Regulation of flagellar mediated motility in the species ***Salmonella enterica***

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Abstract

Salmonella enterica is considered zoonotic pathogen with capability to colonies on range of plants and animals allowing transmission between them. Whole genome sequence analysis of *S. enterica* generates a phylogenetic tree comprising of three clades: A1, A2 and B. These 3 clades encompass the known 2,600 serovars used to type *S. enterica* during clinical outbreaks of salmonellosis. *S. enterica* exploits the bacterial flagellum to be motile in liquid environments and over surfaces. The genetic regulation of flagellar assembly is an elegant and harmonious system driving assembly of the flagellum from the base upwards.

We surveyed the response and changes to flagellar regulation in a cohort of *S. enterica* serovars. Our analysis encompassed examining phenotypic motility, flagellar gene expression and flagellar abundance depending on nutrient composition. We demonstrated that the timing of flagellar gene expression is consistent across the species but the magnitude of flagellar gene expression varies significantly. The *S. enterica* flagellar system is bistable, producing a heterogeneous population of motile cells. Our data suggested that population heterogeneity plays a role in the adaptation of *S. enterica* serovars with respect to motility.

The great similarity of the flagellum systems between *S.enterica* and *E.coli* gave us a reason to study why flagellar regulation in *S.enterica* differed from *E. coli*. Indeed, we replaced the master flagellar regulators, *flhDC* from *E.coli* into the *S. enterica.* We found a significant variation in $FlhD_4C_2$ activity through mixing *flhD* and *flhC* between both organisms. In conclusion, the diversity and changes we observe in just a small subset of *S. enterica* serovars and by introducing *flhDC* homologues has made us reconsider a number of assumptions we make about the regulation of the flagellar system based on model-domesticated strains of *S. enterica*.

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شكر وتقدير

بسم الله الرحمن الرحيم

(سَنُرِيهِمْ آيَاتِنَا فِي الْآفَاقِ وَفِي أَنْفُسِهِمْ حَتَّى يَتَبَيَّنَ لَهُمْ أَنَّهُ الْحَقُّ)

في البداية نشكر الله تعالى على توفيقه على اتمام هذا العمل حيث اتقدم بجزيل الشكر الى كل شخص في مركز البيولوجيا الخلوية البكتيرية / كلية الطب / جامعة نيوكاسيل في بريطانيا واخص بالامتنان والعرفان الى الدكتور فيل الدريج الذي شرفنا بقبوله الاشراف على اطروحة الدكتوراه فله منا كل التقدير والاحترام، حيث كان له الاثر الكبير في توجيهه ودعمه المتواصل للوصول الى اهداف البحث.

اضافة الشكر والتقدير الى السادة رتشارد دانيل و كيفن ولتون على متابعتنا طيلة الثلاث سنوات الماضية.

أيضا، كنت محضوضاً بالتعرف على الدكتور توم اوين و الدكتور جاد ساسين بالتوجيه والمساعدة بالمختبر.

شكر خاص موصول بالفخر الى والدي ووالدتي العزيزين على قلبي الذي لو لا دعاءهما لي طيلة فترة الدراسة لما كان هذا الانجاز، اضافة الى اخوتي واخواتي واخص اسامه، ارقم، اسراء وانعام. لا ننسى ايضاً شكري وتقديري الى عمي د. عبد المنعم الليلة وعمتي نجوى الدباغ دعمهم المتواصل والدعاء لنا.

شكر خاص ايضاً يمله المحبة والامتنان الى د. يحيى المشهداني رئيس قسم علوم البيئة اضافة الى الاستاذ الدكتور أبي الديوه جي رئيس جامعة الموصل، الذي لولا دعمهم لما كنت درست الدكتوراة في بريطانيا.

اخيراً، انا مدين بعمق وشعور بالامتنان الخاص الى زوجتي العزيزة اسيل دعمها وحثها المتواصل لي طيلة فترة دراستي، حيث كانت الاب والام والاخ والصدر الحنون هنا في بريطانيا.

شكر خاص الى بلدي العراق الذي اعطاني الفرصة للدراسة والمتمثل بوزارة التعليم العالي والبحث العلمي والى الملحقية العراقية في لندن لدعمها المتواصل طيلة ايام الدراسة.

أيمن محمد جبر البنا، اب 2017

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List of Abbreviations

3’ 3	prime end of DNA/RNA
5’ 5	prime end of DNA/RNA
ADP	Adenosine Diphosphate
Amp	Ampicillin
ATP	Adenosine-5'-triphosphate
cAMP	Cyclic Adenosine Mono-Phosphate
CAP	Catabolite Activator Protein
CDTs	Cytolethal Distending Toxin
Cm	Chloramphenicol
C-terminus	Peptide carboxy terminus
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
e, ec	*Escherichia coli*
ESMA	Electrophoretic Mobility Shift Assay
FT	Flow Through
GFP	Green Fluorescent Protein
GI	Gastrointestinal Tract
H2O	Water
HACCP	Hazard Analysis and Critical Control Points
HAP	Flagellar Hook-Associated Protein
HBB	Flagellar hook basal body
HIV	Human Immunodeficiency Virus
H-NS	Histone-like nucleoid-structuring protein
Hr/hrs	Hour/hours
IM	Bacterial inner membrane
IPTG	Isopropyl B-D-1-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase pair
LB	Luria Bertani
M	Molar
mg	Milligram

min/mins	Minute/Minutes
MinE	Minimal E salt growth medium
ml	Millilitre
MLST	Multilocus Sequence Typing
mm	Millimeter
mM	Millimolar
mRNA	Messenger ribonucleic acid
ms	Millisecond
MW	Molecular weight
NaAc	Sodium acetate
NaCl	Sodium chloride
NaN_3	Sodium azide
N-terminus	Peptide amino terminus
OM	Bacterial outer membrane
OMP	Outer membrane protein
P	Prompter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
RLU	Relative Light Units
RNA	Ribose nucleic acid
RPM	Revolutions per minute
S, st	*Salmonella enterica*
s/sec/secs	Second/seconds
SDS	Sodium dodecyl sulphate
SPI	*Salmonella enterica* pathogenicity island
T/*t*	Time
T3S	Type three secretion
T3SS	Type three secretion system
TEMED	Tetramethylethylenediamine
Tet, tet	Tetracycline
Tm	Annealing Temperature
TPA	Type strain Phil Aldridge

UV	Ultra violet
V	Volts
V	Volume
w/v	Percentage volume concentration
WHO	World Health Organisation
WT	Wild type
µl	Microliter
µm	Micrometer
µM	Micromolar
σ	Sigma factor

Chapter One: Introduction

1.1 History of *Salmonella*

Salmonellosis has been considered a health problem for years, being a significant economic burden in relation to illness and death. In 1880 Karl Joseph Eberth was the first bacteriologist to recognize *Salmonella* under the microscope naming the cells he observed “typhoid bacilli” (Marineli *et al.*). In 1884, Georg Gaffky was the first to describe *Salmonella enterica* serovar Typhi, identifying his observations as ‘’bacillus typhus’’ (Gaffky, 1884). Later in 1886, the veterinarian D. Salmon and his colleague T. Smith discovered the main cause of swine fever (hog cholera) in the United States (Salmon, 1886). In 1900, the bacterial species was subsequently named *Salmonella* to reflect who discovered it (Salmonella Subcommittee of the Nomenclature Committee of the International Society for, 1934). Further investigation by White in 1925 proposed an antigenic diagram for the classification of Salmonellae based on somatic and flagella antigens. Consequently, scientist Kauffmann developed the *Salmonella* serological scheme defined as the Kauffmann-White chart including 2540 serovars that is still used to this day (Kauffmann, 1947).

1.2 Features of the Genus *Salmonella*

Salmonella is a Gram-negative, facultative anaerobic, motile, non-spore forming bacterium. *Salmonella* are rods in shape ranging in length between 2-5μm with a diameter of 0.7-1.5 μm. *Salmonella* are attributed to the family called *Enterobacteriaceae* (Fàbrega and Vila, 2013). Typically, *Salmonella* are motile in liquid and on semi-solid media because they have flagella. However, some serotypes are non-motile such as serovars Gallinarum and Pullorum (Holt *et al.*, 1986). In principle, *Salmonella* has been divided into two species: *bongori* and *enterica* based upon the hypothesis suggested by White in 1929 (Murray, 2009). Subsequently Kauffmann amended the *Salmonella* serotyping scheme in 1966 to include more than

2,600 serovars (Popoff *et al.*, 1998). *Salmonella* serovars are determined depending upon the expression of flagellar (H) and somatic lipopolysaccharide (O) antigens. After development of methods including DNA-DNA hybridisation (Brenner *et al.*, 2000), it was found that most serotypes could be further collated as subspecies (Reeves *et al.*, 1989). Recently, The World Health Organization (WHO), based on the Kauffmann-White scheme, has updated the Genus of *Salmonella* (Grimont and Weill, 2007) *Salmonella* is therefore divided into two species, *S. enterica* and *S. bongori. S. enterica* is then further divided to six subspecies: *S. enterica* subsp. enterica, *S. enterica* subsp. salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae and *S. enterica* subsp. indica. (Guiney and Fierer, 2011) (figure1). Over the last century, microbiologists have used nutritional and serological properties to characterize bacteria (Urwin and Maiden, 2003). At the present time, new systems for the diagnosis are slowly being adopted. One example is based on Multi-locus Sequence Typing (MLST) as a substitute to describe *Salmonella enterica* (Achtman *et al.*, 2012a). MLST is described as sequencing gene fragments from seven housekeeping genes in order to identify and catalogue organisms (Maiden, 2006). Recently, using MLST instead of serotyping for identification of *Salmonella* species provides a more accurate diagnosis and rapid epidemiological tracking. MLST has given us a better appreciation of *Salmonella* diversity and accurate epidemiology, although not strictly direct diagnosis of cases (Achtman *et al.*, 2012a).

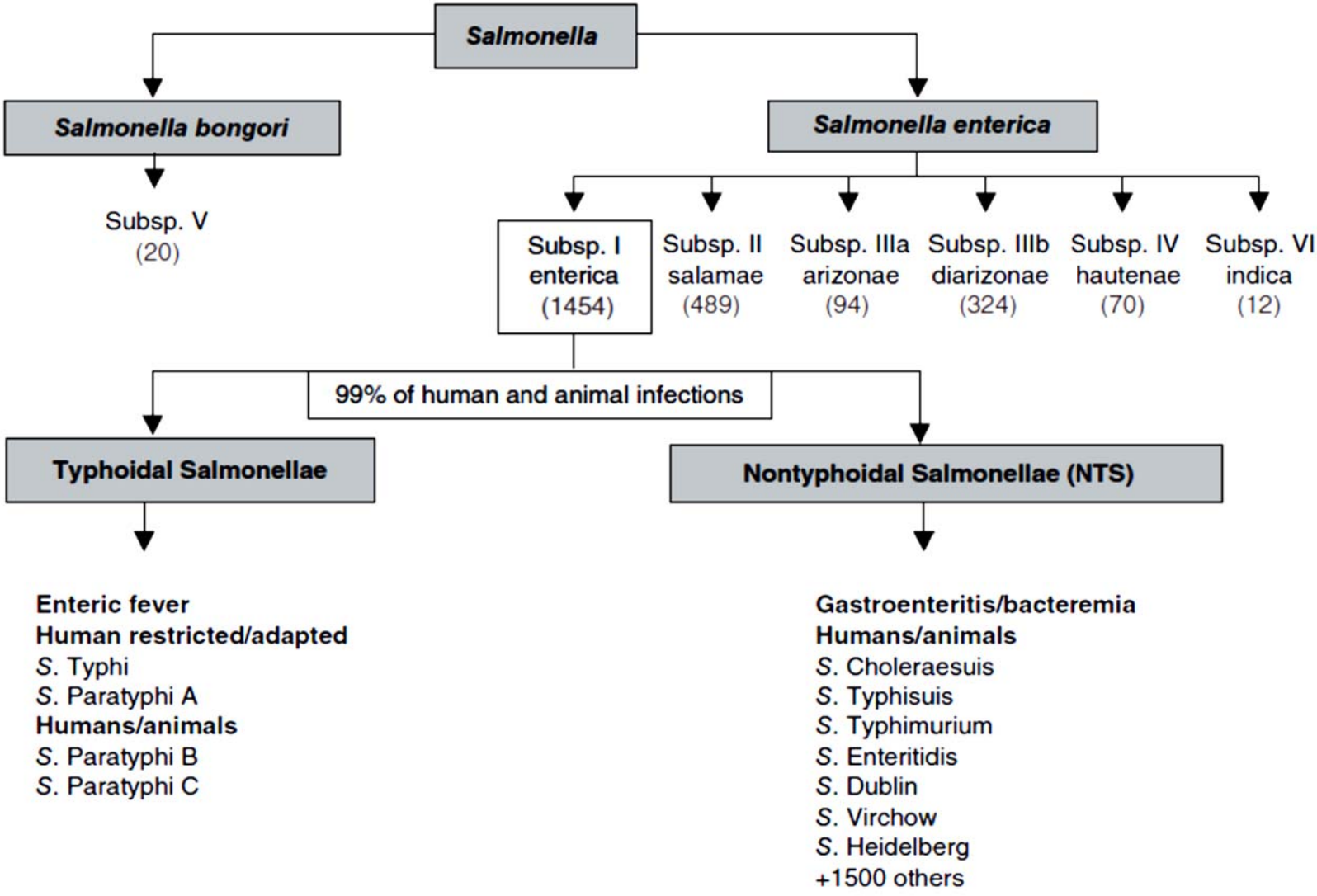


Figure 1. The genus *Salmonella* subspecies taxonomy, adapted from (Langridge *et al.*).

1.3 Animal Reservoirs for *Salmonella* and Routes of Transmission

Salmonellosis is spread through various forms of transmission. Contamination of environmental sources are the most common associated with *Salmonella* infection (figure 2). For example, farms, water and direct contact with animals colonised with *Salmonella* (Pui *et al.*, 2011). Salmonellosis is conveyed across to humans mainly via contaminated or undercooked meats from poultry, cattle, sheep, pigs as they are the most predominant reservoirs (Samuel, 1996). *Salmonella* are able to survive many years in the soil compared to months in water if the environmental circumstances are suitable (Todar, 2015). We as hosts are also considered a reservoir permitting person to person transmission (Mermin *et al.*, 2004).

1.3.1 Salmonellosis in Poultry

Poultry are considered one of the biggest and most crucial reservoirs of *Salmonella* compared to other animals (Khan, 1969). The prevalent serotypes are carried in the poultry reservoir worldwide: Typhimurium, Enteritidis, Gallinarum and Pullorum respectively (Wallis, 2006). For Typhimurium and Enteritidis, infection starts in the digestive system by overrunning and colonising intestinal cells. The consequence is a severe systemic infection in small chicks (Kaiser *et al.*, 2000). As the physiological nature of poultry means that they share the digestive tract and reproductive organ, eggs will be contaminated with *Salmonella* and thus allow direct transmission to the developing chick (Howard *et al.*, 2012). Gallinarum is a source of adult chicken fowl typhoid disease, characterized by an acute septicaemia and haemorrhages (Shivaprasad *et al.*, 2013). For Pullorum infection, chicks develop white diarrhoea and egg infection as Pullorum colonizes the reproductive tract with great efficacy (Wigley *et al.*, 2001; Haider *et al.*, 2014). *Salmonella* in the poultry industry is considered a serious economic burden by decreasing production via mortality and

the associated high cost of treatment prevention. There is also a significant impact on public health as a result of the ease of transmission via food to the human population (McEntire *et al.*, 2014).

1.3.2 Salmonellosis in Cattles

Salmonella in cattle causes fever, decrease production of milk, loss of appetite and severe diarrhoea. The most common *Salmonella* serovars that infect cattle are Typhimurium and Dublin (Wallis, 2006). With respect to Typhimurium infection is frequently associated with indigestion, inflammation of the digestive system, bloody diarrhoea, anaemia, dehydration and death (Elvidge, 2013). Moreover, in Vietnam, Typhimurium is associated with human infectious disease especially via consumption of infected meat (Vo *et al.*, 2006). Occasionally, Typhimurium in the cattle might be an intermediate host (carrier and latent) without any clinical signs. The carrier state is still considered dangerous due to pathogen shedding and the subsequent environmental spread. Shedding has also been implicated with atypical livestock infection routes such as via the respiratory tract and conjunctiva (Wallis and Barrow, 2005). For serovar Dublin, systemic infections in particular in pregnant cows can lead to neonatal infection (Hall and Jones, 1977). The main route of infection is through oral transmission by contaminated fields due to faecal matter (Pell, 1997). Once more, the associated public health problems of *Salmonella* disease among cattle is a significant economic threat especially, like in poultry, the high cost of treatment, increase in the percentage of abortion, decrease a meat production and reduced milk yield (Visser *et al.*, 1997).

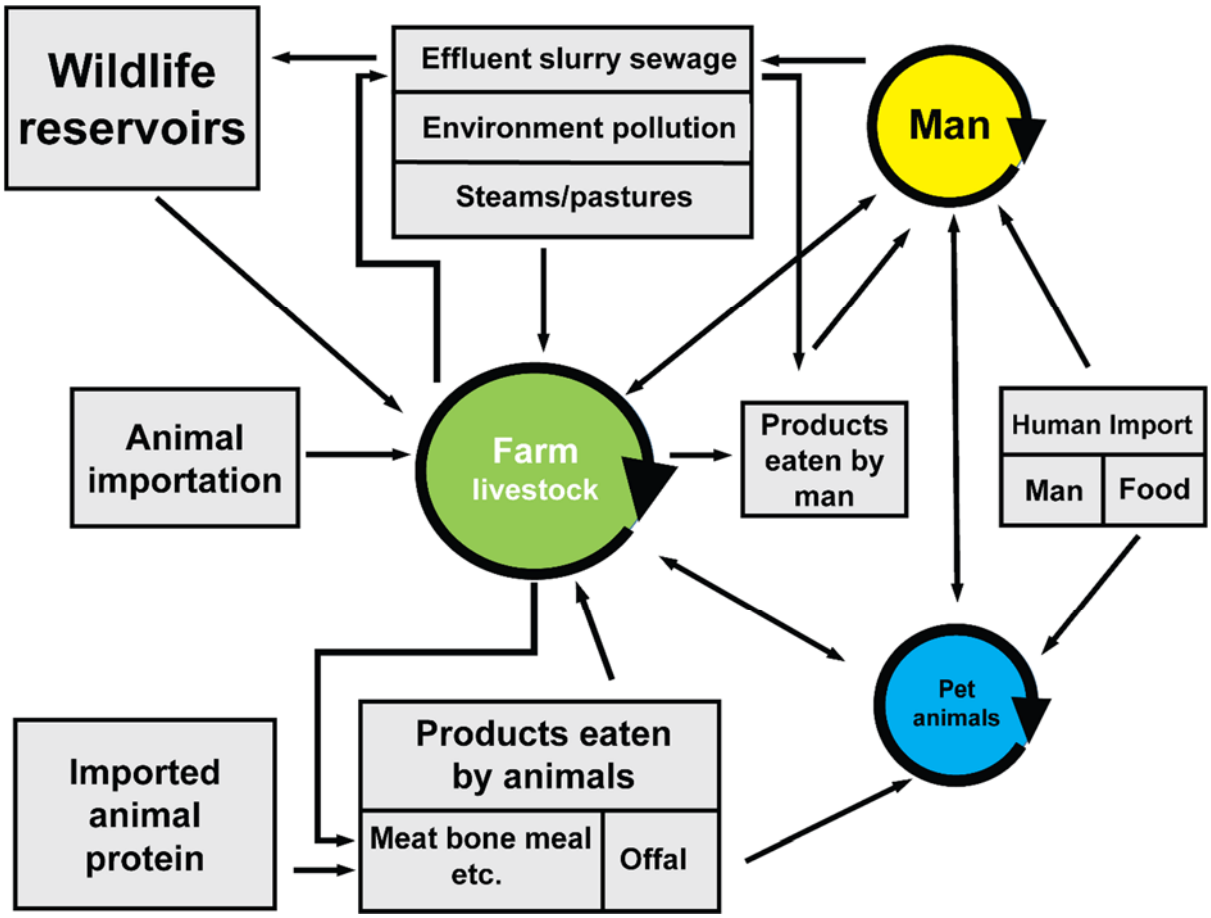


Figure 2. The *Salmonella* life cycle and infection, adapted from (Torrence and Isaacson, 2008).

1.3.3 Salmonellosis in Pigs

The *Salmonella* serovars infectious for the pig are divided into two categories: host-restricted and ubiquitous. The predominant serovar in pigs is Choleraesuis and considered the main problem for pig breeding (Sojka *et al.*, 1977; Wallis and Barrow, 2005). The clinical signs start with general weakness, fever, respiratory infection, digestive infection (enterocolitis), lymphatic infection associated with splenomegaly, hepatomegaly, septicaemia and death (Fedorka-Cray *et al.*, 2000). In contrast, ubiquitous infections include Typhimurium and Derby serovars and are most common in young piglets and very rarely happen in the adult pigs (Wallis, 2006). The pathogenesis of the disease commences from contamination of food and the bacteria enter through the oral cavity directly to infect the tonsils and may cause tonsillitis (Wood, Pospischil *et al*. 1989). The respiratory system can also become infected leading to inflammation of alveolar cells (pneumonia) resulting in difficulty breathing and, without treatment, death. Occasionally the disease goes to the digestive system causing enteritis (Wood *et al.*, 1989; Fedorka-Cray *et al.*, 1995; Boyen *et al.*, 2006).

1.3.4 Salmonellosis in Pets

Salmonellosis in dogs and cats have rarely taken place even though *Salmonella* is isolated from pet faeces (Stevenson and Hughes, 1988). The clinical signs of the disease are uncommon. However, inflammation for the digestive system (enteritis) is thought to be common in puppies and kittens (Carter and Quinn, 2000). On the other hand, pets are considered a reservoir for *Salmonella* serovars and shedding is a key transmission route for the pathogen to human and other animals (Van Immerseel, 2004). Recently, *Salmonella* serovars are being isolated frequently from reptiles like domestic snakes. Once more this is not a recognized transmission route to infect the human being. Java, Stanley, Marina, Poona and Pomona serovars have been

identified in reptiles but they are not considered serovar specific for these animals (D'Aoust *et al.*, 1990; Warwick *et al.*, 2001).

1.3.5 Salmonellosis in Human

Salmonellosis in humans is considered a heavy burden for public health, due to the bacteria having the ability to achieve high-level shedding from infected patients in case of chronic disease (Gordon, 2008b). Shedding has been implicated as being a key means of transmission among a population especially in developing countries. Incidences of non-typhoidal *Salmonella* are the most common human infectious disease caused by Salmonella. Where the incoming *Salmonella* servoar invades endothelial intestinal cells causing enteritis, enterocolitis and severe diarrhoea. In particular for children this can lead to passing through to the bloodstream causing bacteraemia (Huang *et al.*, 2004; DuPont, 2009). Furthermore, Salmonellosis is considered a big problem especially in elderly people and immunocompromised patients, associated with increased mortality rate (Celum *et al.*, 1987). For example, in Africa, *Salmonella* infections are increasingly being observed in association with HIV, causes severe clinical symptoms and leading to death (Graham, 2010).

