Genetic regulation of the Type III Secretion System and its potential effect on the lateral flagella system in *Aeromonas hydrophila* AH-3

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Summary

Aeromonas species are ubiquitous water-borne bacteria that are able to cause a variety of diseases in poikilothermics and humans. *Aeromonas hydrophila* is one of the most pathogenic species, responsible for aeromonad septicaemia in fish, gastroenteritis and wound infections in humans. The T3SS is utilized to inject protein effectors directly into host cells. One of the major genetic regulators of the T3SS in several Gram-negative bacterial species is the AraC-like protein ExsA. Lateral flagella are expressed by bacteria upon contact with host cells or a surface and are required for host cell adherence and biofilm formation. However, no direct link between the T3SS and the lateral flagella system has yet been found in *A. hydrophila*. Moreover, the genetic regulation of the T3SS that involves the master regulator ExsA has not been determined in *A. hydrophila* AH-3.

The aim of this project is to determine the genetic regulation of the T3SS and the potential interaction between the T3SS and the lateral flagella system in *A. hydrophila* AH-3.

The genes encoding the T3SS regulatory components including *exsA*, *exsD*, *exsC* and *exsE* were mutated and the activities of the T3SS promoters were measured in *exs* mutant backgrounds. The interactions between each of the Exs proteins were investigated using BACTH assay and Far-Western Blot. Together, the findings suggested a regulatory cascade that the effector protein ExsE was bound to the chaperone protein ExsC, which sequestered the anti-activator ExsD from inhibiting the T3SS master regulator ExsA via direct protein-protein interactions.

The T3SS regulatory components were also shown to affect the expression of the lateral flagella system using swarming assays. The activities of the lateral flagella promoters were shown to be repressed by the absence of ExsD and ExsE, suggesting that the T3SS master regulator ExsA was a negative regulator of the lateral flagella system.

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Abbreviations

°C	Degrees celsius
µg	micrograms
µl	microlitre
Amp	Ampicillin
APS	Ammonium persulphate
BACTH	Bacterial Adenylate Cyclase Two-Hybrid
BHIB	Brain Heart Infusino Broth
BLAST	Basic local alignment search tool
bp	Base pairs
cAMP	3'-5'cyclic adenosine monophosphate
Cm	Chloramphenicol
cm	Centimeter
Da	Daltons
dH_2O	Sterile MilliQ water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
g	grams
Gm	Gentamycin
HCl	Hydrochloric acid
hr	hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
kDa	kilo Daltons
Km	Kanamycin
L	Litre
LB	Luria-Bertani

M	Molar
mg	miligrams
min	minutes
ml	mililitre
mM	milimolar
MOPS	3-(N-morpholino)-propanesulfonic acid
MU	Miller Unit
nm	nanometers
OD	Optical density
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
p (value)	Probability (value)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
^{R}	Resistant
Rif	Rifampicin
RNA	Ribonucleic acid
RNAse	Ribonuclease
s	seconds
SDS	Sodium dodecul sulphate
Sm	Streptomycin
TEMED	N,N,N,N-tetra-methylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Volts
v/v	Concentration volume by volume
w/v	Concentration weight by volume
xg	times gravity

Chapter 1

Introduction

1.1 The genus *Aeromonas*

Aeromonas species are ubiquitous water-borne Gram-negative facultative anaerobes that are able to cause a variety of diseases in poikilothermics and humans. The genus *Aeromonas*, since the first discovery in the 19th century, has been studied by scientists for over 100 years (Janda & Abbott 2010).

The first report of *Aeromonas* was identified in 1891 in a study of septicemia ('red-leg' disease) of frogs, which was linked with the bacterium *Bacillus hydrophilus fuscus*, now known as *Aeromonas hydrophila* (Farmer et al 2006). The taxonomy then became confusing regarding the species of the genus *Aeromonas*. Although several species were isolated before 1936, including *A. hydrophila*, *Aeromonas caviae* and *Aeromonas salmonicida*, which now belong to the genus *Aeromonas*, they were classified into different genera including *Proteus*, *Pseudomonas*, *Bacterium* and *Vibrio* (Farmer et al 2006). It was Kluyver and van Niel who proposed the name of the genus *Aeromonas* in 1936 based on the morphological and physiological features of *Aerobacter liquefaciens*, which is now known as *A. hydrophila* (Farmer et al 2006). However, *A. hydrophila* has been known as more than 15 synonymous species names such as *Aerobacter liquefaciens*, *Bacillu ichthyosmius* or *Proteus hydrophilus* in different genera. It was Stanier and collegues who replaced this species into genus *Aeromonas* because of the presence of the polar flagella and their ability to ferment carbohydrate (Stanier 1943). This was the first description of the genus *Aeromonas*. They have also clarified that the *A. liquefaciens* and *P. hydrophilus* were identical species.

Another important species of the genus *Aeromonas*, *A. caviae*, which used to be known as *Bacillu punctatus*, *Bacterium punctatum* or *Pseudomonas caviae* was not classified into the genus *Aeromonas* because it was isolated from guinea pigs, which are warm-blooded (Scherago 1937). However, it was later reported by Liu who had demonstrated the ability of *P. caviae* to produce acid from carbohydrates

under strictly anaerobic conditions (Liu 1962). As it was reported by Hugh and Leifson in 1953 that the *Pseudomonas* species could only oxidise carbohydrates but were unable to ferment carbohydrates, this was then utilized to distinguish between the genus *Pseudomonas* and *Aeromonas* (Hugh & Leifson 1953). Thus Liu proposed to relocate *P. caviae* into genus *Aeromonas*. Thereby the presence of polar flagella, the production of oxidase, the ability to ferment carbohydrates and the rod shape were used to characterise the genus *Aeromonus* in Gram-negative bacteria.

The year of 1986 has become a milestone for the taxonomy of *Aeromonas* when Colwell and collegues proposed the removal of genus *Aeromonas* from *Vibrionaceae* family and re-classified into a new-recognised family named *Aeromonadaceae* (Colwell et al 1986). Using molecular genetic tools, including 16S rRNA and 5S rRNA sequences as well as rRNA-DNA hybridisation data, they suggested a phylogenetically distinct family for the genus *Aeromonas*, *Aeromonadaceae*.

The uses of molecular genetic tools expanded the phylogenetic studies of the *Aeromonas* species rapidly. However, 16S rRNA sequence analysis was not sufficient to distinguish the intra-species relationships within the genus *Aeromonas*, since their 16S rRNA sequences were found to be almost identical (Yanez et al 2003). Thereby alternatives have been applied to phylogenetic studies for the past 20 years. It was reported by Martinez-Murcia and collegues in many phylogenetic studies of the genus *Aeromonas* that the utilisation of 16S rRNA group-specific signature using a more variable V2 region of rRNA rather than V3 region, the sequencing of a single house-keeping gene such as *gyrB* or *rpoD*, as well as the multilocus phylogenetic analysis (MLPA) of a series of house-keeping gene (*gyrB*, *rpoD*, *rpoB*, *atpA*, *recA*, *dnaJ* and *gyrA*), provided more powerful tools to incorporate both the intra-species and inter-species phylogenies in bacterial systematics (Carvalho et al 2012, Fontes et al 2010, Martinez-Murcia et al 1992a,

Martinez-Murcia et al 2011, Martinez-Murcia et al 2005, Saavedra et al 2007, Soler et al 2004, Yanez et al 2003).

The number of species in the genus *Aeromonas* has grown rapidly in the molecular genetic era. Only four species including *A. hydrophila*, *Aeromonas punctata* (now known as *A. caviae*), *A. salmonicida* and *Aeromonas sobria* were classified in the genus *Aeromonas* in 1980 (Janda & Abbott 2010). In 2003, there were 13 species in the genus *Aeromonas* and then it was proposed by Janda and Abbott in 2010 that the number of species in genus *Aeromonas* showed increase to 24 including *Aeromonas allosaccharophila* (Martinez-Murcia et al 1992b), *Aeromonas aquariorum* (Martinez-Murcia et al 2008), *Aeromonas bestiarum* (Ali et al 1996), *Aeromonas bivalvium* (Minana-Galbis et al 2007), *Aeromonas culicicola* (Pidiyar et al 2002), *Aeromonas encheleia* (Esteve et al 1995), *Aeromonas enteropelogenes* (Schubert et al 1991), *Aeromonas eucrenophila* (Schubert & Hegazi 1988), *Aeromonas ichthiosmia* (Schubert et al 1990), *Aeromonas jandaei* (Carnahan et al 1991), *Aeromonas media* (Allen et al 1983), *Aeromonas molluscorum* (Minana-Galbis et al 2004), *Aeromonas popoffii* (Huys et al 1997), *Aeromonas schubertii* (Hickman-Brenner et al 1988), *Aeromonas sharmana* (Saha & Chakrabarti 2006), *Aeromonas simiae* (Harf-Monteil et al 2004), *Aeromonas trota* (Husslein et al 1992) and *Aeromonas veronii* (Hickman-Brenner et al 1987).

To date, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN www.bacterio.net), the number of valid *Aeromonas* species in the genus *Aeromonas* has increased to 31, including 7 newly discovered species since 2010, namely *Aeromonas australiensis* (Aravena-Roman et al 2013), *Aeromonas diversa* (Minana-Galbis et al 2010), *Aeromonas fluvialis* (Alperi et al 2010b), *Aeromonas piscicola* (Beaz-Hidalgo et al 2010), *Aeromonas rivuli* (Figueras et al 2011), *Aeromonas sanarellii* (Alperi et al 2010a) and *Aeromonas taiwanensis* (Alperi et al 2010a). However, it was recently reported that the *A. aquariorum* and *A. hydrophila* subsp. *Dhakensis* have now synonymized into the same taxon named

Aeromonas dhakensis species (Beaz-Hidalgo et al 2013).

Since the first DNA hybridisation analysis carried out in 1979, the aeromonads have been classified into two genotypical groups, a more diverse group of motile aeromonads and a more homogeneous non-motile group (Macinnes et al 1979). The motile aeromonads represent the mesophilic strains of *Aeromonas* such as *A.caviae* and *A. hydrophila* that are able to swim in the liquid environments using a single polar flagellum, while the non-motile aeromonads represent the psychrophilic strains of *Aeromonas* such as *A. salmonicida* (Farmer et al 2006). Moreover, in 1985, a Japanese group observed peritrichous lateral flagella in mesophilic *Aeromonas* strains *A. caviae* and *A. hydrophila* by electron microscopy (Shimada et al 1985). The details of the lateral flagella system are discussed later in section 1.5.

1.2 Ecology

The *Aeromonas* speices are ubiquitous in aquatic environments such as marine water, ground water and inhabitants of aquatic environments like fish or frogs, whereas they can also be isolated from domesticated animals, invertibrates, birds, insects and even natural soil (Farmer et al 2006). They are widely distributed in basically every environmental niche of the ecosystem. The *Aeromonas* species are able to adapt to a wide range of environments as they have shown tolerance to various growth factors, including a temperature range from 4°C to 45°C, and a pH range from 3.5 to 9.8. They have also been shown to survive in both oligotrophic and hypereutrophic environments, and a wide range of salinities from 0% to 6% (v/v) (Beaz-Hidalgo & Figueras 2013, Delamare et al 2000, Janda & Abbott 2010, Knochel 1990, Lambert & Bidlas 2007, Palumbo et al 1985, Wang & Gu 2005).

As the first isolated *Aeromonas* species and one of the four earliest classified species in genus *Aeromonas* species in the 20th century, *A. hydrophila* was not

found by chance. In a recent study *A. hydrophila* was shown to be the most frequently isolated *Aeromonas* species from both human clinical material and environmental samples, 54.8% and 45.3% respectively (Aravena-Roman et al 2011). An earlier report by Hazen and collegues indicated that *A. hydrophila* species were isolated from 91.8% of natural aquatic inhabitats in the U.S.A. with a wide range of temperatures, pHs, salinities and conductivities. The numbers of *A. hydrophila* were higher in lotic than in lentic systems, higher in saline systems than in freshwater, only habitats that were extreamly saline, thermal or polluted were excepted (Hazen et al 1978). Similarly, it was reported by Karem and collegues that *A. hydrophila* exhibited an adaptive acid tolerance response to allow the pathogen to survive at a pH as low as 3.5 (Karem et al 1994). The bacteria undergo a physiological change as the pH decreases to 5.0, when the signal triggers an increased production of 28 proteins and decreased synthesis of 10 proteins in order to allow bacteria to withstand the acidic environments, reflecting the fact that they are mostly gastrointestinal pathogens (Karem et al 1994).

Like all aeromonads, *A. hydrophila* species are falcutative anaerobic non-endospore forming rod-shaped Gram-negative bacteria, ranging in size from 1.0-3.5μm in length and from 0.3-1.0μm in width (Horneman et al 2007, Joseph et al 1988). The *A. hydrophila* strains can express dual flagella systems, using a single polar flagellum to swim in liquid environment while expressing peritrichous lateral flagella upon contact with the host cell or solid surfaces. *A. hydrophila* strains can grow on the commonly used noninhibitory laboratory media including Luria-Bertani, MacConkey and blood agar, on which the colonies of *A. hydrophila* are typically circular, 1-3mm in diameter and showing effect of β-haemolysis on blood agar (Janda & Abbott 2010).

1.3 Epidemiology

As introduced before, the *Aeromonas* species consist of two major groups defined by the DNA-hybridisation analysis, including the non-motile pigmented psychrophilc species and the motile mesophilic non-pigmented species. The psychrophilic *Aeromonas* species represented by *A. salmonicida* predominantly causes furunculosis in salmonid fish as well as many other fish species such as cod, carp and goldfish (Cipriano & Austin 2011, McCarthy & Rawle 1975). The fish infected by *A. salmonicida* may develop skin hyperpigmentation, furuncles or ulcers on the skin, anaemia and internal haemorrhage (Beaz-Hidalgo et al 2012, Bernoth et al 1997). On the other hand, infections mostly associated with humans are caused by mesophilic aeromonads. In this case, similar clinical signs are only visible in the skin or the internal organs (Beaz-Hidalgo et al 2012, Bernoth et al 1997). The first association of *Aeromonas* species with human infections was reported in 1951, when *Aeromonas* was recovered from autopsy samples of an acute fulminant metastatic myositis patient (Caselitz 1996). It was reported that three mesophilic *Aeromonas* species, including *A. hydrophila, A. caviae* and *A. veronii biovar sobria*, account for 85% of human infections and clinical isolations (Chopra & Houston 1999). Human infections by aeromonads often result in gastrointestinal disease and wound infection, while septicaemia less commonly occurs in the immunocompromised host (Janda & Abbott 2010). Additionally, skin infections related to aeromonads were discovered in a post-tsunami study in southern Thailand, in which *A. hydrophila* was identified as the major pathogen to infect wounds exposed to contaminated water (Hiransuthikul et al 2005).

1.3.1 Gastroenteritis

The *Aeromonas* species were first associated with gastrointestinal disease in 1961 when *A. punctata* (now known as *A. caviae*) was isolated from 7 out of 8 patients with intestinal disease (Gilardi 1967). However, the role of *Aeromonas*

species in causing gastrointestinal disease is still not conclusive, as there has been no well-defined outbreak of *Aeromonas* gastroenteritis and it has yet to fulfil Koch's postulates, both convential and molecular (Janda & Abbott 2010). A study carried out by Morgan and collegues in 1985 failed to establish gastrointestinal disease in volunteers when they were inoculated with as many as 10^{10} CFU of *A. hydrophila* (Morgan et al 1985). Furthermore, as Koch's postulates require the isolated pathogen to re-induce the disease in a healthy organism, several animal models were attempted but still failed to associate *Aeromonas* with the gastroenteritis. For example, one of the recent attempts made by Kelleher and Kirov in 2000 using *Rattus norvegicus* (clindamycin-pretreated rats) successfully linked *Pseudomonas aeruginosa* with chronic diarrhoea but failed for *Aeromonas* species (Kelleher & Kirov 2000). Although the genes responsible for the production of enterotoxins and haemolysins in *Aeromonas* species were analyzed in order to fulfill the molecular standard of Koch's postulates 1, the genes and the phenotypes were not found to be exclusive in the pathogenic members of the genus *Aeromonas* (von Graevenitz 2007).

Despite the perplexing association of the *Aeromonas* species with gastroenteritis, there have been many reports. For instance, in 2004 an acute diarrhea outbreak was reported in Sao Bento with 2710 cases, in which *Aeromonas* species were the most abundant enteric pathogen isolated from stool samples of diarrhea patients (Hofer et al 2006). Among all types of gastroenteritis, *Aeromonas* species were found to be associated mostly with secretory enteritis, which accounted for nearly 80% of all *Aeromonas* gastroenteritis cases (Chan et al 2003, Essers et al 2000, Sinha et al 2004). It was also reviewed by Ghenghesh and collegues that pathogenic *Aeromonas* species were commonly found in foods and drinking water, and were isolated more from diarrhoeic children than non-diarrhoeic children in developing countries of Africa, Asia and Latin America (Ghenghesh et al 2008). Although there were cases, such as in Libya, where more *Aeromonas* species were found in non-diarrhoeic children (22.3%) than in diarrhoeic children

(17.2%), the level of the virulence factors such as mannose-resistant haemagglutin were found to be significantly higher in *Aeromonas* diarrhoeic children (26%) than in healthy controls (3%) (Ghenghesh et al 2008).

1.4 Pathogenicity and virulence factors

As discussed before, the determination of the virulence factors are perplexing in *Aeromonas* species as the genes encoding for the potential virulence factors such as enterotoxins were found in both the pathogenic and non-pathogenic *Aeromonas* species. However, various approaches and animal models, including *in vitro* cell lines such as HEp-2 or Caco-2, clindamycin-pretreated rats and medicinal leech, have been used to investigate the virulence factors of *Aeromonas* species (Indergand & Graf 2000, Kelleher & Kirov 2000, Silver et al 2007, Thornley et al 1996). For instance, the genes encoding the Type III secretion system (T3SS) were identified in *A. veronii* using the *Hirudo verbena* (medicinal leech) model, since the digestive tract of the leech was inhabited by a simple microbial community (Silver et al 2007). The pathogenicity of *Aeromonas* species is associated with a variety of virulence factors and colonization factors such as the lipopolysaccharide, S-Layer, enterotoxins, the polar and lateral flagella systems as well as the secretion systems (Austin 1996, Vilches et al 2004).

1.4.1 Lipopolysaccharide (LPS)

Lipopolysaccharides are found uniquely in Gram-negative bacteria, including *A. hydrophila* and *A. salmonicida*, and are embedded in the outer membrane of the bacterial cell envelope. They are comprised of three main components, the extremely variable O-antigen, the core oligosaccharide and the most conserved lipid A (Tomas 2012). The hydrophobic lipid A domain, which anchors the LPS to the outer membrane, contributes the most to the pathogenicity of Gram-negative bacteria (Luderitz et al 1978). The immunostimulatory ability of lipid A resulting from

the activation of monocytes or macrophages via Toll-like receptor 4 (TLR4) or CD14 can lead to the release of inflammatory mediators, followed by Gram-negative sepsis, which is clinically recognised by fever, hypotension, respiratory and renal failure as well as disseminated intravascular coagulation (Cohen 2002, Tomas 2012).

1.4.2 S-layers

The surface-layers (S-layers) are found in both Gram-positive and Gram-negative bacteria and are comprised of a single protein or glycoprotein with a size of 40-200kDa (Tomas 2012). In *Aeromonas*, the S-layers form a 5-10nm thick pore-containing tetragonal structure surrounding the bacteria, contributing to adhesion, protection against protease and phagocytes and resistance to the complement-mediate killing (Beveridge et al 1997, Tomas 2012). The S-layers have been described in *A. salmonicida*, *A. hydrophila* and *A. veronii*.The S-layers were identified first in *A. salmonicida*, and reported to be the major virulence factor contributing to the septicaemia in fish (Kay et al 1981). However, the S-layers are less commonly found in mesophilic *Aeromonas* species (Esteve et al 2004, Kokka et al 1990).

1.4.3 Enterotoxins

The exotoxins produced and secreted by *Aeromonas* species are usually found to target intestinal epithelial cells,and are thus refered to as enterotoxins. There are two main types of enterotoxins in *Aeromonas*, cytotoxic and cytotonic (Tomas 2012). The cytotoxic enterotoxins represented by Act isolated from *A. hydrophila* SSU are generally pore-forming proteins, associated with cytotoxicity, enterotoxicity and hemolysis (Asao et al 1984, Chopra & Houston 1999, Galindo et al 2006). The hemolysis ability of Act results from its pore-forming nature, which leads to the swelling of the host cells upon water entry (Sha et al 2002, Tanoue et al 2005).

The cytotonic enterotoxins are divided into two groups, heat-labile (56° for 10 min) and heat stable (100°C for 30 min). The heat-labile cytotonic enterotoxins are represented by Alt from *A. hydrophila* SSU, while the heat-stable cytotonic enterotoxins are represented by Ast from the same species. Both of these can induce cAMP production in the intestinal epithelial cells resulting in the accumulation of fluid via cAMP pathway, thus causing diarrhoea (Chopra & Houston 1999, Ljungh & Kronevi 1982).

1.5 Flagella Systems

In order to help with localization, moving towards attractants and infection of host cells, bacteria exploit several ways to provide them with surface motility, including swimming, swarming, sliding, twitching, gliding and drafting. The colonization approaches that involve the presence of flagella systems are swimming and swarming (Harshey 2003). Rather than the movement of a single bacterium involved in swimming motility, swarming motility involves a group of bacteria moving to colonize solid surfaces, such as laboratory media. *Aeromonas* cells demonstrate swarming motility on 0.5%-1% (w/v) agar (Harshey 1994, Kirov et al 2002).

A single flagellum normally consists of a long helical filament (10-15μm in length and ~20nm in diameter) projecting out from the cell and a hook structure connecting the filament to the basal body, which is embedded in the cytoplasmic membrane. The basal body is comprised of a central rod structure and several surrounding ring structures, on which the motor is attached that rotates the filament in either a counterclockwise or clockwise sense. The motor rotation in counterclockwise causes the flagellar filament, which is usually a left-handed helix, to bundle together along the long axis of the cell and push the bacterium in one direction at a speed of 20-30μm/s until the chemotaxic signal response switches the motor to rotate in a clockwise sense (Surette & Stock 1996). When the motor rotates

in clockwise direction, the bacterial cell tumbles and moves in random directions until it senses environmental signal, such as an increase of attractants or a decrease of repellents. The signal transduction is dependent upon a two-component regulatory system, including a sensor kinase and a response regulator, which involves the movement of a phosphoryl group via phosphorelay (Harshey et al 2003, Hoch 2000, Rowsell et al 1995).

Most Gram-negative bacteria express only one flagella system, either a polar flagella system for swimming motility or a lateral flagella system for swimming and swarming motility, whereas bacteria such as some strains of *Vibrio* and *Aeromonas* can express dual flagella systems and switch between the two flagella systems in response to environmental signals (McCarter 1999, Merino et al 2006).

More than half of the mesophilic *Aeromonas* strains, including *A. caviae* and *A. hydrophila*, contain dual flagella systems, in which the constitutively expressed polar flagella system allows the bacteria to swim in liquid environment, while the inducible lateral flagella system allows the bacteria to swarm over solid surfaces. These two flagella systems not only allow the bacteria to move towards and to colonize onto host cells, but also provide ability to adhere onto the host cell surface and form a biofilm in order to accumulate together (Gavin et al 2002). The accumulation of the bacterial cells will result in a polysaccharide matrix to gain them more resistance to anti-bacterial defences of the host than they have as individual cells (Canals et al 2006a, Costerton et al 1999). Additionally, the massive population of the bacteria may also trigger the quorum sensing system to activate certain virulence genes (Whitehead et al 2001).

1.5.1 Structural composition of the flagella system

The assembly of the flagellum starts from the basal body which is embedded into the bacterial cell membrane, followed by the hook-associated proteins and the

filament (Rabaan et al 2001). The basal body consists of an M ring and S ring in the inner membrane, as well as a periplasmic P ring and an L ring in the outer membrane.The MS ring is assembled first that forms a secretion apparatus associated with the Type III secretion system (T3SS) to export extracytoplasmic components of the flagella (Macnab 2003). The motor structure that is responsible for the rotation of the flagella consists of two components, the stator and the rotor. The stationary stator, such as Mot A and MotB proteins in *E. coli*, is attached to the peptidoglycan layer and the C ring, which is assembled at the cytoplasmic side of the MS ring. The rotor, which is comprised of polymers of FliG in *E. coli*, is attached to the MS ring (Macnab 2003).

The motor can be powered solely by proton-motive force, like in *E. coli* and *Streptococcus spp*, or solely by sodium-motive force like in alkaliphilic *Bacillus* strains and *Caloramator fervidus*, while in *Vibrio parahaemolyticus* the polar flagella system and the lateral flagella system are powered by sodium-motive force and proton-motive force respectively (Atsumi et al 1992, Blair & Berg 1990, Imae & Atsumi 1989, McCarter 1999, Mulkidjanian et al 2008). Recent studies in *A. hydrophila* AH-3 showed that the polar flagella system involves the stator complex, MotXY, with another two redundant set of stator proteins PomAB and PomA2B2, all of which are driven by sodium-motive force, while the lateral flagella system is driven by proton-motive force like in *Vibrio* species (Molero et al 2011, Wilhelms et al 2009).

The flagellar filament structure is comprised of either a homopolymer of a single flagellin protein, as in *E. coli*, or a heteropolymer of different flagellin proteins, as in the polar flagella system in *Campylobacter* or *Vibrio* species (Guerry et al 1991, McCarter 1995, Rabaan et al 2001). In *A. hydrophila* AH-3, the polar flagella system was reported to possess two tandem repeats of flagellin genes, *flaA* and *flaB*, while only one flagellin gene *lafA* was found in the gene loci encoding the lateral flagella system (Canals et al 2006a, Canals et al 2006b, Gavin et al 2002). Although most

lateral flagella systems in *Vibrio* and *Aeromonas* species contain only one flagellin gene, the lateral flagella systems in *A. caviae* and *A. hydrophila* AH-1 were found to possess two flagellin genes, *lafA1* and *lafA2*, indicating intraspecies difference in flagella composition (Gavin et al 2002). The flagellin proteins of both flagella systems are important for the adherence and biofilm formation of the bacteria. In a study carried out by Kirov and collegues, *A. caviae* cells with mutations in the polar flagellin genes *flaA* and *flaB* were non-adherent to the intestinal cell lines Henle 407 and Caco-2, while mutations in the lateral flagellins *lafA1* and *lafA2* resulted in 60% reduction of adherence. Moreover, biofilm formation was reduced by approximately 30% in both a *flaB* mutant and *lafA1A2* mutant strains of *A. caviae* (Kirov et al 2004).

The flagella systems are important colonization factors, but the synthesis of the flagella is highly energy-consuming and requires plentiful resources of cell machinery, thus the production of flagella systems are tightly controlled in bacteria. The details of the genetic composition and regulation of the lateral flagella system in *A. hydrophila* AH-3 are discussed below.

1.5.2 Lateral flagella system in *A. hydrophila*

Early in 1985, a Japanese group observed peritrichous lateral flagella in *A. caviae* and *A. hydrophila* by electron microscopy (Shimada et al 1985). Later in 2002, it was found that more than half of the mesophilic *Aeromonas spp.* possessed both flagella systems. Among these species, *A. caviae* and *A. hydrophila* are the most clinically isolated strains (Kirov et al 2002). However the lateral flagella system was described to be involved in the pathogenic association with the host cells in *E.coli* and *Salmonella typhimurium* in 1994 (Harshey 1994). This led to a rapid progress in understanding the function of the lateral flagella system in other Gram-negative bacteria including *Aeromonas* species. The lateral flagella system in *A. hydrophila* was found to be involved in host cell adherence and biofilm formation, as it was reported by Gavin and collegues that mutations in the lateral flagella gene

lafB and *lafS* led to ~85% reduction in HEp-2 cell adhesion and ~63% reduction of biofilm formation in *A. hydrophila* AH-3 (Gavin et al 2002, Kirov 2003).

As the bacteria which possess the dual flagella systems are relatively rare, the study of the flagella systems in *A. hydrophila* is often compared with *Vibrio parahaemolyticus*, which is the best-studied organism containing the dual flagella systems (Canals et al 2006a) (Figure 1.1). Based on the homology studies with *V.parahaemolyticus*, the genes of the lateral flagella system in *A. hydrophila* were identified and the functions of these genes were proposed (Table 1.1).

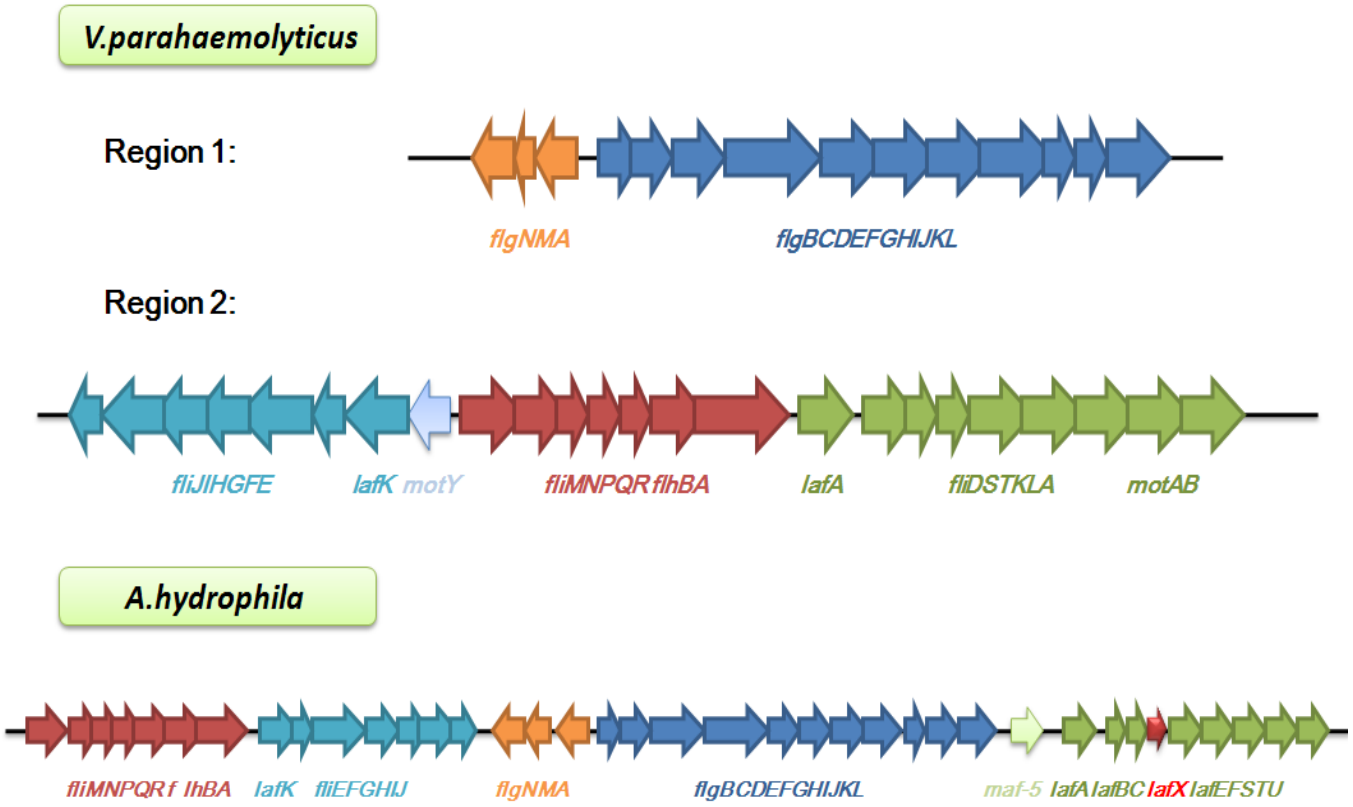


Figure 1.1 Genetic organization of the lateral flagella systems in *V.parahaemolyticus* and *A. hydrophila*. The arrows with the same colour represent homologous genes while unique genes such as *motY* in *V.parahaemolyticus*, *maf-5* and *lafX* in *A. hydrophila* are shown in different colours (Canals et al 2006a, Merino et al 2006, Stewart & McCarter 2003).

Table 1.1 Characteristics of the lateral flagella system genes in *A. hydrophila* AH-3

Gene name	Function of protein product	Homologues
Putative operon *fliM-flhA*		
fliM	Switch (C ring)	*fliM* in *V. parahaemolyticus*
fliN	Switch (C ring)	*fliN* in *V. parahaemolyticus*
fliP	Export/assembly	*flip* in *V. parahaemolyticus*
fliQ	Export/assembly	*fliQ* in *V. parahaemolyticus*
fliR	Export/assembly	*fliR* in *V. parahaemolyticus*
flhB	Export/assembly	*flhB* in *V. parahaemolyticus*
flhA	Export/assembly	*flhA* in *V. parahaemolyticus*
Putative operon *lafK-fliJ*		
lafK	Regulation	*lafK* in *V. parahaemolyticus*
fliE	Basal body component	*fliE* in *V. parahaemolyticus*
fliF	M ring	*fliF* in *V. parahaemolyticus*
fliG	Switch	*fliG* in *V. parahaemolyticus*
fliH	Export/assembly	*fliH* in *V. parahaemolyticus*
fliI	Export ATP synthase	*fliI* in *V. parahaemolyticus*
fliJ	Export/assembly	*fliJ* in *V. parahaemolyticus*
Putative operon *flgM-flgN*		
flgN	Chaperone	*flgN* in *V. parahaemolyticus*
flgM	Anti-σ^{28}	*flgM* in *V. parahaemolyticus*
Putative operon *flgA-flgN*		
flgA	P ring addition protein	*flgA* in *V. parahaemolyticus*
Putative operon *flgB-flgL*		
flgB	Rod	*flgB* in *V. parahaemolyticus*
flgC	Rod	*flgC* in *V. parahaemolyticus*
flgD	Rod	*flgD* in *V. parahaemolyticus*

flgE	Hook	*flgE* in *V. parahaemolyticus*
flgF	Rod	*flgF* in *V. parahaemolyticus*
flgG	Rod	*flgG* in *V. parahaemolyticus*
flgH	L ring	*flgH* in *V. parahaemolyticus*
flgI	P ring	*flgI* in *V. parahaemolyticus*
flgJ	Peptidoglycan hydrolase	*flgJ* in *V. parahaemolyticus*
flgK	HAP1	*flgK* in *V. parahaemolyticus*
flgL	HAP3	*flgL* in *V. parahaemolyticus*
Putative operon *maf-5*		
maf-5	Glycotransferase	No known homologue in *V. parahaemolyticus*
Putative operon *lafA-lafU*		
lafA	Flagellin	*lafA* in *V. parahaemolyticus*
Putative operon *lafB-lafU*		
lafB	HAP2	*fliD* in *V. parahaemolyticus*
lafC	Flagellar protein chaperone	*fliS* in *V. parahaemolyticus*
Putative operon *lafX-lafU*		
lafX	unknown	unknown
lafE	Hook length control	*fliK* in *V. parahaemolyticus*
lafF	Flagellar protein	*fliT* in *V. parahaemolyticus*
lafS	σ^{28}	*fliA* in *V. parahaemolyticus*
lafT	Motor protein	*motA* in *V. parahaemolyticus*
lafU	Motor protein	*motB* in *V. parahaemolyticus*

Table 1.1 Characteristics of the lateral flagella system genes in *A. hydrophila* AH-3. All of the putative operons are transcribed from 5' to 3' except for operon *flgA-flgN* and *flgM-flg*N, both of which are transcribed from 3' to 5' and share the same terminator downstream of *flgN*. The putative operons *lafA-lafU*, *lafB-lafU* and *lafX-lafU* also share the same terminator downstream of *lafU*. This table is based on the information in Gavin et al. 2002, Canals et al. 2006a and Merino et al. 2006.

Although homologues of most of *A. hydrophila* lateral flagella genes can be found in *V. parahaemolyticus*, the lateral flagella system in *A. hydrophila* is different to that of *V. parahaemolyticus* at a genetic level. In *V. parahaemolyticus*, the lateral flagella system consists of 38 genes, located on two distinct regions of chromosomal DNA, while in *A. hydrophila*, the 38 genes of the lateral flagella system remain in one chromosomal region as shown in Figure 1.1 (Canals et al 2006a, Merino et al 2006).

In *A. hydrophila* AH-3, 38 genes of the lateral flagella system are categorized into 9 putative operons, *fliM-flhA*, *lafK-fliJ*, *flgM-flgN*, *flgA-flgN*, *flgB-flgL*, *maf-5*, *lafA-lafU*, *lafB-lafU* and *lafX-lafU* (Table 1.1). Although some of the operons share the same terminators, each of the operons is regulated by a single putative promoter. However, it has been reported in many bacteria including *V. parahaemolyticus* and *A. hydrophila* that the lateral flagella system expression is controlled in a hierarchal fashion (Wilhelms et al 2013).

The Class I genes including *fliM-flhA*, *lafK-fliJ*, *flgA* and *lafTU*, are σ^{70}-dependent and are transcribed first. These genes are involved in encoding the major regulator LafK and structural components such as C ring (FliM and FliN), M ring (FliF) and the motor (LafTU). The enhancer-binding protein LafK, which functions as the major genetic regulator of the lateral flagella system, together with an alternative sigma factor RpoN (σ^{54}) are required for the transcription of the Class II genes, including *flgB-flgL* and *lafX-lafU*. These genes are responsible for encoding the lateral flagella-specific sigma factor LafS (σ^{28}) and the other structural components forming the rod and the hook, such as FlgB, FlgC, FlgD and FlgE. Class III genes, including *flgM-flgN*, *maf-5*, *lafA* and *lafA-lafC*, are σ^{28}-dependent and are involved in encoding the cognate anti-σ^{28} factor FlgM, the filament capping protein (LafB), the putative glycotransferase protein (Maf-5) and and the flagellin protein (LafA) (Canals et al 2006a, Wilhelms et al 2013). The structural composition of the lateral flagella system is shown in Figure 1.2.

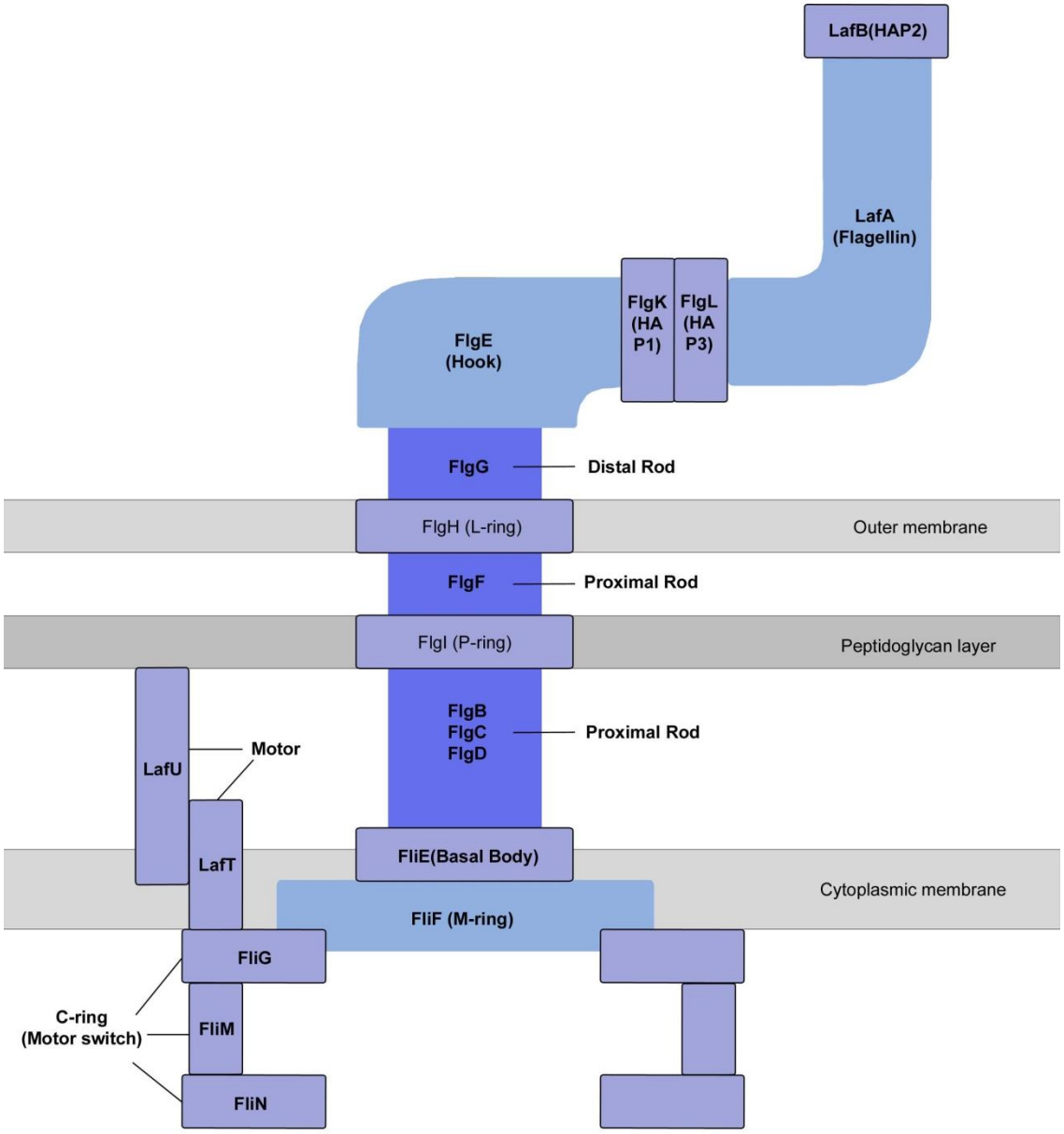


Figure 1.2 Illustration of putative structural composition of the lateral flagella system in *A. hydrophila* AH-3. Protein components were assembled from the cytoplasm outwards. The motor (LafT and LafU) and the basal body with the ring structures (FliM, FliN, FliG, FliF and FliE) are assembled first, followed by the rod (FlgB, FlgC, FlgD, FlgF and FlgG), P-ring (FlgI), L-ring (FlgH) and the hook (FlgE) along with hook-associated proteins (HAP) (FlgK and FlgL). The flagellin protein (LafA) and the capping protein (LafB) are secreted and assembled at last.

The complete mechanism of how the lateral flagella system is regulated remains unclear in *A. hydrophila*. In *V. parahaemolyticus*, the lateral flagella system is under control of the polar flagella system and physical sensors, which sense the disturbance in the environment and trigger the *V. parahaemolyticus* cells to differentiate into an elongated swarmer cell formation and to express the lateral flagella system (McCarter & Silverman 1990). In more recent studies of the regulation between the polar and lateral flagellar in *V. parahaemolyticus* and *A. hydrophila*, it was shown that a mutation in the polar flagellar regulator FlrA abolished the production of the polar flagella system in *A. hydrophila*, as its homologue FlaK also abolished the polar flagella system in *V.parahaemolyticus*. But the mutation in FlaK eliminated the production of the lateral flagella as well as the polar flagella in *V.parahaemolyticus* while the mutation in FlrA did not affect the lateral flagella expression in *A. hydrophila*. Furthermore the lateral flagella regulator LafK is able to complement the lack of FlaK in *V.parahaemolyticus* while the LafK homologue in *A. hydrophila* cannot compensate for the mutation in FlrA (Wilhelms et al 2011). This suggested that the polar flagella system was not responsible for the regulation of the lateral flagella system in *A. hydrophila*, therefore the signal transduction that triggers the expression of lateral flagella system still remains unclear in *A. hydrophila*.

It was recently reported that cyclic di-GMP, produced by diguanylate cyclase that contains GGDEF domains, was directly involved in the regulation of biofilm formation and motility in *V.cholerae* (FlrA) as well as the flagella biosynthesis in *P. aeruginosa* (FleQ) (Hengge 2009, Hickman & Harwood 2008, Krasteva et al 2010). The intracellular concentration of cyclic-di-GMP was responsible for switching the bacteria between the sessile and motile lifestyle. High intracellular concentration of the cyclic-di-GMP is coupled with increased biofilm formation and decreased lateral flagella expression while low concentration of cyclic-di-GMP increases the expression of the lateral flagella system, hence improving swarming motility and reducing biofilm formation. It was reported in *A. hydrophila* SSU that the

cyclic-di-GMP functions as the second messenger modulating biofilm formation and swarming motility in a quorum-sensing-dependent manner (Kozlova et al 2012, Kozlova et al 2011). However, a detailed mechanism of how cyclic-di-GMP regulates the lateral flagella system in *A. hydrophila* still remains unknown.

Moreover, evidence in *A. hydrophila* AH-1 has shown that the expression level of two tandem lateral flagellin gene *lafA1 and lafA2* are significantly decreased in mutants of the T3SS-associated genes, *exsD* and *aopN*. However, Δ*exsA* Δ*exsD* and the Δ*exsA* Δ*aopN* double deletions restored the secretion level of the LafA flagellin protein (Yu et al 2007). This finding indicated potential cross-talk between the lateral flagella system and the T3SS in *A. hydrophila*, which will be discussed and investigated in detail in this project.

1.6. Type III secretion system

The secretion systems in Gram-negative bacteria have been discovered and intensively studied for the last three decades. To date, there are more than six different types of secretion systems discovered, however the Type I to Type VI secretion systems have been studied intensively. The Type I secretion system (T1SS) represented by the *E. coli* haemolysin secretion system that involves only three proteins is the simplest among all secretion systems (Gentschev et al 2002, Letoffe et al 1996). The Type II secretion system (T2SS) and Type V secretion system (T5SS) are both two-step pathways that secret proteins distinctively across the outer membrane after they have been transported across the inner membrane into the periplasm through Sec or Tat (two-arginine translocation) systems (Cianciotto 2005, Stathopoulos et al 2000, Voulhoux et al 2001). The Type IV secretion system (T4SS) built from the conjugation apparatus is involved in genetic exchange and delivery of effectors directly into eukaryotic host cells (Cascales & Christie 2003, Christie & Vogel 2000). The Type VI secretion system (T6SS), which was discovered in *V. cholerae* in 2006, is a bacteriophage-like system that punctures the target cells (either eukaryotic host cells or bacterial competitors) and delivers the effectors directly into their cytoplasm (Bonemann et al 2010, Coulthurst 2013, Hood et al 2010, Pukatzki et al 2006). Recently, a novel Type VII secretion system (T7SS) was discovered in the Gram-positive bacteria, *Mycobacterium marinum*, *Staphylococcus aureus* and *Bacillus subtilis*. The secreted proteins via T7SS are co-dependent on each other and are different from the secreted proteins in other secretion systems (Abdallah et al 2007).

The Type III secretion system has been reported in more than 25 species of Gram-negative bacteria including *Yersinia spp*., *Pseudomonas spp*., *Vibrio spp.*, *Salmonella spp*. and *Shigella spp*. (Cornelis et al 1998, Cornelis & Van Gijsegem 2000, Galan & Collmer 1999, Park et al 2004, Yahr et al 1997). Unlike Type I, Type II or Type V secretion systems, the T3SS can inject protein effectors across the

plasma membrane directly into host cell cytosol or to secrete pore-forming translocators that helps effector proteins to get through (Cornelis & Van Gijsegem 2000). The structural composition of the T3SS is similar to the flagella systems. The T3SS is comprised of more than 20 proteins and forms a needle-like complex, most of which share homology with the export proteins of the flagella systems. The basal body of the T3SS is composed of multiple ring structures with a central rod that function as a syringe to 'pump' protein effectors through the needle, thus the T3SS is often call the injectisome (Galan & Wolf-Watz 2006, Marlovits & Stebbins 2010).

1.6.1 Effector proteins of T3SS in *Aeromonas spp.*

The effector proteins that are injected from the bacteria into the target host cells have been broadly studied in *Vibrio*, *Yersinia* and *Pseudomonas* species. In *Aeromonas* species, *A. salmonicida* is the best-studied model of T3SS-secreted toxins. To date, there are seven different kinds of toxins discovered in *A. salmonicida*, including AexT, AopP, Ati2, AopN, ExsE, AopO and AopH, of which AexT and AopP have been investigated intensively.

The exoenzyme T (AexT), which is homologous to the exotoxin ExoT/S in *P.aeruginosa* and cytotoxin YopE in *Yersinia spp.*, is a bifunction toxin that possesses an ADP-ribosylating domain and a GTPase-activating domain. As an ADP-ribosyltransferase or a GTPase-activating protein, AexT targets the host cell cytoskeleton to depolymerize the actin filament, which leads to cell morphological change and cytotoxicity. Furthermore, it was reported that the muscular and non-muscular actin were the substrates of the ADP-ribosylating domain while the monomeric GTPase of the Rho family is the target of the GTPase-activating domain (Braun et al 2002, Burr et al 2003, Fehr et al 2007).

The second T3SS-secreted toxin identified in *A. salmonicida* is AopP, which affects the nuclear factor kappa B (NF-*κ*B) signaling pathways downstream of I*κ*B*α*

phosphorylation, preventing NF-κB entering the nucleus and leading to apoptosis of the host cells. Unlike its homologue YopJ in *Yersinia spp.*, AopP does not interfere with the mitogen-activated protein kinase (MAPK) signaling pathway (Fehr et al 2006).

Recent studies by Bergh and collegues have identied the effector proteins Ati2, AopN and ExsE in *A. salmonicida*. The effector protein Ati2 and its homologue VPA0450 in *V. parahaemolyticus* disrupt the cytoskeletal binding site on the inner surface of the host cell membrane, compromising host membrane integrity and leading to cell lysis (Broberg et al 2010, Vanden Bergh et al 2013). AopN, which was reported to be the gate protein controlling the secretion of translocator proteins, has now been shown to be a secreted effector as well, potentially suppressing host immune response based on the homology studies in *Pseudomonas*, *Chlamydia* and *Bordetella* species (Archuleta et al 2011, Crabill et al 2012, Nagamatsu et al 2009, Vanden Bergh et al 2013). The effector protein ExsE was intensively studied in *P.aeruginosa*, in which the ExsE was shown to be involved in the regulation of the T3SS but the effect of ExsE within the host cell is still unknown (Rietsch et al 2005, Urbanowski et al 2005). The regulatory role of ExsE effector protein will be discussed and investigated in detail in this study.

However, in *A. hydrophila* AH-3, the first protein effector AexT was identified in 2007 and was found to be smaller than the AexT protein in *A. salmonicida*. The putative function of *A. hydrophila* AexT is similar to *A. salmonicida* AexT, both of which possess an ADP-ribosylating domain and a GTPase-activating domain (Vilches et al 2008). In another mesophilic strain of *Aeromonas*, *A. hydrophila* SSU, an AexT-like protein effector was identified in the same year, named as AexU. The N-terminal of AexU shares high homology with the N-terminal of AexT in *A.salmonicida*, both of which exhibit the ADP-ribosylating activity. A study carried out by Sierra and collegues showed reduced ADP-ribosylating activity of the N-terminal truncated AexU (amino acid residues 232-512) (Sierra et al 2007). So far, no other

T3SS-secreted effector proteins have been identified in *A. hydrophila*.

