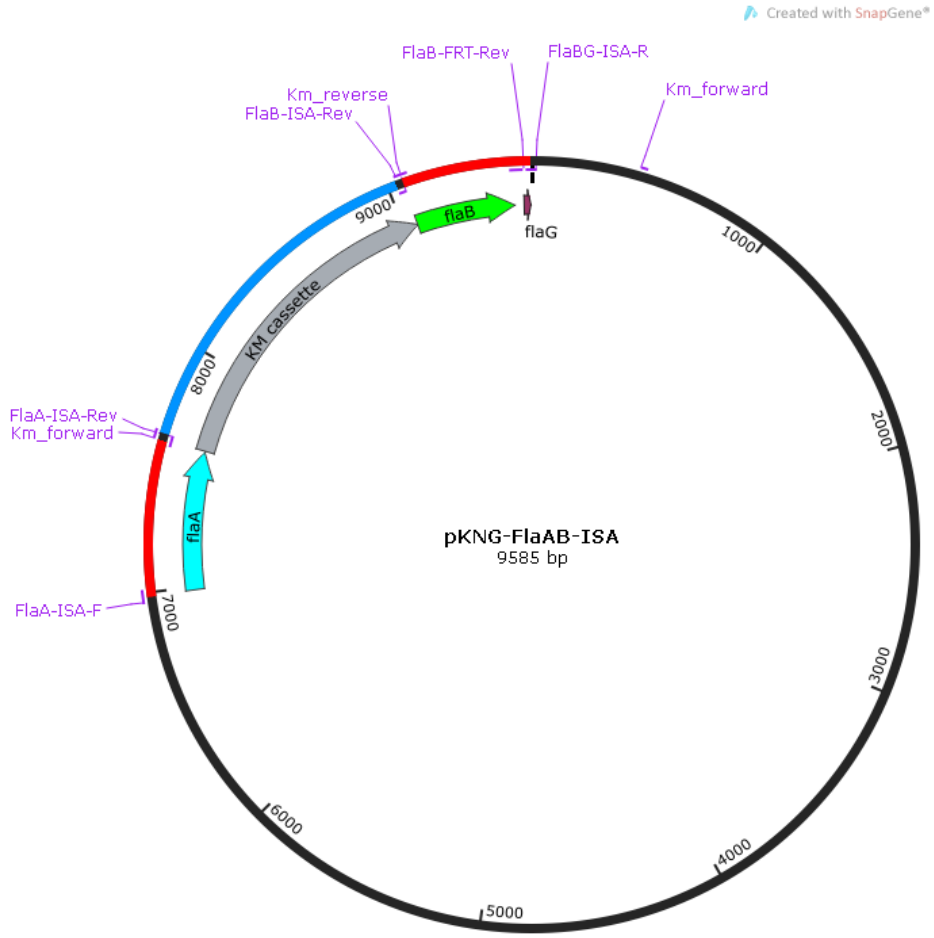
The DNA fragments and plasmid used in Isothermal Assembly are listed in Table 3.1. The concentrations of all DNA samples were measured using nanodrop to ensure they were equimolar. After that, ISA was applied and the reaction mix was transformed into *E. coli* CC118λ*pir* to be selected (Sm^R and Km^R). Resistant colonies were isolated and the possible recombinant vectors were analysed by PCR screening using primers including flaAISAF1 and flaBISAR2 (section 2.7.4) (Figure 3.4). Then the selected pKNG101::*flaA*::Km::*flaB* (Figure 3.2) was confirmed by DNA sequencing and retransformed into *E. coli* S17-1 λ*pir* for the conjugation with *A. caviae* Sch3N (Nal^R). The strains obtained from conjugation were resistant to nalidixic acid and kanamycin and were replica plated on two TSA plates. They were tested to see if they were oxidase positive (*Aeromonas*) versus negative (*E. coli*) One TSA plate contained Nal and Km, the other plate contained Km and Sm. Strains that were Km resistant but Sm sensitive, were thought to have undergone allelic exchange through a double homologous recombination event. Those were subsequently checked by a diagnostic PCR using primers including flaAISAF1 and flaBISAR2 to confirm the result (Figure 3.5). This resulted in the *flaA-flaB* mutant strain *A. caviae* Sch3N *flaA*::Km::*flaB*.

The size of PCR product using flaAISAF1 and flaBISAR2 as primers and *A. caviae* Sch3N (WT) genomic DNA as template is about 2500 bp. The size of PCR product using flaAISAF1 and flaBISAR2 as primers and *A. caviae flaA*::Km::*flaB* genomic DNA as template is about 2600 bp. There is a restriction cutting site which can be recongnized by HindIII between *flaA* gene and *flaB* gene in *A. caviae* Sch3N and this restriction cutting site is removed in *A. caviae flaA*::Km::*flaB.* In order to tell the difference between the above two PCR products, both of the PCR products were digested by HindIII. It can be observed that the PCR product (2500 bp) using *A. caviae* Sch3N (WT) genomic DNA as template was digested while the PCR product (2600 bp) using *A. caviae flaA*::Km::*flaB* genomic DNA as template was not digested. This result indicates that the *flaA* and *flaB* genes in *A. caviae flaA*::Km::*flaB* were knocked out by kanamycin resistance cassette insertion (Figure 3.5B).

Table 3.1 DNA fragments and plasmid used in ISA and primers of *flaA1* and *flaB2.* Complementary termini of 40bp sequence homology were added to the original Km cassette, *flaA1* and *flaB2* genes.

Names	Sizes (bp)	Primers
flaA1	676 bp	*flaA*ISAF1: 5' CCCCCCCCCTGCAGGTCGACGGATCTCAATACCAACGTTTCATCGC
		*flaA*ISAR1: 5' TCAAACATGAGAACCAAGGAGAATACTCTGAGTGCTAATACTGATCC
flaB2	510 bp	*flaB*AISAF2:5' GTTTTAGTACCTAGCCAAGGTGTGCTTAGCTTGTCTCAAGCCGGAG
		*flaB*ISAR2: 5' GCTGCTCCTCATATCAATGATGTTGACGTTAAGGCCCGCTTAACCCAGCAGCTGCAG
Km cassette	1492bp	Kmfor: 5' TTCTCCTTGGTTCTCATGTTTGACAGCTT
		Kmrev: 5' GCACACCTTGGCTAGGTACTAAAACAATTC
pKNG101	6986bp	N/A
pKNG101::*flaA*::Km::flaB	9585bp	N/A

(A)


Created with SnapGene®
FlaB-FRT-Rev
FlaBG-ISA-R
Km_reverse
FlaB-ISA-Rev
Km_forward
9000
flaB
flaG
1000
KM cassette
8000
2000
FlaA-ISA-Rev
Km_forward
flaA
pKNG-FlaAB-ISA
9585 bp
FlaA-ISA-F
7000
3000
6000
4000
5000

(B)

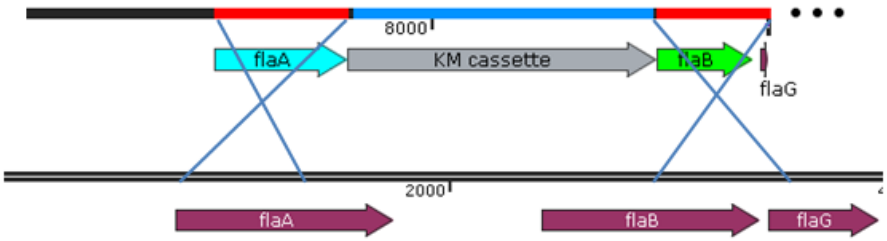

8000
flaA
KM cassette
flaB
flaG
2000
flaA
flaB
flaG

Figure 3.2 Isothermal assembly (ISA) method for construction of pKNG101::*flaA*::Km::*flaB* and construction of *A. caviae flaA*::Km::*flaB*

(A) This plasmid was constructed using Isothermal assembly of the 676bp 5' *flaA1* fragment the 1492bp KmR cassette, and the 510bp 3' *flaB2* fragment assembled into BamHI digested pKNG101. The PCR fragments *flaA1* and *flaB2* were generated with primers that had overhangs with the pKNG101 and kanamycin cassette (*flaA1*) and the kanamycin cassette and pKNG101 (*flaB2*) respectively. The four DNA fragments *flaA1*, KmR, *flaB2* and pKNG101 were mixed together at equimolar concentrations and assembled together using ISA.

(B) A diagrammatic representation of allelic exchange between the suicide vector construct pKNG101::*flaA*::Km::*flaB* and the *flaA* and *flaB* genes on the chromosome of *A. caviae* Sch3N following transfer of suicide plasmid from *E. coli* CC118λ*pir* into the *Aeromonas* cells. Potential double crossover mutants were selected by replica plating. Each colony was patched on to two LB agar plates, one with kanamycin and nalidixic acid and the other one with kanamycin and streptomycin. Those that were streptomycin sensitive were taken for further investigation. The features of the plasmid vector are shown by arrows, the *flaA* gene is shown by a blue arrow, the *flaB* gene is shown by a green arrow and the Km cassette is shown by a grey arrow. The figure was created using SnapGene®software.

1 2 3

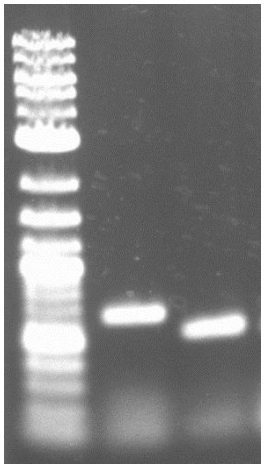


Figure 3.3 Analysis of PCR products following amplification of *flaA* gene fragment and *flaB* gene fragment using *A. caviae* Sch3N genomic DNA as template.

PCR was carried out using Q5 DNA polymerase and *A. caviae* Sch3N genomic DNA. 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products are shown in Table 3.1. Lane 1, 2-Log linear DNA ladder (Appendix 1); lane 2, *flaA1* gene fragment (676bp); lane 3, *flaB2* gene fragment (510bp).

1 2

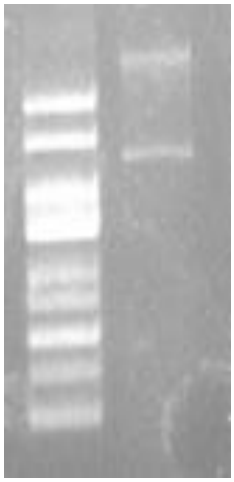


Figure 3.4 Analysis of mini-preparation of the pKNG101::*flaA*::Km::*flaB* plasmid.

Plasmid DNA product was carried out using mini-preparation of plasmid DNA following section 2.12 protocol. 5 ul of the plasmid DNA products were observed following electrophoresis in a 1% agarose gel. Lane 1, supercoiled DNA ladder (Appendix 1); lane 2, pKNG101::*flaA*::Km::*flaB* (9585bp).

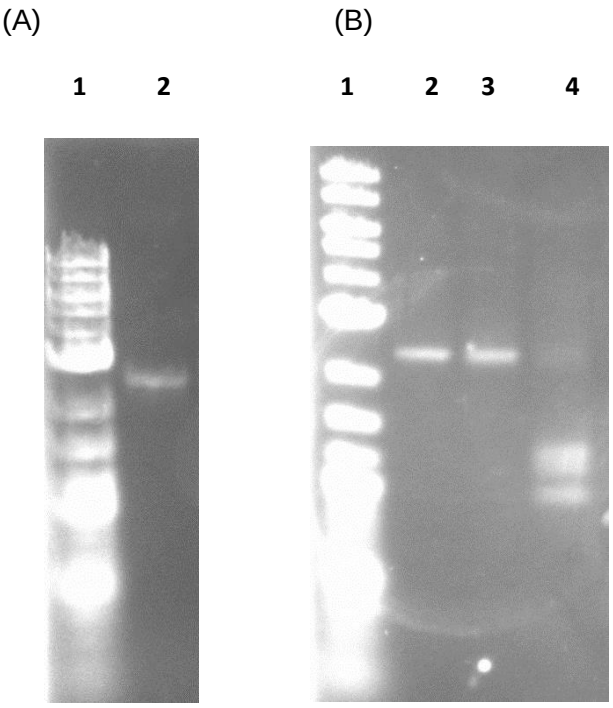


Figure 3.5 Analysis of PCR products following amplification of a DNA fragment containing the *flaA* and *flaB* genes using *A. caviae* Sch3N (WT) and *A. caviae flaA*::Km::*flaB* genomic DNA as template.

PCR was carried out using Q5 DNA polymerase and *A. caviae* Sch3N genomic DNA and *A. caviae* Sch3N *flaA*::Km::*flaB* genomic DNA. 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products were shown in Table 3.1.

(A) Lane 1, 2-Log linear DNA ladder; lane 2, PCR product containing *flaA1* gene and *flaB2* gene from *A. caviae* Sch3N (WT) (~2500bp) using *flaA*ISAF1 and *flaB*ISAR2 as primers;

(B) Lane 1, 2-Log linear DNA ladder; lane 2, PCR product containing *flaA1* gene and *flaB2* gene from *A. caviae flaA*::Km::*flaB* (~2600bp) using *flaA*ISAF1 and *flaB*ISAR2 as primers; lane 3, PCR product containing *flaA1* gene and *flaB2* gene from *A. caviae flaA*::Km::*flaB* after restriction enzyme digestion with HindIII; lane 4, *flaA1* gene and *flaB2* gene from *A. caviae* Sch3N (WT) after restriction enzyme digestion with HindIII.

3.3 Construction of a series of *flaA* gene mutants

In order to express various flagellin deletion derivatives in the *A. caviae flaA-flaB* mutant, primers were designed to result in a sequentially smaller flagellin encoding PCR fragment reducing from the 3' end. The PCR's all used the same forward primer, but different reverse primers that incorporated a new stop codon that would stop translation of the flagellin in each of the constructs. Giving rise to a series of flagellin that were sequentially 6 amino acids smaller than the previous construct. Both sets of primers incorporated with HindIII cutting site to help with cloning.

Construction of pBBR1-MCS containing *flaA* gene deletion constructs was required in this project. A series of *flaA* gene fragments were cloned into pBBR-1MCS via restriction enzyme digestion and ligation. There are 17 primers designed in this project (Appendix 1) and 16 kinds of *flaA* gene fragments that were amplified via PCR (Figure 3.6). After digestion with HindIII and ligation, 32 kinds of pBBR1-MCS *flaA* gene insertion were created (Table 3.2). The *flaA* gene fragments were cloned into pBBR1-MCS in both orientations due to digestion of the plasmid with a single enzyme and both sets of constructs would be tested in the subsequent experiments.

The *flaA* gene and *flaA* gene deletions which have the same orientation as the plasmid *lacZα* gene were labeled with "-" and the *flaA* gene and *flaA* gene deletions which have the opposite orientation as *lacZα* gene were labeled with "+". There is a *lac* promoter at the upstream region of the multiple cloning site (MCS) which possibly regulates the expression of *flaA* gene and *flaA* gene deletions inserted in the "-" orientation.

The constructs reduced the *A. caviae* FlaA flagellin from its wild-type length of 306 amino acids in construct FlaA0 to 220 amino acids in length in construct FlaA15 (Figure 3.6 and Table 3.3).

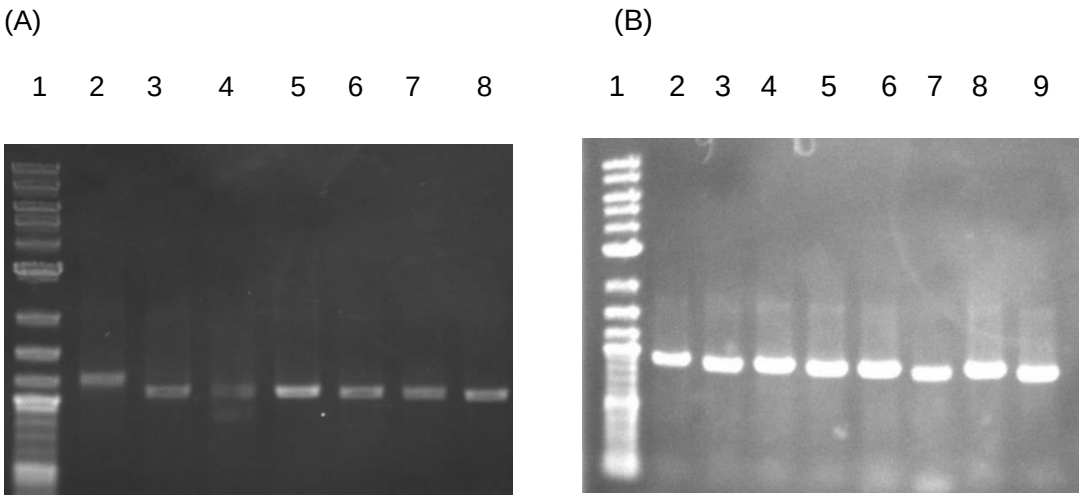


Figure 3.6 Analysis of PCR products following amplification of *flaA* gene fragment using pBBR1-MCS-*flaA* series plasmids (minus orientation) as template.

PCR was carried out using Q5 DNA polymerase and pBBR1-MCS-flaA series plasmids (minus orientation). 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products are M13 forward primer and M13 reverse primer.

(A) Lane 1, 2-Log linear DNA ladder; lane 2, *flaA1*- gene fragment (1187bp); lane 3, *flaA2*- gene fragment (1169bp); lane 4, *flaA3*- gene fragment (1151bp); lane 5, *flaA4*- gene fragment (1133bp); lane 6, *flaA5*- gene fragment (1115bp); lane 7, *flaA6*- gene fragment (1097bp); lane 8, *flaA7*- gene fragment (1079bp). lane 9, *flaA8*- gene fragment (1061bp).

(B) Lane1, 2-Log linear DNA ladder; lane 2, *flaA9*- gene fragment (1043bp); lane 3, *flaA10*- gene fragment (1025bp); lane 4, *flaA11*- gene fragment (1007bp); lane 5, *flaA12*- gene fragment (989bp); lane 6, *flaA13*- gene fragment (971bp); lane 7, *flaA14*- gene fragment (953bp); lane 8, *flaA15*- gene fragment (935bp).

Table 3.2 pBBR1-MCS derivatives containing *flaA* fragments.

flaA gene fragment	Opposite to *lacZα* gene orientation	Same as *lacZα* gene orientation
flaA	pBBR1-MCS-*flaA0*+	pBBR1-MCS-*flaA0*-
flaA1	pBBR1-MCS-*flaA1*+	pBBR1-MCS-*flaA1*-
flaA2	pBBR1-MCS-*flaA2*+	pBBR1-MCS-*flaA2*-
flaA3	pBBR1-MCS-*flaA3*+	pBBR1-MCS-*flaA3*-
flaA4	pBBR1-MCS-*flaA4*+	pBBR1-MCS-*flaA4*-
flaA5	pBBR1-MCS-*flaA5*+	pBBR1-MCS-*flaA5*-
flaA6	pBBR1-MCS-*flaA6*+	pBBR1-MCS-*flaA6*-
flaA7	pBBR1-MCS-*flaA7*+	pBBR1-MCS-*flaA7*-
flaA8	pBBR1-MCS-*flaA8*+	pBBR1-MCS-*flaA8*-
flaA9	pBBR1-MCS-*flaA9*+	pBBR1-MCS-*flaA9*-
flaA10	pBBR1-MCS-*flaA10*+	pBBR1-MCS-*flaA10*-
flaA11	pBBR1-MCS-*flaA11*+	pBBR1-MCS-*flaA11*-
flaA12	pBBR1-MCS-*flaA12*+	pBBR1-MCS-*flaA12*-
flaA13	pBBR1-MCS-*flaA13*+	pBBR1-MCS-*flaA13*-
flaA14	pBBR1-MCS-*flaA14*+	pBBR1-MCS-*flaA14*-
flaA15	pBBR1-MCS-*flaA15*+	pBBR1-MCS-*flaA15*-

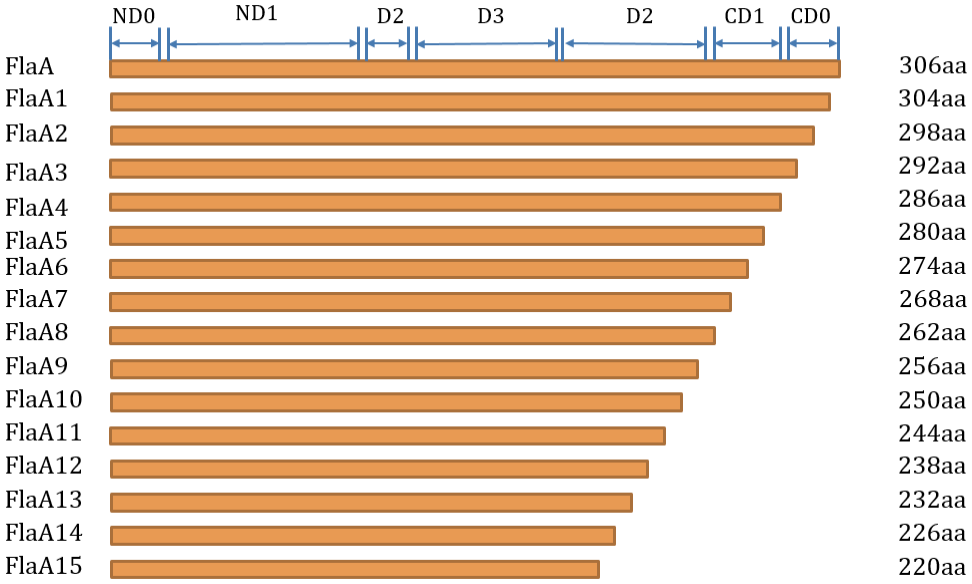


Figure 3.7 The amino acid number of each expressed FlaA deletion derivative from pBBR1-MCS-*flaA* series plasmids

Starting with the N-terminal chain there are four domains including D0, D1, D2 and D3, the C-terminal chain is back to D0 to form a coiled coil. FlaA and FlaA deletions are listed in order of decreasing size and the amino acid number of each FlaA and FlaA deletions are described too. The deletions sequentially remove the CD0 and CD1 domain components from the C-terminal end of the flagellin.