Salmonella serovars identified in humans include, Arizonae, Choleraesuis, Enteritidis, Typhi, Paratyphi and Typhimurium (Farmer *et al.*, 1984). Non-typhoidal *Salmonella* disease significantly impacts our economy, especially related to foodborne disease resulting from contaminated food requiring increased food security and the associated healthcare costs of treating *Salmonella* infections (Rabsch *et al.*, 2001). The clinical signs in adults on a primary infection irrespective of the invading Serovar are associated with the onset of disease during 6–72 hours, then developing a fever, abdominal pain, nausea, diarrhoea, dehydration and occasional vomiting (Yates, 2011).

1.4 *Salmonella* and the Public Health

Salmonellosis is a significant international public health issue causing subclinical morbidity, and consequently also has an important economic influence. In spite of Salmonellosis being considered a self-limited disease causing mild and moderate infection, severe infections occasionally occur and may lead to morbidity (Jones, 2005). Although strict laws are associated with public health and hygiene issues like hazard analysis and critical control points (HACCP) that are updated periodically especially related to food processing and education of customers, the problem of foodborne Salmonellosis is still globally prevalent (De Buck *et al.*, 2004; Food and Drug, 2012). Animals are defined as the final host of the disease that have the ability to transmit the pathogenic bacteria to human via the environment and consumer markets (Solari *et al.*, 2003; Martins *et al.*, 2013). Even in developed countries, foods are identified as the most repeated problem, ultimately causing a huge financial impact on society (De Jong and Ekdahl, 2006). For example, in the United States of America, there were 1.4 million reported cases of non-typhoidal *Salmonella* infection annually and the expenses for treatment close to 3 billion dollars (Domınguez *et al.*, 2002). Another burden responsible with epidemiological transmission in *Salmonella* is the emergence of antimicrobial resistance (Ohl and Miller, 2001).

1.5 Pathogenicity

S. enterica is considered part of the zoonotic bacteria with the ability to be transmitted across a broad scale of animals. Zoonotic or zoonosis is a Greek word derived from zoo- ‘of animals’ and nosos ‘the disease’ defining a disease that is naturally passed between animals, whether domestic or wild, and humans, with or without the need of a vector (Palmer *et al.*, 1998). For example, human infection can

results from the consumption of contaminated water and/or food (Murray, 2009). The pathogenesis of *S. enterica* is dependent on the kinetics of progression in the body and whether it is acute, chronic and / or recurrent. In general, after *S. enterica* enters the digestive system, it can cross directly into the lymphatic system via the small intestine and then into the bloodstream, this systemic infection in humans is called Typhoid fever (Gordon, 2011). This systemic infection of *Salmonella,* if allowed to persist in the lymphatic system will allow colonization of the liver, spleen and kidney (Voedisch *et al.*, 2009). Symptoms are associated with the production of endotoxins that act on the vascular and nervous systems represented by vasodilation leading to a blood rash accompanied with fever, vomiting and diarrhoea (da Silva *et al.*, 1993). Salmonellosis, also causes general dehydration leading to increased viscosity of blood, hypertension and septic shock. Thus the systemic infection is considered dangerous stage in the severe disease because will leading to for example, kidney failure, hypoxia and death (Ryan and Ray, 2004; Coburn *et al.*, 2007).

1.5.1 Acute Systemic Disease

There are a small number of serovars able to cause systemic Salmonellosis in humans. This subset of serovars colonise the healthy adult but, exhibit a limited host range compared to the rest of known serovars. The route of transmission is usually by consumption of contaminated food or water via a faecal to oral route. Bacterial proliferation exploits macrophages which are largely exploited to achieve dissemination around the body by *S. enterica* (Gyles *et al.*, 2008). In acute systemic disease, *S. enterica* passes through the small intestinal epithelium to the reticulo-endothelial system allowing the bacteria to migrate and colonise hepatic cells, the spleen, the kidneys, the lymph nodes, the gallbladder, the lungs and bone marrow. During systemic infection *S. enterica* crosses into the blood stream resulting in bacteraemia (Blackwell *et al.*, 2001; Valdez *et al.*, 2008). Usually bacteraemia without

related enteric symptoms is itself a serious illness and might be fatal especially in immunocompromised patients (Acheson and Hohmann, 2001). Complications in Salmonellosis particularly in immunocompromised patients, include meningitis (Varaiya *et al.*, 2001), pneumonia (Samonis *et al.*, 2003), neonatal septic arthritis (Sarguna and Lakshmi) and osteomyelitis (Kamel, 2006).

1.5.2 Gastroenteritis

Gastroenteritis is typically related with enterocolitis, as a result of consumption food or water which was contaminated with *Salmonella* (Mead *et al.*, 1999). The incubation period of *S. enterica* that caused gastroenteritis is short between 6-72 hours. The dose require to cause disease in healthy people is greater than 10^6 cells (Blaser and Newman, 1982). *S. enterica* has the ability to tolerate the acidity of the stomach through high population numbers and the bacteria passages through to reaches the intestine. The clinical signs that accompany this localized disease are sudden abdominal pains, cramp, nausea, vomiting, headaches and diarrhoea. The clinical signs of the disease last several days until recovery. Treatment and recovery are also depend on host factors including the immune system, health and age (Fluit, 2005).

1.5.3 The Carrier State

Patients may suffer from recurrent infection of *Salmonella* which are associated with repeated enteric fever and thus systemic Salmonellosis (Glaser *et al.*, 1985). The chronic state of *S. enterica* is known to be as a result of a systemic infection (Worley *et al.*, 2006). Chronic carriage of *Salmonella* has a historical place in our experience of *Salmonella* infections relating to the case of Typhoid Mary. Mary was a household cook at the turn of the $19^{th}/20^{th}$ century who is now accepted to have been an asymptomatic carrier of *Salmonella* (Pui *et al.*, 2011). Even today there are strict

recommendations that known carriers do not work within a food preparation environment (Bhan *et al.*, 2005).

It is documented that 2 to 5% of typhoid cases can lead to a chronic carriage of *S. enterica* Typhi (Bhan *et al.*, 2005). Not all carriers remain asymptomatic and clinical signs of recurrent fever, muscle pains, headache and general weakness have been reported in carriers (Acheson and Hohmann, 2001). Serovar Typhi is isolated frequently over a period of three months from the stool of people recovering from an acute systemic infection. The carrier state, however, is associated with Typhi being shed in stool samples for over 12 months.

Chronic carriage of Typhi, and potentially other serovars, is associated with biofilm growth in specific niches of our bodies (Gunn *et al.*, 2014). There is strong evidence that infection of the gall bladder and attachment to gall stones plays a crucial role in the chronic carriage of Tyhpi (Adcox *et al.*, 2016)Gall bladder infection occurs during an acute phase of infection. The route of infection is primarily via the liver during a systemic infection, although direct infection via an ascending route can potentially occur (Gunn *et al.*, 2014).

The carrier state is very similar to accepted nature of *Salmonella* as a zoonotic bacterial species. Similar carrier states are well established in animals of significant economic impact thus driving public and veterinary health initiatives to deal with the control of *Salmonella* infections. Chronic carriage of *Salmonella* in livestock is associated with abortion and a high rate of neonatal *Salmonella* infections. Consequently, new-born animals typically suffering from gastroenteritis and / or severe systemic diseases such as fever and loss of appetite leading finally to death (Uzzau *et al.*, 2000). One example is in pigs, where serotype Choleraesuis is

associated with infected female pigs leading to high rates of mortality in newborn piglets (Uzzau *et al.*, 2000).

1.5.4 An Africa Issue

Salmonella disease is considered a global problem because of the increasing number of cases. The annual incidence for the disease is estimated at approximately 22 million cases including 216510 fatalities, according to a survey in 2000 (Crump *et al.*, 2004). It has also been observed that the morbidity rate in African countries are significantly higher compared to other continents. (Okoro *et al.*, 2012). Non-typhoidal Salmonellosis is extremely prevalent across Africa (Graham, 2010; Reddy *et al.*, 2010). However, non-typhoidal systemic Salmonellosis is associated with other diseases such as severe anaemia, malaria, malnutrition and HIV in adults (Gordon *et al.*, 2002; Berkley *et al.*, 2009; Graham, 2010). The clinical signs of non-typhoidal systemic *Salmonella* disease is distinguished by a fever, which cannot be differentiated from malaria and other causes of diarrhoea (Kingsley *et al.*, 2009). The researchers found that the mortality rate for non-typhoidal systemic *Salmonella* infection in both adults and children reached (22 – 45%) particularly in those suffering from HIV disease (Cheesbrough *et al.*, 1997; Gordon, 2008a; Gordon *et al.*, 2008). Obviously there is a strong correlation between HIV and non-typhoidal systemic *Salmonella* disease as well as the rise of mortality rate (Okoro *et al.*, 2012).

1.**6** The Life-cycle of a Salmonella infection

On infection with non-typhoidal *Salmonella* (NTS) or invasive serovars such as Typhi the early stages of the pathogenicity cycle are very similar. The outcome of this initial phase, after ingestion, is inflammatory diarrhea. Inflammatory diarrhea is the outcome of an immune reaction to the invading pathogen (Tsolis *et al.*, 2008). In contrast, diarrhea associated with, for example, *Vibrio cholera* is defined as secretory

via the action of the cholera toxin on the gut epithelia(Faruque *et al.*, 1998). For NTS these early stages of host-pathogen interaction are associated with innate immune recognition of pathogen associated molecular patterns, such as lipopolysaccharide and flagellin (to be discussed later in Section 1.10) (Gunn *et al.*, 2014). The resulting production of the inflammatory cytokine IL-8 and the net influx of neutrophils plays a key role in fluid accumulation (Zhang *et al.*, 2003). Although this is occurring within even 6 hours of ingestion of NTS serovars, for Typhi diarrhea onset is delayed and only occurs in up to a third of cases (Gunn *et al.*, 2014).

The resulting diarrhea symptoms stems from how *Salmonella* interacts and ultimately crosses the intestinal epithelial layer. On reaching the small intestine Salmonella exploits the natural properties of a subset of epithelial cells known as M (microfold) cells (Sansonetti, 2002). M cells have the ability to take up bacterial cells and antigens via, for example, phagocytosis. They are found within regions of the small intestine epithelial layer known as Peyer patches. Traversing the epithelial barrier via M cells by Salmonella can be either an active process requiring a key virulence factor encoded by the *Salmonella* pathogenicity island 1 (SPI-1 – see section 1.8.1 later) or allowing the M cells to naturally phagocytosis the bacterial cells. The net result is that both NTS and Typhi servoars cross the epithelial layer. Typhi exploits this process to then go on to establish a systemic infection often by either gaining entry to the blood stream or hijacking macrophages surviving in vacuoles via the action of the second Salmonella pathogenicity island SPI-2 (Tsolis *et al.*, 2008).

Localised infection of the small intestine results also from NTS serovars escaping M cells and subsequently invade adjacent epithelial cells from the basal side. However, apical invasion has been implicated in the process of establishing the localized infection. These early stages of host-pathogen interaction provide evidence that NTS is not evading the immune system. Immune evasion becomes important during

invasive systemic infection of other sites within the body. Other than macrophage vacuole survival via the action of SPI-2 invasion of dendritic cells also found within the basolateral face of the small intestine is another means to transduce to other organs. A key step in the pathogenicity cycle of Typhi is a bacteremia that is associated with enteric fever. NTS serovars are reported to have the ability to also achieve access to the blood stream. However, Typhi possess further virulence factors such as the Vi antigen that increases its survival chances during this phase while our immune system can to an extent overcome NTS bacteremia (Tsolis *et al.*, 2008). A key issue rising globally is the impact NTS strains have especially in immunocompromised individuals that are unable to combat the entry of *Salmonella* into the blood stream.

1.7 Bacterial Motility

Bacterial movement is principally driven by the bacterial flagellum (Bray, 2001). However, less than 50 % of the bacterial kingdom encode the flagellar system (Faulds-Pain *et al.*, 2011). Even though bacteria lack a flagellum this does not mean these species are non-motile as other types of motility, such as sliding, gliding, swarming and twitching exist (Kearns, 2010).

Firstly, swarming is a particular form of movement related to the arrangement rows of bacteria moving together exploiting flagellar (Harshey, 2003; Partridge and Harshey, 2013). In comparison, swimming differs from swarming in that the bacterial cells run through liquid media as individuals (Kearns, 2010). However, swarming is a harmonic translocation of groups of bacteria, usually as rafts, across a wet surface. Swarming is also a slower means of translocation than swimming. For example, the range of speeds measured for swarming are between 2-10 μm/s (Fraser and Hughes, 1999; Männik *et al.*, 2009; Mayola Coromina *et al.*, 2014). In contrast,

swimming speeds have been measured between 20-50 μm/s within the liquid media (Männik *et al.*, 2009; Kearns, 2010). Interestingly, it has been noted that increasing the number of flagellar per cell drives the swarming phenotype (Wang *et al.*, 2004). Even though flagellar drives swimming and swarming motility, there are other means for cells to drive motility over surfaces. The other types of motility kinetics include twitching, gliding and sliding (figure 3).

Twitching, sometime called social gliding, is the movement facilitated by type IV pili stretching and shrinkage thus pulling cells across a surface. Twitching movement is typically defined as very slow movement with a speed of 0.06-0.3 μm/s over the surface. Twitching and gliding occurs in certain species like *Streptococcus*, *Pseudomonas*, *Pasteurella* and *Actinobacteria* (Harshey, 2003; Kaiser, 2007). The behaviour of twitching mechanism resembles swarming motility, because it is a group movement consisting of extremely organized rafts of attached cells.

Gliding, can also describe adventurous gliding, defined as an energetic motility and moving smoothly through the axis of the bacteria which is not using flagella or pili at all (Yu and Kaiser, 2007). It is a mode of movement found originally in *Flavobacterium*, *Myxobacteria* and *Cyanobacteria* (Hoiczyk, 2000; Wolgemuth *et al.*, 2002; McBride, 2004). The average speed of gliding is also broader ranging between 0.1-10 μm/s across a solid surface (Harshey, 2003). Gilding typically represents the motion of the entire cell body on a mucous coated surface central body of the bacterial cell to slip across allowing the surface and this movement acting as the momentum for motility (Bardy *et al.*, 2003; Kearns, 2010).

In terms of the sliding phenotype or spreading, this is defined as a bacterial cell movement across the solid surface, based on surfactant force through helping reduce molecular tension outside of the bacteria cell wall (Martínez *et al.*, 1999). This motion

is not dependent on the flagella. Instead type II protein secretion plays a significant role is secreting surfactants of proteineous nature that facilitate this mode of movement. One example is surfactin production by *Bacillus subtilis*, which is capable sliding out from a growing colony in a thin layer across a surfactin covered surface (Stewart *et al.*, 2009). Furthermore, sliding motility is generated from the internal generated by the growing colony. Similar to twitching this sliding movement is slow with an a speed of 0.3-6 μm/s (Murray and Kazmierczak, 2008). *Salmonella*, is another example that can exploit sliding movement in particular through low-level of magnesium environments and the surface protein PagM (Park *et al.*, 2015; Shrout, 2015).

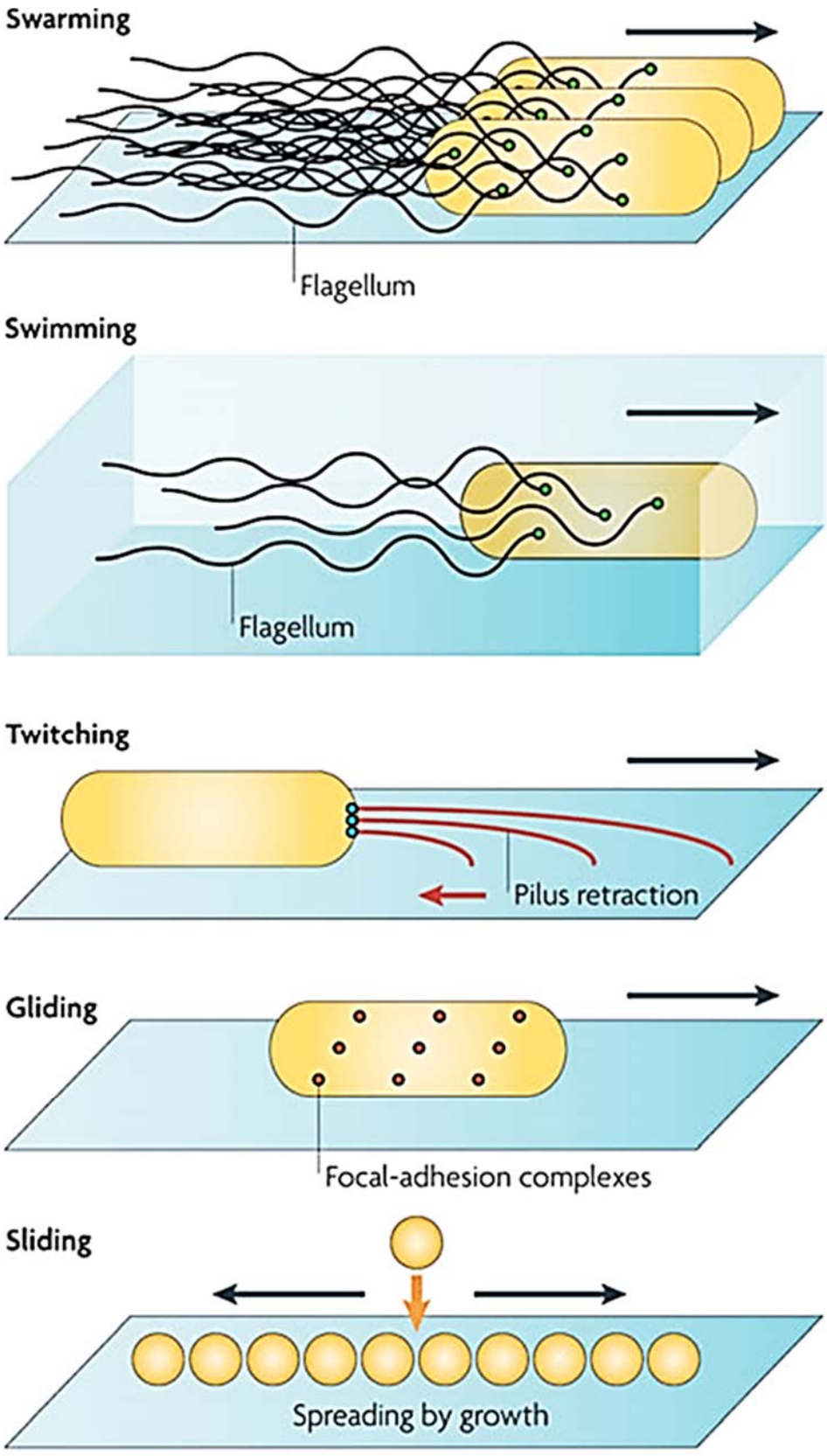


Figure 3. Classification of bacterial motility based on the nature of environment condition, adapted from (Kearns, 2010)

1.8 Virulence Factors

1.8.1 Salmonella Pathogenicity Island (SPI)

Pathogenicity Islands are specific areas of a bacterial chromosome that encode virulence factors (Groisman and Ochman, 1996). There is strong evidence that many pathogenicity islands are the result of horizontal gene transfer. A typical SPI has a differential GC content than the flanking DNA sequence. Typical events associated with the acquisiation of SPIs have been identified from bioinformatics analysis include transposon insertions and / or bacteriophage integration events (Groisman and Ochman, 1996). These observations are further strengthened by flanking genes and some of internal SPI genes showing similarity to phage genes. *S. enterica* has been defined to have up to seven SPIs (Ochman and Groisman, 1996). Here 2 key SPIs are discussed in relation to their roles in localized and systemic Salmonella infections.

a) *Salmonella* Pathogenicity Island 1(SPI1)

SPI1 is composed of approximately 29 genes which include different elements required to create a type III secretion system (figure 4) (Collazo and Galán, 1997). SPI1 is essential for invasion of intestinal epithelia and commencement of enteropathogenesis. This involves the secretion of 13 effectors proteins into non-phagocytic intestinal epithelial cells (Zeng *et al.*, 2003). The effector proteins intentionally modulate cellular functions such as the actin cytoskeleton leading to changes in the host membrane and subsequently invasion of *S. enterica* into the intestinal epithelial cells (Willse *et al.*, 2004; Zhang *et al.*, 2004).

b) *Salmonella* Pathogenicity Island 2 (SPI2)

SPI2 has been identified to a particular region of the Salmonella chromosome. SPI2 is a 39.8 Kb that was first identified through signature-tagged transposon (STM)

mutagenesis. STM analysis uses pools of transposon mutants for pathogenicity experiments where the pool of mutants is compared to what is extracted from the model organism after a given period of time (Martinez-Argudo and Jepson, 2008). SPI2 has the capability to encode a second T3SS. This time SPI2 controls *S. enterica* intracellular pathogenesis playing a role in colonization of the host and systemic infections (figure 4) (Hensel, 2000). SPI2 is essential in order for *Salmonella* to survive in a vacuole (*Salmonella* Containing Vacuole) and within incidence synchronization of infection (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Karasova *et al.*, 2010). SPI2 has 44 genes (Schmidt and Hensel, 2004; Thomson *et al.*, 2008). SPI2 contributes to survival and proliferation of bacteria intracellular existing in tissues such as liver and spleen thus is needed during systemic infection (Gyles *et al.*, 2008).

1.8.2 Flagella

Flagella have a pivotal role in pathogenesis within the host not just through motility but as well as by different pathways including:

i. Enhancing the ability of bacteria to adhere on host cells (Arora *et al.*, 1998).
ii. Encouraging bacterial to create a biofilm allowing the pathogen to persist within the host (O'Toole *et al.*, 2000).
iii. Potentially deliver effector proteins from the bacteria (Konkel *et al.*, 2004).
iv. Cause a pro-inflammatory immune response for the host by recognition via Toll-like receptor 5 (TLR5) (Hayashi *et al.*, 2001).
v. Flagellin has the ability trigger a adaptive immunity (Honko and Mizel, 2005).