1.6.2 The chaperone proteins and the constitution of T3SS in *A. hydrophila*

The formation of the injectisome involves three classes of T3SS chaperone proteins. The first class of chaperones is to prevent the premature polymerization of the extracellular structural subunits of the T3SS, and is similar to the lateral flagellar chaperone protein FlgN for hook-associated proteins FlgK (HAP1) and FlgL (HAP3) in *A. hydrophila* (Auvray et al 2001, Fraser et al 1999).

The second class of the chaperone proteins is associated with the pore-forming proteins in T3SS. These pore-forming proteins are not only capable of penetrating target cell membranes but also toxic to bacteria themselves, hence the chaperone proteins are required to neutralize the toxicity of these pore-forming proteins before they are secreted (Neyt & Cornelis 1999b).

The final class of the chaperone proteins is the dedicated chaperones for the effector proteins. Many of these chaperones and their cognate effector proteins are encoded adjacent to each other, and they interact with each other through the chaperone binding domain, which is located just downstream of the N-terminal export signal of the effector protein (Cornelis 2006). This class of chaperones does not share any sequence similarity but a conserved structure that consists of 5 β-strands and 3 α-helices (Evdokimov et al 2002, Luo et al 2001, Stebbins & Galan 2001). Additionally these chaperones are often dimerized and interact with the chaperone binding domain of the effector proteins via the hydrophobic surfaces (Birtalan et al 2002).

So far, the putative chaperone proteins identified in *A. hydrophila* and encoded from the T3SS regulon include Acr2, AcrG, AcrH, ExsC, AscB, AscE and AscG, of which the chaperone proteins Acr2, AcrG, AscB, AscE and AscG are responsible for

binding the extracellular structural subunits, the AcrH is coupled with the pore-forming proteins, while the ExsC is a chaperone for the effector protein.

The chaperone protein Acr2 has not been studied intensively in *Aeromonas*, but its homologue Pcr2 has been investigated in detail in *P. aeruginosa*. It was reported that Pcr2 interacted with an important translocator PopN, which acted as a gate blocking the T3 secretion when the T3SS was not triggered. Furthermore, the mutation in *pcr2* gene resulted in reduced T3 secretion (Yang et al 2007a).

The AcrG chaperone protein is coupled with AcrV protein, which is a needle-tip protein involved in the translocation of the effector protein. It was reported in *A. salmonicida* that the *acrV* mutant was unable to translocate the toxin AexT into the host cell but constantly secrete the toxin regardless of the calcium effect.This suggested that both the chaperone protein AcrG and the translocon protein AcrV are involved in low-calcium response (Burr et al 2003).

The AcrH chaperone protein is responsible for binding and inhibiting the activities of two proteins, AopB and AopD, which are encoded downstream of the *acrH* gene. The homologues of AopB and AopD in *Yersinia spp.* are YopB and YopD, which are known to be the pore-forming proteins that facilitate effector proteins to translocate into the host cell cytosol (Hakansson et al 1996, Neyt & Cornelis 1999a). The binding of AcrH to AopB and AopD is extensively studied by Leung's group, who suggested a stable 1:1 monomeric complex of AcrH-AopD while the 1:1 monomeric complex of AcrH-AopB is metastable (Tan et al 2009).

Both of the chaperone proteins AscE and AscG bind the structural subunit AscF, which polymerizes and forms the needle structure of the injectisome. A recent study of the crystal structure of the AscE-AscG hetero-molecular chaperone suggested that the the AscE functions as a chaperone of the chaperone protein AscG in order to bind the AscF protein (Chatterjee et al 2011). This model correlates with the

complex formation of their homologues YscE-YscF-YscG and PscE-PscF-PscG in *Yersina* and *Pseudomonas* respectively (Quinaud et al 2005, Sun et al 2008).

Little is known about the chaperone protein AscB in *Aeromonas*, except that it binds to an extracellular T3SS structural protein AscC. The homologues of AscC in *Yersinia*, YscC is a channel-forming protein that forms a secretin complex on the outer membrane of the bacteria (Koster et al 1997).

Most of the T3SS genes in *A. hydrophila* were identified and studied in comparison with their homologues in *P. aeruginosa*. The genetic organization of the *A. hydrophila* T3SS is shown in Figure 1.3 and the putative functions of each gene are listed in Table 1.2. The putative structural composition of the T3SS in *A. hydrophila* AH-3 is shown in Figure 1.4.

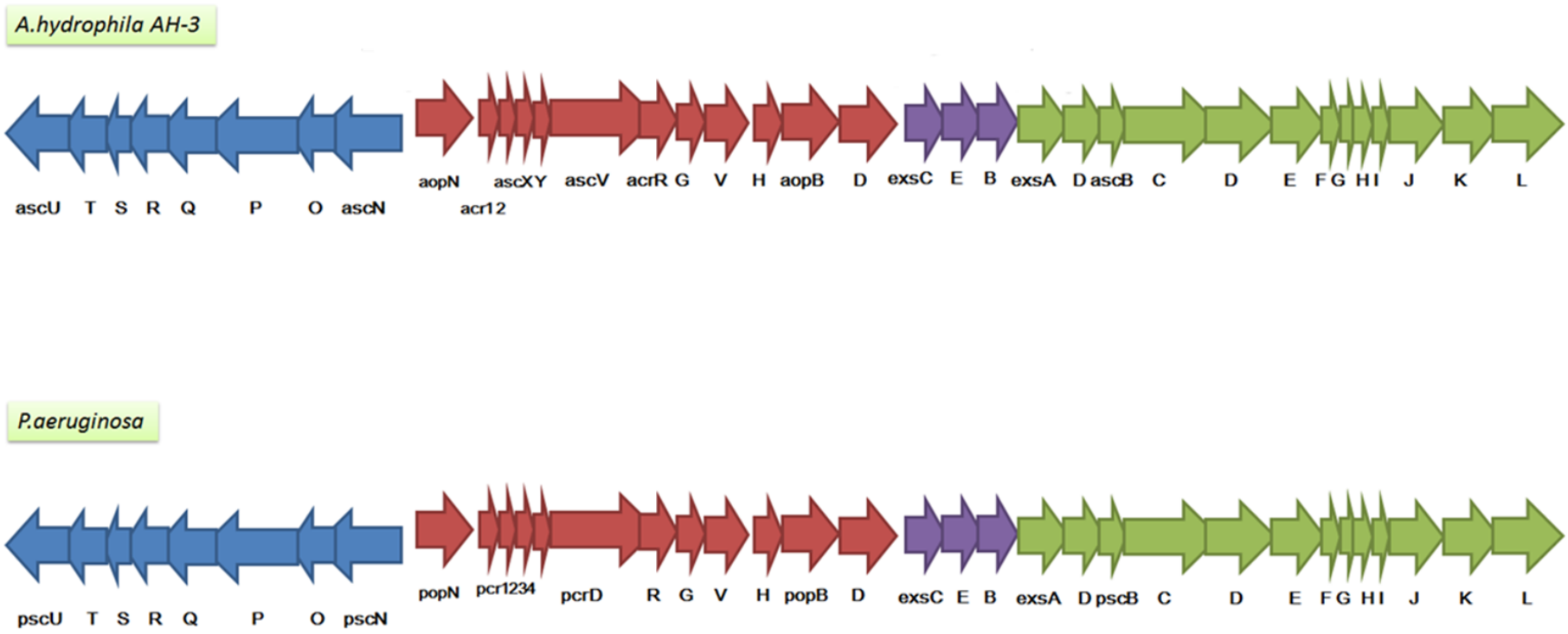


Figure 1.3 Genetic organizations of the T3SS in *A. hydrophila* AH-3 and *P. aeruginosa*. The arrows with the same colour represent homologous genes. The T3SS regulon in both bacteria contains five putative operons, *ascN-ascU* (*pscN-pscU*), *aopN-aopD* (*popN-popD*), *exsC-exsB*, *exsA-ascL* and *exsD-ascL*, in which the *exsA-ascL* and *exsD-ascL* share the same terminator. All putative operons are transcribed from 5' to 3' except for *ascN-ascU* (*pscN-pscU*), which is transcribed from 3' to 5'. This figure is adapted from Vilches et al. 2004.

Table 1.2 Characteristics of the T3SS genes in *A. hydrophila* AH-3

Gene names	Putative function of proteins	Homologues
Putative operon *ascN-ascU*		
ascU	Regulation of secretion	*pscU* in *P. aeruginosa*
ascT	T3SS apparatus	*pscT* in *P. aeruginosa*
ascS	T3SS apparatus	*pscS* in *P. aeruginosa*
ascR	T3SS apparatus	*pscR* in *P. aeruginosa*
ascQ	Secretion apparatus	*yscQ* in *Yersinia pestis*
ascP	Regulation of secretion	*pscP* in *P. aeruginosa*
ascO	Regulation of secretion	*pscO* in *P. aeruginosa*
ascN	ATPase	*yscN* in *Y. pestis*
Putative operon *aopN-aopD*		
aopN	Translocation regulation, effector	*yopN* in *Y.pestis*
acr1	Secretion apparatus	*pcr1* in *P. aeruginosa*
acr2	Chaperone	*pcr2* in *P. aeruginosa*
ascX	T3SS apparatus	*pcr3* in *P. aeruginosa*
ascY	T3SS apparatus	*pcr4* in *P. aeruginosa*
ascV	Secretion apparatus	*yscV* in *Y. pestis*
acrR	Low-Ca^{2+} response	*lcrR* in *Y. pestis*
acrG	Translocation regulation, chaperone	*acrG* in *A. salmonicida*
acrV	Secreted protein	*acrV* in *A. salmonicida*
acrH	Chaperone	*acrH* in *A. hydrophila* AH-1
aopB	Pore-forming protein	*aopB* in *A. hydrophila* AH-1
aopD	Pore-forming protein	*aopD* in *A. hydrophila* AH-1
Putative operon *exsC-exsB*		

exsC	Chaperone	*exsC* in *P. aeruginosa*
exsE	Secreted protein	*exsE* in *P. aeruginosa*
exsB	Regulatory mRNA	*exsB* in *P. aeruginosa*
Putative operon *exsA-ascL*		
exsA	Transcriptional activator of T3SS	*exsA* in *P. aeruginosa*
Putative operon *exsD-ascL*		
exsD	Regulatory protein	ExsD in *P.aeruginosa*
ascB	Chaperone	*yscB* in *Y. pestis*
ascC	Secretion apparatus, channel-forming	*yscC* in *Y. pestis*
ascD	T3SS apparatus	*pscD* in *P.aeruginosa*
ascE	Chaperone	*pscE* in *P.aeruginosa*
ascF	Translocon, needle-forming	*pscF* in *P.aeruginosa*
ascG	Chaperone	*pscG* in *P. aeruginosa*
ascH	Translocon	*yscH* in *Y. pestis*
ascI	T3SS apparatus	*yscI* in *Y. pestis*
ascJ	T3SS apparatus	*yscJ* in *Y. pestis*
ascK	T3SS apparatus	*yscK* in *Y. pestis*
ascL	ATPase regulator	*yscL* in *Y. pestis*

Table 1.2 Characteristics of the T3SS genes in *A. hydrophila* AH-3. The putative function of the protein products are based on information of their homologues in *Y.pestis*, *P. aeruginosaI* or other strains of *Aeromonas spp.* (Blaylock et al 2006, Burr et al 2003, Chatterjee et al 2011, Goranson et al 1997, Koster et al 1997, Quinaud et al 2005, Silva-Herzog et al 2008, Sun et al 2008, Tan et al 2009, Yang et al 2007a, Yang et al 2007b). The T3SS apparatus proteins are involved in the formation of the T3SS basal body, while the secretion apparatus and translocons are required for the export and translocation of the extracellular structural subunits, pore-forming proteins and the effector proteins. It should be noted that only *exsB* gene is not translated into protein but functions as an mRNA in regulating *exsA* mRNA stability and translation.

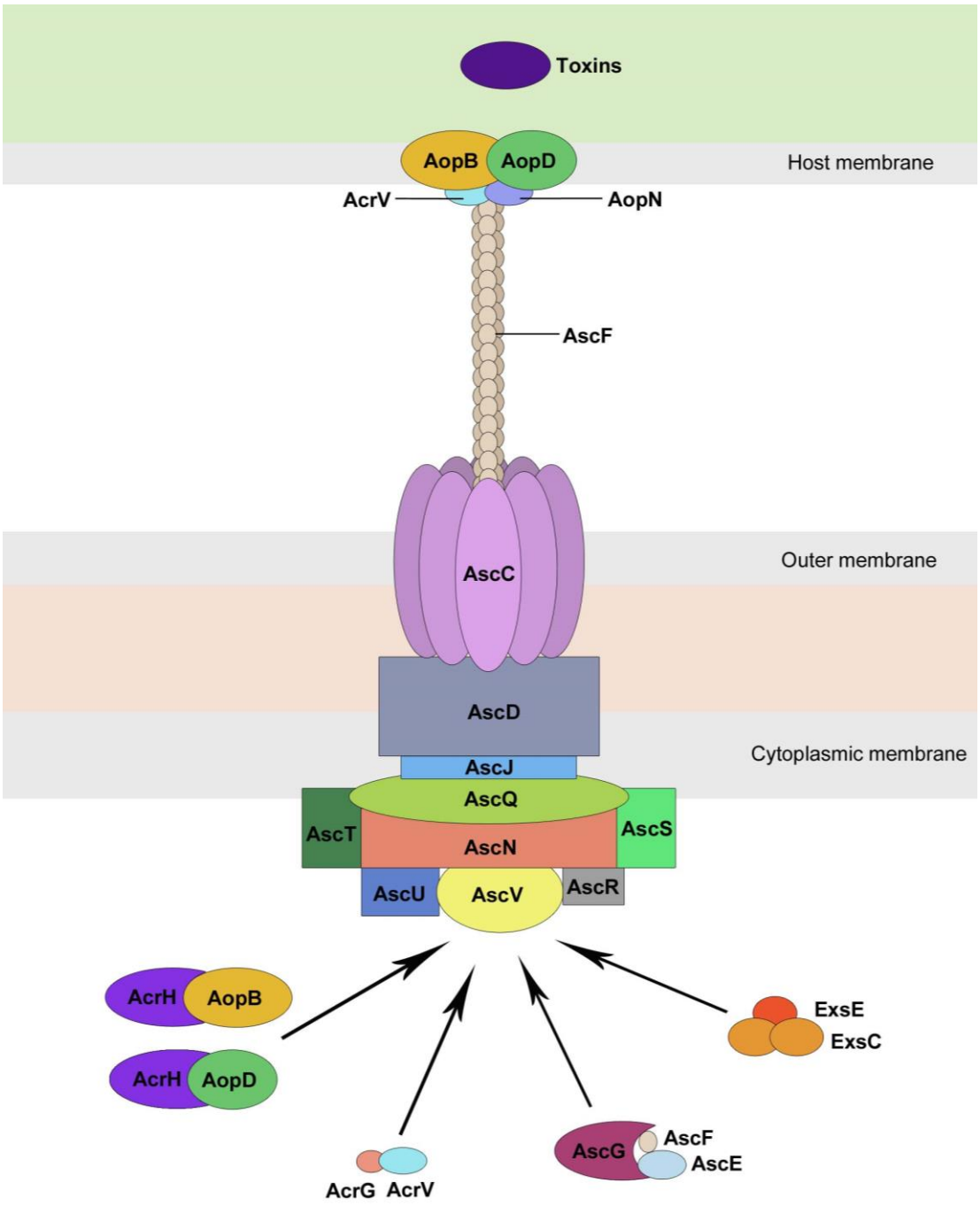


Figure 1.4 Illustration of putative structural composition of the T3SS in *A. hydrophila* AH-3. The proteins involved in the assembly of the T3SS apparatus are shown in squares, while the proteins in oval represent secreted proteins or proteins responsible for export and translocation (secretion apparatus and translocons). The exported extracellular structural subunits (AscF and AscV), pore-forming proteins (AopB and AopD) and effector proteins (ExsE) are bound to their cognate chaperones before they are secreted.

1.6.3 Regulation of the T3SS

The assembly of the T3SS requires a high level of energy and resources; thus it is regulated by many intracellular and extracelluar factors. One of the major transcriptional factors in *P. aeruginosa* is the AraC family protein ExsA. As the transcriptional activation of ExsA was required for the expression of the T3SS secretion apparatus, translocation machinery and secreted effectors, ExsA has been described as the master regulator of the *P. aeruginosa* T3SS (Hovey & Frank 1995, Yahr et al 1995). Moreover, ExsA in *A. hydrophila* shares 76% amino acid homology with ExsA in *P. aeruginosa*, both of which belong to the AraC family of transcriptional activators (Frank & Iglewski 1991, Vilches et al 2009, Yahr & Frank 1994).

In both *P. aeruginosa* and *A. hydrophila* AH-3, the ExsA-dependent promoters usually contain a consensus ExsA binding site centred 15bp upstream of the -35 RNA polymerase binding site and the sequence of the ExsA consensus binding site is TNAAAANA (Vilches et al 2009, Yahr & Wolfgang 2006). In *P. aeruginosa*, the recruitment of RNA polymerase (RNAP) onto the promoters requires sigma factor σ^{70}, and the primary function of ExsA is to facilitate RNAP-σ^{70} binding onto the T3SS promoter sequences (Vakulskas et al 2009). As the role of ExsA in *P.aeruginosa* was defined as the transcriptional activator by facilitating the RNAP-σ^{70} binding onto the T3SS promoter regions, the role of ExsA in *A. hydrophila* was proposed to be similar.

As in most AraC/XylS family proteins, *P. aeruginosa* ExsA consists of a carboxyl-terminal helix-turn-helix (HTH) DNA-binding motif and an amino-terminal oligomerization and ligand binding domain (Brutinel et al 2009, Martin & Rosner 2001). The potential ExsA DNA-binding model proposed by Brutinel and collegues involves the binding of a monomeric ExsA onto the -35 RNA polymerase site and then the recruitment of another ExsA molecule binding the ExsA-consensus binding

site (TNAAAANA) located 15bp upstream -35 region (Brutinel et al 2008). This model suggested potential ExsA-ExsA cooperative binding of the T3SS promoter sequences in *P. aeruginosa*.

It was reported in 2002 that the master regulator ExsA was negatively regulated by an anti-activator protein named ExsD in *P. aeruginosa* through direct protein-protein interaction. This was demonstrated using bacterial LexA two-hybrid assay (McCaw et al 2002). Later on, structural analysis of ExsA protein in *P. aeruginosa* suggested that the C-terminal domain (CTD) of ExsA consisted of 2 HTH DNA-binding motifs while the N-terminal domain (NTD) functions in oligomerization and ligand binding. The same study also demonstrated that the C-terminal HTH domain of *P.aeruginosa* ExsA is incapable of cooperative DNA-binding and unaffected by the inhibition of ExsD, suggesting that the N-terminal domain of ExsA is required for interactions with ExsD and ExsA itself (Brutinel et al 2009). Thereby it is proposed that in *P. aeruginosa* the binding of ExsD to the NTD of ExsA disrupts the self-association of ExsA, which is required to bind to the second binding site on the ExsA-dependent promoter sequences.

Meanwhile, the chaperone protein ExsC was found to be an anti-anti-activator of T3SS in *P. aeruginosa*. Mutagenesis and complementation experiments have shown that ExsC de-represses the T3SS transcription via ExsD, since Bacterial LexA two-hybrid assay and co-purification assay have suggested that ExsC interacts with ExsD through direct protein-protein interactions (Dasgupta et al 2004).

Later in 2005, the same group discovered that the secreted protein ExsE sat at the top of the signalling cascade by binding to its cognate chaperone protein ExsC through direct protein-protein interactions (Urbanowski et al 2005). Thus the regulatory cascade of the *P. aeruginosa* T3SS is fully uncovered. The secreted protein ExsE binds to the chaperone protein ExsC, which antagonizes the anti-activation activity of ExsD on the master regulator ExsA (Figure 1.5).

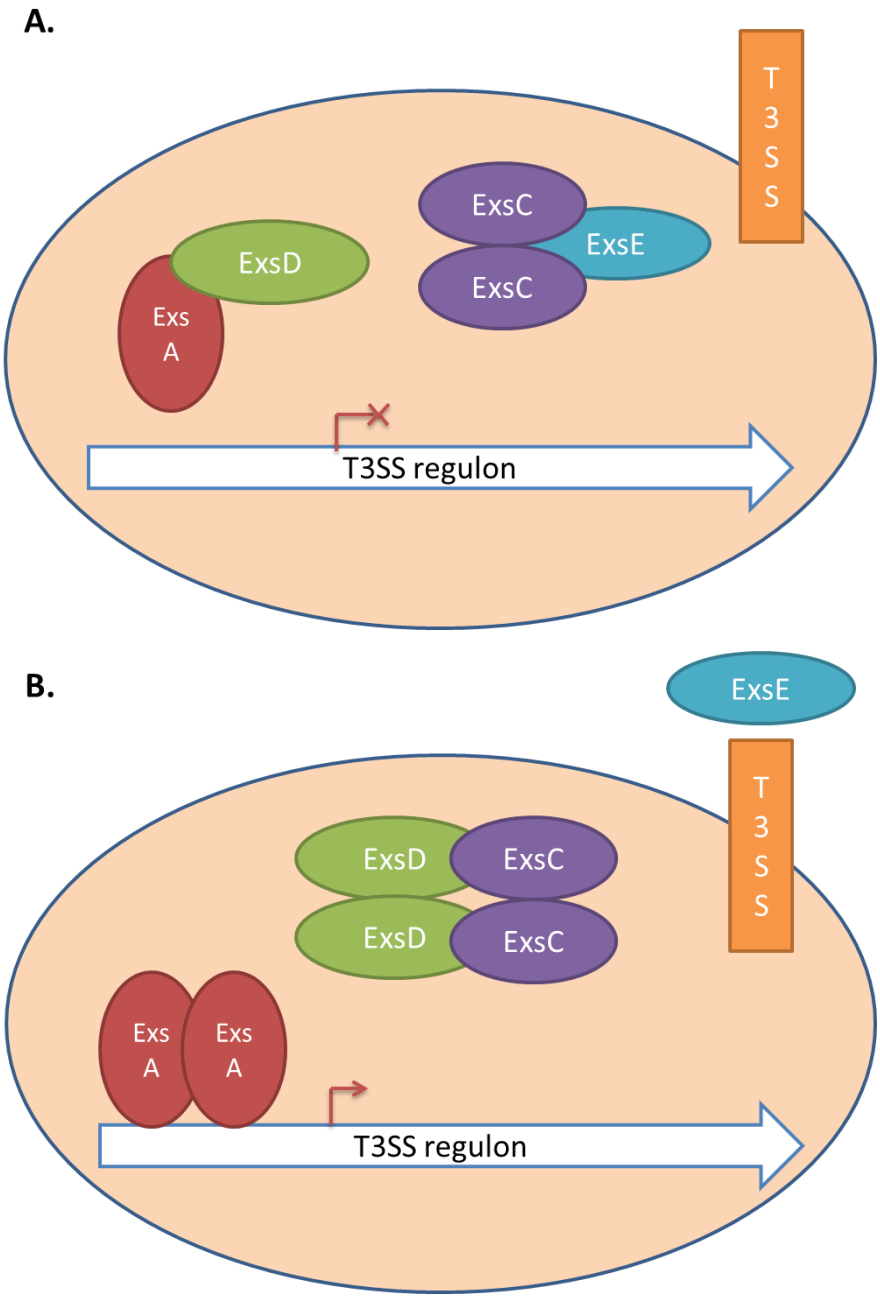


Figure 1.5 The regulatory cascade of the T3SS in *P. aeruginosa*. **A.** When the T3SS is not activated, the effector protein ExsE is bound to its cognate chaperone ExsC, allowing the de-activator protein ExsD to bind the activator protein ExsA, thus no transcription activation of the T3SS. **B.** When the T3SS is triggered upon contact with host cells or by other stimulus, the effector protein ExsE is secreted out from the bacteria, allowing the chaperone protein ExsC to bind to the de-activator ExsD, thus releasing ExsA to activate the transcription of the T3SS. This model is also proposed in *A. hydrophila* AH-3.

In *P. aeruginosa*, Gel Filtration Chromatography (GFC) and Analytical Ultracentrifugation (AUC) analysis of co-expressed ExsA-ExsD suggested the complex formation at 1:1 ratio (Thibault et al 2009). Moreover, Gel shift assays and a monohybrid study indicated that the anti-activator ExsD inhibit ExsA-dependent activation by affecting the ExsA self-association but not blocking the DNA target sites (Brutinel et al 2010). It was also reported that ExsD could only bind to ExsA when they were synthesized at the same time as both proteins were folding-intermediates (Bernhards et al 2013, Brutinel et al 2010, Thibault et al 2009).

Furthermore, a recent study carried out by Bernhards and collegues indicated that ExsD forms a homotrimer at 30°C, which prevent ExsD from inhibiting the activator ExsA. The trimerization of ExsD disrupts when the temperature rises to 37°C. Also the engineered monomeric ExsD is fully capable of inhibiting ExsA at 30°C. Taken together the findings, they suggested that the self-association of ExsD is temperature-sensitive and competes with the inhibition for ExsA in *P. aeruginosa* (Bernhards et al 2013).

In *P.aeruginosa*, the ExsA-ExsD complex is dissociated by the addition of ExsC, which has a higher affinity to bind ExsD to form a 2:2 heterocomplex, thus releasing ExsA to activate the T3SS regulon (Figure 1.5). As it was reported in *P. aeruginosa*, the size of the ExsC-ExsD complex consisted of two molecules of both ExsC and ExsD proteins using GFC and AUC, together with the Isothermal Titration Calorimetry (ITC) data, which indicated an equal molar binding ratio of ExsC and ExsD, suggesting a heterotetramer complex formation of ExsC-ExsD at 2:2 ratio, with a binding affinity of 18nM (Zheng et al 2007). The binding affinity of ExsA to ExsD is still unknown, but given the fact that ExsA-ExsD complex is readily dissociated by ExsC, the binding affinity of ExsA to ExsD may be more than 100nM.

It was first identified by Urbanowski and collegues in *P. aeruginosa* that ExsC, as the chaperone for the effector protein ExsE, released ExsD in the presence of

ExsE (Urbanowski et al 2005). This corresponds to the Isothermal Titration Calorimetry studies carried out by Zheng's group, who has determined the binding affinities for ExsC-ExsD (18nM) and ExsC-ExsE (1nM) in *P. aeruginosa* (Zheng et al 2007). This suggests that the ExsC chaperone protein prefers to bind to the effector protein ExsE rather than the anti-activator ExsD. When the concentration of ExsE decreases, the abundant ExsC proteins bind to ExsD and antagonize the inhibition of ExsD on ExsA. Thereby the concentration of the effector protein ExsE plays a crucial role in the regulation of the ExsA transcriptional activation.

In comparison with *P .aeruginosa*, the regulation of the T3SS is not intensively studied in *A. hydrophila*. In 2004, the complete T3SS that consisted of 35 genes in *A. hydrophila* AH-3 was sequenced but the regulation of the T3SS in *A. hydrophila* still remained unknown (Vilches et al 2004). It was then reported by Sha and collegues in 2007 that the Exs proteins in *A. hydrophila* SSU strain had similar effects on the regulation of the T3SS to those found in *P. aeruginosa* using mutagenesis and over-expression assays (Sha et al 2007). In the meantime, proteomic study in *A. hydrophila* AH-1 has shown that the T3SS secreted translocon protein AcrV was abundant in Δ*exsD* and Δ*aopN* mutants while absent in Δ*exsD* Δ*exsA* and Δ*aopN* Δ*exsA* double mutants (Yu et al 2007). Furthermore, in *A. hydrophila* AH-3, quantitative RT-PCR results for *aopN* and *aexT* mRNA production showed 60-85% decrease in Δ*exsA* mutant background compared with the results of the wild type. In the same study, the activities of promoters upstream of *aopN* and *aexT* genes were significantly decreased in the *exsA* mutant when compared to the wild type strain (Vilches et al 2009). All these findings suggest that ExsA, like its homologue in *P.aeruginosa*, is likely to be the master regulator of the T3SS in *A. hydrophila* as well. However, there is no evidence of ExsC and ExsE involvement in the regulation of the T3SS in *Aeromonas* species. In this study, the putative regulatory cascade that involves ExsA, ExsD, ExsC and ExsE proteins will be investigated in detail.

1.7 Project hypothesis and objectives

A previous study in our laboratory investigating the lateral flagella system of *A. hydrophila* discovered two non-swarming transposon mutants that were found to be mutated in the *exsD* gene, which encodes one of the regulators of the *A. hydrophila* T3SS. Since the T3SS regulatory cascade that involves the ExsA, ExsD, ExsC and ExsE proteins was well-described in *P. aeruginosa*, the aim of this study is to determine whether the regulatory cascade of the T3SS in *A. hydrophila* is the same as in *P. aeruginosa* and if the T3SS cross-talks with the lateral flagella system via the T3SS regulatory proteins.

Thereby, insertional knockouts of the regulatory components of the T3SS and the lateral flagella system are required. Mutations in *exsA* and *lafK* have already been constructed previously in the laboratory, but the insertional knockouts of *exsC*, *exsD* and *exsE* genes were required. To understand what roles these *exs* genes played in the regulation of the T3SS, the activities of the T3SS promoters were to be measured in the wild type and *exs* mutant backgrounds.

Furthermore, protein-protein interactions interactions within the T3SS were investigated. The Exs proteins were to be over-expressed using the pET system and the pMAL system, followed by the investigation of direct protein-protein interactions using Far-Western Blot. In addition to protein over-expression, the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system was utilized to study the protein-protein interactions in between Exs proteins *in vivo*.

Finally, when the T3SS regulatory cascade was determined in *A. hydrophila* AH-3, the relationship between the T3SS and the lateral flagella system was investigated by carrying out the swarming assay on the *exs* mutants and measuring the lateral flagella promoter activities in the *exs* mutant backgrounds.

Chapter 2
Methods and Materials

2.1 Media, bacterial strains, plasmids and antibiotics used in this study

All media components used in this project were purchased from Oxoid™, Fisher™ or Difco™. Horse blood added Columbia Blood Agar (CBA) was provided by the Department of Infection and Immunity in the Medical School of the University of Sheffield. All media were autoclaved at 121°C and 15 psi for 20 min. Appropriate antibiotics and supplements were added when required.

2.1.1 Luria Bertani Agar (LB agar)

10g	Tryptone
10g	NaCl
5g	Yeast Extract
15g	Bacteriological Agar

(Made up to 1000ml with dH_2O and autoclaved to sterilize)

2.1.2 Luria Bertani Broth (LB broth)

2g	Tryptone
2g	NaCl
1g	Yeast Extract

(Made up to 200ml with dH_2O and autoclaved to sterilize)

2.1.3 Brain Heart Infusion Broth (BHIB)

37g of Brain Heart Infusion powder was dissolved in 1L of distilled water and autoclaved to sterilize.

2.1.4 Swarming agar

Tryptone	1% (w/v)
Glucose	0.5% (w/v)
NaCl	0.5% (w/v)
Eiken Agar	0.6% (w/v)

(Dissolve in dH_2O and autoclave to sterilize)

Table 2.1 Bacterial strains used in the study

Name of strains	Description	Reference
Aeromonas hydrophila AH3 strains		
AH3R	Wild type *Aeromonas hydrophila* AH3 strain, *Rif*^{R}	Dr. J. Tomás University of Barcelona
exsA mutant	AH3R strain with *exsA* knocked out by the insertion of Km^{R} cassette	Laboratory collection
exsC mutant	AH3R strain with *exsC* knocked out by the insertion of Km^{R} cassette	This study
exsD mutant	AH3R strain with *exsD* knocked out by the insertion of Km^{R} cassette	This study
exsE mutant	AH3R strain with *exsE* knocked out by the insertion of Km^{R} cassette	This study
lafK mutant	AH3R strain with *lafK* knocked out by the insertion of Km^{R}	Laboratory collection
AH3R-*PascN*	Reporter plasmid pKAG-P*ascN* conjugated into AH3R wild type	This study
AH3R-*PaopN*	Reporter plasmid pKAG-P*aopN* conjugated into AH3R wild type	This study
AH3R-*PexsC*	Reporter plasmid pKAG-P*exsC* conjugated into AH3R wild type	This study
AH3R-*PexsA*	Reporter plasmid pKAG-P*exsA* conjugated into AH3R wild type	This study
AH3R-*PexsD*	Reporter plasmid pKAG-P*exsD* conjugated into AH3R wild type	This study
AH3R-*PfliM*	Reporter plasmid pKAG-P*fliM* conjugated into AH3R wild type	This study

AH3R-P*lafK*	Reporter plasmid pKAG-P*lafK* conjugated into AH3R wild type	This study
AH3R-P*flgM*	Reporter plasmid pKAG-P*flgM* conjugated into AH3R wild type	This study
AH3R-P*flgA*	Reporter plasmid pKAG-P*flgA* conjugated into AH3R wild type	This study
AH3R-P*flgB*	Reporter plasmid pKAG-P*flgB* conjugated into AH3R wild type	This study
AH3R-P*maf*	Reporter plasmid pKAG-P*maf* conjugated into AH3R wild type	This study
AH3R-P*lafA*	Reporter plasmid pKAG-P*lafA* conjugated into AH3R wild type	This study
AH3R-P*lafB*	Reporter plasmid pKAG-P*lafB* conjugated into AH3R wild type	This study
AH3R-P*lafX*	Reporter plasmid pKAG-P*lafX* conjugated into AH3R wild type	This study
Escherichia coli strains		
DH5α	F–, Φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *rec*A1, *end*A1, *hsd*R17, (rK–, mK+), *pho*A, *sup*E44, λ– *thi*-1, *gyr*A96, *rel*A1	(Hanahan 1983)
CC118-λ*pir*	*phoA20*, *thi-1*, *rspE*, *rpoB*, *argE*, (Am)*, recA1*, phage λ*pir*	(Herrero et al 1990)
S17-1-λ*pir*	Sm^R, F^-, *recA*, *hsdR*, RP4-2 (Tc::Mu) (Km::Tn*7*), phage λ*pir*	(Miller 1972)
Sm10-λ*pir*	KmR, *thi*-1, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu, phage λ*pir*	(Miller & Mekalanos 1988)
HB101	F^- *mcrB mrr hsd*S20(r_B^- m_B^-) *recA*13 *leuB*6 *ara*-14 *proA*2 *lacY*1 *galK*2	(Hanahan 1983)

	xyl-5 *mtl*-1 *rpsL*20(Sm^R) *gln*V44 λ^-	
BL21(DE3)	F^- *omp*T *gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3)	Novagen™
C41(DE3)	*ompT hsdSB (rB- mB-) gal dcm* (DE3)	Lucigen™
BL21Star™(DE3)	F– *omp*T *hsd*S_B(r_B–, m_B–) *gal dcm rne*131 (DE3)	Invitrogen™
BTH101	F-, *cya*-99, *araD*139, *galE*15, *galK*16, *rpsL*1(*Str*^{r}), *hsdR*2, *mcrA1*, *mcrB1*	Euromedex™
E. coli ER2523 (NEB Express)	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--*TetS*)2 [dcm] R(zgb-210::Tn10--*TetS*) endA1* Δ *(mcrC-mrr)114::IS10*	New England BioLabs™

Table 2.1 Bacterial strains used in this study.

2.1.5 Bacterial Growth

All bacterial strains used in this study are listed in table 2.1 while plasmids used are listed in table 2.2. *A. hydrophila* strains were incubated at 30℃ on LB agar, CBA or in broth cultures with shaking at 200rpm, in which antibiotics were added when required (Table 2.3), while no antibiotics were present in swarming agar.

E. coli strains were grown at 37℃ on LB agar or in broth cultures with shaking at 200rpm, in which antibiotics were added when required.

Table 2.2 Plasmids used in the study

Name of plasmids	Characteristics	Reference
pBBR1MCS	Broad-host-range plasmid, Cm^R	(Kovach et al 1994)
pBB*exsA*	*exsA* cloned into pBBR1MCS	Laboratory collection
pBB*exsCEB*	*exsCEB* region cloned into pBBR1MCS	Laboratory collection
pBB*exsCKm*^{R}	*exsC::*Km^R knockout ligated with pBBR1MCS	This study
pBBR1MCS-5	Broad-host-range plasmid, Gm^R	(Kovach et al 1994)
pBBR5*exsA*	*exsA* ligated with pBBR1MCS-5	This study
pUC4KIXX	Source of Km^R cassette *(npt*II)	Amersham Pharmacia™
pJMK30	pUC19 derivative, containing a Km^R cassette (AphA-3) from *Campylobacter coli*	(van Vliet et al 1998)
pKNG101	RK6 derived suicide plasmid, *SacB*, Sm^R	(Kaniga et al 1991)
pKNG*exsA*Km^R	*exsA*::Km^R knockout ligated with pKNG101 suicide plasmid	This study
pKNG*exsC*Km^R	*exsC*::Km^R knockout ligated with pKNG101 suicide plasmid	This study
pKNG*exsD*Km^R	*exsD*::Km^R knockout ligated with pKNG101 suicide plasmid	This study
pKNG*exsE*Km^R	*exsE*::Km^R knockout ligated with pKNG101 suicide plasmid	This study

pGEM-3Zf(+)	Standard cloning vector, used for ISA-cloning in this study, Amp^R	Promega™
pGEM*exsD*Km^R	*exsD:*Km^R knockout ligated with pGEM-3Zf(+)	This study
pGEM*exsE*Km^R	*exsE:*Km^R knockout ligated with pGEM-3Zf(+)	This study
pET28a	Over-expression vector, T7 promoter, N-terminal His tag, Km^R	Novagen™
pET28*exsA*	*exsA* ligated in frame with His-tag in pET28a	This study
pET28*exsC*	*exsC* ligated in frame with His-tag in pET28a	This study
pET28*exsD*	*exsD* ligated in frame with His-tag in pET28a	This study
pET28*exsE*	*exsE* ligated in frame with His-tag in pET28a	This study
pMAL-c5x	Over-expression vector, *malE*-, MBP5 coding, Amp^R	New England BioLabs™
pMAL*exsA*	*exsA* ligated in frame with MBP5 gene in pMAL-c5x	This study
pMAL*exsC*	*exsC* ligated in frame with MBP5 gene in pMAL-c5x	This study
pMAL*exsD*	*exsD* ligated in frame with MBP5 gene in pMAL-c5x	This study
pMAL*exsE*	*exsE* ligated in frame with MBP5 gene in pMAL-c5x	This study
pKAGb-2(-)	Broad-host-range reporter vector, ori_{1600} carrying promoter-less *lacZ* gene, Cm^R	M.S.Thomas, unpublished
pKAG-P*ascN*	Promoter upstream of *ascN* cloned into	This study

	pKAG-b2(-)	
pKAG-P*aopN*	Promoter upstream of *aopN* cloned into pKAG-b2(-)	This study
pKAG-P*exsC*	Promoter upstream of *exsC* cloned into pKAG-b2(-)	This study
pKAG-P*exsA*	Promoter upstream of *exsA* cloned into pKAG-b2(-)	This study
pKAG-P*exsD*	Promoter upstream of *exsD* cloned into pKAG-b2(-)	This study
pKAG-P*fliM*	Promoter upstream of *fliM* cloned into pKAG-b2(-)	This study
pKAG-P*lafK*	Promoter upstream of *lafK* cloned into pKAG-b2(-)	This study
pKAG-P*flgM*	Promoter upstream of *flgM* cloned into pKAG-b2(-)	This study
pKAG-P*flgA*	Promoter upstream of *flgA* cloned into pKAG-b2(-)	This study
pKAG-P*flgB*	Promoter upstream of *flgB* cloned into pKAG-b2(-)	This study
pKAG-P*maf*	Promoter upstream of *maf-5* cloned into pKAG-b2(-)	This study
pKAG-P*lafA*	Promoter upstream of *lafA* cloned into pKAG-b2(-)	This study
pKAG-P*lafB*	Promoter upstream of *lafB* cloned into pKAG-b2(-)	This study
pKAG-P*lafX*	Promoter upstream of *lafX* cloned into pKAG-b2(-)	This study
pKT25	pSU40 derivative, *lac* promoter, allow in-frame fusion at C-terminal of T25,Km^R	Euromedex™

pKNT25	pSU40 derivative, *lac* promoter, allow in-frame fusion at N-terminal of T25,Km^R	Euromedex™
pUT18	pUC19 derivative, *lac* promoter, allow in-frame fusion at N-terminal of T18, Amp^R	Euromedex™
pUT18C	pUC19 derivative, *lac* promoter, allow in-frame fusion at C-terminal of T18, Amp^R	Euromedex™
pKT25-zip	pKT25 derivative, leucine zipper of GCN4 fused at C-terminal of T25,Km^R	Euromedex™
pUT18C-zip	pUT18C derivative, leucine zipper of GCN4 fused at C-terminal of T25,Km^R	Euromedex™
pKT25-*exsA*	*exsA* cloned in-frame with pKT25	This study
pKT25-*exsC*	*exsC* cloned in-frame with pKT25	This study
pKT25-*exsD*	*exsD* cloned in-frame with pKT25	This study
pKT25-*exsE*	*exsE* cloned in-frame with pKT25	This study
pKNT25-*exsA*	*exsA* cloned in-frame with pKNT25	This study
pKNT25-*exsC*	*exsC* cloned in-frame with pKNT25	This study
pKNT25-*exsD*	*exsD* cloned in-frame with pKNT25	This study
pKNT25-*exsE*	*exsE* cloned in-frame with pKNT25	This study
pUT18-*exsA*	*exsA* cloned in-frame with pUT18	This study
pUT18-*exsC*	*exsC* cloned in-frame with pUT18	This study
pUT18-*exsD*	*exsD* cloned in-frame with pUT18	This study
pUT18-*exsE*	*exsE* cloned in-frame with pUT18	This study
pUT18C-*exsA*	*exsA* cloned in-frame with pUT18C	This study
pUT18C-*exsC*	*exsC* cloned in-frame with pUT18C	This study
pUT18C-*exsD*	*exsD* cloned in-frame with pUT18C	This study
pUT18C-*exsE*	*exsE* cloned in-frame with pUT18C	This study

Table 2.2 Plasmids used in this study.

All plasmids were stored at -20°C and further stored in glycerol stock at -80°C after transformation into *E. coli* DH5α with the exception that suicide plasmids were transformed into *E. coli* CC118λ*pir.*

Table 2.3. Antibiotics and other additives used in this study

Antibiotics/Additives	Working concentration	Concentration made
Kanamycin (Km)	50µg/ml	50mg/ml (dH2O)
Streptomycin (Sm)	50µg/ml	50mg/ml (dH2O)
Ampicillin (Amp)	100µg/ml	100mg/ml (dH2O)
Rifampicin (Rif)	50µg/ml	50mg/ml (DMSO)
Chloramphenicol (Cm)	50µg/ml	50mg/ml (Ethanol)
Gentamycin (Gm)	50µg/ml	50mg/ml (dH2O)
IPTG	0.3mM to 1mM	1M (dH_2O)

Table 2.3 Antibiotics and other supplements used in this study

2.2 Chromosomal DNA extraction

Bacteria were cultured overnight in 10ml broth culture with appropriate antibiotics at its appropriate temperature. They were then harvested by centrifugation at 1600xg for 15 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 1ml Solution A (section 2.2.1), in which 20µl of lysozyme (100mg/ml) was added. The sample was incubated at 37°C for 20 min followed by freezing at -80°C for 5 min. Then 1.5ml of Solution B (section 2.2.1) was added and mixed by inversion. The sample was incubated at 37°C for 5 min until viscous. Then 10µl of RNAse (20mg/ml) was added followed by 20 min incubation at 37°C. Then 10µl proteinase K (10mg/ml) was added followed by 20 min incubation at 37°C again. A volume of 0.5ml of buffered phenol pH7.4 (bottom layer) was added to 0.5ml of sample and mixed, followed by centrifugation at 15,000xg for 10 min. The top aqueous layer was transferred into a fresh Eppendorf tube. This was repeated once with phenol and twice with phenol-chloroform.

It was then followed by ethanol precipitation. A 1/10 volume of 3M sodium acetate and 2 times volume of 100% ethanol were added and mixed by inversion. The sample was centrifuged at 15,000xg for 5 min and the supernatant was discarded. A volume of 0.5ml of 70% ethanol was added carefully not to disturb the DNA pellet and was followed by centrifugation at 15,000xg for 5 min. The supernatant was discarded and the DNA pellet was left to dry. Finally the DNA pellet was dissolved in 200µl of dH_2O.

2.2.1 Buffer Preparation

Solution A:

10mM Tris-HCl pH7.2	(78.8mg in 50ml of dH_2O)
150mM NaCl	(0.438g in 50ml of dH_2O)
100mM EDTA	(1.86g in 50ml of dH_2O)

Solution B:

100mM Tris-HCl pH 8.8	(0.78g in 50ml of dH_2O)

1% (w/v) SDS	(2.5ml of 20% (w/v) SDS)
100mM NaCl	(0.29g in 50ml of dH_2O)

2.3 Polymerase Chain Reaction (PCR)

Reagents of PCR listed in 2.3.1 were mixed together to make a Master Mix, except the DNA template, which was added separately into each tube. The polymerases utilized in this study were KOD DNA polymerase (Toyobo Life Science™), Platinum® Pfx DNA polymerase (Invitrogen™), Phusion High-Fidelity DNA polymerase (New England Biolabs™) and Taq DNA polymerase (New England Biolabs™). The PCR samples were then put in the thermal cycler and the reaction was started under appropriate conditions listed in table 2.4.

2.3.1 Normal PCR conditions

10X Buffer	5µl
2mM dNTPs	5µl
25mM $MgCl_2$	4µl
Primers	1µl +1µl
Polymerase	0.5µl
DNA	5µl

Distilled H_2O was added up to 50µl of total volume

2.3.2 colony PCR screen condition

10X Buffer	5µl
2mM dNTPs	5µl
50mM $MgCl_2$	2µl
DMSO	2µl
Primers	1µl+1µl
Taq Polymerase	0.5µl

Distilled H_2O was added up to 50μl of total volume.

Bacterial colonies were picked up by sterilized tips and were transferred into each PCR tube independently.

Table 2.4 PCR cycle conditions

		Taq polymerase	KOD polymerase	Pfx polymerase
Heated lid		105°C	105°C	105°C
Denaturing	1 cycle	95°C 3 min	95°C 3 min	95°C 3 min
Denaturing	30 cycles	95°C 40 sec	95°C 40 sec	95°C 40 sec
Annealing	30 cycles	55°C 30 sec	60°C 30 sec	55°C 30 sec
Extension	30 cycles	72°C variable	70°C variable	68°C variable
Final extention	1 cycle	72°C 2 min	70°C 2 min	68°C 2 min
Hold	Infinite	10°C	10°C	10°C

Table 2.4 PCR conditions for different polymerase. Annealing temperature was altered according to different primers. A gradient of annealing temperature might be required for optimization. Annealing temperature was set to 55°C in the presence of 5μl of 10X Enhancer Buffer provided for Pfx polymerase. Extension time was set up according to the length of the target fragment following the manufacturer's instructions. The default extension rates for all polymerase were 1kb/min.

2.4 Agarose Gel Electrophoresis

2.4.1 Buffer Preparation

Preparation of 50X TAE:		6X Loading Buffer:	
Tris	2M	Bromophenol Blue	0.25%(w/v)
Glacial acetic acid	5.7% (v/v)	Xylene cyanole	0.25%(w/v)
EDTA	0.3M	Glycerol	50% (v/v)

1X TAE was made by diluting 20ml 50X TAE in 1000ml dH_2O.

2.4.2 Preparation of agarose gel

To 100ml 1X TAE Buffer 0.8g-1.0g of agarose powder was added to make a 0.8%-1.0% agarose gel. It was heated in the microwave untill the agarose was dissolved completely. When it cooled down to approximately 56°C, it was poured into a gel tray with a comb and sealed by tape. It was left for 20-30 min to solidify and put into a gel tank filled up with 1X TAE buffer. Then the DNA samples with loading dye (5:2) were loaded into the gel wells. The gel was usually run at 80V for 75 min and stained with ethidium bromide (0.5μg/ml) for 15 min. Finally the gel photo was taken on an ultraviolet trans-illuminator.

2.4.3 Gel extraction using QIAgen agarose gel extraction kit

The desired DNA fragment could be excised from agarose gel with a sterile scalpel and transferred to a sterile pre-weighted Eppendorf tube. To 1 volume of the agarose gel (100mg~100μl), 3 volumes of Buffer QG was added. It was then incubated at 50°C for 10-15 min and mixed by inverting every 2-3 min until the gel slice was completely dissolved. Then 1 gel volume of isopropanol was added and mixed. The sample was transferred to a QIAquick spin column and centrifuged at 14,000 xg for 1 min. The flow-through was discarded and 0.5ml of Buffer QG was added to the spin column to remove any residual agarose, followed by centrifugation for 1 min. The flow-through was discarded again and 0.75ml of Buffer PE was added to wash the sample. It was centrifuged for 1 min and the flow-through was discarded, followed by further centrifugation for 1 min to remove any further wash buffer. The spin column was then placed in a fresh Eppendorf tube and 50μl of Elution Buffer was added to the centre of the spin column, followed by centrifugation for 1 min to elute the desired DNA sample.

2.5 Restriction enzyme digestion

All components were mixed together according to the manufacturer's guidance. The sample was usually purified following the PCR purification protocol in 2.6.

2.5.1 Standard reaction conditions:

DNA samples	5µl-26µl (0.5-2µg)
Restriction enzyme	1µl (10 units)
10X Buffer	3µl

dH2O was added up to 30ul of total volume.

2.6 PCR purification using the QIAgen PCR purification kit

Five volumes of Buffer PBI was added to the PCR sample and mixed by inversion. A QIAquick spin column was placed in a provided 2ml collection tube and the sample was applied to the QIAquick spin column, followed by centrifugation at 15,000xg for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. A volume of 0.75ml of Buffer PE was added to the column followed by centrifugation at 15,000xg for 1 min. The flow-through was discarded and the column was placed back into the collection tube followed by centrifugation for an additional 1 min. The column was placed in a sterile Eppendorf tube. A volume of 50µl of Buffer EB was added to the centre of the column membrane and it was left to stand for 1 min. To eluted DNA, the sample was centrifuged at 15,000xg for 1 min.

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

Bacteria were cultured overnight in 10ml BHIB or LB broth with appropriate antibiotics at the appropriate temperature with shaking at 200 rpm.

To harvest the cells, the samples were centrifuged at 1,600xg for 15 min to pellet the cells. The supernatant was discarded and the cell pellets were

resuspended in 250µl of Buffer P1. The sample was then transferred into a fresh Eppendorf tube and 250µl of Buffer P2 was added and mixed by inversion, 350µl of Buffer N3 was then added and gently mixed by inversion.