Table 3.3 The pBBR1-MCS-*flaA* series plasmids and components amino acid number of each expressed FlaA deletion.

Name of plasmid	Name of expressed protein	Number of amino acids
pBBR1-MCS-*flaA 0*-	FlaA	306
pBBR1-MCS-*flaA1*-	FlaA1-	304
pBBR1-MCS-*flaA2*-	FlaA2-	298
pBBR1-MCS-*flaA3*-	FlaA3-	292
pBBR1-MCS-*flaA4*-	FlaA4-	286
pBBR1-MCS-*flaA5*-	FlaA5-	280
pBBR1-MCS-*flaA6*-	FlaA6-	274
pBBR1-MCS-*flaA7*-	FlaA7-	268
pBBR1-MCS-*flaA8*-	FlaA8-	262
pBBR1-MCS-*flaA9*-	FlaA9-	256
pBBR1-MCS-*flaA10*-	FlaA10-	250
pBBR1-MCS-*flaA11*-	FlaA11-	244
pBBR1-MCS-*flaA12*-	FlaA12-	238
pBBR1-MCS-*flaA13*-	FlaA13-	232
pBBR1-MCS-*flaA14*-	FlaA14-	226
pBBR1-MCS-*flaA15*-	FlaA15-	220

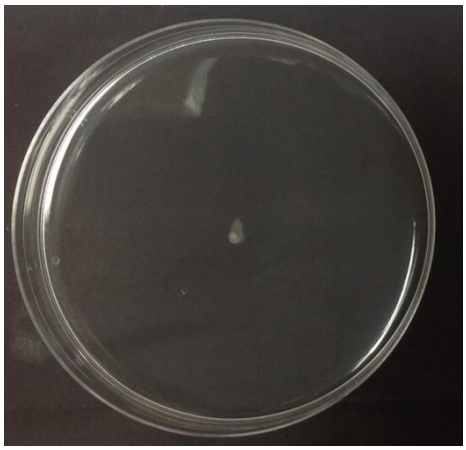
3.4 Swimming motility assay of *A. caviae flaA-flaB* mutant and complemented strains.

Mutation of the *fla* locus genes have been shown to affect motility (Rabaan et al. 2001). In order to determine the influence of the *flaA* constructs on swimming motility of *A. caviae* Sch3N, swimming motility assays were carried out with the *A. caviae* Sch3N wild-type (WT), the *A. caviae flaA-flaB* mutant and the mutant complemented with pBBR1-MCS-*flaA* series plasmids.

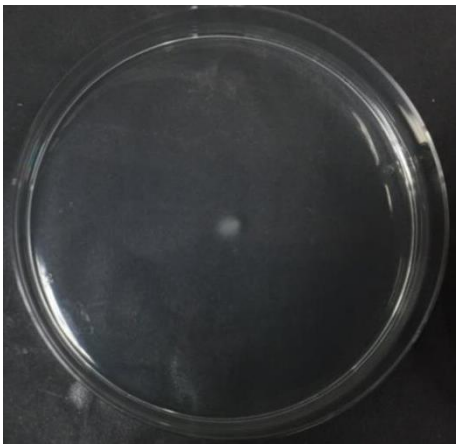
The *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*-) strain was created via conjugation of the *A. caviae flaA-flaB* mutant and S17-1 λ*pir* (pBBR1-MCS-*flaA0*-). The *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*+) was created via conjugation of *A. caviae flaA-flaB* mutant and S17-1 λ*pir* (pBBR1-MCS-*flaA0*+). The plasmids pBBR1-MCS-*flaA0*+ and pBBR1-MCS-*flaA0*- were transfered into the *A. caviae flaA-flaB* mutant to see if these plasmids can compensate the swimming ability of *A. caviae flaA-flaB* mutant and to determine if the presentation of the gene in the plasmid had any effect on the complementation assay.

When tested for the ability to swim though semi-solid motility agar as expected the *A. caviae flaA-flaB* mutant strain was found to be non-motile compared to wild-type (WT) (Figure 3.8 and Figure 3.9). *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*+) strain was found to be non-motile which means the motility deficiency of *A. caviae flaA-flaB* mutant was not compensated. The *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*-) was found to be motile which means the motility deficiency of the *A. caviae flaA-flaB* mutant was compensated (Figure 3.8 and Figure 3.9). Six motility plates were set up and three repeats were carried out (18 plates in total). Plasmids containing flaA1+~flaA15+ and flaA1-~flaA15- did not show the ability to compensate the motility deficiency of *A. caviae flaA-flaB* mutant (Data not shown).

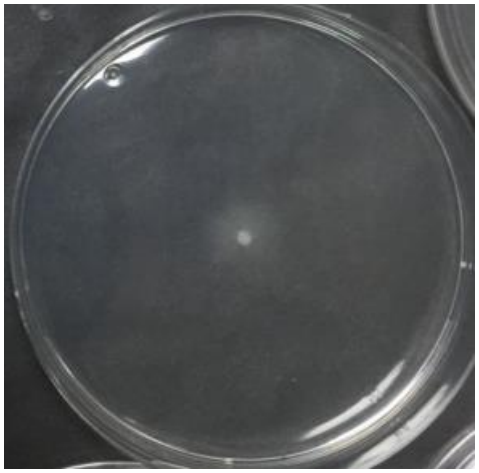
(A)



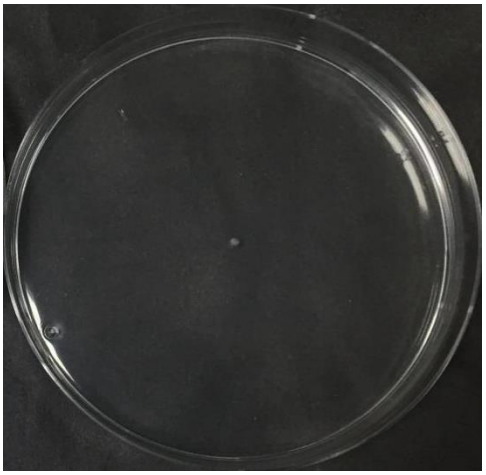
(B)



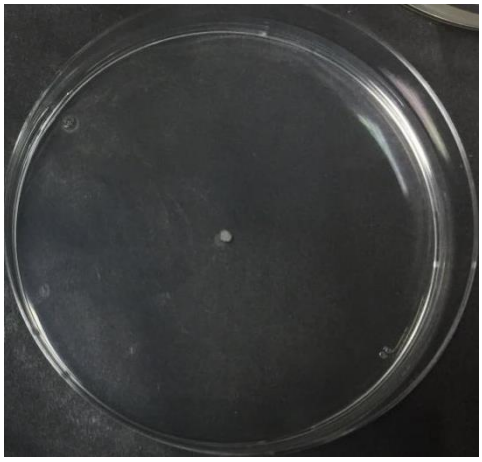
(C)



(D)



(E)



(F)

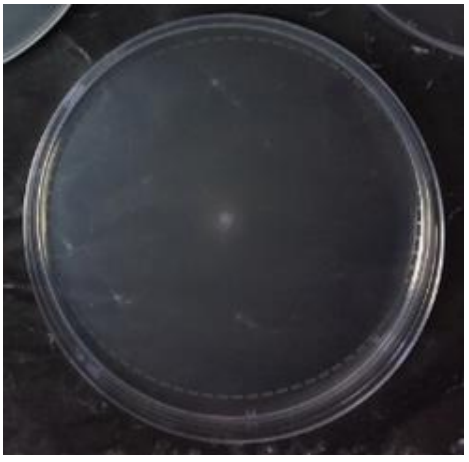


Figure 3.8 Swimming motility assay of *A. caviae* Sch3N (WT), *A. caviae flaA-flaB* mutant and complemented strains

Strains were incubated at room temperature for 16 hours. Motility was assessed by the production of a halo caused by bacterial migration.Strains of *A. caviae* Sch3N tested for motility on 0.25% motility agar.

(A) shows *A. caviae flaA-flaB* mutant is non-motile on swimming motility agar.

(B) shows *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*-) is motile on swimming motility agar.

(C) shows *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*+) is non-motile on swimming motility agar.

(D) shows *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*-) is non-motile on swimming motility agar.

(E) shows *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*+) is non-motile on swimming motility agar.

(F) shows *A. caviae* Sch3N (WT) is motile on swimming motility agar.

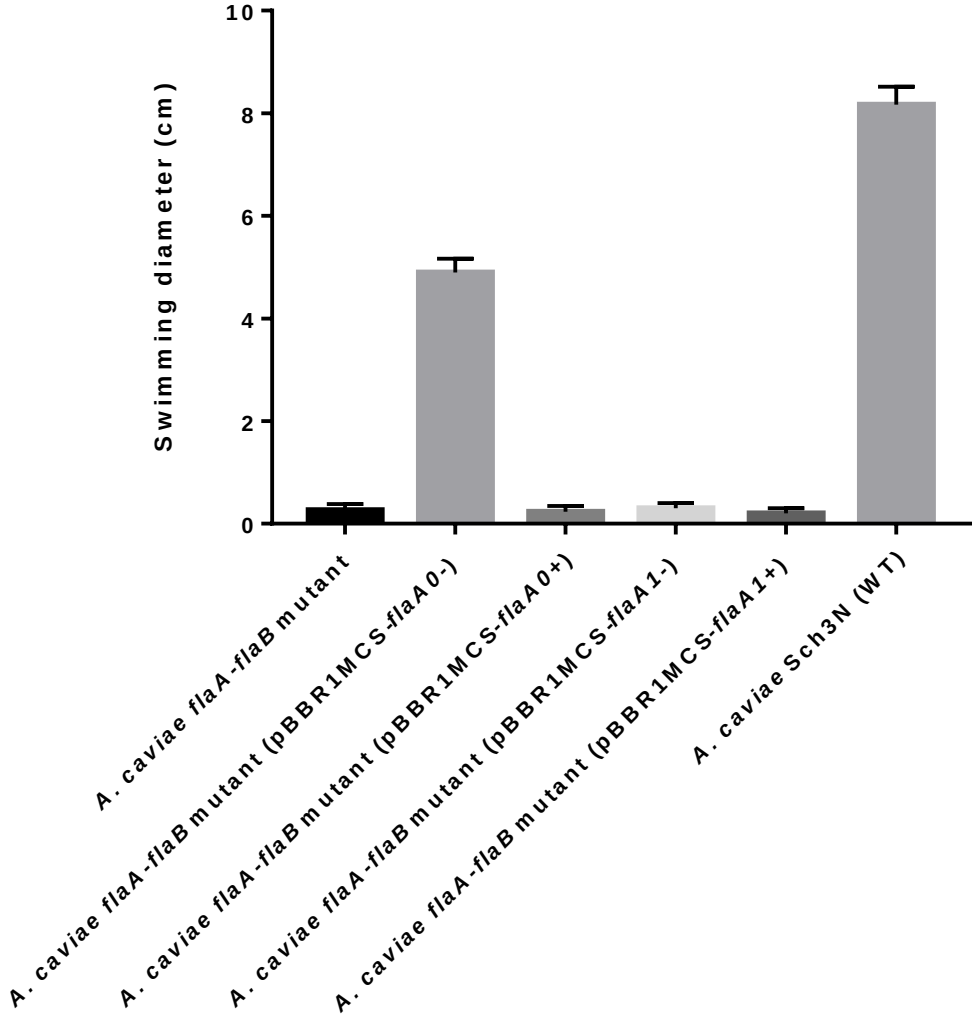


Figure 3.9 Quantification of the swimming assays of *A. caviae* Sch3N (WT) and mutant strains.

The swimming diameter of each strain was measured on the swimming agar after overnight incubation at 37°C. The swimming diameters were significantly lower in the *flaA-flaB* mutant when compared to the *A. caviae* Sch3N wild type ($p<0.001$). The swimming diameters of *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*+) was considerably greater than the untransformed mutant, thereby demonstrating complementation. *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*-) and *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*+) were significantly decreased when compared to the *A. caviae* Sch3N wild type ($p<0.001$). The swimming diameters were measured 3 times for each strain. The error bars showed Standard Error of the Mean (SEM). The significance was determined using Student's t-test.

3.5 Western blotting of FlaA deletion derivatives in complemented strains

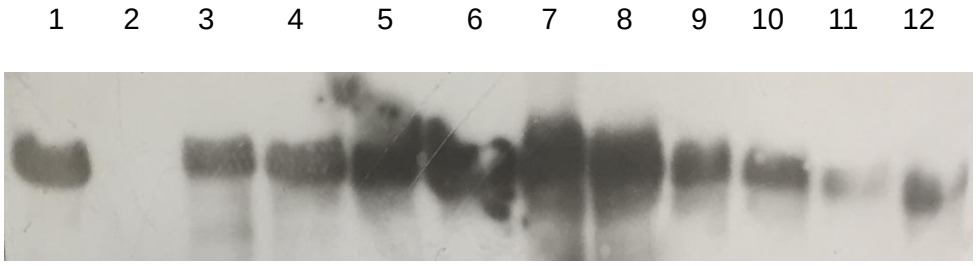
In order to determine how much of the C-terminal section of the flagellin is required for glycosylation and to begin to determine where Maf1 binds flagellin western blot analysis was performed to see if the deletion constructs were still able to be glycosylated.

In order to test the functions of FlaA deletion derivatives in glycosylation in the *A. caviae* Sch3N, protein expressions of the *flaA* gene deletions were attempted. Plasmids containing modified *flaA* genes have been constructed and transferred into *A. caviae flaA-flaB* mutant. The construct pBBR1-MCS-*flaA0-* has been shown to be able to to compensate the motility deficiency of *A. caviae flaA-flaB* mutant and this finding suggests the plasmids containing flaA0-~flaA15- are functional in the *A. caviae flaA-flaB* mutant and these conjugated strains are all capable of expressing FlaA. Based on that, all these 16 plasmids were selected to be conjugated with *A. caviae flaA-flaB* mutant and tested by Western blotting. Each conjugated strain will express a particular modified FlaA protein which is missing a small part of amino acid sequence at its C-terminal. There are 2 antibodies applied in this experiment, anti-polar flagellin antibody is designed to recognize glycosylated FlaA and anti-FlaA antibody is designed to recognize total FlaA, glycosylated or unglycosylated.

The wild-type, mutant and complemented mutant strains were grown in broth overnight at 37°C, the OD of each culture was normalised and the load sample harvested by centrifugation. The pellet was boiled in SDS-PAGE sample buffer and then the proteins were separated by 12% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was then probed with the rabbit anti-polar flagellin antibody that only recognises the glycosylated version of the *Aeromonas* polar flagellin. The polar flagellins are present in the wild-type sample but absent in the mutant as expected (Figure 3.10). The glycosylated flagellin is detected in the mutant expressing FlaA0- at the same size as the wild type (Figure 3.10). The FlaA deletion derivatives expressed by pBBR1-MCS-*flaA1-* to pBBR1-MCS-*flaA15-* were partially detected by anti-polar flagellin antibody. There were only 5 samples including FlaA15, FlaA13, FlaA11, FlaA9 and FlaA 7 detected (Figure 3.10). The FlaA deletion derivatives expressed by pBBR1-MCS-*flaA10-*~ pBBR1-MCS-*flaA15-* were probed with the Anti-FlaA antibody to confirm the expressions of these proteins and part of these proteins were detected by Anti-FlaA antibody (Figure 3.11).

The cultures of *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-) and *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-) were sonicated and separated into insoluble fraction and soluble fraction to determine the solubility of FlaA8- expressed by pBBR1-*flaA8*- and FlaA9- expressed by pBBR1-MCS-*flaA9*- (Figure 3.12).

(A)



(B)

Figure 3.10 Analysis of FlaA and FlaA deletion derivatives from *A. caviae* strains.

All strains were grown in BHIB at 37°C for overnight. 20 µl of total proteins from each strain were electrophoresed in a 12% SDS polyacrylamide gel. The gel was applied to be analysed by Western Blot following section 2.22 protocol and flagellins (~35Da) from *A. caviae* strains were selected via rabbit anti-polar flagellin antibody and anti-rabbit HRP. The flagellin FlaA in the mutant background was observed to be sequentially smaller in size from FlaA1 to FlaA9 after which the glycosylated Flagellin is not detected.

(A) lane 1, *A. caviae* Sch3N wild-type (WT); lane 2, *A. caviae flaA-flaB* mutant; lane 3, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA0*-); lane 4, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*-); lane 5, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA2*-); lane 6, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA3*-); lane 7, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA4*-); lane 8, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA5*-); lane 9, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA6*-); lane 10, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA7*-); lane 11, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-); lane 12, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-)

(B) lane 1, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-); lane 2, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-); lane 3, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA10*-); lane 4, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA11*-); lane 5, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA12*-); lane 6, *A. caviae flaA-flaB*

mutant (pBBR1-MCS-*flaA13*-); lane 7, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA14*-); lane 8, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA15*-); lane 9, *A. caviae* Sch3N wild-type (WT).

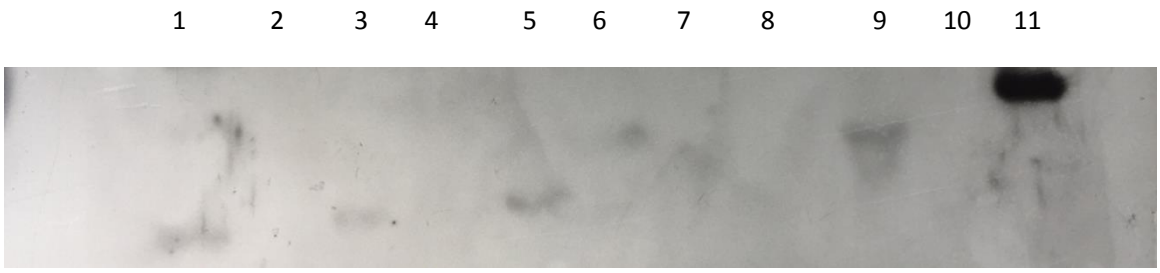


Figure 3.11 Analysis of FlaA and FlaA deletion derivatives from *A. caviae* strains.

All strains were grown in BHIB at 37°C for overnight. 20 ul of total proteins from each strain were electrophoresed in a 12% SDS polyacrylamide gel. The gel was applied to be analysed by Western Blot following section 2.22 protocol and flagellins (~35Da) from *A. caviae* strains were selected via rat anti-FlaA antibody and anti-mouse HRP. The flagellin FlaA in the mutant background to be observed to sequentially get large in size from FlaA15 to FlaA7. Lane 1, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA15*-); lane 2, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA14*-); Lane 3, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA13*-); Lane 4, *A. caviae flaA-flaB* mutant (pBBR1-MCS -*flaA12*-); lane 5, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA11*-); lane 6, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA10*-); lane 7, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-); lane 8, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-); lane 9, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA7*-); lane 10, *A. caviae flaA-flaB* mutant; lane 11, *A. caviae* Sch3N wild-type (WT).

1 2 3 4

Figure 3.12 Analysis of FlaA deletion derivatives from *A. caviae* strains.

FlaA8- and FlaA9- were expressed from pBBR1-MCS-*flaA8*- and pBBR1-MCS-*flaA9*- in *A. caviae flaA-flaB* mutant background. Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. Cells were disrupted by sonication and separated into soluble and insoluble fractions (supernatant and pellet) by centrifugation. 20 ul of total proteins from each strain were electrophoresed in a 12% SDS polyacrylamide gel. The gel was applied to be analysed by Western Blot following section 2.22 protocol and flagellins (~35Da) from *A. caviae* strains were selected via rabbit anti-polar flagellin antibody and anti-rabbit HRP. Lane 1, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-) (insoluble fraction); Lane 2, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-) (insoluble fraction); Lane 3, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA8*-) (soluble fraction); Lane 4, *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA9*-) (soluble fraction).

3.6 Discussion

The *fla* locus of *A. caviae* Sch3N is linked to the biosynthesis of polar flagella filament. This locus includes *flaA*, *flaB*, *flaG*, *flaH* and *flaJ* which have been listed in genetic order (Rabaan et al. 2001).

The *flaA* and *flaB* of *A. caviae* Sch3N encode two flagellin subunits. Strain that lacks *flaA* and *flaB* shows no motility and further study proved that production of flagellins in this strain is absolutely terminated (Rabaan et al. 2001). Motility and adherence drop to about 50% in the strain with single mutation in *flaA* or *flaB* in which flagella are still expressed. This finding indicates that *flaA-flaB* compose flagellin with a 1:1 ratio (Rabaan et al. 2001).

In this project, *A. caviae flaA-flaB* mutant was constructed. This mutant shows no motility therefore, it agrees with the findings of Rabaan et al (2001) and it is suitable

for the investigation of *flaA*.