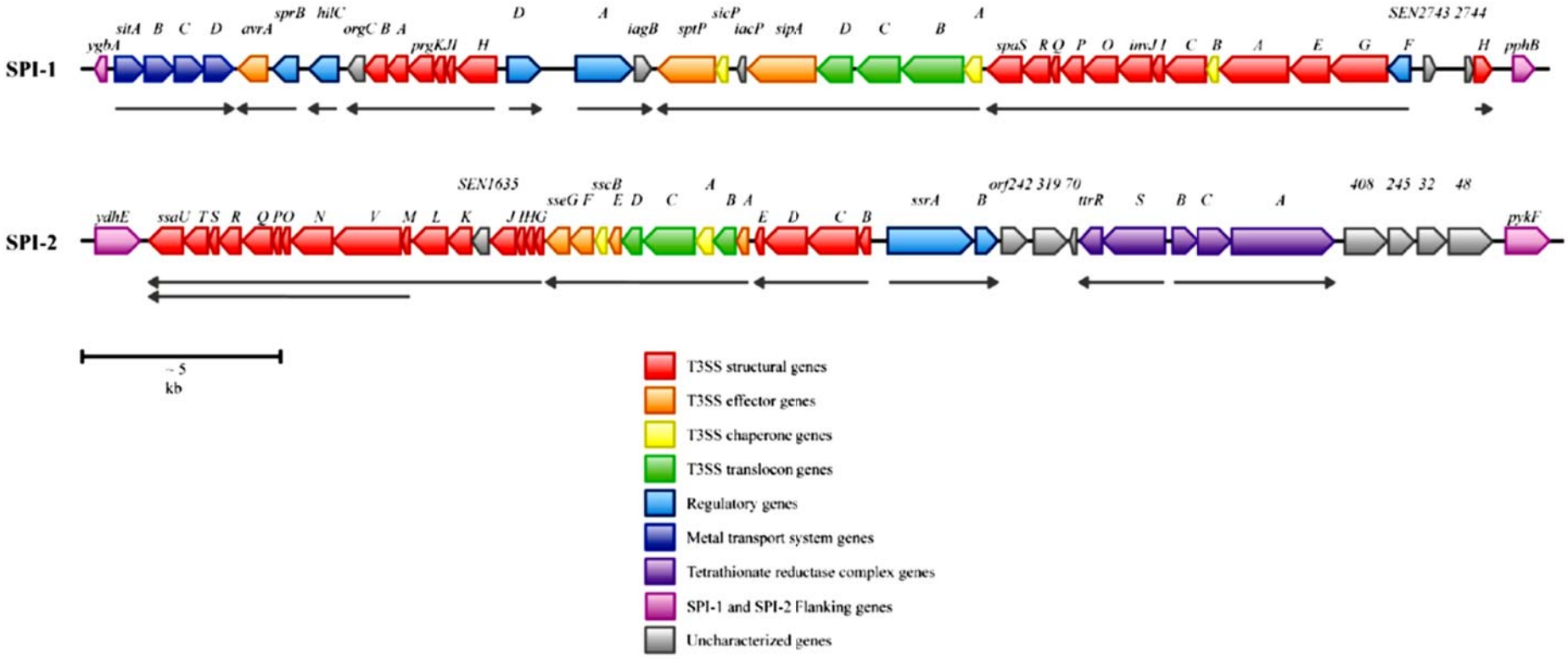


Figure 4. Genetic arrangement of *Salmonella* Pathogenicity Island (SPI1 & SPI2), adapted from (Wisner, 2011).

1.8.3 Fimbriae

Fimbriae, also known as pili, are usually between 0.5-10 μm in length and ~8 nm in width that protrude from the cell surface (Townsend *et al.*, 2001). Fimbriae functions are significant for many bacteria involved in biofilm formation bacterial survival in different circumstances, and also play a pivotal role in adhesion on to the eukaryotic cell (Zeiner *et al.*, 2012). In *Salmonella* serovars, thirteen fimbrial operons have been identified (*fim, pef, lpf, bcf, saf, sef, stb, stc, std, stf, sth, sti, stj* and *csg*) (Betancor *et al.*, 2012). They all contribute to adherence and colonization of epithelial intestinal cells (De Buck *et al.*, 2005; Clayton *et al.*, 2008). For example, the SEF14 fimbriae has been identified in Berta, Gallinarum, Enteritidis and Dublin serovars. SEF14 plays an important role in bacterial adhesion, in particularly it has high affinity for cells within the reproductive tract (Turcotte and Woodward, 1993; Doran *et al.*, 1996). In spite of *Salmonella* fimbriae playing a role in the colonization of specific cells (Klemm, 1994; Collinson *et al.*, 1996) the pathogenesis pathway of the *Salmonella* fimbriae is still opaque and unclear partly as a result of encoding for so many variants (Lockman and Curtiss, 1992; Van Der Velden *et al.*, 1998; Folkesson *et al.*, 1999; Edwards *et al.*, 2000).

1.8.4 Toxins

In *Salmonella* toxins are classified into endotoxin and exotoxin, which contribute to pathogenicity (Ashkenazi *et al.*, 1988). Endotoxins are components of the cell wall and outer membrane of Gram-negative bacteria represented by lipid and lipopolysaccharide. They also elicit different immune responses (Hitchcock *et al.*, 1986). During Salmonellosis, endotoxins contribute to the adherence of bacteria to particular tissues (epithelial tissues), resistance to phagocytosis and withdrawal of water from the epithelial cells (Todar, 2009).

In contrast, exotoxins, including enterotoxins and cytotoxins, have the capability to disable functionality of eukaryotic cells (Ashkenazi *et al.*, 1988). *Salmonella* encodes an exotoxin operon across a limited small area of the genome. One example of a *Salmonella* toxin is the Cytolethal Distending Toxin (CDT) produced by Serovar Typhi (Spanò and Galán, 2008). CDT is produced by a diverse range of bacteria including *Salmonella*. CDT interaction with host cells provokes a dramatic cellular distension leading to cell cycle arrest and apoptosis (Gelfanova *et al.*, 1999; Lara-Tejero and Galán, 2002; Heywood *et al.*, 2005; Shenker *et al.*, 2006). Part of the CDT complex is encoded by cdtB which has been shown to exhibit similarity to DnaseI. Recently Gao *et al.,* (2017) have proposed that *Salmonella* CDT genes of Typhi have evolved from the *artAB* locus identified in a wider range of serovars (Gao *et al.*, 2017). There is supporting evidence that CDT plays an important role in the acute phase of Typhoid fever and potentially establishing the carriage state (Galán, 2016). Interestingly many exotoxins produced by pathogens are secreted into the in vivo environment and thus are able to attack host cells. There is strong evidence that for the Typhi CDT secretion requires host cell invasion by Typhi (Haghjoo and Galán, 2004). Galan (2016) even proposes this may have been one reason why it took researchers a long time to discover this toxin and its role in Typhi pathogenesis (Galán, 2016).

1.8.5 Virulence Plasmids

Some *Salmonella* virulence genes are encoded by plasmids (Figueiredo, 2016). Plasmids have been classified that included virulence genes significant for invasion and colonization of the disease (Gulig, 1990). These plasmids have been identified in many *Salmonella* serovars, particularly in Typhi, Typhimurium, Dublin, Enteritidis, Gallinarum, and Choleraesuis (Rotger and Casadesús, 2010). Usually, virulence plasmids are low copy number plasmids of a size between 30 and 100 kb depending on serovar (Gulig *et al.*, 1993). For example, the s*pv* region (7.8 kb) is a plasmid

encoded locus that is important for infectious disease in the reticuloendothelial system in the rodents. Otherwise, in humans, the mechanisms of action of virulence plasmids for digestive system disease is still indistinct. However, researchers referred to the probability that the virulence plasmid (*spv*ABCD) is involved in invasion and bacterial spread into the human endothelial tissue of the intestine (Guerra *et al.*, 2002; Raupach *et al.*, 2003).

1.8.6 Other Virulence Factors

In *Salmonella,* there are several of the other virulence factors that participate in invasion and resistance of bacteria against the immune system. Surface polysaccharides play a role for adhesion and settle down bacteria on to intestinal cell (Foley *et al.*, 2013). These cases have been identified in the intestinal cells of some calves and chickens infected with Salmonellosis (Turner *et al.*, 1998; Morgan *et al.*, 2004). On the other hand, some prophages encode and leading to integrated with *Salmonella* chromosome and consequences raising the ferocity of bacteria like *gifsy*-1, *g*ifsy-2 and *gtgE* regions contributed in prevention against oxidative stress (De Groote *et al.*, 1997; Farrant *et al.*, 1997; Figueroa‐Bossi *et al.*, 2001; Ho *et al.*, 2002).

1.9 The Bacterial Flagellum

1.9.1 Flagellum Biogenesis Pathways

1.9.1.1 Stages Assembly of the MS Ring and Export Apparatus

Flagella are nanomachines possessed by approximately 50% of sequence bacteria helping them in locomotion and colonisation (Faulds-Pain *et al.*, 2011). The base of flagellum is located within the cell envelope, it is able to rotate via energy supplied by either sodium or proton motive force (Terashima *et al.*, 2008). Each flagellum

consists of three parts: a basal body, hook and filament (figure 5). However, every part has a function and requires specific proteins to create it (Macnab, 2003). Generally, the construction of the flagellum is from inside the bacteria starting from the basal body and extending to the outside with the filament.

The first section of each flagellum to be constructed is the MS ring and proximal rod (Ueno *et al.*, 1992). The MS ring is the base of the rotor and is sits in the inner membrane of the Gram-negative bacteria (figure 5). The MS-ring consists of a multimeric complex of 26 subunits of the single protein FliF. In contrast the axial framework leading into the filament has 5.5 subunits per rotation of the basic helix (Ueno *et al.*, 1994). The MS-ring is then used as the foundation for the rest of the basal body. Under the MS ring, on its cytoplasmic face, the C-ring is assembled. The main components of the C-ring are two proteins FliM and FliN (Francis *et al.*, 1994; Zhao *et al.*, 1996). The C-ring interacts with the MS ring via FliG, that participates as part of the rotary system (Thomas *et al.*, 2006). The role played by FliG was defined using genetic fusions of FliF and FliG in both *E. coli* and *Caulobacter crescentus*. Such chimeric fusions produce a functional flagellum emphasizing the interaction interface of the MS and C-ring (Francis *et al.*, 1992; Jenal and Shapiro, 1996).

On generating a MS-C ring complex, the flagellar associated Type III export apparatus system assembles within the C-ring inner space making contact to the underside of the MS-ring. From this point onwards, during assembly, the majority of flagellar proteins will be synthesized in the cytoplasm and then exported via the flagellum base into the periplasmic space and outside the cell until completion of the flagellum through the structure itself (Macnab, 2004). The export apparatus is comprised of six proteins, FlhA, FlhB, FliO, FliP, FliQ, and FliR that generate an export gate/channel in the MS-ring body. Three cytosolic proteins FliH, FliI, and FliJ are for export specificity, efficiency and delivery of the flagellum components

participating transiently as part of the export apparatus (table 1). Structural biology resolution of the FliH, FliI and FliJ structures has identified significant structural similarity to the Sec system and F_0F_1-ATPase (Kishikawa *et al.*, 2013). This has led to the proposal that the export apparatus for Type II secretion has evolved from these two ancestral and essential cellular macromolecular structures (Imada *et al.*, 2016).

FliI is an ATPase and is a member of the Walker-type ATPase family (Fan and Macnab, 1996). FliI creates a hexameric ring- for protein export (Claret *et al.*, 2003; Minamino *et al.*, 2006). The ATPase FliI is negatively regulated through FliH interactions creating a complex with $FliH_2FliI_1$ stoichiometry (Minamino and Macnab, 2000a). FliH also interacts with FliN via a hydrophobic patch, this interaction functions to localize FliI within the C-ring (Minamino and Macnab, 2000a; Fraser *et al.*, 2003a). Recently, Minamino (2008) was able to show that FliI and FliH were not strictly essential for flagellar assembly, as previously assumed (Minamino and Namba, 2008). A Δ*fliHI* deletion assembled one flagellum inefficiently if the strain carried a third mutation in a third export protein, FlhB. This led to the proposal that the role of the ATPase FliI was to drive efficient export rather than the export process itself (Minamino and Namba, 2008).

FlhA and FlhB are membrane associated components of the Type III export channel that possess a large cytoplasmic C-termini domains(Macnab, 2004). This was imaged how the export apparatus embedded inside the C-ring by using freeze-fracture technique (Katayama *et al.*, 1996). It is believed they play a significant role in providing a location for the other components to interact with (Minamino and Macnab, 2000). FlhB has been shown to undergo a self-regulated proteolytic cleavage in response to flagellar assembly (Fraser *et al.*, 2003). This cleavage event is associated with substrate specificity regulation (see later). Importantly the cleavage of FlhB and the net outcome has led to the proposal that FlhAB form the self-

regulating gate that controls protein export via the flagellar associated Type III secretion apparatus (Minamino, 2014).

1.9.1.2 Assembly the Flagellar Rod

On completing the MS/C-ring structure, the continuation of flagellar assembly requires the addition of the majority of the subsequent proteins at the growing tip until completion (Karlinsey *et al.*, 2000a). Above the MS ring the proximal and distal rod are next to be assembled (figure 5). The first secreted subunit utilizing the type III pathway is thought to be FliE. FliE protein is a special protein that is proposed to form the linker between the rod and the MS-ring protein FliF (Müller *et al.*, 1992). The presence of some type of connection region between the MS ring and the axial proteins appears in the top view of annular symmetry and is required to allow the generation of the helical foundation that can be traced right through the rest of the structure from the rod in to the external filament (Müller *et al.*, 1992). As well as needing to generate a transition between the MS-ring and the rod assembly from this point onwards requires a capping structure. There are five proteins associated with rod assembly: FlgB, FlgC, FlgF,FlgG and FlgJ (Homma *et al.*, 1990; Nambu *et al.*, 1999). One of these proteins, FlgJ, is the rod cap during assembly through the periplasmic gap (Kubori *et al.*, 1992b). As well as acting as a cap, FlgJ has the essential function to permit the rod to perforate the peptidoglycan layer by hydrolyzing it (Hirano *et al.*, 2001). FlgJ achieves this role by possessing a protein domain with muramidase activity (Hirano *et al.*, 2001a). Rod length is assumed to be dictated by the size of the periplasmic space and is assumed not to be controlled directly as is hook-length-control (Kubori *et al.*, 1992a). The actual rod structure is built from FlgB, FlgC, FlgF and FlgG, where FlgBC form the proximal rod closest to the MS-ring and FlgFG are the distal rod proteins (Macnab, 2003).

1.9.1.3 Assembly of P and L Ring

The FlgI protein assembles the P-ring around the distal rod. Thereafter, FlgH forms the L-ring on the P-ring around the distal rod (figure 5) (Chevance *et al.*, 2007). FlgI and FlgH are the only two flagellar subunits not secreted by the type III secretion system (Macnab, 2003). Instead they are secreted by the sec pathway into the periplasmic space so they can assemble around the growing structure. Researchers believe these two proteins are produced and secreted early before rod assembly. However, they must be held in a monomeric state until the suitable time to assemble around the growing rod. As these subunits are usually in the periplasmic space where proteolysis is used to prevent unwanted proteins in this space FlgH and FlgI require protection (Minamino *et al.*, 2008). FlgI has been proposed to work as the FlgH/I chaperone in the periplasmic state (Nambu and Kutsukake, 2000).

1.9.1.4 Assembly of Hook

Approximately 120 units of FlgE forms the hook with assembly coordinated by the hook cap protein, FlgD (Hirano *et al.*, 1994) (figure 5). The optimal length of the hook is 55 nm, this length is required in order to generate a hook structure that can function correctly to convert the rotational forces into the need torque to drive motility by spinning the filament (Hirano *et al.*, 1994). Two proteins determine the length of hook FliK and FlhB (Vogler *et al.*, 1991). Mutants in *fliK* have a much broader hook length range althought the majority of observed hooks are shorter than the optimal length (Williams *et al.*, 1996). In contrast, if a mutant of *flhB* is present substrate specificity is unable to be switched leading to a greater number of cells with longer hooks (Macnab, 2003).

1.9.1.5 Junction Proteins Assembly

When hook assembly is complete, the cap protein FlgD is swapped with a complex that includes three proteins defined as the hook-associated proteins. These three

proteins are the first to be secreted after the FlhB/FliK induced substrate specificity switch. Two short segments consist of HAP1 (FlgK) and HAP3 (FlgL) (Ikeda *et al.*, 1989). The 3rd HAP is the filament-capping protein HAP2 (FliD), FilD subunits form a pentameric complex revealed by electron microscopy (Ikeda et al., 1996). HAP1 and HAP3 generate a transitional zone that acts to allow flagellin subunit incorporation to continue until a mature filament exists. Interestingly, the structural detail of FlgK and FlgL has shown that FlgK is more hook like while FlgL is more filament like further exemplifying their role as transitional proteins (Samatey *et al.*, 2004).

1.9.1.6 Assembly of Filament

Ultimately, the filament (flagellin) is assembled from at least 20,000 subunits of FliC or FljB (Yonekura *et al.*, 2002; Adkins *et al.*, 2006). Flagellin subunit incorporation is controlled by FliD, the filament cap at the distal end of the growing filament (figure 5 and 6) (Yonekura *et al.*, 2000). The filament does not possess a length-control mechanism as seen for hook assembly. This in part is to allow for a long filament to drive efficient motility (Macnab, 2003). The mechanism of action of FliD can be argued to act by knitting flagellin monomers into a filament (Yonekura *et al.*, 2000). The filament can be fragile and easily broken, however, the flagellar system is coordinated to allow immediate rebuilding by further delivery of FliD and flagellin monomers through a filament permissive flagellum structure (Homma and Iino, 1985). This is assumed to be on further explanation why the flagellar assembly substrate specificity switch exists.

1.9.1.7 Assembly of Mot proteins

Two further accessory proteins MotA and MotB associate with the outer face of the C-ring. These proteins form the flagellar stator (Armstrong and Adler, 1967). MotA and MotB are responsible for the torque-generating movement of the flagellum via confirmation changes in MotA versus MotB allowing the uptake protons to generate

the power stroke triggering the motion of the flagellum (Zhou *et al.*, 1998; Braun *et al.*, 1999). MotA and MotB are 8 complexes distributed around of the flagellar motor system, but could accommodate 2 or 3 more (Thomas *et al.*, 2006). FliG is proposed to be a key interaction site for MotA to produce torque (Garza *et al.*, 1995). The current model argues that FliG/MotA interact allowing the stators to sit in the vicinity of the FliM/N section of the C-ring. Conformational changes due to proton flux then allows MotA/B to transiently interact with FliM/N thus rotating the flagellum basal body(Kojima and Blair, 2004). Overall, the membrane proteins MotA and MotB play a vital role in the flagellar movement.

Table 1. The flagellum proteins assembly, functions and locations.

Name of protein	Functions	Location
MotA	Stator protein; exerts torque against rotor/switch	Cytoplasmic membrane
MotB	Stator protein; converts proton energy into torque	Cytoplasmic membrane
FliF	MS-ring protein; mounting flange for rotor/ switch and rod; housing for export apparatus	Cytoplasmic membrane
FliI	ATPase; drives type III flagellar export	Cytoplasm
FliH	Negative regulator of FliI	Cytoplasm
FliJ	General chaperone	Cytoplasm
FlgN	FlgK-, FlgL-specific chaperone	Cytoplasm
FliS	FliC-specific chaperone	Cytoplasm
FliT	FliD-specific chaperone	Cytoplasm
FliG	Rotor/switch protein; torque generation; strong interaction with MS ring	Peripheral
FliM	C ring; rotor/switch protein; target for CheY-P binding	Peripheral
FliN	C ring; rotor/switch protein	Peripheral
FlhA	Export component; target for soluble export complex	Center of MS ring
FlhB	Export component; substrate specificity switch; target for soluble export complex	Center of MS ring
FliO	Export component	Center of MS ring
FliP	Export component	Center of MS ring
FliQ	Export component	Center of MS ring
FliR	Export component	Center of MS ring
FliE	MS-ring rod junction protein; export gate	Periplasmic space
FlgB	Rod protein; transmission shaft	Periplasmic space
FlgC	Rod protein; transmission shaft	Periplasmic space
FlgF	Rod protein; transmission shaft	Periplasmic space
FlgG	Distal rod protein; transmission shaft	Periplasmic space
FlgJ	Rod capping protein; muramidase	Periplasmic space
FlgI	P-ring protein; part of bushing; internal disulfide bridge	Periplasmic space
FlgA	Chaperone for P-ring protein	Periplasmic space
FlgH	L-ring protein; part of bushing; lipoprotein	Outer membrane
FlgD	Hook-capping protein	Outside cell
FlgE	Hook protein	Outside cell
FliK	Hook-length-control protein	Outside cell
FlgK	HAP1; first hook-filament junction protein	Outside cell
FliD	HAP2; filament-capping protein; flagellin folding chaperone	Outside cell
FlgL	HAP3; second hook-filament junction protein	Outside cell
FliC	Filament protein; flagellin	Outside cell

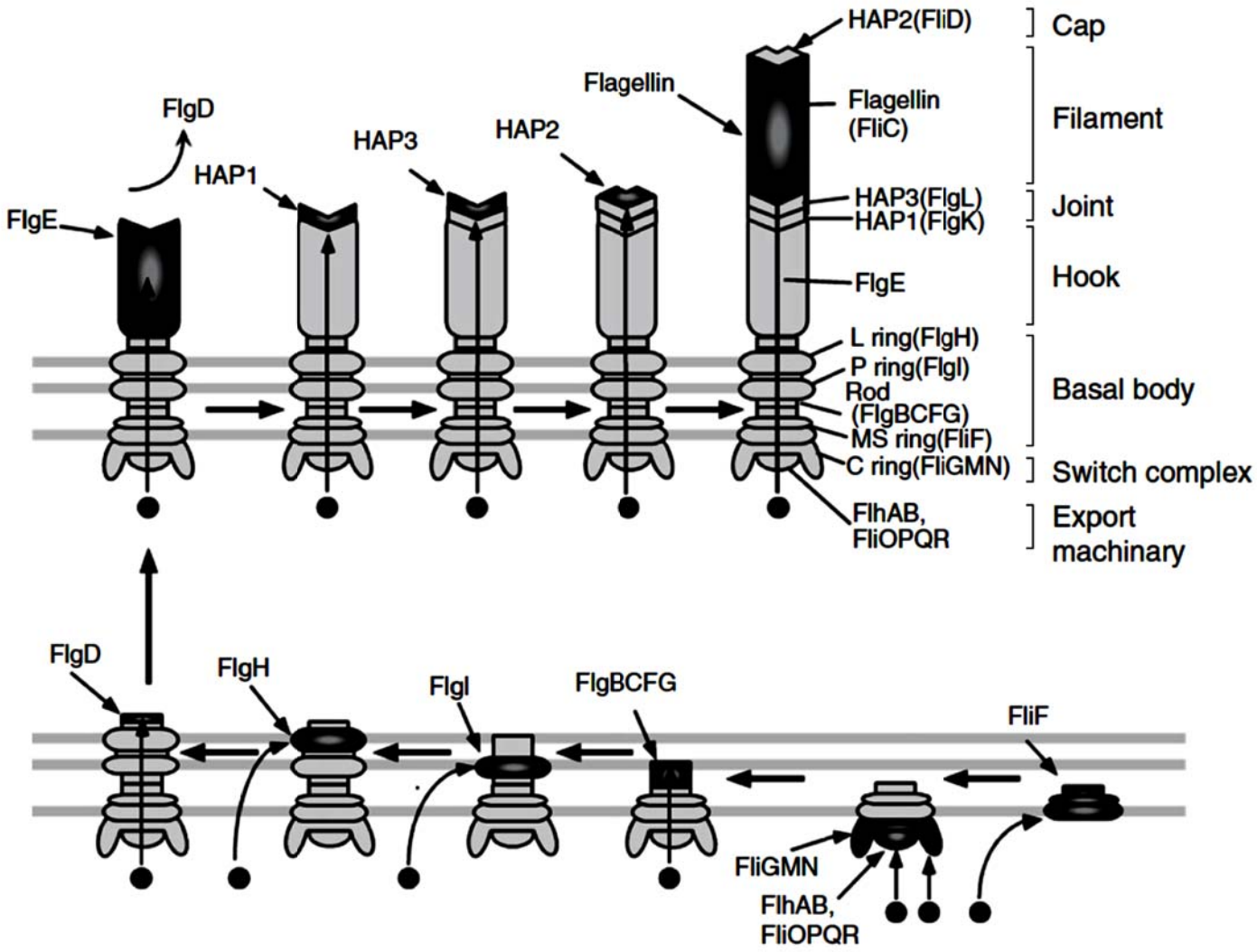


Figure 5. The paradigm of flagellar assembly pathway steps, adapted from (Terashima *et al.*, 2008).