The sample was centrifuged at 15,000xg for 10 min and a white pellet was formed at the bottom of the tube. The supernatant was transferred to the QIAprep spin column. To increase the concentration of the products, each spin column might contain supernatants from more than one tube (of the same sample). The sample was then centrifuged at 15,000xg for 1 min and the flow-through was discarded. A volume of 0.5ml of Buffer PB was added followed by centrifugation at 15,000xg for 1 min and the flow-through was discarded. A volume of 0.75ml of Buffer PE was added followed by centrifugation at 15,000xg for 1 min and the flow-through was discarded. The sample was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep spin column was placed into a fresh Eppendorf tube, 50µl of Buffer EB (pre-warmed) was added to the centre of the column membrane and it was left to stand for 1 min. To elute plasmid DNA, the sample was centrifuged at 15,000xg for 1 min.

2.8 Ligation

Purified PCR fragments and purified linearized plasmids with corresponding restriction sites on both ends can be ligated together using T4 DNA ligase according to the manufacturer's guidance. A typical ligation reaction is shown in table 2.5. All samples and controls were incubated overnight at 15℃.

Table 2.5 Ligation reaction and controls.

	Ligation Reaction	+Ligase Control	No Ligase Control
Linearized plasmid	100ng-200ng	100ng-200ng	100ng-200ng
PCR fragment	50ng-100ng	Null	Null
Ligase Buffer	1.5µl	1.5µl	1.5µl
T4 Ligase	1 unit	1 unit	Null

dH_2O	Added up to 15µl of total volume

Table 2.5 Ligation reaction conditions. T4 DNA ligase from Promega™ was used throughout the study.

2.9 Preparation of competent cells

E. coli strains (DH5α, CC118-λ*pir*, etc.) were incubated overnight in 10ml broth culture with 200 rpm shaking at 37°C.

1ml suspension of bacterial cells from the pre-culture was transferred into 100ml of sterile fresh broth to make a 1:100 dilution. Then this culture was incubated at 37℃ with 200rpm shaking for approximately 2 hours until the OD_{600nm} reached 0.3 (approx. 4-7 x 10^7 cfu/ml). The culture was collected into two 50ml plastic centrifuge tubes, chilled on ice for 15 min and it was then centrifuged at 1600xg for 20 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 33ml (1/3 of the original volume) of RF1 solution. It was incubated on ice for 15 min. The cell culture was then centrifuged at 1600xg for 20 min at 4°C.The cell pellet was then resuspended in 8ml (1/12.5 of the original volume) of RF2 solution and incubated on ice for 15 min. The cell suspension was then aliquoted (200µl or 400µl) into pre-chilled sterile Eppendorf tubes and stored at -80°C.

2.12.1 Buffer Preparation

RF1 solution

100mM KCl	0.745g of KCl in 100ml of dH_2O
50mM $MnCl_2$	0.99g of $MnCl_2{\cdot}4H_2O$ in 100ml of dH_2O
30mM Potassium acetate	0.295g of KCH_3CO_2in 100ml of dH_2O
10mM $CaCl_2$	0.147g of $CaCl_2{\cdot}2H_2O$ in 100ml of dH_2O

RF2 solution

10mM MOPS	0.21g of MOPS in 100ml of dH_2O
10mM KCl	75mg of KCl in 100ml of dH_2O
75mM $CaCl_2$	1.10g of $CaCl_2{\cdot}2H_2O$ in 100ml of dH_2O

15% glycerol (v/v)	15ml in 100ml of dH_2O

2.10 Transformation

A volume of 60-100µl of competent cells were added into sterile Eppendorf tubes, to each of which 200ng of plasmid DNA (or all 15µl of the ligation sample) was added and mixed by pipetting up and down. The sample was chilled on ice for 30 min. It was then heat shocked in a 42°C water bath for 90 seconds. Then 1ml of sterile BHIB or LB broth was added into each tube and the sample was incubated at 37°C for 60 min. Then 100µl of the sample was spread on LB agar plates with appropriate antibiotics and incubated overnight at 37°C.

2.11 Bacterial conjugation

Bacterial strains to be conjugated were pre-cultured overnight in 10ml broth culture with appropriate antibiotics at the appropriate temperature with shaking at 200rpm.

To harvest the cells, the samples were centrifuged at 1600xg for 15 min. The supernatants were discarded and the pellets were washed with 5ml sterile PBS. They were re-centrifuged and washed with 5ml PBS again. Then the samples were centrifuged at 1600xg for another 15 min and the supernatants were poured off. The cell pellets were resuspended in their own volume (supernatant left in the tube, around 0.3ml).The suspensions of the two conjugal bacterial strains (*E. coli* donor and *A. hydrophila* recipient) were mixed in a sterile Eppendorf tube. The mixed strains were pipetted onto a sterile 25mm diameter 0.45µm nitrocellulose filter disc placed on the CBA plate.

The sample was incubated at 30°C for 6 hours. The filter disc was then removed and washed in a sterile universal tube with 3ml sterile PBS. Serial dilutions (10^{-2} and 10^{-3}) were made and 100ul of each was spread onto LB agar plates with appropriate antibiotics.

2.11.1 Buffer preparation

PBS

Dissolve 5 PBS tablets (Sigma-Aldrich™) in 1L dH_2O then autoclaved to sterilize, to generate 0.01M phosphate buffer (pH7.4), 0.0027M KCl and 0.137M NaCl.

2.12 β-Galactosidase assay

2.12.1 Buffer Preparation:

Z-Buffer (final concentration of 1X)

Na_2HPO_4 (0.06M)	16.1g of $Na_2PO_4 \cdot 7H_2O$
NaH_2PO_4 (0.04M)	5.5g of $NaH_2PO4 \cdot H_2O$
KCl (0.01M)	0.75g of KCl
$MgSO_4$ (0.001M)	0.24g of $MgSO_4 \cdot 7H_2O$
dH_2O	added up to 1000ml

Final pH=7.0

(β-Mercaptoethanol (0.05M), which is 270µl in 100ml of Z-Buffer, to be added on the day of experiment.)

1L of Z-Buffer was made up and stored at 4°C. β-Mercaptoethanol was not added because it is very volatile and would evaporate upon storage at 4°C.

1M Na_2CO_3	10.6g/100ml
0.1% (w/v) SDS	Make up 1% (w/v) stock solution and dilute 10X
Chloroform	
(5) ONPG (ortho-Nitrophenyl-β-galactoside)	4mg/ml in Z-Buffer with 0.05M β-Mercaptoethanol, to be prepared on the day of experiment

2.12.2 Protocol of β-galactosidase assay:

Bacterial strains containing the reporter plasmids in which the promoter of interest was fused to *lacZ* were incubated in 10ml broth culture overnight with appropriate antibiotics at appropriate temperature (for promoters that were thought to be activated on swarming agar, samples were cultured on swarming agar overnight first and then scraped into 1ml sterile PBS until the OD_{600nm} was 0.3-0.7). Antibiotics with colour (e.g. Rifampicin) may not be added to avoid interference of absorbance reading.

A 50µl suspension of the bacterial pre-culture was added into 5ml of BHI broth containing the appropriate antibiotics. For each strain, three tubes were prepared (cultured triplicate) and incubated in the shaker (200rpm) at the appropriate temperature. The OD was checked after approximately 3 hours at 600nm, every 15 min, until they reached the OD_{600} 0.3-0.7 for each tube. If any of them reached the OD before others, they were stored on ice to stop bacterial growth.

In the meanwhile, ONPG solution containing the substrate of β-galactosidase was prepared. A volume of 54µl of mercaptoethanol was added to 20ml of Z-Buffer to make up a 0.05M solution, into 6ml of which, 24mg of ONPG was added to make a 4mg/ml ONPG solution.

Two 13ml glass tubes (β-galactosidase assay tubes) were labelled for each sample and two controls contained only sterile broth culture with antibiotics (PBS only for bacteria that were cultured on swarming agar). Thus for one bacterial strain, there were 6 tubes, duplicates of the strains that had been grown in triplicates.

A volume of 900µl of Z-Buffer containing Mercaptoethanol was added to each tube and then 30µl of chloroform was shot quickly into the buffer to make a dome at the bottom of the tube, to prevent quick evaporation of chloroform. Then 100µl of each bacterial culture that had reached the OD_{600} 0.3-0.7 was added into each tube. The OD_{600} of the bacterial culture should be read again just before adding into the tubes. Then 30µl of 0.1% SDS was added to each tube and vortexed for 10 seconds

to permeabilize the cells. They were then incubated in the 30°C water bath for 15 min and all the steps afterwards were carried out at 30°C.

To each tube, 200µl of ONPG (4mg/ml) was added and vortexed for 3 seconds and each tube was treated the same in 30 sec intervals including the controls. A Timer was used to record the time when the substrate was added.

When an appropriate yellow colour had developed, the reaction was stopped by adding 500µl of 1M Na_2CO_3 and mixed. The exact time of adding Na_2CO_3 was recorded as the stopping time. The stopping should be carried out in the same way as starting that all tubes were treated in 30 seconds intervals.

The optical density of the samples were read at two wavelengths, 420nm and 550nm.The two control tubes were used to set the reference for each of the two wavelengths. Absorbance at 420nm was the absorbance of the yellow O-nitrophenol. The absorbance at 550nm was the scatter of cell debris, when multiplied by 1.75; it approximated the scatter observed at 420nm. Thus OD_{550} was a light scattering factor for the contribution of the cells to the value at 420nm. The absorbance measured at 420nm should between 0.2-0.8 to be a significant value.

Miller Unit was a standard to measure the activity of β-galactosidase in β-galactosidase assay. Miller Unit was calculated as:

1 Miller Unit = 1000 x [OD420 - (1.75 x OD550)] / (Time x Volume x OD600)

in which, time was the reaction time = (stopping time – starting time) in min. Volume was the volume of cells added into the glass test tubes in milliliters (i.e. 0.1ml). OD600 reflected the cell density.

2.13 Oxidase test

A small amount (around 1mg) of N,N,N'N,N-tetramethyl-p-phenylene diamine dihydrochloride was added to 1ml dH_2O and pipetted onto a filter disc. Colonies were picked up by either a tip or a toothpick stick and then scratch on the filter disc. If the colony turned purple instantly (within 5 seconds), it indicated that this bacterial colony was oxidase positive (e.g. *Aeromonas spp.*). Otherwise it indicated that the

colony was oxidase negative (*e.g. E. coli*).

2.14 Protein Overexpression

The *E. coli* protein expression strain containing the plasmid of interest was incubated overnight in 10ml LB broth with appropriate antibiotics and 1% (w/v) filter sterilized glucose. On the next day, it was transferred into 1L of LB broth with appropriate antibiotics and 1% (w/v) filter-sterilized glucose. It was then incubated at 37°C for approximately 2 hours until the OD_{600} was around 0.2. At this point, 0.3mM-1mM IPTG was added to induce the protein expression. After 2-3 hours incubation, the cells were harvested by centrifugation at 8,000xg for 15 min at 4°C. The pellet was resuspended in Binding Buffer and frozen overnight at -20°C. It was then sonicated using Jencons Vibracell at 20 kHz (20% amplitude) for 8x 20sec with 1 min intervals on ice. The sonicated sample was then centrifuged at 30,000xg for 30 min at 4°C to separate soluble and insoluble proteins. After sonication, the supernatant that contained soluble proteins was transferred into a fresh universal tube while the pellet left in the centrifuge tube and both were stored at -20°C.

2.14.1 Buffer Preparation

Binding Buffer (1L)

10mM Imidazole	5ml of 2M imidazole stock solution
500mM NaCl	30g
5% (v/v) Glycerol	50ml
20mM Phosphate Buffer	200ml of 0.1M Phosphate Buffer

Phosphate Buffer stock (1L)

0.2M $NaH_2PO_4 \cdot H_2O$	27.6g in 1L dH_2O
0.2M of $Na2HPO_4 \cdot 7H_2O$	53.6g in 1L dH_2O

Mix 95ml of 0.2M $NaH_2PO_4 \cdot H_2O$ and 405ml of 0.2M of $Na2HPO_4 \cdot 7H_2O$, and 500ml dH_2O was added to make 1L 0.1M Phosphate Buffer

1M IPTG stock 2.38g in 10ml of dH_2O

2.15 Polyacrylamide Gel Electrophoresis (PAGE)

Table 2.6 SDS-PAGE Gel components.

Resolving Gel (Pour first)

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

Stacking Gel (Pour last)

Acrylamide (30% w/v)	Upper Tris 0.5M pH 6.8	H_2O	TEMED	AP (10% w/v)	SDS (10% w/v)
1.3ml	2.1ml	4.4ml	17 µl	100 µl	100µl

Table 2.6 SDS-PAGE Gel components. The percentage of resolving gel used was dependent upon the expected size of the protein sample. The smaller the size of the protein, the higher percentage of resolving gel was used.

The protein samples were prepared by boiling with 1X Laemmli Buffer at 100°C for 10 min, followed by chilling on ice for 2 min and centrifuged for 5 min. To each well of SDS-PAGE gel, 10-15µl of the sample was loaded. The gel was run at 160V for 100 min in 1X SDS Running Buffer. The gel was stained with Coomassie Blue Stain Solution overnight and then destained with Destain Solution until the bands

were visible.

2.15.1 Buffer Preparation

Upper Tris (0.5M pH 6.8)	19.7g in 250ml of dH_2O
Lower 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	
250mM Tris-Base	30.2g in 1L dH_2O
1.92M Glycine	144g in 1L dH_2O
1% (w/v) SDS	10g in 1L dH_2O

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

2.16 Histidine-tagged Protein Purification Protocol

2.16.1 Column Preparation

The 1ml HisTrap HP Column (GE Healthcare™) was washed first with 5ml dH_2O using the syringe with luer adaptor. Then the syringe was filled with 0.5ml of provided 0.1M nickel salt solution ($NiSO_4$) and loaded onto the column. Finally the column was washed with 5ml dH_2O again to remove any air trapped inside.

2.16.2 Purification protocol

Sonicated samples were taken out from the -20°C freezer, if the desired protein was insoluble, it was first dissolved in Binding Buffer supplied with 8M Urea. Then the sample was first prepared by filtration through a 0.45µm filter to remove any debris.

When the column was prepared according to 2.16.1 column preparation, it was equilibrated with 10ml Binding Buffer using the syringe. Then the protein sample was loaded onto the column and the flow-through (FT) was collected. Any Histidine-tagged proteins should now be bound to the nickel ions in the column. The column was then washed with 10x column volumes of Binding Buffer and the wash fraction was collected as well.

The His-tagged protein was then eluted with 5ml Binding Buffer with increasing imidazole concentration (20mM to 500mM) and the elutant was collected in 1ml fractions. All fractions were checked by SDS-PAGE, including the flow-through and the wash fractions.

After the protein elution, the column was regenerated by washing with 10ml Binding Buffer and could be used to purify the same protein to avoid cross-contamination.

2.16.3 Buffer Preparation

8M Urea Buffer	48g in 100ml of Binding Buffer

2M imidazole stock	6.8g in 50ml dH_2O and Buffered to pH7.8

2.17 Protein Purification for MBP-tagged proteins

The over-expression and purification of MBP-tagged proteins was performed according to the pMAL™ Protein Fusion and Purification System manual (New England BioLabs™). The IPTG induced cell culture was harvested follow 2.14 protein expression protocol but resuspended in 25ml of Column Buffer instead.

The 1ml MBPTrap HP columns supplied from GE Healthcare™ was first prepared by washing with 10 column volumes (10ml) of Column Buffer. When the MBP-tagged protein sample was sonicated and centrifuged, the supernatant which contained the fusion protein was loaded on the column and the flow-through (FT) was collected. The column was then washed with 15ml Column Buffer and the wash fraction was collected as well. Then the fusion protein was eluted with Column Buffer + 10mM maltose and 10 fractions of 1ml aliquots were collected. All fractions including the flow through and the wash were checked by SDS-PAGE. Finally the column was regenerated by washing with 10ml Column Buffer.

2.17.1 Buffer Preparation

Column Buffer (1L)	
20mM Tris-HCl (pH7.4)	20ml of 1M Tri-HCl pH7.4
200mM NaCl	11.7g
1mM EDTA	2ml of 0.5M EDTA
1mM sodium azide	1ml of 1M sodium azide
10mM β-mercaptoethanol	0.7ml

1M Tris-HCl pH7.4	31.52g in 200ml of dH_2O and buffered to pH7.4
0.5M EDTA	3.72g in 20ml of dH_2O and buffered to pH8.0
1M sodium azide	0.65g in 10ml of dH_2O

10mM Maltose	0.36g in 100ml of Column Buffer

2.18 Western Blot

After SDS-PAGE, one of the two duplicate gel was stained with Coomassie as usual while the other one was soaked in 1X Transfer Buffer for 15 min in a square 15cm petri dish with enough buffer to cover the gel, to remove any SDS left on the gel. Then the blotting cassette (Bio-Rad™) together with 4 filter paper and 1 nitrocellulose membrane (Geneflow™ 0.45μm pore size) of similar size with the gel were soaked in 1X Transfer Buffer for 15 min as well. Then the blotting cassette was set up and any air bubble was removed. It was then put into a tank filled up with 1X Transfer Buffer and run at 10V overnight (or 60V for 1hour) with stirring. The cassette should be put in the direction that the electrons passed from the gel towards the membrane to allow blotting.

On the next day, the membrane was taken out and the pre-stained ladder should have transferred onto the membrane. The membrane was then soaked in 20ml of PBS + 5% (w/v) dry skimmed milk powder (Marvel™) for 1hour (or overnight) to block any non-specific binding sites. After blocking, the membrane was washed 10 min in 20ml PBS Buffer with 0.1% v/v Tween20 (PBST). Then 10μl (1:2000) of primary antibody was added to 20ml of PBST + 5% milk, in which the membrane was soaked for 1 hour. After primary antibody binding, the membrane was washed 3 times 10 min in 20ml PBST Buffer. Then the membrane was probed with 20ml PBST + 5% milk with 5μl secondary antibody (1:4000) for 1 hour. After secondary antibody binding, the membrane was washed 3 times 10 min in 20ml PBST Buffer again and was ready for developing.

Pierce™ ECL Western Blotting Substrate (Thermo Scientific™) was used to develop the membrane. 1ml of Solution I was mixed with 1ml of Solution II and poured evenly on the membrane. Then the western blot photo was taken using

Chemidoc™ XRS+ System (Bio-Rad™).

2.18.1 Buffer Preparation

10X Transfer Buffer (1L)	
Glycine	144g
Tris-Base	30.2g
dH_2O added up to 1L	

PBST Buffer	
10mM PBS Buffer	1L
Tween 20	0.1% (v/v)

2.19 Far-Western Blot

Far-Western blotting was based on Western Blot and used to investigate protein-protein interactions. After blocking, the membrane was probed with a non-antibody protein with a different fusion tag as the loaded proteins. The membrane was probed for 1 hour followed by 3 times 10 min wash with PBST Buffer. Then the membrane was then incubated with the primary antibody against the probing protein for 1 hour followed by 3 times 10 min wash with PBST Buffer. Then the secondary antibody binding and developing were carried out the same way as the standard Western Blot in 2.18.

2.20 Bacterial Adenylate Cyclase Two-Hybrid (BACTH) Assay

The BACTH Assay was carried out using the Euromedex™ BACTH System Kit. The target genes were first cloned into the BACTH plasmids in frame with either T25 or T18 fragments of the *cyaA* gene at the 5' or 3' end of the gene of interest (See Table 2.2 plasmids used in this study), to allow co-expression of fusion proteins. Then one of the T25 derived plasmid constructs was co-transformed with a T18

derived plasmid construct into *E. coli* BTH101 reporter competent cells and incubated on MacConkey/maltose agar for 2 nights at 30°C.

When the two proteins of interest interacted with each other, heterodimerization of the fusion proteins allowed the complementation of the T25 and T18 fragments to form a catalytic domain of adenylate cyclase (CyaA), thus cAMP was synthesized. Then the *mal* operon was activated by cAMP/CAP complex. Therefore the maltose metabolism pathway was switched on in the *E. coli* BTH101 reporter strain, which allowed the fermentation of maltose and the production of acid that turned the pH indicator in MacConkey agar pink. Therefore positive interaction colonies were pink/red in colour whereas negative colonies were white.

2.20.1 Media Preparation

MacConkey agar

MacConkey agar base (Difco™)	40g in 1L dH_2O

Dissolved and autoclave sterilized. Components below were added when it was cooled down to approximately 56°C.

1% (w/v) maltose	50ml of 20% (w/v) maltose
0.5mM IPTG	0.5ml of 1M IPTG
Ampicillin (100μg/ml)	1ml of ampicillin (100mg/ml)
Kanamycin (50μg/ml)	1ml of kanamycin (50mg/ml)

2.21 Isothermal Assembly (ISA)

The ISA was used to assemble multiple DNA fragments into one plasmid vector in a single reaction. Each fragment assembled in this reaction should have an approximately 30bp adapter sequence overlapping each other. Then the fragments, which were equal-molar when added, were mixed together with ISA Buffer and three enzymes: T5 exonuclease, Phusion polymerase and Taq ligase. Then it was incubated at 50°C overnight and transformed into *E. coli* DH5α to select desired resistance.

2.21.1 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	0.25gµl
100mM NAD	50µl
dH_2O	335µl

2.21.2 ISA reaction (5 tubes)

5X ISA Buffer	20µl
Diluted (8X, 1µl to 7µl of 1X ISA Buffer) T5 exonuclease	0.5µl
Phusion Polymerase	1.25µl
Taq Ligase	10µl
dH_2O	43.3µl
Then aliquot into 5x 15µl in each reaction	

2.22 Statistics

The statistical analysis carried out in this study was using Microsoft™ Excel 2010 and GraphPad™ Prism 5, in which Student's t-test and One-way ANOVA were used to determine the significance of the data.

2.23 Software and website

Apart from the statistical software, other software and websites were used in this study. For example, SnapGene® was used to illustrate DNA maps and design primers; FinchTV® was used to display DNA sequencing traces; BLAST® was used in sequencing and gene mapping; NEBcutter (V2.0) was used to determine restriction sites.

Chapter 3
Regulation of Type III Secretion System

3.1 Introduction

The Type III secretion system has been reported in many Gram-negative bacteria such as *Yersinia spp*., *Pseudomonas spp*., *Vibrio spp.*, *Salmonella spp*. and *Shigella spp*. (Cornelis et al 1998, Cornelis & Van Gijsegem 2000, Galan & Collmer 1999, Park et al 2004, Yahr et al 1997). Unlike Type I, Type II or Type V secretion systems, T3SS is known to inject protein effectors across the plasma membrane directly into host cell cytosol or to secrete translocators that help effector proteins to get through (Cornelis & Van Gijsegem 2000). The T3SS was not reported in *Aeromonas spp.* until 2002, when Burr and colleagues first identified seven open reading frames that encoded homologues of *Yersinia* T3SS in *Aeromonas salmonicida* (Burr et al 2002). Later on, the T3SS was found to be present in more *Aermonas* species, in most of which the gene cluster that encoded T3SS was located on a single chromosomal region like in *Pseudomonas spp.*, except for *A. veronii* which had two distant T3SS gene clusters on its single chromosome (Chacon et al 2004, Makino et al 2003, Yu et al 2004).

Similar to *P. aeruginosa*, previous evidence has shown that the T3SS in *A. hydrophila* was also under control of the master regulator ExsA (Vilches et al 2009, Yahr & Wolfgang 2006). Moreover, ExsA in *A. hydrophila* shares 76% amino acid homology with ExsA in *P. aeruginosa*, both of which belong to AraC family of transcriptional activators (Frank & Iglewski 1991, Yahr & Frank 1994). Like ExsA in *P. aeruginosa*, ExsA in *A. hydrophila* is also thought to recognize a specific DNA sequence (TNAAAANA), which is shared in all promoter regions of the T3SS gene cluster (Vilches et al 2004). This consensus sequence that recruits ExsA is normally found 15 bp upstream of the -35 RNA polymerase binding site in *P. aeruginosa* (Hovey & Frank 1995). Similar strategies were utilized in this project to identify promoters of the T3SS in *A. hydrophila*.

Further evidence in *P. aeruginosa* has shown that the T3SS master regulator ExsA is controlled by a regulatory cascade of proteins ExsD, ExsC and ExsE (Dasgupta et al 2004, McCaw et al 2002, Rietsch et al 2005, Urbanowski et al 2005, Zheng et al 2007). ExsD is reported to be an anti-activator protein that negatively regulates the T3SS master regulator ExsA through direct protein-protein interaction (McCaw et al 2002). ExsC is an anti-anti-activator protein that binds to ExsD and stop it from inhibiting ExsA (Dasgupta et al 2004). Also, ExsC is reported to be the chaperone protein for the small secreted protein ExsE, which is found to be a negative regulator of the T3SS (Rietsch et al 2005). ExsE negatively regulates the T3SS via its physical interactions with ExsC, preventing ExsC from binding to ExsD, thus allowing ExsD to be free to bind and inhibit the transcriptional activation from ExsA (Urbanowski et al 2005).

A previous study in our laboratory investigating the lateral flagella system of *A. hydrophila* discovered several mutants using a transposon library screen that were inhibited for swarming motility. Two of the non-swarming transposon mutants were discovered to be mutated in the *exsD* gene that encodes one of the regulators of the *A. hydrophila* T3SS. This finding correlated with the work of the Leung laboratory in Singapore who had suggested regulatory cross-talk between the aeromonad T3SS and the lateral flagella system (Yu et al 2007). As very little is known about the regulatory cascade of the aeromonad T3SS or indeed its potential role in regulating the lateral flagella system, this project was undertaken.

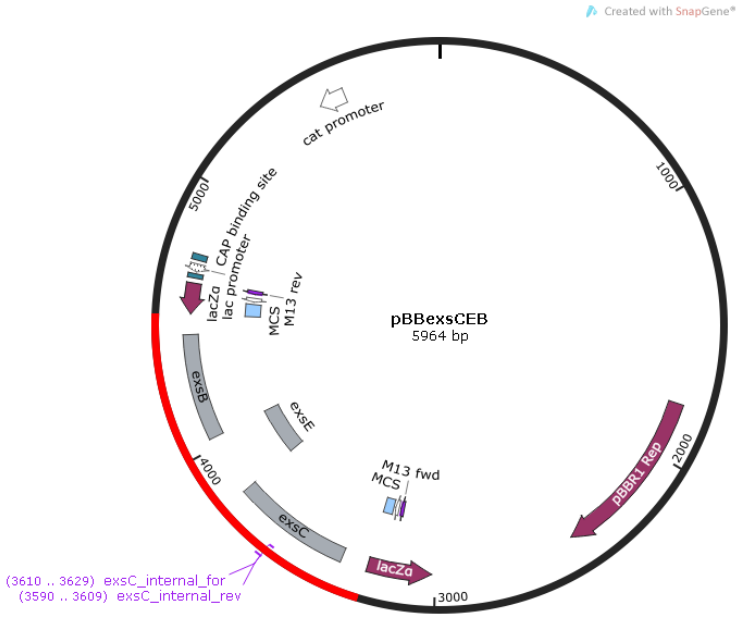
3.2 Construction of an *exsC* mutant

The aim of these experiments was to produce insertional knockouts of the potential regulatory components of the *A. hydrophila* T3SS. Since the *exsA* mutant had already been created in the lab previously (Shaw, unpublished), an *exsC* mutant was the first to be constructed.

In order to facilitate a knockout in the *exsC* gene, a selectable marker was inserted in the middle of the *exsC* gene. This is achieved by PCR amplification using Pfx DNA polymerase with *exsC* forward and reverse internal primers (Appendix 1) on the template pBB*exsCEB* plasmid, obtained from laboratory collection (Shaw, unpublished) (Figure 3.1 A). The size of *exsCEB* region was 1080bp and the two inverse primers were located adjacent to each other near the middle of the *exsC* gene, but pointing different orientations, to allow PCR amplification of the full plasmid (5964bp). After PCR amplification, the *exsCEB* region was divided into two parts centred within the *exsC* gene, one was 249bp while the other one was 831bp. Thus the plasmid was opened up to allow a blunt-end insertion (Figure 3.1 B). The PCR product was checked by agarose gel electrophoresis (Figure 3.2).

In order to provide the kanamycin resistance cassette for the insertional mutagenesis, the plasmid pUC4KIXX was digested with the restriction enzyme *Sma*I. This resulted in a blunt end DNA fragment of 1.4kb that contained the kanamycin resistant cassette. This was separated on a 1% agarose gel from the plasmid backbone and purified by gel extraction (Figure 3.3).

A.



B.

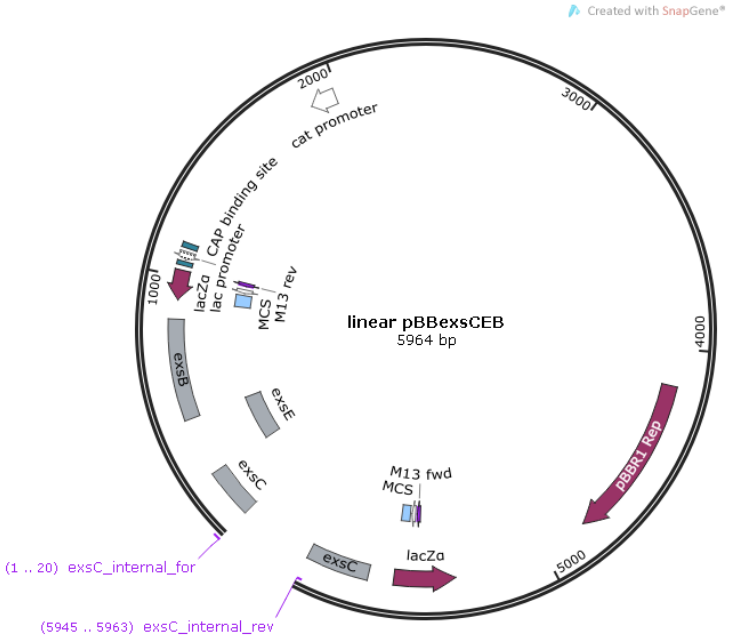


Figure 3.1 Depiction of the inverse PCR of pBB*exsCEB* using primers *exsC* internal forward and *exsC* internal reverse. **A,** plasmid map of pBB*exsCEB* and the *exsC* internal primers (shown in purple) located near the middle of *exsC* gene. **B,** linearized pBB*exsCE*B with a blunt-end insertional site sitting internal of *exsC* to allow insertion of an antibiotic resistance selectable marker. Key features of the plasmid construct were shown in arrows. The *exsC*, *exsE* and *exsB* genes were shown in grey boxes. Created using SnapGene® software.

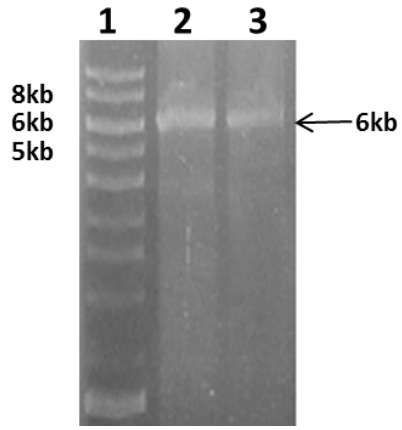


Figure 3.2 A 1% agarose gel showing the PCR product of pBB*exsCEB* with primers *exsC* internal forward and reverse, using Pfx DNA polymerase. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience). Lane 2 and 3, two independent PCR products of linearized pBB*exsC*EB (~6kb), amplified with *exsC* internal primers.

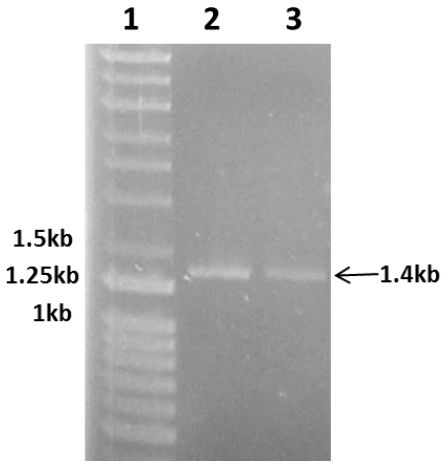


Figure 3.3 A 1% agarose gel showing the 1.4kb gel extracted kanamycin resistance cassette from pUC4KIXX. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience). Lane 2 and 3, two independent Km^R cassettes (~1.4kb) obtained by digesting pUC4KIXX plasmid with *Sma*I restriction enzyme, followed by gel extraction.

As the pBB*exsCEB* PCR product had been amplified using Pfx DNA polymerase, that had resulted in the production of blunt ends, this allowed its ligation to the *Sma*I generated 1.4kb Km^R resistance cassette. The ligation was transformed into *E. coli* DH5α and a number of colonies were isolated that were kanamycin resistant from the Km^R cassette and Chloramphenicol resistant from the plasmid backbone.

However it was essential to check the orientation of Km^R insertion in these potential clones as it should be the same orientation as the *exsCEB* operon to avoid polar effects. The constructed plasmid pBB*exsCKm*^{R} was sequenced and checked on agarose gel, which shows an expected band of 7.3kb (Figure 3.4).

Then the *exsCEB*::Km^R fragment was excised from pBB*exsC*Km^R by digestion with the *Bam*HI restriction enzyme, as *Bam*HI sites were located upstream and downstream of the *exsCEB* fragment. This was subsequently ligated into *Bam*HI-digested suicide plasmid pKNG101 to construct pKNG*exsC*Km^R. The *E. coli* competent cell CC118-λ*pir* was used to select the constructed suicide plasmid pKNG*exsC*Km^R (Figure 3.5). Then the constructed suicide plasmid was transformed into *E. coli* S17-1-λ*pir* donor cells in order for conjugation with *A. hydrophila* AH3R strains to allow allele exchange. After conjugation, a double crossover event was selected by picking up colonies that were streptomycin sensitive and kanamycin resistant, since the streptomycin resistance gene was carried by the suicide plasmid backbone. The putative mutants were checked by PCR screening using *exsC* amplification primers (Appendix 1) and Taq DNA polymerase (Figure 3.6). The expected size of the PCR screening product should be around 2kb, which was comprised of 1.4kb kanamycin resistance cassette and 600bp of *exsC* amplification product. The PCR screening products were sent for sequencing provided by Core Genomic Facility, University of Sheffield. The sequencing results were viewed using FinchTV and analysed using BLAST search to ensure correct orientation of Km^R insertion and double crossover of the *exsC* knockout.

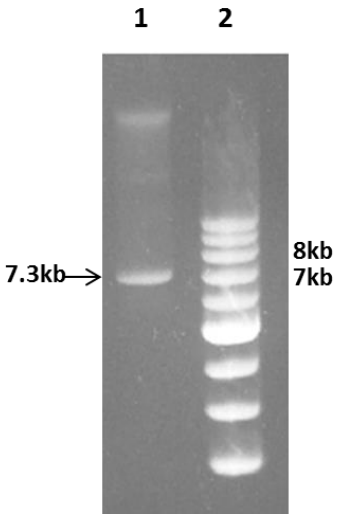


Figure 3.4 A 1% agarose gel showing plasmid construct of pBB*exsCKm*^{R}. Lane 1, the plasmid construct of pBB*exsC*Km^{R} (~7.3kb). Lane 2, Promega™ Supercoiled DNA Ladder (2-10kb).

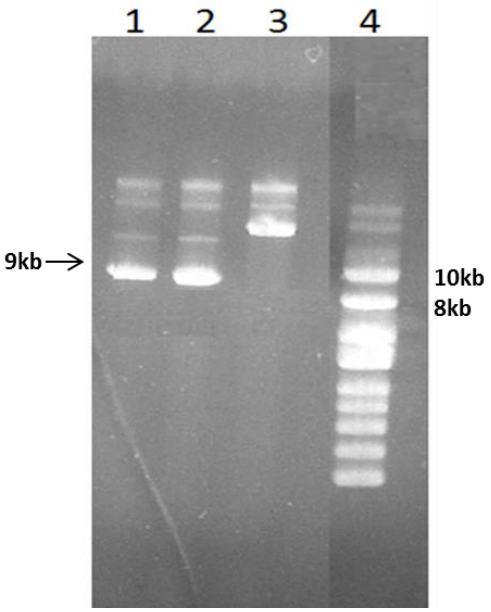


Figure 3.5 A 1% agarose gel showing plasmid construct of pKNG*exsC*Km^{R}. Lane 1-3, independent isolations of plasmid pKNG*exsC*KmR (~9kb), while lane 3 was a possible dominant dimer of the plasmid; Lane 4, Promega™ Supercoiled DNA Ladder (2-10kb).

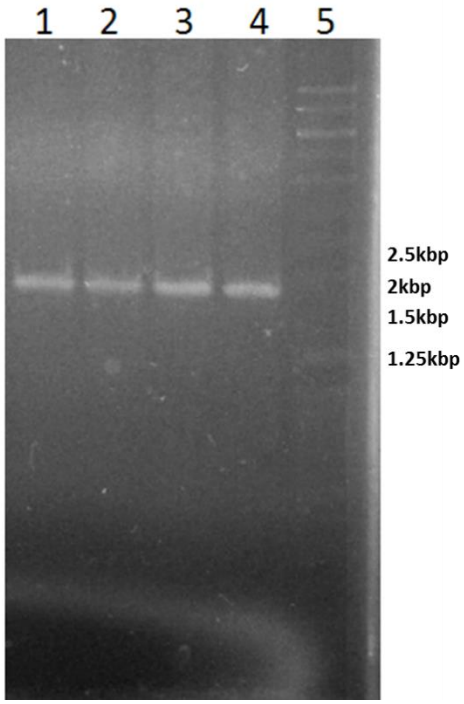


Figure 3.6 A 1% agarose gel showing PCR screening of potential *exsC* mutants. Lane 1-4, PCR screening products *exsC*:KmR (~2kb), amplified from both pKNG*exsC*Km^{R} positive control (lane1) and from potential *exsC* mutant chromosomal DNA (lane 2-4); Lane 5, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience).

3.3 Construction of the *exsD* and *exsE*⁻ mutants

After the mutagenesis of *exsC*, the construction of *exsD* and *exsE* mutants were carried out following the same protocol but after several attempts no successful suicide vector constructs were obtained. Thereby another mutagenesis method, Isothermal Assembly (ISA), also known as Gibson assembly, was utilized to construct the *exsD* and *exsE* mutants.

ISA required three DNA fragments and one digested plasmid for the assembly, in which *exsD* was amplified by PCR into two fragments using primers *exsD* F1 forward/reverse and *exsD* F2 forward/reverse (Appendix 1), each containing adapter sequence overlapping with the plasmid or the kanamycin cassette (Figure 3.7 and Figure 3.8). The kanamycin cassette was amplified by PCR from plasmid pJMK30 using Pfx DNA polymerase and primers Kan forward/reverse (Appendix 1), while the plasmid pGEM-3Zf(+) was digested with the restriction enzyme *Hinc*II (Figure 3.9). The fragments were then assembled together following the ISA protocol described in section 2.21. After transformation, ampicillin and kanamycin resistant *E.coli* DH5α colonies were selected to harvest the plasmid construct pGEM*exsD*Km^R, while pGEM*exsE*Km^R was assembled in the meantime using the same method (Figure 3.10). The expected size of the *exsD* ISA products cloned in the plasmid was 5.4kb, which was comprised of 3.2kb pGEM-3Zf(+) plasmid vector, 1.4kb of kanamycin resistance cassette and 800bp of both *exsD* fragments. Then the *exsD*::Km^R fragment was extracted from the pGEM*exsD*Km^R constructs by PCR using Pfx DNA polyermase with *exsD*_pGEM forward and *exsD*_pGEM reverse primers (Appendix 1) (Figure 3.11). A similar PCR amplification was carried out to obtain the *exsE::*Km^R fragment. Pfx DNA polymerase was used with primers *exsE*_pGEM forward and *exsE*_pGEM reverse primers on the plasmid construct pGEM*exsE*Km^R (Figure 3.11). Both PCR fragments of *exsD*::Km^R and *exsE*::Km^R were blunt-end and were ready to ligate into *Sma*I-digested suicide plasmid pKNG101 (Figure 3.11).

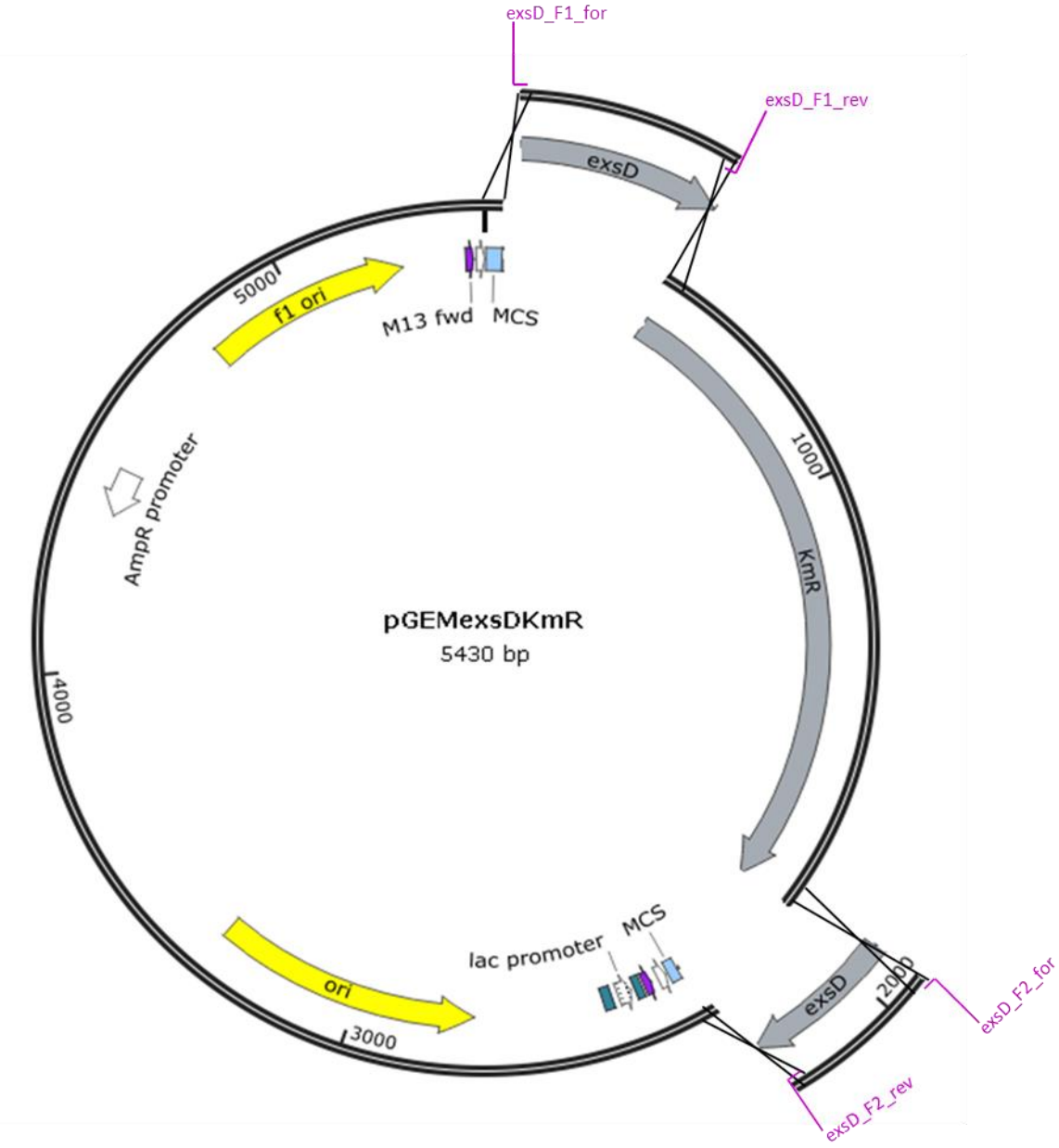


Figure 3.7 Illustration of Isothermal Assembly to construct pGEM*exsD*Km^R plasmid. PCR fragments of 460bp for 5' *exsD* (fragment 1, amplified by *exsD* F1 forward/reverse primers), 350bp for 3' *exsD* (fragment 2, amplified by *exsD* F2 forward/reverse primers) and 1.4kb of the kanamycin resistance cassette from pJMK30 were mixed together with the *Hinc*II-digested pGEM-3Zf(+) plasmid using Isothermal Assembly. Each fragment was designed to contain a 30bp adapter sequence overlapping each other. Key features of the plasmid were shown in arrows while primers were shown in purple. Created using SnapGene software.

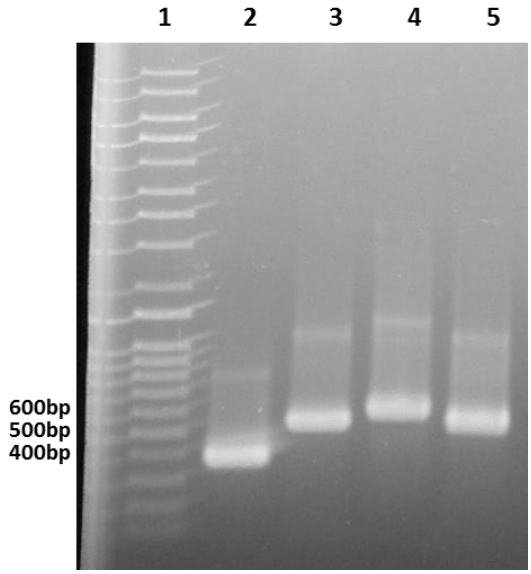


Figure 3.8 A 1% agarose gel showing PCR amplification of *exsD* fragments and *exsE* fragments for ISA. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 2, 3' fragment of *exsD* amplified by *exsD* F2 forward/reverse primers (360bp); Lane 3, 5' fragment of *exsD* amplified by *exsD* F1 forward/reverse primers (460bp); Lane 4, 3' fragments of *exsE* amplified by *exsE* F2 forward/reverse primers (600bp); Lane 5, 5' fragment of *exsE* amplified by *exsE* F1 forward/reverse primers (500bp).

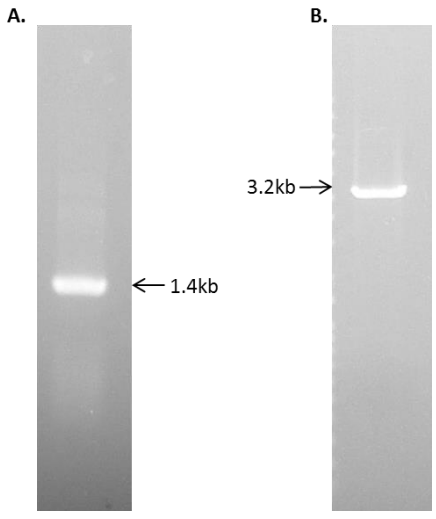


Figure 3.9 A 1% agarose gel of kanamycin cassette and linearized pGEM-3Zf(+). A, kanamycin resistance cassette amplified from pJMK30 using Kan1 forward/reverse primers (1.4kb); B, linearized pGEM-3Zf(+) resulted from digestion with *Hinc*II restriction enzyme.

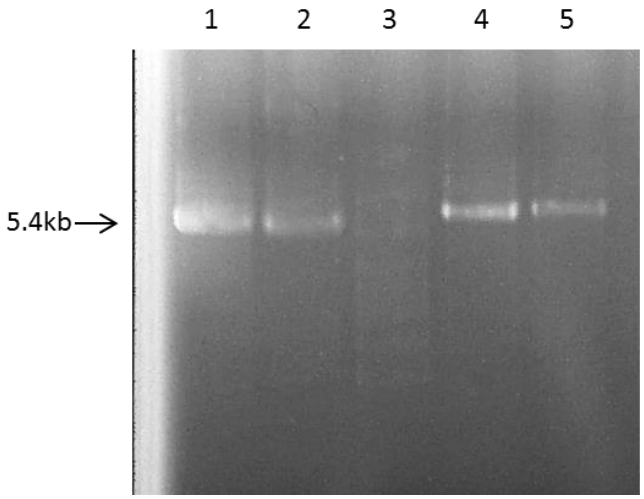


Figure 3.10 A 1% agarose gel of ISA constructs isolated after transformation and propagated in *E. coli* DH5α. Lane 1 and 2, two independent isolates of pGEM*exsD*Km^R; Lane 3, Promega™ Supercoiled DNA Ladder (2-10kb); Lane 4 and 5, two independent isolates of pGEM*exsE*Km^R.

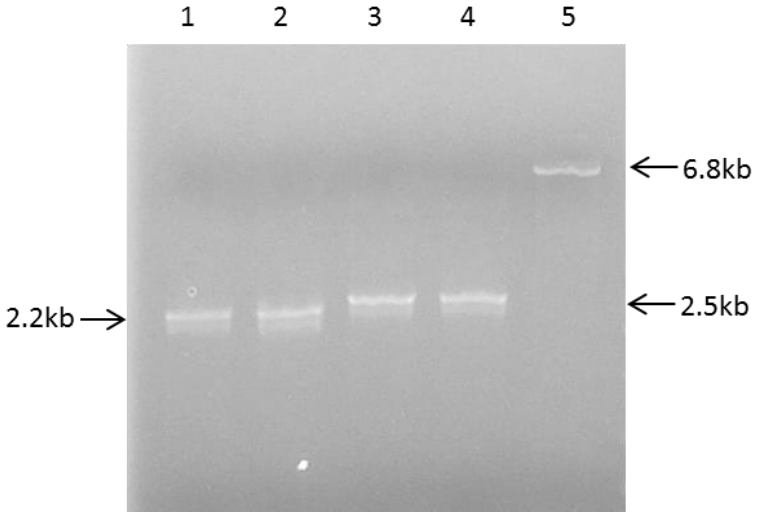


Figure 3.11 A 1% agarose gel of PCR fragments isolated after amplification from pGEM*exsD*Km^R and from pGEM*exsE*Km^R together with *Sma*I-digested pKNG101. Lane 1 and 2, two independent PCR products of *exsD*:Km^R from pGEM*exsD*KmR using primers *exsD*_pGEM forward and *exsD*_pGEM reverse (2.2kb); Lane 3 and 4, two independent PCR products of *exsE*:Km^R from pGEM*exsE*KmR using primers *exsE*_pGEM forward and *exsE*_pGEM reverse (2.5kb); Lane 5, *Sma*I-digested pKNG101 (6.8kb).

After ligation, the samples were transformed into competent cells of *E. coli* CC118-λ*pir* to select both kanamycin resistant and streptomycin resistant colonies, which was carried by the plasmid backbone. The plasmid constructs pKNG*exsD*Km^R and pKNG*exsE*Km^R were then harvested from those isolates by mini-prep (Figure 3.12). Once the suicide plasmids pKNG*exsD*Km^R and pKNG*exsE*Km^R were constructed, they were transformed into *E. coli* donor cell S17-1-λ*pir* and then conjugated into *A. hydrophila* AH3R to allow allele exchange (Figure 3.13). After conjugation, the transconjugants were incubated on LB agar with rifampicin and kanamycin. Each colony was then patched onto replica plates, one with rifampicin and kanamycin, while the other one with kanamycin and streptomycin. The ones that were rifampicin and kanamycin resistant but streptomycin sensitive were potential *exsD* mutant or *exsE* mutant.