Expression of the complete *flaA* gene (*flaA0*) in the minus orientation resulted in complementation, whereas in the plus orientation it did not, this suggests expression of the flagellin is better or greater in the minus orientation. The possible reason for this result is the regulation of *lac* promoter.

Based on the result from this project, pBBR1-MCS-*flaA0*- can partially restore the swimming ability of *A. caviae flaA-laB* mutant and pBBR1-MCS-*flaA0*+ can not restore the swimming ability of *A. caviae flaA-flaB* mutant. Besides, pBBR1-MCS-*flaA1*+~ pBBR1-MCS-*flaA15*+ are all failure to restore the swimming ability of *A. caviae flaA-flaB* mutant and the same result was observed from pBBR1-MCS-*flaA1*-~pBBR1MCS-*flaA15*-. This finding indicates that pBBR1-MCS-*flaA0*+~pBBR1MCS-*flaA15*+ are not functional in the conjugated strains and pBBR1-MCS-*flaA0*-~pBBR1-MCS-*flaA15*- are all functional and producing FlaA or modified FlaA in the conjugated strains. The failure to restore the swimming ability for pBBR1-MCS-*flaA1*-~pBBR1-MCS-*flaA15*- is probably caused by the deletion of chaperone binding domain of FlaA (parker 2014). The deletion of chaperone binding domain of FlaA leads to the failure of binding of FlaJ and result in the failure of export of flagellin.

For the next step, the strains including *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*-) to *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA15*-) are all selected for Western Blotting of flagellin detection. According to the Western Blotting results, the FlaA deletion expressed by pBBR1-MCS-*flaA8*- was glycosylated and FlaA deletion expressed by pBBR1-MCS-*flaA10*- was unglycosylated.

The N and C-terminal D0 and D1 domains of bacterial flagellins are usually essential for the chaperone binding, export and polymerization and these domains are usually conserved(Auvray et al. 2001). It has been proved that the FlaA without the C-terminal chaperone-binding domain (CBD amino acids 261–306) still can be glycosylated but it is unable to bind FlaJ in *A. caviae* which means the chaperone binding domain of FlaA is not required for glycosylation of flagellin (Parker et al. 2014). In this experiment, the amino acids 220–306 from C-terminal chaperone-binding domain were sequentially deleted to determine the function of these amino acids. Based on the result of Western Blotting in section 3.15, it has been confirmed that

FlaA which lacks the CBD amino acids 220-250 can not be glycosylated and FlaA which lacks the CBD amino acids 256-306 can be glycosylated. This result suggested that the Site of Maf bind is required is around amino acids 256-250. Further experiment will be applied to determine if Maf1 can bind these FlaA deletion derivatives. Wether the FlaA deletion derivatives which lack the CBD amino acids 256-306 can bind the Maf1 will be investigated in the future.

With the help of Anti-FlaA antibody, FlaA deletions expressed by pBBR1-MCS-*flaA7*- to pBBR1-MCS-*flaA15*- have been partially detected and this finding confirmed that the flagellins after deletion of CBD amino acids 220-250 were still expressed but unglycosylated. However, FlaA14-, FlaA12- and FlaA10- were not detected by anti-FlaA antibody and the reason for that may be the samples were not prepared properly or the plasmids did not express well. It can be observed form figure 3.11 that the bands are getting shallow from FlaA to FlaA15- which indicates that the abilities of binding anti-FlaA antibody of FlaA deletion derivatives are reducing. The explanation for this result may be the loss of C-terminal amino acids.

In order to reconstruct the glycosylation of FlaA in vitro, a source of soluble FlaA deletion derivative which still can be glycosylated is required. The FlaA8- expressed by pBBR1-MCS-*flaA8*- and FlaA9- expressed by pBBR1-MCS-*flaA9*- have been tested and the result indicates that the FlaA8- and FlaA9- were both detected in the soluble fractions. This finding suggests that FlaA8- and FlaA9- are soluble and glycosylated which are suitable for the further experiments.

3.7 Conclusion

However, expression of FlaA0- only resulted in partial (50%) recovery of motility in the mutant strain, this is in agreement with Rabaan et al 2001 that created better single mutant in both *flaA* gene and *flaB* gene instead of double mutant in *flaA* gene and *flaB* gene. This suggests that both flagellins are required for optimal flagella filament function. Or that the expression leads of the flagellins are not optimal. However, on comparation with the normal western blot the flagellin levels of the *flaA0*- expressing strain do not look great selected compared to the wild type (Figure 3.7).

The plasmids including pBBR1-MCS-*flaA0*+~pBBR1-MCS-*flaA15*+ and

pBBR1-MCS-*flaA0*-~pBBR1-MCS-*flaA15*- are all functional and producing FlaA or modified FlaA in the conjugated strains. pBBR1-MCS-*flaA0*- can produce fully functional FlaA and partialy restore the swimming ability of *A. caviae flaA-flaB* mutant. pBBR1-MCS-*flaA0*+ can not restore the swimming ability of *A. caviae flaA-flaB* mutant (Figure 3.8). The rest of all plasmids with flaA gene deletion can not restore the swimming ability of *A. caviae flaA-flaB* mutant. This result indicates that complete FlaA encoded by pBBR1-MCS-*flaA0*- is essential for the flagella assembly and deletion in *flaA* gene will result in absence of functional flagella.

Deletion strains including *A. caviae flaA-laB* mutant (pBBR1-MCS-*flaA1*-) ~*A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA15*-) are non-motile. This result is probably caused by not bound correctly by FlaJ (CBD removed) (Parker et al. 2012). Western Blotting of these FlaA deletions shows that FlaA0- to FlaA9- were all glycosylated and FlaA10- to FlaA15- were all unglycosylated (Figure 3.10 and Figure 3.11). This result suggested that the Site of Maf bind is required is around amino acids 256-250. The flagellins after deletion of CBD amino acids 220-250 were still expressed but unglycosylated and FlaA10- to FlaA15 did not decomposed. The FlaA8- expressed by pBBR1-MCS-*flaA8*- and FlaA9- expressed by pBBR1-MCS-*flaA9*- have been tested and the result indicates that the FlaA8- and FlaA9- were both detected in the soluble fractions (Figure 3.12). This finding suggests that FlaA8- and FlaA9- are soluble and glycosylated which are suitable for the further experiments.

Chapter 4 Investigation of pseudaminic acid biosynthesis in *Aeromonas caviae*

4.1 Introduction

Recently, a *flm* locus of *A. caviae* Sch3N was identified. This locus which includes *flm* genes consists of *flmA(PseB)*, *flmB(PseC)*, *neuA(PseF)*, *flmD(PseG/H)*, and *neuB(PseI)*. Following the *flm* locus, there are *lsg* and *lst* which encode the LPS O-antigen flippase and transferase respectively (Tabei et al. 2009). In *A. caviae* Sch3N, the *flm* locus is involved in both flagellin glycosylation and lipopolysaccharide (LPS) O-antigen(O-Ag) biosynthesis, the mutants without these genes lost motility, flagella, and their LPS O-antigen (Gryllos et al. 2001).

Based on the papers published before, LPS O-antigen biosynthesis and flagellin glycosylation can be cross correlated. In *A. caviae* Sch3N, the flagellin is O-glycosylated with Pse5Ac7Ac while the LPS contains the same sugar (Tabei et al. 2009; Parker et al. 2012). Based on the two mechanisms summarized above, it is clear that Pse5Ac7Ac acts as a pivotal factor which connects the two mechanisms with glycosylation. Because of that, the investigation of the proteins involved in Pse5Ac7Ac biosynthetic pathway becomes very valuable. NeuA which directly interacts with Pse5Ac7Ac is a crucial target that has been applied in this project.

LPS is consists of a lipid portion, a core oligosaccharide (OS) and an O-specific polysaccharide which is known as the O-antigen (Nikaido 1996; Whitfield and Valvano 1993). The surface-exposed LPS O-antigen which is required for virulence in many pathogenic bacteria prevents the cell from elimination by immune system (Tomás 2012).

The genes and proteins for the synthesis of Pse5Ac7Ac have been studied in the pathogen *C. jejuni* and *H. pylori* and the biosynthetic pathway has been elucidated and in order to investigate the Pse5Ac7Ac glycosylation of the *Aeromas* flagellin further, a source of CMP-Pse5Ac7Ac was needed. Therefore, attempts to reconstruct the biosynthetic pathway were undertaken.

4.2 Construction of *A. caviae flmA* mutant and *A. caviae neuA* mutant

NeuA of *A. caviae* which is CMP-sugar synthetase is an important target of this project and the function of this protein has been investigated via the homologous proteins in other bacteria. In order to determine the role of *neuA* in *A. caviae*, a mutant strain that lacks *neuA* was constructed in this project. As the previous mutant made in the lab was shown to be polar and effect the expression of downstream genes.

Furthermore, we wished to see the effect of an N-terminal His-tag on the function of the enzyme in *A. caviae*. As a previous attempt to over-express the NeuA protein in *E. coli* had resulted in an insoluble protein. Therefore, an alternative approach to express His-containing NeuA in *A. caviae* was considered.

In order to create this mutant, 2 DNA fragments, *neuA1* and *neuA2,* were designed and obtained by PCR first and these DNA fragments represented the 5' and 3' portions of *neuA* respectively (Figure 4.2A). Then the Km cassette which is a PCR amplified Tn5-derived kanamycin resistance cartridge (*nptII*)) and pKNG101 digested with BamHI were prepared. The DNA fragments and plasmid used in ISA are listed in Table 4.1. The concentrations of all DNA samples were measured using nanodrop. After that, ISA was applied and the reaction mix was transformed into *E. coli* CC118λ*pir* to be selected (Sm^R and Km^R). Resistant colonies were isolated and the possible recombinant vectors were analysed by PCR screening (section 2.7.4). Then the selected pKNG101::*neuA*::Km construct (Figure 4.3) was confirmed by DNA sequencing and retransformed into *E. coli* S17-1 λ*pir* for the conjugation with *A. caviae* Sch3N (Nal^R) (Figure 4.3). The strains obtained from conjugation were grown on two TSA plates. One TSA plate contains kanamycin and nalidixic acid, the other plate contains kanamycin and streptomycin. Strains that were kanamycin resistant but streptomycin sensitive, were thought to have undergone allelic exchange through a double homologous recombination event. Those strains were subsequently checked by a diagnostic PCR to confirm the result (Figure 4.4). This resulted in the *neuA* mutant strain *A. caviae* Sch3N *neuA*::Km.

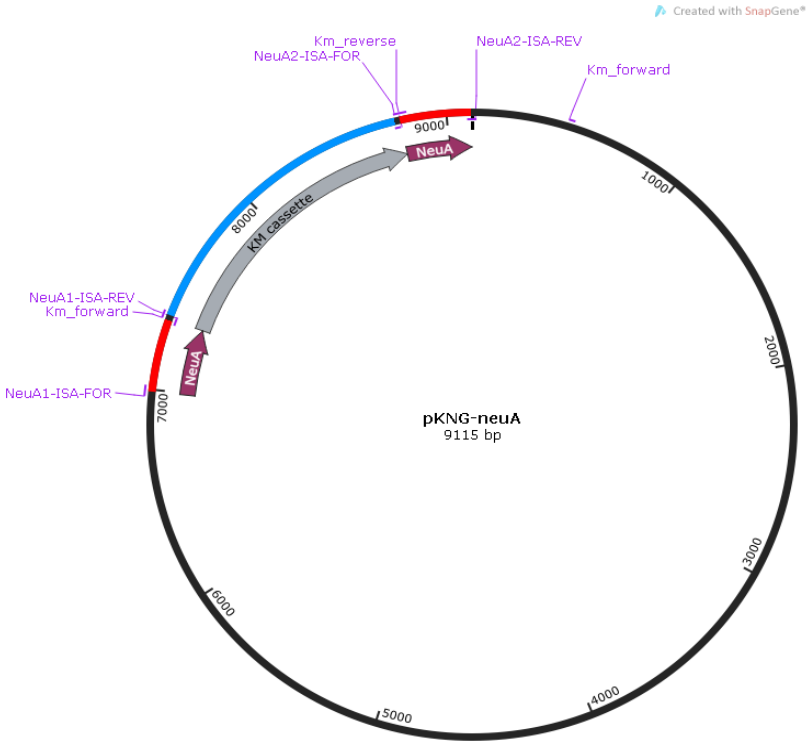
The function of *flmA* was also been looked into. The strain *A. caviae* Sch3N *flmA*::Km was constructed in this project via a similar way. 2 DNA fragments, *flmA1* and *flmA2,* were designed and obtained by PCR and these DNA fragments

represented the 5’ and 3’ portions of *flmA* respectively (Figure 4.1). Then the strain *A. caviae* Sch3N *flmA*::Km was created in a similar way as above strain *A. caviae* Sch3N *neuA*::Km.

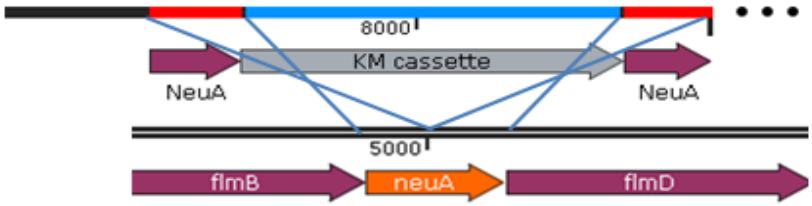
Table 4.1 DNA fragments and plasmid used in ISA and primers of *neuA1* and *neuA2.* Complementary termini of 40bp sequence homology were added to the original Km cassette, *neuA1* and *neuA2* genes*.*

Names	Sizes (bp)	Primers
neuA1	345+40bp	*Neu*AISAF1: 5’CCCCTGCAGGTCGACGGATCAATATTGCCATCATCCCTGC
		*Neu*AISAR1: 5’ACATGAGGACCAAGGAGAATATCATCAGGCTCAACAAAAGGT
neuA2	328+40bp	*Neu*AISAF1:5’AGTACCTAGCCAAGGTGTGCTGCCAGGGATTAGAATTGTTAACGT
		*Neu*AISAR2: 5’ACTTATGGTACCCGGGGATCGCGTAAATAGCTTCTCAGCAC
flmA1	539bp	*FlmA*-ISA1-F:5’ CCCCCCCCCTGCAGGTCGACGGATCATTAATCACAGGTGGTACGGG
		FlmA-ISA1-R:5’ GTCAAACATGAGAACCAAGGAGAATCCATAGCGAACTACACTGAATC
flmA2	553bp	*FlmA*-ISA2-F:5’ GTTTTAGTACCTAGCCAAGGTGTGCAATGTAATGGGGTCTCGGG
		FlmA-ISA2-R:5’ CTTCTACTTATGGTACCCGGGGATTCACACTTCAAAGTTGGCATCT
Km cassette	1452+40bp	Kmfor: 5’ TTCTCCTTGGTTCTCATGTTTGACAGCTT
		Kmrev: 5’ GCACACCTTGGCTAGGTACTAAAACAATTC
pKNG101	6986bp	N/A
pKNG101::*neuA*::Km	9115bp	N/A
pKNG101::*flmA*::Km	9434bp	N/A

(A)


Created with SnapGene®
Km_reverse
NeuA2-ISA-FOR
NeuA2-ISA-REV
Km_forward
9000
NeuA
1000
8000
KM cassette
2000
NeuA1-ISA-REV
Km_forward
NeuA
NeuA1-ISA-FOR
7000
pKNG-neuA
9115 bp
3000
6000
5000
4000

(B)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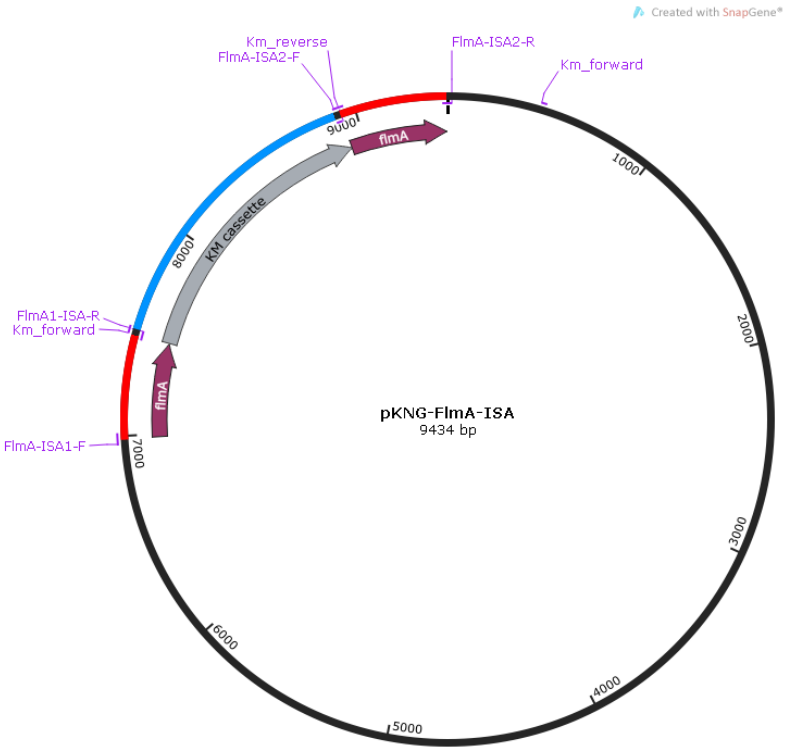
KM cassette
NeuA
NeuA
5000
flmB
neuA
flmD

(C)


Created with SnapGene®
Km_reverse
FlmA-ISA2-F
FlmA-ISA2-R
Km_forward
9000
flmA
1000
KM cassette
8000
2000
FlmA1-ISA-R
Km_forward
flmA
pKNG-FlmA-ISA
9434 bp
FlmA-ISA1-F
7000
3000
6000
4000
5000

(D)

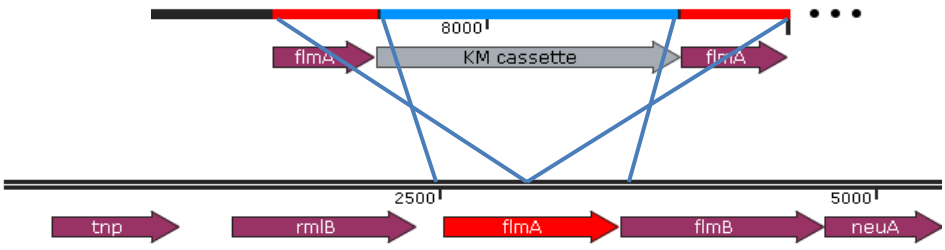

8000
flmA
KM cassette
flmA
2500
5000
tnp
rmlB
flmA
flmB
neuA

Figure 4.1 Isothermal assembly (ISA) method for constructions of pKNG101::*neuA*::Km and pKNG101::*flmA*::Km and constructions of *A. caviae neuA*::Km and of *A. caviae flmA*::Km.

.

(A) This plasmid was constructed using Isothermal assembly of the 385bp 5' neuA fragment the 1452+40bp Km cassette, and the 368bp 3' neuA fragment assemble into BamHI digested pKNG101. Arrows show the direction of selected open reading frames and the sites of primers are induced. The PCR fragments *neuA1* and *neuA2* were generated with primers that had overhangs with the pKNG101 and kanamycin cassette (*neuA1*) and the kanamycin cassette and pKNG101 (*neuA2*) respectively. The four DNA fragments *neuA1*, Km, *neuA2* and pKNG101 were mixed together at equimolar concentrations and assembled together using ISA.

(B) A diagrammatic representation of allelic exchange between the suicide vector construct pKNG101::*neuA*::Km and the *neuA* gene on the chromosome of *A. caviae* Sch3N following conjugation of suicide plasmid from *E. coli* S17-1-λ*pir* into the *Aeromonas* cells. Potential double crossover mutants were selected by replica plating. Each colony was patched on to two LB agar plates, one with kanamycin and nalidixic acid and the other one with kanamycin and streptomycin. Those that were streptomycin sensitive were taken for further investigation. The features of the plasmid vector were shown in arrows. The *neuA* gene was shown in purple arrow and the KmR cassette was shown in grey arrow. The figure was created using SnapGene®software

(C)This plasmid was constructed using Isothermal assembly of the 539bp 5' *flmA* fragment, the 1452+40bp Km cassette, and the 553bp 3' *flmA* fragment to be assembled into BamHI digested pKNG101. Arrows show the direction of selected open reading frames and the sites of primers are induced. The PCR fragments *flmA1* and *flmA2* were generated with primers that had overhangs with the pKNG101 and Km cassette (*flmA1*) and the Km cassette and pKNG101 (*flmA2*) respectively. The four DNA fragments *flmA1*, Km cassette, *flmA2* and pKNG101 were mixed together at equimolar concentrations and assembled together using ISA.

(D) A diagrammatic representation of allelic exchange between the suicide vector construct pKNG101::*flmA*::Km and the *flmA* gene on the chromosome of *A. caviae* Sch3N following conjugation of suicide plasmid from *E. coli* S17-1-λ*pir* into the

Aeromonas cells. Potential double crossover mutants were selected by replica plating. Each colony was patched on to two LB agar plates, one with kanamycin and nalidixic acid and the other one with kanamycin and streptomycin. Those that were streptomycin sensitive were taken for further investigation. The features of the plasmid vector were shown in arrows. The *flmA* gene was shown in purple arrow and the Km^R cassette was shown in grey arrow. The figure was created using SnapGene®software.