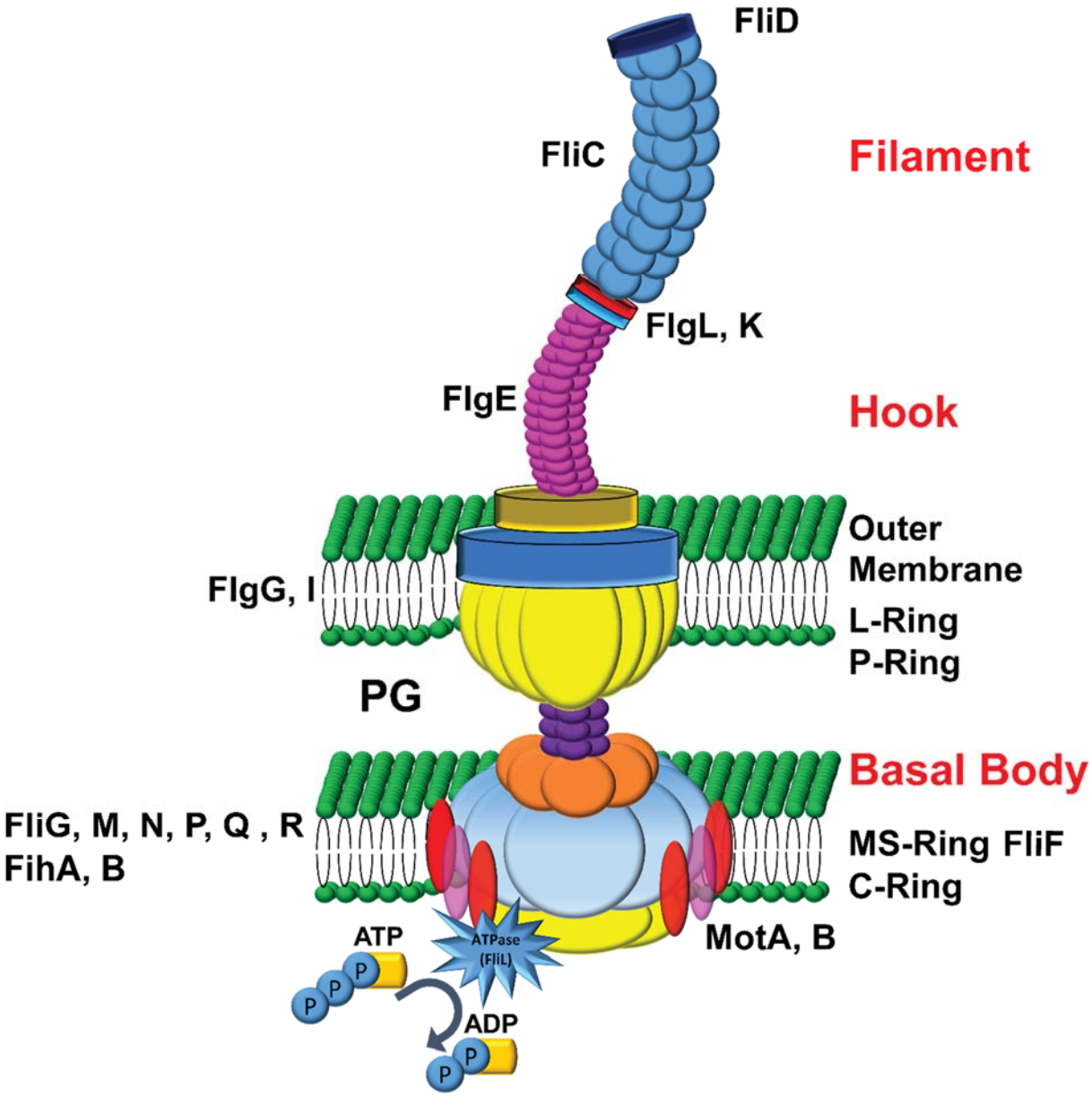


Figure 6. Paradigm description of the whole flagellum structure, embedded into the gram-negative bacteria cell wall, the structure are divided into the three sections: Starting from the basal body, hook and filament. Adapted and modified from (Pallen *et al.*, 2005).

1.9.2 The Flagella Regulon

Sixty genes are responsible for *S. enterica* assembling it's flagella, their function and chemotaxis (figure 7) (Chilcott and Hughes, 2000). Constructional genes have been described as three assembly phases: early genes, middle genes and late genes based on when their products are needed in flagellar assembly (Chilcott and Hughes, 1998). Moreover, there are three classes of promoters driving expression flagellar genes: class I, class II, and class III depending on their chronological expression after stimulation by the flagellar regulon (Karlinsey *et al.*, 2000b). The chronological activation of flagellar promoters generates a transcriptional hierarchy. In the upper part of the hierarchy is the master operon of the flagellar system, *flhDC*. FlhDC serves to coordinate making the ultimate decision for flagellar production.

1.9.2.1 The Master Regulator $FlhD_4C_2$

In *S. enterica* and *E. coli,* the master regulator is encoded by the *flhDC* operon. The *flhDC* operon is found in the family with the phyla defined by the gamma Proteobacteria known as the *Enterobacteriacae* that includes *S. enterica* and *E. coli* (Kutsukake *et al.*, 1990; Liu and Matsumura, 1994). The *flhDC* operon can also be found in members of the betaproteobacteria such as the genus *Burkholderia* (Aldridge and Hughes, 2002). The *flhDC* operon plays an essential role in motility for *S. enterica* and *E. coli*. Without expression of *flhDC* no flagella genes will be transcribed. It is not surprising then that the ultimate output of FlhDC activity is regulated at multiple stages during its expression. Furthermore, FlhDC itself is an uncommon transcriptional activator found across the bacterial kingdom. The majority of transcriptional regulators found across the bacterial kingdom are homodimers of a one protein produced by a single gene (Wang *et al.*, 2006). In contrast FlhDC forms a

heteromeric complex that on resolution of the protein structure was defined as a $FlhD_4C_2$ complex.

Original biochemical studies of the FlhDC complex presented a strong case for a 1:1 interaction generating an $FlhD_2FlhC_2$ complex (Liu and Matsumura, 1994; Claret and Hughes, 2002). However, the solving the crystal structure of the FlhDC complex identified the known accepted form as a hexameric FlhD4C2 complex (figure 7). Both FlhD and FlhC are alpha-helical structures with FlhC possessing a special zinc binding domain (Wang *et al.*, 2006). Interestingly Wang et al (2006) proposed FlhD as the DNA binding component, however, biochemical data of the DNA binding ability of the complex and individual components argued for FlhC to be the DNA binding factor of the complex (Claret and Hughes, 2002; Aldridge *et al.*, 2010).

$FlhD_4C_2$ interacts with DNA sequences 28-88 bp upstream of transcription start site of $FlhD_4C_2$-dependent promoters thus flanking the -35 promoter region (Liu and Matsumura, 1994). There is a conserved $FlhD_4C_2$ binding site comprised of a 17-18 bp imperfect palindrome with a spacer region of either 10 or 11 bp (Claret and Hughes, 2002). When bound to its DNA binding sites, $FlhD_4C_2$ interacts with the C-terminal domain of RNA polymerase α helical subunit as a class I transcriptional activator (Ishihama, 1993). This class of activators directly interact with σ^{70}-RNA polymerase holoenzyme ,unwinding the DNA promoter to initiate the transcription (Liu *et al.*, 1995; Wang *et al.*, 2006). The $FlhD_4C_2$ complex activates the genes that encode the flagellar protein export system apparatus, basal body, hook and the main regulatory proteins FliA and FlgM (Gillen and Hughes, 1993; Liu *et al.*, 1995; Prüß *et al.*, 2001; Frye *et al.*, 2006). These gene represent the middle genes of the flagellar regulon.

$FlhD_4C_2$ is also considered a global transcription activator as it has affinity to promoter regions of others genes not related to flagella synthesis. Examples include, anaerobic aspiration using dimethyl sulfoxide, nitrate as terminal electron stimulation and the gene *wzz* (Prüß *et al.*, 2001; Stafford *et al.*, 2005). $FlhD_4C_2$ binds and stimulates transcription directly non-flagellar genes via an identical mechanism as seen for the flagella gene hierarchy. However, the activity is not as robust as seen for flagellar genes (Stafford *et al.*, 2005). An important gene of the flagellar system dependent on $FlhD_4C_2$ transcriptional activation is *fliA*, that encodes the sigma factor σ^{28}. FliA protein activates transcription from class III promoters that drive expression of late genes including is *fliC* (see later) (Kutsukake *et al.*, 1990; Ohnishi *et al.*, 1990).

There are very important differences between when considering the transcription of *flhDC* in *E.coli* versus *S. enterica* based on the number of promoters that transcribe *flhDC*. *E.coli* has a single promoter upstream of flhD. In contrast, in *S. enterica* there are seven promoters that have the potential to drive transcription of *flhDC* (Mouslim and Hughes, 2014). Further dissection of the P_{flhDC} region from *S. enterica* has subsequently shown that two of the seven promoters are the primary source of *flhDC* transcription. *flhDC* transcription is controlled by many regulators (figure 8) inclusive of, however not restricted to, the heat shock proteins (DnaK, DnaJ and GrpE), which stimulated by changes in temperature (Li *et al.*, 1993; Shi *et al.*, 1993). Furthermore, there are others cues that dictate *flhDC* expression such as quorum sensing (Sperandio *et al.*, 2002). As well as environmental inputs, *flhDC* expression is regulated via cell cycle inputs (Smith and Hoover, 2009). *E.coli* mutants in *flhD* were observed have an altered phenotype during stationary phase (Prüß and Matsumura, 1997). Recently Sim *et al* (2017) revealed a relationship between the growth rate and flagellar assembly in *E. coli* using steady-state chemostat conditions. Previous studies using microarray analysis have argued that *E. coli* prefers low nutrient slow

growth conditions for optimal flagellar gene expression (Wada *et al.*, 2011). However, using steady-state growth kinetics Sim et al (2017) observed the opposite that fast growing *E. coli* produced more flagellar per cell, while in slow growth only a small proportion of cells produce a flagellar. This suggests that growth kinetics impacts the regulation flhDC transcription (Sim *et al.*, 2017).

External regulatory cues include osmolality represented by control of *flhDC* transcription by OmpR. Osmolality have been shown to negatively affect *flhDC* expression in particular when it increases in the surrounding medium (Shin and Park, 1995). OmpR has been shown to interact with the *flhDC* promoter region in both *E. coli* and *S. enterica* (Shin and Park, 1995). Temperature also plays an important role, controlling the levels of the $FlhD_4C_2$ production via three heat shock proteins (DnaK, DnaJ and GrpE). Temperature regulation is more pronounced in *E.coli* strains compared to *S. enterica*. A number of model strains of *E. coli* are only motile at 30°C while *S. enterica* is motile at both 30 and 37°C (Soutourina *et al.*, 2002). As well as downregulation of the *flhDC* operon, leading to a decrease in flagellar gene expression, positive regulatory stimuli also impact *flhDC* transcription (Chilcott and Hughes, 2000). Two examples of regulation include the input of the cyclic AMP catabolite activator protein (CAP) and Histone-like nucleoid-structuring protein (H-NS) (Soutourina *et al.*, 1999; Soutourina *et al.*, 2002). *flhDC* production is very susceptible to the availability of carbon sources, based on cyclic AMP catabolite activator protein (CAP) which induces *flhDC* expression (Kutsukake, 1997; Soutourina *et al.*, 1999). For *S. enterica* specifically *flhDC* transcription is also regulated by LrhA, RtsB, HilD, RcsB and FimZ (Wozniak *et al.*, 2009). RtsB and HilD link *flhDC* transcription to SPI1 expression. This dual regulatory loop allows S. enterica to coordinate movement with the desire to also invade host-cells during host-pathogen interactions. Similarly, FimZ and $FlhD_4C_2$ counter regulate each other's

system to either promote movement (flagella) and inhibit adherence (fimbrae) or vice versa (Clegg and Hughes, 2002).

1.9.2.2 Regulation of $FlhD_4C_2$ Activity

The flagellar master regulator *flhDC* is essential for driving the whole of flagellar system and thus is considered as an important regulatory target for many flagellar regulatory genes. Other than multiple transcription regulatory inputs the FlhDC proteins themselves are controlled by a range of regulators which exert either a positive or negative impact on the function of $FlhD_4C_2$. These include direct regulation via the proteins FliT, FliZ, YdiV and ClpP.

The ATP-dependent protease ClpP functions as a negative regulator of the $FlhD_4C_2$ protein by degrading the complex (Tomoyasu *et al.*, 2003). In *clpP* mutants the flagellar system is overproduced due to increased $FlhD_4C_2$ activity driving transcription of flagellar genes. For example, FliC subunit production exhibits a fourfold increase compared to $clpP^+$ (Tomoyasu *et al.*, 2002). Moreover, the ClpP protein is working high efficient against of DNA - bound $FlhD_4C_2$ complex in particular in cohort with the YdiV protein (Takaya *et al.*, 2012).

Nutrient availability regulation via YdiV acts to down-regulate class II promoter activity via preventing $FlhD_4C_2$ activity, especially in low nutrient conditions (Takaya *et al.*, 2012). *ydiV* is considered a non-flagellar gene but it represents a pivotal control point for the quantity of $FlhD_4C_2$ activity through the repressing the $FlhD_4C_2$ function. *ydiV* expression is sensitive and affected by low nutrient media and consequences, allowing increase the $FlhD_4C_2$ activity, thus significantly impact the whole flagellar system (Wada *et al.*, 2011). In contrast, when absent $FlhD_4C_2$ activity will be increased and the flagellar system significantly impacts the activity, and also YdiV when missing into the bacteria lead to decrease the growth proliferation into the poor

medium because of YdiV responsible directly with growth control (Takaya *et al.*, 2012). YdiV acts by interacting with both $FlhD_4C_2$ and ClpXP. The model argues that YdiV will bind to FlhD4C2 then the YdiV:$FlhD_4C_2$ complex is able to interact with a ClpXP complex more efficiently than $FlhD_4C_2$ itself. The net result is a rapid turnover of $FlhD_4C_2$ (Takaya *et al.*, 2012).

In *S. enterica,* $FlhD_4C_2$ regulation occurs via the flagellar specific regulators FliZ and FliT. FliZ is considered a positive regulator for the $FlhD_4C_2$ network, acting as a DNA-binding protein as a negative regulator of *ydiV* expression (figure 8) (Koirala *et al.*, 2014a). FliT has been defined as a negative regulator of class II promoter activity, via measurement of P*fliA* activity (Kutsukake *et al.*, 1999). Through *fliT* deletion, the strains are more motile, because FliT is unable to downregulate $FlhD_4C_2$ activity (Aldridge and Hughes, 2002; Yamamoto and Kutsukake, 2006a). Like YdiV, FLiT can interact with the $FlhD_4C_2$ complex via direct interactions with FlhC. Aldridge et al (2010) showed that addition of FliT to $FlhD_4C_2$ complexes led to the disruption of the complex releasing FlhD and FlhC. The mechanism of action for FliT has recently been shown to focus on $FlhD_4C_2$ not bound to DNA. Aldridge *et al* (2010) showed that $FlhD_4C_2$ bound to DNA is resistant to FliT regulation. This is different from YdiV regulation, where interaction with $FlhD_4C_2$:DNA complexes leads to $FlhD_4C_2$ falling off the DNA (Takaya *et al.*, 2012). When not bound to the DNA, FliT interaction with $FlhD_4C_2$ the result of the complex falling apart reduces its availability to bind DNA until it can reform the $FlhD_4C_2$ complex. Moreover, FliT protein act as a chaperone for the filament capping protein (FliD) across binding together to prevent oligomerization before export out of the flagellum export passage (Fraser *et al.*, 1999; Bennett *et al.*, 2001). Overall, FliT protein has dual function either acting as an anti-FlhDC factor by interaction with FlhC and disruption the flagellar master regulator or as an export

carrier for FliD protein and contributes to filament assembly (Yamamoto and Kutsukake, 2006a; Sato *et al.*, 2014).

Figure.7 Pardigm of FlhD4C2 complex by using crystallography technique. Adapted from S.Wang *et. al* (2006).

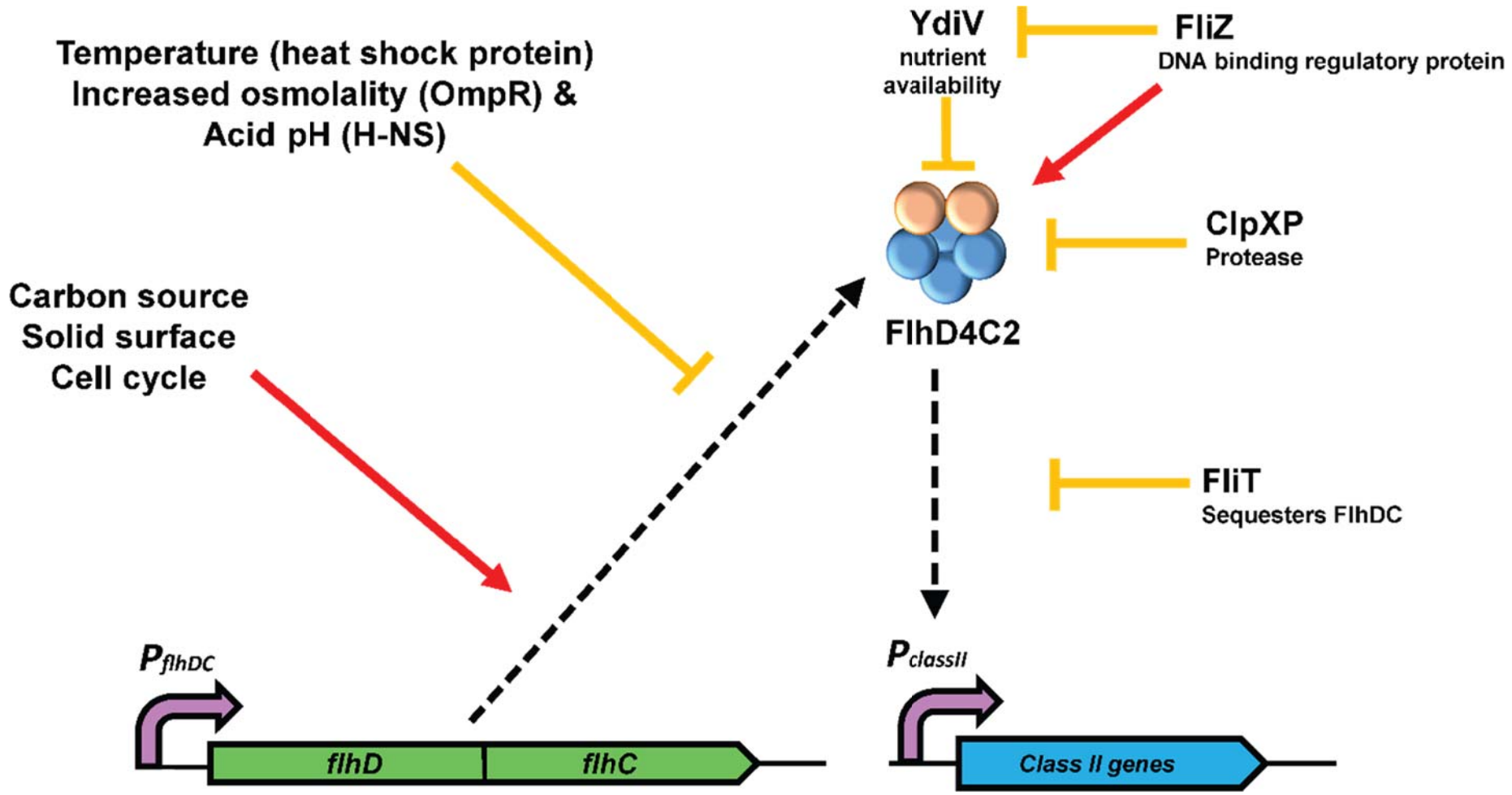


Figure 8. Flagellar master regulator network. Many factors interact positively and negatively with FlhDC stability, including external signals such as nutrients, temperature and pH. In contrast internal factors like ClpP, FliZ and FliT proteins. Adapted and modified from (Smith and Hoover, 2009)

1.9.2.3 The FliA and FlgM regulatory feedback loop

In the *Salmonella* flagellar system, expression from class III flagellar promoters requires the alternative sigma factor σ^{28} (FliA). The flagellar protein export system plays a vital role in directing σ^{28} action. The activity of σ^{28} is dependent upon the flagellar substrate specificity switch regulated by FlhB and FliK (Minamino *et al.*, 1999). On the export system switch the flagellum gains increased affinity for the filament type substrates, which contain the filament cap protein, flagellins and the regulatory protein FlgM (Chilcott and Hughes, 1998). FlgM acts as an anti-σ^{28} factor preventing the system making late subunits until the hook-basal body complex has been completed (Kutsukake and Iino, 1994).