The chromosomal DNA of these potential *exsD* mutants were then extracted and used as templates for PCR screening using Taq DNA polymerase with *exsD* F1 forward and *exsD* F2 reverse primers. The expected size of the wild type was approximately 800bp, which was solely the size of the *exsD* fragment, while the expected size of a double crossover or allelic exchange mutant should be 2.2kb, with an extra 1.4kb of the kanamycin resistance cassette (Figure 3.14). The same PCR screening was carried out on potential *exsE* mutant chromosomal DNA using Taq DNA polymerase with *exsE* F1 forward and *exsE* F2 reverse primers. The expected size of the double crossover should be 2.5kb with additional kanamycin resistance cassette (Figure 3.15). The PCR screening of a single crossover might result in multiple bands due to the integration of the whole suicide plasmid construct into the *A. hydrophila* AH3R chromosome, however only the size of the *exsD* or *exsE* fragment by itself was normally shown due to the limit of elongation time during PCR screening. The PCR screening products of double crossovers were sent for sequencing provided by Core Genomic Facility, University of Sheffield. The sequencing results were analyzed by FinchTV and BLAST search to double check the insertional knockout of the *exsD* and *exsE* genes.

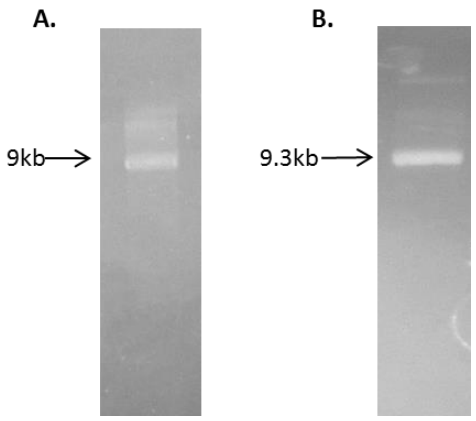


Figure 3.12 A 1% agarose gel showing suicide plasmid constructs pKNG*exsD*Km^R and pKNG*exsE*Km^R harvested from kanamycin and streptomycin resistant *E. coli* CC118-λ*pir* cells. A. suicide plasmid construct pKNG*exsD*Km^R (9kb), comprised of *exsD*:Km^R (2.2kb) and *Sma*I-digested pKNG101 (6.8kb); B, suicide plasmid construct pKNG*exsE*Km^R (9.3kb), comprised of *exsE*:Km^R (2.5kb) and *Sma*I-digested pKNG101 (6.8kb).

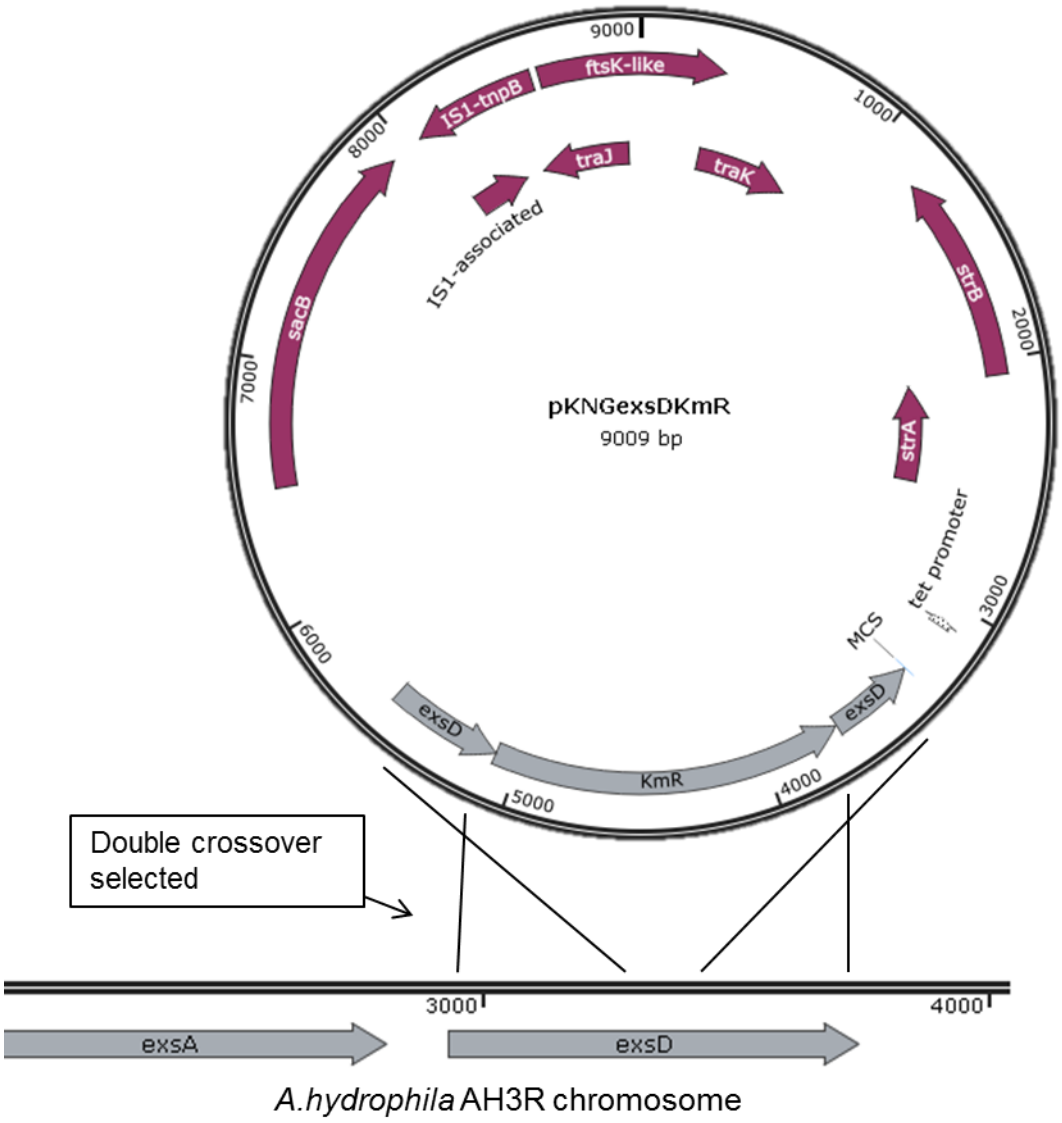


Figure 3.13 A diagrammatic representation of allelic exchange between the suicide vector construct pKNG*exsD*Km^R and the *exsD* gene on the chromosome of *A. hydrophila* AH3R following conjugation of suicide plasmid from *E. coli* S17-1-λ*pir* into *the Aeromonas* cells*.* Potential double crossover mutants were selected by replica plating. Each colony was patched on two LB agar plates, one with kanamycin and rifampicin and the other one with kanamycin and streptomycin. Those that were streptomycin sensitive were taken for further investigation. Key features of the plasmid vector were shown in red arrows, while the *exsA* gene, *exsD* gene and the Km^R cassette were shown in grey arrows. The figure was created using SnapGene® software.

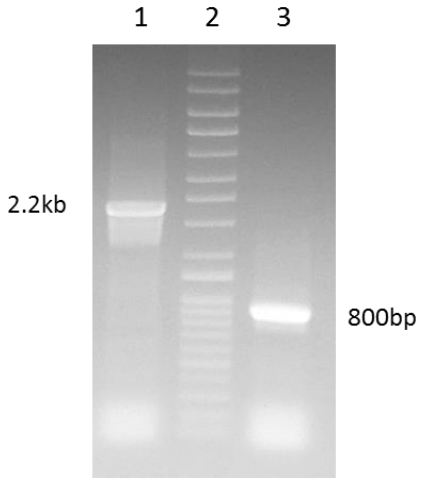


Figure 3.14 A 1% agarose gel showing PCR screening of potential *exsD* mutants. Lane 1, the PCR screening product of an aeromonad trans-conjugant that has a potential double crossover. The size of the band consisted of the size of *exsD* fragment (800bp) and the kanamycin cassette (1.4kb); Lane 2, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 3, the PCR screening product of a potential single crossover, only the size of the *exsD* fragment was shown (800bp) due to the limit of elongation time during PCR screening.

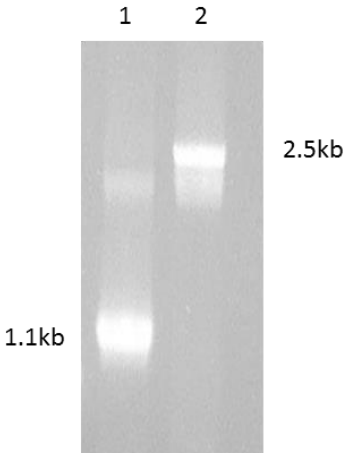


Figure 3.15 A 1% agarose gel showing PCR screening of potential *exsE* mutants. Lane 1, the PCR screening product of an aeromonad trans-conjugant that has a potential single crossover, only the size of *exsE* fragment on its own was shown (1.1kb); Lane 2, the PCR screening product of an aeromonad trans-conjugant that has a potential double crossover with an additional 1.4kb of the kanamycin resistance cassette.

3.4 β-Galactosidase assays for the T3SS promoter activity

The putative promoter regions of the T3SS from *A. hydrophila* AH3R were previously cloned into broad host range promoter probing plasmid pKAGb-2(-) to fuse with the promoter-less *lacZ* gene in order to measure the promoter activity by β-galactosidase assays (Shaw, unpublished). Some of the putative promoter regions were predicted by searching for the specific ExsA binding site TNAAAANA among the T3SS regulon (Hovey & Frank 1995, Vilches et al 2004). As a result, a total of 5 putative promoter regions of the *A. hydrophila* AH3R T3SS were cloned into the pKAGb2(-) and were conjugated independently into the constructed *A. hydrophila exsA, exsC*, *exsD, exsE* mutants and the AH3R wild type strain. Each promoter region cloned was 400-800bp in length and was named as *PascN*, *PaopN*, *PexsC*, *PexsA* and *PexsD*, after the first gene immediately downstream of the putative promoter (Figure 3.16).

Once the individual reporter plasmids were conjugated into *A. hydrophila* AH3R cells, the promoter activities were measured through the β-galactosidase assay for each strain. Before measuring the β-galactosidase activities of the *A. hydrophila* T3SS promoters in different mutant backgrounds, the conditions in which the bacteria were cultured were optimized in the *A. hydrophila* AH3R wild type strain. It was suggested by Vilches and colleagues that the addition of 20mM $MgCl_2$ and 10mM EGTA to the media was the optimal inducing condition for the expression of *A. hydrophila* T3SS (Vilches et al 2009). However, in order to determine the effect of $MgCl_2$ and EGTA on the *A. hydrophila* T3SS promoter activities, the promoter activities were still measured in both inducing and non-inducing conditions, to which no $MgCl_2$ or EGTA was added (Figure 3.17).

The β-galactosidase activity of *PascN* in *A. hydrophila* AH3R wild type strain grown in non-inducing conditions was 808 MU while the activity was 2098 MU when grown with additional $MgCl_2$ and EGTA. The promoter activity of *PascN* in inducing

condition was a significant increase compared to the activity in non-inducing condition ($p<0.001$) (Figure 3.17). However, when promoter *PaopN* was assayed, the β-galactosidase activities for non-inducing condition and inducing condition were 2357 MU and 1470 MU respectively. The promoter activity of *PaopN* assayed in inducing condition was significantly lower than in non-inducing condition ($p<0.01$). A similar discovery was found for promoter *PexsA*, of which the promoter activity assayed in inducing condition was significantly lower than in non-inducing condition ($p<0.001$). The promoter activity of *PexsA* in non-inducing condition was 10066 MU while the activity was 7377 MU in inducing condition (Figure 3.17). However, the promoters *PexsC* and *PexsD* showed no significant difference when assayed in either inducing condition or non-inducing condition (Figure 3.17). The promoter activity of *PexsC* was 352 MU in non-inducing condition while it was 418 MU in inducing condition. The activity of *PexsD* in non-inducing condition was 494 MU while in inducing condition the promoter was 649 MU (Figure 3.17).

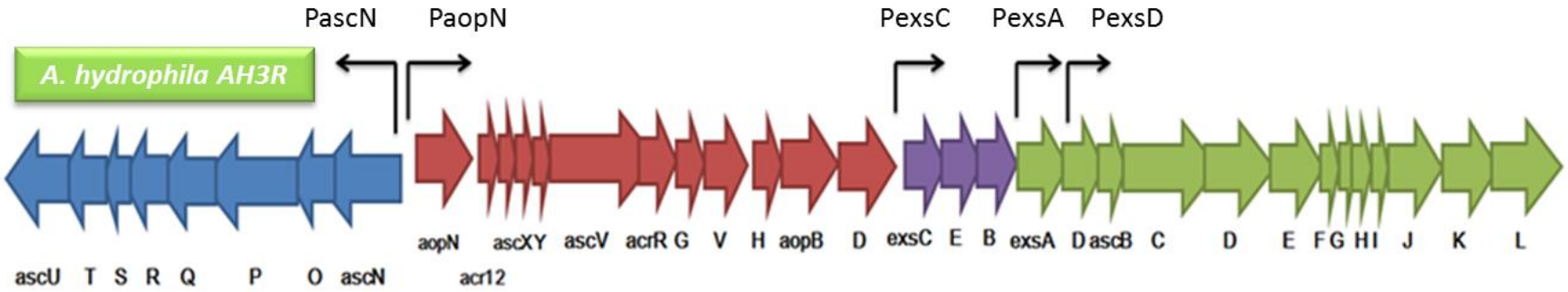


Figure 3.16 Illustration of the T3SS regulon in *A. hydrophila* AH3R. The putative promoter regions were shown in bent arrows and named as *PascN, PaopN, PexsC, PexsA* and *PexsD* after the name of first gene downstream. Each promoter region was amplified by PCR and cloned into *lacZ*-fusion plasmid pKAGb-2(-) to measure the activity in different mutant backgrounds. This figure is adapted from Vilches, et al. (2004).

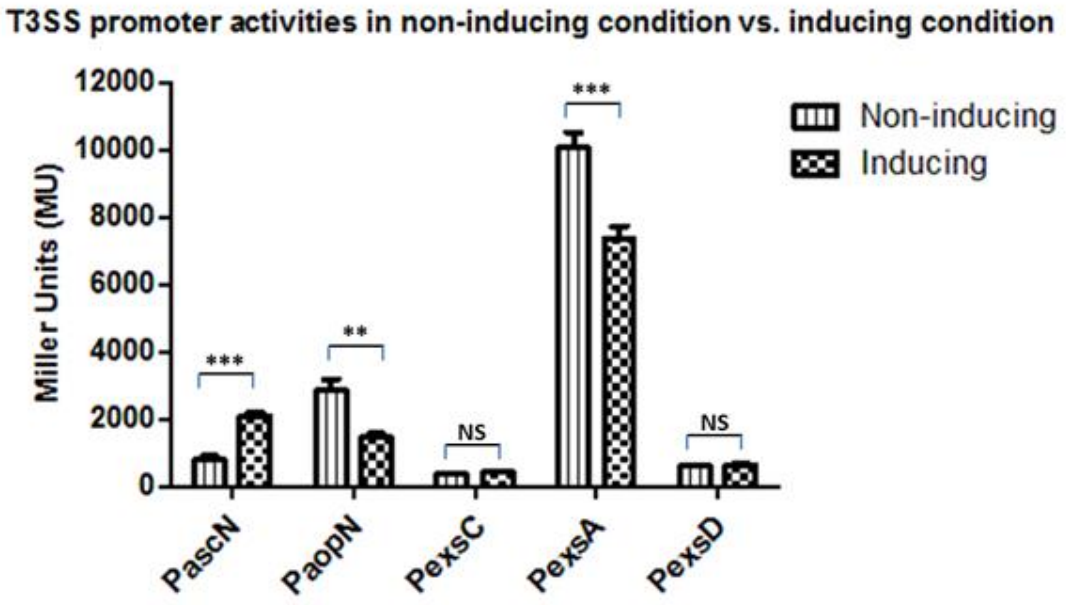


Figure 3.17 β-galactosidase activities of *A. hydrophila* AH3R T3SS promoters assayed in non-inducing and inducing conditions. All of the promoter activities were measured in *A. hydrophila* AH3R wild type strains, which were grown in LB broth to log phase at 30°C with shaking at 200rpm. Additional 20mM $MgCl_2$ and 10mM EGTA were added in inducing condition. Only PascN showed increased promoter activity in inducing condition, while promoter activities of PaopN and P*exsA* were significantly decreased with addition $MgCl_2$ and EGTA. The promoter activity of *PexsC* and *PexsD* showed no significant difference in non-inducing condition or inducing condition. Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test ($p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$).

The activities of *A. hydrophila* T3SS promoters were then measured in different mutant backgrounds using non-inducing conditions to avoid more variations. The promoter activity of *PascN* in the *A. hydrophila* AH3R wild type was 808 MU, but the promoter activity was almost completely suppressed in the *A. hydrophila exsA* mutant, in which the promoter activity was down to approximately 15 MU (Figure 3.18). In the *exsD* mutant, the PascN promoter activity increased to 1551 MU, which was significantly different from the activities in both *A. hydrophila* AH3R wild type and *exsA* mutant strains ($p<0.001$). The *PascN* promoter activity was repressed, but not completely shut down, to 444 MU in the *A. hydrophila exsC* mutant (Figure 3.18). While in the *A. hydrophila exsE* mutant strain, the promoter activity was further increased to 2059 MU, which was a significant increase compared to the activities in the *A. hydrophila exsA* and *exsC* mutants ($p<0.001$).

The other aeromonad T3SS promoters such as *PexsC* and *PexsD* showed similar trend of activities as in *PascN* promoter in different backgrounds. (Figure 3.19 and Figure 3.20). The β-galactosidase activity of *PexsC* in the *A. hydrophila* AH3R wild type was 352 MU, while it was almost completely switched off in the *A. hydrophila exsA* mutant. When *exsD* was knocked out, the promoter activity of *PexsC* increased to 1424 MU, which was significantly higher than the activities measured in both the wild type and the *exsA* mutant strains ($p<0.001$). In *A. hydrophila exsC* mutant, the promoter activity of *PexsC* decreased to 347 MU but remained at a similar level to the *A. hydrophila* AH3R wild type. While in the *A. hydrophila exsE* mutant, the *PexsC* promoter activity showed an increase to 604 MU, which was significantly differently from which measured in the wild type, *exsA* mutant and *exsC* mutant ($p<0.001$) (Figure 3.19)

The promoter activity of *PexsD* in *A. hydrophila* wild type strain was 494 MU and it was significantly reduced to 121 MU when *exsA* gene was knocked out ($p<0.001$) (Figure 3.20). In the *A. hydrophila exsD* mutant, the promoter activity of *PexsD* increased to 744 MU, which is significantly higher than the promoter activity

measured in the wild type strain ($p<0.05$). While in *exsC* mutant the promoter activity was reduced to 413 MU, which was close to the wild-type value but significantly lower than in the *exsD* mutant strain ($p<0.001$). When *exsE* was absent, the promoter activity of *PexsD* reached 1109 MU, which was a significant increase compared to the activities in all other backgrounds ($p<0.01$) (Figure 3.20).

The promoter activity of *PaopN* in the wild type strain *A. hydrophila* AH3R was 2357 MU, which was significantly higher than the promoter activities of *PascN*, *PexsC* and *PexsD* in the wild type strain ($p<0.001$). When the master regulator ExsA was knocked out, the activity of *PaopN* promoter was reduced down to 43 MU. However, the promoter activity of *PaopN* in the *exsD* mutant was 1067 MU, which was significantly lower than the activity in the wild type strain but higher than the activity in the *exsA* mutant ($p<0.01$) (Figure 3.21). Unlike *PascN*, *PexsC* and *PexsD*, the promoter activity of *PaopN* was not reduced in the *exsC* mutant or increased in the *exsE* mutant. The β-galactosidase activity of *PaopN* was 1708 MU in *exsC* mutant, which was significantly higher than in the *exsD* mutant but lower than in the wild type ($p<0.05$). In the *exsE* mutant, the *PaopN* promoter activity was 609 MU, which was significantly lower than the activities measured in the wild type ($p<0.001$), *exsD* mutant ($p<0.05$) and *exsC* mutant strains ($p<0.001$) (Figure 3.21)

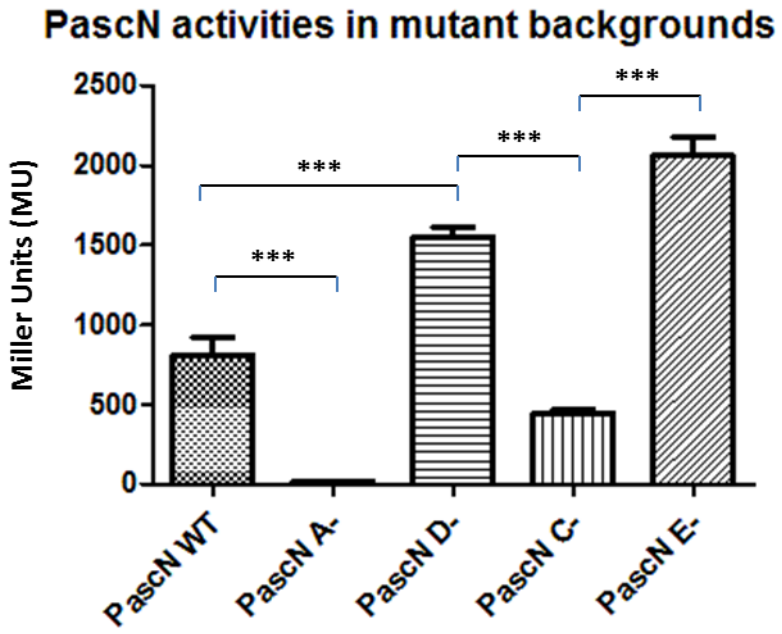


Figure 3.18 β-galactosidase activities of promoter *PascN* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown in LB broth to log phase at 30°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. The promoter activity of *PascN* was significantly decreased in the *exsA* mutant and *exsC* mutant when compared to the promoter activities in *exsD* mutant and *exsE* mutant ($p<0.001$). Also, the promoter activities in the wild type strain was significantly lower than both the *exsD* mutant and the *exsE* mutant strains ($p<0.001$). Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test ($p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$).

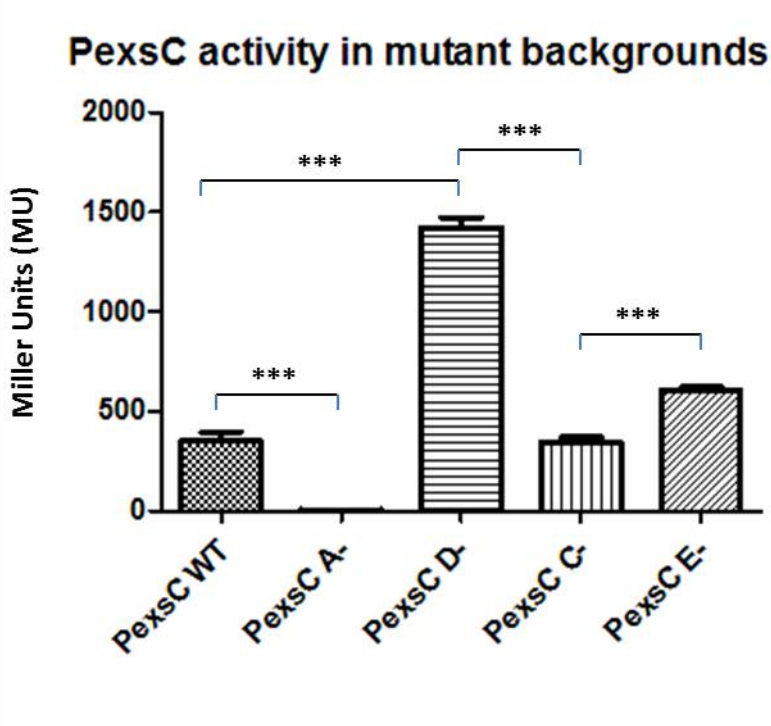


Figure 3.19 β-galactosidase activities of promoter *PexsC* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown in LB broth to log phase at 30°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. The promoter activity of *PexsC* was significantly decreased in *exsA* mutant and *exsC* mutant when compared to the promoter activities in *exsD* mutant and *exsE* mutant ($p<0.001$). The promoter activity of *PexsC* in the *exsD* mutant was significantly higher than in the wild type strain ($p<0.001$) while the promoter activity in the *exsC* mutant remained at a similar level to the wild type strain. Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test ($p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$).

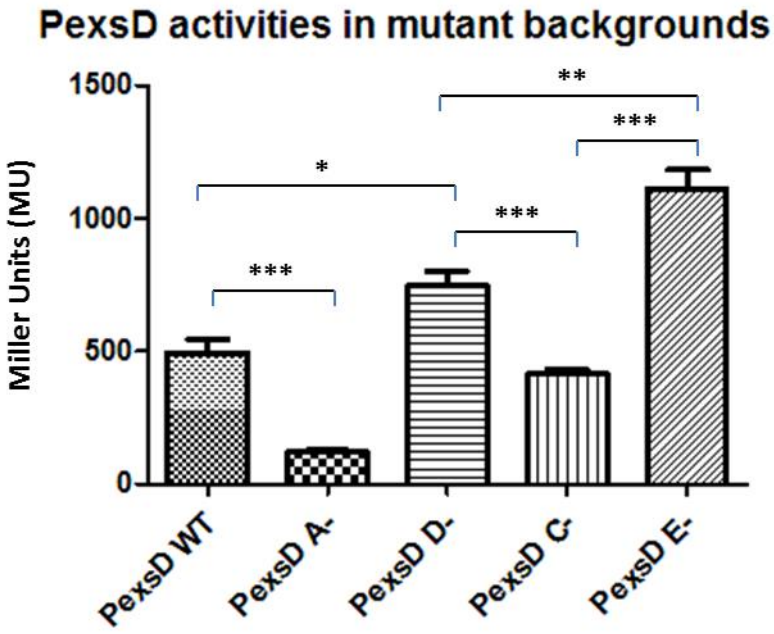


Figure 3.20 β-galactosidase activities of promoter *PexsD* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown in LB broth to log phase at 30°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. The promoter activity of *PexsD* was significantly decreased in *exsA* mutant and *exsC* mutant when compared to the promoter activities in *exsD* mutant and *exsE* mutant ($p<0.001$). The promoter activity in the *exsD* mutant was a significant increase when compared to the wild type strain ($p<0.05$). However, the promoter activity of *PexsD* in the *exsC* mutant remained at a similar level to the wild type strain. In the *exsE* mutant, the promoter activity was significantly increased compared to the *exsC* mutant ($p<0..001$), *exsD* mutant ($p<0.01$), *exsA* mutant ($p<0.001$) and the wild type strains ($p<0.001$). Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test (p*<0.05, p**<0.01, p***<0.001).

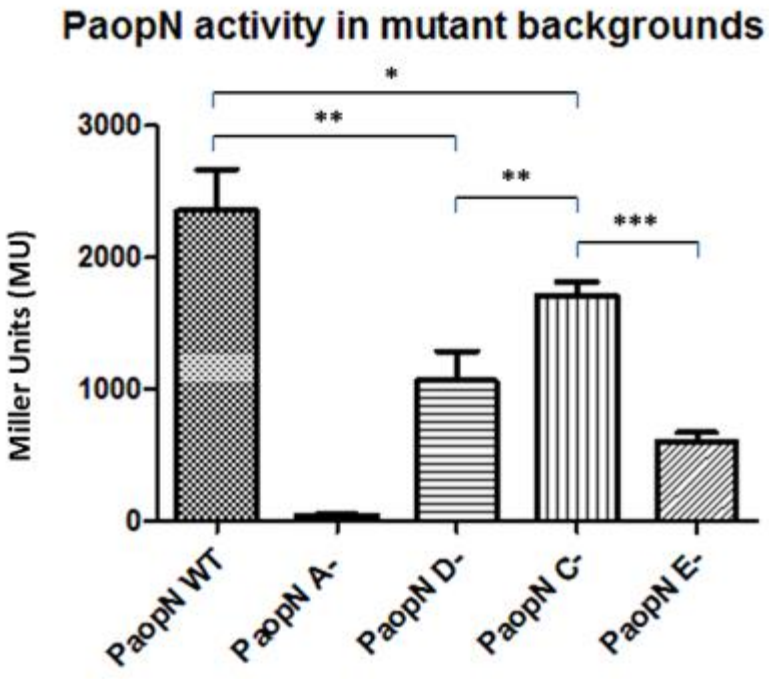


Figure 3.21 β-galactosidase activities of promoter *PaopN* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown in LB broth to log phase at 30°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. The promoter activity of *PaopN* was significantly decreased in *exsA* mutant when compared to the promoter activities in the other backgrounds ($p<0.001$). Surprisingly the promoter activity of *PaopN* in *exsD* mutant was significantly lower than both the wild type strain and the *exsC* mutant strain ($p<0.01$). However, the promoter activity of *PaopN* in the *exsC* mutant was significantly lower than in the wild type strain ($p<0.05$). In *exsE* mutant, the promoter activity of *PaopN* was significantly lower than the activities measured in the *exsC* mutant ($p<0.001$), *exsD* mutant ($p<0.05$) and the wild type strain ($p<0.001$). Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test ($p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$).

As for the promoter *PexsA*, which was responsible for the expression of the putative T3SS master regulator ExsA in *A. hydrophila*, the promoter activity was much higher than the other promoters of T3SS. In *A. hydrophila* wild type strain AH3R, the β-galactosidase activity of the promoter *PexsA* was 10,066 MU. Unlike the other promoters of T3SS, all of which were repressed when *exsA* was knocked out, the promoter activity of *PexsA* was not suppressed but demonstrated a significant increase in activity in the *exsA* mutant to 15,546 MU ($p<0.001$) (Figure 3.22). More surprisingly, the promoter activity of *PexsA* decreased to 5,030 MU in the *exsD* mutant background, which was significantly lower than the promoter activities of both the wild type and the *exsA* mutant strains ($p<0.001$). In the *exsC* mutant, the promoter activity of *PexsA* increased to 12,826 MU, which was significantly higher than both the activities in the wild type ($p<0.05$) and in the *exsD* mutant strains ($p<0.001$), but not significantly different from the promoter activity in the *exsA* mutant. The promoter activity of *PexsA* in the *exsE* mutant was 7,860 MU, which was significantly lower than both the activities in the *exsA* mutant ($p<0.001$) and in the *exsD* mutant strains ($p<0.01$) (Figure 3.22).

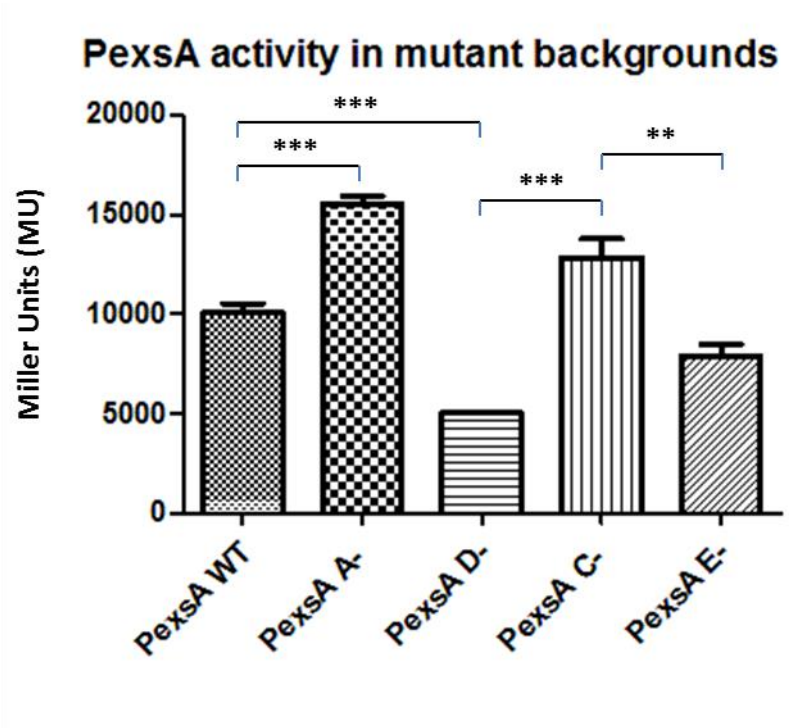


Figure 3.22 β-galactosidase activities of promoter *PexsA* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown in LB broth to log phase at 30°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. Unlike the other promoters of the T3SS, the promoter activity of *PexsA* demonstrated a significant increase in the *exsA* mutant and *exsC* mutant backgrounds when compared to the promoter activities measured in the *exsD* mutant and *exsE* mutant backgrounds ($p<0.01$). The promoter activity in the *exsD* mutant was a significant decrease when compared to the wild type strain ($p<0.001$). Each bar represents each strain grown in triplicate with duplicate samples. The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test ($p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$).

3.5 Re-constitution of T3SS regulation in *Escherichia coli*

Due to the unexpected results of the *PexsA* promoter activities in different *A. hydrophila* backgrounds, another approach was required to investigate the regulation of the T3SS. Thereby, *E. coli* was utilized to re-constitute T3SS regulation by co-transforming two plasmids into this cell background (Figure 3.23). The reporter plasmid pKAGb-2(-) contains a promoter-less *lacZ* gene and a chloramphenicol resistant gene. When the T3SS promoter was fused upstream of the promoter-less *lacZ* gene, the β-galactosidase activity of the promoter could then be measured in the same way it was measured in *A. hydrohphila* backgrounds. In the meantime, the gene encoding the master regulator of the *A. hydrophila* T3SS, *exsA* was cloned into the broad host range pBBR1MCS-5 plasmid by digesting both the *exsA* fragment and the plasmid with restriction enzyme *Hin*dIII and ligating using T4 DNA ligase (Figure 3.24). The orientation of the cloning was checked by sequencing to ensure the expression of *exsA* gene *in-trans*. Once both plasmids were ready, they were co-transformed into *E. coli* DH5α cells. Then chloramphenicol and gentamycin resistant colonies were selected to perform β-galactosidase assays.

Each of the T3SS promoter activities was measured in the presence or absence of *exsA* in *E. coli* cells by co-transforming the promoter-fused pKAGb-2(-) reporter plasmid with pBBR5*exsA* plasmid construct or pBBR1MCS-5 empty plasmid respectively (Figure 3.25). All of the T3SS promoters, except for promoter *PexsA*, had no activity or very low activity in *E. coli* DH5α cells, whether or not *exsA* was present *in-trans*. When the promoter activity of *PexsA* was measured with empty pBBR1MCS-5, the β-galactosidase activity was approximately 2,806 MU, but when measured with pBBR5*exsA* construct, the promoter activity of *PexsA* was decreased significantly to approximately 1,962 MU ($p<0.001$) (Figure 3.25). This suggested that the master regulator ExsA might negatively regulate its own promoter *PexsA*, which agreed with our findings of *PexsA* promoter activities in *A.*

hydrophila wild type and mutant backgrounds.

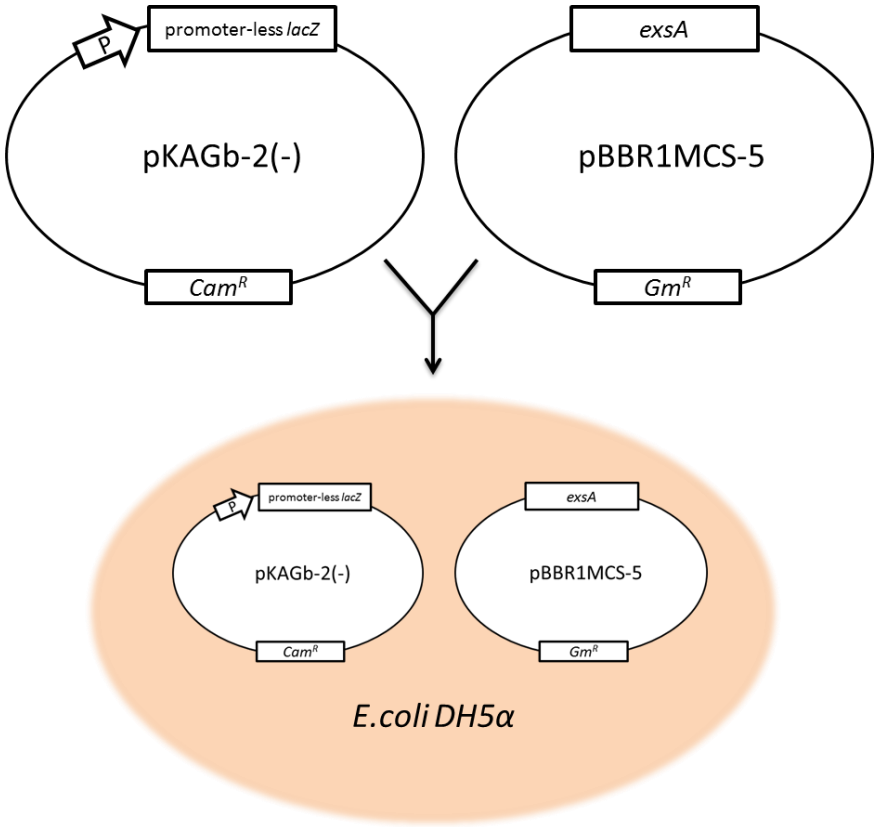


Figure 3.23 Re-constitution of T3SS regulation in *E. coli* DH5α.

The promoters (P) of T3SS were cloned into reporter plasmid pKAGb-2(-) and fused upstream of a promoter-less *lacZ* gene. The *A. hydrohphila* AH3R *exsA* gene was cloned into pBBR1MCS-5 plasmid and co-transformed into *E. coli* DH5α with pKAGb-2(-) reporter plasmid constructs. The T3SS promoter on the reporter plasmid could then be regulated by the *exsA in-trans*. β-galactosidase assay was carried out to measure the promoter activity in the presence or absence of *exsA in-trans*.

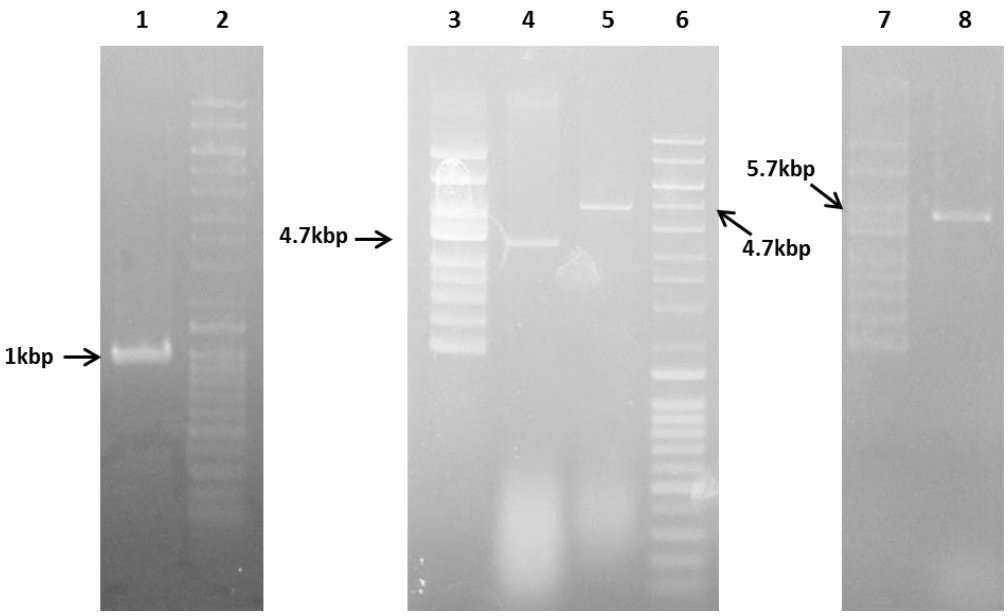


Figure 3.24 A 1% agarose gel showing cloning of *exsA* into pBBR1MCS-5. Lane 1, *Hin*dIII-digested *exsA* fragment (~1kb); Lane 2, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 3, Promega™ Supercoiled DNA Ladder (2-10kb); Lane 4, Undigested pBBR1MCS-5 plasmid (~4.7kb); Lane 5, *Hin*dIII-digested pBBR1MCS-5 (~4.7kb); Lane 6, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 7, Promega™ Supercoiled DNA Ladder (2-10kb); Lane 8, ligated plasmid construct pBBR5*exsA* (~5.7kb).

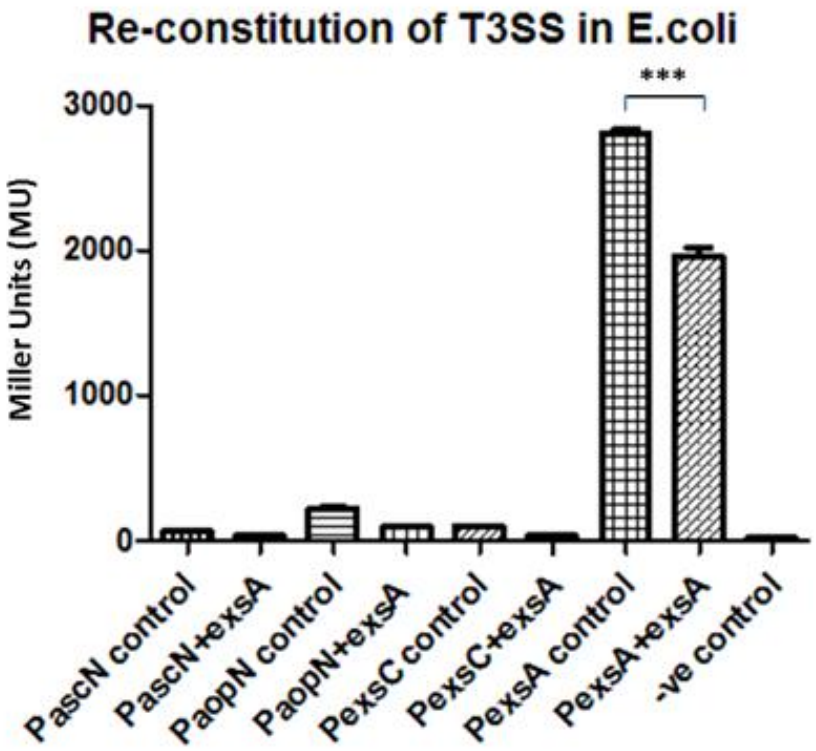


Figure 3.25 β-galactosidase activities of T3SS promoters in the *E. coli* re-constitution system. The promoter activities were measured when each strain was grown in LB broth to log phase at 37°C with shaking at 200rpm without adding any $CaCl_2$ or EGTA. Each T3SS promoter activity was measured in the presence of *exsA* (+*exsA*) or absence of *exsA* (control) *in-trans*. Negative control was measured using promoter-less pKAGb-2(-) plasmid. All of the *A. hydrophila* T3SS promoters had no or very low activities in the *E. coli* re-constitution system except for the promoter *PexsA*. The promoter activity of *PexsA* was significantly higher in the absence of *exsA* rather than in the presence of *exsA in-trans* ($p<0.001$). The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test (p*<0.05, p**<0.01, p***<0.001).

3.6 Discussion

The aim of this study was to investigate the role of each *exs* gene in the regulation of the T3SS in *A. hydrophila* and find out if the regulatory cascade is present in *A. hydrophila* AH3R through knocking out the *exs* genes and measuring the activities of putative T3SS promoters in the wild type and mutant backgrounds.

Before measuring the activities of *A. hydrophila* T3SS promoters in the mutant backgrounds, the assay condition was optimized first, as there was evidence showing that the expression of the *A. hydrophila* T3SS was induced significantly with additional 20mM $MgCl_2$ and 10mM EGTA. Vilches and colleagues demonstrated that the expression level of two T3SS mediated genes *aopN* and *aexT* was up-regulated with additional $MgCl_2$ and EGTA using *gfp*-fusions (Vilches et al 2009). However in this study, the β-galactosidase activities of *PaopN* and *PexsA* were decreased in the inducing condition, in which additional $MgCl_2$ and EGTA were added. Moreover, no significant difference of promoter activity between inducing condition and non-inducing condition was found for promoters *PexsC* and *PexsD* (Figure 3.17).

The reason why the findings in this study were contradictory to Vilches' was unknown but there were limitations in the methods used in both this project and the Vilches' study. In this study, as the putative T3SS promoters were cloned in the multi-copy *lacZ*-fusion plasmid pKAGb-2(-), the β-galactosidase assay carried out was possibly influenced by the transcriptional factor titration effect. The potential repressors might be 'outnumbered' by the high copy number of the promoter, which was supposed to be repressed but still showing activities during the assay (Brewster et al 2014, Guido et al 2006).

As in Vilches' study, the activity of the *PaopN* promoter was obtained by measuring the expression level of *gfp*-fusion. The *gfp* reporter gene was integrated

into the *A. hydrophila* AH-3 chromosomal DNA downstream of the *PaopN* promoter and upstream of the *aopN* gene. The integration of *gfp* gene might affect the expression of the downstream operon, in which at least three genes, *acrR*, *acrG* and *acrV* were possibly involved in the low-Ca^{2+} response as their homologues had similar activities in *Y. pestis*, *P. aeruginosa* or *A. salmonicida* (Table 1.2). What was not indicated in Table 1.2 was that not only AcrR was involved in low-Ca2+ response as its homologues LcrR in *Y.pestis*, but also the homologues of AcrG and AcrV were reported to be involved in calcium response in *A. salmonicida* as well as in *Y. pestis* (Barve & Straley 1990, Burr et al 2003, Matson & Nilles 2001).

Furthermore, the promoter activity of *PaopN* measured later on in mutant backgrounds also suggested potential secondary regulations on some of the T3SS promoters. The genes in the operon downstream of *PaopN* will be dicussed in more detail when investigating the promoter activity of *PaopN* in mutant backgrounds. To avoid more variations, the β-galactosidase activities of the T3SS promoters measured afterwards in the mutant backgrounds were all assayed in non-inducing conditions.

As shown in figure 3.18, the promoter activity of *PascN* was repressed down to 15 MU in the *exsA* mutant, suggesting that the promoter was not activated in the absence of ExsA. While in the *exsD* mutant, the promoter activity was boosted up to 1551 MU, which was significantly increased when compared to the activities in both *exsA* mutant and the wild type ($p<0.001$). This suggested that PascN promoter activity was increased in the absence of ExsD, which was proposed to be the anti-activator of the T3SS in this study. Then the promoter activity was suppressed down to 444 MU in the *exsC* mutant, which was a significant decrease compared to the wild type or the *exsD* mutant strains ($p<0.01$). In the *exsE* mutant, the promoter activity was increased up to 2059 MU, indicating that the promoter was activated in the absence of ExsE.

The patterns of the *PascN*, *PexsC* and *PexsD* promoter activities in different mutant backgrounds were in consensus with the hypothesis that ExsA was the master regulator of the T3SS. Therefore, in the *exsA* mutant, the promoters were not activated due to the absence of the master regulator. While in the *exsD* mutant, the absence of the anti-activator ExsD allowed the activator ExsA to switch on the promoters, thus the promoter activities of *PascN*, *PexsC* and *PexsD* were increased significantly compared to in the wild type or in the *exsA* mutant strains.

In the *exsC* mutant, the hypothesis proposed that the absence of ExsC allowed ExsD to bind ExsA, thus the promoter should be suppressed. However, the β-galactosidase activities of *PascN*, *PexsC* and *PexsD* promoters in the *exsC* mutant strain remained at similar levels of activity to the wild type strain, indicating that there was likely to be secondary regulation. One of the possible reasons is that the anti-activator ExsD may self-associate, which prevents the inhibitory function of ExsD on ExsA. As reported in *P. aeruginosa*, the ExsD trimerized at 30°C and was unable to inhibit ExsA until the trimerization was disrupted at 37°C. Moreover, the presence of ExsC protein overcomes the ExsD trimerization, suggesting a higher binding affinity of ExsC-ExsD than ExsD self-association (Bernhards et al 2013). In this case, the self-association of ExsD could explain the similar promoter activities of *PascN*, *PexsC* and *PexsD* when measured in the *exsC* mutant and the wild type strains. Hence in the absence of ExsC, the ExsD self-association competed with the inhibition of ExsA, thus the promoter activities of *PascN*, *PexsC* and *PexsD* were reduced when compared to the activities measured in the *exsD* mutant but remained at a similar level to the activities in the wild type strain.

However, the fact that *P. aeruginosa* grows optimally at 37°C while the optimal growth temperature for *A. hydrophila* AH-3 is 30°C, makes the temperature-sensitive self-association activity of ExsD unclear in *A. hydrophila* AH-3 strains. There was evidence from previous studies showing that many other factors were

also involved in the regulation of the T3SS, including quorum sensing system, environmental factors like Mg^{2+} or Ca^{2+} concentration, σ^{54} factor RpoN and the translocation regulator AopN and AcrR (Hendrickson et al 2000, Vilches et al 2009, Yu et al 2007, Yu et al 2004). The complete picture of all factors that affects the regulation of *A. hydrophila* T3SS still remains unknown.

In the *exsE* mutant, the absence of ExsE allowed the chaperone protein ExsC to bind ExsD, thus ExsA was released to switch on the promoter, hence the activity of *PascN* and *PexsD* was further increased. In the environment, when the bacteria such as *A.salmonicida* or *P.aeruginosa*, were in contact with the host cells and the effector proteins were secreted, in this case ExsE, the T3SS regulon was then further activated to inject more toxins into the host cells (Rietsch et al 2005, Urbanowski et al 2007, Urbanowski et al 2005, Vanden Bergh & Frey 2013).

The promoter activity of *PexsC* was not elevated in the absence of ExsE, and was much lower than the promoter activity of *PexsD*. It possibly suggests that when ExsE is absent, more ExsD was made in contrast with ExsC due to higher promoter activity. The increase in the pool of ExsD proteins provide the bacteria with another level of regulation for ExsA activation. Moreover, as it was recently reported in *P. aeruginosa* that the ExsD could only bind to ExsA when they were synthesized at the same time as folding intermediates, the ExsD protein released from ExsC binding could not re-bind the ExsA protein (Bernhards et al 2013). Thereby, it was not surprising that ExsD was required to be synthesized at a higher level than ExsC. In fact, the expression of ExsD was also driven by the *PexsA* promoter, as both the *exsA-ascL* and *exsD-ascL* operons share the same terminator, to allow simultaneous expression of ExsD and ExsA.

The promoter activity of *PaopN* in the wild type was around 2357 MU, which was higher than the activities of *PascN*, *PexsC* and *PexsD*, suggesting that *PaopN* is a stronger promoter. Although the activity of *PaopN* was completely suppressed in

the *exsA* mutant, the absence of ExsD and ExsE did not increase the promoter activity compared to the wild type while the absence of ExsC did not repress the promoter activity like it did to *PascN*, *PexsC* and *PexsD*.

The reason why this promoter was regulated differently from the others is unknown but it is possibly related to the genes in the operon downstream of *PaopN*. The first gene of the operon that was regulated by *PaopN* was *aopN* and the function of its protein product AopN was known to be similar to its homologue YopN in *Yersinia spp*., which functions as a valve to form a protein complex that blocks Yop secretion from a cytosolic location of the Type III secretome (Ferracci et al 2005, Vilches et al 2008). The other genes downstream in the same operon were also reported to be involved in the regulation of the T3SS, such as *acrR*, *acrG* and *acrV*.