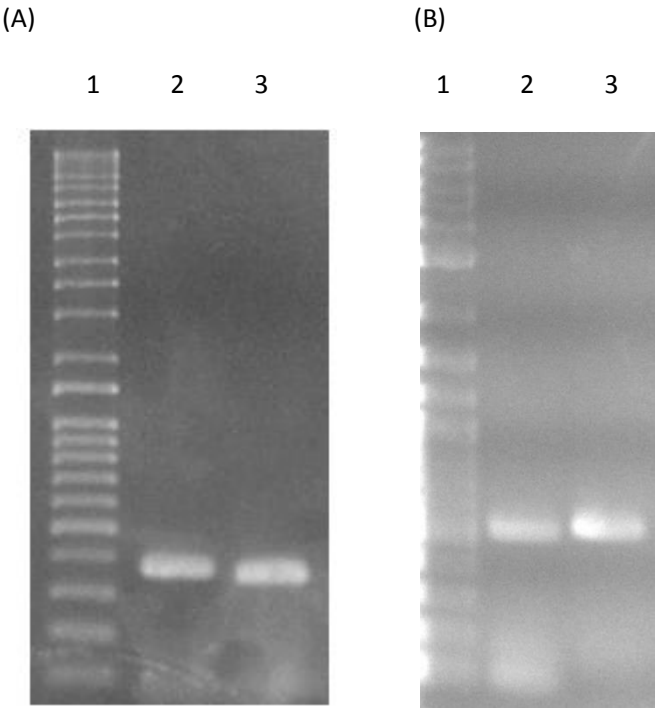


Figure 4.2 Analysis of PCR products following amplification of (A) *neuA* gene fragment 1 and *neuA* gene fragment 2, (B) *flmA* gene fragment 1 and *flmA* gene fragment 2 using *A. caviae* Sch3N genomic DNA as template.

PCR was carried out using Pfx DNA polymerase and *A. caviae* Sch3N genomic DNA. 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products are shown in Table 3.1. (A) Lane1, Q-step4 linear DNA ladder; lane 2, *neuA* gene fragment 1 (~380bp); lane 3, *neuA* gene fragment 2 (~360bp); (B) Lane1, Q-step4 linear DNA ladder; lane 2, *flmA* gene fragment 1 (539bp); lane 3, *flmA* gene fragment 2 (553bp).

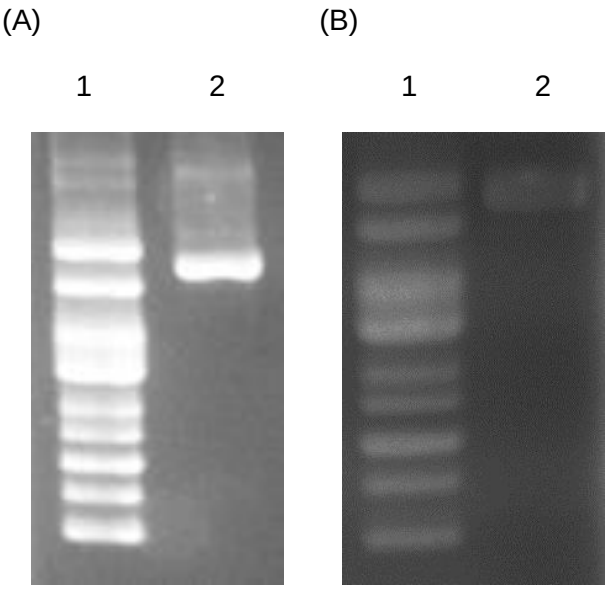


Figure 4.3 Analysis of mini-preparation of the pKNG101::*neuA*::Km and pKNG101::*flmA*::Km plasmids.

Plasmid DNA product was purified using the mini-preparation protocol (section 2.12). 5 ul of the plasmid DNA products were observed following electrophoresis in a 1% agarose gel. (A) Lane1, supercoiled DNA ladder; lane 2, pKNG101::*neuA*::Km; (B) Lane1, supercoiled DNA ladder; lane 2, pKNG101::*flmA*::Km.

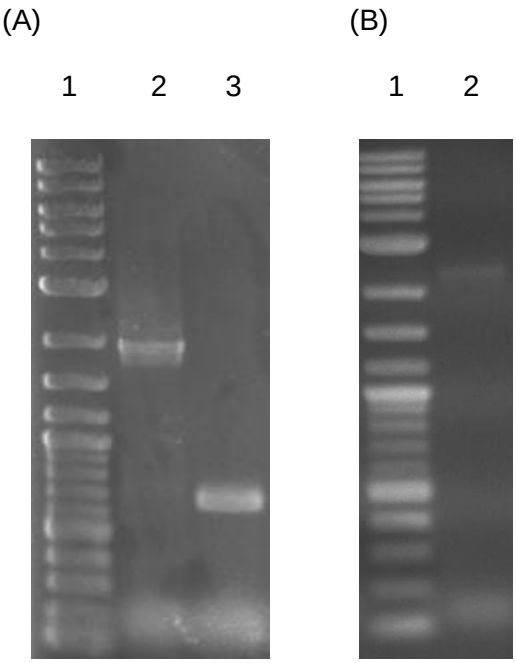


Figure 4.4 Analysis of (A) *neuA* gene from *A. caviae* Sch3N (WT) and *A. caviae neuA*::Km strain, (B) *flmA* gene from *A.caviae flmA*::Km strain.

PCR was carried out using Taq DNA polymerase and *A. caviae* Sch3N (WT) genomic DNA and *A. caviae* Sch3N *neuA*::Km genomic DNA. 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products are shown in Table 3.1. (A) Lane1, 2-Log linear DNA ladder; lane 2, *neuA* gene from *A. caviae neuA*::Km strain (~2000bp); lane 3, *neuA* gene from *A. caviae* Sch3N (WT) (~700bp); (B) Lane1, 2-Log linear DNA ladder; lane 2, *flmA* gene from *A. caviae flmA*::Km strain (~2500bp) .

4.3 Swimming motility assay of *A. caviae neuA* mutant and *A. caviae flmA* mutant

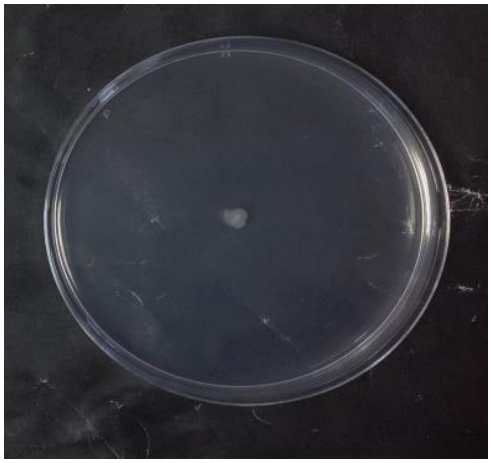
Mutation of the *flm* locus genes have been shown to affect motility and the LPS of *A. caviae* (Gryllos et al. 2001). In order to determine the influence of *neuA* on swimming motility of *A. caviae* Sch3N, swimming motility assays were carried out with the *A. caviae* Sch3N wild-type (WT), the *A. caviae neuA* mutant and the previously constructed complementary strains *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His). The plasmid pBBR1-MCS-*neuA*-His indicates that the *neuA* gene was attached with a polyhistidine-tag at the N-terminal. *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) was created via conjugation of *A. caviae neuA* mutant and S17-1 λ*pir* (pBBR1-MCS-*neuA). A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) was created via conjugation of *A. caviae neuA* mutant and

S17-1 λ*pir* (pBBR1-MCS-*neuA*-His). The plasmids including pBBR1-MCS-*neuA* and pBBR1-MCS-*neuA*-His were provided by former researcher and they were conjugated with the *A. caviae neuA* mutant to see if these plasmids can compensate the swimming ability of *A. caviae neuA* mutant. Four motility plates were set up and three repeats were carried out (12 plates in total).

The *A. caviae neuA* strain was found to be non-motile compared to wild-type (WT). *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) strain was found to be motile which means the motility deficiency of *A. caviae neuA* mutant was compensated. The *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) was found to be motile which means the motility deficiency of *A.* caviae *neuA* mutant was compensated (Figure 4.5). Furthermore, the addition of the N-terminal His-tag to NeuA did not affect the functionality of the enzyme.

In order to determine the influence of *flmA* on swimming motility of *A. caviae* Sch3N, swimming motility assays were carried out with the *A. caviae* Sch3N wild-type (WT), the *A. caviae flmA* mutant. The *A. caviae flmA* mutant strain was found to be non-motile compared to wild-type (WT) (Figure 4.5 and Figure 4.6).

(A)



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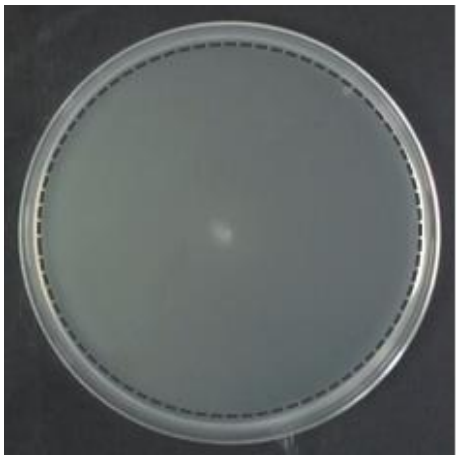

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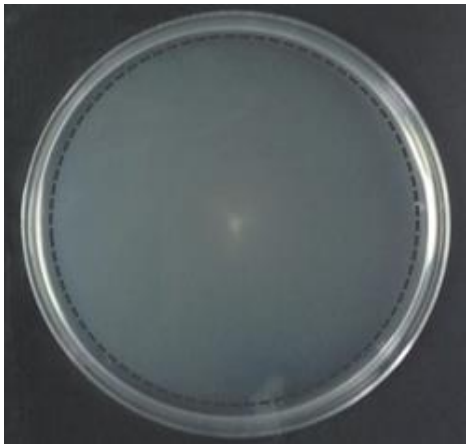
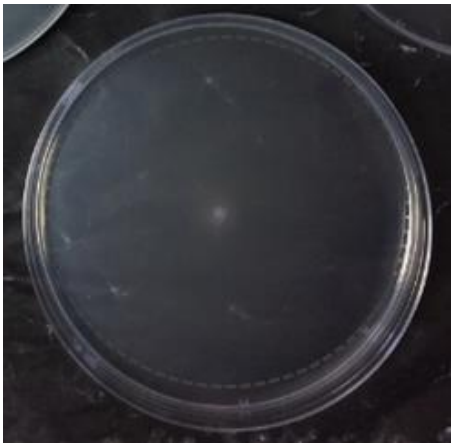


(D)



(E)

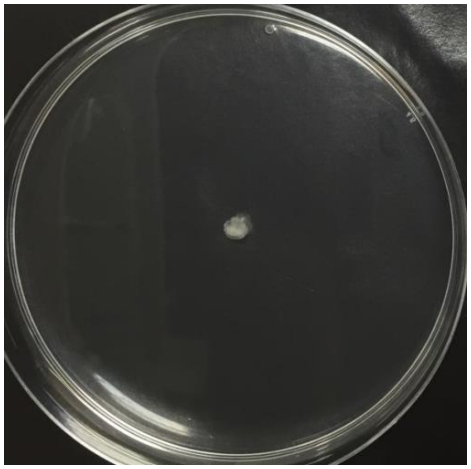


Figure 4.5 Swimming motility assay of *A. caviae* Sch3N (WT), *A. caviae flmA* mutant, *A. caviae neuA* mutant and complemented strains.

Strains were incubated at room temperature for 16 hours. Motility was assessed by the production of a halo caused by bacterial migration. Strains of *A. caviae* Sch3N tested for motility on 0.25% motility agar.

(A) Shows *A. caviae neuA*::Km is non-motile on swimming motility agar. Figure

(B) Shows *A. caviae neuA*::Km (pBBR-*neuA*) is motile on swimming motility agar.

(C) Shows *A. caviae neuA*::Km (pBBR-*neuA*-His) is motile on swimming motility agar.

(D) Shows *A. caviae* Sch3N (WT) is motile on swimming motility agar.

(E) Shows *A. caviae flmA*::Km is non-motile on swimming motility agar.

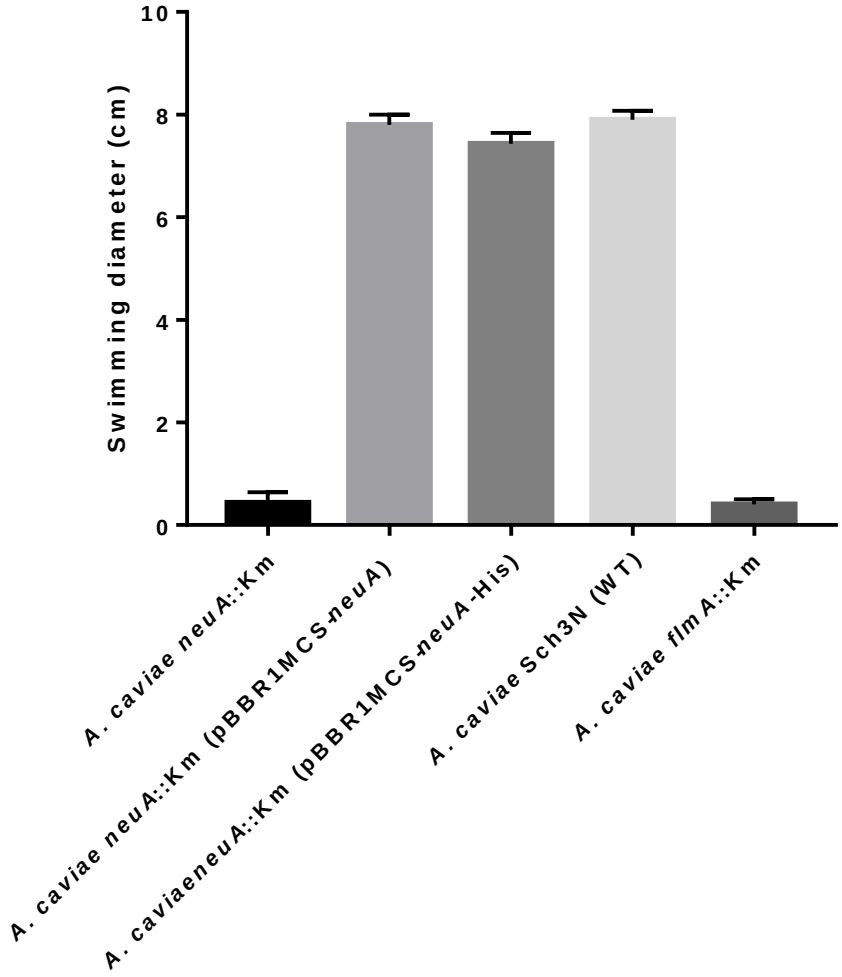


Figure 4.6 Quantification of the swimming assays of A. caviae Sch3N (WT) and mutant strains.

The swimming diameter of each strain was measured on the swimming agar after overnight incubation at 37°C. The swimming diameters were significantly lower in the *neuA* mutant when compared to the *A. caviae* Sch3N wild type ($p<0.001$), while there was no significant difference of swimming diameter among the *A. caviae neuA*::Km (pBBR1-MCS-*neuA*) strain, *A. caviae neuA*::Km (pBBR1-MCS-*neuA*-His) strain and *A. caviae* Sch3N wild type strain ($p>0.05$). The swimming diameters of *A. caviae flmA* mutant was significantly decreased when compared to the *A. caviae* Sch3N wild type ($p<0.001$). The swimming diameters were measured 3 times for each strain. The error bars showed Standard Error of the Mean (SEM). The significance was determined using Student's t-test.

4.4 LPS analysis and flagellin analysis of *A. caviae* Sch3N *neuA*::Km

As previously shown, mutation of the *flm* locus affects the LPS of *A. caviae* (Tabei et al. 2009; Gryllos et al. 2001). LPS of the wild-type, mutant strain *A. caviae* Sch3N *neuA*::Km and complemented strains were investigated. In order to determine the role of *neuA* in LPS biosynthesis of *A. caviae* Sch3N, LPS extraction was applied in this project. The products of LPS extraction of *A. caviae* Sch3N wild-type (WT), *A. caviae neuA* mutant (pBBR1-MCS-*neuA*), *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) and *A. caviae neuA* mutant were loaded and subjected to SDS-PAGE. The gel was then silver stained (Figure 4.7). The wild-type strain and the complemented strains possess both the A and B bands as previously described by Gryllos et al (2001) and Tabei et al (2009). Band B that represents the core plus O-antigen is missing from the *A. caviae neuA* mutant strain.

To confirm the lack of motility phenotype observed for the mutant strain, the strains were probed for the production of flagellin protein, the whole-cell proteins of these mutants and wild-type were separated by SDS-PAGE. Then western blot was applied with rabbit anti-polar flagellin antibody and anti-rabbit HRP conjugate antibody. Figure 4.8 of Western bolt showed polar flagellins were absent in *A. caviae neuA* mutant and polar flagellin was present in the *A. caviae* Sch3N wild-type (WT), *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) strains (Figure 4.7).

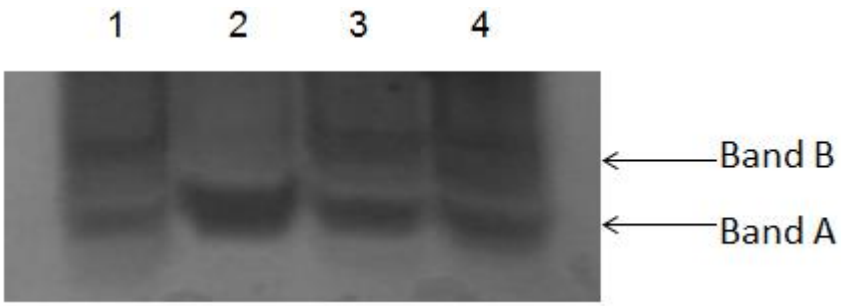


Figure 4.7 Analysis of LPS extracted from WT and *neuA* mutant *A. caviae* strains.

LPS was extracted from *A. caviae* Sch3N wild-type (WT), *A. caviae neuA* mutant, *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) and analysed by SDS-PAGE (12%) and silver stained according to section 2.17 and 2.18 protocols. Cells were grown in BHIB at 37°C for overnight. Lane 1, LPS from *A. caviae* Sch3N wild-type (WT); Lane 2, LPS from *A. caviae neuA* mutant; Lane 3, LPS from *A. caviae neuA* mutant (pBBR1-MCS-*neuA*); lane 4, LPS from *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His).

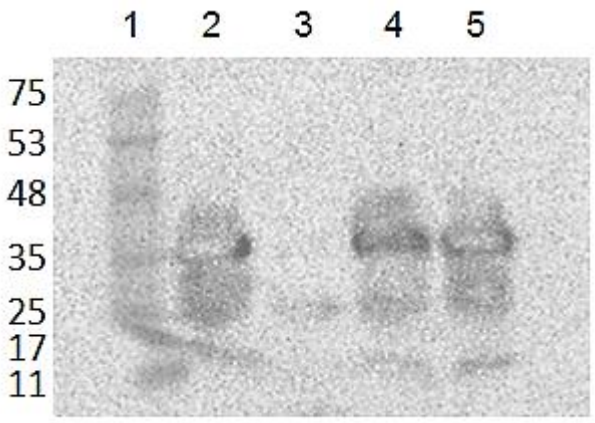


Figure 4.8 Analysis of flagellins from WT and *neuA* mutant *A. caviae* strains.

Whole-cell protein prepared from *A. caviae* Sch3N wild-type (WT), *A. caviae neuA* mutant, *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His). All strains were grown in BHIB at 37°C for overnight. 20 ul of total proteins from each strain were electrophoresed in a 12% SDS polyacrylamide gel and then blotted onto nitrocellulose membrane. The gel was applied to be analysed by Western Blot following section 2.22 protocol and polar flagellins (~35Da) from *A. caviae* strains were selected via rabbit anti-polar flagellin antibody and anti-rabbit HRP. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2 polar flagellins from *A. caviae* Sch3N wild-type (WT); Lane 3, polar flagellins from *A. caviae neuA* mutant; Lane 4, polar flagellins from *A. caviae neuA* mutant (pBBR1-MCS-*neuA*); Lane 5, polar flagellins from *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His).

4.5 Protein overexpression of *flm* genes

Part of this project was to try and biochemically reconstitute the flagellin glycosylation process using pure *A. caviae* flagellin, the glycosyltransferase Maf1 and CMP-Pse5Ac7Ac. Therefore, a source of CMP-Pse5Ac7Ac is required. Previous workers have shown that this is possible using the equivalent Pse/Flm enzymes of *H. pylori* (Schoenhofen et al. 2006).

In order to investigate pseudaminic acid biosynthetic pathway in *A. caviae* Sch3N, protein expression of the *flm* genes was attempted. Plasmids containing part modified *flm* genes were constructed in our laboratory and each *flm* gene when expressed

would produce a protein with N-terminal His_6-tag. Due to solubility problems with FlmD proteins, *flmD* was cloned into pMAL-c5X in order to produce a protein with N-terminal MBP-tag. Both of the pET-28a-*flmD* which was constructed before and pMAL-c5X were digested with NdeI and BamHI then the products of two digestions were mixed together and ligated with ligase. After ligation, ampicillin resistant colonies were screened by colony PCR screen using pMAL screening primers with Taq DNA polymerase. Selected pMAL-c5X-*flmD* plasmid vector was then sequenced using pMAL screening primers to ensure the in-frame insertion, the orientation of the inserts and no mutation in the inserts. After examination with sequencing and BLAST search, pMAL-c5X-*flmD* plasmid was then transformed into *E. coli* (NEB Express) for the over-expression of the MBP-fused FlmD protein.