σ^{28} is encoded by the flagellar gene *fliA* and is transcribed from a class II and class III promoter defining it as a middle flagellar gene. Expression from both promoters generates an auto-regulatory feedback loop that allows, when σ^{28} becomes active, *fliA* expression to be increased further. The net result is the strong induction of class III promoters allowing strong expression of late genes such as *fliC* that encodes the flagellin. σ^{28} recognises -35 and -10 sequences that differentiate its target promoters from the major sigma σ^{70} (Kutsukake *et al.*, 1990). The consensus sequence for a σ^{28} promoter is TAAA-N15-GCCGATAA, that generates a short -35 region and a long -10 recognition site (Chilcott and Hughes, 2000).

flgM gene is also expressed from a class II and class III promoter. Class II promoter produced FlgM protein acts as an internal checkpoint. The result of the FlgM / σ^{28} interaction is an essential component of the flagellar system to establish the transcriptional hierarchy. When the HBB structure is completed, FlgM expressed from the class III promoter has an increase opportunity to inhibit σ^{28} to counteract the auto-regulatory feedback loop σ^{28} generates, thus modulating σ^{28} activity (Hughes *et al.*, 1993; Kutsukake, 1994; Chevance and Hughes, 2008).

The regulatory mechanism dictated by FlgM has been studied in some detail. When present in the cytoplasm FlgM binds to free σ^{28} molecules (Karlinsey *et al.*, 2000). FlgM has a very strong affinity to σ^{28} leading to two outcomes a) rapid binding to free σ^{28} and b) FlgM can out compete RNA polymerase for σ^{28} (Chadsey and Hughes, 2001). These properties and nature of FlgM's actions means that sensing of flagellum status can be rapidly feedback into the system via a quick shutdown of σ^{28} activity. FlgM is a small protein approximately 10kDa in size. Surprisingly structural analysis has shown that the N-terminal region possesses very little defined structure, while the C-terminal region forms non-rigid α-helices (Daughdrill *et al.*, 1998). It is this C-terminal region that defines the binding site of FlgM to σ^{28} (Daughdrill *et al.*, 1997). The structural analysis using NMR of FlgM:σ^{28} complexes showed that FlgM wraps itself around σ^{28} interacting with sequences in the sigma factor 2.1, 3.1, 4.1 and 4.2 (Kutsukake *et al.*, 1994; Chadsey and Hughes, 2001).

The interaction between FlgM and σ^{28} has two further functions, as well as inhibiting σ^{28} sigma factor activity (Aldridge *et al.*, 2006b). The first of these roles is the Type III chaperone activity of σ^{28}. Using point mutations in σ^{28} Aldridge et al (2006) showed that the FlgM/ σ^{28} interaction was required for efficient secretion of FlgM. A key group of mutants in σ^{28} used in defining it as the T3S chaperone of FLgM were all in region 4 that prevented σ^{28} acting as a sigma factor but still were effienct at facilitating FlgM secretion (Chadsey and Hughes, 2001; Aldridge *et al.*, 2006). The second role of the FlgM/σ^{28} interaction functions to modulate the degradation of both proteins (Barembruch and Hengge, 2007). In the absence of FlgM, σ^{28} is degraded more rapidly arguing that FlgM protects σ^{28}. In contrast, loss of σ^{28} argues that FlgM would be more stable, however loss of σ^{28} also reduces FLgM secretion. Overall, the FlgM/σ^{28} has an intricate interplay of modulating σ^{28} activity by sensing the assembly

status of FlgM while modulating the availability of both proteins either via protein secretion or protein degradation.

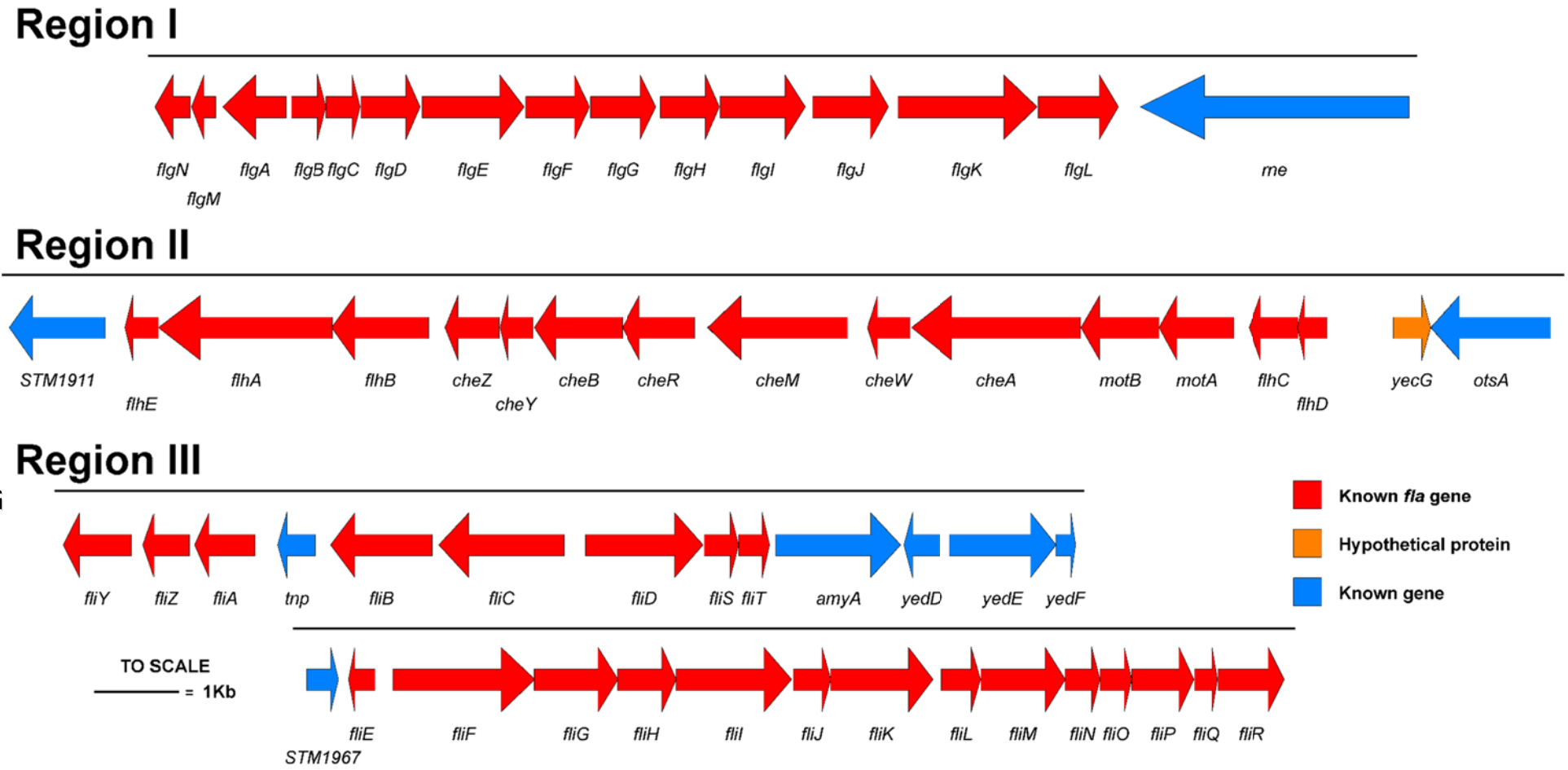


Figure 9. Schematic diagram of *Salmonella* flagellar genes distributed across the whole genome (provided by Dr. P. Aldridge).

1.10 Flagella Immune Response Adaptive versus Innate Recognition

Salmonella has two key antigens associated with the flagella encoded by the flagellin genes *fliC* and *fljB.* The flagellins are recognised by both the innate and adaptive arms of the immune system during infection (Salazar-Gonzalez and McSorley, 2005). Flagellin triggers three inducers of alternative immune system signaling pathways: Toll-like receptor 5 (TLR5), the cytosolic proteins Birc1e/Naip5, and the NOD-like receptor (Sun *et al.*, 2007).

Flagellin innate immune response is stimulated by Toll-like receptor TLR5 recognition which activates pro-inflammatory gene expression (Gewirtz *et al.*, 2001). Furthermore, flagellin proteins released into dendritic cells are directly sensed via the NLR receptor (figure 9). NLR recognition directs the splenic dendritic cell to activate the inflammasome complex (NALP), leading to production of pro-inflammatory interleukins (IL-1 and IL18). IL-18 plays a role to trigger cytotoxic T cell ($CD8^+$ T) via interaction with the IL-18 receptor (figure 9). Subsequently, the cytotoxic T cell produces IFN-λ which is bactericidal to *Salmonella* growth (figure 9) (Ayres and Vance, 2012; Kupz *et al.*, 2012). Flagellin represents a class of pathogen produced molecules defined as pathogen-associated molecular patterns (PAMPs) when considering the ability of our immune system to recognize invading pathogens. Other PAMPs include for example lipopolysaccharides and peptidoglycan, both components of the bacterial cell wall. Indeed, flagellin, LPS and peptidoglycan all have been implicated in the stimulated immune defence responses of animal host-pathogen interactions, as well as human infections (McDermott *et al.*, 2000; Eaves-Pyles *et al.*, 2001; Sierro *et al.*, 2001). Interestingly, with respect to flagellin, not all flagellin types are recognised by TLR-5 and thus bacterial pathogens possessing such derivatives are able to avoid recognition by the innate immune response. For

example, *Campylobacter jejuni* and *Helicobacter pylori* avoid TRL5 recognition having evolved a sequence change in the TLR5 recognition motif found within region D1 of their flagellins (Andersen-Nissen *et al.*, 2005).

1.11 Summary

In summary, *Salmonella* serovars are considered a key pathogen and involved in public health implications. Transmission occurs across a wide range of different hosts causing a variety of diseases from localized infection of the intestine which in time can become systemic. The flagellar system generates a rotational nanomachine via the highly coordinated process. In this study our attention focussed on the flagella by studying how flagellar gene expression varies across the *S. enterica* species.

In the following chapters, different *Salmonella* serovars were assessed for flagellar gene expression and motility to assess the diversity among them. The results have shown how that all serovars regulate the temporal activation of flagellar gene expression. However, serovars can be differentiated by the magnitude of gene expression. Data suggests that the change in magnitude is in part a result of population heterogeneity. Subsequently, we utilized our knowledge of *flhDC* regulation for *E.coli* and *S. enterica* to swap *flhDC* between them using *S. enterica* as our chosen model. This process allowed the investigation on how this key regulatory complex responds in different species.

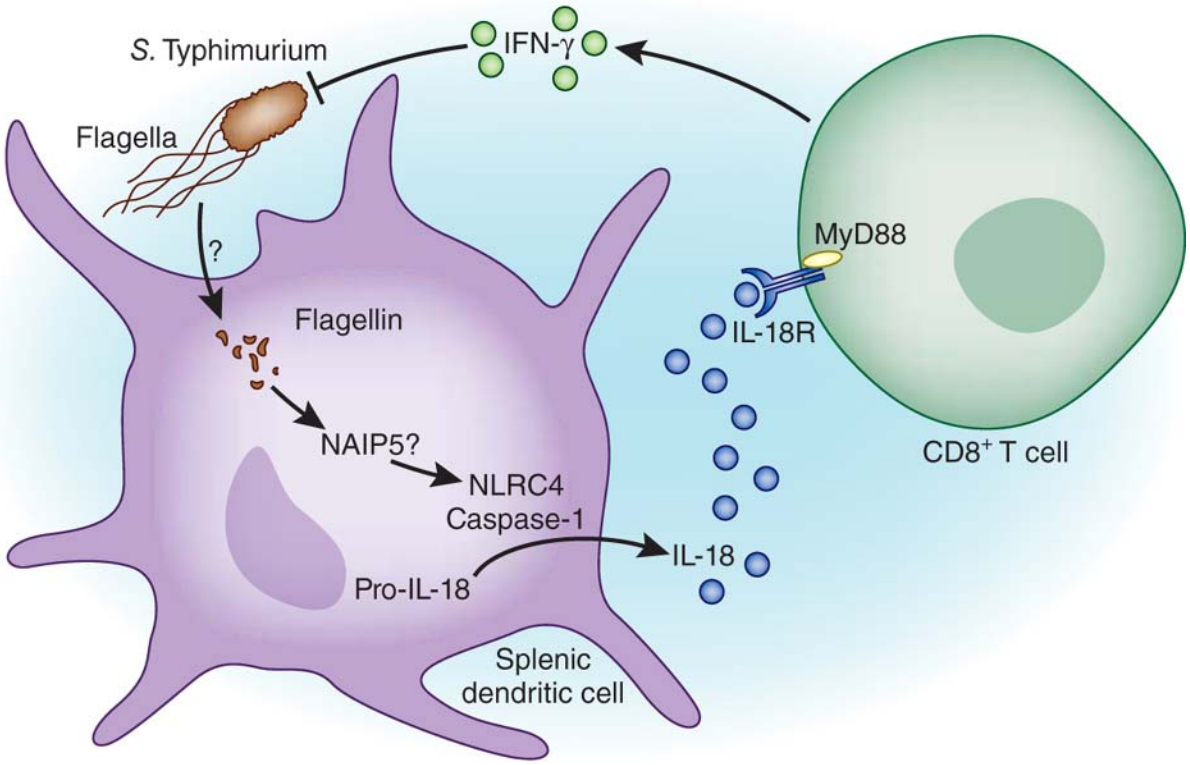


Figure 10. In-depth, the pathway of innate immune response of the flagellin starting from bacterial invasion the mammalian host and inducing the immune response via Toll-like receptor has located on splenic dentric cell and then triggered the inflammasome complex (NALP) to producing IL18 and thus goes to killer cell ($CD8^+$ T) to induce IFN-λ, this is act as inhibition of *Salmonella* proliferation, adapted from (Ayres and Vance, 2012).

Chapter Two: Aims of study

2.1 Aim of study

Flagella are one of the best examples to study evolution and the self-assembly of a complex organelle. A previous PhD thesis, in the Aldridge laboratory, has shown that when growth is controlled by continuous culture in a chemostat *E.coli* and *S. enterica* respond in a similar manner with respect to the output of the flagellar system. Interestingly, at the fast growth rate, 15 % of the *S. enterica* population did not produce a flagellum while in an *E.coli* population the response was homogenous. This important observation has led to the crucial question: why does S.*enterica* behave in this way?

On the other hand, a significant foundation of our understanding on flagellar gene expression in *S. enterica* is biased towards results obtained for the serovar Typhimurium. Therefore, a number of key conclusions and assumptions made with respect to flagellar regulation and output are assumed to hold for all serovars of *S. enterica.* Previous studies have shown that the majority of Typhimurium isolates are motile. Indeed, *S. enterica* exhibits a robust motility phenotype in that many strains are declared motile. We therefore aimed to ask the second question: do a range of serovars isolated from different hosts isolated from around the world reflect the robustness of their motile phenotype when considering flagellar gene expression activation and magnitude?

To achieve the two aims of this study the objectives included

1) Define the method of use for the tetracycline inducible system
2) Characterise flagellar gene expression with respect to its temporal activation and magnitude in *S. enterica* serovars

3) Replace *flhDC* from *S. enterica* with *flhDC* from *E. coli* on the chromosome to investigate the impact of the output of the flagellar system and the growth rate control of motility.

In the following result chapters, we present the data generated from the projects aims and objectives. We will show that while all motile, some serovars do indeed have a different response with respect to flagellar gene expression. Further investigation leads us to explore the impact of population heterogeneity across the serovars. While replacing *flhDC* between *E. coli* and *S. enterica* we further explore the impact of replacing each gene individually and how known regulators of $FlhD_4C_2$ perceive *flhDC* homologues from the close relative *E. coli*.

Chapter Three: Methodology

3.1 Bacterial Growth Conditions

All strains used in this study are from a lab TPA collection stored within the Centre for Bacterial Cell Biology. All strains were frozen at -80 °C in 10 % DMSO. Bacterial strains were activated on Luria Bertani (L.B) medium and where needed specific antibiotics were added to conserve plasmids. Strains were incubated at either 30°C or 37°C depending on the plasmid. The plasmids pKD46 and pWRG99 are temperature sensitive, growth at 30 °C will allow replication of these plasmids.. Antibiotics used during this study included Ampicillin, Chloramphenicol, Tetracycline and Kanamycin (table 1).

Antibiotic	Stock Con.	Final con.	Add to 100 ml
Ampicillin	20 mg /ml	100 µg/ml	50µl
Chloramphenicol	2.5 mg/ml	12.5 µg/ml	50µl
Tetracycline	2.5 mg/ml	12.5 µg/ml	50µl
Kanamycin	10 mg/ml	50 µg /ml	200µl

Table 2: Antibiotic concentrations profile

3.2 Techniques Associated with DNA

3.2.1 Genomic DNA Extraction

Bacterial DNA was isolated using Sigma-Aldrich genomic DNA kit according to the manufacturer's protocol. After overnight incubation at appropriate temperatures, 1.5 ml culture was centrifuged at 12000 rpm for 2 minutes. The supernatant was discarded and the pellet was re-suspended thoroughly in 180 µl of Lysis solution T/buffer. To suspension was added 20 µl of Proteinase K and incubated for 30 minutes at 55 °C. Two hundred microliters of Lysis solution C was then added. After vortexing for 15 seconds, the mixture was incubated at 55 °C for further 10 minutes. A 200 µl of ethanol 100% was added into the mixture and vortexed for 5-10 seconds.

The mixture was transferred to a column and centrifuged at 6500 rpm for 1 minute. The column was washed with 500 µl of Wash solution 1 and centrifuged for 1 minute at 6500 rpm. A second wash with 500 µl of Wash solution followed, but centrifuged for 3 minutes at 12000 rpm. The genomic DNA was eluted by using 200 µl of the sterile Milli-Q filtered PCR water. Finally, DNA quantification was determined via a Nanodrop NA-1000 spectrophotometer and genomic DNA was kept at -20 °C.

3.2.2 Plasmid Extraction

A. Extraction of Plasmid Using Miniprep Kit

The procedure was used the Sigma-Aldrich NA0150 GenElute HP Plasmid Kit according to manufacturer's protocol. A single colony was picked from a freshly streaked plate into the 5 ml LB broth with the appropriate antibiotic and incubated an overnight with shaking. The culture was centrifuged for 10 minutes at 4500 rpm. The supernatant was discarded, the pellet was suspended with 200 µl Resuspension Solution and mixed well by vortex. A 200 µl of Lysis buffer was added into the suspension and directly inverted until the solution became clear and slimy. Neutralization/Binding Buffer (350 µl) was then added and inverted eight times to precipitate the cell debris. The mixture was centrifuged at 12000 rpm for 10 minutes. The cleared lysate was transferred directly into the column and centrifuged for 1 minute at 12000 rpm. The column was washed with 500 µl Wash Solution 1 and centrifuged for 1 minute at 12000 rpm. The column was washed again with 750 µl Wash Solution 2 and centrifuged at 12000 rpm for 1 minute. The column was further centrifuged for 2 minutes at 12000 rpm in order to remove residual Wash Solution 2. The plasmids were eluted by using 100µl sterile Milli-Q filtered PCR water. Plasmid DNA concentration was quantified (Nanodrop NA1000) and the sample was stored at -20 °C.

B. Extraction of Plasmid Using Midiprep Kit

The aim of using this kind of kit to get a high quantity of plasmid. The procedure was used the Sigma-Aldrich NA0200 GenElute HP Plasmid Kit according to manufacturer's protocol. All chemicals solutions and reagents were prepared as described in the manufacturer's booklet.

3.2.3 Gel Electrophoresis

DNA was verified by using 1% agarose gel electrophoresis. Four grams of agarose powder was dissolved within 400 ml 1X TAE buffer and added 9 µl DNA-stain Nancy-520. On melting, molten agarose was kept at 60°C until use it if necessary. DNA samples were diluted 5:1 in 6 X Loading Dye (Promega). All DNA samples were assessed against a DNA ladder (Promega 1kb or 100bp) as a control. Typically, the power conditions used were: constant voltage of 120 V, electrical charge of 400 MA and a time between 40-50 mins. Gels were imaged via a UV-Transilluminator cabinet (Syngene Ingenius) and Genesnap software.

3.2.4 Amplification Of DNA By Using Polymerase Chain Reaction (PCR)

PCR was conducted using Q5 High-Fidelity DNA Polymerase (N.E.B.) and used by manufacturer's standard protocols. Primers were synthesized by Integrated DNA Technologies (IDT) design utilizing Serial Cloner 2.6.1. The reactions were performed using a Biometra T3000 thermocycler. To determine the proper reaction annealing temperature (TM) forward and reverse primers were input information: http://tmcalculator.neb.com/#!/. The total volume of all PCR reactions was 50µl (Table 2, 3).

Cycling Step	Temperature(°C)	Time	# of Cycles
Initial Denaturation	92°C	2 min	1
Denaturation	92 °C	30 sec	30
Annealing	50-65 °C	30 sec	30
Extension	72 °C	2:30 min	30
Final Extension	72 °C	5 min	1
Hold	4 °C	Pause	1

Table 3: PCR temperature condition and cycle timings

Ingredients list	Volume (µl)
(5xQ5) Reaction buffer	10
dNTP mix 2mM	5
Forward Primer 20 pmol / µl	2.5
Reverse Primer 20 pmol / µl	2.5
Template DNA	VARIED
High Fidelity DNA Polymerase	0.5
H2O	To final volume of 50

Table 4: PCR reaction components

3.2.5 Purification PCR DNA Fragment

PCR DNA fragments were purified to remove impurities such as oil, salt, primers and nucleotides via a PCR Clean up Kit. The kit used was the Sigma-Aldrich NA1020 GenElute Clean up Kit according to the manufacturer's protocol.

3.2.6 DNA Gel Extraction

A UV-Transilluminator (Syngene) was used to determine the desired DNA fragment on gel. The correct DNA fragment within an agarose gel was cut out and solubilized and extracted by using a Sigma-Aldrich Gel Extraction Kit in accordance with the manufacturer's protocol.

3.2.7 Ethanol Precipitation Of DNA Fragment

Ethanol precipitation technique was used to concentrate PCR DNA fragments and plasmids. For example, a 50 µl PCR DNA solution was mixed with 5 µl 3M NaAc (pH 5.2) and 140 µl 100 % ethanol. The mixture was left at room temperature for 30 mins before spinning at 13000 rpm for 15 minutes. The supernatant was disposed and the pellet washed with 500 µl 70 % ethanol followed by centrifugation at 13000 rpm for 10 min. The pellet was dried using a vacuum evaporator (Scanvac) under pressure at a temperature of -104°C. Finally, the pellet was resuspended in required volume of sterile filtrated Milli-Q H_2O.

3.2.8 DNA Sequencing

The sequencing of DNA was performed by Source Bio-Science Company. Each reaction required a 5 µl PCR fragment (1ng/µl per 100bp) and specific primers 3.2pmol/ µl.

3.3 Real Time Quantitative Polymerase Chain Reaction

Real time-qPCR was used to define ratio of reporter plasmids for pRG51 and pRG39 to chromosomal DNA content per cell.