The homologues of AcrG and AcrV in *Y. pestis*, LcrG and LcrV were reported to be involved in the secretion of the T3SS. The LcrG protein functions as a negative regulator of T3SS similar to YopN that block the T3SS secretion but from the cytoplasmic side of the secretion apparatus on the inner membrane. It also functions as the cognate chaperone protein for the needle-tip secreted protein LcrV. When the T3SS is not activated in *Y. pestis*, the intracellular level of LcrV is low thus allowing the chaperone protein LcrG to block the secretion apparatus. But when the T3SS is triggered, the level of LcrV is increased to titrate out the LcrG protein from secretion apparatus thus allowing secretion (Matson & Nilles 2001). The homologues of *acrG* and *acrV* in *A. salmonicida* were also involved in the regulation of the T3SS in response to calcium as discussed before in introduction (Burr et al 2003).

Like the homologue of AcrR in *Y. pestis*, LcrR is a bifunctional transcriptional factors that down-regulates the expression of LcrG in the presence of Ca^{2+}, while it is necessary for LcrG expression in the absence of Ca^{2+} (Barve & Straley 1990). All three gene products of *acrG*, *acrV* and *acrR* are possibly involved in the regulation of T3SS, thus It is likely that there are secondary regulations affecting the activity of

PaopN promoter.

The promoter activity of *PascN*, *PaopN*, *PexsC* and *PexsD* suggested that they were not strong promoters when compared to *PexsA*, which was responsible for the transcription of the T3SS master regulator ExsA. The promoter activity of *PexsA* was significantly increased when *exsA* was knocked out, suggesting that the master regulator ExsA might negatively regulate its own promoter *PexsA*.

In 1976, AraC was reported to repress the expression of its own gene *araC* by binding to its own promoter region and blocking the recruitment of the RNA polymerase (Casadaban 1976, Hahn & Schleif 1983). As a member of AraC family proteins, there has been no report of ExsA that it can repress its own transcription so far. However, many members of the AraC family proteins share this feature that repress their own expression, such as XylR, one of the regulators for xylene metabolism in *Pseudomonas putida* and YbtA, a pesticin receptor regulator in *Yersinia pestis* (Fetherston et al 1996, Inouye et al 1987).

The observations of *PexsA* activities in the *exsD*, *exsC* and *exsE* mutant strains further contributed to the hypothesis that ExsA was under control of a regulatory cascade. When *exsD* was knocked out, ExsA was freely allowed to repress its own promoter *PexsA*, thus a decrease of the *PexsA* promoter activity in the *exsD* mutant strain (Figure 3.22). In the *exsC* mutant, the promoter activity of P*exsA* was increased significantly when compared to the promoter activities in the wild type and the *exsD* mutant strains, but to a similar extent as the activity measured in *exsA* mutant (Figure 3.22). This was consistent with our hypothesis that in the absence of ExsC, ExsD was free to bind ExsA, which was then unable to repress the promoter P*exsA*, thus similar phenotypes were observed in the *exsC* mutant and the *exsA* mutant strains. Similarly, in the *exsE* mutant, the absence of the effector protein ExsE allowed its chaperone protein ExsC to bind to the de-activator ExsD thus allowing the activator protein ExsA to repress its own promoter *PexsA*, thereby the

promoter activity of *PexsA* was decreased significantly in the *exsE* mutant (Figure 3.22).

Due to the lack of T3SS in *E. coli DH5α* re-constitution system, all of the T3SS promoters, except for *PexsA*, had similar β-galactosidase activity as the negative control, suggesting that these promoters required certain T3SS-specific factors other than ExsA to be activated. This contributed to the previous suspicion that there might be secondary regulation or regulators other than the master regulator ExsA.

However, the promoter *PexsA* was still highly active in *E. coli* DH5α cells in the absence of *exsA in-trans*, suggesting that this promoter was likely to be activated by housekeeping sigma factor σ^{70} in *E. coli*. While in the *E. coli* DH5α cells with induced *exsA* expression, the promoter activity of *PexsA* was down-regulated significantly. This confirmed our previous suspicion that ExsA might negative regulate its own promoter *PexsA*. Given all the results obtained so far, it was suggested that *PexsA* was constantly activated but under a negative feedback control of the master regulator ExsA, which was also constitutively expressed. Therefore, it could be deduced that the bacteria *A. hydrophila* AH3R maintained a minimal level of T3SS expression in the environment due to the constitutive expression of ExsA. When the bacteria encountered host cells, according to our hypothesis, ExsE was secreted out and ExsC was free to bind to ExsD that released abundant ExsA to further activate the T3SS in *A. hydrophila* AH3R.

The inhibition of ExsA on its own promoter *PexsA* provided another level of the T3SS regulation. As the *exsA-ascL* and *exsD-ascL* operons share the same terminator, the only gene different is the *exsA* gene, which is under control of *PexsA* but not *PexsD* promoter (Figure 3.16). The fact that the activation of *PexsD* promoter requires the T3SS master regulator ExsA may suggest a scenario where the bacteria switch off the T3SS and release from the host cell. In this situation, the accumulation of ExsA restrains the expression of itself by inhibiting its own promoter as well as synthesizing more anti-activator ExsD, reducing the expression of the

T3SS overall, which results in the closure of the injectisome and detaching from the host cells. It also makes sense as the closure of the T3SS channel leads to the accumulation of the secreted protein ExsE, which sequester the chaperone protein ExsC from binding the anti-activator ExsD.

3.7 Conclusion

- The T3SS of *A. hydrophila* is regulated by a cascade of regulators that involves ExsA, ExsD, ExsC and ExsE.
- The T3SS master regulator ExsA is inhibited by ExsD, which is repressed by ExsC while ExsC is inhibited by ExsE.
- The T3SS master regulator ExsA also has a negative feedback that represses its own promoter *PexsA*.

Chapter 4
Interactions Studies of T3SS Regulators

4.1 Introduction

The hypothesis in this study that the master regulator ExsA of the *A. hydrophila* T3SS was under control of a cascade of proteins was largely based on the well-described T3SS regulatory cascade in *Pseudomonas aeruginosa*, in which ExsA, ExsD, ExsC and ExsE interact with each other through direct protein-protein interactions.

ExsA was first identified in *P. aeruginosa* and was deduced to be a transcriptional factor due to its extensive homology shared with VirF in *Yersinia enterocolitica*, which was an AraC family transcriptional activator (Frank & Iglewski 1991). As in most AraC/XylS family proteins, *P. aeruginosa* ExsA consists of a carboxyl-terminal helix-turn-helix DNA-binding motif and an amino-terminal oligomerization and ligand binding domain (Brutinel et al 2009, Martin & Rosner 2001). As the transcriptional activation of ExsA was required for the expression of the T3SS secretion apparatus, translocation machinery and secreted effectors, ExsA was described as the master regulator of the *P. aeruginosa* T3SS (Hovey & Frank 1995, Yahr et al 1995).

It was then reported in 2002 that the master regulator ExsA was negatively regulated by another protein named ExsD in *P. aeruginosa* through direct protein-protein interaction using bacterial LexA two-hybrid assay (McCaw et al 2002). Later on, Brutinel and Yahr's group demonstrated that the C-terminal domain of *P.aeruginosa* ExsA is incapable of cooperative DNA-binding and unaffected by the inhibition of ExsD, suggesting that the N-terminal domain of ExsA is required for interactions between ExsD and ExsA. (Brutinel et al 2009).

Meanwhile, ExsC was found to be an anti-anti-activator in *P. aeruginosa* by Dasgupta and Yahr's group in 2004. Mutagenesis and complementation experiments have suggested that ExsC functions in de-repression of the T3SS,

while Bacterial LexA two-hybrid assay and co-purification assays have suggested that ExsC interacts with ExsD through direct protein-protein interactions (Dasgupta et al 2004). They have also suggested that ExsC is a potential T3SS-specific chaperone protein due to the low molecular weight, the acidic isoelectric point and the putative C-terminal amphipathic α-helix (Dasgupta et al 2004).

Later in 2005, the same group discovered that the secreted protein ExsE sat at the top of the signalling cascade by inhibiting its chaperone protein ExsC through direct protein-protein interactions (Urbanowski et al 2005). Therefore, the regulatory cascade of the *P. aeruginosa* T3SS is fully uncovered, that the T3SS-secreted protein ExsE binds to the chaperone protein ExsC, which antagonizes the anti-activation activity of ExsD on the master regulator ExsA.

Moreover, the T3SS was reported in clinical *Aeromonas* isolates including *A. hydrophila*, *A. caviae* and *A. veronii* in 2004, by the identification of the T3SS genes *ascF* and *ascG* (Chacon et al 2004). In the same year, the complete T3SS that consisted of 35 genes in *A. hydrophila* AH-3 was sequenced but the regulation of the T3SS in *A. hydrophila* still remained unknown (Vilches et al 2004). It was then reported by Chopra's group in 2007 that Exs proteins in *A. hydrophila* SSU strain, like in *P. aeruginosa*, had similar effect on the regulation of the T3SS using mutagenesis and over-expression assays (Sha et al 2007). In the meantime, Leung's group demonstrated similar anti-activation activity of ExsD on ExsA in *A. hydrophila* AH-1 strain using mutagenesis assays (Yu et al 2007).

There was no evidence of direct protein-protein interactions in the putative regulatory cascade of T3SS in *A. hydrophila* AH3R. Thus in this study, direct protein-protein interactions between each of the Exs proteins were investigated using Bacterial Adenylate Cyclase Two-Hybrid System (BACTH) and Far-Western Blot.

4.2 Cloning of the T3SS regulatory components (*exs* genes) into the BACTH system

The Bacterial Adenylate Cyclase Two Hybrid (BACTH) assay was carried out using the Euromedex™ BACTH System Kit (Section 2.20). The target genes were first cloned into the BACTH plasmids in frame with either T25 or T18 fragments of the *cyaA* gene at the 5' or 3' end of the gene of interest (Table 2.2 plasmids used in this study), to allow co-expression of fusion proteins. There were 4 different BACTH plasmids allowing the target genes to fuse in different orientations, for instance, *exsA* was cloned into pKT25 and pKNT25 for both C-terminal and N-terminal fusion with the T25 fragment respectively.

For cloning into pKT25 plasmid, the *exsA* gene was amplified from the *A. hydrophila* AH3R chromosomal DNA using *exsA*_pKT25 forward and *exsA*_pKT25 reverse primers (Appendix 1) with Platinum Pfx DNA polymerase, introducing an *Xba*I restriction site at the 5' end and an *Eco*RI restriction site at the 3' end for directional cloning (Figure 4.1). In order to insert the *exsA* gene in frame with the T25 fragment, there was a 6bp spacer, designed in the *exsA*_pKT_forward primer, located in between the 3' end of T25 fragment and the 5' end of the *exsA* gene (Figure 4.2).The plasmid pKT25 and the amplified *exsA* gene were then digested with both *Xba*I and *Eco*RI restriction enzymes to allow ligation with T4 DNA ligase. After ligation, it was transformed into *E. coli* DH5α cells and kanamycin resistant colonies were selected for screening using colony PCR screen (section 2.3.2). The screening primers pKT25_screen forward and pKT25_screen reverse (Appendix 1) were used together with Taq DNA polymerase and the colony PCR screening products were checked by agarose gel electrophoresis (Figure 4.3). Potential successful constructs were sequenced by Core Genomic Facility, University of Sheffield. The sequencing results were analysed using FinchTV and BLAST search to ensure the in-frame insertion.

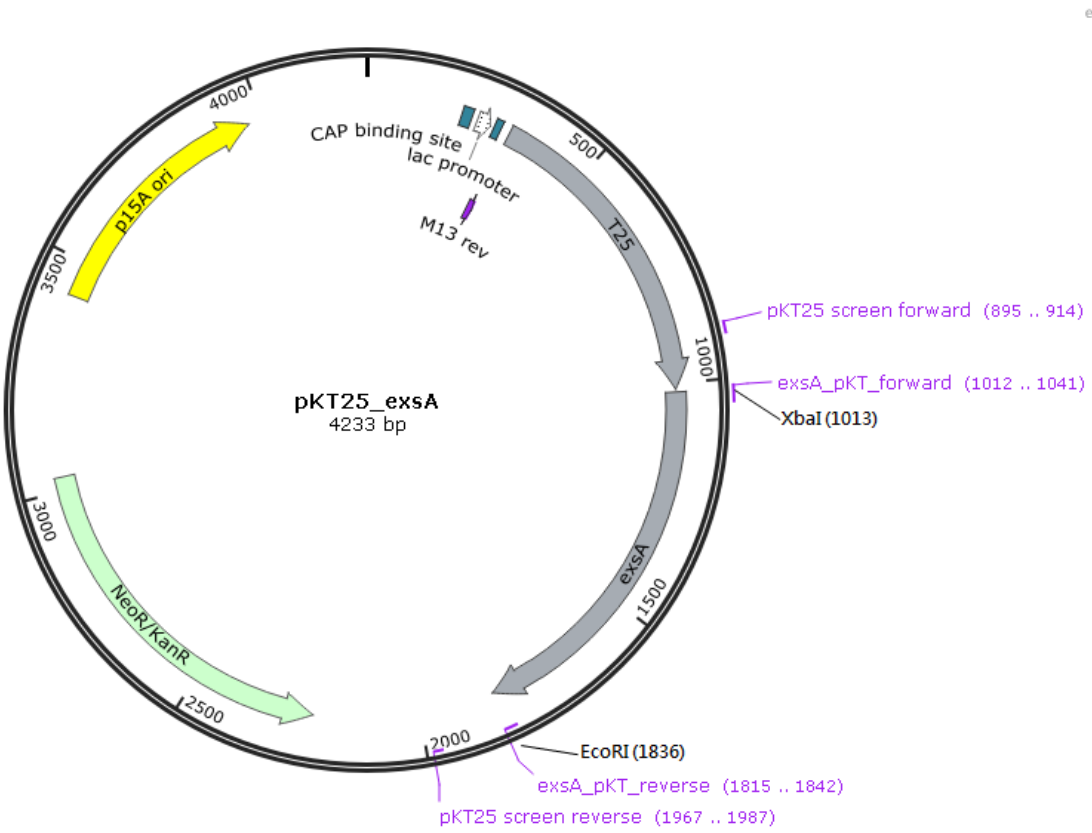


Figure 4.1 Illustration of the plasmid construct pKT25-*exsA*. The *exsA* gene was amplified using Platinum Pfx DNA polymerase with *exsA*_pKT_forward primer, which introduced an *Xba*I site at 5' end, and *exsA*_pKT_reverse primer, which introduced an *Eco*RI site at 3' end. The pKT25 plasmid and the amplified *exsA* fragment were digested with both of the restriction enzymes and ligated together with T4 ligase. A successful insert was screened by colony PCR screen using pKT25_screen forward and pKT25_screen reverse primers with Taq polymerase. The *exsA* gene was inserted in frame with the T25 fragement at 3' end, allowing the ExsA protein fused to the C-terminal of the T25 fragment. Key features of the plasmid construct were shown in arrows while primers were shown in purple. Created using SnapGene software.

Figure 4.2 Demonstration of the in-frame insertion of the *exsA* gene into the pKT25 plasmid. The *Xba*I restriction site (TCTAGA) was located at the 3' end of the T25 fragment but the first nucleotide of the *Xba*I restriction site (T) overlapped with the last nucleotide of the T25 fragment. In order to insert *exsA* in frame with the T25 fragment, the spacer in between must be multiples of 3 (number of codons). Thereby an extra G was designed downstream of the *Xba*I site in the *exsA*_pKT_forward primer to make a 6bp spacer in between thus allowing in-frame insertion of the *exsA* gene into the pKT25 plasmid. Generated using SnapGene software.

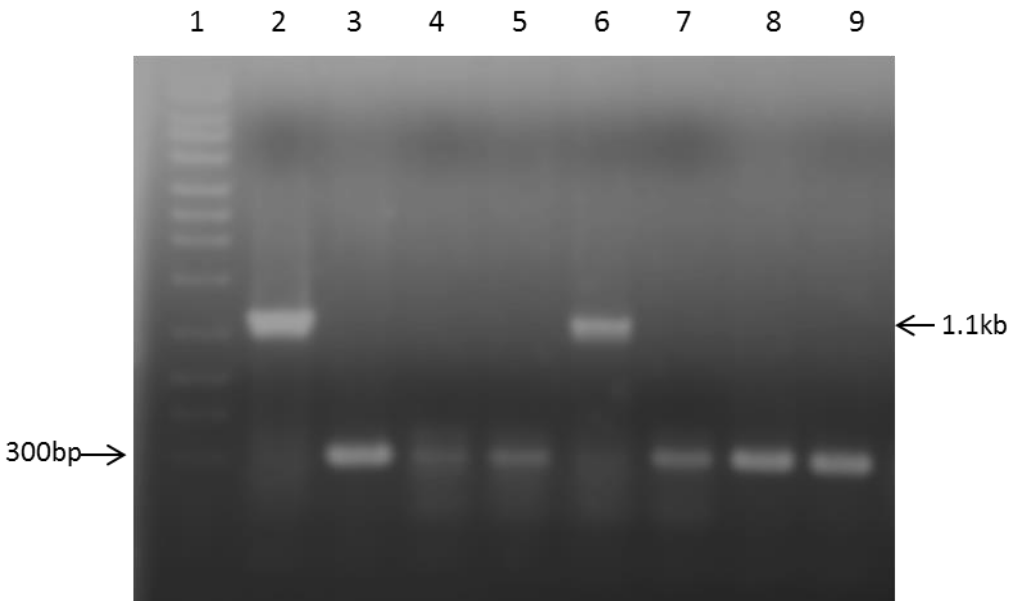


Figure 4.3 A 1% agarose gel showing colony PCR screen of pKT25-*exsA*. There was an approximately 300bp distance between the pKT25_screen forward and pKT25_screen reverse primers, thus the screening of successful constructs should be 1.1kb, which comprised of 300bp and the size of the inserted *exsA* gene (800bp). Lane 1, Highranger 1kb DNA ladder (Norgen Biotek); Lane 3-5 and 7-9, empty pKT25 plasmid with no insert; Lane 2 and 6, potential successful pKT25-*exsA* plasmid construct.

A similar approach was used to clone each of the *exsA*, *exsD*, *exsC* and *exsE* genes into each of pKT25, pKNT25, pUT18 and pUT18C plasmids to obtain 16 plasmid constructs in total. Examples of pKNT25-*exsC*, pUT18-*exsD* and pUT18C-*exsD* were shown to illustrate different orientation of the T25/T18 fusion provided by different BACTH plasmids (Figure 4.4, Figure 4.5, and Figure 4.6).

Once all 16 of the BACTH plasmid constructs were obtained, then one of the T25 derived plasmid constructs was co-transformed with a T18 derived plasmid construct into *E. coli* BTH101 reporter competent cells and incubated on MacConkey/maltose agar for 2 nights at 30°C.

When the two proteins of interest interacted with each other, heterodimerization of the fusion proteins allowed the complementation of the T25 and T18 fragments to form a catalytic domain of adenylate cyclase (CyaA), thus cAMP was synthesized. Then the *mal* operon was activated by cAMP/CAP complex. Therefore the maltose metabolism pathway was switched on in the *E. coli* BTH101 reporter strain, which allowed the fermentation of maltose and the production of acid that turned the pH indicator in MacConkey agar pink. Therefore positive interaction colonies were pink/red in colour whereas negative colonies were white. Control plasmids pKT25-zip and pUT18C-zip, which expressed T25-zip and T18-zip fusion proteins with leucine zipper motifs, were co-transformed into *E.coli* BTH101 reporter strain to present a positive control of the interaction, while plasmid pKT25 and pUT18 were co-transformed into *E.coli* BTH101 as a negative control (Figure 4.7).

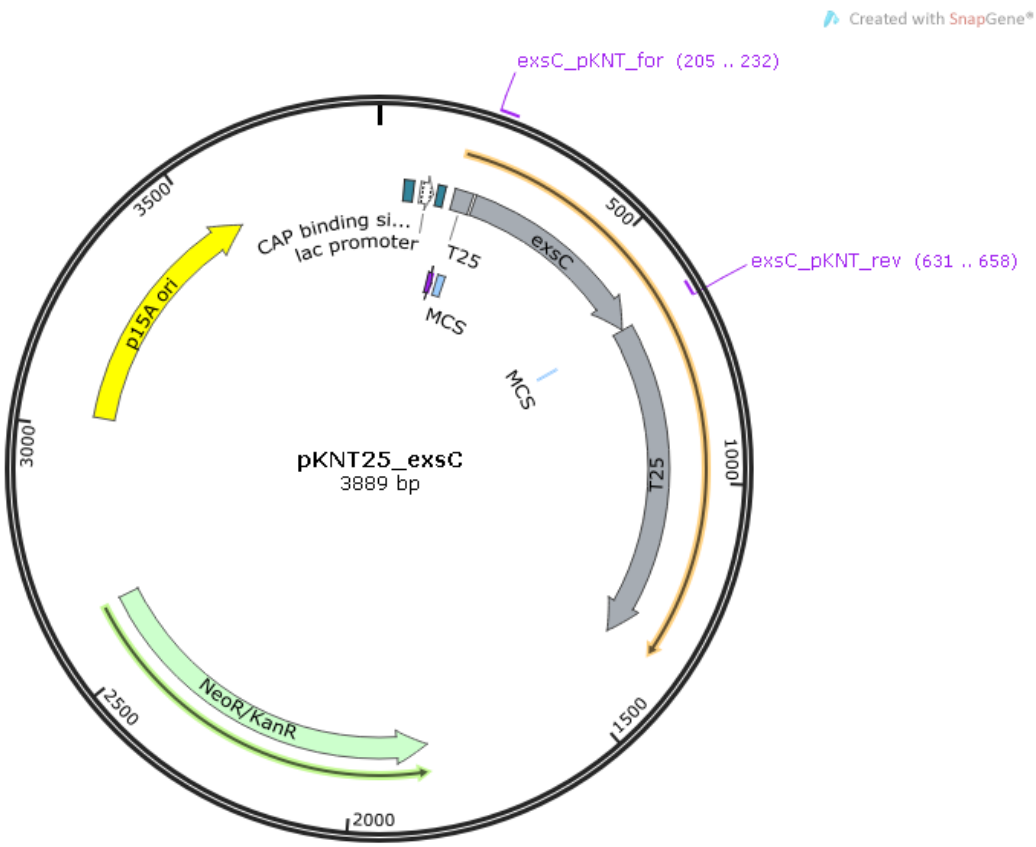


Figure 4.4 Illustration of the plasmid construct pKNT25-*exsC*. The *exsC* gene was amplified using Platinum Pfx DNA polymerase with *exsC*_pKNT_forward and *exsC*_pKNT_reverse primers. The *exsC* gene was inserted in frame at 5' end of the T25 fragment, allowing ExsC protein fused to the N-terminal of the T25 fragment. Key features of the plasmid construct were shown in thick arrows while ORFs were shown in thin arrows. Primers were shown in purple. Created using SnapGene software.

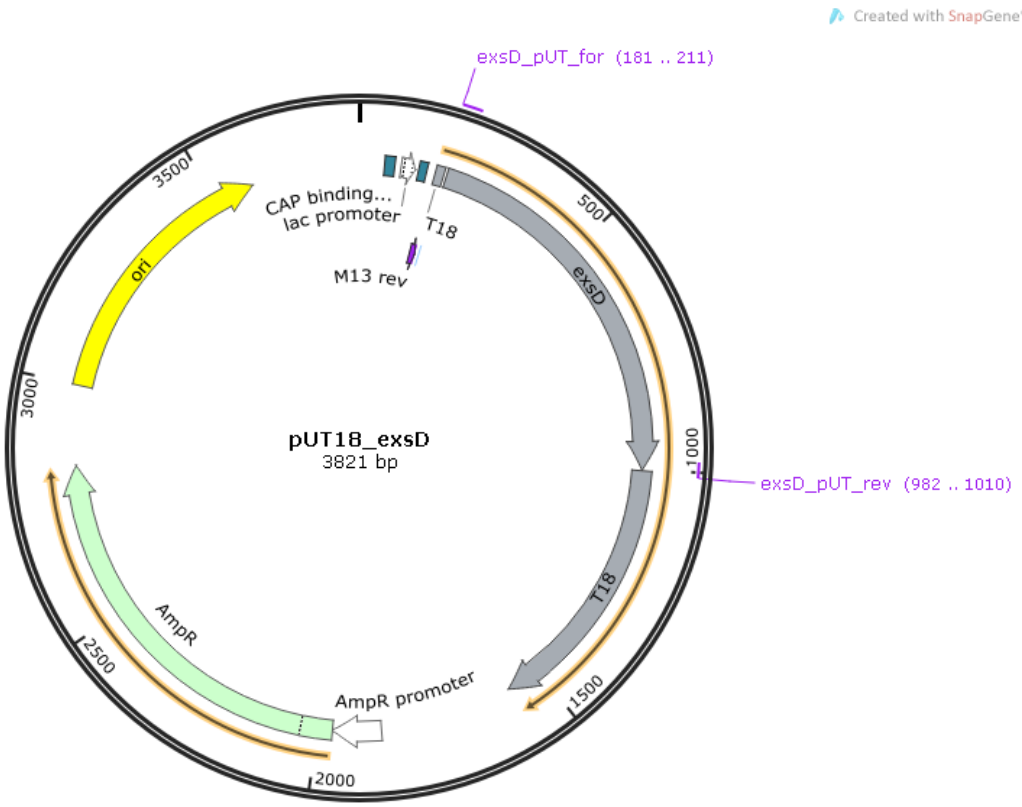


Figure 4.5 Illustration of the plasmid construct pUT18-*exsD*. The *exsD* gene was amplified using Platinum Pfx DNA polymerase with *exsD*_pUT_forward and *exsD*_pUT_reverse primers. The *exsD* gene was inserted in frame at 5' end of the T18 fragment, allowing ExsD protein fused to the N-terminal of the T18 fragment. Key features of the plasmid construct were shown in thick arrows while ORFs were shown in thin arrows. Primers were shown in purple. Created using SnapGene software.

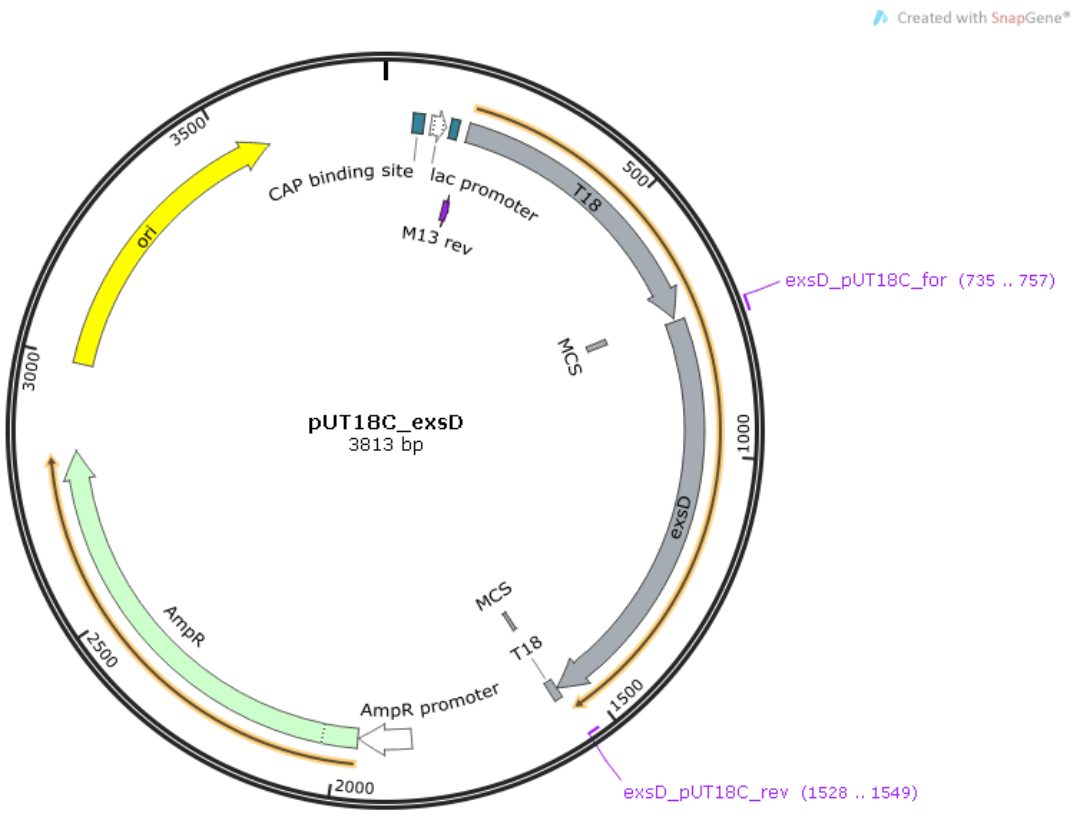


Figure 4.6 Illustration of the plasmid construct pUT18C-*exsD*. The *exsD* gene was amplified using Platinum Pfx DNA polymerase with *exsD*_pUT18C_forward and *exsD*_pUT18C_reverse primers. The *exsD* gene was inserted in frame at 3' end of the T18 fragment, allowing ExsD protein fused to the C-terminal of the T18 fragment. Key features of the plasmid construct were shown in thick arrows while ORFs were shown in thin arrows. Primers were shown in purple. Created using SnapGene software

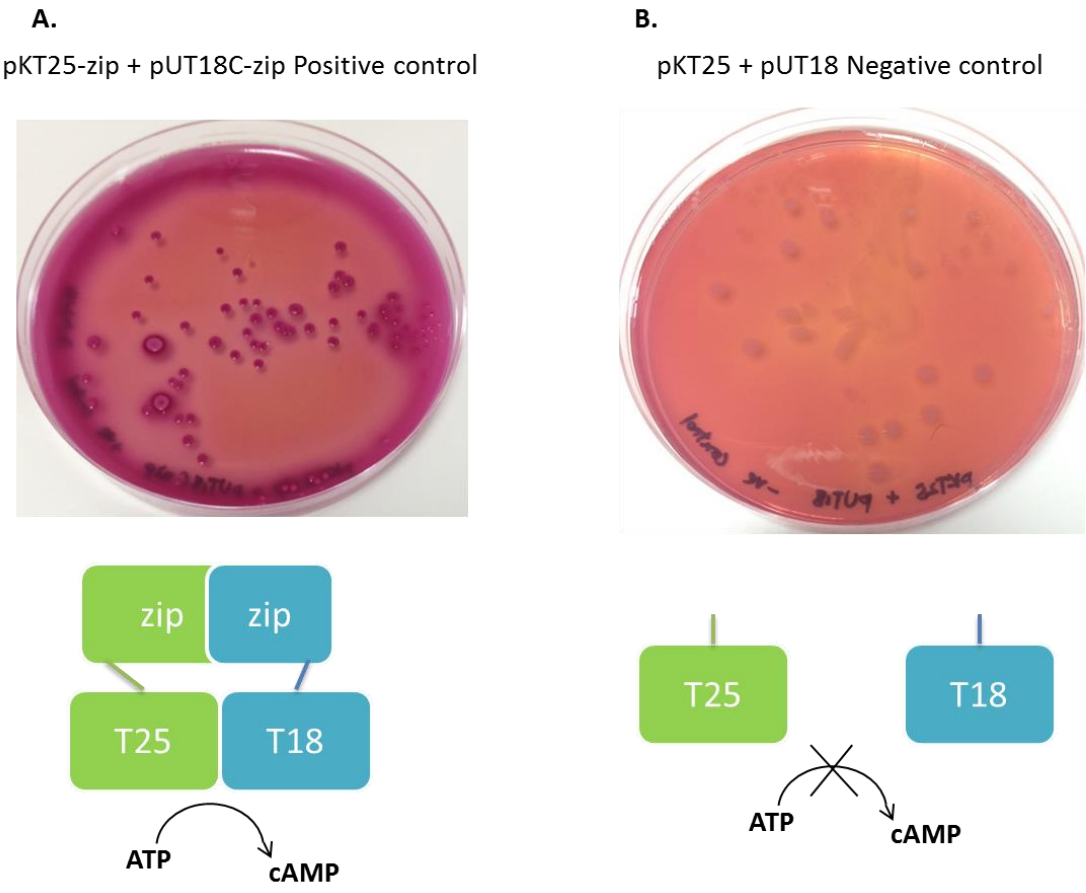


Figure 4.7 Illustration of the BACTH controls. **A.** Plasmid pKT25-zip and pUT18C-zip expressed fusion proteins T25-zip and T18-zip that interacted with each other through leucine zipper motifs, allowing complementation of T25 and T18 fragment to form a catalytic domain of adenylate cyclase (CyaA), thus cAMP was synthesized from ATP. When *E.coli* BTH101 containing these plasmids was grown on MacConkey-maltose agar for 2 days at 30°C, the MacConkey agar turned pink/red as the result of the activation of the *mal* operon. **B.** Plasmid pKT25 and pUT18 were co-transformed into *E.coli* BTH101 reporter strain as a negative control. T25 and T18 fragments were apart from each thus no complementation of the adenylate cyclase. Therefore the colonies and the MacConkey Agar remained white.

4.3 Investigation of the interactions between Exs proteins using BACTH

As the hypothesis suggested, the master regulator of the T3SS in the *A. hydrophila* AH3R strain was ExsA. It is thought to be able to self-interact for cooperative activation of the T3SS promoters as well as interacting with the anti-activator ExsD, while not interacting with ExsC or ExsE. When co-transforming pKT25-*exsA* with pUT18-*exsA* or pUT18C-*exsA*, strong interactions were shown in both cases. Moreover, when co-transforming pKNT25-*exsA* with pUT18-*exsA* or pUT18C-*exsA*, the interactions were very strong as well. Thereby only one figure of *exsA*-*exsA* self-interaction was shown here (Figure 4.8). The interactions between ExsA and ExsD were as strong as the self-interaction of ExsA. Each of the *exsA*-fused BACTH plasmid constructs showed positive interaction when co-transformed with each of the *exsD*-fused BACTH plasmid constructs, except for *exsD*-pKT25, which showed no interaction with either pUT18-*exsA* or pUT18C-*exsA* (Figure 4.9). Only pKT25-*exsA* with pUT18C-*exsD* is shown as an example in Figure 4.8. On the other hand, ExsA showed no interaction with ExsC in any case; the co-transformation of pUT18-*exsA* with pKNT25-*exsC* is shown as an example (Figure 4.8). Similarly, ExsA showed no interaction with ExsE in any case; pUT18C-*exsA* with pKNT25-*exsE* is shown as an example (Figure 4.8).

ExsD which had been shown to interact with ExsA, showed no evidence of ExsD-ExsD self-interaction when co-transforming pKT25-*exsD* or pKNT25-*exsD* with either pUT18-*exsD* or pUT18C-*exsD*. Only pUT18-*exsD* with pKT25-*exsD* is shown here as an example (Figure 4.10). However, ExsD was shown to interact with ExsC in every combination. Thus pUT18C-*exsD* with pKT25-*exsC* is shown as an example of strong ExsD-ExsC interaction (Figure 4.10). In contrast no evidence of ExsD-ExsE interaction was found in any case, as shown in co-transformation of pUT18C-*exsD* with pKNT25-*exsE* (Figure 4.10).

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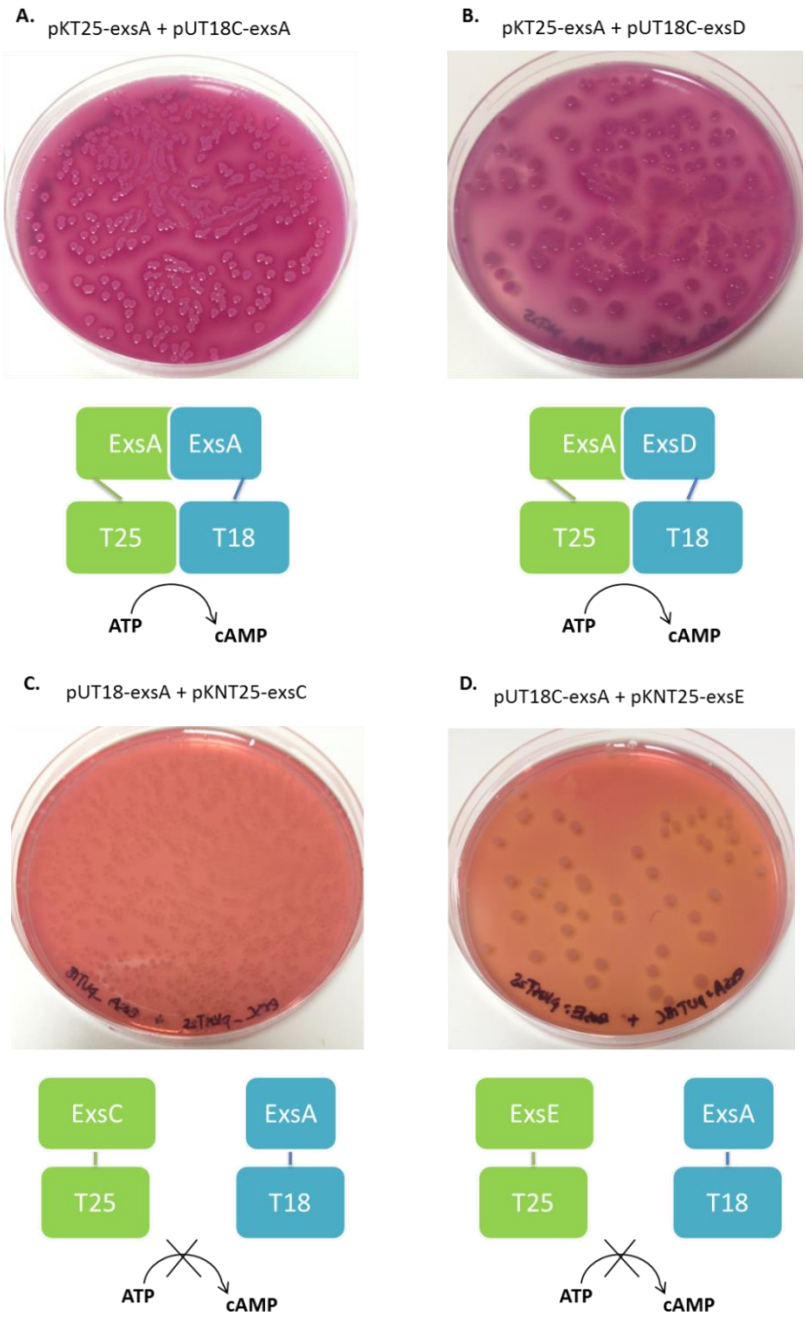

Figure 4.8 Illustration of BACTH interactions between ExsA and each of the Exs proteins. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A,** pKT25-*exsA* with pUT18C-*exsA* showing strong ExsA-ExsA self-interaction **B,** pKT25-*exsA* with pUT18C-*exsD* showing strong ExsA-ExsD interaction; **C,** pUT18-*exsA* with pKNT25-*exsC* showing no interaction between ExsA and ExsC; **D,** pUT18C-*exsA* with pKNT25-*exsE* showing no interaction between ExsA and ExsE. The BACTH assay was carried out three times for each combination.

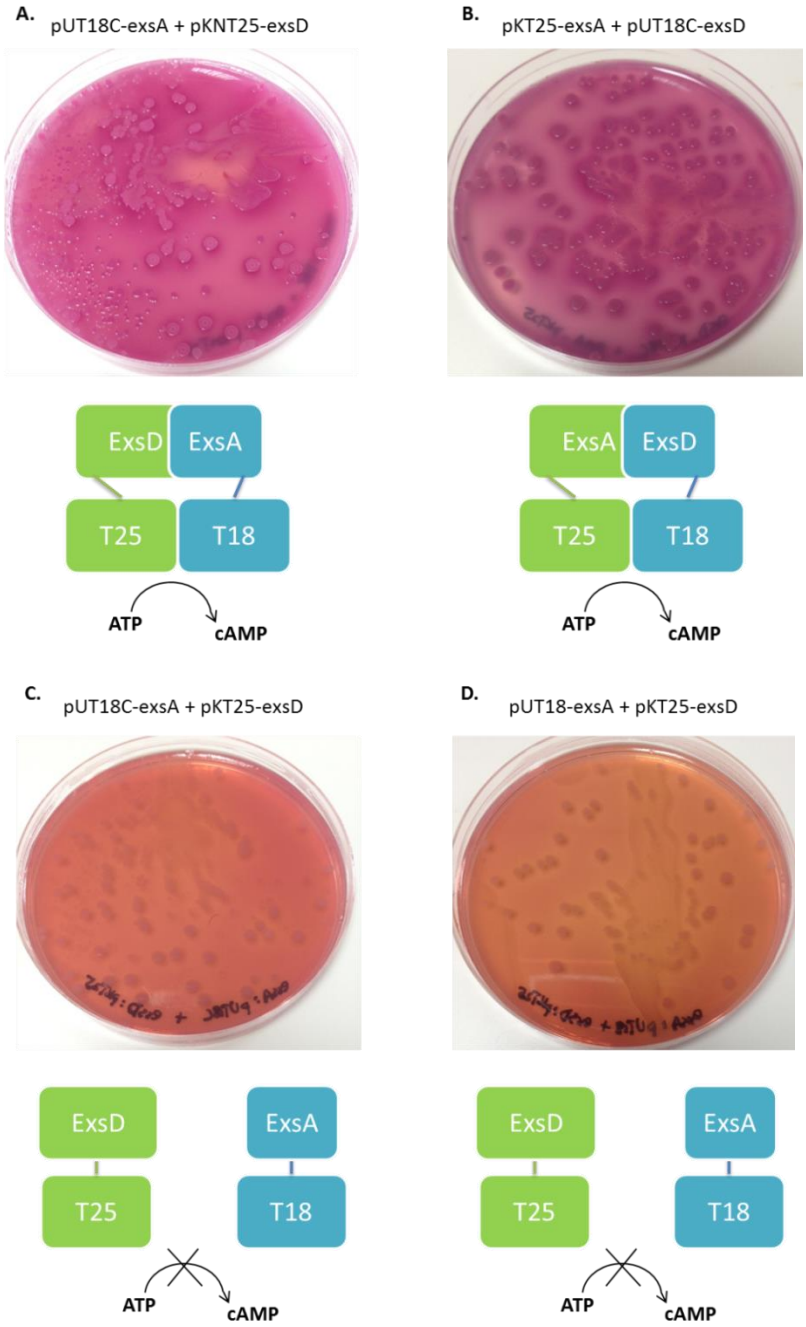

Figure 4.9 Illustration of BACTH interactions between ExsA and ExsD. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A,** pUT18C-*exsA* with pKNT25-*exsD* showed strong ExsA-ExsD interaction; **B,** pKT25-*exsA* with pUT18C-*exsD* showed strong ExsA-ExsD interaction as well; **C and D,** pKT25-*exsD* plasmid construct showed no ExsA-ExsD interaction when co-transformed with either pUT18-*exsA* or pUT18C-*exsA* plasmid construct. The BACTH assay was carried out three times for each combination.

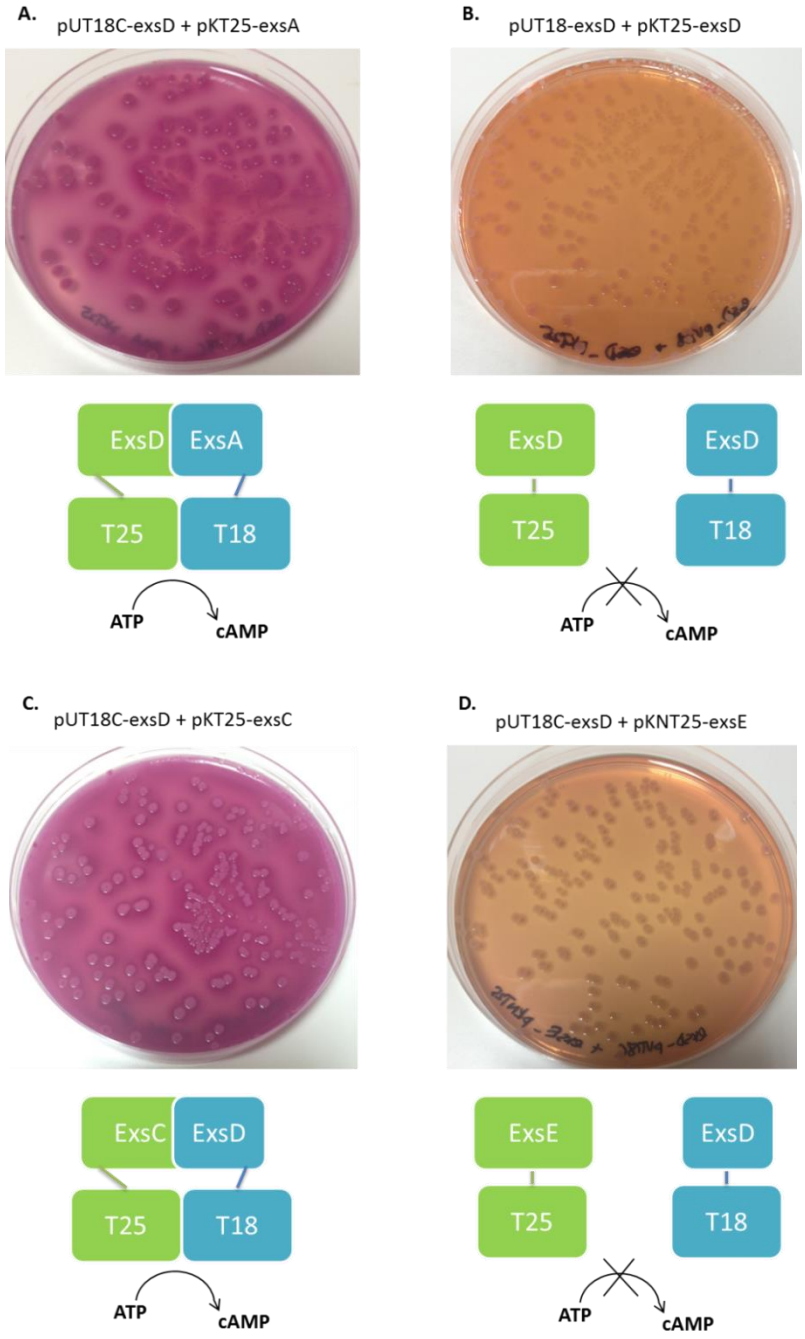

Figure 4.10 Illustration of BACTH interactions between ExsD and each of the Exs proteins. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A,** pUT18C-*exsD* with pKT25-*exsA* showed strong ExsD-ExsA interaction; **B,** pUT18-*exsD* with pKT25-*exsD* showed no ExsD-ExsD self-interaction; **C,** pUT18C-*exsD* with pKT25-*exsC* showed strong ExsD-ExsC interaction; **D,** pUT18C-*exsD* with pKNT25-*exsE* showed no evidence of ExsD-ExsE interaction. The BACTH assay was carried out three times for each combination.

Moreover, ExsC protein was shown not to interact with the ExsA protein in any combination of BACTH plasmid constructs. Co-transformation of pKT25-*exsC* with pUT18-*exsA* is shown here as an example of negative interaction (Figure 4.11). Strong ExsC-ExsD interaction was shown in every case; hereby pUT18-*exsC* with pKNT25-*exsD* is shown as an example (Figure 4.11). Although pKT25-*exsC* and pKNT25-*exsC* were shown to interact with pUT18-*exsC* and pUT18C-*exsC*, the pink/red colouration of MacConkey Agar in ExsC-ExsC self-interaction was much weaker compared to the colouration in ExsC-ExsD or ExsC-ExsE. The co-transformation of pUT18C-*exsC* with pKNT25-*exsC* into the *E. coli* BTH101 reporter strain is shown as an example (Figure 4.11). Very strong ExsC-ExsE interaction was observed in every combination of *exsC*-fused BACTH plasmid constructs with *exsE*-fused BACTH plasmid constructs. pKT25-*exsC* with pUT18C-*exsE* is shown as an example (Figure 4.11).

As for ExsE protein, which was shown to interact with ExsC protein only, the *exsE*-fused BACTH plasmid constructs showed no positive interaction when co-transformed with *exsA*-fused or *exsD*-fused BACTH plasmid constructs. The co-transformation of pUT18C-*exsE* with pKT25-*exsA* and pKNT25-*exsE* with pUT18C-*exsD* showed negative interaction in ExsE-ExsA and ExsE-ExsD (Figure 4.12). On the other hand, as demonstrated in ExsC interactions, ExsE-ExsC interaction was very strong in every case, as shown in pUT18-*exsE* with pKNT25-*exsC* co-transformation (Figure 4.12). However, ExsE-ExsE self-interaction was shown to be very weak, as no interaction was observed in co-transformation of pKT25-*exsE* with pUT18-*exsE* or pUT18C-*exsE*, while weak interaction was observed in co-transformation was observed in co-transformation of pKNT25-*exsE* with pUT18-*exsE* or pUT18C-*exsE* (Figure 4.12 and Figure 4.13).

A summary of the overall interactions between each two of the T3SS regulatory components (Exs proteins) are shown in Table 4.1. Strong interactions were

observed between ExsA-ExsA, ExsA-ExsD, ExsD-ExsC and ExsC-ExsE, which agreed with the hypothesis that ExsA-ExsD-ExsC-ExsE regulatory cascade was based on direct protein-protein interactions.