The primers used to amplify *flmA, flmB, neuA* and *neuB* introduced an Nde1 restriction site and a HindIII restriction site and a stop codon at either end of each gene allowing in-frame cloning into the pET-28a vector to ensure an N-terminal His-tag. The PCR products of the *flm* genes were then digested by NdeI and HindIII and ligated with the pET-28a plasmids digested by the same pair of restriction enzymes. The ligations were transformed into *E. coli* DH5α competent cells and kanamycin resistant colonies were screened by colony PCR using T7 promoter and T7 terminator primers. Plasmids were extracted from *E. coli* and all plasmids were then transformed into *E. coli* overexpression strains (BL21 DE3) to overexpress the histidine-tagged proteins. The solubility of each protein was double checked with SDS-PAGE.

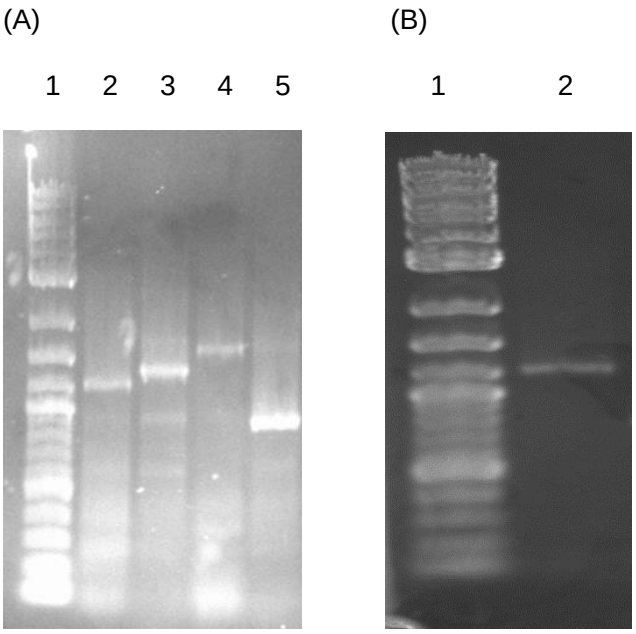


Figure 4.9 Analysis of PCR products following amplification of *flm* locus gene fragments using pET-28a-*flmA*-His, pET-28a-*flmB*-His, pMAL-c5X-*flmD,* pET-28a-*neuB*-His and pET-28a-*neuA*-His as template.

PCR was carried out using Q5 DNA polymerase and pET-28a-*flmA*-His, pET-28a-*flmB*-His, pMAL-c5X-*flmD*, pET-28a-*neuB*-His and pET-28a-*neuA*-His. 5 ul of the PCR products were observed following electrophoresis in a 1% agarose gel. Primers used to amplify these products are T7 forward primer and T7 reverse primer and pMAL sequence forward primer and pMAL sequence reverse primer. (A) Lane1, 2-Log linear DNA ladder; lane 2, *flmA* gene fragment (1299bp); lane 3, *flmB* gene fragment (1450bp); lane 4, *flmD* gene fragment (1718bp); lane 5, *neuA* gene fragment (973bp); (B) Lane1, 2-Log linear DNA ladder; lane 2, *neuB* gene fragment (1345bp).

Table 4.2 Plasmids with *flm* gene insertions applied in this project and expected expressed Proteins (Tabei et al. 2009).

Name of plasmids	Protein expressed	Protein size (kDa)
pBBR1-MCS-*neuA*-His	His-NeuA	27.8
pET-28-*flmA*-His	His-FlmA	40
pET-28-*flmB*-His	His-FlmB	45
pMAL-c5X-*flmD*	MBP-FlmD	98.8
pET-28-*neuB*-His	His-NeuB	40.6
pET-28-*neuA*-His	His-NeuA	27.8

Firstly, each plasmid was collected from *E. coli* DH5α by mini-preparation and they were retransformed into *E. coli* BL21 (DE3). Each strain was grown in LB broth with appropriate antibiotics for 2 hours and then the culture was split in two and 1mM IPTG was added to one culture (+IPTG) and one culture without IPTG (-IPTG) (figure 4.10, figure 4.11 and figure 4.12). After that, each strain was grown in LB broth following section 2.19 protein overexpression protocol and the supernatant and pellet of each protein was checked by SDS-PAGE to make sure that all the proteins were soluble (figure 4.10, figure 4.11 and figure 4.12). The overexpressed protein in the supernatant and pellet were collected. It has been confirmed that all proteins obtained from protein purification were soluble. Based on the results of SDS-PAGE, FlmA, FlmB, NeuA and NeuB have been prepared successfully. Each protein was isolated and purified by application to a His-trap column to which a gradient of imidazole was applied.

FlmA which is about 40kDa was purified and most of the protein was eluted in EB with 150mM of imidazole and 200mM of imidazole (figure 4.13). FlmB which is approximately 45kDa was purified and most of the protein was eluted in EB with 100mM imidazole, 150mM imidazole and 200mM imidazole (figure 4.14). NeuA which is about 27.8kDa was purified and most of the protein was eluted in EB with 150mM imidazole and 200mM imidazole (figure 4.15). NeuB which is about 40.6kDa was purified and most of the protein was eluted in Elution Buffer with 150mM of imidazole and 200mM of imidazole (Figure 4.16). The protein overexpression of FlmD was carried out by MBP-tag protein purification protocol via 2.22 and purified FlmD was eluted into 50mM maltose buffer (Figure 4.17). All 6 enzymes are shown in Figure 4.18.

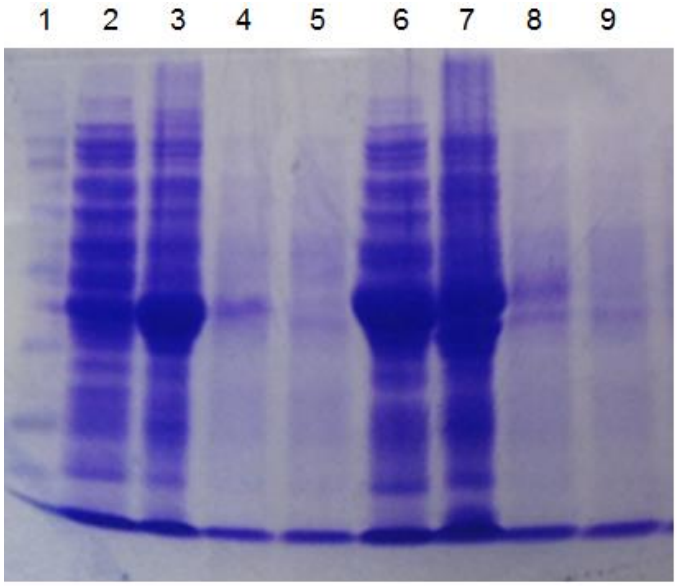


Figure 4.10 Analysis of protein overexpression and solubility of His-tagged FlmA and His-tagged NeuB.

His-tagged FlmA and NeuB were expressed from pET-28a-*flmA*-His and pET-28a-*neuB*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with or without 1mM IPTG addition for 3 hours. Cells were disrupted by sonication and separated into soluble and insoluble fractions (supernatant and pellet) by centrifugation.

10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa) (Appendix 1); Lane 2, total protein from induced cells with pET-28a-*flmA*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*flmA*-His in pellet (insoluble fraction); Lane 4, total protein from induced cells with pET-28a-*flmA*-His; Lane 5, total protein from uninduced cells with pET-28a-*flmA*-His. Lane 6, total protein from induced cells with pET-28a-*neuB*-His in supernatant (soluble fraction); Lane 7, total protein from induced cells with pET-28a-*neuB*-His in pellet (insoluble fraction); Lane 8, total protein from induced cells with pET-28a-*neuB*-His; Lane 9, total protein from uninduced cells with pET-28a-*neuB*-His. The difference between lane 4 and 5 is caused by the addition of IPTG. Induced cells expressed more His-tagged FlmA than uninduced cells. The difference between lane 8 and 9 is caused by the addition of IPTG. Induced cells expressed more His-tagged NeuB than uninduced cells.

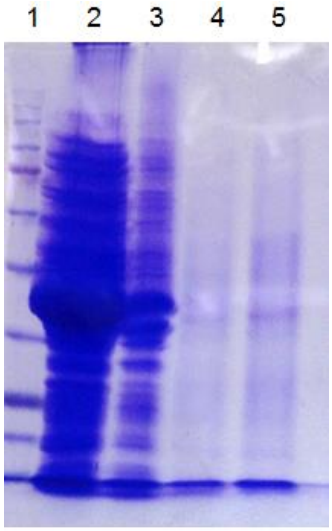


Figure 4.11 Analysis of protein overexpression and solubility of His-tagged FlmB.

His-tagged FlmB were expressed from pET-28a-*flmB*-His and in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with or without 1mM IPTG addition for 3 hours. Cells were disrupted by sonication and separated into soluble and insoluble fractions (supernatant and pellet) by centrifugation. 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET-28a-*flmB*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*flmB*-His in pellet (insoluble fraction); Lane 4, total protein from induced cells with pET-28a-*flmB*-His; Lane 5, total protein from uninduced cells with pET-28a-*flmB*-His. The difference between lane 4 and 5 is caused by the addition of IPTG. Induced cells expressed more His-tagged FlmB than uninduced cells.

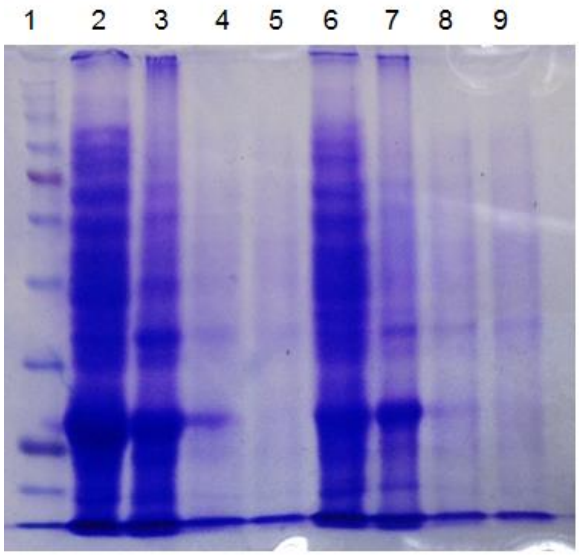


Figure 4.12 Analysis of protein overexpression and solubility of His-tagged NeuA and NeuA.

His-tagged NeuA and NeuA were expressed from pET-28a-*neuA*-His and pBBR1-MCS-*neuA*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with or without 1mM IPTG addition for 3 hours. Cells were disrupted by sonication and separated into soluble and insoluble fractions (supernatant and pellet) by centrifugation. 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET-28a-*neuA*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*neuA*-His in pellet (insoluble fraction); Lane 4, total protein from induced cells with pET-28a-*neuA*-His; Lane 5, total protein from uninduced cells with pET-28a-*neuA*-His. Lane 6, total protein from induced cells with pBBR1-MCS-*neuA*-His in supernatant (soluble fraction); Lane 7, total protein from induced cells with pBBR1-MCS-*neuA*-His in pellet (insoluble fraction); Lane 8, total protein from induced cells with pBBR1-MCS-*neuA*-His; Lane 9, total protein from uninduced cells with pBBR1-MCS-*neuA*-His. The difference between lane 4 and 5 is caused by the addition of IPTG. Induced cells expressed more His-tagged NeuA than uninduced cells. The difference between lane 8 and 9 is caused by the addition of IPTG. Induced cells expressed more NeuA than uninduced cells.

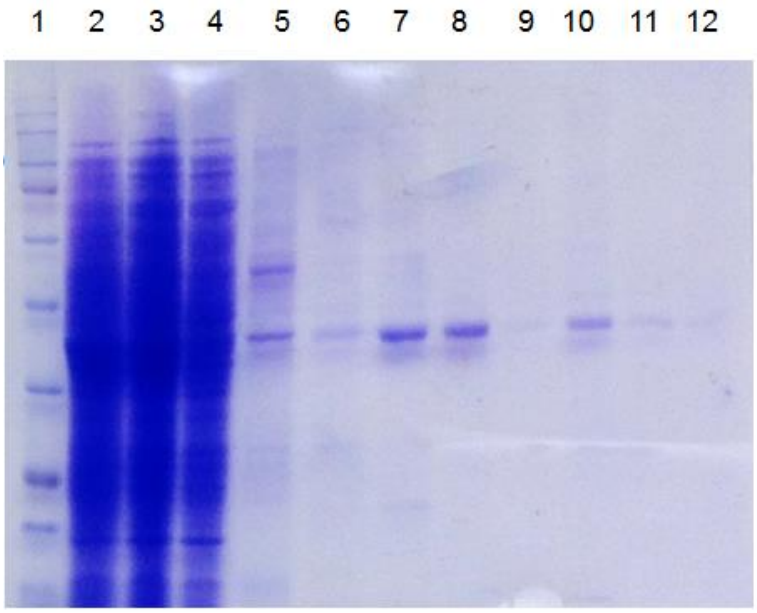


Figure 4.13 Analysis of protein purification of the His_6-tagged FlmA.

12% SDS-PAGE gel showing the purification of His-FlmA protein. His-tagged FlmA was expressed from pET-28a-*flmA*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. The soluble supernatant was applied to a HisTrap™ HP Column. Unbound proteins were washed off in elution buffer (flow-though and wash). His-tagged FlmA (40kDa) was eluted in increasing concentrations of imidazole (50mM-500mM) elution buffer (EB). 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET-28a-*flmA*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*flmA*-His in flow-though; Lane 4, total protein from induced cells with pET-28a-*flmA*-His after filtration; Lanes 5-12, protein from induced cells containing pET-28a-*flmA*-His eluted in 50,100, 150, 200, 250, 300, 400 and 500 mM of imidazole elution buffer, respectively.

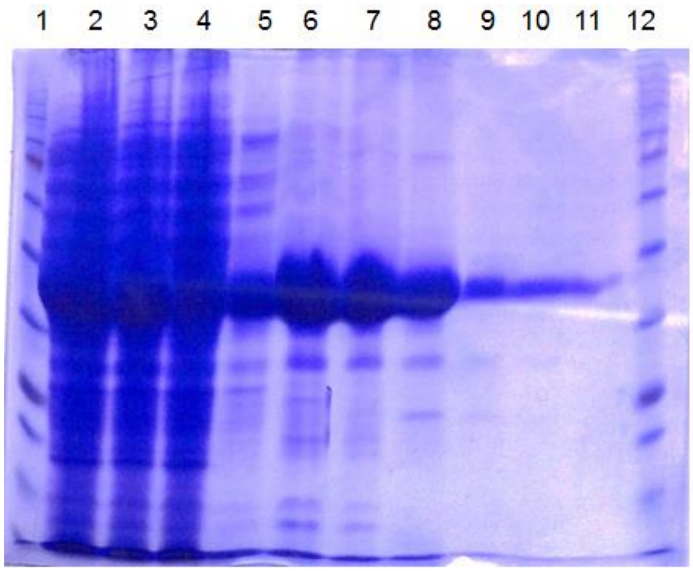


Figure 4.14 Analysis of protein purification of the His_6-tagged FlmB.

His-tagged FlmB was expressed from pET28a-*flmB*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. The soluble supernatant was applied to a HisTrap™ HP Column. Unbound proteins were washed off in elution buffer (flow-though and wash). His-tagged FlmB (45kDa) was eluted in increasing concentrations of imidazole (50mM-500mM) elution buffer (EB). 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET28a-*flmB*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET28a-*flmB*-His in flow-though; Lane 4, total protein from induced cells with pET28a-*flmB*-His after filtration; Lanes 5-12, protein from induced cells containing pET-28a-*flmB*-His eluted in 50,100, 150, 200, 250, 300, 400 and 500 mM of imidazole elution buffer, respectively.

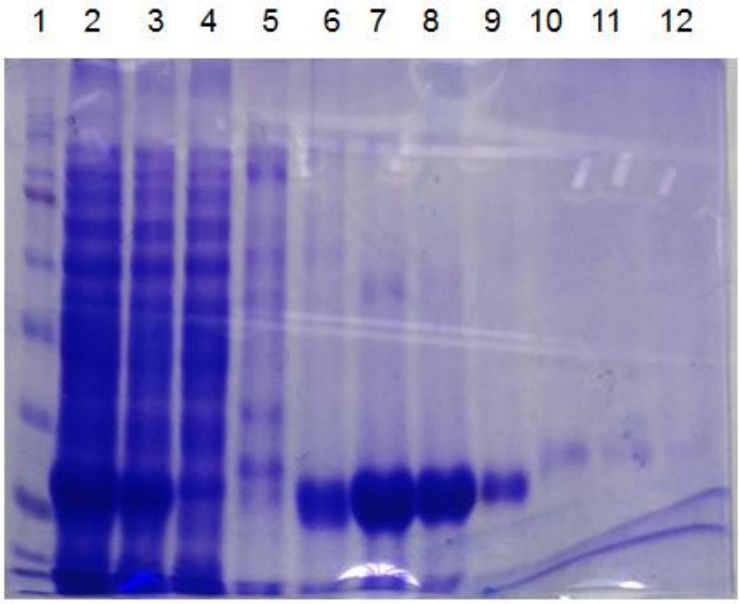


Figure 4.15 Analysis of protein purification of the His_6-tagged NeuA.

His-tagged NeuA was expressed from pET-28a-*neuA*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. The soluble supernatant was applied to a HisTrap^{TM} HP Column. Unbound proteins were washed off in elution buffer (flow-though and wash). His-tagged NeuA (27.8kDa) was eluted in increasing concentrations of imidazole (50mM-500mM) elution buffer (EB). 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET-28a-*neuA*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*neuA*-His in flow-through; Lane 4, total protein from induced cells with pET-28a-*neuA*-His after filtration; Lanes 5-12, protein from induced cells containing pET-28a-*neuA*-His eluted in 50,100, 150, 200, 250, 300, 400 and 500 mM of imidazole elution buffer, respectively.

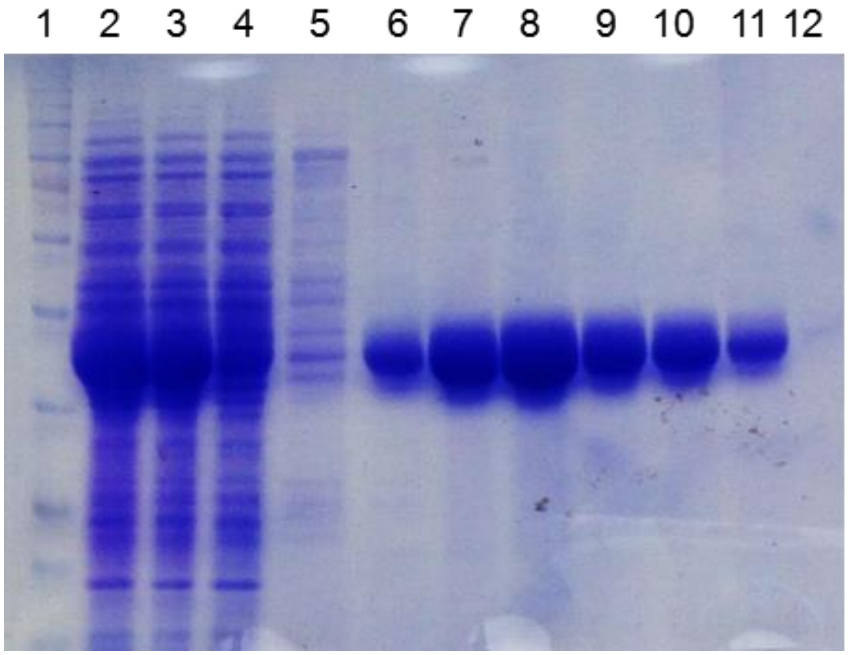


Figure 4.16 Analysis of protein purification of the His_6-tagged NeuB.

His-tagged NeuB was expressed from pET-28a-*neuB*-His in *E. coli* strain BL21 (DE3). Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. The soluble supernatant was applied to a HisTrap™ HP Column. Unbound proteins were washed off in elution buffer (flow-though and wash). His-tagged NeuB (40.6kDa) was eluted in increasing concentrations of imidazole (50mM-500mM) elution buffer (EB). 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pET-28a-*neuB*-His in supernatant (soluble fraction); Lane 3, total protein from induced cells with pET-28a-*neuB*-His in flow-though; Lane 4, total protein from induced cells with pET-28a-*neuB*-His after filtration; Lanes 5-12, protein from induced cells containing pET-28a-*neuB*-His eluted in 50,100, 150, 200, 250, 300, 400 and 500 mM of imidazole elution buffer, respectively.