3.3.1 Isolation Condition Genomic / Plasmid DNA

All cultures were inoculated from a single colony in 5 ml of L.B medium and incubated at 37 °C overnight with shaking. The next day cultures were diluted OD_{600} of 0.05 in 5ml fresh L.B medium and grown up until reached OD_{600} of 0.5. In order to growth inhibit, all strains were treated with 0.25 % Sodium azide (NaN_3). Genomic and plasmid DNA were extracted using Sigma-Aldrich genomic DNA kit in accordance with the manufacturer's protocol. The DNA concentration was quantified by using a Spectrophotometer (Nanodrop NA1000).

3.3.2 Determination Standard Curve

A standard curve was determined by using different serial dilutions of genomic and plasmid DNA (Table 5), in order to define a template DNA concentration inside the linear zone for quantification (figure11). Quantification analysis of unknown samples used ΔΔCT qPCR relative DNA quantification technique based on the initiation frequency of template of DNA deletion (Lee *et al.*, 2006).

3.3.3 Samples analysis

Experiments were performed using a Calibration Robot (Qiagen) and a Qiagen-Rotor gene Q real time PCR machine. The final reaction volume was 20 µl that included 0.5 µl of each forward and reverse primer (20 pmol / µl), 9 µl SYBR Green qPCR Master Mix (Promega) and 10 µl chromosome or plasmid template. The rotor disc was sealed with heat sealing film via a Rotor disc heat sealer (Qiagen) before reactions were placed in the thermocycler qPCR machine. All qPCR experiments were repeated as biological triplicates.

3.3.4 Statistical Data Analysis for qPCR

Standard curves were designed in order to plot unknown samples versus threshold cycle (CT) (figure 11), resulting from the reference gene or target gene for a comparison of PCR amplification efficiencies. Finally, the data were analysed by finding a ratio between reference gene and target gene via simple equation depend upon (Pfaffl, 2012) as a following:-

$$\text{ratio} = \frac{(\text{Etarget})\ \Delta\text{CP target}\ (\text{MEAN control} - \text{MEAN sample})}{(\text{ERef})\ \Delta\text{CP Ref}\ (\text{MEAN control} - \text{MEAN sample})}$$

Tukey multiple comparison test was used for statistical analysis of data.

Solution number	Dilution factor	µl H2O PCR	Final concentration
1	1	1	1
2	5	1	0.2
3	25	1	0.04
4	125	1	0.008
5	625	1	0, 0016
6	3125	1	0.00032

Table 5: The serial dilution of Genomic DNA and plasmid to create standard curve

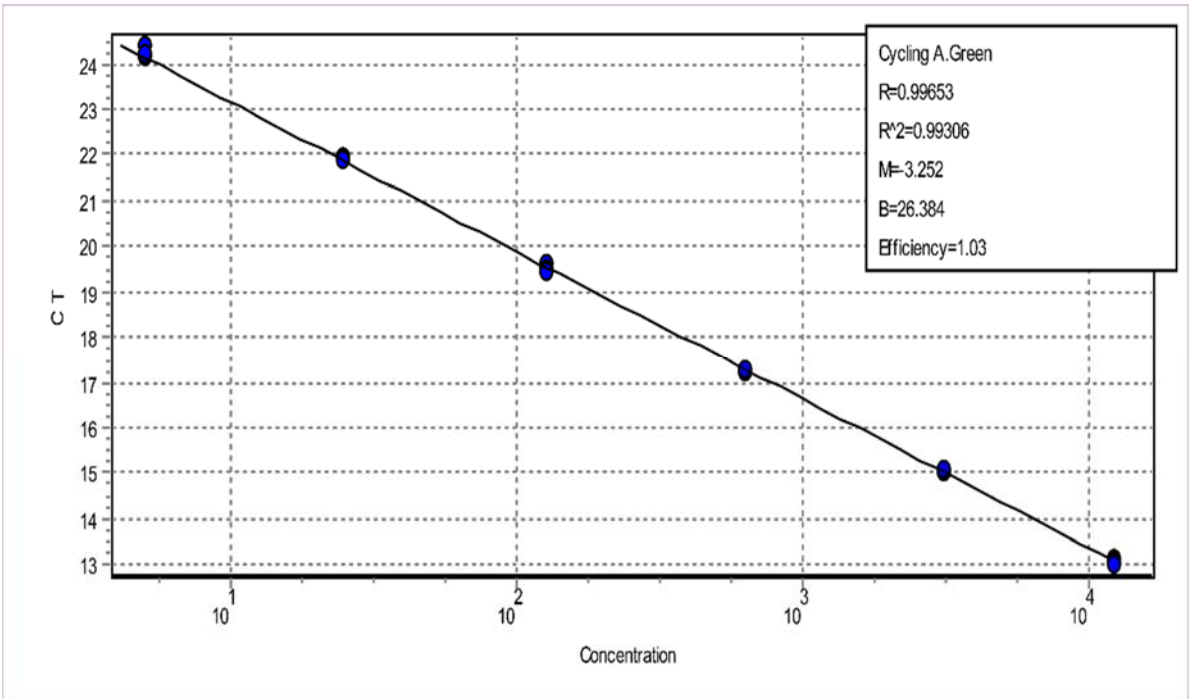


Figure 11. A standard curve produced from the serial dilutions of Genomic DNA data. The slope of the heel up line is represented the points of gathering between the concentrations of the DNA upwards.

3.4 Micro-plate Assay to Measure The Activation Of Flagellar Gene Expression

Depending on the necessities of each experiment, strains were streaked out at 37°C or 30°C on a selective antibiotic plate. Overnight cultures were inoculated in test tubes and incubate with rigorous shaking. Pre-cultures were prepared to start growth at an OD_{600} = 0.02 in 3 ml of liquid media and permitted to grow until reaching OD_{600} = 0.15. Each microplate well was prepared with 10 µl tetracycline (50 µg / ml), the tetracycline acted as an inducer for the *tetRA* promoter to initiate *flhDC* operon transcription. Growing cultures were aliquoted as 200 µl volumes into the 96 well flat-bottomed plate taking care and consideration that no bubbles were present. The plate was sealed using a gas-permeable membrane. The plate reader (Fluostar-Omega) was configured with two protocols measuring optical density and luminescence using the BMG LABTECH's computer's software. All experiments were performed at 30°C in LB media unless specifically stated in the figure legend. Finally, the results were analysed by using a Microsoft Excel 2013.

3.5 Chromosomal Mutagenesis and Modification of *Salmonella* Strains

In order to knock-out and knock-in desirable genes into the chromosome, The Lambda Red recombination system was exploited. Lambda Red is considered a robust technique for insertion of heterologous DNA into the chromosome designed originally by Datsenko & Wanner, (2000). Knock-out steps for the Lambda Red recombination system are shown in Figure 12. The pKD46 plasmid was introduced into the bacteria cells by electroporation (BIORAD MicroPulser electroporator). Bacteria were recovered in LB media at 30°C for one hour with shaking and plated LB plate / ampicillin for 24 hours at 30°C. One colony was picked into a 30 ml LB / ampicillin and 0.1% arabinose culture. The culture incubated at 30°C with shaking

until it was reached an OD_{600} = 0.6-0.8. The cells were pelleted and washed with cold sterile filtrated Milli-Q water after centrifugation at 4500 rpm for 15 minutes twice. PCR products were transformed into the bacteria via electroporation (BIORAD MicroPulser electroporator) and recovered the cells were plated out onto specific antibiotic agar as described above. The antibiotic resistance cassette was removed via transforming a FLP plasmid into the cell using electroporation or transduction. Transformations were plated out on to ampicillin LB plates at 30°C. Eight colonies were randomly picked on to LB plates (No antibiotics) and grown at 42 °C overnight in order to induce plasmid loss. Colonies were screened on LB with a specific antibiotic to ensure the colonies were sensitive for the antibiotic, that meant the antibiotic cassette had been removed all constructs were confirmed using PCR.

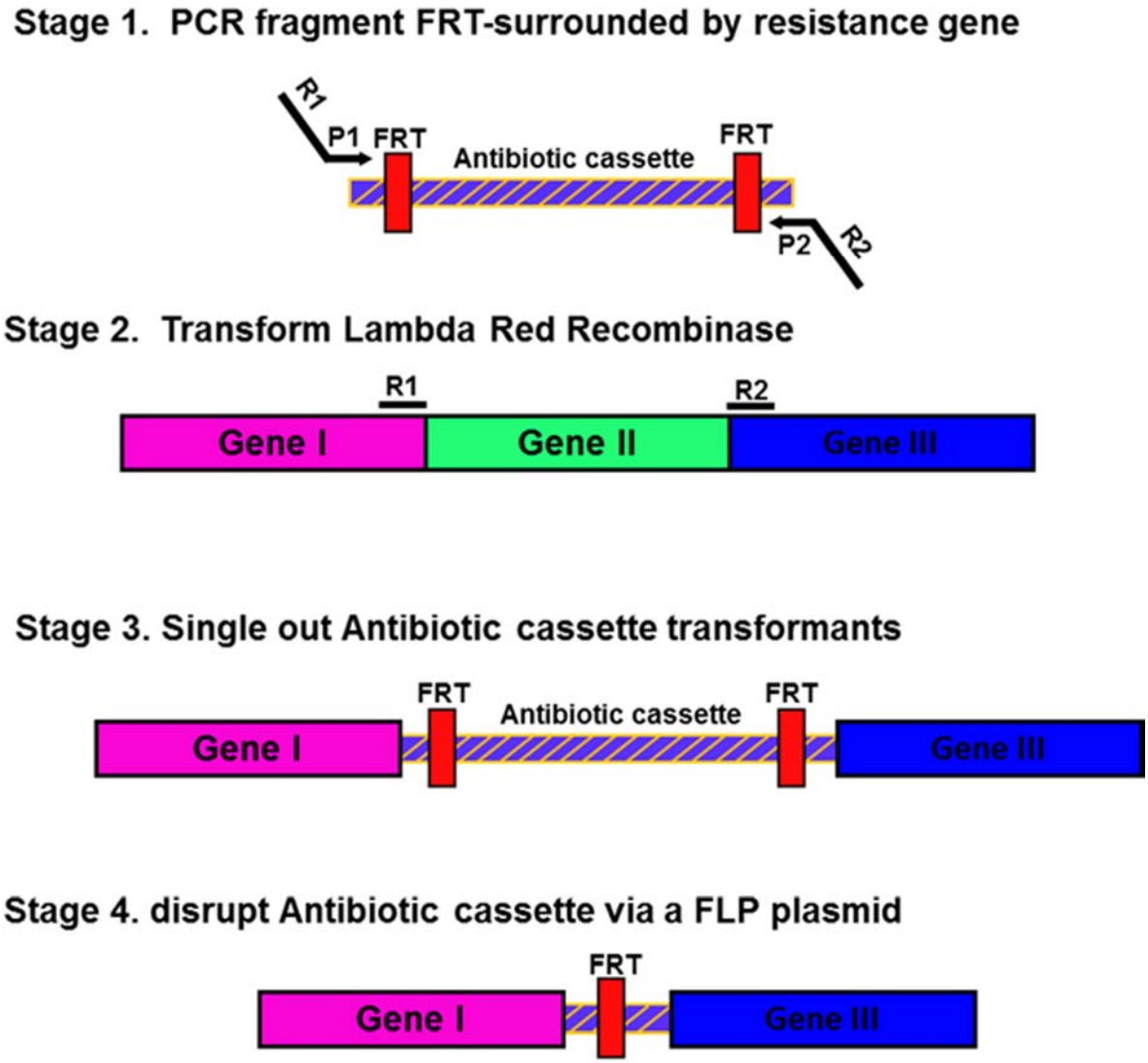


Figure 12. Strategic way to remove the gene precisely. R1 and R2 indicate to the homology sites. P1 and P2 indicate to primers regions. Adapted and modified from (Datsenko & Wanner, 2000).

In order to replace certain genes with other gene on the chromosome, the Blank *et al* (2011) protocol was used. The method was followed by using the specific plasmid pWRG100 a derivative of pkD3 which encoded the chloramphenicol antibiotic cassette and the *I-SceI-XbaI-rev* enzyme restriction sites (figure 13). The *I-SceI* enzyme is encoded by the pWRG99 plasmid, under control of tetracycline induction. The rationale of the Blank *et al* system is that when *I-SceI* is expressed from pWRG99 in cells in which the pWRG100 cassette has been inserted into the chromosome, the double stranded break produced is lethal. However, if a PCR product is introduced and swapped successfully using lambda red recombination for the region deleted by pWRG100, the cells will grow as a normal cell colony and be sensitive to chloramphenicol. This means that the chromosome has already disposed of *I-SceI* restriction site with chloramphenicol cassette and replaced the region with the desirable gene. We have confirmed gene substitution via sequence analysis. All steps of this gene replacement strategy are described in figure 13.

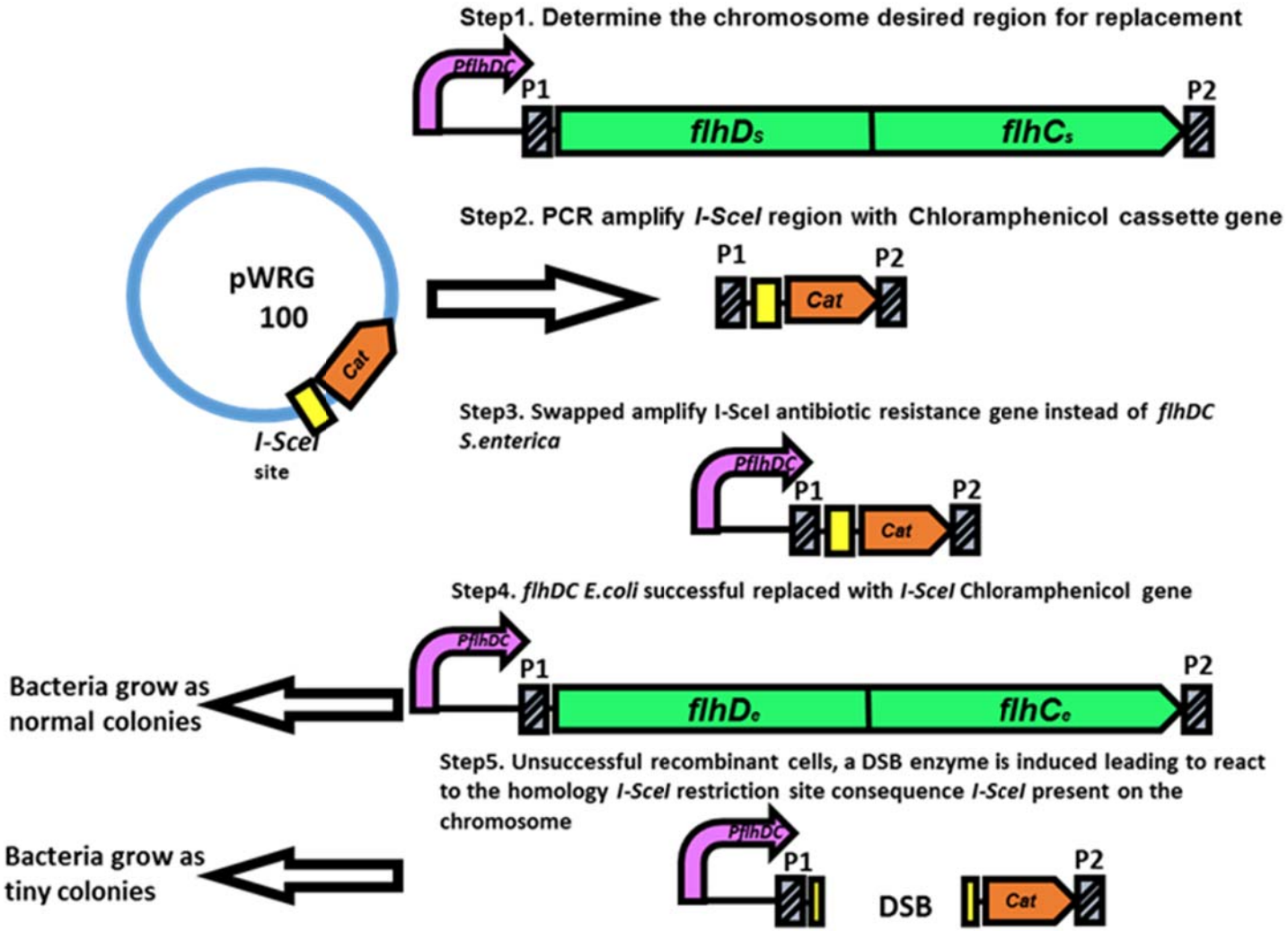


Figure 13. A replacement strategy for *flhD* and *flhC* genes from *E.coli* into the *S.enterica* in vivo. P1 and P2 indicate to primers initiate regions, Cat represented to the chloramphenicol antibiotic cassette was used to homologous recombination site instead of *flhDC* gene. Adapted and modified from (Blank *et al.*, 2011)

3.6 DNA Molecular Cloning

In order to a clone certain gene into the certain plasmid, we have used Gibson cloning technique. It included preparation material and reagents according to manufacturer's standard protocols. Primers designed for each DNA and vector fragments. We have calculated the quantities as flowing the NEB equation to make the balance of concentration between DNA or vector fragments. The DNA and vector amplified have mixed with Gibson Assembly Master Mix at 50°C /fifteen minutes to one hour in order to give the compatibility being completed. Then transformed into the NEB 5 alpha competent *E.coli* and plated it out on specific antibiotic LB at a certain temperature overnight. Finally, we have checked the cloned plasmid via sequencing them as we mentioned earlier.

3.7 Preparation of Competent Cells

A single colony of DH5α *E. coli* was inoculated into the 200 ml LB media Erlenmeyer flask at 37°C on an orbital shaker. The bacterial culture was grown to an OD_{600} = 0.1-0.2. Bacterial cells were decanted into a 50 ml falcon tube and chilled on ice for 15 min and centrifuged at 4500 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 40 ml pre-chilled $CaCl_2$ 100mM and chilled on ice for 40 minutes. The pellet resuspended again in 1ml pre-chilled $CaCl_2$100mM with 0.1% glycerol and was centrifuged at 4500 rpm for 10 minutes at 4°C. Aliquots of 100 µl in 1.5 ml Eppendorf tubes and placed in liquid nitrogen and stored at -80°C.

3.8 Transformation Techniques

Different techniques was used for transformation bacteria either using DNA fragments or plasmids to create mutant bacteria or new strains.

3.8.1 Electroporation

Cells were grown overnight in 5 ml LB-media with a specific antibiotic, if necessary, and required temperature. Overnight cells were diluted to OD_{600} = 0.05 into 30 ml of LB media with antibiotics if needed. The culture was harvested at mid-log phase by centrifugation at 4500 rpm for 10 min. cells were washed twice with cold sterile filtrated Milli-Q water and the supernatant discarded. The DNA was added into a 50 µl suspension of vortexed cells and carefully pipetted into the electroporation cuvette. Bio-Rad MicrioPluser electroporator on a channel one which suitable pulses for *S. enterica* and *E. coli* cells. After electroporation, the cells were recompensed by using 1 ml LB and transferred to a 1.5 ml Eppendorf tube for incubation for one hour at 30°C or 37°C. The cells were the cultured on the LB plates with a specific antibiotic.

3.8.2 Heat Shock

For transformation, 100 µl of competent DH5α *E. coli* cells were thawed on ice then the desirable plasmid added and mixed gently before placing on ice for 30 minutes. Cells were heat-shocked in a 42-45^{o}C water bath for 50 seconds and then transferred directly on ice for 2 minutes. Then, 900 µl LB medium was added to the pellet and incubated at 30 / 37°C for 1h with shaking. Finally, the culture was plated out on LB agar with suitable antibiotic at 30 / 37°C.

3.8.3 Transduction

Phage stocks were prepared from 5 ml LB medium at 37°C overnight cultures. 1ml of bacterial culture was added to 4 ml phage buffer and incubated at 37°C for 8-16 hours. The mixture was centrifuged at 4500 rpm for 10 minutes. The supernatant transferred into a new tube and 500 µl chloroform was added. The mixture was vortexed and left one hour for settlement. The phage solution was diluted into serial dilution to $1X10^{-3}$, considered the stock of the certain phage ready to use for

transduction. For transduction, we were took 200 µl of the culture OD_{600}= 0.6-0.8 and mixed with the 200 µl phage stock and incubated one hour at 37°C. For a controls, phage stock was mixed with LB broth and 200 µl culture was added to 200 µl saline to make sure the phage stock and culture were not contaminated. Transductions were plated on to specific antibiotic LB plates and incubated overnight at 30 / 37°C.

3.9 Motility Assay

All strains were examined in semisolid agar in order to define bacterial motility. The concentration of agar approximately 0.3%. One colony was picked via sterile wooden sticks from freshly grown colonies (LB plate) and stabbed directly into the centre of the motility agar and incubated for 6-8 hours at 37°C. The motility diameter was visualized using a Syngene Bioimaging cabinet. ImageJ software program was used to measuring the diameter of the bacterial swim. All experiments were done in triplicate.

3.10 Fluorescent Microscopy

500 µl molten 1% agarose was placed onto Multi-spot microscope slide and covered with a plain microscopic slide to get a flat clear surface. 1 µl bacterial culture at OD_{600} = 0.6-0.8 was pipetted on the slide and after drying at 42°C covered with a cover slip. A Nikon-Ti inverted microscope was used to acquire two different types of a pictures: a phase contrast image (100 ms) and Green fluorescent light GFP (500 or 1000ms). All images were acquired via MetaMorph v7.7.80 software and processed using ImageJ software to merge the images into one RGB image.

3.11 Matlab software for Image analysis

3.11.1 Microbetracker

Mirobetracker software was downloaded from the http://microbetracker.org/ website. Phase contrast images were used to determine the outside border of each cell leading to cell numbers being calculated. The options have used in the program including load phase, contour-green-phase for displaying image, join-refine-split-add for manual improving image, and particular parameter has loaded (alg4ecoli.set for *E. coli* detection) are showing in figure 14. ‘’This frame’’ option started the image analysis process. After process, save analysis was chosen leading to the analysed image being saved within a mesh image (figure 14).