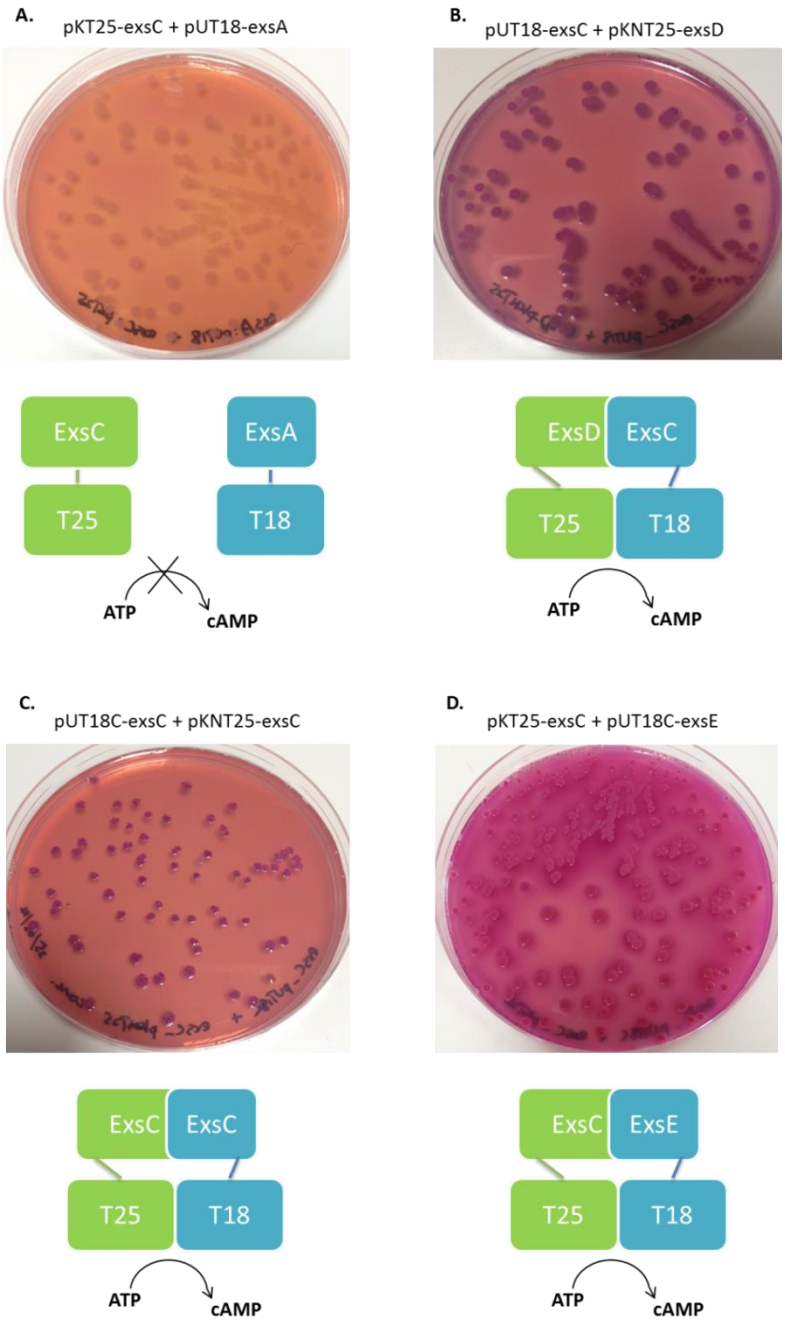

Figure 4.11 Illustration of BACTH interactions between ExsC and each of the Exs proteins. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A,** pKT25-*exsC* with pUT18-*exsA* showed no ExsC-ExsA interaction; **B,** pUT18-*exsC* with pKNT25-*exsD* showed strong ExsC-ExsD interaction; **C,** weak ExsC-ExsC self-interaction was shown as in pUT18C-*exsC* with pKNT25-*exsC* co-transformation; **D,** pKT25-*exsC* with pUT18C-*exsE* showed very strong ExsC-ExsE interaction. The BACTH assay was carried out three times for each combination.

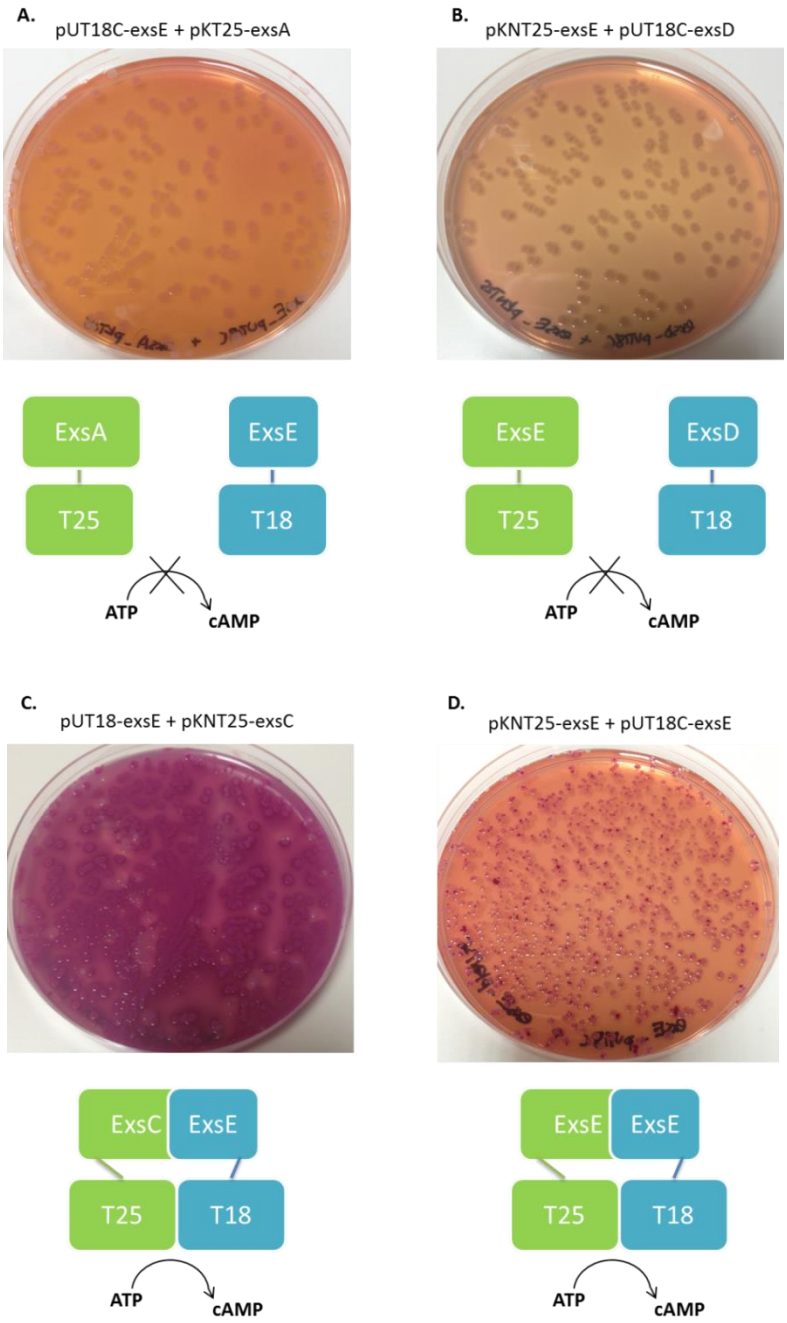

Figure 4.12 Illustration of BACTH interaction between ExsE and each of the Exs proteins. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A,** pUT18C-*exsE* with pKT25-*exsA* showed no ExsE-ExsA interaction; **B,** no ExsE-ExsD interaction was shown as in pKNT25-*exsE* with pUT18C-*exsD*; **C,** very strong ExsE-ExsC was shown as in pUT18-*exsE* with pKNT25-*exsC*; **D,** weak ExsE-ExsE self-interaction was shown as in pKNT25-*exsE* with pUT18C-*exsE*. The BACTH assay was carried out three times for each combination.

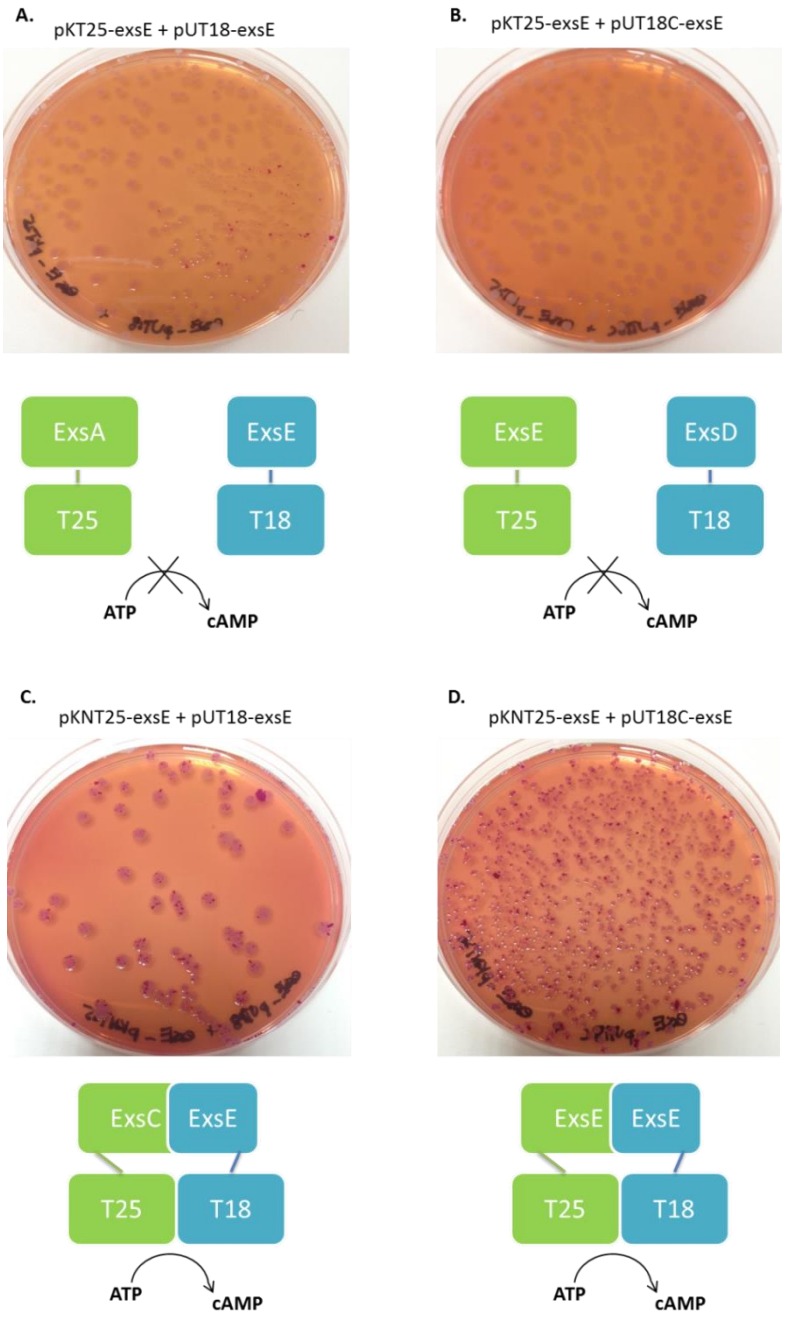

Figure 4.13 Illustration of BACTH assay of ExsE-ExsE self-interaction. Plasmid combinations were co-transformed into *E. coli* BTH101 and grown on MacConkey-maltose agar for 2 days at 30°C. **A and B,** no interaction was observed in co-transformation of pKT25-*exsE* with pUT18-*exsE* or pUT18C-*exsE*; **C and D,** weak ExsE-ExsE self-interaction was shown in co-transformation of pKNT25-*exsE* with pUT18-*exsE* or pUT18C-*exsE*. The BACTH assay was carried out three times for each combination.

		T25-ExsA	ExsA-T25	T25-ExsD	ExsD-T25	T25-ExsC	ExsC-T25	T25-ExsE	ExsE-T25
		pKT25-exsA	pKNT25-exsA	pKT25-exsD	pKNT25-exsD	pKT25-exsC	pKNT25-exsC	pKT25-exsE	pKNT25-exsE
ExsA-T18	pUT18-exsA	+	+	-	+	-	-	-	-
T18-ExsA	pUT18C-exsA	+	+	-	+	-	-	-	-
ExsD-T18	pUT18-exsD	+	+	-	-	+	+	-	-
T18-ExsD	pUT18C-exsD	+	+	-	-	+	+	-	-
ExsC-T18	pUT18-exsC	-	-	+	+	+/-	+/-	+	+
T18-ExsC	pUT18C-exsC	-	-	+	+	+/-	+/-	+	+
ExsE-T18	pUT18-exsE	-	-	-	-	+	+	-	+/-
T18-ExsE	pUT18C-exsE	-	-	-	-	+	+	-	+/-

Table 4.1 Investigation of the interactions between each two of the Exs proteins using BACTH system. Positive interactions were shown in '+' while negatives were shown in '-', weak interactions were shown in '+/-'. The orientation (from N-terminal to C-terminal) of the T25/T18 fusion was shown in green boxes. Positive interactions were observed in ExsA-ExsA, ExsA-ExsD, ExsD-ExsC and ExsC-ExsE, which agreed with the hypothesis of ExsA-ExsD-ExsC-ExsE regulatory cascade of the T3SS in *A. hydrophila* AH3R.

4.4 Overexpression of Exs proteins using the pET system

Apart from the BACTH assay, the *in-vitro* investigation of the protein-protein interactions in the regulatory cascade of the T3SS in *A. hydrophila* AH3R requires the overexpression of the Exs proteins.

The over-expression vector pET28a was used to over-express Exs proteins. Since a pET28*exsA* plasmid construct had already been created in the lab (Shaw, unpublished), only the plasmid construct pET28*exsD*, pET28*exsC* and pET28*exsE* were required. To begin with, the T3SS regulatory genes *exsD*, *exsC* and *exsE*, were amplified independently by PCR from the *A. hydrophila* AH3R chromosomal DNA using Platinun Pfx DNA polymerase. The primers used to amplify these genes (Appendix 1) introduced a start codon and a stop codon at either end of each gene allowing in-frame cloning into the pET28a vector to ensure an N-terminal His-tag. The PCR products of the *exs* genes were then digested by *Nde*I and *Hin*dIII (*Nde*I and *EcoR*I for *exsE*) and ligated with the pET28a plasmids digested by the same pair of restriction enzymes (Figure 4.14). The ligations were transformed into *E.coli* DH5α competent cells and kanamycin resistant colonies were screened by colony PCR screen using T7 promoter (forward) and T7 terminator (reverse) primers (Appendix 1). Plasmid constructs pET28*exsC*, pET28*exsD* and pET28*exsE* screened with successful inserts were extracted from the E. coli DH5α cells (Figure 4.15).

As all *exs* genes were cloned into the pET28a plasmids, they were then sequenced using T7 promoter (forward) and T7 terminator (reverse) primers to ensure the in-frame insertion, the orientation of the inserts and that no mutation was present in the inserts. After examination with sequencing and BLAST search, all plasmids were then transformed into *E. coli* over-expression strains to overexpress the Histidine-tagged Exs proteins.

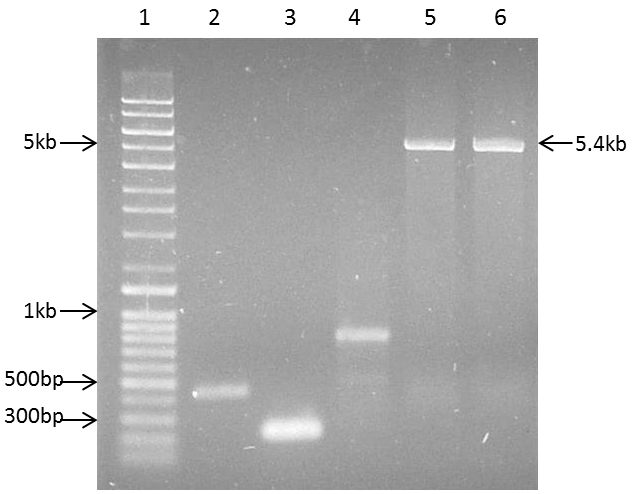

Figure 4.14 A 1% agarose gel showing the double-digested *exsC*, *exsE*, *exsD* and pET28a. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 2, *Nde*I and *Hin*dIII digested *exsC* fragment (~450bp); Lane 3, *Nde*I and *Eco*RI digested *exsE* fragment (~250bp); Lane 4, *Nde*I and *Hin*dIII digested *exsD* fragment (~800bp); Lane 5, *Nde*I and *Hin*dIII digested pET28a plasmid vector (~5.4kb); Lane 6, *Nde*I and *Eco*RI digested pET28a plasmid vector (~5.4kb).

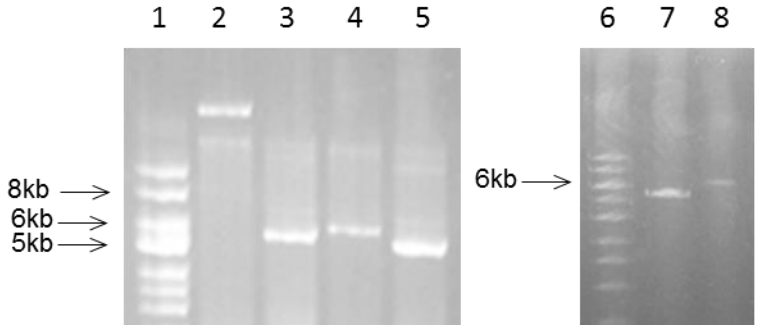

Figure 4.15 A 1% agarose gel showing the plasmid constructs of pET28*exsA*, pET28*exsC*, pET28*exsD* and pET28*exsE*. Lane 1, Promega™ Supercoiled DNA Ladder; Lane 2, plasmid construct pET28*exsA* (~6.2kb), which was possibly dimerized; Lane 3, plasmid construct pET28*exsC* (~5.8kb); Lane 4, plasmid construct pET28*exsD* (~6.2kb); Lane 5, plasmid construct pET28*exsE* (~5.6kb); Lane 6, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 7, empty pET28a vector linearized by *Hin*dIII digestion (~5.4kb); Lane 8, *Hin*dIII-digested plasmid construct pET28*exsA* (~6.2kb).

Because the over-expression of His-tagged Exs proteins was unstable in *E.coli* BL21(DE3) and C41(DE3) strains, the *E.coli* BL21Star™ (DE3) strain was used for stable overexpression of the Exs proteins. Due to the mutation in the RNaseE gene (*rne131*) of the *E.coli* BL21Star™ (DE3) strain, mRNA stability was increased and protein overexpression was enhanced.

The His-tagged Exs protein over-expression was carried out following the methods in section 2.14. After sonication, both the soluble fraction and the insoluble fraction were loaded on SDS-PAGE gel to determine the solubility of the His-tagged Exs proteins (Figure 4.16). However, all of the His-tagged Exs proteins were present in the insoluble fraction. Several approaches were tried to solubilize the His-tagged Exs proteins, such as inclusion body preparation, but after dialyzing out the guanidine HCl or Urea, the proteins precipitated again and were not soluble until at least 3.2M Urea was added. Thereby 3.2M Urea was added to the Binding Buffer to solubilize the His-tagged Exs proteins for protein purification by affinity chromatography (section 2.16). After solubilizing the His-tagged Exs proteins in 3.2M Urea, the samples were centrifuged at 30,000xg for 30min at 4°C to obtain the supernatant crude extracts, which were then loaded on HisTrap HP Column (GE Healthcare™) for protein purification.

During protein purification of the His-tagged Exs proteins from the nickel column, the samples were collected at each point, such as crude extract, flow-through, wash fraction and elutions with different concentration of imidazole-added Binding Buffer (imidazole-BB). Then 10µl of each sample was loaded on a 12% SDS-PAGE gel (section 2.15) together with 1X Laemmli Buffer to determine the purified His-tagged Exs proteins (Figure 4.17, Figure 4.18, Figure 4.19 and Figure 4.20).

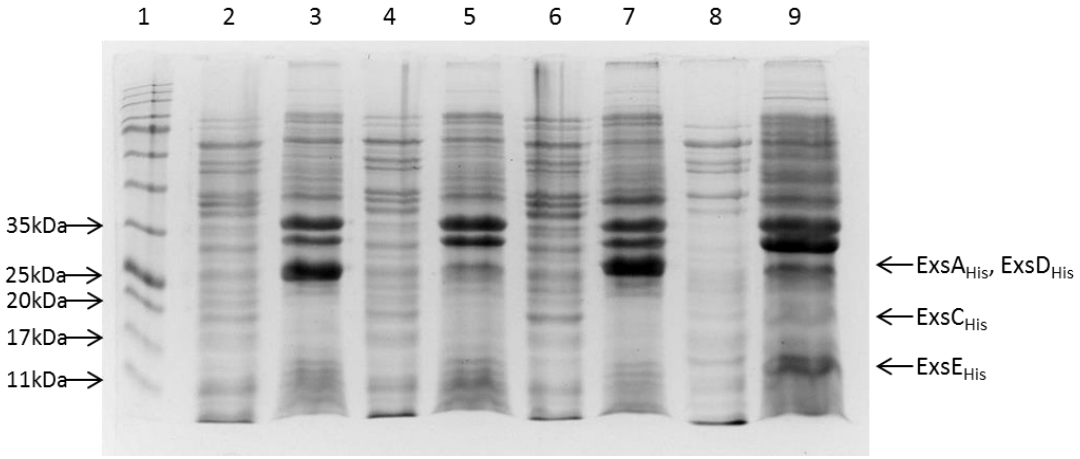

Figure 4.16 A 12% SDS-PAGE gel showing the solubility of the His-tagged Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, soluble fraction of $_{His}$ExsA overexpression; Lane 3, insoluble fraction of $_{His}$ExsA overexpression (~33kDa); Lane 4, soluble fraction of $_{His}$ExsC overexpression; Lane 5, insoluble fraction of $_{His}$ExsC overexpression (~18.5kDa); Lane 6, soluble fraction of $_{His}$ExsD overexpression; Lane 7, insoluble fraction of $_{His}$ ExsDoverexpression (~33kDa); Lane 8, soluble fraction of $_{His}$ExsE overexpression; Lane 9, insoluble fraction of $_{His}$ExsE overexpression (~11kDa). All of the His-tagged Exs proteins were present in the insoluble fraction. The $_{His}$ExsC was hardly visible on this gel, but present later on after protein purification.

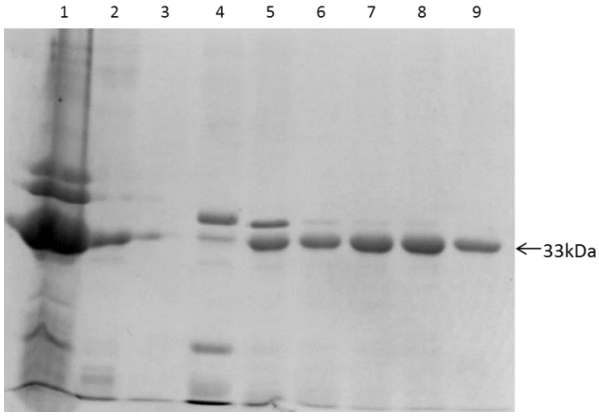

Figure 4.17 A 12% SDS-PAGE gel showing the purification of $_{His}$ExsA protein. Lane 1, the crude extract of $_{His}$ExsA; Lane 2, the flow-through collected when loading crude extract; Lane 3, the wash fraction collected when washing the nickel column with Binding Buffer (BB); Lane 4, elution with 100mM imidazole-BB; Lane 5, elution with 150mM imidazole-BB; Lane 6, elution with 200mM imidazole-BB; Lane 7, elution with 300mM imidazole-BB; Lane 8, elution with 400mM imidazole-BB; Lane 9, elution with 500mM imidazole-BB. The purified $_{His}$ExsA (~33kDa) was obtained from lane 7, 8 and 9.

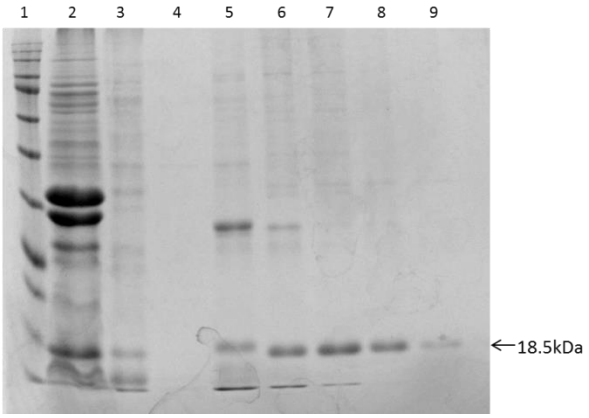

Figure 4.18 A 12% SDS-PAGE gel showing the purification of $_{His}$ExsC protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, the crude extract of $_{His}$ExsC; Lane 3, the flow-through collected when loading crude extract; Lane 4, the wash fraction collected when washing the nickel column with Binding Buffer (BB); Lane 5, elution with 100mM imidazole-BB; Lane 6, elution with 200mM imidazole-BB; Lane 7, elution with 300mM imidazole-BB; Lane 8, elution with 400mM imidazole-BB; Lane 9, elution with 500mM imidazole-BB. The purified $_{His}$ExsC (~18.5kDa) was obtained from lane 7, 8 and 9.

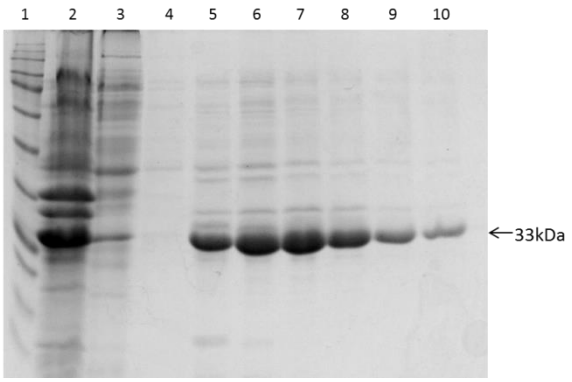

Figure 4.19 A 12% SDS-PAGE gel showing the purification of $_{His}$ExsD protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, the crude extract of $_{His}$ExsD; Lane 3, the flow-through collected when loading crude extract; Lane 4, the wash fraction collected when washing the nickel column with Binding Buffer (BB); Lane 5, elution with 100mM imidazole-BB; Lane 6, elution with 150mM imidazole-BB; Lane 7, elution with 200mM imidazole-BB; Lane 8, elution with 300mM imidazole-BB; Lane 9, elution with 400mM imidazole-BB; Lane 10, elution with 500mM imidazole-BB. The purified $_{His}$ExsD (~33kDa) was obtained from lane 9 and 10.

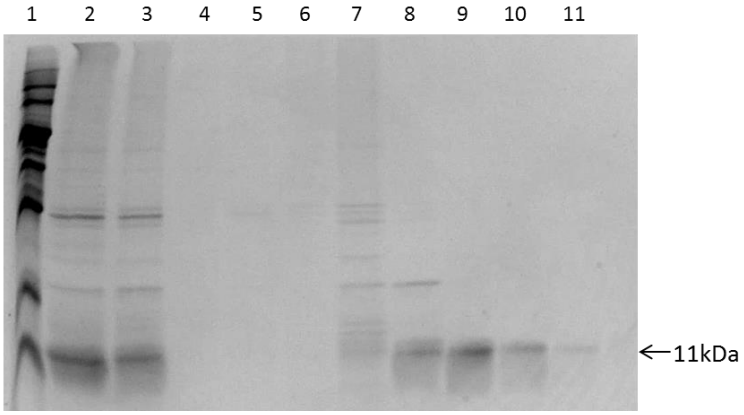

Figure 4.20 A 12% SDS-PAGE gel showing the purification of $_{His}$ExsE protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2 and 3, the crude extract of $_{His}$ExsD; Lane 4, the flow-through collected when loading crude extract; Lane 5, the wash fraction collected when washing the nickel column with Binding Buffer (BB); Lane 6, elution with 100mM imidazole-BB; Lane 7, elution with 150mM imidazole-BB; Lane 8, elution with 200mM imidazole-BB; Lane 9, elution with 300mM imidazole-BB; Lane 10, elution with 400mM imidazole-BB; Lane 11, elution with 500mM imidazole-BB. The purified $_{His}$ExsE (~11kDa) was obtained from lane 9, 10 and 11.

4.5 Overexpression of Exs proteins using the pMAL™ system

In order to determine the protein-protein interactions, the proteins were needed to be tagged with another detectable domain that could also possibly enhance the protein solubility. Therefore the maltose-binding protein (MBP) tag was chosen.

To overexpress MBP-fused Exs proteins using pMAL™ Protein Fusion and Purification System, the genes encoding the Exs proteins were required to be cloned into the pMAL-c5X plasmid. Since the *exs* genes had already been inserted in-frame with the pET28a plasmids vector, it was easier to cut out the *exs* inserts from the pET28*exs* plasmid constructs and then ligated into digested pMAL-c5X, where they would be inserted in-frame to create an N-terminal MBP-fusion protein. The plasmid constructs pET28*exsA*, pET28*exsC*, pET28*exsD* and pET28*exsE* were digested with *Nde*I and *Eco*RI, then the inserts were gel extracted (section 2.4.3) to obtain double-digested *exs* fragments. In the meantime, the pMAL-c5X plasmid vector was digest with *Nde*I and *Eco*RI as well to allow ligation with the double-digested *exs* fragments. After ligation, ampicillin resistant colonies were screened by colony PCR screen using pMAL screening primers (Appendix 1) with Taq DNA polymerase (Figure 4.21). Once all *exs* genes were cloned into the pMAL-c5X plasmid vector, they were 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, all plasmids were then transformed into *E. coli* ER2523 (NEB Express) for the over-expression of the MBP-fused Exs proteins.

Once transformed, The *E. coli* expression strain ER2523 was induced with 0.3mM IPTG for 2 hours at 37°C. The cells were harvested and sonicated followed by centrifugation to separate soluble and insoluble fractions. The whole-cell extracts, soluble fractions and insoluble fractions were loaded on a 12% SDS-PAGE gel to find out the solubility of the MBP-fused Exs proteins. The solubility was double

checked with Western Blot using murine anti-MBP antibody and HRP-conjugated goat anti-mouse IgG (Figure 4.22). Most of the MBP-fused Exs proteins were present in the soluble fraction while only MBP5 protein, which was expressed by the MBP-encoding gene on the pMAL-c5X plasmids, was present in the insoluble fractions (Figure 4.22).

The soluble MBP-fused Exs proteins were then purified by affinity chromatography using MBPTrap HP columns supplied from GE Healthcare™ (section 2.17). The MBP-Exs fusion proteins were eluted from the MBPTrap HP columns by 10mM maltose and each fraction of the elution was collected together with the flow-through and the wash fraction. Then 10µl of each sample was mixed with 1X Laemmli Buffer to be loaded on SDS-PAGE gels (Figure 4.23, Figure 4.24, Figure 4.25 and Figure 4.26).

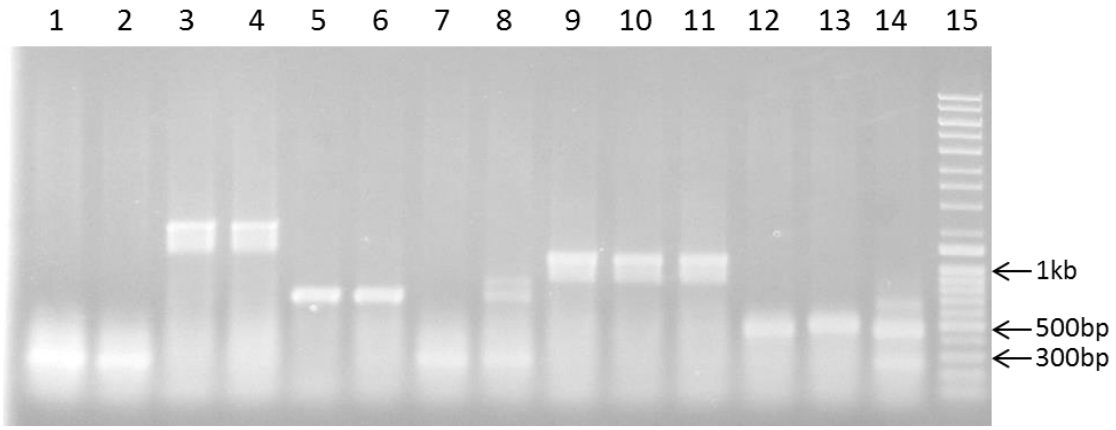

Figure 4.21 A 1% agarose gel showing the colony PCR screen of the pMAL*exs* plasmid constructs. Lane 1-4, colony PCR screen of pMAL*exsA*, in which lane 3 and 4 were potential positive constructs with an extra 900bp of the *exsA* inserts; Lane 5-8, colony PCR screen of pMAL*exsC*, in which lane 5 and 6 were potential positive constructs with an extra 500bp of the *exsC* inserts; Lane 9-11, colony PCR screen of pMAL*exsD*, in which all lanes were potential positive constructs with an extra 800bp of the *exsD* inserts; Lane 12-14, colony PCR screen of pMAL*exsE*, in which lane 12 and 13 were potential positive constructs with an extra 200bp of the *exsE* inserts.

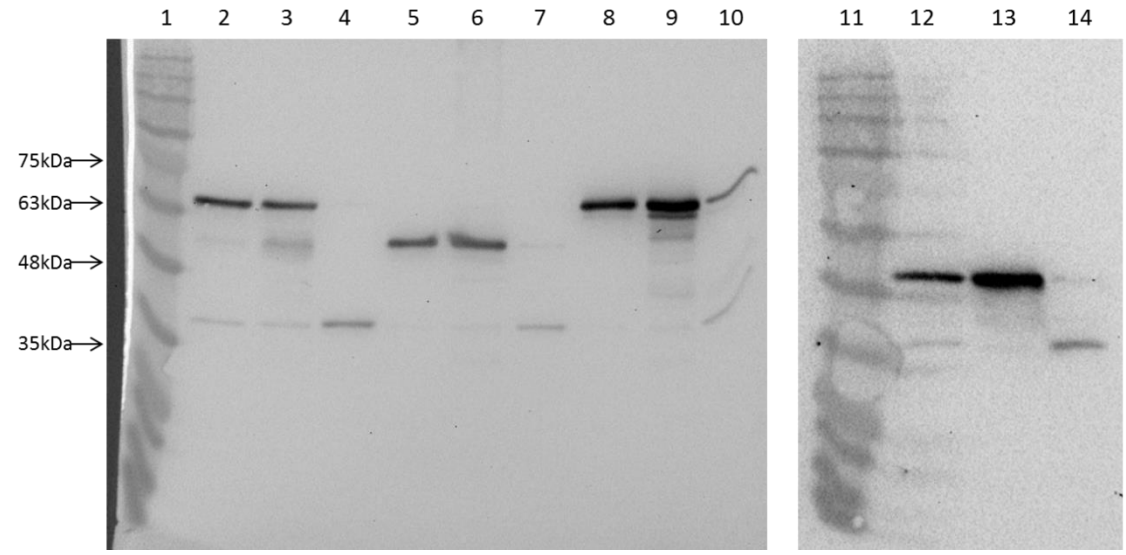

Figure 4.22 Western Blot using murine anti-MBP antibody to show the solubility of the MBP-fused Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, whole-cell extract of MBP-ExsA from NEB express; Lane 3, soluble fraction of MBP-ExsA, the expected size was 75kDa, which comprised of the size of ExsA (~33kDa) and MBP5 (~42kDa); Lane 4, insoluble fraction of MBP-ExsA; Lane 5, whole-cell extract of MBP-ExsC; Lane 6, soluble fraction of MBP-ExsC (~61kDa, 19kDa+42kDa); Lane 7, insoluble fraction of MBP-ExsC; Lane 8, whole-cell extract of MBP-ExsD; Lane 9, soluble fraction of MBP-ExsD (~75kDa, 33kDa+42kDa); Lane 10, insoluble fraction of MBP-ExsC; Lane 11, BLUeye Prestained Protein Ladder (Geneflow); Lane 12, whole-cell extract of MBP-ExsE; Lane 13, soluble fraction of MBP-ExsE (~53kDa, 11kDa+42kDa); Lane 14, insoluble fraction of MBP-ExsE. The sizes of MBP-ExsA and MBP-ExsD were smaller than expected, which might be because the exact size of a fusion protein was not equal to the sum of its protein components.

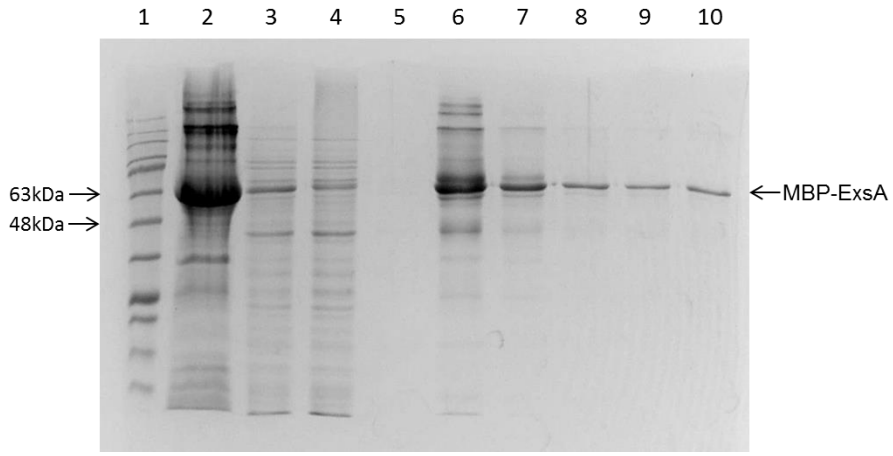

Figure 4.23 A 10% SDS-PAGE gel showing the purification of MBP-ExsA fusion protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, crude extract of MBP-ExsA before purification; Lane 3 and 4, the flow-through collected when loading crude extract; Lane 5, the wash fraction collected when wash the column with Column Buffer (section 2.17.1); Lane 6-10, elution with 10mM maltose. Purified MBP-ExsA fusion protein (~75kDa) was obtained from lane 8, 9 and 10.

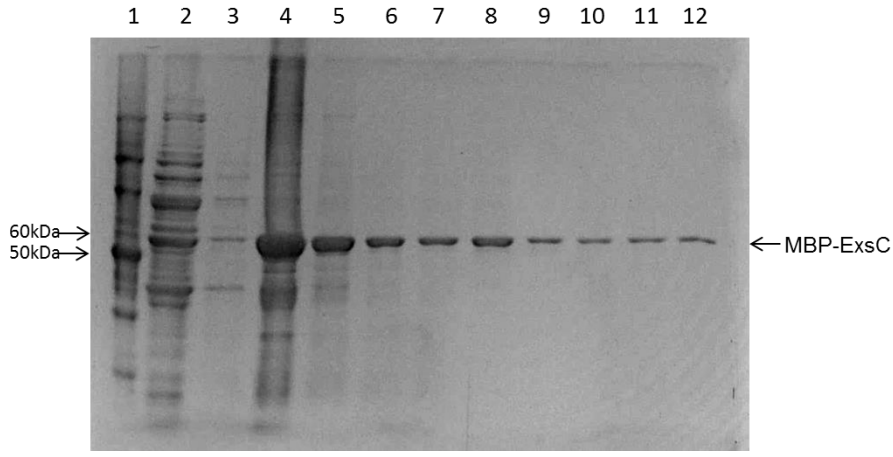

Figure 4.24 A 10% SDS-PAGE gel showing the purification of MBP-ExsC fusion protein. Lane 1, PageRuler Unstained Protein Ladder (Thermo Scientific); Lane 2, crude extract of MBP-ExsA before purification; Lane 3, the flow-through collected when loading crude extract; Lane 4, the wash fraction collected when wash the column with Column Buffer; Lane 5-12, elution with 10mM maltose. Purified MBP-ExsC fusion protein (~61kDa) was obtained from lane 6-12.

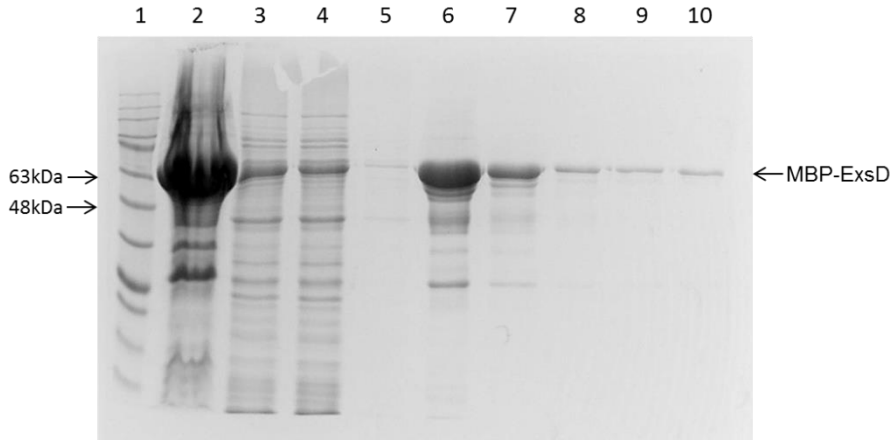

Figure 4.25 A 10% SDS-PAGE gel showing the purification of MBP-ExsD fusion protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, crude extract of MBP-ExsD before purification; Lane 3 and 4, the flow-through collected when loading crude extract; Lane 5, the wash fraction collected when wash the column with Column Buffer; Lane 6-10, elution with 10mM maltose. Purified MBP-ExsD fusion protein (~75kDa) was obtained from lane 8, 9 and 10.

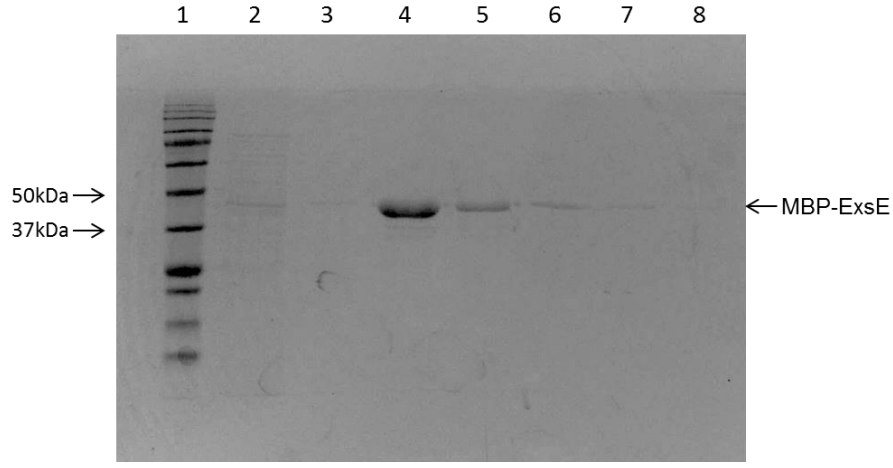

Figure 4.26 A 10% SDS-PAGE gel showing the purification of MBP-ExsE fusion protein. Lane 1, Precision Plus Protein™ Unstained standards (Bio-Rad); Lane 2, the flow-through collected when loading crude extract; Lane 3, the wash fraction collected when wash the column with Column Buffer; Lane 4-8, elution with 10mM maltose. Purified MBP-ExsE fusion protein (~53kDa) was obtained from lane 4-6.

4.6 Investigation of the Exs protein interactions using Far-Western Blot

As both the His-tagged and MBP-fused Exs proteins were obtained, the interactions between each of the Exs proteins were determined using Far-Western Blot (section 2.19). In Far-Western Blot, the target proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane. Then the target proteins were probed with a putative interacting protein, which had a different tag with the target proteins on the nitrocellulose membrane. The interactions between two proteins could then be detected by Western Blot using antibodies against the tagged putative interacting protein.

In this case, the purified His-tagged Exs proteins were loaded on an SDS-PAGE gel together with a MBP-fused Exs protein as a positive control (Figure 4.27). They were then transferred onto a nitrocellulose membrane, which was probed with the MBP5 protein without Exs fusion as a negative control. The over-expression and purification of the MBP5 protein were carried out following the exact methods used for preparation of MBP-fused Exs proteins (Figure 4.28). Once the nitrocellulose membrane was probed with the MBP5 protein, the murine anti-MBP antibody was used followed by HRP-conjugated goat anti-mouse IgG to allow the detection of the MBP fusions (Figure 4.29). Only the positive control MBP-ExsC fusion protein was detected by anti-MBP antibody when probed with MBP5 protein, suggesting that the His-tagged Exs proteins were unable to interact with MBP5 protein without Exs fusions.

Then the nitrocellulose membrane with His-tagged Exs proteins was probed with MBP-ExsA fusion protein to investigate the interactions between ExsA and each of the Exs proteins (Figure 4.30). The $_{His}$ExsD protein was detected in the Far-Western Blot when probed with MBP-ExsA, indicating the interaction between ExsA and ExsD, although the interaction appeared weak. No interaction with ExsC or ExsE was seen. Furthermore, no self interaction with ExsA was observed.

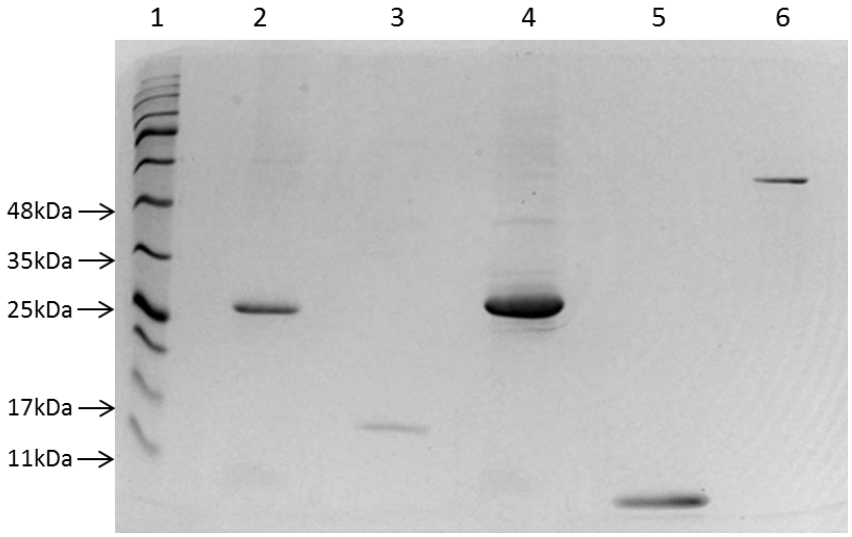

Figure 4.27 A 12% SDS-PAGE gel showing the purified Exs proteins and a MBP-fused Exs protein as positive control for Far-Western Blot. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified $_{His}$ExsA protein (~33kDa); Lane 3, purified $_{His}$ExsC protein (~18.5kDa); Lane 4, purified $_{His}$ExsD protein (~33kDa); Lane 5, purified $_{His}$ExsE protein (~11kDa); Lane 6, purified MBP-ExsC protein (~61kDa). The proteins on this SDS-PAGE gel were then transferred onto a nitrocellulose membrane for Far-Western Blot.

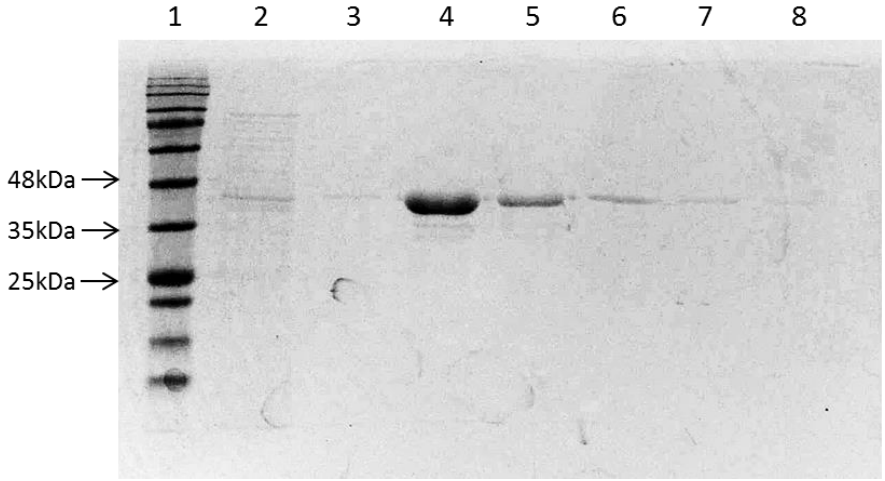

Figure 4.28 A 10% SDS-PAGE gel showing the purification of the MBP5 protein. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, the flow-through collected when loading crude extract; Lane 3, the wash fraction collected when wash the column with Column Buffer; Lane 4-8, elutions with 10mM maltose. Purified MBP5 protein (~42.5kDa) without Exs fusion was obtained from lane 4-6.

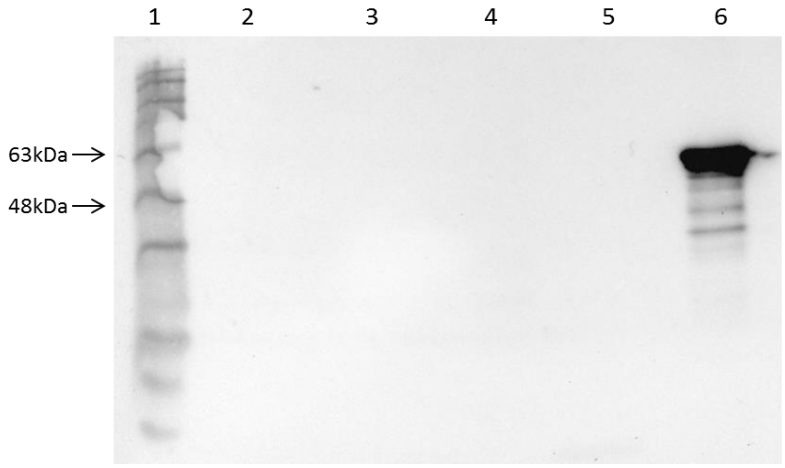

Figure 4.29 Far-Western Blot of MBP5 probing His-tagged Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified $_{His}$ExsA protein (~33kDa); Lane 3, purified $_{His}$ExsC protein (~18.5kDa); Lane 4, purified $_{His}$ExsD protein (~33kDa); Lane 5, purified $_{His}$ExsE protein (~11kDa); Lane 6, purified MBP-ExsC protein (~61kDa) as a positive control. Only MBP-ExsC was observed after blotting with murine anti-MBP antibody, suggesting that MBP5 protein without Exs fusions was unable to interact with His-tagged Exs proteins.

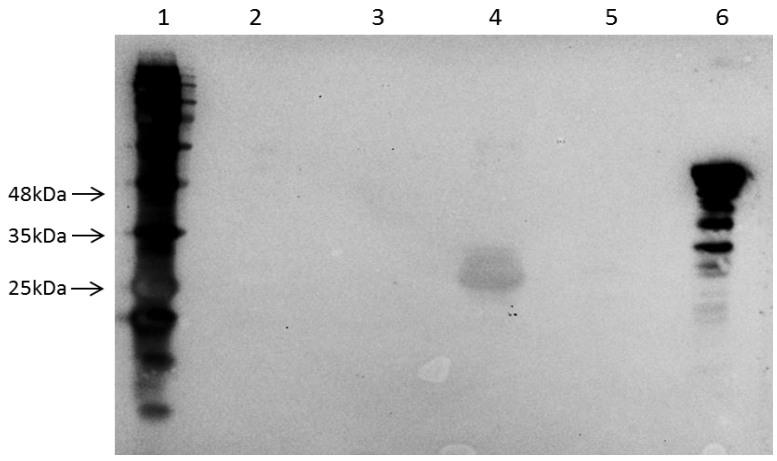

Figure 4.30 Far-Western Blot of MBP-ExsA probing His-tagged Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified $_{His}$ExsA protein (~33kDa); Lane 3, purified $_{His}$ExsC protein (~18.5kDa); Lane 4, purified $_{His}$ExsD protein (~33kDa); Lane 5, purified $_{His}$ExsE protein (~11kDa); Lane 6, purified MBP-ExsC protein (61kDa) as a positive control. The $_{His}$ExsD protein was detected, suggesting ExsA-ExsD interaction.

When probing the His-tagged Exs proteins with MBP-ExsC in the Far-Western Blot, the $_{His}$ExsD and $_{His}$ExsE were detected by murine anti-MBP antibody and HRP-conjugated goat anti-mouse IgG (Figure 4.31). There were two bands of different sizes detected for $_{His}$ExsD, one was the size of the $_{His}$ExsD (~33kDa) while the other one was possibly the size of dimerized $_{His}$ExsD (~66kDa). Apart from $_{His}$ExsD, $_{His}$ExsE was also detected when probed with MBP-ExsC, suggesting ExsC-ExsD interaction and ExsC-ExsE interaction. No interaction was seen for ExsA or self-interaction with ExsC.