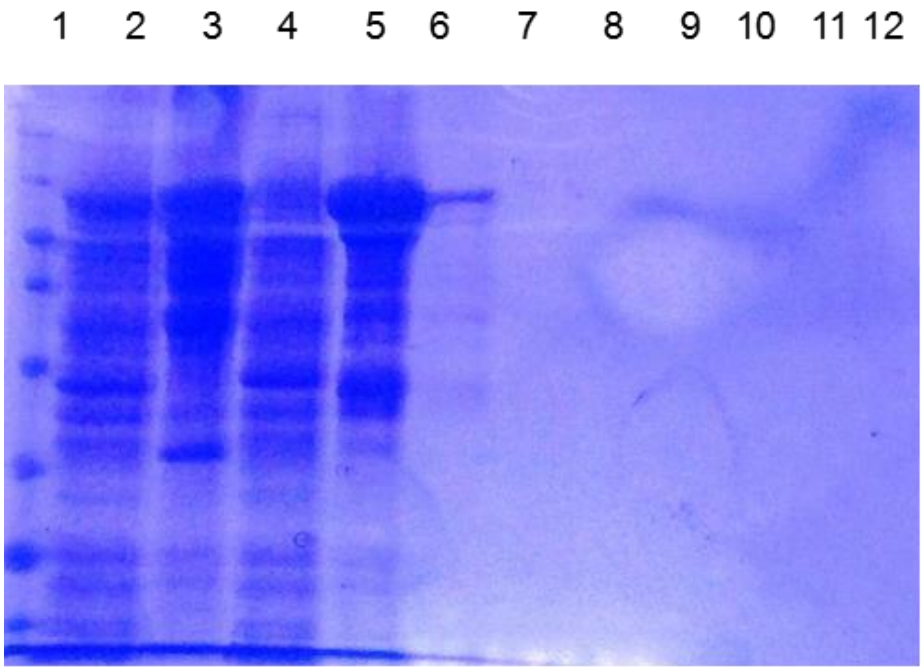


Figure 4.17 Analysis of protein purification of the MBP-tagged FlmD.

MBP-tagged FlmD was expressed from pMAL-c5X-*flmD* in *E. coli* strain ER2523 (NEB Express). Cells were grown in LB broth at 37°C with 1mM IPTG addition for 3 hours. The soluble supernatant was applied to a MBPTrap™ HP Column. Unbound proteins were washed off in elution buffer (flow-though and wash). MBP-tagged FlmD (100kDa) was eluted in increasing concentrations of maltose (20mM-300mM) column buffer (CB). 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, total protein from induced cells with pMAL-*c5x-flmD* in supernatant (soluble fraction); Lane 3, total protein from induced cells with pMAL-*c5x-flmD* in flow-through; Lane 4, total protein from induced cells with pMAL-*c5x-flmD* after filtration; Lanes 5-12, protein from induced cells containing pMAL-c5X-*flmD* eluted in 20, 50, 100, 150, 200, 250 and 300 mM of column buffer, respectively.

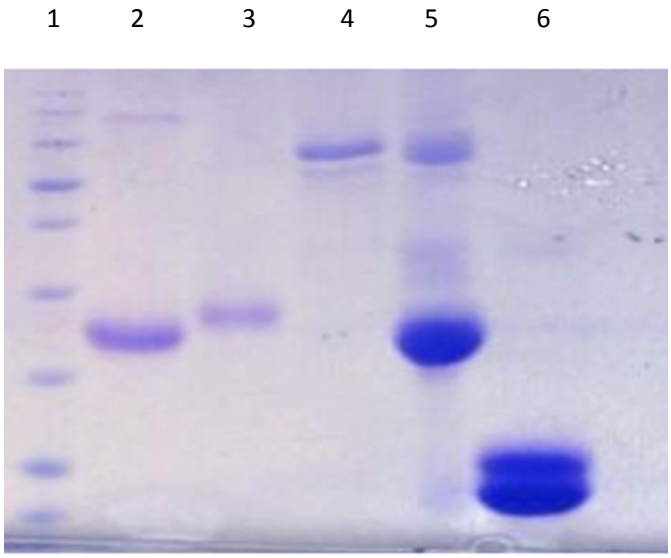


Figure 4.18 Analysis of protein overexpression of purified FlmA, FlmB FlmD, NeuB and NeuA.

The proteins including His-tagged FlmA, His-tagged FlmB, His-tagged NeuB and His-tagged NeuA were expressed from pET-28a-*flmA*-His, pET-28a-*flmB*-His, pET-28a-*neuA*-His and pET-28a-*neuB*-His in *E. coli* strain BL21 (DE3). MBP-tagged FlmD was expressed from pMAL-c5X-*flmD* in *E. coli* (NEB Express). The proteins were then purified either using His-trap or MBP-trap column.

Cells were grown in LB broth at 37°C with or without 1mM IPTG addition for 3 hours. 10 µl of the protein samples were electrophoresed in a 12% SDS polyacrylamide gel. Lane 1, Prestained protein ladder (sizes shown in kDa); Lane 2, purified FlmA from induced cells with pET-28a-*flmA*-His in supernatant (soluble fraction); Lane 3, purified FlmB from induced cells with pET-28a-*flmB*-His in supernatant (soluble fraction); Lane 4, purified FlmD protein from induced cells with pMAL-c5X-*flmD* in supernatant (soluble fraction); Lane 5, purified NeuB from induced cells with pET-28a-*neuB*-His in supernatant (soluble fraction). Lane 6, purified NeuA from induced cells with pET-28a-*neuA*-His in supernatant (soluble fraction).

4.6 CMP-Pse5Ac7Ac biosynthetic reactions

This project aimed to produce CMP-Pse from the starting substrate UDP-GlcNAc. Previous workers have reconstituted the CMP-Pse5Ac7Ac biosynthesis pathway from *H. pylori* (Schoenhofen et al. 2006). The starting substrate was shown to be UDP-GlcNAc. We wished to do the same for the *A. caviae* pathway to allow us to

access CMP-Pse5Ac7Ac the final product of the pathway. In collaboration with a fellow PhD student Joe Ferner the enzymatic activity of FlmA (PseB) was investigated by NMR. FlmA is thought to have UDP-N-acetylglucosamine 5-inverting 4, 6-dehydratase activity. Previous workers have shown the activity of this enzyme can be followed by NMR (McNally et al. 2008).

In this project, one of the aims was to reconstitute the CMP-Pse5Ac7Ac biosynthesis pathway and generate CMP-Pse5Ac7Ac from UDP-GlcNAc, L-Glu, Acetyl-CoA and PEP via enzymes encoded by *flm locus* from *A. caviae*. The reactions occurred in NMR tube and whole reaction process is monitored by Nuclear magnetic resonance spectroscopy (NMR).

However, the NMR trace for UDP-GlcNAc did not change (Figure 4.19). There is no reaction detected after 4 hours, therefore the FlmA enzyme appeared to be inactive. The enzyme was soluble when purified, therefore circular dichroism was performed to see if it had folded correctly (Figure 4.20). The change in signal between 200-230nm depicts alpha helix formation, suggesting the protein is folded.

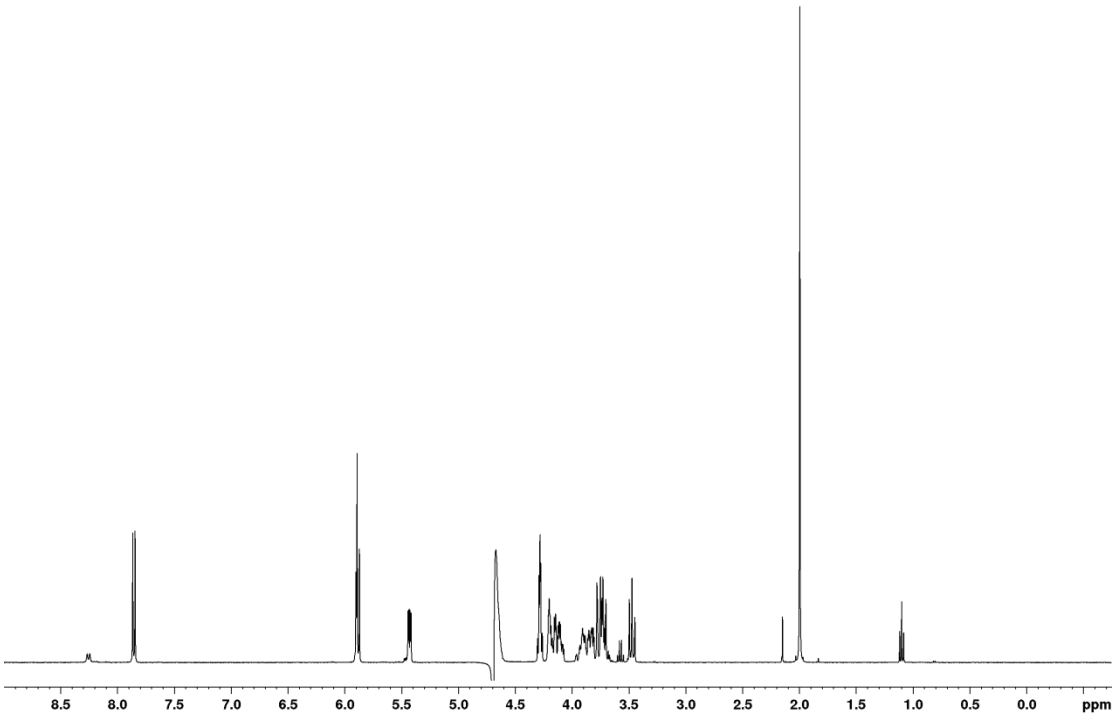


Figure 4.19 data of NMR spectrum of UDP-GlcNAc in 10% D_2O phosphate buffer.

The figure shows 1H NMR spectrum of UDP-GlcNAc in 10% D_2O phosphate buffer. The first enzyme in the biosynthesis pathway is FlmA (PseB). However when purified FlmA was incubated with UDP-GlcNAc and the reaction followed for 4 hours using 1 ml NMR reaction mix as previously described, there was no change in the UDP-GlcNAc spectra (McNally et al. 2008).

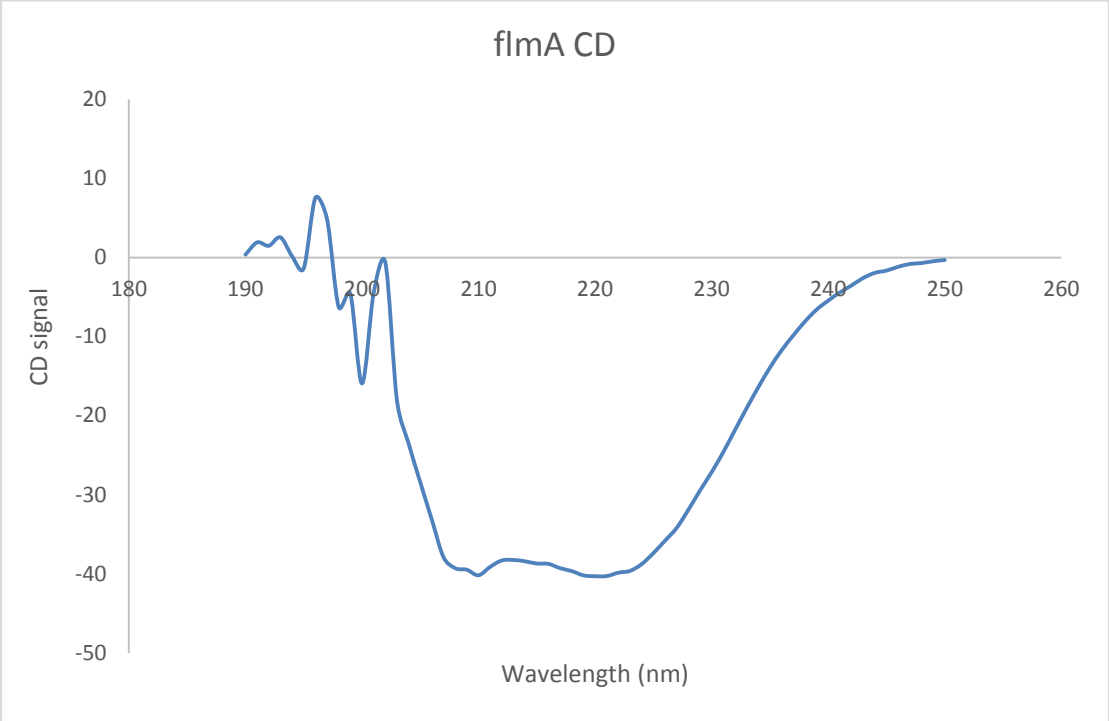


Figure 4.20 CD spectra of FlmA

The data of CD spectra of FlmA shows that FlmA has negative peaks at 220nm and 210nm and a positive one at 196nm. The characteristicsof these CD signals are corresponded to alpha helix which is secondary structure of protein.

Based on the result of NMR reaction of UDP-GlcNAc and FlmA, there is no product detected after overnight reaction. This result suggests that the FlmA is inactive and further NMR reactions are all held due to the lack of UDP-2-acetamido-2,6-dideoxy-b-L-arabinohexos-4-ulose.

Emily Flack (University of York) and her co-worker were investigating a similar CMP-Pse5Ac7Ac biosynthetic pathway in *Helicobacter pylori* (Table 4.3). The pseudaminic acid can be generated from UDP-GlcNAc via purified PseB, PseC, PseH, PseG and PseI of *H. pylori*. PseB from *H. pylori* is homologue of FlmA from *A. caviae* and UDP-2-acetamido-2,6-dideoxy-b-L-arabinohexos-4-ulose has been proved to be generated from UDP-GlcNAc via PseB (Schoenhofen et al. 2006). All 5 enzymes were taken to York University for further testing. The activity of each enzyme has been tested via a series of reactions and followed by LC-MS. The reactions involved in the CMP-Pse5Ac7Ac biosynthetic pathway are shown in Figure 4.21. FlmA (PseB) which is a dehydratase initiates the biosynthesis of pseudaminic acid via catalyzing the conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) (607.35 g/mol) to

UDP-2-acetamido-2,6-dideoxy-b-L-arabino-hexos-4-ulose (589.34 g/mol). FlmB (PseC) which is aminotransferase catalyzes UDP-2-acetamido-2,6-dideoxy-b-L-arabino-hexos-4-ulose and L-glutamine and result in UDP-4-amino-4,6-dideoxy-b-L-AltNAc (590.372 g/mol) and a-ketoglutarate. After processing by FlmD (PseH/PseG) which is acetyltransferase and nucleotidase, the UDP-4-amino-4,6-dideoxy-b-L-AltNAc was catalyzed to 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose (246.26 g/mol). The acetyl group was induced and UDP group was removed in this process. NeuB (PseI) which is pseudaminic acid synthetase catalyzes PEP and 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose and result in pseudaminic acid (334.32g/mol)and phosphate (Schoenhofen et al. 2006; Schirm et al. 2003). NeuA (PseF) is acts as CMP-sugar synthetase and catalyzes pseudaminic acid and CTP and result in CMP-Pse5Ac7Ac (639.50 g/mol) and pyrophosphate(Schirm et al. 2003).

Table 4.3 Enzymes from *A. caviae* and corresponding homologues from *H. pylori*.

Enzyme in *A. caviae*	Homologues in *H. pylori*
FlmA	PseB
FlmB	PseC
FlmD	PseH/PseG
NeuB	PseI
NeuA	PseF

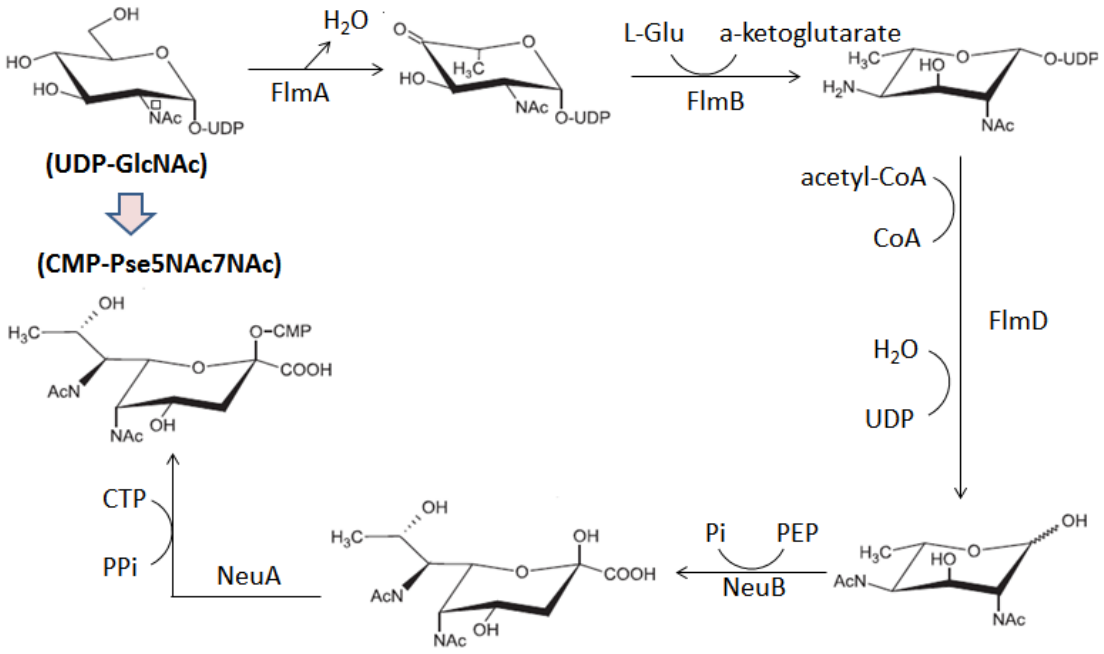
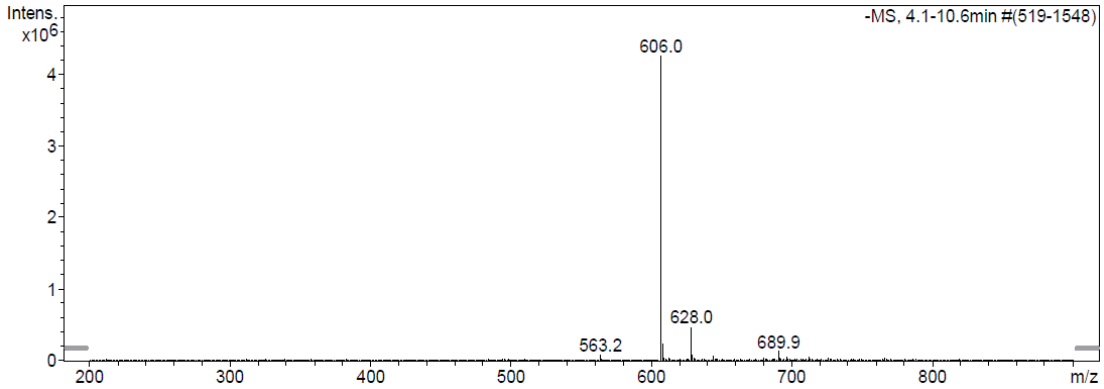


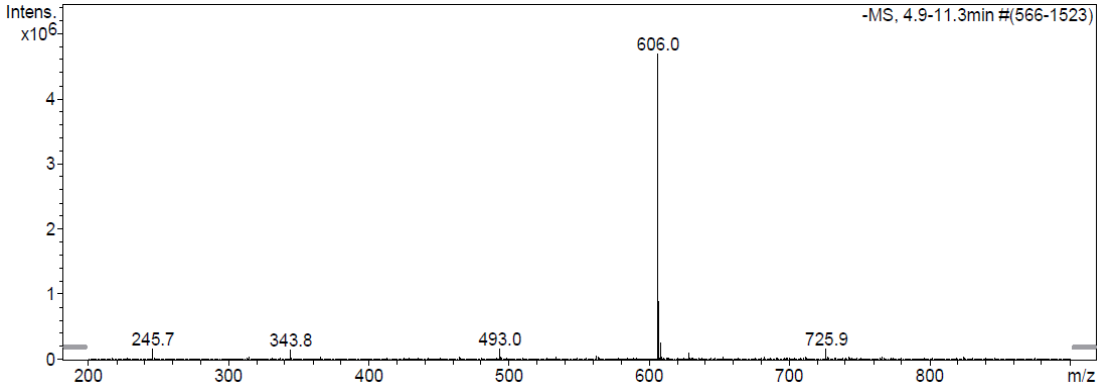
Figure 4.21 The proposed CMP-Pse5Ac7Ac biosynthetic pathway in *A. caviae*.