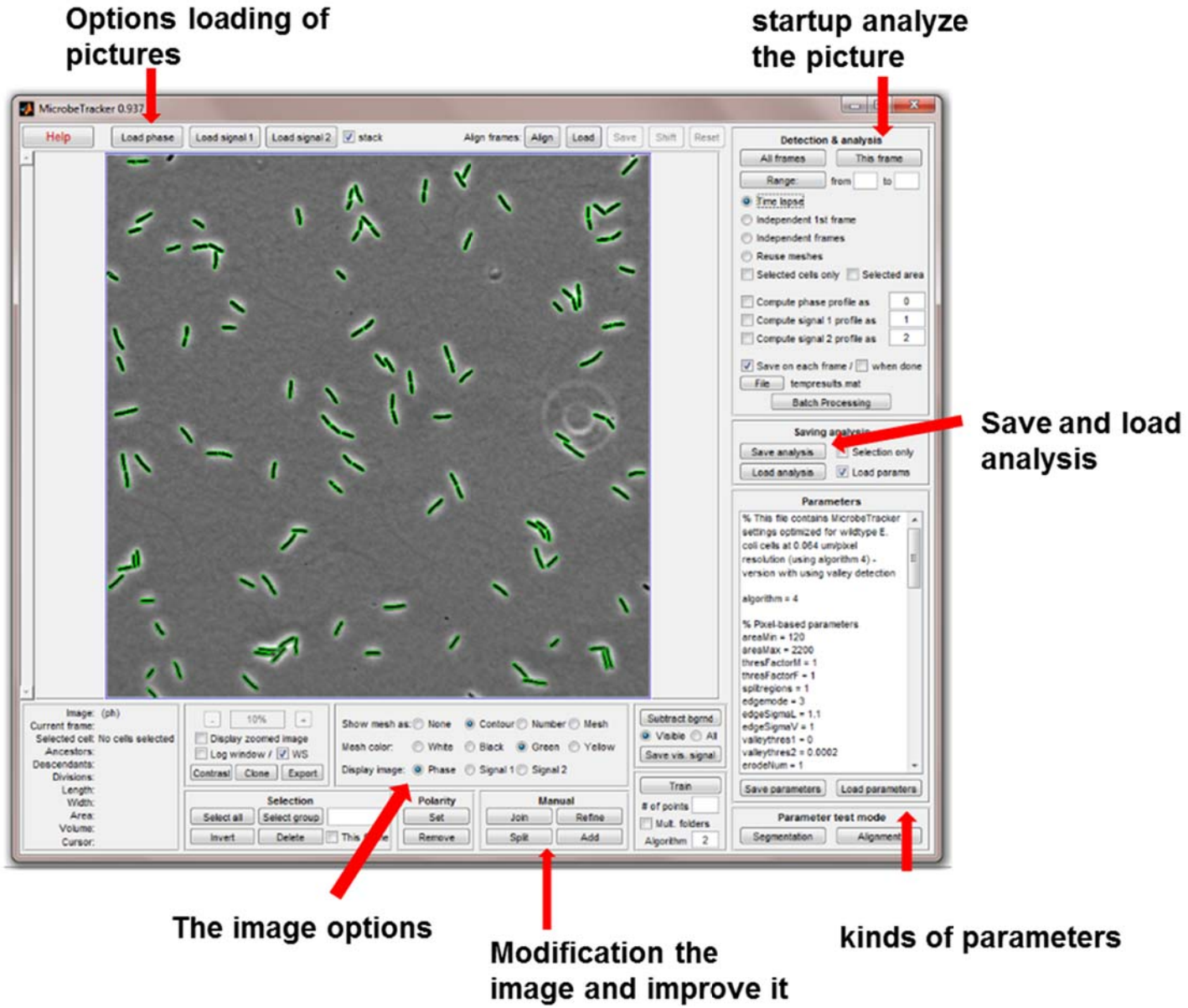


Figure 14. Image caption how to use Microbetracker program for determining the number of cells and counting flagellar foci.

3.11.2 Spot-finder

GFP images were uploaded directly after phase contrast analysis image (mesh) using Spot-finder Z in software to count and recognizing GFP flagellar foci. All options were setup on in program (figure 15).

3.11.3 Export file

All mesh matlab files were exported to Microsoft excel files via the ‘’exportlgthxls’’ option responsible for exporting cell length data and a ‘’exportspots2xls’’ option regarding to flagellar foci.

3.11.4 Intensity image analysis

Mirobetracker software was used to recognizing GFP Intensity for each cell. After boarded and counted a GFP image was uploaded using the signal 1 option and the ‘’subtract bgrnd’’ option selected. Analysis used the options ‘’Resue mesh’’ option and ‘’compute signal one’’. Analysed GFP intensity for each cell and exported as an excel file (exportcells2xls). The analysis used either Excel of R program.

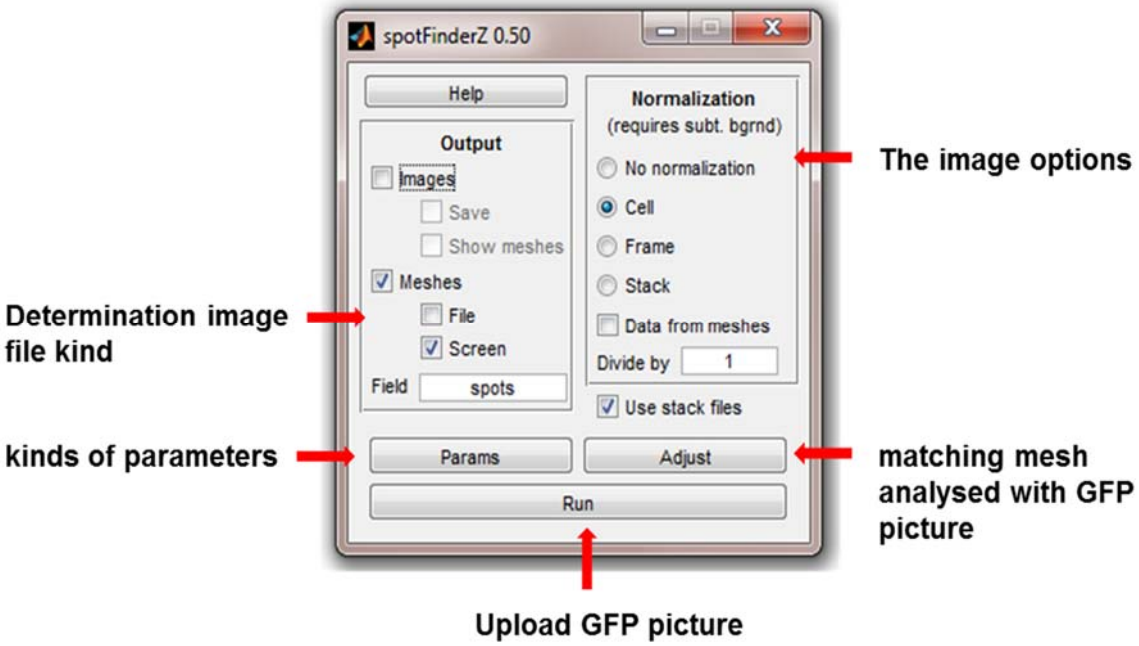


Figure 15. Image caption how to setup properly Spot-Finder option into the Matlab program.

3.12 Freezing Strains

One colony was picked from fresh LB agar plates and inoculated into 5 ml LB media with or without antibiotic if necessary and grown at 30 or 37°C overnight with shaking. 150 µl sterile DMSO was added into 1.5 ml of culture and then mixed them and placed directly into the -80°C freezer.

3.13 Techniques Associated With Protein-Protein Interaction

3.13.1 Overexpression FlhDC

A 5ml LB media overnight culture was used to inoculate a 1 litter LB with kanamycin calculate at an OD_{600} = 0.05 and incubated with shaking at 30°C. When the culture reached OD600 = 0.6, 1ml 1M of IPTG was added as an inducer to produce the FlhDC protein complexes and incubated for a further three hours. The culture was centrifuged at 7000 rpm for 15 minutes. The pellets resuspended with 25 ml sterile LB media and centrifuged at 4500 rpm for 10 minutes. The pellet was kept directly at -80°C. The pellet was resuspended in 25 ml His-Loading buffer, and the bacterial cells lysed via a Cell Disruptor under pressure a 25Kp, in order to release all proteins. The protein sample was centrifuged at 18000 rpm for 40 min using JA25.50 rotor (Beckman coulter centrifuge) at 4°C. The supernatant was directly loaded onto the Äkta purification column.

3.13.2 Purification of FlhDC Procedure

The Äkta Prime machine was used to purify the His-FlhDC protein complexes. 20% ethanol was injected through the Äkta Prime System in order to remove protein debris, bubbles and wash the system. The Äkta Prime was prepared with a His-Trap column and equilibrated with His-loading buffer. The protein sample was uploaded into the system and monitored using the analogue chart recorder (Biotech). After His-loading buffer was used to re-equilibrate system collected of the His-FlhDC protein

complexes as 2ml fractions via injection of fractions were His-elution buffer as a 0-100% concentration gradient. Tricine SDS gel was used to visualise all purified proteins by mixing 10 µl sample with 5 µl SDS buffer (10%) and prepared at 100°C for 5 minutes. Samples were loaded on SDS gel electrophoresis at 40 volts for 45 minutes.

3.13.3 Heparin-affinity chromatography

The aim of using heparin column was to isolate mimics DNA to ache FlhDC complexes as heparin. The Heparin column was connected to the Äkta Prime and equilibrated Heparin loading buffer. Cell lysates were loaded into the Äkta Prime system and loading equilibrated using fresh Heparin loading buffer. Samples were eluted by injecting Heparin elution buffer using a 0-100% can concentration gradient over 30 tubes, 2 ml each fraction.

3.13.4 Protein Gel Staining

After completed migration of protein on a SDS gel staining used a Coomassie Blue solution for 30 minutes. The gel was destained in the Destaining solution tray for 30 minutes. After that, the SDS gel was transferred into sterile Mill-Q water for 1 hour. Finally, the gel was visualized using a scanner digital (Epson 3490).

3.13.5 Electrophoretic Mobility Shift Assay (EMSA)

Purified proteins were serial diluted 10 times then mixed with same quantity of DNA fragment. The reactions were loaded after added 1µl DNA stain into a acrylamide gel (40%), and 1% TBE running buffer was used into the electrophoresis tray at 60 v for 3 hours at 4 °C. The acrylamide gel was stained by adding 1µl DNA stain into 50ml sterile H2O with shaking 1 hour. The gel was filmed a UV Transilluminator cabinet (Syngene Ingenius). ImageJ software was used to quantification the DNA bond with the protein.

Chapter Four: Evaluation of Flagellar Gene Expression in *Salmonella enterica* Serovars

4.1 Introduction

This chapter will describe how to control flagellar gene expression through different inducible promoters. The tetracycline inducible system is able to drive the entire the flagellum transcription via an on and off switch system (Karlinsey *et al.*, 2000). There are different regulatory structures and functional outputs of the tetracycline system based on the orientation of the *tetR* and *tetA* genes. *tetR* and *tetA* are transcribed from divergent promoters P_{tetR} and P_{tetA} (figure 16). TetR is the repressor of the system when tetracycline is not available. The gene *tetA* encodes the resistance gene and acts as an efflux pump (Hillen and Berens, 1994). The scenario for promoter activation starts with TetR binding to both P_{tetR} / P_{tetA} until tetracycline is in the environment / cell. On binding tetracycline, TetR is unable to stay bound to the DNA. Loss of the TetR:DNA interaction allows for transcription from both promoters (figure 16). Importantly P_{tetA} is fourfold stronger than P_{tetR}, leading to strong expression of the efflux pump (Wray *et al.*, 1981; Bertrand *et al.*, 1983; Saini *et al.*, 2008). This study will exploit the advantage that the tetracycline system is titratable (Gossen and Bujard, 1992). Furthermore, the tetracycline system is able to respond not just to tetracycline but also tetracycline structural derivatives such as anhydrotetracycline (figure 17). This allows us to control the magnitude of *flhDC* expression by different concentrations of antibiotic inducer and promoter orientation, without the stress of antibiotic activity playing a role (Koirala *et al.*, 2014b).

We have measured the motility phenotypes of *S. enterica* serovars when *flhDC* are under control of their native promoter and the tetracycline promoters, comparing class 2 and class 3 promoter activity. We have determined a change in the magnitude of gene expression contributing to flagellar assembly in multiple serovars of *S. enterica*. The activity of $FlhD_4C_2$ is tightly regulated and is considered the main

regulator of the flagellar system. $FlhD_4C_2$ with σ^{70} act together to operate the class 2 promoters of middle genes (Chilcott and Hughes, 2000). The middle genes are responsible for producing the hook and basal body. In addition $FlhD_4C_2$ with σ^{70} drive expression of σ^{28} (*fliA*) that specially activates the transcription of class 3 promoters.

In this chapter we complimented gene expression assays by counting flagellar numbers per cell using fluorescence microscopy. Having observed different magnitudes of flagellar gene expression across the *S. enterica* serovars, we correlate the phylogeny of the flagellar system, using bioinformatics analysis, for all serovars in order to identify varieties and patterns with respect to the *Salmonella* species phylogenetic tree.

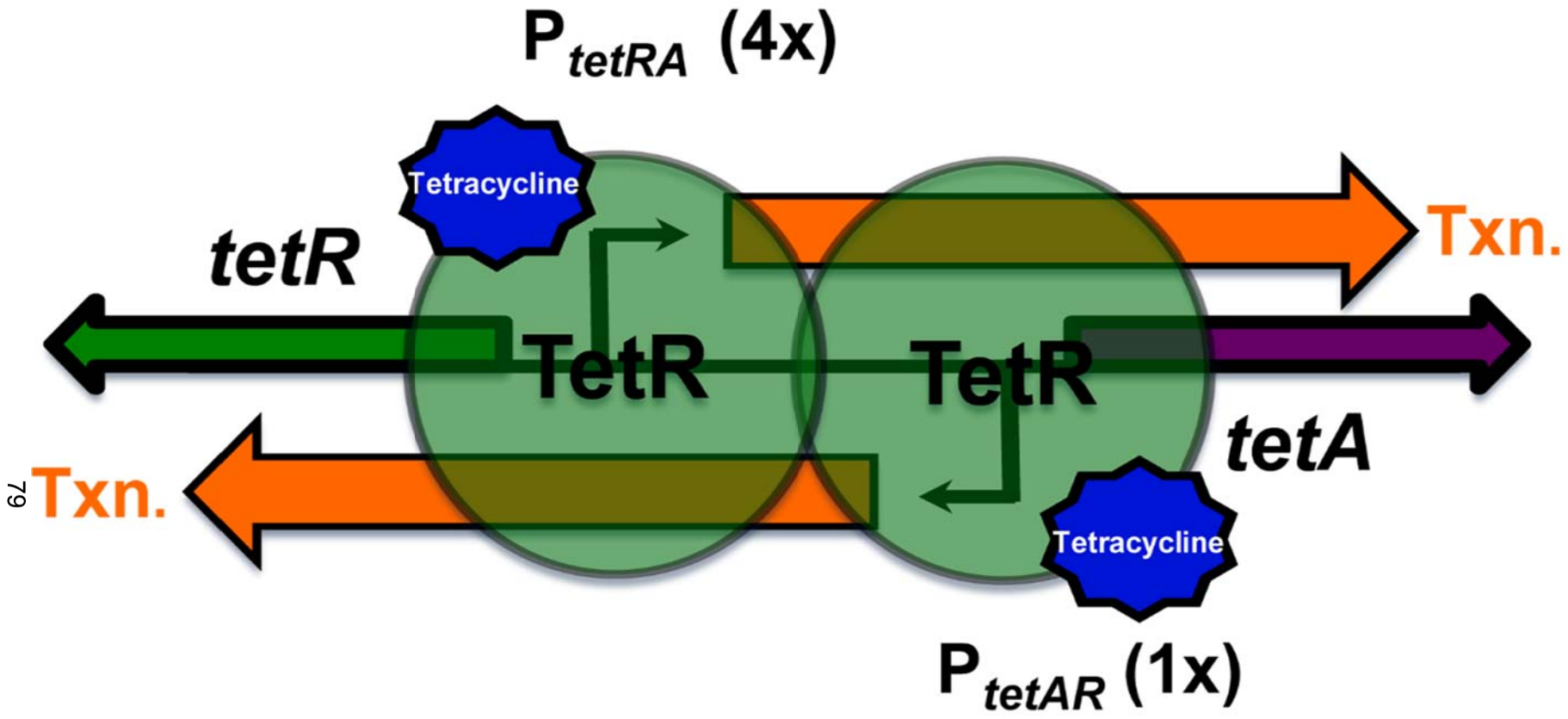
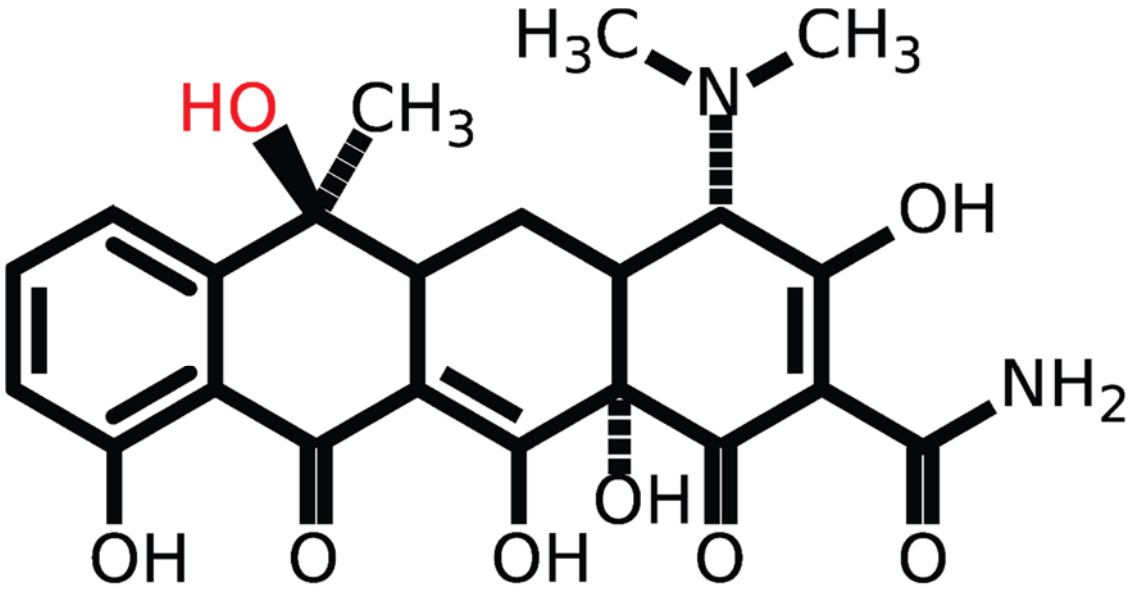
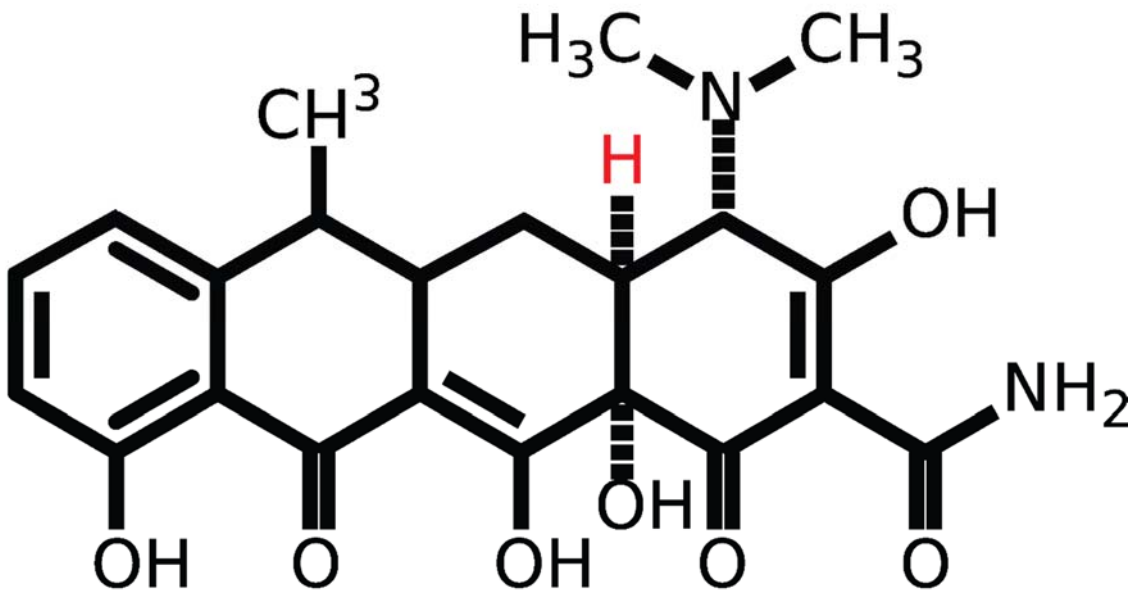


Figure 16. The layout of the tetracycline system. The system has two genes with divergent promoters, *tetA* is the tetracycline resistance gene. TetR acts as suppressor of *tetA* via bind *tetR* with *tetA* pending tetracycline release into the environment. Then the promoter system starts transcription in both directions after removing TetR with tetracycline from the DNA. The two promoters have different expression responses, P_{tetRA} expression fourfold more powerful than P_{tetAR}.



Tetracycline



Anhydrotetracycline

Figure 17. **Chemical structures of tetracycline compared to anhydrotetracycline.** The difference between the two forms are highlighted in red where tetracycline possesses one hydroxyl group more than anhydrotetracycline. Anhydrotetracycline has one more hydrogen group and missing one hydroxyl group.

4.2 Tetracycline Inducible System

4.2.1 Comparison the Motility Output Between P_{tetRA} and P_{tetAR}

Previous studies have used the tetracycline inducible promoter to control and drive the flagellar system in order to determine the temporal activation of flagellar gene expression (Karlinsey *et al.*, 2000b; Brown *et al.*, 2008). We have replaced P_{flhDC} from *S. enterica* with the two promoters P_{tetRA} and P_{tetAR}, to define the effect on the flagellar system when driving the expression at different levels and how this impacts the motility output phenotype.

The motility assay was an obvious starting point to compare between the strains that have different promoters. Promoter activities of the P_{tetRA} and P_{tetAR} were quantified by measuring the movement of bacteria through semi-sold agar 0.3% (figure 18). No significant variation was found across the strains tested with respect to their motility phenotype in motility agar (P=0.22). However, even though the strains were comparable, the weaker P_{tetAR} showed an expected drop in motility. This suggests that in general the two promoters are able to sustain motility in *S. enterica*.

4.2.2 Stimulation of Flagellar Gene Expression by Different Types of Tetracycline Promoters

The tetracycline promoter system used for this study is derived from Tn10 and has been used previously to control flagellar gene expression (Karlinsey *et al.*, 2000b). The T-POP transposon designed from Tn10*d*Tc was the original method of driving flagellar gene expression (Rappleye and Roth, 1997). T-POP was modified to allow transcription out of the transposon cassette. We have exploited this technology to measure the punctuality of flagellar gene expression activation and the magnitude of expression. The genetic design of the tetracycline systems used in this study remove

the IS elements of T-POP (figure 19). This is because T-POP has additional sequences downstream of *tetA* and *tetR (Rappleye and Roth, 1997).*

A comparison was carried out between the three promoters (P_{tetRA}, P_{tetAR} and TPOP-P_{tetRA}), driving transcription of *flhDC* (figure 19). Interestingly, flagellar gene promoter activity changes with respect to timing and magnitude for the synthetic constructs correlating to P_{tetRA} being stronger. In contrast, P_{tetAR} behaved similar to T-POP-P_{tetRA} (figure 20). Here the flagellar gene expression for both promoters are comparable to each other with respect to timing and magnitude level. These results suggests that the extra DNA in T-POP reduces transcription activity of P_{tetRA}. In turn this impacts both the timing and magnitude of *flhDC* dependent activation of the flagellar system.