However, when MBP-ExsD was used to probe His-tagged Exs proteins, nothing was detected except for the positive control. Thereby, the Far-Western Blot was carried out reversely, using $_{His}$ExsD to probe the MBP-fused Exs proteins. Thus MBP-fused Exs proteins were loaded on the SDS-PAGE gel and transferred onto the nitrocellulose membrane, which was probed with $_{His}$ExsD followed by detection using mouse anti-His antibody and HRP-conjugated goat anti-mouse IgG (Figure 4.32). Only MBP-ExsC protein was detected apart from the positive control, showing the interaction between ExsD and ExsC.

In the meantime, the Far-Western Blot of MBP-ExsE probing His-tagged Exs proteins was carried out, in which the $_{His}$ExsC and the $_{His}$ExsE proteins were detected by anti-MBP antibody and HRP-conjugated goat anti-mouse IgG (Figure 4.33). The presence of $_{His}$ExsC and the $_{His}$ExsE proteins in the Far-Western Blot probed with MBP-ExsE protein suggested the interactions between ExsE and ExsC as well as the ExsE-ExsE self-interaction.

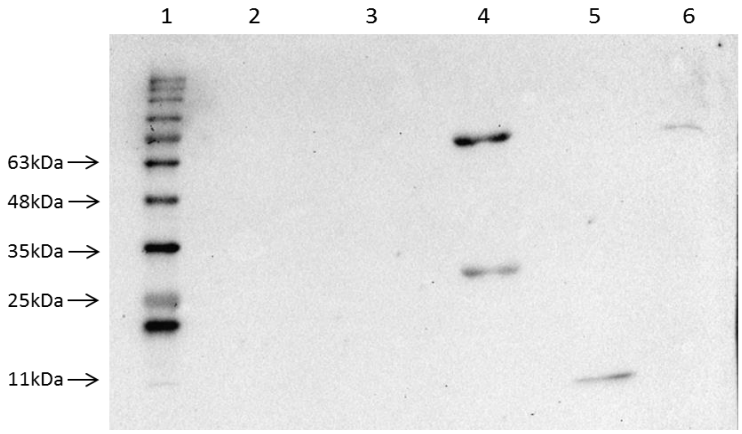

Figure 4.31 Far-Western Blot of MBP-ExsC probing His-tagged Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified $_{His}$ExsA protein (~33kDa); Lane 3, purified $_{His}$ExsC protein (~18.5kDa); Lane 4, purified $_{His}$ExsD protein (~33kDa); Lane 5, purified $_{His}$ExsE protein (~11kDa); Lane 6, purified MBP-ExsC protein (~61kDa) as a positive control. The $_{His}$ExsD and $_{His}$ExsE proteins were detected, suggesting ExsC-ExsD and ExsC-ExsE interactions. The upper band showing in lane 4 was possibly the size of a dimerized $_{His}$ExsD (~66kDa), implying possible dimerization of ExsD protein in the presence of ExsC.

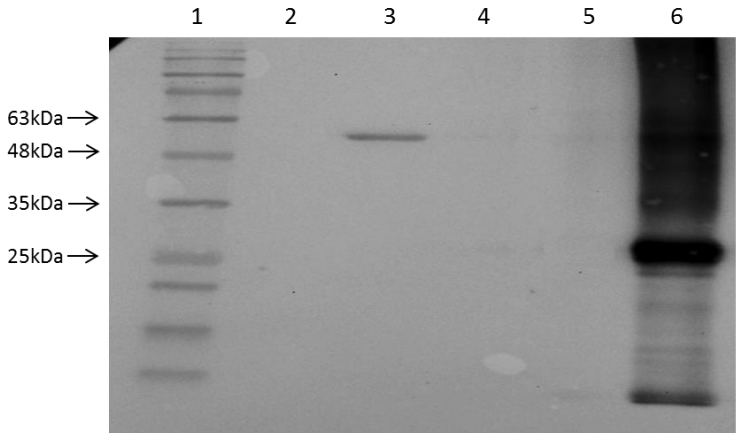

Figure 4.32 Far-Western Blot of $_{His}$ExsD probing MBP-fused Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified MBP-ExsA protein (~75kDa); Lane 3, purified MBP-ExsC protein (~61kDa); Lane 4, purified MBP-ExsD protein (~75kDa); Lane 5, purified MBP-ExsE protein (~53kDa); Lane 6, purified $_{His}$ExsD protein (~61kDa) as a positive control. Only MBP-ExsC was detected when probed with $_{His}$ExsD protein.

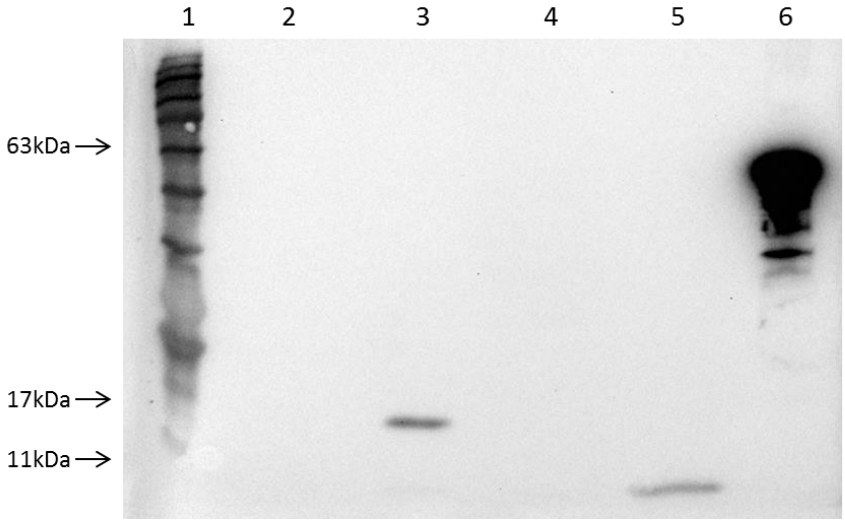

Figure 4.33 Far-Western Blot of MBP-ExsE probing His-tagged Exs proteins. Lane 1, BLUeye Prestained Protein Ladder (Geneflow); Lane 2, purified $_{His}$ExsA protein (~33kDa); Lane 3, purified $_{His}$ExsC protein (~18.5kDa); Lane 4, purified $_{His}$ExsD protein (~33kDa); Lane 5, purified $_{His}$ExsE protein (~11kDa); Lane 6, purified MBP-ExsC protein (~61kDa) as a positive control. The $_{His}$ExsC and $_{His}$ExsE were detected when probed with MBP-ExsE, suggesting ExsE-ExsC interaction and ExsE-ExsE self-interaction.

4.7 Discussion

The protein-protein interactions among the Exs proteins were investigated both *in-vivo* and *in-vitro* through BACTH assay and Far-Western Blot respectively. The overall results of the interaction studies agreed with the hypothesis that ExsA-ExsD-ExsC-ExsE regulatory cascade was based on direct protein-protein interactions.

The evidence in *P. aeruginosa* has suggested that the C-terminal domain of ExsA has two helix-turn-helix DNA binding motifs but lacks the ability to self-interact for cooperative binding of the DNA (Brutinel et al 2009). Furthermore, in *P. aeruginosa*, ExsD was shown to inhibit the DNA-binding ability of ExsA by interacting with the N-terminal domain of ExsA, which is involved in the ExsA-ExsA self-interaction (Brutinel et al 2010). However in this study, the C-terminal domain of the ExsA protein was shown to interact with both N-terminal and C-terminal domain of itself in the BACTH assay (Figure 4.8A). Rather than over-expressing only 120 amino acid of C-terminal domain of ExsA (278 amino acids in total) in Brutinel's study, the T25/T18 fusion to the N-terminal domain of ExsA might not be sufficient to block the interactions between ExsA and ExsD or ExsA-ExsA self-interaction.

The reason why pKT25-*exsD* showed no ExsA-ExsD interaction when co-transformed with either pUT18-*exsA* or pUT18C-*exsA* was unclear, since C-terminal domain of ExsD showed strong interaction with either C-terminal or N-terminal domain of ExsA when co-transforming pUT18C-*exsD* with either pKT25-*exsA* or pKNT25-*exsA* (Figure 4.9). Also the T25 fragment was unlikely to be the reason of no interaction because pKNT25-*exsD* showed strong interaction with both pUT18-*exsA* and pUT18C-*exsA* (Figure 4.9).

In Far-Western Blot, when MBP-ExsA was used to probe His-tagged Exs proteins, only $_{His}$ExsD was detected although the interaction seemed weak. This

correlates with the recent findings in *P. aeruginosa* that ExsD is found to bind ExsA only as folding intermediate when these two proteins were synthesized together (Bernhards et al 2013). Thereby, strong interactions between ExsD and ExsA were observed *in vivo* using BACTH assay when they were co-expressed whereas weak interactions were shown *in vitro* using Far-Western Blot.

However, in Far-Western Blot, when MBP-ExsD was used to probe His-tagged Exs proteins, no evidence of an interaction with any Exs protein was observed (Figure not shown), but when $_{His}$ExsD was used to probe MBP-fused Exs proteins in Far-Western Blot, the interaction between ExsD and ExsC was observed (Figure 4.32). As shown in Figure 4.1, in pKT25-*exsD* plasmid construct, the T25 fragment was fused to the N-terminal of the ExsD protein that allowed the C-terminal of ExsD to interact with other proteins. It might indicate that when large subunits such as T25 fragment or MBP5 protein was fused to the N-terminal of ExsD protein, the interactions between ExsD and other Exs proteins were disabled, suggesting that the N-terminal domain of ExsD was required for protein-protein interactions.

Furthermore, the BACTH assay showed no evidence of ExsD-ExsD self-interaction, which was also observed in the Far-Western Blot. However, when the $_{His}$ExsD protein was probed with MBP-ExsC protein in Far-Western Blot, possible ExsD dimerization was observed, suggesting that ExsD dimerization might require the involvement of ExsC proteins. As it was reported in *P. aeruginosa*, the size of the ExsC-ExsD complex consisted of two molecules of both ExsC and ExsD proteins using Gel Filtration Chromatography and Analytical Ultracentrifugation, together with the Isothermal Titration Calorimetry data, which indicated an equal molar binding ratio of ExsC and ExsD, suggesting a heterotetramer complex of ExsC-ExsD at 2:2 ratio with a binding affinity of 18nM (Zheng et al 2007). However, the finding in this study does not correlate with the work of Berhards and collegues, who suggested the formation of ExsD homotrimer at 30°C in *P. aeruginosa* (Bernhards et al 2013). It might be because that the self-association requires

full-length of ExsD and both His-tagged and MBP-fused proteins were over-expressed in *E. coli* at 37°C. But the assay was not carried out at 37°C.

The interactions of ExsC with each of the Exs proteins shown in BACTH assay revealed a potential hierarchy of ExsC interactions based on the binding affinity. The potential strongest interaction was observed in ExsC-ExsE, which was a typical T3SS chaperone-effector interaction described in *P. aeruginosa* (Dasgupta et al 2004, Urbanowski et al 2005, Vogelaar et al 2010). Although in this study the binding kinetics was not yet carried out, the ExsC-ExsD interaction was thought to be weaker than the ExsC-ExsE interaction but stronger than the ExsC-ExsC self-interaction from the observation of the BACTH assay. This finding corresponds to the Isothermal Titration Calorimetry studies carried out by Zheng's group, who has determined the binding affinities for ExsC-ExsD (18nM) and ExsC-ExsE (1nM) in *P. aeruginosa* (Zheng et al 2007). This suggests that the ExsC chaperone protein prefers to bind to the effector protein ExsE rather than the anti-activator ExsD. When the concentration of ExsE decreases, the abundant ExsC proteins bind to ExsD and antagonize the inhibition of ExsD on ExsA. Thereby the concentration of the effector protein ExsE plays a crucial role in the regulation of the ExsA transcriptional activation.

However, quantitative assays are required to determine how strong the interactions are between ExsC and ExsD or between ExsC and ExsE in *A. hydrophila*. The BioLayer Interferometry (BLI) was carried out trying to investigate the binding kinetics of the Exs proteins using ForteBio BLItz™ system (Data not shown). The BLI assay utilizes glass-fibre based biosensors that immobilize protein X, which is able to bind protein Y. The protein binding in each case changes the wavelength of the reflection light that can be measured to calculate the binding affinity of the protein X to protein Y. However, in this study the binding of the second protein to the first protein was unsuccessful, thus the binding affinity between the Exs proteins remained unknown. The reason why BLItz assay failed was unclear but

it might be due to the insolubility of some of the His-tagged Exs proteins, which had to be dissolved in at least 3.2M Urea, hence an interference of the BLItz assay.

The ExsC-ExsC self-interaction was shown to be the weakest in the BACTH assays while no evidence of the ExsC self-interaction was found using Far-Western Blots, suggesting that the self-interaction of ExsC was likely to be based on the interaction of ExsC with other Exs proteins. Both ExsD and ExsE were reported to bind to dimerized ExsC in *P. aeruginosa*, in which the ExsC-ExsD forms a 2:2 heterotetramer while ExsC binds to ExsE in a 2:1 form (Vogelaar et al 2010, Zheng et al 2007).

ExsE-ExsE self-interaction was shown to be very weak in BACTH assays, as pKT25-*exsE* showed no interaction with neither pUT18-*exsE* nor pUT18C-*exsE* while pKNT-*exsE* showed weak interaction with pUT18-*exsE* and pUT18C-*exsE* (Figure 4.13). It was hard to determine whether ExsE-ExsE self-interaction presents until the Far-Western Blot showed clear ExsE-ExsE interaction when $_{His}$ExsE protein was detect by ExsE-MBP probing.

The interactions that was shown in BACTH assay but not shown in Far-Western Blot were ExsA-ExsA, ExsD-ExsA and ExsC-ExsC. There were several possible reasons why these interactions were not present in Far-Western Blot. First of all, Far-Western Blot is an *in-vitro* method to investigate protein-protein interactions, thus some of the interactions may require the *in-vivo* environment to take place. Also, the size of MBP5 fusion protein is approximately 42.5kDa, which is fused to the N-terminal of the Exs protein and is much larger than the sizes of both T25 and T18 fragment of CyaA protein. Thus the interactions that involved N-terminal domains of the Exs proteins, especially for ExsA and ExsD proteins, were possibly blocked by the MBP-fusion. Although there were approaches that utilized factor Xa to cleave the MBP proteins off the MBP-fused Exs proteins, this was not feasible due to the stability and solubility of the Exs proteins.

The reason why the His-tagged Exs proteins were insoluble was unknown. It was not because of pH of the Binding Buffer, as the isoelectric points (pI) of the Exs proteins (6.53 for $_{His}$ExsA, 5.44 for $_{His}$ExsC, 6.13 for $_{His}$ExsD and 11.54 for $_{His}$ExsE) were not close to the pH of the Binding Buffer (pH 7.9). If the pI of the proteins were close to the pH of the Binding Buffer, the net charges of the proteins were close to zero, thereby rather than interacting with the buffer to become solubilized, the proteins preferred to interact with each other to precipitate. However, as several of the proteins appear to be chaperones, a co-expression approach using pET-Duet should be attempted. This should allow observation of direct protein-protein interactions in pull-down assays.

4.8 Conclusion

- Direct protein-protein intections were found between ExsA-ExsA, ExsA-ExsD, ExsD-ExsC and ExsC-ExsE using BACTH system
- His-tagged and MBP-fused Exs proteins were overexpressed and purified for Far-Western Blot.
- Direct protein-protein interactions were found between ExsA-ExsD, ExsD-ExsC, ExsC-ExsE and ExsE-ExsE using Far-Western Blot.

Chapter 5.
Regulation of Lateral Flagella System

5.1 Introduction

The flagellated Gram-negative bacteria *A. hydrophila* consists of two distinct flagella system, polar flagella and lateral flagella systems, in which the polar flagella system allows the bacteria to swim in liquid environments while the lateral flagella system allows the bacteria to swarm over the solid surfaces. Both flagella systems provide the bacteria with the ability to move towards more promising environments or away from less favourable conditions. Early in 1985, a Japanese group observed peritrichous lateral flagella in *A. caviae* and *A. hydrophila* by electron microscopy (Shimada et al 1985). Later in 2002, it was found that more than half of the mesophilic *Aeromonas* spp. possessed both flagella systems. Among these species, *A. caviae* and *A. hydrophila* are the most clinically isolated strains (Kirov et al 2002). However the lateral flagella system was described to be involved in the pathogenic association with host cells in *E.coli* and *Salmonella typhimurium* in 1994 (Harshey 1994). This led to a rapid progress in understanding the function of the lateral flagella system in Gram-negative bacteria including *Aeromonas* species, in which the lateral flagella system was reported to be involved in host cell adherence and biofilm formation (Gavin et al 2002, Kirov 2003).

It was reported that the lateral flagella gene clusters of *A. hydrophila* are comprised of 38 genes, located in a single chromosomal region rather than two distinct chromosomal regions as is seen in *V. parahaemolyticus* (Canals et al 2006a, Kirov 2003). The regulation of the lateral flagella system is mediated by the master regulator LafK and is dependent upon many other factors, such as T3SS and c-di-GMP levels (Gavin et al 2002, Merino et al 2006, Tamayo et al 2007, Yu et al 2007).

Unlike *V. parahaemolyticus*, in which the mutations in polar flagellum result in constitutive expression of lateral flagella, mutagenesis of polar flagellum production in *Aeromonas* does not affect the expression of the lateral flagella (Gavin et al 2002,

Merino et al 2006). However the intracellular c-di-GMP level has been shown to play a role in the regulation of the lateral flagella system in *Vibrio* and in *Aeromonas*, in both of which high intracellular c-di-GMP concentration represses the expression of the lateral flagella while low c-di-GMP level enhance the swarming motility caused by increased lateral flagella expression (Tamayo et al 2007).

It was also reported in the *A. hydrophila* AH-1 strain that the expression levels of two tandem lateral flagellin genes *lafA1* and *lafA2* were decreased drastically when *exsD* or *aopN* genes were knocked out (Yu et al 2007). Similar findings were discovered in a previous study of the *A. hydrophila* AH-3 strain in our laboratory, where two of the non-swarming transposon mutants were found to be mutated in the *exsD* gene (Shaw unpublished). As it was indicated before, the *exsD* gene encoded an anti-activator protein ExsD that inhibited the T3SS master regulator ExsA. Thus the mutation in *exsD* gene resulted in activation of the T3SS, suggesting potential cross-talk between the lateral flagella system and the T3SS in *A. hydrophila*.

In this study, the potential cross-talk between the two systems was investigated by swarming assays of different mutants and measurement of the lateral flagella promoters in various backgrounds.

5.2 Swarming assays of *A. hydrophila* AH3R wild type and different mutants

Different strains of *A. hydrophila* AH3R including the *exsA* mutant, *exsC* mutant, *exsD* mutant, *exsE* mutant and a previously constructed *lafK* mutant were plated on the swarming agar (section 2.1.4) together with the *A. hydrophila* AH3R wild type (Figure 5.1). The wild type strain of *A. hydrophila* AH3R was able to swarm on the surface of the 0.6% (w/v) semi-solid agar, but when the major regulator of the *Aeromonas* lateral flagella system was knocked out in the *lafK* mutant, the swarming motility was completely lost. On the other hand, the swarming motility was not affected in the *exsA* mutant and *exsC* mutant as the mutant strains were able to swarm as much as in wild type strain. However, when the *exsD* gene or *exsE* gene was knocked out, the swarming motility was repressed as it was in the *lafK* mutant (Figure 5.1).

The phenotypes of the swarming assays were quantified by measuring the swarming diameters of each strain on the swarming agar after overnight incubation at 30°C (Figure 5.2). The average swarming diameter of the *A. hydrophila* AH3R wild type strain was 6.9cm while it was significantly decreased to 1.1cm in the *lafK* mutant strain ($p<0.001$). The swarming motility of *exsA* mutant and *exsC* mutant strains were not significantly different from the wild type strain, with an average swarming diameter of 5.7cm and 6.3cm respectively ($p>0.05$). Moreover the swarming motility was significantly repressed in *exsD* mutant and *exsE* mutant strains when compared to the wild type strain, the *exsA* mutant and *exsC* mutant strains ($p<0.001$). The average swarming diameters of *exsD* mutant and *exsE* mutant strains were 2.7cm and 2.6cm respectively. The swarming diameters were measured at least 15 times for each strain and each time the different strains were plated on the same 220mm x 220mm square petri dishes with swarming agar to minimize the variations.

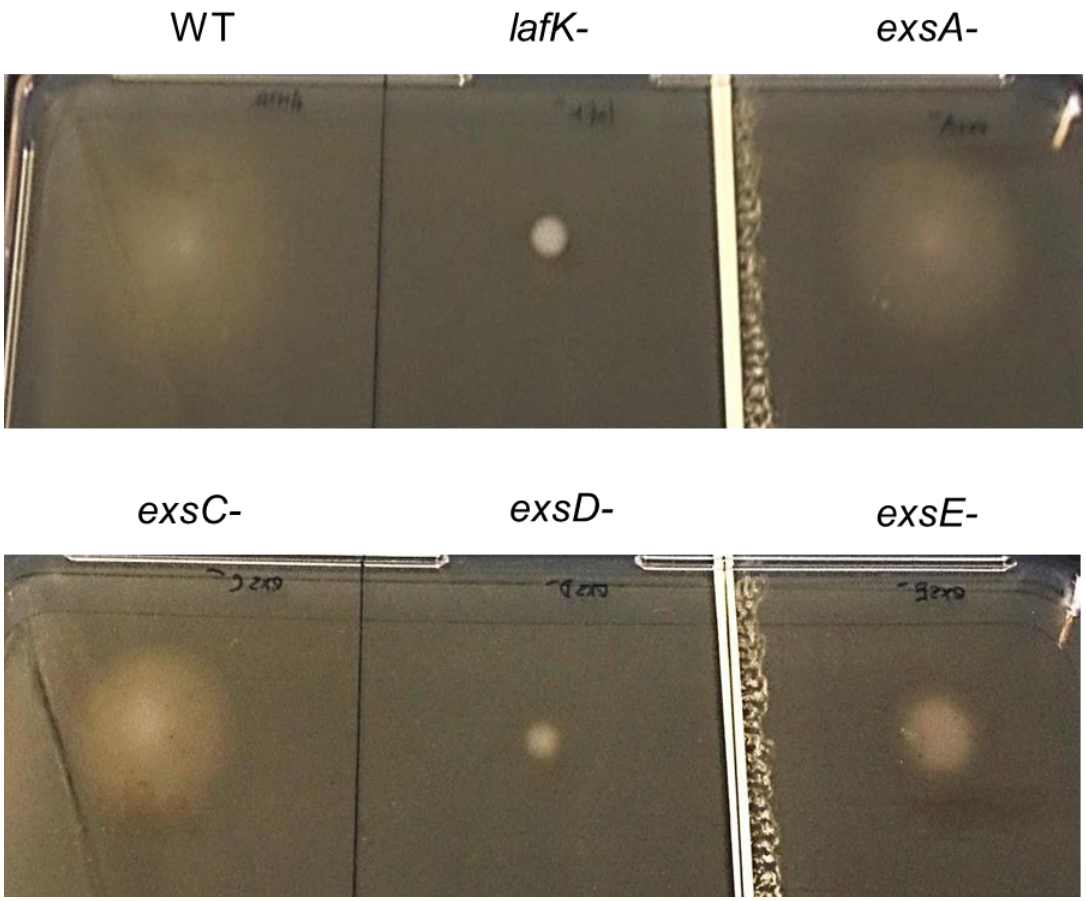

Figure 5.1 Swarming assays of different mutant strains of *A. hydrophila* AH3R. The *exsA* mutant and *exsC* mutant had similar swarming motility as the *A. hydrophila* AH3R wild type, while the swarming motility was repressed in the *lafK* mutant, *exsD* mutant and *exsE* mutant on the surface of 0.6% (w/v) swarming agar. Bacteria were incubated on the swarming agar at 30°C overnight. The experiment was repeated at least 15 times.

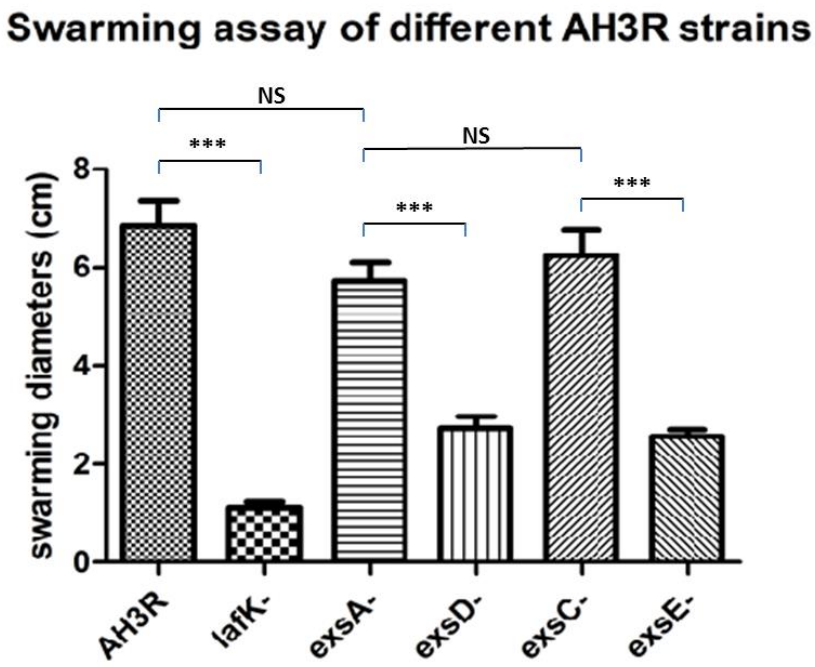

Figure 5.2 Quantification of the swarming assays of *A. hydrophila* AH3R wild type and mutant strains. The swarming diameter of each strain was measured on the swarming agar after overnight incubation at 30°C. The swarming diameters were significantly lower in the *lafK* mutant when compared to the *A. hydrophila* AH3R wild type ($p^{***}<0.001$), while there was no significant difference of swarming diameters among the wild type strain, the *exsA* mutant and *exsD* mutant ($p>0.05$). However, the swarming diameters of *exsD* mutant and *exsE* mutant was significant decreased when compared to the *exsA* mutant and *exsC* mutant ($p^{***}<0.001$). The swarming diameters were measured at least 15 times for each strain. The error bars showed Standard Error of the Mean (SEM). The significance was determined using Student's *t*-test.

5.3 Construction of *lacZ*-fusion vector with lateral flagella promoters

In order to measure the changes of lateral flagella promoter activities in different mutant backgrounds, the putative promoters of lateral flagella system were cloned with a promoter-less *lacZ* gene on the reporter plasmid vector pKAGb-2(-).

First of all, the putative promoter regions of the lateral flagella system were identified according to the designation of the open reading frames (ORFs) reported in *A. hydrophila* AH3. A total number of 9 putative promoters of the lateral flagella system were proposed according to the homology with other bacteria and confirmed by RT-PCR of the ORFs in previous studies (Canals et al 2006a, Gavin et al 2002). Each putative promoter region was 400-800bp in length and was named as *PfliM*, *PlafK*, *PflgM*, *PflgA*, *PflgB*, *Pmaf*, *PlafA*, *PlafB* and *PlafX*, after the first gene immediately downstream of the putative promoters (Figure 5.3).

The construction of the plasmids pKAG-*PfliM* pKAG-*PlafK*, pKAG-*PflgM*, pKAG-*PflgA*, pKAG-*PflgB*, pKAG-*Pmaf*, pKAG-*PlafA*, pKAG-*PlafB* and pKAG-*PlafX* (pKAG-P_{LF} constructs) were achieved by the fusion of the lateral flagella promoter regions with the promter-less *lacZ* gene in the reporter plasmid vector pKAGb-2(-) (Figure 5.4). First, the putative promoter regions were amplified by PCR from the *A. hydrophila* AH3R chromosomal DNA using amplification primers for each promoter regions (Appendix 1) together with Platinum Pfx DNA polymerase (Figure 5.5). The forward amplification primer of each promoter regions introduced a *Hin*dIII restriction site at 5' end of the PCR product while the reverse primers introduced a *Bam*HI restriction site at 3' end. The PCR products of each lateral flagella promoter regions were then digested with *Hin*dIII and *Bam*HI in order to ligate with the *Hin*dIII and *Bam*HI double-digested pKAGb2(-) plasmid vector (Figure 5.5).

The ligated samples were transformed into *E. coli* DH5α competent cells and successful constructs were selected by colony PCR screen using the pKAGb2(-)

screening primers (*bla* forward and *lacZ* reverse, Appendix 1) and Taq DNA polymerase. The screen of the pKAG-*PlafX* construct was shown as an example of successful colony PCR screen (Figure 5.6).

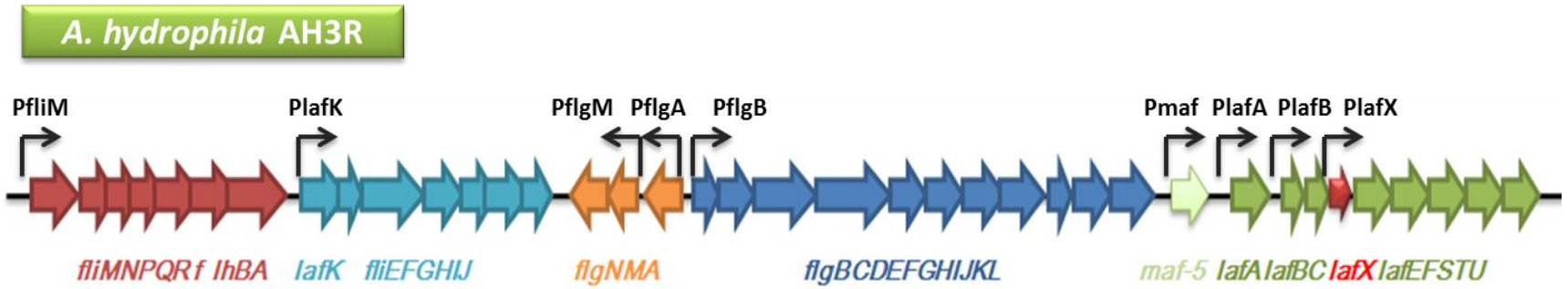

Figure 5.3 Illustration of the lateral flagella gene region in *A. hydrophila* AH3R. The putative promoter regions were shown in bent arrows and named as *PfliM*, *PlafK*, *PflgM*, *PflgA*, *PflgB*, *Pmaf*, *PlafA*, *PlafB* and *PlafX* after the name of first gene downstream. Each promoter region was 400-800bp in length and were amplified by PCR and cloned into *lacZ*-fusion plasmid pKAGb-2(-) to measure the activity in different backgrounds. The figure is adapted from Canals, et al. (2006).

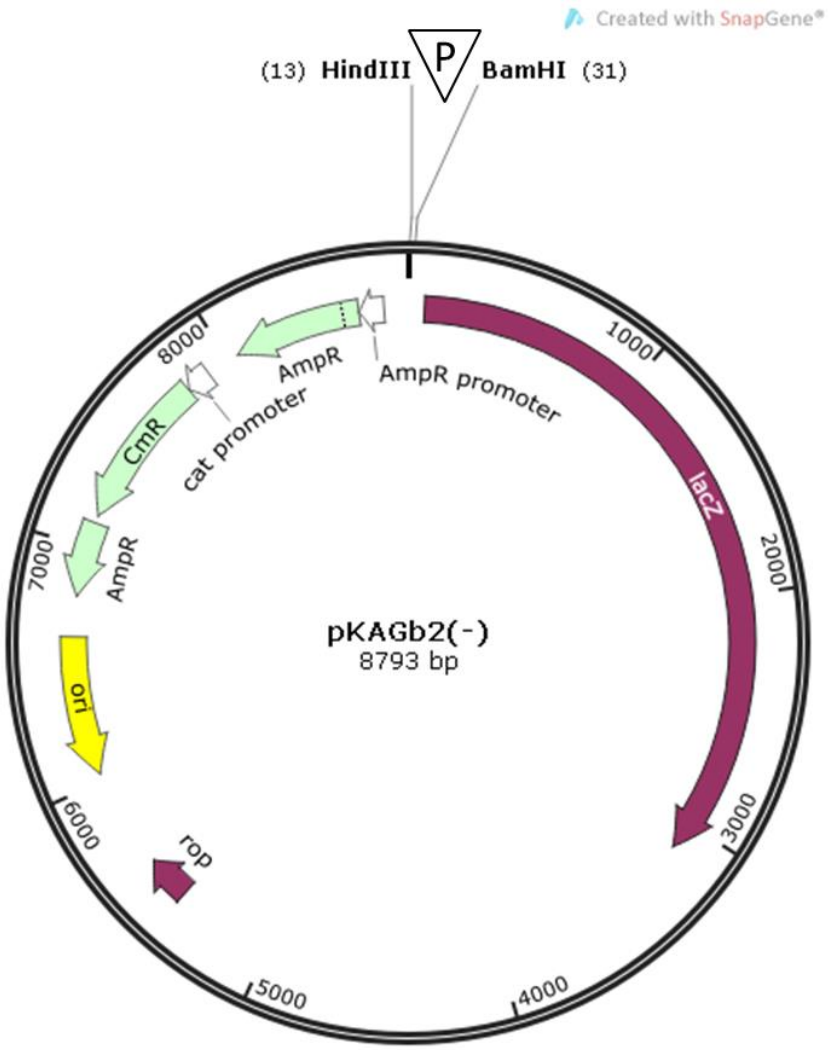

Figure 5.4 Illustration of the lateral flagella promoter regions cloning with a promoter-less *lacZ* gene in the reporter plasmid vector pKAGb-2(-). The insertion of the lateral flagella promoter regions were represented by the triangle. Both the lateral flagella promoter inserts and the pKAGb-2(-) plasmid vector were digested with *Hin*dIII and *Bam*HI restriction enzymes. The putative promoter was inserted upstream of a promoter-less *lacZ* gene in order to measure the promoter activity by β-galactosidase assay. The plasmid carried a chloramphenicol resistance gene which was used to select successful transformants or conjugants. Key features of the plasmid were shown in large arrows. This figure was created using SnapGene software.

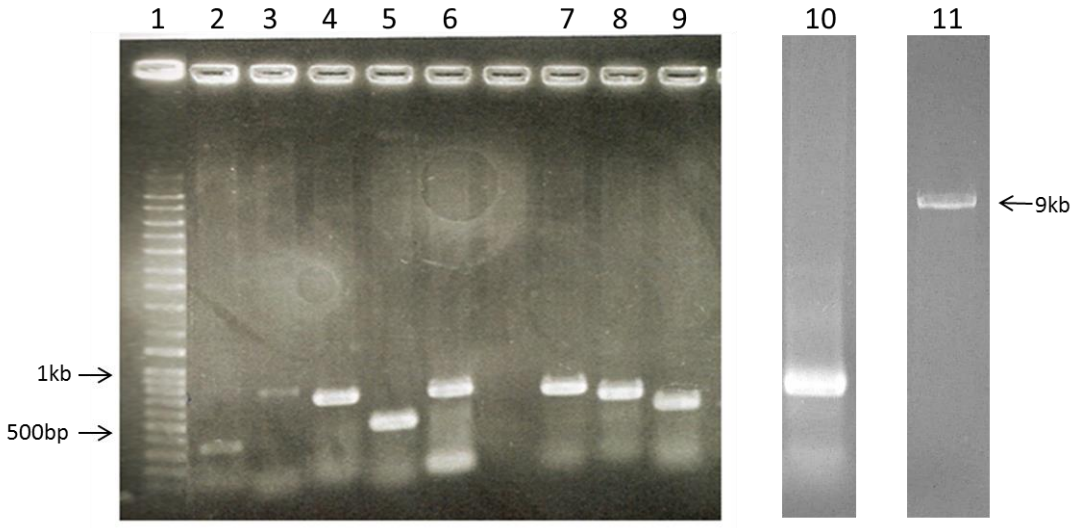

Figure 5.5 A 1% agarose gel picture showing the PCR products of lateral flagella putative promoters. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 2, PCR product of *PfliM* region (~400bp); Lane 3, PCR product of *PlafK* region (~800bp); Lane 4, PCR product of *PflgM* region (~800bp); Lane 5, PCR product of *PflgA* region (~600bp); Lane 6, PCR product of *PflgB* region (~800bp); Lane 7, PCR product of *PlafA* region (~800bp); Lane 8, PCR product of *PlafB* region (~700bp); Lane 9, PCR product of *PlafX* region (~600bp); Lane 10, PCR product of *Pmaf* region (~800bp); Lane 11, *Hin*dIII and *Bam*HI double digested pKAGb-2(-) plasmid vector (~9kb).

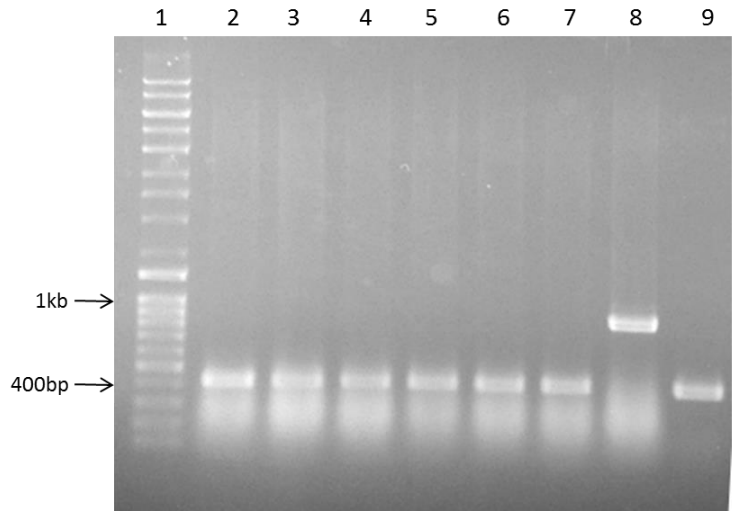

Figure 5.6 A 1% agarose gel showing the colony PCR screen pKAG-P_{LF} constructs. Lane 1, Q-step 4 quantitative DNA ladder (Yorkshire Bioscience); Lane 2, colony PCR screen of the pKAG-*PfliM* construct, Lane 3, colony PCR screen of the pKAG-*PflgM* construct ; Lane 4, colony PCR screen of the pKAG-*PflgB* construct ; Lane 5, colony PCR screen of the pKAG-*Pmaf* construct ; Lane 6, colony PCR screen of the pKAG-*PlafA* construct ; Lane 7, colony PCR screen of the pKAG-*PlafB* construct ; Lane 8, colony PCR screen of the pKAG-*PlafX* construct ; Lane 9, colony PCR screen of the pKAGb-2(-) plasmid control. Only pKAG-*PlafX* screen was a successful construct with an extra 600bp of the *PlafX* insertion.

5.4 β-Galactosidase assay of the lateral flagella promoters

The successful constructs were extracted from the *E. coli DH5α* cells (Figure 5.7) and were sequenced using the pKAGb-2(-) screening primers (*bla* and *lacZ*) by Core Genomic Facility, University of Sheffield. The sequencing results were analysed using FinchTV and BLAST search to ensure the insertion of the lateral flagella promoter regions into the *lacZ*-fusion plasmid vector pKAGb-2(-).

The pKAG-P_{LF} plasmid constructs were then transformed into *E. coli* S17-*λpir* cells in order for conjugation into *A. hydrophila* AH3R. Followed by conjugation, the chloramphenicol resistant *A. hydrophila* AH3R colonies were selected, which possessed the pKAG-P_{LF} plasmid constructs independently. Then the β-galactosidase activities of the lateral flagella promoters were measured either in liquid conditions, in which the bacteria were grown in LB broth at 30°C with 200rpm shaking overnight, or in solid conditions, in which the bacteria were grown on swarming agar at 30°C overnight and the edge of the swarmer cells were resuspended in sterile PBS for β-galactosidase assay (Figure 5.8).

The β-galactosidase activity of promoter *PflgM* was significantly increased from 54 MU to 436 MU on swarming agar when compared to broth culture ($p<0.001$). Same pattern was also found for promoter *PflgB*, the β-galactosidase activity of which was 375 MU in liquid conditions but significantly increased to 970 MU when grown in solid conditions ($p<0.001$). The promoter activity of *PlafA* when grown in broth culture was 318 MU and it was significantly increased to 976 MU when grown on swarming agar ($p<0.001$). Furthermore, the promoter activity of *PlafX* was significantly increased from 145 MU in liquid condition to 349 MU in solid conditions ($p<0.001$) (Figure 5.8).

However, there was no significant difference of the promoter activities when grown in liquid or solid conditions for promoters *PfliM*, *PflgA*, *Pmaf* and *PlafB*

(p>0.05). The β-galactosidase activity of promoter *PfliM* was 190 MU when grown in broth culture while it was 247 MU when grown on swarming agar (p>0.05). The β-galactosidase activity of promoter *PflgA* was 358 MU when grown in broth culture while it was 297 MU when grown on swarming agar. The promoter activity of *Pmaf* was 118 MU in liquid condition while it was 90 MU in solid condition and the promoter activity of *PlafB* was 41 MU in liquid condition while it was 59 MU in solid condition (Figure 5.8).

Only the promoter activity of *PlafK* was significantly decreased when grown on swarming agar compared to grown in broth culture (p<0.001). The β-galactosidase activity of *PlafK* in liquid conditions was 139 MU while it was decreased to 26 MU in solid condition, which was almost completely suppressed (Figure 5.8).

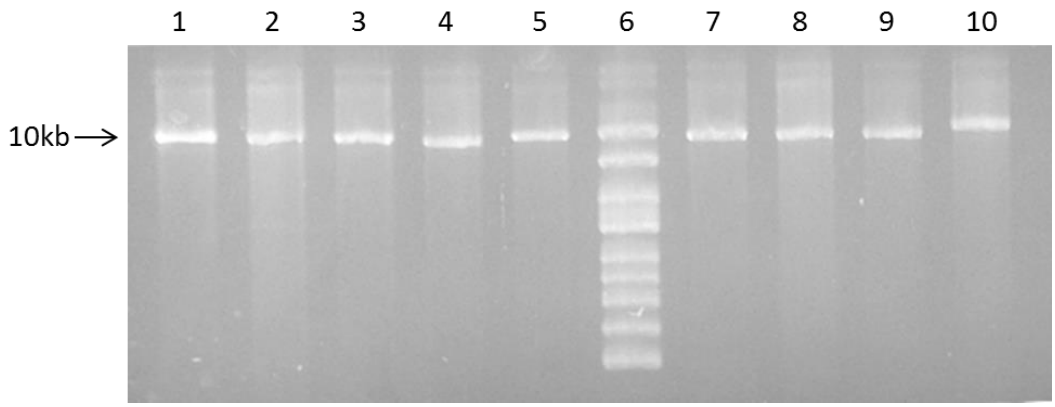

Figure 5.7 A 1% agarose gel showing successful pKAG-P_{LF} plasmid constructs. Lane 1, plasmid construct of pKAG-*PfliM* (~9.5kb); Lane 2, plasmid construct of pKAG-*PlafK* (~9.5kb); Lane 3, plasmid construct of pKAG-*PflgM* (~9.5kb); Lane 4, plasmid construct of pKAG-*PflgA* (~9.5kb); Lane 5, plasmid construct of pKAG-*PflgB* (~9.5kb); Lane 6, Promega™ Supercoiled DNA Ladder (2-10kb); Lane 7, plasmid construct of pKAG-*Pmaf* (~9.5kb); Lane 8, plasmid construct of pKAG-*PlafA* (~9.5kb); Lane 9, plasmid construct of pKAG-*PlafB* (~9.5kb); Lane 10, plasmid construct of pKAG-*PlafX* (~9.5kb).

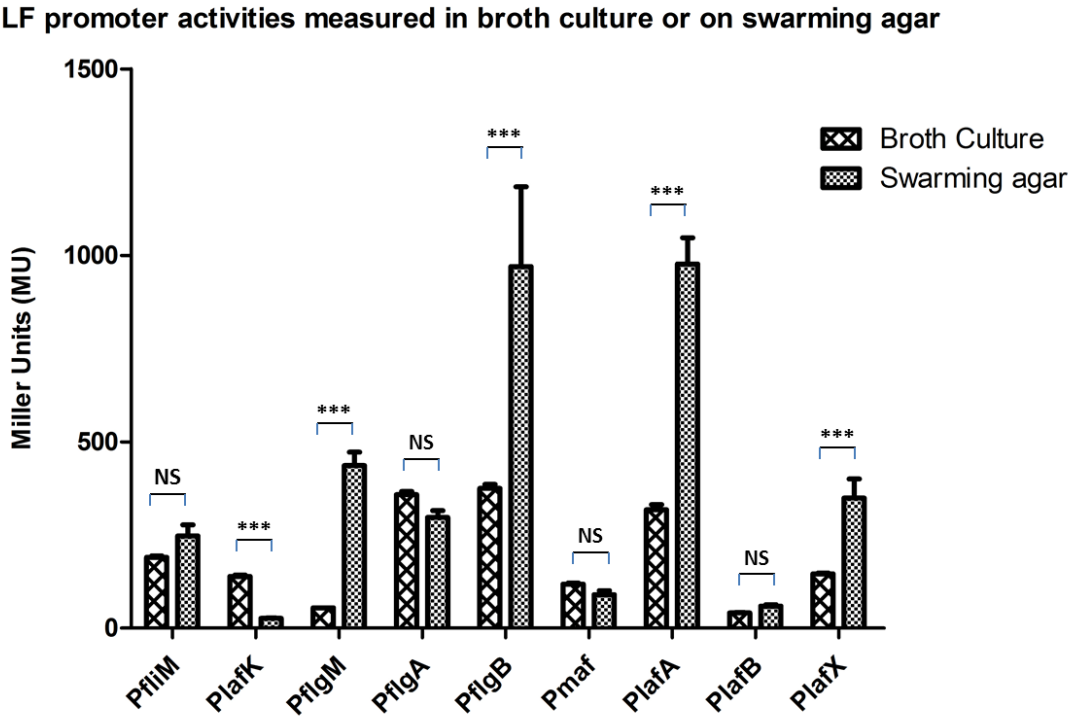

Figure 5.8 β-galactosidase activities of lateral flagella promoters measured in liquid condition (in LB broth) or in solid condition (on swarming agar) in *A. hydrophila* AH3R wild type. The β-galactosidase activities of promoters *PflgM*, *PflgB*, *PlafA* and *PlafX* were increased significantly when grown on swarming agar compared to grown in LB broth (p***<0.001), while there was no significant difference of the promoter activities when grown in broth culture or on swarming agar for *PfliM*, *PflgA*, *Pmaf* and *PlafB* promoters (p>0.05). Only the promoter activity of *PlafK* was significantly decreased when grown on swarming agar (p<0.001). The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test.

Among the putative promoters, *PfliM*, *PflgB* and *PlafA* were further analysed to investigate the potential cross-talk between the lateral flagella system and the T3SS by measuring the β-galactosidase activities of these promoters in different mutant backgrounds, including the *lafK* mutant and all of the *exs* mutants.

The plasmid constructs pKAG-*PfliM*, pKAG-*PflgB* and pKAG-*lafA* were conjugated into the *exsA* mutant, *exsD* mutant, *exsC* mutant and *exsE* mutant and chloramphenicol resistant mutant strains were selected. The mutant strains that carried pKAG-P_{LF} constructs were then incubated at 30°C overnight on the swarming agar in order for the β-galactosidase assay.

The β-galactosidase activity of promoter *PfliM* measured in the *A. hydrophila* AH3R wild type strain was 247 MU while it was significantly decreased to 22 MU in the *lafK* mutant ($p<0.001$). The promoter activity of *PfliM* was 215 MU in the *exsA* mutant but significantly decreased to 88 MU when measured in the *exsD* mutant strain ($p<0.001$). Similarly, in the *exsC* mutant strain, the promoter activity of *PfliM* was 225 MU while decreased significantly to 143 MU in the *exsE* mutant strain ($p<0.001$). There was no significant difference of the promoter activities among the *exsA* mutant, the *exsC* mutant and the wild type strains ($p>0.05$). However, the promoter activity in the *exsD* mutant was significantly higher than in the *lafK* mutant while it was significantly lower than in the *exsE* mutant ($p<0.001$) (Figure 5.9).

The β-galactosidase activity of promoter *PflgB* in the *A. hydrophila* AH3R wild type strain was 970 MU while significantly decreased to 20 MU in the *lafK* mutant ($p<0.001$). The promoter activity was 684 MU in the *exsA* mutant strain, which was not significantly different with the promoter activities in the wild type and the *exsC* mutant strain ($p>0.05$) while it was significantly higher than the promoter activities in the *lafK* mutant, *exsD* mutant and *exsE* mutant strains ($p<0.001$). The promoter activity of *PflgB* was 105 MU in the *exsD* mutant while it was significantly increased to 665 MU in the *exsC* mutant strain ($p<0.001$). The β-galactosidase activity of

promoter *PflgB* was 435 MU in the *exsE* mutant, which was significantly lower than in the wild type, the *exsA* mutant and the *exsC* mutant strains but significantly higher than in the *lafK* mutant and *exsD* mutant strains ($p<0.001$) (Figure 5.10).

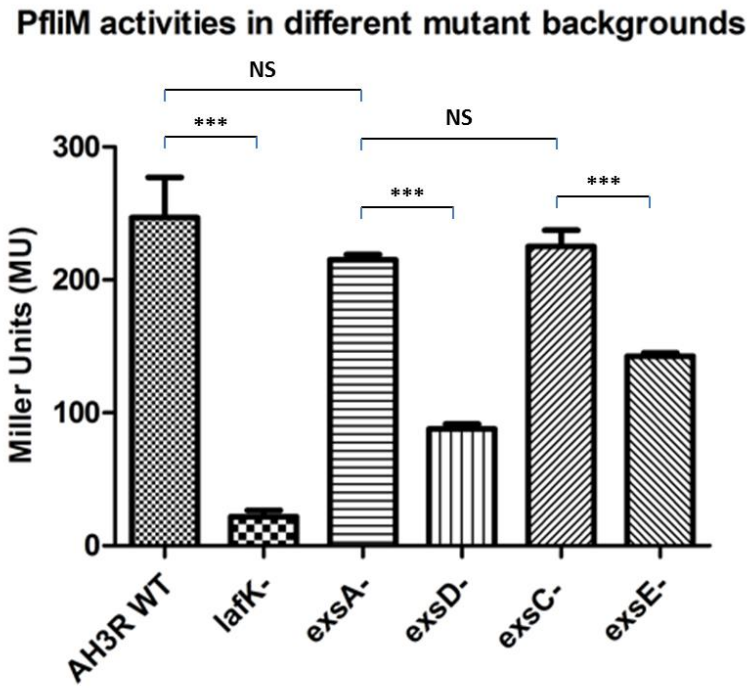

Figure 5.9 β-galactosidase activities of promoter *PfliM* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown on swarming agar at 30°C overnight. Compared to the wild type strain, the promoter activity of *PfliM* was significantly decreased when the lateral flagella major regulator *lafK* was knocked out (p***<0.001), but there was no significant difference between the promoter activities in *exsA* mutant and the wild type strain (p>0.05). The promoter activity of *PfliM* was significantly decreased in the *exsD* mutant when compared to the *exsA* mutant (p***<0.001). Smiliarly in the *exsE* mutant, the promoter activity of *PfliM* was a significant decreased compared to the *exsC* mutant (p***<0.001). There was no significant difference of the promoter activities between the *exsA* mutant and the *exsC* mutant (p>0.05). The promoter activity of *PfliM* was significantly higher in the *exsD* mutant than in the *lafK* mutant, moreover, the promoter activity was significantly higher in the *exsE* mutant than in the *exsD* mutant (p***<0.001). The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test.