This diagram shows CMP-Pse5Ac7Ac is synthesized from UDP-N-acetylglucosamine via FlmA, FlmB, FlmD, NeuB and NeuA (Schoenhofen et al. 2006). FlmA (PseB) initiates the biosynthesis of pseudaminic acid via catalyzes UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-2-acetamido-2,6-dideoxy-b-L-arabino-hexos-4-ulose. Subsequently, the product modified by FlmB (PseC) and UDP-4-amino-4,6-dideoxy-b-L-AltNAc is generated. After processed by FlmD (PseH/PseG), 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose is generated. Eventually, 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose is catalyzed by NeuB (PseI) and results in pseudaminic acid (Schoenhofen et al. 2006; Schirm et al. 2003). The pseudaminic acid is activated by NeuA (PseF) and results in CMP-Pse5Ac7Ac (Schirm et al. 2003)

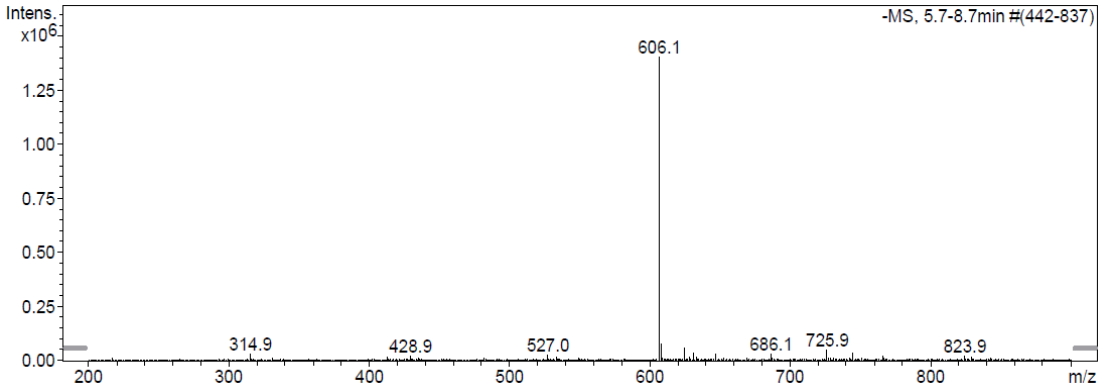
(A)


Intens.
x10^6
-MS, 4.1-10.6min #(519-1548)
606.0
628.0
563.2
689.9
m/z

(B)


Intens.
x10^6
-MS, 4.9-11.3min #(566-1523)
606.0
245.7
343.8
493.0
725.9
m/z

(C)


Intens.
x10^6
-MS, 5.7-8.7min #(442-837)
606.1
314.9
428.9
527.0
686.1
725.9
823.9
m/z

(D)

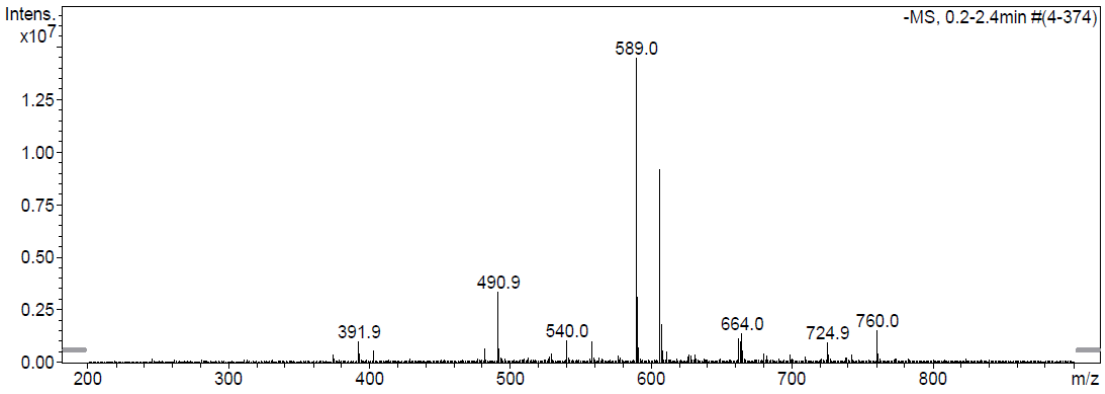


Figure 4.22 LCMS data of reactions of PseB and PseC from *H. pylori* and FlmA and FlmB from *A. caviae*.

Peak 606 m/z indicates UDP-GlcNAc and peak 589 m/z indicates UDP-4-amino-4,6-dideoxy-b-*L*-AltNAc. All data was collected after 4 hours of reaction.

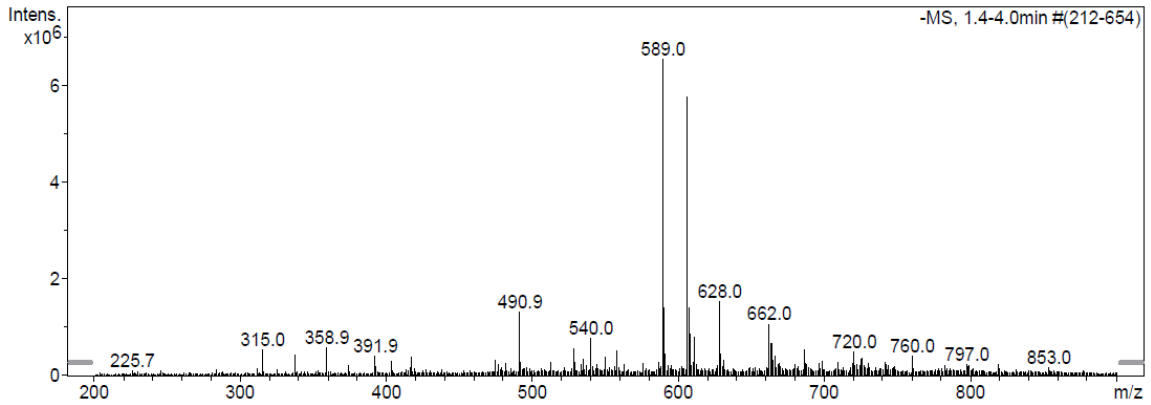
(A) The reaction mixture with PseC from *H. pylori*, showing no conversion to product.

(B) The reaction mixture with FlmA from *A. caviae* and PseC from *H. pylori* after 4 hours reacting, showing no conversion to product.

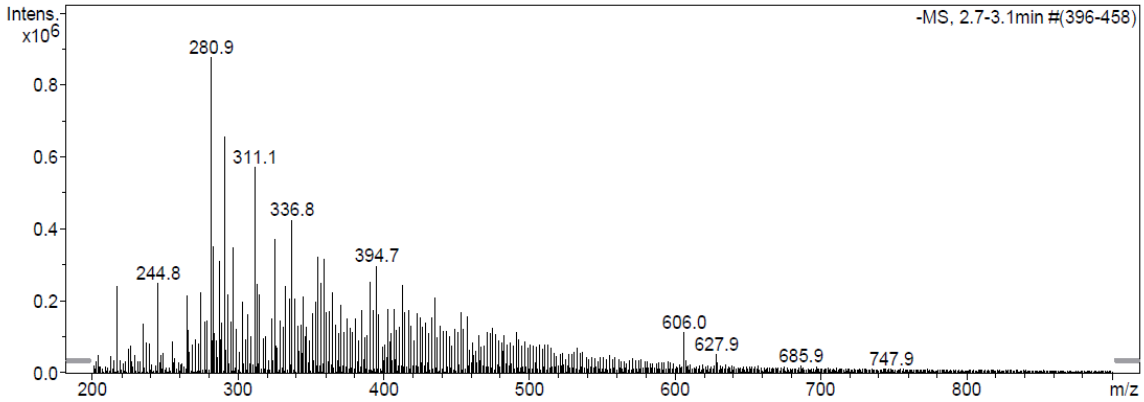
(C) The reaction mixture with FlmA from *A. caviae* and PseC from *H. pylori* after leaving overnight (more FlmA from *A. caviae* had been added after 6 hours), showing no conversion to product.

(D) The reaction mixture with PseB from *H. pylori* and FlmB from *A. caviae* after leaving overnight, showing some conversion to product (UDP-4-amino-4,6-dideoxy-b-*L*-AltNAc).

(A)


Intens.
x10^6
-MS, 1.4-4.0min #(212-654)
589.0
628.0
662.0
490.9
540.0
315.0
358.9
391.9
225.7
720.0
760.0
797.0
853.0
m/z

(B)


Intens.
x10^6
-MS, 2.7-3.1min #(396-458)
280.9
311.1
336.8
394.7
244.8
606.0
627.9
685.9
747.9
m/z

(C)

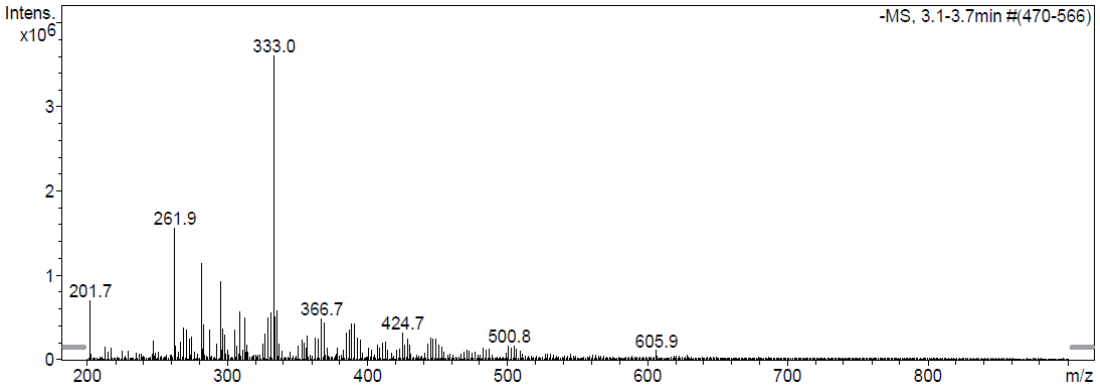

Intens.
x10^6
-MS, 3.1-3.7min #(470-566)
333.0
261.9
201.7
366.7
424.7
500.8
605.9
m/z

Figure 4.23 LC-MS data of reactions of PseB, FlmB, FlmD and NeuB

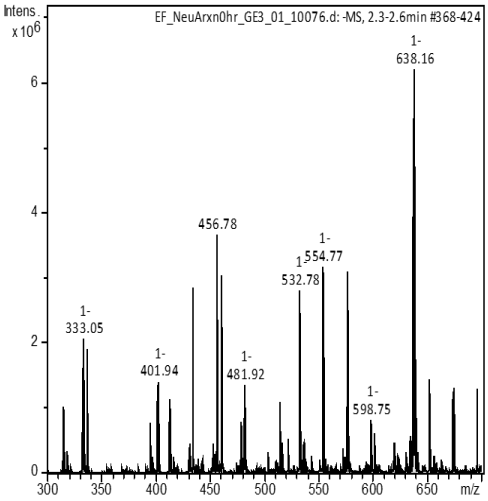
Peak 244 m/z indicates 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose; peak 333 m/z indicates pseudaminic acid; peak 606 m/z indicates UDP-GlcNAc and peak 589 m/z indicates UDP-4-amino-4,6-dideoxy-b-*L*-AltNAc. All data was collected after 4 hours of reaction.

(A) The reaction mixture with PseB from *H. pylori* and FlmB from *A. caviae* after leaving overnight. There is no loss of the FlmB product (UDP-4-amino-4,6-dideoxy-b-*L*-AltNAc) without FlmD.

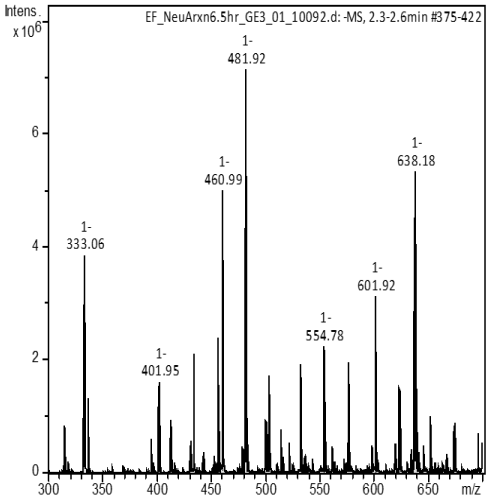
(B) The reaction mixture with PseB from *H. pylori* and FlmB and FlmD from *A. caviae* after leaving overnight showing complete loss of the FlmB product and some residual UDP-GlcNAc.

(C) The reaction mixture with PseB from *H. pylori* and FlmB, FlmD and NeuB from *A. caviae*, showing formation of pseudaminic acid.

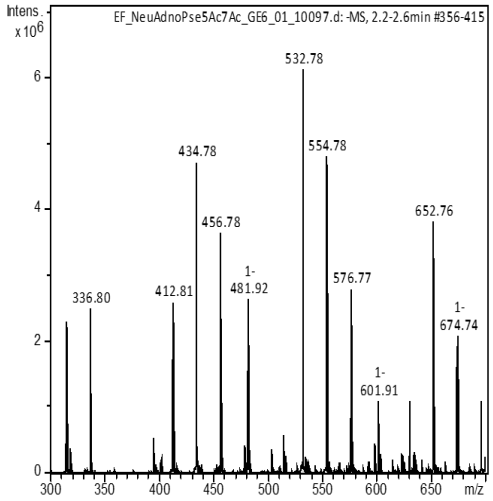
(A)


EF_NeuArxn0hr_GE3_01_10076.d: -MS, 2.3-2.6min #368-424
Intens.
x 10^6
1-
638.16
456.78
1-
554.77
532.78
1-
333.05
1-
401.94
1-
481.92
1-
598.75
m/z

(B)


EF_NeuArxn6.5hr_GE3_01_10092.d: -MS, 2.3-2.6min #375-422
Intens.
x 10^6
1-
481.92
1-
460.99
1-
638.18
1-
333.06
1-
601.92
1-
554.78
1-
401.95
m/z

(C)


EF_NeuAdnoPse5Ac7Ac_GE6_01_10097.d: -MS, 2.2-2.6min #356-415
Intens.
x 10^6
532.78
434.78
554.78
456.78
652.76
1-
481.92
576.77
336.80
412.81
1-
674.74
1-
601.91
m/z

(D)

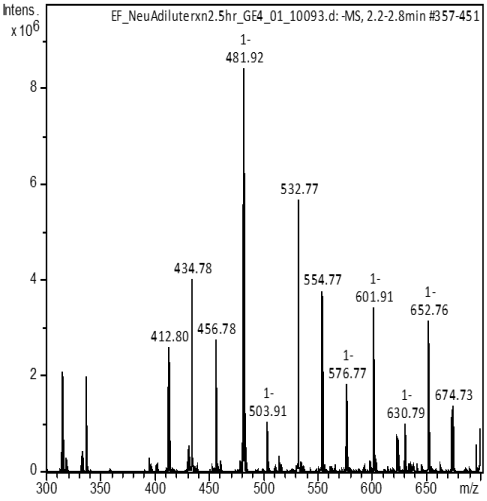

EF_NeuAdiluterxn2.5hr_GE4_01_10093.d: -MS, 2.2-2.8min #357-451
Intens.
x 10^6
1-
481.92
532.77
434.78
554.77
1-
601.91
1-
652.76
412.80
456.78
1-
576.77
1-
503.91
1-
630.79
674.73
m/z

Figure 4.24 LC-MS data of reaction of NeuA from *A. caviae*

Peak 333 m/z indicates pseudaminic acid, peak 638 m/z indicates CMP-pseudaminic acid and peak 481 m/z indicates CTP. Reaction conditions: 5 mM Pse5Ac7Ac, 1.5 mM CTP, 10 mM $MgCl_2$, 50 mM NaCl, 25 mM sodium phosphate, pH 7.4, 360 μgmL^{-1} NeuA.The enzyme control was not included.

(A) Initially reactions were performed at 5 mM Pse5Ac7Ac (4 hours), showing formation of CMP-pseudaminic acid.

(B) Initially reactions were performed at 5 mM Pse5Ac7Ac (4+6.5 hours, at 25 °C), showing formation of CMP-pseudaminic acid.

(C) Reaction mix without Pse5Ac7Ac (control group), showing no formation of CMP-pseudaminic acid.

(D) Initially reactions were performed at 0.5 mM Pse5Ac7Ac (4+2.5 hours, at 25 °C), showing no formation of CMP-pseudaminic acid.

4.7 Discussion

NeuA which is encoded by the *neuA* gene is the CMP-sugar synthetase in *A. caviae* Sch3N. The function of *neuA* was to be investigated in this project. Firstly, a mutant strain *A. caviae* Sch3N *neuA*::Km was constructed via PCR, ISA, conjugation and allelic exchange. This strain is a *neuA* insertional knockout and is kanamycin resistant. Swimming ability of this strain has been confirmed to be abolished which means *neuA* is essential for the biosynthesis of the functional polar flagella (Figure 4.5A). Then, two complementing strains which are *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) were constructed via conjugation of these plasmids into the mutant strain. The strain *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) contains pBBR1-MCS-*neuA* which was able to compensate the swimming ability of *A. caviae neuA* mutant (Figure 4.5B). The strain *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) contains pBBR1-MCS-*neuA*-His which was able to complement the swimming ability of *A. caviae neuA* mutant (Figure 4.5C). This finding indicates that His-NeuA which is encoded by pBBR1-MCS-*neuA*-His has the same ability of NeuA and that the addition of an N-terminal His-tag to this protein does not affect its activity. The previous reported *neuA* mutant had a polar effect on downstream genes including *flmD* and

neuB, as the introduction of pBBR1-MCS-*neuA* or pBBR1-MCS-*neuA*-His into this strain did not rescue the phenotype of the mutant (Tabei et al. 2009). These results suggest we would be able to purify an active His-tagged NeuA enzyme from A*. caviae* if the pET-28a-*neuA*-His overexpression did not work and resulted in soluble protein. However, we are able to recover soluble His-tag NeuA from *E. coli*, this enzyme was subsequently shown to be active (Figure 4.18).

Secondly, the LPS of *A. caviae neuA* mutant was analyzed. The result from LPS extraction confirmed that the LPS from this mutant lacked O-specific polysaccharides which contains pseudaminic acid (Tabei et al. 2009) (Figure 4.7). According to Figure 4.7, only the core oligosaccharide (OS) was detected and O-specific polysaccharides were absent in *A. caviae neuA* mutant. The LPS of *A. caviae neuA* mutant (pBBR1-MCS-*neuA*) and *A. caviae neuA* mutant (pBBR1-MCS-*neuA*-His) were as same as the LPS of *A. caviae* Sch3N wild type which means the LPS biosynthesis in these stains were restored. This finding indicates that *neuA* is essential for the biosynthesis of functional LPS and it is related to O-specific polysaccharide as previously Tabei et al 2009 showed Pse5Ac7Aac to be present in the LPS of the strain. Based on the result of western blot of polar flagellin of these strains, the biosynthesis of functional flagellin was abolished in *A. caviae neuA* mutant (Figure 4.8). This finding is match to the result of swimming ability assay and the previously reported findings of Tabei et al (2009).

Based on the information above, the results of swimming ability assay, western blot and LPS extraction are all in accordance with the hypothesis. The NeuA-His has been expressed in *A. caviae* and produces an active enzyme. Which make it is possible to generate CMP-Pse5Ac7Ac via NeuA-His and Pse5Ac7Ac.

The *A. caviae flmA* mutant strain was found to be non-motile compared to wild-type (WT) (figure 4.5E). Swimming ability of this strain has been confirmed to be abolished which means *flmA* is essential for the biosynthesis of the functional polar flagella (Figure 4.5E).

The *flm* locus which is involved in pseudaminic acid biosynthesis is an important target of this study. With the help of the proteins encoded by the *flm* genes, it is possible to reconstruct the pseudaminic acid biosynthetic pathway and generate Pse5Ac7Ac and CMP-Pse5Ac7Ac. The plasmids which contain the *flm* genes some

of which have been constructed by former researchers, but whether the proteins encoded by these plasmids are soluble were still needed to be confirmed. Based on the progress of experiment, all of the plasmids with *flm* gene insertion were extracted via mini-preparation successfully. The overexpression and purification of each protein have been has been shown. Based on the figure 4.13, figure 4.14, figure 4.15, figure 4.16 and figure 4.17, it is obvious that the bands of FlmA, FlmB, FlmD, NeuA and NeuB were thick and high amount of nearly pure protein is visible on SDS-PAGE gels. Based on the results of protein purification, a protein of about 40kDa was purified according to Figure 4.13 and the presumed protein is FlmA. A protein which is about 45kDa was purified according to Figure 4.14 and the presumed protein is FlmB. A protein of about 100kDa was purified according to Figure 4.17 and the presumed protein is FlmD. A protein of about 28kDa was purified according to Figure 4.15 and the presumed protein is NeuA. A protein of about 41kDa was purified according to Figure 4.16 and the presumed protein is NeuB.

With the help of these 5 enzymes, generation of CMP- Pse5Ac7Ac was attempted. His-FlmA did not catalyzed UDP-GlcNAc to UDP-2-acetamido-2,6-dideoxy-b-*L-arabino*hexos-4-ulose. The purified FlmA was confirmed to be soluble and well folded. The reason why it is inactivated could be the His-tag affected the function of FlmA. Previous research has been proved that FlaA1 from *H. pylori* can complement *A. caviae flmA* mutant(Tabei et al. 2009).

It has been revealed that FlaA1 oligomers that are consistent with the hexameric structure derived from crystallographic analysis, this finding suggests that the FlaA1 enzyme exists as a hexamer in solution(Noboru Ishiyama et al. 2006). Based on that result, the reason why FlmA is inactivated could also be the FlmA protomers did not combined properly to form a hexamer. The *A. caviae flmA* mutant strain is suitable for conjugation with plasmid expressing FlmA protein. The pBBR1-MCS-*flmA* will be constructed in the further experiment to investigate the function of flmA gene.

His-FlmB, His-FlmD His-NeuA, and His-NeuB were all tested to be soluble. Based on the results of enzymatic reactions of His-FlmB, MBP-FlmD His-NeuA, and His-NeuB, all of these enzymes are functional and CMP-Pse5Ac7Ac has been successfully generated from UDP-2-acetamido-2,6-dideoxy-b-*L-arabino*hexos-4-ulose via these enzymes.

FlmB (PseC) which is aminotransferase catalyzes UDP-2-acetamido-2,6-dideoxy-b-L-arabino-hexos-4-ulose and L-glutamine and result in UDP-4-amino-4,6-dideoxy-b-L-AltNAc (590.372 g/mol) and a-ketoglutarate. His-FlmB has been proved to be functional and this finding indicates that His-tag did not affect the function of FlmB. After processed by FlmD (PseH/PseG) which is acetyltransferase and nucleotidase, the UDP-4-amino-4,6-dideoxy-b-L-AltNAc was catalyzed to 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose (246.26 g/mol). The acetyl group was induced and UDP group was removed in this process. MBP-FlmD has been proved to be functional and this finding indicates that MBP-tag did not affect the function of FlmD. Besides, FlmD has the same ability of PseH and PseG from *H. pylori* or Cj1312 and Cj1313 from *C. jejuni* (Liu and Tanner 2006; McNally et al. 2006). NeuB (PseI) which is pseudaminic acid synthetase catalyzes PEP and 2,4-diacetamido-2,4,6-trideoxy-b-L-altropyranose and result in pseudaminic acid(334.32g/mol) and phosphate (Schoenhofen et al. 2006; Schirm et al. 2003). His-NeuB has been proved to be functional and this finding indicates that His-tag did not affect the function of NeuB. NeuA (PseF) is acts as CMP-sugar synthetase and catalyzes pseudaminic acid and CTP and result in CMP-Pse5Ac7Ac and pyrophosphate(Schirm et al. 2003). His-NeuA has been proved to be functional which is capable to produce CMP-Pse5Ac7Ac from Pse5Ac7Ac. This finding indicates that His-NeuA has the same function as NeuA and His-tag did not affect the function of NeuA. Furthermore, CMP- Pse5Ac7Ac has been generated successfully and it can be applied to glycosylate FlaA with the help of Maf1.

4.8 Conclusion

FlmA, FlmB, FlmD, NeuA and NeuB were all successfully expressed from plasmids. Based on the result of SDS-PAGE, 5 proteins were all purified and proved to be soluble, but His-FlmA proved to be well folded but inactivated (Figure 4.18 and Figure 4.19).

It has been confirmed that pseudaminic acid can be generated from UDP-GlcNAc via PseB from *H. pylori*, FlmB, FlmD and NeuB from *A. caviae.* His-FlmB has been proved to be functional and this finding indicates that His-tag did not affect the function of FlmB. MBP-FlmD has been proved to be functional and this finding indicates that MBP-tag did not affect the function of FlmD. Besides, FlmD has the same ability of PseH and PseG from *H. pylori* or Cj1312 and Cj1313 from *C. jejuni.* His-NeuB has been proved to be functional and this finding indicates that His-tag did

not affect the function of NeuB. CMP-Pse5Ac7Ac has been successfully generated from Pse5Ac7Ac which was catalyzed by His-NeuA. His-NeuA has the same function as NeuA and His-tag did not affect the function of NeuA (Figure 4.22, Figure 4.23 and Figure 4.24).

The biosynthesis of functional flagellin was abolished in *A. caviae neuA* mutant and this strain did not have polar effect. The palsmids including pBBR1-MCS-*neuA*-His and pBBR1-MCS-*neuA* can both complement *A. caviae neuA* mutant. His-NeuA which is encoded by pBBR1-MCS-*neuA*-His has the same ability of NeuA and that the addition of an N-terminal His-tag to this protein does not affect its activity (Figure4.5 and Figure 4.6).

The *A. caviae flmA* mutant strain was found to be non-motile compared to wild-type (WT) (Figure 4.5E). Swimming ability of this strain has been confirmed to be abolished which means *flmA* is essential for the biosynthesis of the functional polar flagella (Figure 4.5E).

LPS from *A. caviae neuA* mutant lacked O-specific polysaccharides which contain pseudaminic acid and the plasmids including pBBR1-MCS-*neuA*-His and pBBR1-MCS-*neuA* can restore this defect in *A. caviae neuA* mutant (Figure 4.7). This finding suggests that neuA is essential for the biosynthesis of functional LPS and it is related to O-specific polysaccharide. This result is corresponding to the result that his-NeuA encoded by pBBR1-MCS-*neuA*-His has the same ability as NeuA encoded by pBBR1-MCS-*neuA*. Based on the result of western blot of polar flagellin of these strains, the biosynthesis of functional flagellin was abolished in *A. caviae neuA* mutant (Figure 4.8). This finding is match to the result of swimming ability assay and the previously reported findings of Tabei et al (2009).

Chapter 5 Conclusions

The aim of this project was to investigate proteins associated with flagellar glycosylation in *Aeromonas caviae*.

Maf1 the motility-associated factor is involved in polar flagellar biosynthesis and it is thought to be the glycosyl-transferase that directly transfer of pseudaminic acid onto the polar flagellin proteins in *A. caviae* Sch3N (Tabei et al. 2009; Parker et al. 2012). The Maf1 protein is deemed as a putative flagellin pseudaminyl-transferase (Parker et al. 2012). Theoretically, Maf1 of *A. caviae* Sch3N has the ability to transfer activated pseudaminic acid (CMP-Pse5Ac7Ac) to the hydroxyl group of serine and threonine residues in the central immunogenic D2/D3 domain of flagellin of *A. caviae* Sch3N (Parker et al. 2012; Tabei et al. 2009). Flagellin proteins, are usually insoluble, previous work has shown that the C-terminal chaperone binding domain of the *A. caviae* flagellin FlaA is not involved in the glycosylation process (Parker et al. 2014)(Parker et al 2014). Identification of the site where Maf1 binds is crucial for the investigation of glycosylation of flagellin. Attempts have been made to create deletion derivatives of flagellin that are still glycosylated and are more soluble to provide more suitable substrate for future glycosylation assays.

The plasmids including pBBR1-MCS-*flaA0*+~pBBR1-MCS-*flaA15*+ and pBBR1-MCS-*flaA0*-~pBBR1-MCS-flaA15- are all functional and producing FlaA or FlaA deletion derivatives in the conjugated strains. pBBR1-MCS-flaA0- can produce fully functional FlaA and partially restore the swimming ability of *A. caviae flaA-flaB* mutant. The pBBR1-MCS-*flaA0*+ can not restore the swimming ability of *A. caviae flaA-flaB* mutant. The rest of all plasmids with *flaA* gene deletion can not restore the swimming ability of *A. caviae flaA-flaB* mutant. This result indicates that complete FlaA encoded by pBBR1-MCS-flaA0- is essential for the flagella assembly and deletion in *flaA* gene will result in absence of functional flagella. Expression of the complete *flaA* gene (*flaA0-*) in the minus orientation with the *lacZ* promoter resulted in complementation, whereas in the plus orientation it did not, this suggests expression of the flagellin is better or greater in the minus orientation. The possible reason for this result is better expression from the *lac* promoter.

However, expression of FlaA0- only resulted in partial (50%) recovery of motility

in the mutant strain, this is in agreement with the result from Rabaan et al (2001). This suggests that both flagellins are required for optimal flagella filament function, even though both flaA and flaB are almost identical to each other (Rabaan et al. 2001). Or that the expression leads of the flagellins are not optimal. However, on comparation with the normal western blot the flagellin levels of the *flaA0*- expressing strain do not look to be expressed to the high levels when compared to the wild type (Figure 3.8B).

Deletion strains including *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA1*-) to *A. caviae flaA-flaB* mutant (pBBR1-MCS-*flaA15*-) are non-motile. This result is probably caused by not being bound correctly by FlaJ (CBD removed) (Parker et al. 2012).

Western Blotting of these FlaA deletion derivatives shows that FlaA0- to FlaA9- were all glycosylated and FlaA10- to FlaA15- were all unglycosylated. This result suggested that the site of Maf1 bind is required is around amino acids 256-250. The flagellins after deletion of CBD amino acids 220-250 were still expressed but unglycosylated and FlaA10- to FlaA15 did not decompose. However, the abilities of binding anti-FlaA antibody of FlaA deletion derivatives are reducing. The explanation for this result may be the loss of C-terminal amino acids. Further experiment will be applied to determine if Maf1 can bind these FlaA deletion derivatives. Whether the FlaA deletion derivatives which lack the CBD amino acids 256-306 can bind the Maf1 will be investigated in the future.

In order to reconstruct the glycosylation of FlaA *in vitro*, a source of soluble FlaA deletion derivative which still can be glycosylated is required. Based on the results, the FlaA8- expressed by pBBR1-MCS-flaA8- and FlaA9- expressed by pBBR1-MCS-flaA9- have been tested and the result indicates that the FlaA8- and FlaA9- were both detected in the soluble fractions (Figure 3.12). This finding suggests that FlaA8- and FlaA9- are soluble and glycosylated which are suitable for the further experiments.

Furthermore, to prove that Maf-1 is indeed the glycosyl-transferase the substrate for the reaction need to be isolated or created, namely CMP-Pse5Ac7Ac which is the activated form of pseudaminic acid. Therefore, in order to create CMP-Pse5Ac7Ac, the Flm proteins from the pseudaminic biosynthesis pathway have been overexpressed and purified. Hence the biosynthesis of Pse5Ac7Ac and CMP-Pse5Ac7Ac can be looked into via these reactions. According to the results of

SDS-PAGE of Flm proteins, FlmA, FlmB, FlmD, NeuA and NeuB were all successfully expressed from plasmids and all of the proteins were purified and proved to be soluble. Unfortunately, initially reaction was performed with UDP-GlcNAc and FlmA which turned out to be inactivated. The CD spectra was applied to examine the structure of FlmA and the result indicates that FlmA is well folded. In the meanwhile, swimming ability assay of *A. caviae flmA*::Km suggests that the swimming ability of this strain has been confirmed to be abolished which means *flmA* is essential for the biosynthesis of the functional polar flagella (Figure 4.5E).

Based on the data of LC-MS, it has been confirmed that FlmB, FlmD, NeuB and NeuA are all activated and CMP-Pse5Ac7Ac has been successfully generated from UDP-2-acetamido-2,6-dideoxy-b-*L-arabino*hexos-4-ulose. His-FlmB has been proved to be functional and this finding indicates that His-tag did not affect the function of FlmB. MBP-FlmD has been proved to be functional and this finding indicates that MBP-tag did not affect the function of FlmD. Besides, FlmD has the same ability of PseH and PseG from *H. pylori* or Cj1312 and Cj1313 from *C. jejuni.* His-NeuB has been proved to be functional and this finding indicates that His-tag did not affect the function of NeuB. CMP-Pse5Ac7Ac has been successfully generated from Pse5Ac7Ac which was catalyzed by His-NeuA. His-NeuA has the same function as NeuA and His-tag did not affect the function of NeuA.

It has been proved to be feasible to prepare CMP-Pse5Ac7Ac via Flm protein and substrates including UDP-GlcNAc. The unglycosylated FlaA deletion can be produced from pBBR1-MCS containing *flaA* gene deletion (pBBR1-MCS-*flaA9*-). Maf1 has been investigated and prepared by former researchers. With the help of above substrates and enzyme, it is possible to reconstruct the FlaA glycosylation in vitro via Maf1, CMP-Pse5Ac7Ac and unglycosylated FlaA deletion.

The connections between glycosylation in flagellin biosynthesis and LPS O-antigen biosynthesis were summarized. The biosynthesis of functional flagellin was abolished in *A. caviae neuA* mutant and this strain did not have polar effect. The palsmids including pBBR1-MCS-*neuA*-His and pBBR1-MCS-*neuA* can both complement *A. caviae neuA* mutant. His-NeuA which is encoded by pBBR1-MCS-*neuA*-His has the same ability of NeuA and that the addition of an N-terminal His-tag to this protein does not affect its activity. LPS from *A. caviae neuA* mutant lacked O-specific polysaccharides which contain pseudaminic acid and the plasmids including pBBR1-MCS-*neuA*-His and pBBR1-MCS-*neuA* can restore this

defect in *A. caviae neuA* mutant. This finding suggests that neuA is essential for the biosynthesis of functional LPS and it is related to O-specific polysaccharide. This result is corresponding to the result that His-NeuA encoded by pBBR1-MCS-*neuA*-His has the same ability as NeuA encoded by pBBR1-MCS-*neuA*.

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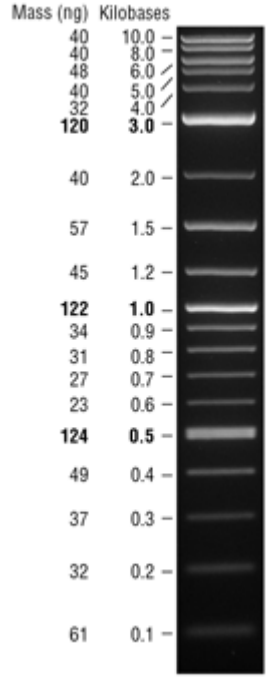
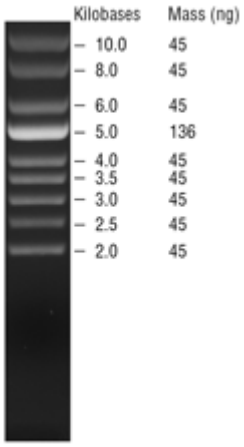
Appendix 1

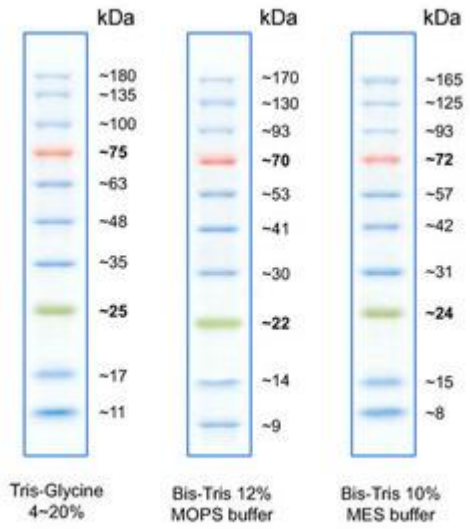
2-Log linear DNA ladder:



Supercoiled ladder:



Prestained protein ladder:



Primers used in the project

flmA-forward: GCGCATATGCTGAATAATAAAACAGTATTAATCAC

flmA-reverse: GCGGGATCCTCACACTTCAAAGTTGGCAT

flmD-forward: GCGCATATGAAGTTTTTTATTCGCACC

flmD-reverse: GCGAGGCTTTTATTTTGTCCATAATGG

flaA-forward: GCGCAAGCTTGTTAAATTTTAATAGCTTAGGAGAAAAATCAT

flaA0-reverse: GCGAAGCTTGAACCTTAGTTCTGCAGCAG

flaA1-reverse: GCGAAGCTTCAGCAGGGACTACGCAGACTG

flaA2-reverse: GCGAAGCTTCTGCGGACGCTAATTGGCCTG

flaA3-reverse: GCGAAGCTTCTGGGCCAGTCAGCTGGAAGC

flaA4-reverse: GCGAAGCTTAGCGGCCTGTTACAGGATATT

flaA5-reverse: GCGAAGCTTATTCTGCTTTCACATATTGGC

flaA6-reverse: GCGAAGCTTGGCGGTCTCTCATGCAAAATC

flaA7-reverse: GCGAAGCTTATCTGCATCTTAGATACGAGA

flaA8-reverse: GCGAAGCTTAGAACGGGCTTAGCTTACGTT

flaA9-reverse: GCGAAGCTTGTTTTCCGATTAATTGGCCTG

flaA10-reverse: GCGAAGCTTCTGGTTGCGTTAGGTGGAATC

flaA11-reverse: CGCAAGCTTATCCAGACGTTACTGCACCGC

flaA12-reverse: CGCAAGCTTCGCACCCAGTTATGCTCGCTT

flaA13-reverse: CGCAAGCTTCTTGCTATCTTAGACTTCCAA

flaA14-reverse: CGCAAGCTTCAACATAGCTTAAGCAGCAGC

flaA15-reverse: GCGAAGCTTAAGGCCCAATTAGTTTTGGCT

Appendix 2

Sequence of flm genes and flaA gene

flmA:

TCACACTTCAAAGTTGGCATCTACATGAGTTTTTATCAATTCCCGCAGAGACTCCA
CGGTCTCCCACTCAGTGTTAGTACCTGAGTTATATTTGAAACCAAACGGTACTTTT
ACGGCACTATGATGCTTAATATAGTCTTCTTCTGTATGATTGAATGACACAGAAGG
TAAAATTGCGTAATAGCGGCCCAAATCAATTGTGTTCAATGAATCGGTATCTGTAA
TCATCTCTTCATGCAGCTTTTCTCCTGGACGAATACCAACTACCTTCGTCTTGCAT
TCAGGAGATATTGCTGTTGCTATATCTAAAATACGATATGATGGGATTTTTGGCACA
AAAATCTCACCACCGAGATGATGCTCCAGAGCATACATCACCATATTAACACCATC
CTGCAGTGAAATATTAAAACGTGTCATTTCTTCATGGGTGATCGGCAACACACCC
TCAGCACGTTTTTTCAAGAAAAATGGTATTACAGAACCCCGAGACCCCATTACATT
TCCATAGCGAACTACACTGAATCTTATATTTCGCGAACCTTTGATATTATTTGCTGC
TGTAAACAACTTGTCAGAGGTCAGCTTTGTCGCTCCATAAAGATTGATCGGCGCA
CAAGCTTTATCTGTTGACAACGCTACAACTTCTTTTACTCCACACTGCAAAGCAG
CGTGGATAACATTTTCAGCACCATCGACGTTAGTGCGAATGCACTCTGTCGGATT
ATACTCGGCTGTATCTACTTGTTTAATAGCAGCAGCATGAATAATGACATCGATTCC
TTCGCACGCTTGAACCATTCGATTACGATCACGTACATCACCAATGAAAAAGCGC
AGTTGTGGATAATCTTTTTGCGGATAATTTAAACGTAATTCAGATTGCTTTAGTTCA
TCTCGAGAAAAAATGACAATGCGCTTAACTTGGGGGTAGCGCTCAAGGATAGTTT
TTATAAATTGTTTACCAAATGATCCCGTACCACCTGTGATTAATACTGTTTTATTATT
CAGCAT

flmB:

TTATTTCCCTTCCAATATCTCAGACAATACGCATACTACTTTATCTTGCTGCTCTTC
TGTCATGCCGTGGAACATCGGCAAAGAAATAGCTTCACGATAATATTGCTCTGCC
TCAGGAAAATCACCAGATTTGAATCCCATATCTTCGTAATAAGGCTGTGTATGTAC
AGGTATGTAATGAACATTCACCCCGATACCATTTTCACGTAACGCTTCAAAAACCT
CTCGGTGCGTTAATGAAATATTATTAAGCTGAAGTCGTATAACGTAAAGATGCAAT
CCGGAATGGGTATTTTCTAACTGGAATGGTAAGACTATCGGTAACCGCTTCAAAA
GTCTGTTATAACGGTCAGCCAAGCGATGACGAGCAGCGATAAATTCATCAAGGC
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CCCAAGTCAATCTGTTGATAATACCAACCACCATGACTTTCGCCTTTCATCTGCTC
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AACTACCGCCGATAGCATGTGATGCATCCTCGATAACTTTAAAGCCATATTCAGTT
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GCCACAATACAAACCGCAATTTGCTGATGCAACAAAAGTAACTGGGCTTGTCCAT
AAAAAATCGCCAGGACCAAGACCCAAAGCCAAACAGGCAATGTGTAGTGCCGAA
GTAGCACTATTTACAGCCAACGCATACGCTGCGCCAGTATGGGTTGTTAAATATTT
TTCAAATTCAGGGACTTTTGGTCCCTGAGTTAGAAAATCAGATTGTAACACCGCA
ACCACGCCATCAATATCTTGCTGGGTGATCTCTTGTTTACCGTATGGAATCAC

flmD:

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ATCCTGATTTTTGGTAGGCATCTATCAAGTAATTTCAAACCTGTTAATCCAATACCC
TGACCATGCTTATTAGGAGCAACAAAAATTGAGATAAGGTAATTGTCAGTACCTAG
TTCATCTAGTCTAACTGCACCACAGGGTTCGCTACTTTCAGCATTAATTATCAAAA
AGAAATAATCTTGCTGGCACTGTATTTTTTTTTCCATCCAGCTTTTGTGCTCTACC
CAACTAGGGGGATTGGGATTAAGCGCATAACGTCTGGTTATTGGATCAGATTGCC
ACTGATAAATCAGTAGAGTATCATCGATTGATACCGGCCTTAGCCTAAATCCTGATA
TTTCTGTATTAGCCATAATAGTATTAATGACTCGTTGCACCCCCTTGCCATCACACA
ACTCGAGATTATTATCTACGTATTCTTTATAATTATTCAACAAGTCATGCAGTGCAG
GGATTAATCTACTTTCAATATCAGACTGTTGTACTAATAAAGCAGCTTGATGTGCCA
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CACATTTGGCTCATCAATACCACCAAAGGAGAGTAAAATCCTAGGCTGACGACTG
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AGGCGTATGCAACTCCACTTAATACTACAGGCTGCTTGTCTGATATATTAAAACAC
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CGTATACCACACTTTCTAAAACATCTGCAGCGTCTTGTTGCCATGGCACTTGCAA
CCAAGAAGAATAATCAGCCCCATGCTCAGGTCTGATAACAGTTGTCAGAGATGGC
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neuB:

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TGAAGATGGTGATCCCCAACTCTTTGGCTTTCTCAAACAACGGTTGGTGCCACT
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neuA:

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GCACCATATTCGAGTGCAACAGCAGCAATTTCAGCATCATCGGTTGAAACAATTAT
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flaA:

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AA