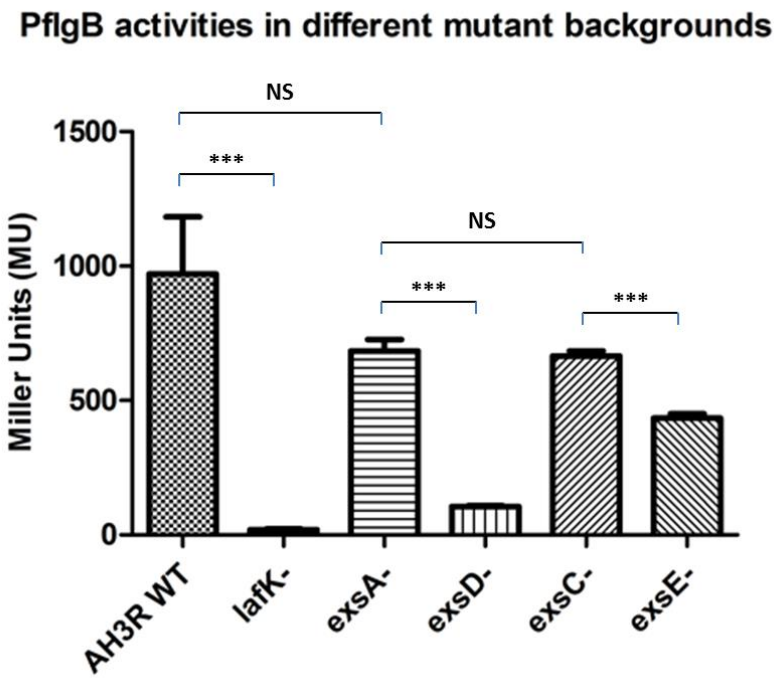

Figure 5.10 β-galactosidase activities of promoter *PflgB* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown on swarming agar at 30°C overnight. Compared to the wild type strain, the promoter activity of *PflgB* was significantly decreased when the lateral flagella major regulator *lafK* was knocked out (p***<0.001), but there was no significant difference between the promoter activities in *exsA* mutant and the wild type strains (p>0.05). The promoter activity of *PflgB* was significantly decreased in the *exsD* mutant when compared to the *exsA* mutant or *exsC* mutant strains (p***<0.001). In the *exsE* mutant, there was a significant decrease of the promoter activity of *PfliM* when compared to the *exsC* mutant or the *exsA* mutant strains (p***<0.001). There was no significant difference of the promoter activities between *exsA* mutant and *exsC* mutant strains (p>0.05). The promoter activity of *PflgB* was significantly higher in the *exsD* mutant than in the *lafK* mutant, moreover, the promoter activity was significantly higher in the *exsE* mutant than in the *exsD* mutant (p***<0.001). The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test.

The pattern of the β-galactosidase activity of promoter *PlafA* was similar to *PfliM* and *PflgB*. In the *A. hydrophila* AH3R wild type strain, the promoter activity of *PlafA* was 976 MU while it was decreased significantly to 22 MU in the *lafK* mutant strain (Figure 5.11). The promoter activity of *PlafA* in the *exsA* mutant was 771 MU, which was significantly lower than in the wild type ($p<0.05$), but significantly higher than in the *lafK* mutant ($p<0.001$), *exsD* mutant ($p<0.001$) and *exsE* mutant strains ($p<0.01$). The promoter activity in the *exsD* mutant was 240 MU, which was approximately 4-fold lower than in the wild type strain and 3-fold lower than in the *exsA* mutant strain. The β-galactosidase activity of *PlafA* was 734 MU in the *exsC* mutant strain, which was not significantly different with the promoter activity in the *exsA* mutant ($p>0.05$) but significantly lower than in the wild type strain ($p<0.01$). Unlike *PfliM* and *PflgB*, the promoter activity of *PlafA* in the *exsE* mutant strain was 638 MU, which was not significantly different with the promoter activity measured in the *exsC* mutant ($p>0.05$) but significantly lower than the *exsA* mutant ($p<0.01$). The promoter activity of *PlafA* in the *exsE* mutant was also significantly higher than in the *exsD* mutant ($p<0.001$), which was approximately 2.5-fold higher (Figure 5.11).

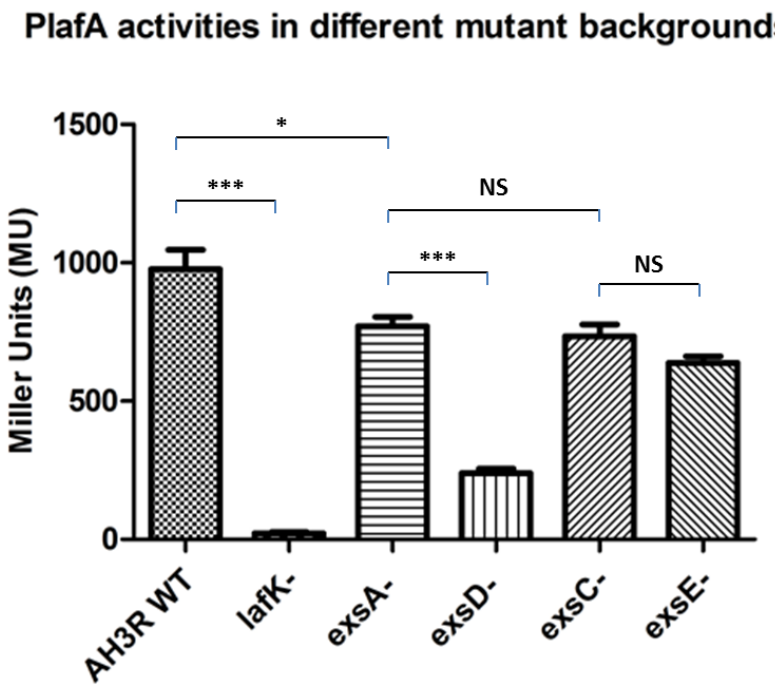

Figure 5.11 β-galactosidase activities of promoter *PlafA* in *A. hydrophila* AH3R wild type and mutant backgrounds. The promoter activities were measured when each strain was grown on swarming agar at 30°C overnight. Compared to the wild type strain, the promoter activity of *PlafA* was significantly decreased in the *lafK* mutant (p***<0.001), in the *exsA* mutant (p*<0.05) and in the *exsC* mutant (p**<0.01). The promoter activity of *PflgB* was significantly decreased in the *exsD* mutant when compared to the *exsA* mutant or *exsC* mutant strains (p***<0.001). In *exsE* mutant, the promoter activity of *PlafA* was a significant decrease when compared to the the *exsA* mutant strains (p**<0.01), but not significantly different from the promoter activity in *exsC* mutant (p>0.05). There was no significant difference of the promoter activities between *exsA* mutant and *exsC* mutant strains (p>0.05). The promoter activity of *PlafA* in the *exsD* mutant was significantly higher than in the *lafK* mutant, but significantly lower than in the *exsE* mutant (p***<0.001). The experiment was repeated at least three times for each bar. The error bars showed Standard Error of the Mean (SEM). The graph was created using GraphPad™ Prism 5. The significance was determined using Student's *t*-test.

5.5 Discussion

It was not surprising that the swarming motility was completely repressed in the *lafK* mutant as shown in the swarming assay, since *lafK* is responsible for encoding the major regulator of the *A. hydrophila* lateral flagella system, LafK. Moreover, the significant decrease of swarming motility was also observed in the *exsD* insertional knockout as well as in the *exsE* insertional knockout. It was in consensus with the hypothesis that there was potential cross-talk between the T3SS and the lateral flagella system of *A. hydrophila*. When the anti-activator ExsD was knocked out, the decrease of swarming motility suggested that the T3SS master regulator ExsA might play a negative regulatory role on the lateral flagella system. Thereby the swarming motility was de-repressed in the *exsC* mutant strains, in which the ExsA protein was inhibited by the abundant anti-activator ExsD. However, in the absence of ExsE, the effector-chaperone interaction was abolished so that the chaperone protein ExsC was bound to the anti-activator ExsD, thus allowing abundant ExsA to repress the lateral flagella system. This finding agrees with the previous study of transposon library screen, in which the *exsD* gene was mutated in two of the non-swarming transposon mutants. It also correlates to the findings of Leung's group, who has demonstrated decreased expression and secretion level of the lateral flagellins LafA1 and LafA2 in the absence of ExsD protein in *A. hydrophila* AH-1 strain (Yu et al 2007).

It was reported in many bacteria including *V. parahaemolyticus* and *A. hydrophila* that the lateral flagella system is hierarchically controlled at three levels (Wilhelms et al 2013) (Figure 5.12). The Class I promoters, including *PfliM*, *PlafK* and *PflgA*, were σ^{70}-dependent and responsible for the transcription of the foremost genes, encoding the major regulator LafK and structural components such as C ring and P ring (Figure 5.12). The enhancer-binding protein LafK together with an alternative sigma factor RpoN (σ^{54}) were required for the activation of the Class II promoters, including *PflgB* and *PlafX*, which were responsible for the transcription of

the lateral flagella-specific sigma factor LafS (σ^{28}) and the other structural components forming the rod and the hook (Figure 5.12). Class III promoters, including *PflgM*, *Pmaf*, *PlafA* and *PlafB*, were LafS (σ^{28})-dependent and were involved in the transcription of the cognate anti-σ^{28} factor FlgM and the flagellin proteins (Canals et al 2006a) (Figure 5.12).

When compared to the β-galactosidase activities of lateral flagella promoters measured in either liquid conditions or solid conditions, 4 out of 9 putative lateral flagella promoters (*PflgM*, *PflgB*, *PlafA* and *PlafX*) were significantly more active when grown on swarming agar compared to growth in LB broth (p***<0.001) (Figure 5.8). This finding suggested that the promoters of the lateral flagella system were regulated in response to the growth conditions of the bacteria and were more active when grown on solid surfaces. It correlates with the polymorphic lifestyle of the *A. hydrophila*, which utilizes the polar flagella to swim in the liquid environment and expresses lateral flagella to swarm over the solid surfaces. On the other hand, there was no significant difference of the promoter activities when grown in broth culture or on swarming agar for *PfliM*, *PflgA*, *Pmaf* and *PlafB* promoters (p>0.05) (Figure 5.8), suggesting that these promoters were possibly not responding to the environmental signal when the bacteria changing its lifestyle. The promoter PlafK was the only promoter showed significantly higher activity in the broth culture than on the swarming agar, suggesting possible negative feedback on the major regulator LafK. This also supports the findings of Wilhelms' and collegues, who have demonstrated that a number of lateral flagella promoters, such as PlafK and PflgA are transcribed and are active in liquid conditions (Wilhelms et al 2013).

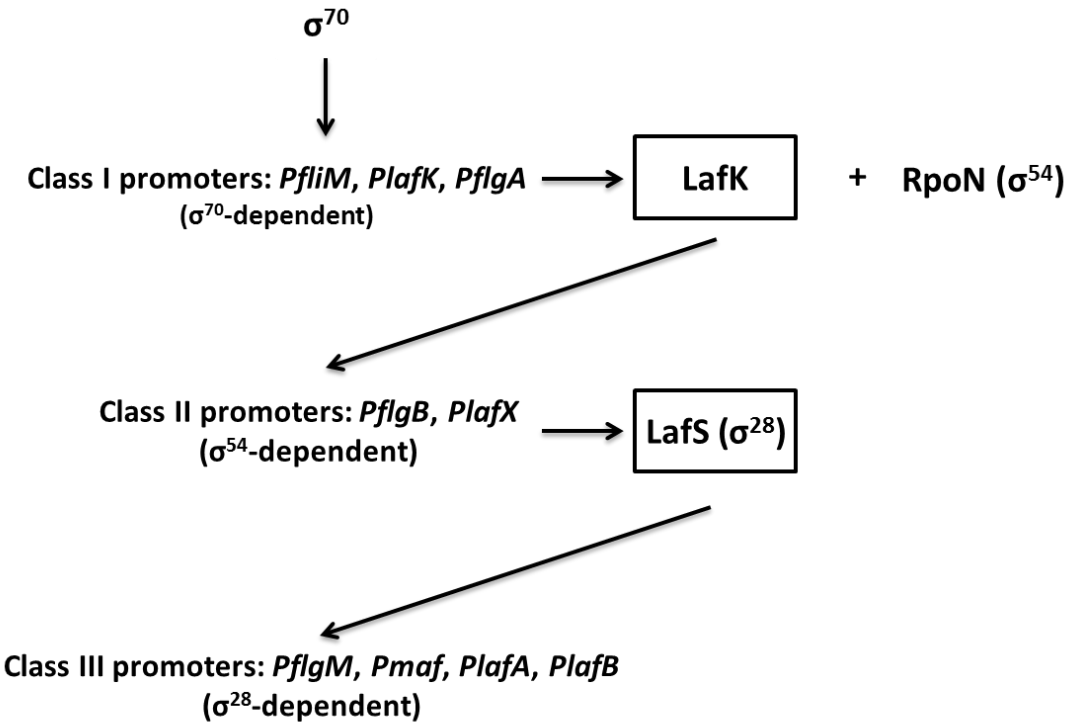

Figure 5.12 The transcriptional hierarchy of the lateral flagella system in *Aeromonas* species. The operon comprised of *fliMNPQRflhAB* genes is regulated by the promoter *PfliM*. The *flgA-flgN* operon is under control of the *PflgA* promoter. The operon of *lafK-fliJ* is regulated by the promoter *PlafK*. These 3 promoters are σ^{70}-dependent and categorized as Class I promoters. The lateral flagella system major regulator LafK is expressed under control of the Class I promoters. Together with RpoN (σ^{54}), LafK is required to activate the σ^{54}-dependent promoters. The promoter *PflgB* that regulates the operon *flgB-flgL* and the promoter *PlafX* that regulates the operon *lafX-lafU* are σ^{54}-dependent and categorized as Class II promoters. The promoter *PlafX* is responsible for the expression of the LafS (σ^{28}), which is required for the activation of σ^{28}-dependent promoters. The promoter *PflgM* that regulates *flgMN* genes, *Pmaf* that regulates *maf-5* gene, *PlafA* that regulates the *lafA* gene and *PlafB* that regulates *lafBC* genes are σ^{28}-dependent and categorized as Class III promoters. This figure was adapted from Wilhelms, et al. (2013)

The promoters *PfliM*, *PflgA*, *Pmaf* and *PlafB* that were not elevated in solid conditions were responsible for the activation of the genes encoding the structural components or involved in the assembly of the flagella. Among these four promoters, *PfliM* and *PflgA* were Class I promoters, which were σ^{70}-dependent and are constitutively activated. A total of 8 genes were under control of these two promoters, *fliM*, *fliN*, *fliP*, *fliQ*, *fliR*, *flhB* and *flhA* which were regulated by promoter *PfliM*; *flgA* under control of *PflgA*. Based on homology studies to *V.parahaemolyticus*, the genes in the operon *fliM-flhB* that were regulated by the promoter *PfliM* were reported to encode either structural components of the C-ring (*fliM* and *fliN*) or involved in the export and assembly of the flagella (*flip*, *fliQ*, *fliR* and *flhB*) while FlgA that was encoded by *flgA* was a P-ring structural protein (Canals et al 2006a). Thereby the expression of the lateral flagella system when the bacteria were grown on the swarming agar did not affect the promoter activities of *PfliM* and *PflgA*.

As for the *Pmaf* promoter, it is responsible for the transcription of the *maf-5* gene, which encodes the Maf-5 (modification accessory factor) protein (Wilhelms et al 2012). As Maf-5 is a lateral flagella glycosyl-transferase it is involved in the glycosylation of the flagellin protein LafA with pseudamic acid. It was also reported recently that the glycosylation by Maf proteins was required for full function of the polar flagellin in *A. caviae* (Parker et al 2012, Parker et al 2014). Therefore, the protein product of *maf-5* was possibly required when the bacteria were grown in the broth culture as well, thus no significant difference of the *Pmaf* promoter activity was observed in between liquid or solid conditions.

On the other hand, the promoter *PlafB*, which was not elevated in solid conditions, was responsible for the expression of LafB protein. LafB was reported to be a HAP2 flagella capping protein and mutation in *lafB* repressed the swarming motility of *A. hydrophila* while not affecting the polar flagella (Gavin et al 2002). The reason why the promoter activity of *PlafB* was not increased when grown on swarming agar was unclear, but the promoter activities *PlafB* was significantly lower

than the other lateral flagella promoters when grown in solid conditions (except for *PlafK*), suggesting that the putative promoter regions of *PlafB* cloned into the *lacZ*-fusion plasmid vector might not include the intact promoter sequence.

As for the the other Class I promoter *PlafK*, which was responsible for the expression of the lateral flagella major regulator LafK, the promoter activity of *PlafK* was not increased but significantly decreased when grown on swarming agar ($p<0.001$). This pattern was similar to what was found earlier for *PexsA* promoter, which was responsible for the transcription of the T3SS master regulator ExsA. It was possible that the lateral flagella major regulator LafK was under control of a negative feedback loop similar to ExsA, that the expression of which was repressed by itself. This speculation can be tested by measuring the promoter activity of *PlafK* while over-expressing the *lafK* gene from an IPTG-inducible plasmid vector, such as pBBR1MCS-5 or pSRKGm, in *A. hydrophila* AH3R, using an empty plasmid vector as a negative control.

One promoter was picked up from each of the Class I (*PfliM*), Class II (*PflgB*) and Class III (*PlafA*) promoters and the promoter activities were measured in the wild type, the *lafK* mutant and the *exs* mutant strains in order to investigate the potential cross-talk between the lateral flagella system and the T3SS. The promoter activities of *PfliM*, *PflgB* and *PlafA* measured in the *exs* mutants correlated with the phenotypes observed in the swarming assay. The patterns of the promoter activities were similar to the patterns of the swarming diameters of different mutant strains. It supported the hypothesis that there was potential cross-talk between the T3SS and the lateral flagella system. The lateral flagella promoter activities were significantly repressed when *exsD* and *exsE* genes were knocked out, while not affected in the absence of *exsA* and *exsC* genes. It suggests that the T3SS master regulator ExsA functions as a negative regulator of the lateral flagella system. When the anti-activator ExsD is missing, the abundant ExsA protein suppresses the expression of the lateral flagella system. Similarly, when ExsE is absent, the

chaperone protein ExsC binds to the anti-activator ExsD thus allowing ExsA to repress the lateral flagella system.

Although this potential negative cross-talk between the T3SS and the lateral flagella system has not been reported in *A. hydrophila* AH-3 before, it has been described in *P. aeruginosa* and *Y. enterocolitica* (Bleves et al 2002, Soscia et al 2007, Vilches et al 2009). It was reported in *Y. enterocolitica* by Bleves and collegues that the mutation in the *flhDC* operon, which was the master regulatory operon of the flagellum, resulted in the up-regulation of the the Yop secretion (Bleves et al 2002). Similar negative cross-talk between the T3SS and the lateral flagella system was determined later in *P. aeruginosa* as well. Soscia and collegues demonstrated that the T3SS expression, secretion and cytotoxicity of the bacterial strain were increased when flagellar assembly and/or mobility were compromised. Furthermore, in the same study, the flagella gene expression and motility were repressed when over-producing the T3SS master regulator ExsA in P. aeruginosa (Soscia et al 2007).

It was suprising to observe that the promoter activity of *PfliM* was down-regulated in the *lafK* mutant, since the promoter *PfliM* was categorized as Class I promoter, the activation of which was in-dependent of the major regulator LafK (Figure 5.12) (Wilhelms et al 2013). However, when the promoter activity of *PfliM* was measure in the *lafK* mutant in broth culture, the promoter activity was restored to 225MU (Figure not shown) at a similar level as measured in the wild type strain on swarming agar (247 MU) or in broth culture (190 MU) ($p>0.05$). It indicates that the promoter *PfliM* is indeed in-dependent of the lateral flagella major regulator LafK in the broth culture but requires LafK for the activation on swarming agar, suggesting a dual function of the promoter *PfliM*. As the bacteria were unable to swim on the swarming agar, the promoter *PfliM* was possibly involved in the expression of the polar flagella system.

However, all three lateral flagella promoter activities were significantly higher in the *exsD* mutant when compared to the *lafK* mutant, in which the promoter activities were completely repressed. It suggests that the ExsA protein was not sufficient to completely shutdown the expression of the lateral flagella system. Furthermore, the lateral flagella promoter activities were significantly increased in the *exsE* mutant when compared to the *exsD* mutant, suggesting possible secondary regulation involved in the inhibition of the lateral flagella system. This correlated with the findings of T3SS promoter activities (e.g. *PaopN*), the pattern of which also indicated possible secondary regulations on the ExsA-ExsD-ExsC-ExsE cascade.

The overall results also correlates with the findings in *V. parahaemolyticus*, which possesses the lateral flagella system and two T3SSs (T3SS1 and T3SS2). It was reported by Gode-Prtratz and collegues that the expression level of the lateral flagella gene $flgB_L$ was significantly repressed when over-expressing ExsA, and the swarming motility of *V. parahaemolyticus* was inhibited by *exsA* induction *in vivo* (Gode-Potratz et al 2010). The same study also suggested that the lateral flagella major regulator LafK was required for the regulation of the T3SS1 in response to calcium. Unlike *P. aeruginosa* or *Yersinia* spp., in which the T3SS is induced by the absence of calcium, the T3SS1 of *V. parahaemolyticus* has been shown to be induced by the presence of calcium or EGTA, in which the effect of EGTA induction has been shown to be the result of iron-chelating (Gode-Potratz et al 2010).

However, it is still unclear how ExsA represses the expression of the lateral flagella system, since the lateral flagella promoter activities were decreased in the absence of ExsD despite the promoter class. The Class I promoter *PfliM*, the Class II promoter *PflgB* and the Class III promoter *PlafA* were all affected by the absence of ExsD and ExsE. The fact that the Class I promoter is affected suggests that the ExsA protein represses the expression of the lateral flagella system prior to the lateral flagella major regulator LafK.

It is also unknown whether the ExsA protein regulates the lateral flagella system directly or indirect. As the ExsA protein has already been over-expressed using the pET system and the pMAL system, the direct protein-DNA interaction can be investigated using electrophoretic mobility shift assays (EMSA) to determine whether ExsA binds to the lateral flagella promoters in a direct protein-DNA interaction manner. Moreover, the interactions between LafK and Exs proteins can be carried out to determine whether there is a protein-protein level of regulation.

5.6 Conclusion

- The swarming ability was shown to be significantly repressed in the *lafK* mutant, *exsD* mutant and *exsE* mutant when compared to the *A. hydrophila* AH3R wild type, *exsA* mutant and *exsC* mutant using swarming assays.
- In *A. hydrophila* AH3R wild type, the activities of Class II and Class III lateral flagellar promoters were up-regulated when grown in solid conditions, except for *PlafB* and *Pmaf*. The activities of Class I promoters were not affected whether grown in liquid or solid conditions.
- The activities of lateral flagellar promoters were repressed in the *lafK* mutant, *exsD* mutant and *exsE* mutant when compared to the *A. hydrophila* AH3R wild type, *exsA* mutant and *exsC* mutant.

Chapter 6.
Conclusions

The results of this study suggest that the regulatory cascade of the T3SS which involves the ExsA, ExsD, ExsC and ExsE proteins in *A. hydrophila* AH-3 is similar to the T3SS regulatory cascade in *P. aeruginosa*. The transcriptional activator ExsA functions as the master regulator of the T3SS in *A. hydrophila* AH-3. The anti-activator protein ExsD inhibits the master regulator ExsA through direct protein-protein interactions. The chaperone protein ExsC is able to sequester the anti-activator ExsD from ExsA by direct binding to ExsD. While the effector protein ExsE binds to its cognate chaperone protein ExsC directly thus releasing ExsD to inhibit ExsA. The Exs regulatory cascade is also shown to be involved in the regulation of the lateral flagella system, in which ExsA possibly functions in down-regulating the lateral flagella promoter activities and suppressing the swarming motility.

Five promoter sequences, *PascN*, *PaopN*, *PexsC*, *PexsA* and *PexsD* were identied in the T3SS regulon and four of them were shown to have no activity in the absence of ExsA, except for *PexsA*, which was responsible for the expression of the master regulator ExsA itself. The promoter activity of *PexsA* was up-regulated in the *exsA* mutant while in the *E. coli* reconstitution system the *PexsA* promoter activity was down-regulated with *exsA in-trans*. This finding indicates that the master regulator ExsA is under control of a negative feedback by inhibiting its own promoter.

It was noted that the promoter activity of *PexsC* was not elevated and was much lower than the promoter activity of *PexsD* in the absence of ExsE. It possibly suggests that when ExsE is absent (secreted), more ExsD was made in contrast with ExsC due to higher promoter activity. The increase in the pool of ExsD proteins provide the bacteria with another level of regulation for ExsA activation. Moreover, as it was recently reported in *P. aeruginosa* that ExsD could only bind to ExsA when they were synthesized at the same time as folding intermediates, the ExsD protein released from ExsC binding could not re-bind the ExsA protein (Bernhards et al

2013). Thereby, it was not surprising that more ExsD was required to be synthesized than ExsC. In fact, the expression of ExsD was also driven by the *PexsA* promoter, as both the *exsA-ascL* and *exsD-ascL* operons share the same terminator, to allow simultaneous expression of ExsD and ExsA (Figure 3.16) (Bernhards et al 2013).

Therefore, the intracellular concentrations of the ExsA, ExsD, ExsC and ExsE proteins play a vital role in the regulation of the T3SS as the pools of ExsA, ExsD, ExsC and ExsE proteins are changing in response to the environmental signals. When the T3SS is trigged, the effector protein ExsE is thought to be secreted out from the cell thus the intracellular concentration of ExsE is decreased, resulting in the increase of a free ExsC pool due to the releases of the chaperone protein ExsC from ExsE. The increase of the free ExsC pool results in the capture of more ExsD protein, leading to the decrease of the intracellular concentration of free ExsD. Thereby more of the master regulator ExsA are released to up-regulate the expression of the T3SS.

The fact that the activation of *PexsD* promoter requires the T3SS master regulator ExsA may suggest a scenario where the bacteria switch off the T3SS and detach from the host cells. In this situation, the accumulation of ExsA suppresses the expression of itself by inhibiting its own promoter and synthesizing more anti-activator ExsD, reducing the expression of the T3SS overall. The increase of the ExsD pool results in the titration of the chaperone protein ExsC, thus the ExsC proteins are ‘outnumbered’ by ExsD, which results in the repression of the T3SS expression and the closure of the injectisome. It also makes sense as the closure of the T3SS channel leads to the accumulation of the secreted protein ExsE, which sequester the chaperone protein ExsC from binding the anti-activator ExsD.

However, the promoter activity of *PaopN* does not fit into the pattern. The promoter activity of *PaopN* is significantly decreased in every *exs* mutant strain when compared to the promoter activity measured in the wild type strain. As the

operon *aopN-aopD* regulated by *PaopN* involves at least three genes *acrR*, *acrG* and *acrV* which are possibly related to the T3SS regulation in response to calcium, it may suggest possible secondary regulation involved in the T3SS expression.

Both the BACTH assay and the Far-Western Blot indicate strong interactions in between ExsA-ExsD, ExsD-ExsC and ExsC-ExsE, supporting the hypothesis of the ExsA-ExsD-ExsC-ExsE regulatory cascade. Moreover, the self-interaction of ExsA protein was observed from BACTH assay while the self-interaction of ExsE protein was observed from Far-Western Blot. However, the self-association of ExsD and ExsC reported in *P. aeruginosa* was not observed in this study. As it has been reported in *P. aeruginosa*, ExsC dimerises to form a 2:2 heterotetramer with ExsD while bind to ExsE at a 2:1 ratio (Bernhards et al 2013, Zheng et al 2007). This might be due to the fusion of T25/T18, His_6 or MBP onto the N-terminal of these proteins during over-expression or because the self-association of ExsC and ExsD required the presence of chaperones.

Moreover, the regulatory components of the T3SS (Exs proteins) has been shown to be involved in the regulation of the lateral flagella system. The swarming assay of the *exs* mutants has shown reduced swarming motility in *exsD* mutant and *exsE* mutant strains similar to the *lafK* mutant strain. It suggests that the T3SS master regulator ExsA functions in repressing the expression of the lateral flagella system in *A. hydrophila* AH-3. It correlates with the findings in *V. parahaemolyticus*, which possess lateral flagella system as well as two T3SSs. The expression level of the lateral flagella gene $flgB_L$ was significantly repressed when over-expressing ExsA and the swarming motility of *V. parahaemolyticus* was inhibited by *exsA* induction *in vivo* (Gode-Potratz et al 2010).

The lateral flagella promoter activities measured in liquid conditions and solid conditions correlate with the lateral flagellar gene transcriptional hierarchy proposed by Wilhelms and collegues, who has classified the lateral flagella promoters into three classes (Wilhelms et al 2013). In general, Class I promoters, including *PfliM*,

PlafK and *PflgA*, have shown no significant difference of activities or even down-regulated when measured on swarming agar compared to in broth culture. In contrast, the activities of Class II and Class III promoters were up-regulated when measured on swarming agar compared to be measured in broth culture, except for *Pmaf* and *PlafB* promoters. The reason why these two promoters failed to fit into the pattern is unclear. The *Pmaf* promoter, which is reported to be a Class III promoter in the lateral flagella system and is required for the expression of the Maf-5 protein (Wilhelms et al 2013). As Maf-5 is a glycosyl-transferase, it is involved in the glycosylation of the lateral flagellin protein LafA with pseudamic acid. (Wilhelms et al 2012). However, Maf proteins may also be required for the polar flagella system as it is reported that Maf proteins are required for full function of the polar flagellin in *A. caviae* (Parker et al 2012, Parker et al 2014). As for *PlafB*, The activity of *PlafB* promoter was too low to be considered as an intact promoter.

The patterns of the promoter activities (*PfliM*, *PflgB* and *PlafA*) measured in the *exs* mutant backgrounds were similar to the patterns of the swarming diameters of different mutant strains. It indicates that the lateral flagella promoter activities are significantly repressed in the absence of ExsD and ExsE, while not affected in the absence of ExsA and ExsC, supporting the hypothesis that the T3SS master regulator ExsA functions as a repressor of the lateral flagella system. When the anti-activator ExsD is absent, the abundant ExsA proteins suppress the expression of the lateral flagella system. Similarly, when ExsE is absent, the chaperone protein ExsC binds to the anti-activator ExsD thus allowing ExsA to repress the lateral flagella system. Although this potential negative cross-talk between the T3SS and the lateral flagella system has not been reported in *A. hydrophila* AH-3 before, there was evidence of cross-talk between the T3SS and the flagella system in *P.aeruginosa* and *Y. enterocolitica* (Bleves et al 2002, Soscia et al 2007, Vilches et al 2009).

An overview of the genetic regulation between the T3SS and the lateral flagella system is shown in Figure 6.1. The T3SS master regulator ExsA activates the transcription of the T3SS by inducing the T3SS promoter activities, except that ExsA negative regulates its own promoter *PexsA*. The master regulator ExsA is controlled by a cascade of proteins including ExsD, ExsC and ExsE. The de-activator ExsD inhibits ExsA by direct binding. ExsC inhibits ExsD and ExsE inhibits ExsC via direct protein-protein interactions as well. ExsA was also shown to negatively regulate the lateral flagella system since the absence of ExsD or ExsE represses the swarming ability and the activities of lateral flagellar promoters.

It was only shown in this study that the regulatory components of the T3SS were involved in the regulation of the lateral flagella system in *A. hydrophila* AH-3. Whether the lateral flagella system affects the T3SS in *A. hydrophila* AH-3 is still unknown, although there was evidence showing that the lateral flagella major regulator LafK was required for T3SS1 expression in *V. parahaemolyticus* (Gode-Potratz et al 2010).

Given the tools prepared in this study so far, the T3SS promoter activities can be measured in the *lafK* mutant background in the presence of Ca^{2+} and/or EGTA. Moreover, the effect of iron in correlation to the T3SS and the lateral flagella system can also be investigated in *A. hydrophila* AH-3. As for the Exs proteins, a pET_{DUET} system may be utilitzed to co-express two proteins in the same time as most of them are chaperones of the others (e.g. ExsA-ExsD, ExsD-ExsC and ExsC-ExsE). Thereby, more protein assays such as Pull-down assay can be used to investigate direct protein-protein interactions between Exs proteins. Also, EMSA can be used to inviestigate the interactions between ExsA and promoter sequences of both T3SS and lateral flagella system.

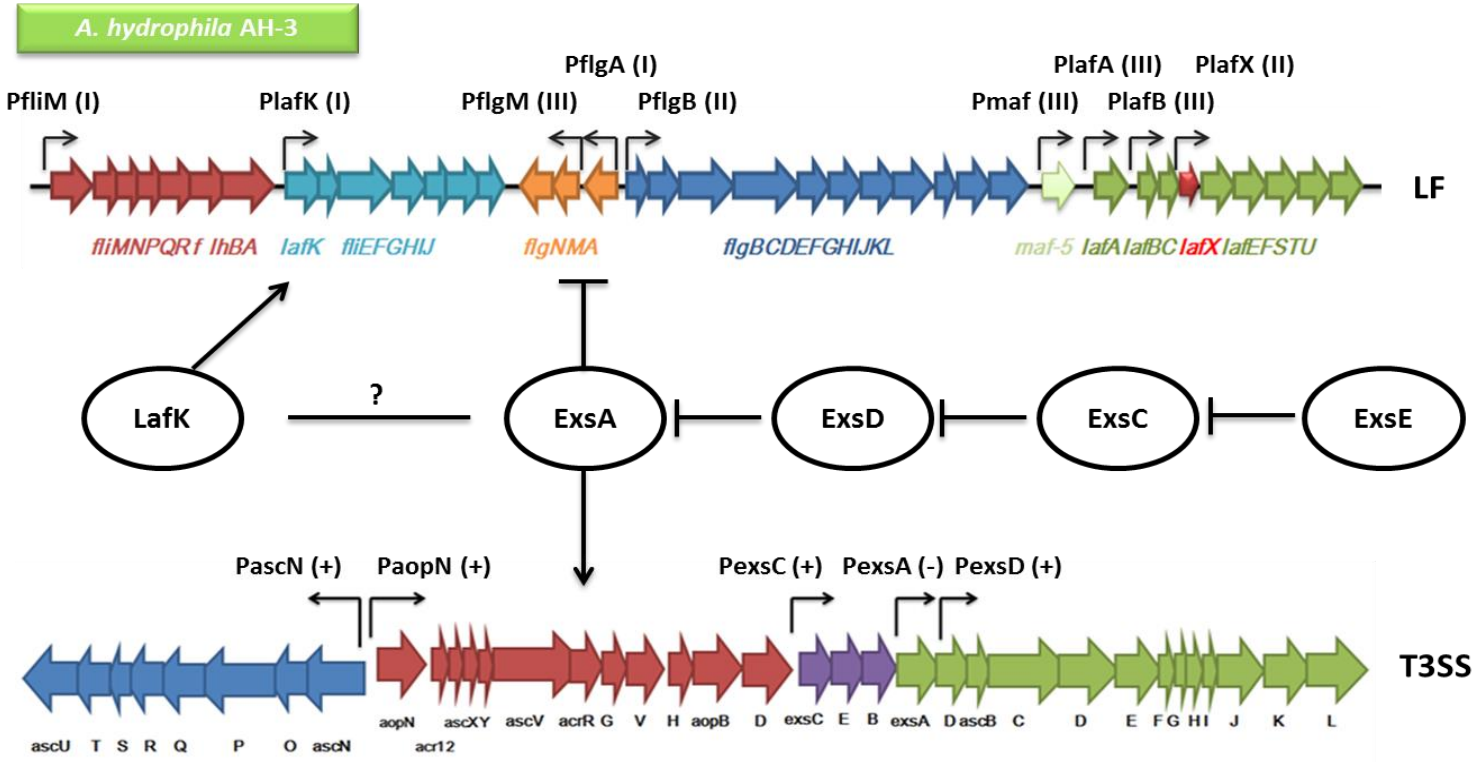

Figure 6.1 An overview of the genetic regulation between the T3SS and the lateral flagella (LF) system in *A. hydrophila* AH-3. The regulators including ExsA, ExsD, ExsC, ExsE and LafK are shown in circles. Each two of the Exs protein are connected by blunt arrows showing the inhibitory interactions in between. The promoters in both the T3SS and the LF system are shown in bent arrows. The promoters in the T3SS are either (+) up-regulated by ExsA or (-) down-regulated by ExsA. The LF promoters are categorized into (I) Class I, (II) Class II or (III) Class III, in which only Class II and Class III promoters are up-regulated by the LF major regulator LafK. The T3SS master regulator ExsA has been shown to down-regulate the expression of the LF system but whether there is interaction between ExsA and LafK is unknown. .

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Appendix 1

Primers used in the project:

exsC internal forward: CTCCACTTTGGTTTCGATGA

exsC internal reverse: ATTGAACTGATACCAGTGAC

exsC amplification forward: GGAGGAAATCATGGATGTAA

exsC amplification reverse: ATCTTCATGGTTATACCCGC

exsD F1 forward:

GAGCTCGGTACCCGGGGATCCTCTAGAGTCATGAGTCAGCAAGATCACAA

exsD F1 reverse:

AAGCTGTCAAACATGAGAACCAAGGAGAATGATGCGATCTCCCAGCTGT

exsD F2 forward:

GAATTGTTTTAGTACCTAGCCAAGGTGTGCTCGCGCCTTGCTGTGGCACT

exsD F2 reverse:

AGAATACTCAAGCTTGCATGCCTGCAGGTCGCTATCTAGGGCTCGGCAGG

exsE F1 forward:

GAGCTCGGTACCCGGGGATCCTCTAGAGTCTCAATCGGCTGCTCACTGAG

exsE F1 reverse:

AAGCTGTCAAACATGAGAACCAAGGAGAAACTTGGCTCGCACTGGCTGG

exsE F2 forward:

GAATTGTTTTAGTACCTAGCCAAGGTGTGCGCACTTCTGCGACGCTCCAT

exsE F2 reverse:

AGAATACTCAAGCTTGCATGCCTGCAGGTCCAATGTTTGGGCTGCAATTG

Kan forward: TTCTCCTTGGTTCTCATGTTTGACAGCTT

Kan reverse: GCACACCTTGGCTAGGTACTAAAACAATTC

exsD_pGEM forward GCGAATTCATGAGTCACCAAGATCACAA

exsD_pGEM reverse GCGAATTCGCTATCTAGGGCTCGGCAGG

exsE_pGEM forward GCGAATTCTCAATCGGCTGCTCACTGAG

exsE_pGEM reverse GCGAATTCCAATGTTTGGGCTGCAATTG

*exsA*_pKT_forward GCCTCTAGAGATGAATGGCATTACTACTGCAG

*exsA*_pKT_reverse GCGAATTCTTAATCAGTGCCATGTCTGGC

*exsC*_pKT_forward GCTCTAGAGGATGTAACTGTCATCATCAA

*exsC*_pKT_reverse GCGAATTCTTATACCCGCACTCCCATCA

*exsD*_pKT_forward GCGCTCTAGACATGAGTCAGCAAGATCACAATTC

*exsD*_pKT_reverse GAGAATTCCTAGGGCTCGGCAGGCTGCCA

*exsE*_pKT_forward GCTCTAGAGAAGATTCAGGAATCACAAGG

*exsE*_pKT_reverse GCGAATTCTCATAACACCCGGATCCGAC

*exsA*_pKNT_forward GCGCAAGCTTGATGAATGGCATTACTACTGCAG

*exsA*_pKNT_reverse GCTCTAGAGAATCAGTGCCATGTCTGGC

*exsC*_pKNT_forward GCTCTAGAGGATGTAACTGTCATCATCAA

*exsC*_pKNT_reverse GCGAATTCGATACCCGCACTCCCATCACCT

*exsD*_pKNT_forward GCAAGCTTGATGAGTCAGCAAGATCACAATTC

*exsD*_pKNT_reverse GCTCTAGATAGGGCTCGGCAGGCTGCCAGT

*exsE*_pKNT_forward GCTCTAGAGAAGATTCAGGAATCACAAGG

*exsE*_pKNT_reverse GCGAATTCGATAACACCCGGATCCGACGTT

*exsA*_pUT_forward GCGCAAGCTTGATGAATGGCATTACTACTGCAG

*exsA*_pUT_reverse GCTCTAGAGAATCAGTGCCATGTCTGGC

*exsC*_pUT_forward GCTCTAGAGGATGTAACTGTCATCATCAA

*exsC*_pUT_reverse GCGAATTCGATACCCGCACTCCCATCACCT

*exsD*_pUT_forward GCAAGCTTGATGAGTCAGCAAGATCACAATTC

*exsD*_pUT_reverse GCTCTAGATAGGGCTCGGCAGGCTGCCAGT

*exsE*_pUT_forward GCTCTAGAGAAGATTCAGGAATCACAAGG

*exsE*_pUT_reverse GCGAATTCGATAACACCCGGATCCGACGTT

*exsA*_pUT18C_forward GCCTCTAGAGATGAATGGCATTACTACTGCAG

*exsA*_pUT18C_reverse GCGAATTCTTAATCAGTGCCATGTCTGGC

*exsC*_pUT18C_forward GCTCTAGAGGATGTAACTGTCATCATCAA

*exsC*_pUT18C_reverse GCGAATTCTTATACCCGCACTCCCATCA

*exsD*_pUT18C_forward GCGCTCTAGACATGAGTCAGCAAGATCACAATTC

*exsD*_pUT18C_reverse GAGAATTCCTAGGGCTCGGCAGGCTGCCA

*exsE*_pUT18C_forward GCTCTAGAGAAGATTCAGGAATCACAAGG

*exsE*_pUT18C_reverse GCGAATTCTCATAACACCCGGATCCGAC

pKT25_screen forward GCACATGTTCGCCATTATGCCG

pKT25_screen reverse GCATTCAGGCTGCGCAACTGTT

pKNT25_screen forward GCACAGGTTTCCCGACTGGAAA

pKNT25_screen reverse GCCGGAACATCAATGTGGCGTT

pUT18_screen forward GCCACCCCAGGCTTTACACTTT

pUT18_screen reverse GCTCACGCCGATATTCATGTGG

pUT18C_screen forward GCAAAAGCCTGTTCGACGATGG

pUT18C_screen reverse GCTCACAGCTTATCTGTAAGCG

exsC for pET forward GCCATATGGATGTAACTGTC

exsC for pET reverse GCAAGCTTTTATACCCGCAC

exsD for pET forward GCCATATGAGTCAGCAAGAT

exsD for pET reverse GCAAGCTTCTAGGGCTCGGC

exsE for pET forward GCCATATGAAGATTCAGGAATCACAAGG

exsE for pET reverse GCGAATTCTCATAACACCCGGATCCGACG

T7 promoter (forward): TAATACGACTCACTATAGGG

T7 terminator (reverse): GCTAGTTATTGCTCAGCGG

pMAL screening primer forward GGTCGTCAGACTGTCGATGAAGCC

pMAL screening primer reverse TGTCCTACTCAGGAGAGCGTTCAC

PfliM amplification forward GCAAGCTTAGATCCATGGCGTCAAGAAG

PfliM amplification reverse GCGGATCCCAAAGTAACCGAGAAGGTGT

PlafK amplification forward GCAAGCTTTGGATAGCTTTCCGGTTGAT

PlafK amplification reverse GCGGATCCCGCAGGTCTGATCAATAACA

PflgM amplification forward GCAAGCTTCGCAGTGACCCATTTGCCAC

PflgM amplification reverse GCGGATCCCTGCAGCTGGGTTTGAACAT

PflgA amplification forward GCAAGCTTGCAGTATTGCCATCCATGGA

PflgA amplification reverse GCGGATCCGGGAAACAACACTTCCCCTT

PflgB amplification forward GCAAGCTTTTCTCGACGAGATCTCCGGT

PflgB amplification reverse GCGGATCCTAATCGACATCTCGGGCCAG

Pmaf amplification forward GCAAGCTTTACAGTTCAGAGCGACTCGA

Pmaf amplification reverse GCGGATCCGGTACGCTGTTGACATCCTG

PlafA amplification forward GCAAGCTTGGAGCTCTATATCAAGACCC

PlafA amplification reverse GCGGATCCCCAGCATCTTGTTGGTAGAG

PlafB amplification forward GCAAGCTTTTCGATGCATCCACCAAAGT

PlafB amplification reverse GCGGATCCCTCTGCTGACCTTTGATGCC

PlafX amplification forward GCAAGCTTAGGCCATATTCTTGCCAAGC

PlafX amplification reverse GCGGATCCGTTGCAAGATCAGACGACCC

pKAGb2(-) screening primer *bla* (forward) TGCACCCAACTGATCTTCAG

pKAGb2(-) screening primer *lacZ* (reverse) TTTCCCAGTCACGACGTTGT

Appendix 2

DNA sequence of *exs* genes

exsC 16742-17185(444bp):

ATGGATGTAA CTGTCATCAT CAATCGGCTG CTCACTGAGT TTGCCACCAA ATATGGTTTG
CCATCCCTGA CCCTGAATCA GGAGGGGGTT GCTGCGCTCT GTTTTGACGA GCAACTGCAA
CTCAGCCTGA TCCTGGTATC GGAGCGGGAT CAGCTGGTGT TGCAGGCCGA CGTGGCCGAG
CTCCATCAGG TAGGTGAGGG GATTTTTCGT CAGTTGGCCA GCTTCAATCG TCACTGGTAT
CAGTTCAATC TCCACTTTGG TTTCGATGAA GAGAGCCTGA CGGTGCAACT TTATCGGCAG
ATGACGGCAA GCCGGTTGAC CCTGGCATAC CTTGAAGAGA GTCTCGCCAG CATGCTGGAG
CATGCCGAGT TCTGGCAAGA GTTGCTGCAA CCTGGTACTC GGGACGCAGG CGGTAGTGAT
CAGGTGATGG GAGTGCGGGT ATAA

exsE 17188-17421 (234bp):

ATGAAGATTC AGGAATCACA AGGCGCACTG GCCCTGCATG CGGCAGAGCC GGGCAAGGTG
GGGGGATTTG CTGGCAGAAC CATGAGTGCA CAGCCAGCCA GTGCGAGCCA AGTTCCCCTG
TCGGCACTTC TGCGACGCTC CATTACCCTC AATCAAGTAC AGGAGCTGGC GCTTCAGCGA
CTGCAACAGG GCGAGCATAC CTCACTGGCC GAACGTCGGA TCCGGGTGTT ATGA

exsA 18165-18980 (816bp):

ATGAATGGCA TTACTACTGC AGAGAAGGGC GATATGGCCC TGCTCCAGTG GTGCATGTCT
GCTTTCAACG TCATCGAACA TCCGCAAGAG GGAATATATA TCCTTCTTGA AGGTTCGATA
ACCTGGCAGG ACTGTACCGA TACCTACGAA CTCACCCCCA ACCAACTCCT GTTCGTGCGC
CGTGGCAACT ACGCGGTTTG TACCGCTGGC AGCCCCTGCC GCCTGCTTTG GCTGCCCCTG
TCAAACAGCT TTTTGCAAGG ATTCTTGCAA CGTTTCGGTT CCCTGTTGAG CGAAGTCGCC
CGGCTGGAGG GGATGGCCCC GACGCTGTTG CCGTTTCACT CTTCCCCCCT GCTGACCCAA
TGTATTCAGG GGCTGTACGG TTTGATTGAC CATGAGCATC CGCCCGCACT GGCCCAATTA
CGCACCGAAG AGCTGCTGTT TCTACTCGCC TTTGGTGAGC AGGGGCCTCA ACTGATGTCA
ATTTTGCGTC AGCTGAGCAA TCGTCAGGTC GAGCGGTTGC AACAGTTCAT GGAAAAGCAC
TACTTGATGG AGTGGAAGCT CAGCGAATTT TCCAAAGAGT TCGGCATGGG GCTGACCACC
TTCAAGGAGC TATTCGGCTC GATATATGGT GTCTCACCGC GAGCCTGGAT CAGTGAACGG
CGGATCCTGT TCGCTCATCA GTTATTGCTC AATAGCCCTT CTAGCATCGT TGATATTGCG
ATGGAGGCCG GTTTTTCCAG CCAATCTTAT TTTACCCAGA GTTATCGCCG TCGTTTTGGT
TGTACGCCAA GTCGTGCCAG ACATGGCACT GATTAA

exsD 19100-19915 (816bp):

ATGAGTCAGC AAGATCACAA TTCATCTAAC CAAGGCCTGT TTGCAGGTCG TCGGGTTACC
GTTGTTCAGC CAGATACCTT GAGTCGGGAT CGTCTGGTCG GCCAACTGTC CGTGCTCCGT
TATCAGGATG CCGGCGTGAT CACCTCGCAG CAAATGGACC TGTTGCAACG GCTGTTGCCA
AGAACCCGGC TGGAGAGCCT GCTGGAATCC CTCTGGTTCC AGCGTCGTCT GGATGCCGCG
CTGAGCGTCT CTCGCGAAGA GCTGCAACAA ATTCTGCGGC TGGCGGGCAG TGAGCGATAC

GACTGGCTGC AACAGCTGGG AGATCGCATC AATCTGGCGG ATCGCGCCTT GCTGTGGCAC
TGGGTCCTTC ACCCTTTGCA TCGCTGGTGG GTGCAGCGTC TGGAACCGCT CTACGGCGCT
TGGCGCAACG AGCTGGTACA GCTGCAGGTT ATGCGCCGTC AACTCAATGC CCAGGCGGTG
TTCTGGCAGA CAGTGGTCGA TGTGCCGGCG GATCTGGAGA GCCGGATCAC CGACCAGCTT
GCGCAGTTGA GCCAGCGCGA GCAGGAGCTT ACACAACTGC ATTCCGATTG CGAAGCGCGT
CTTCAGCTGG CTTGGCCCGC CTGGTATGGG CAGACCAGTC AGGAAGGCGA CCCGGCACTT
CTTATGCCGG TACCGCTGGA GCTTGGCGTA TTCTGGCACG CCCTGCTGGC GCTGCCCCAC
CAGGACGACG TGGCCCTTAC GTTGCACGAG TGGCTGGTCG GTCGGGGTAT TGCCCTGGGT
CAGGATCACT TCTACTGGCA GCCTGCCGAG CCCTAG