The data shown in figure 20, and all subsquent expression figures, use the plasmid based bioluminescnce reporter system derived from pSB401 (Goodier and Ahmer, 2001; Brown *et al.*, 2008). The plasmids used have a selection of flagellar promoters driving the lux operon situated within pSB401. The source of the flagellar promoters is the *S. enterica* serovar Typhimurium strain 14028s (Goodier and Ahmer, 2001). As will become clear later in this chapter using these promoters in different strains is feasible due to the high level of conservation observed amongst class II and class III promoters across the species *S. enterica*. Furthermore, this collection of plasmids have been used regualry in a range of studies (Brown *et al.*, 2008; Aldridge *et al.*, 2010).

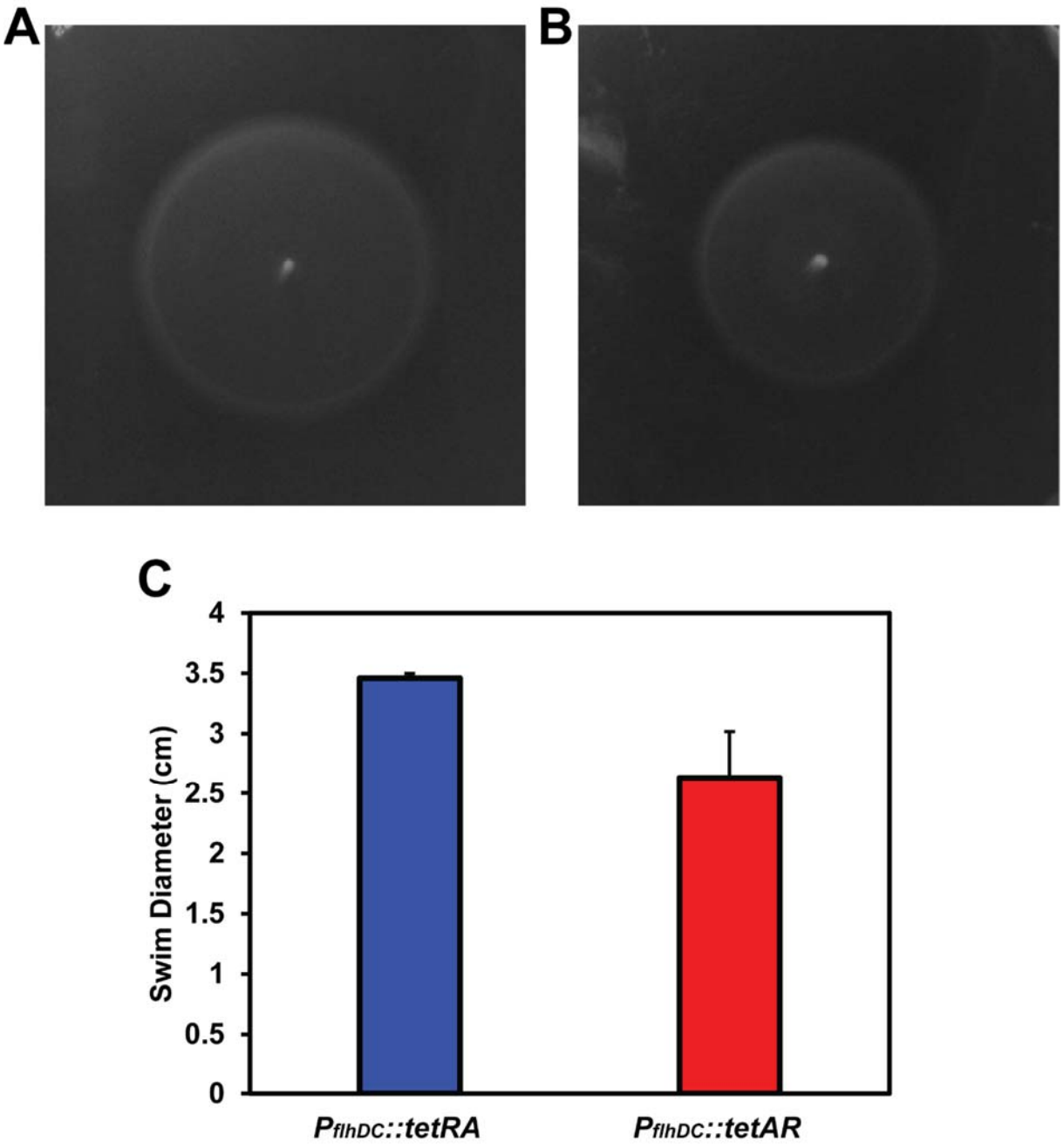


Figure 18. Motility quantified for *S. enterica* under control of the tetracycline system P_{flhDC}*::tetRA and* P_{flhDC}*::tetAR*. (A) Images of examples of the motility phenotype produced by *S. enterica* P_{flhDC}*::tetRA.* (B) Image of *S. enterica* P_{flhDC}*::tetAR*. All strains were grown at 37°C for 6-8 hours in motility agar (0.3% agar). (C) Quantification of the motility phenotype measured by the diameter of the observed outer swimming ring from three repeats. Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3959 and P_{flhDC}*::tetAR* =TPA3789.

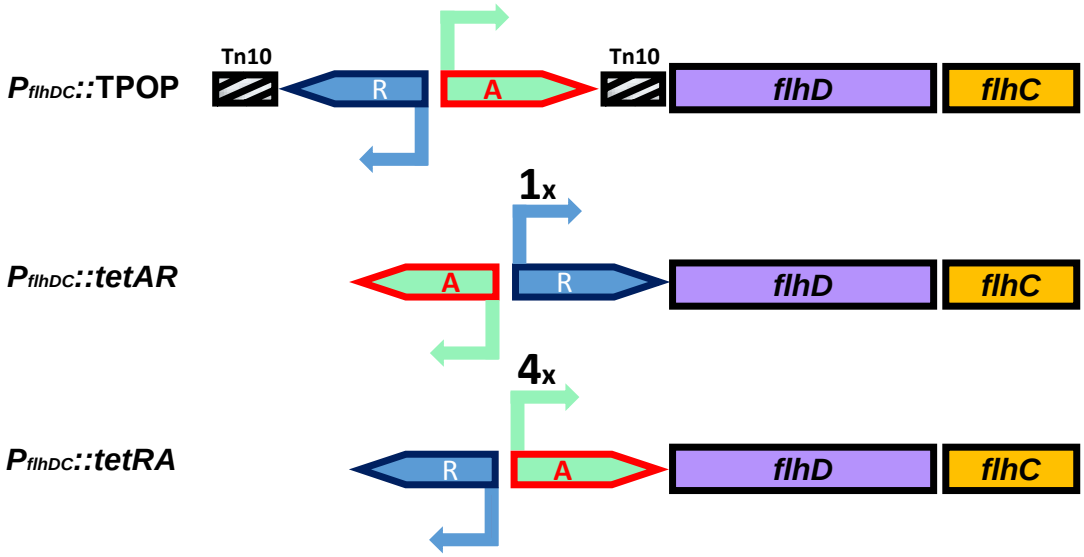


Figure 19. Overview of the genetic differences between the three promoters: TPOP-P_{tetRA}, P_{tetAR} and P_{tetRA}. The designation of each promoter based on the direction of *tetA* and *tetR* genes, with or without Tn10-encoded is emphasised. For P_{tetR} and P_{tetA} the relative strength is also indicated.

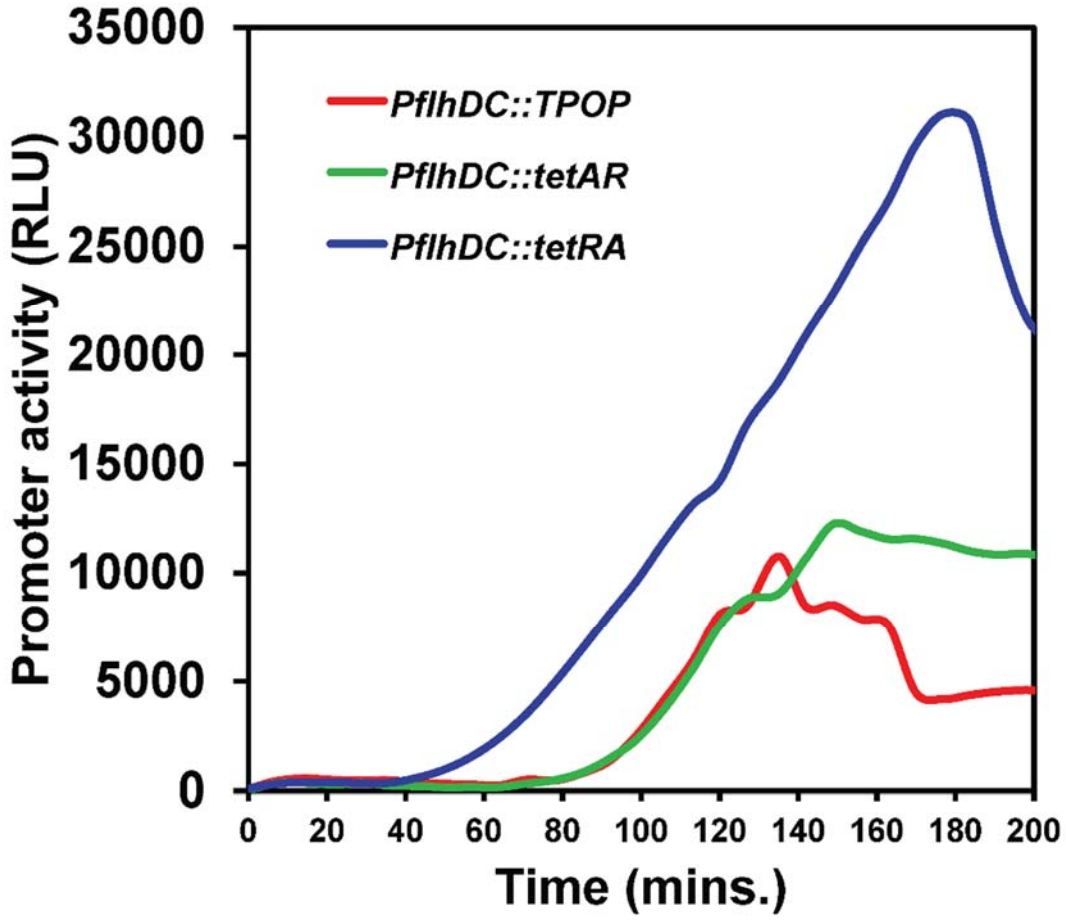


Figure 20. Experimental data, illustrating the variation of class III gene expression for *S. enterica* (LT2) by using different promoters (T-POP-P_{tetRA}, P_{tetAR} and P_{tetRA}). The expression behavior of T-POP compared to P_{tetAR} were identical with respect to the timing and magnitude. In contrast, P_{tetRA} expression of *flhDC* led to expression at an earlier time point and a stronger magnitude compared to the other promoters. Experimental data represents a minimal of three independent repeats. Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3967, P_{flhDC}*::tetAR* = TPA3803 and P_{flhDC}*::T-POP* = TPA86.

4.2.3 Comparison A Titration Of Class II Gene Expression Activity Between Tetracycline And Anhydrotetracycline Under Control tetRA and tetAR promoters

Having the master regulators of flagellar biosynthesis under control of the tetracycline inducible system, we are able to synthetically change the transcription of *flhDC* gene expression taking advantage of the fact that the tetracycline inducible system is titratable. Previous research has indicated that levels of tetracycline concentrations have a titration impact on gene expression (Hamann *et al.*, 1997; Bateman *et al.*, 2001). For all strains in this section, *flhDC* are expressed using variable concentrations of either tetracycline or anhydrotetracycline in order to generate titration curves of *flhDC* activity (figures 21 and 22). The maximum concentrations used for induction were for tetracycline 2.5 µg/ml and anhydrotetracycline 100 ng/ml based on previous studies (Brown *et al.*, 2008; Koirala *et al.*, 2014b). A clear significant change between P_{tetRA} and P_{tetAR} was observed (figures 21C and 22C). For tetracycline the serial dilution range of 2.5 down to 0.0025 µg/ml was compared to no antibiotic (figure 21A). In contrast, the anhydrotetracycline range used was 100 down to 1 ng/ml (figure 22). These ranges show a similar response profile in terms of induction for both P_{tetRA} and P_{tetAR} (figures 21C and 22C). Levels of flagellar gene expression gradually increased with increasing concentrations of inducer (figures 21 and 22). The results revealed that maximum activity of P_{tetRA} was stronger than P_{tetAR} regardless of which inducer was used (figures 21C and 22C). Finally, for both constructs a 50% maximum of activity for P_{tetAR} compared to P_{tetRA} was observed. In contrast, tetracycline was a more efficient inducer producing a higher level of flagellar gene expression in comparison to anhydrotetracycline for both P_{tetRA} and P_{tetAR} (figure 23 A and B).

One of the more significant findings to emerge from this analysis is that when compared, the titration of the flagellar gene expression is different dependent on the

inducer used. We found the relative maximum activity of expression for P_{tetRA} is higher compared to P_{tetAR}. Furthermore, tetracycline can be considered a more powerful inducer of flagellar gene expression than anhydrotetracycline. For example, the P_{tetRA} titration curves look very similar. However, P_{tetAR} curves have a different shape exemplifying the differences between inducers and system dynamics (figure 21C and 22C).

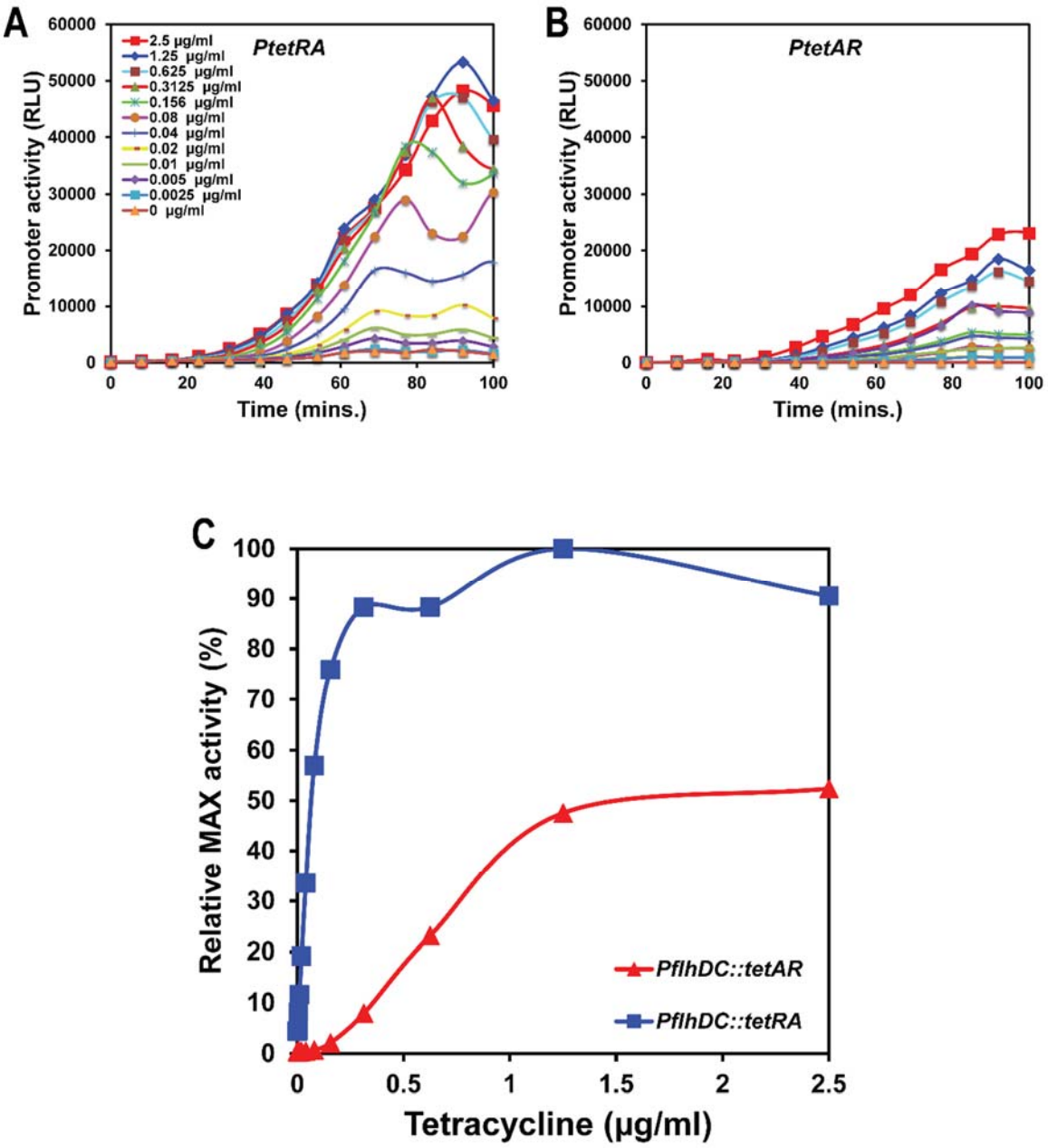


Figure 21. (A & B) Gradual influence of different concentrations of tetracycline for class III gene expression in both promoter P_{tetRA} and P_{tetAR} respectively. The magnitude of *flhDC* expression was affected directly proportional to the concentration of tetracycline. (C) Comparison the relative maximum activity between strains being driven by different promoters (P_{tetAR}, P_{tetRA}). The data further strengthens the argument that P_{tetRA} is fourfold stronger than P_{tetAR}. For tetracycline, a concentration of 0.3125 µg/ml has given the highest level of flagellar gene expression for P_{tetRA}, while, for P_{tetAR} (red line) maximal flagella gene expression has been reached at 1.25 µg/ml tetracycline. Experiment represents a minimal of three independent repeats. Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3967 and P_{flhDC}*::tetAR* =TPA TPA3803.

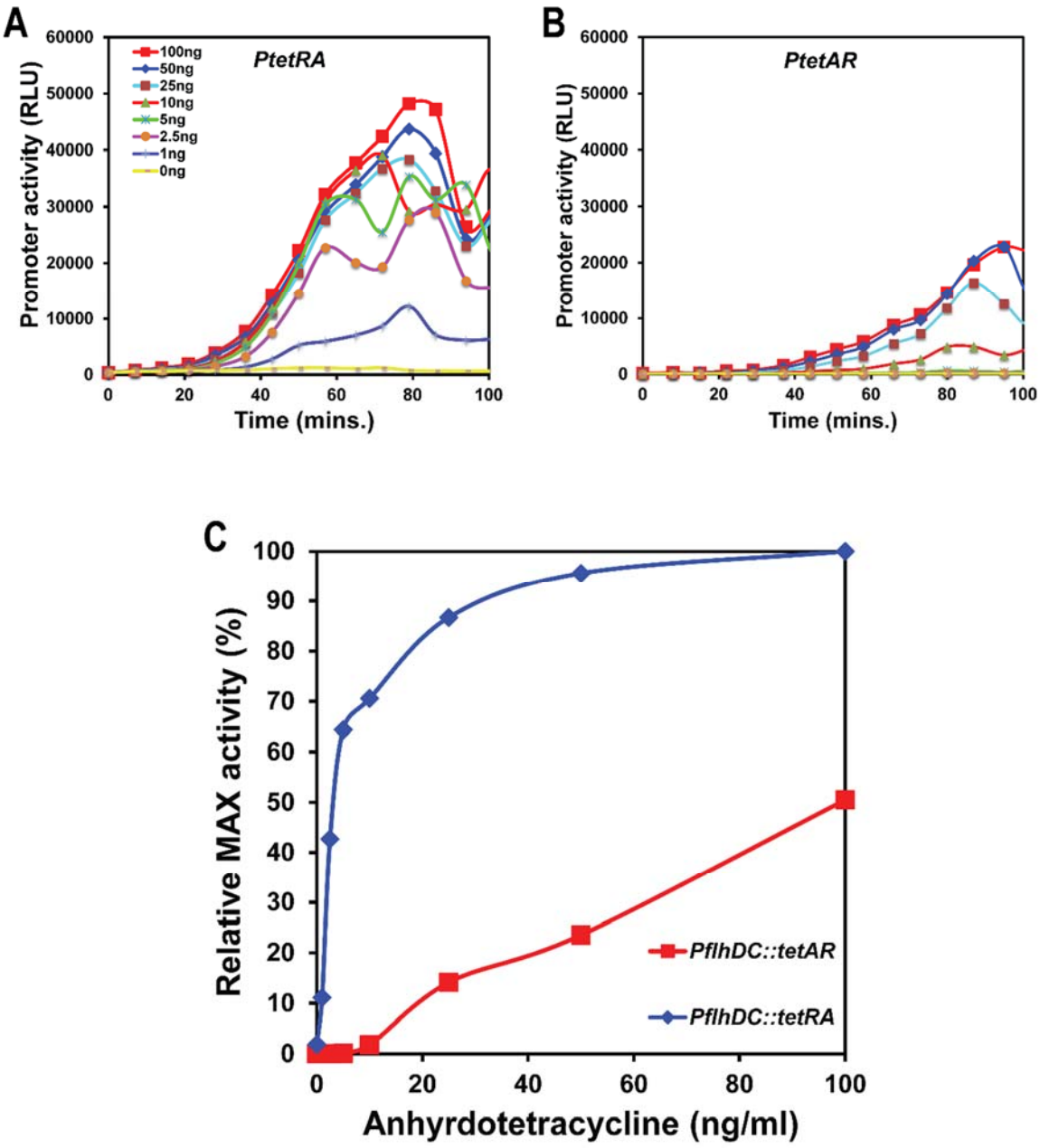


Figure 22. (A & B) Gradual influence of different concentrations of anhydrotetracycline for class III gene expression in both promoter P_{tetRA} and P_{tetAR} respectively. (C) Comparison the relative maximum activity between P_{tetRA} versus P_{tetAR}. The highest level of expression at for P_{tetRA} (blue line) was between 25-50 ng/ml anhydrotetracycline. In contrast, for P_{tetAR} (red line) maximum activity may not have been reached even with 100 ng/ml. Experiment represents a minimal of three independent repeats. Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3967 and P_{flhDC}*::tetAR* = TPA3803.

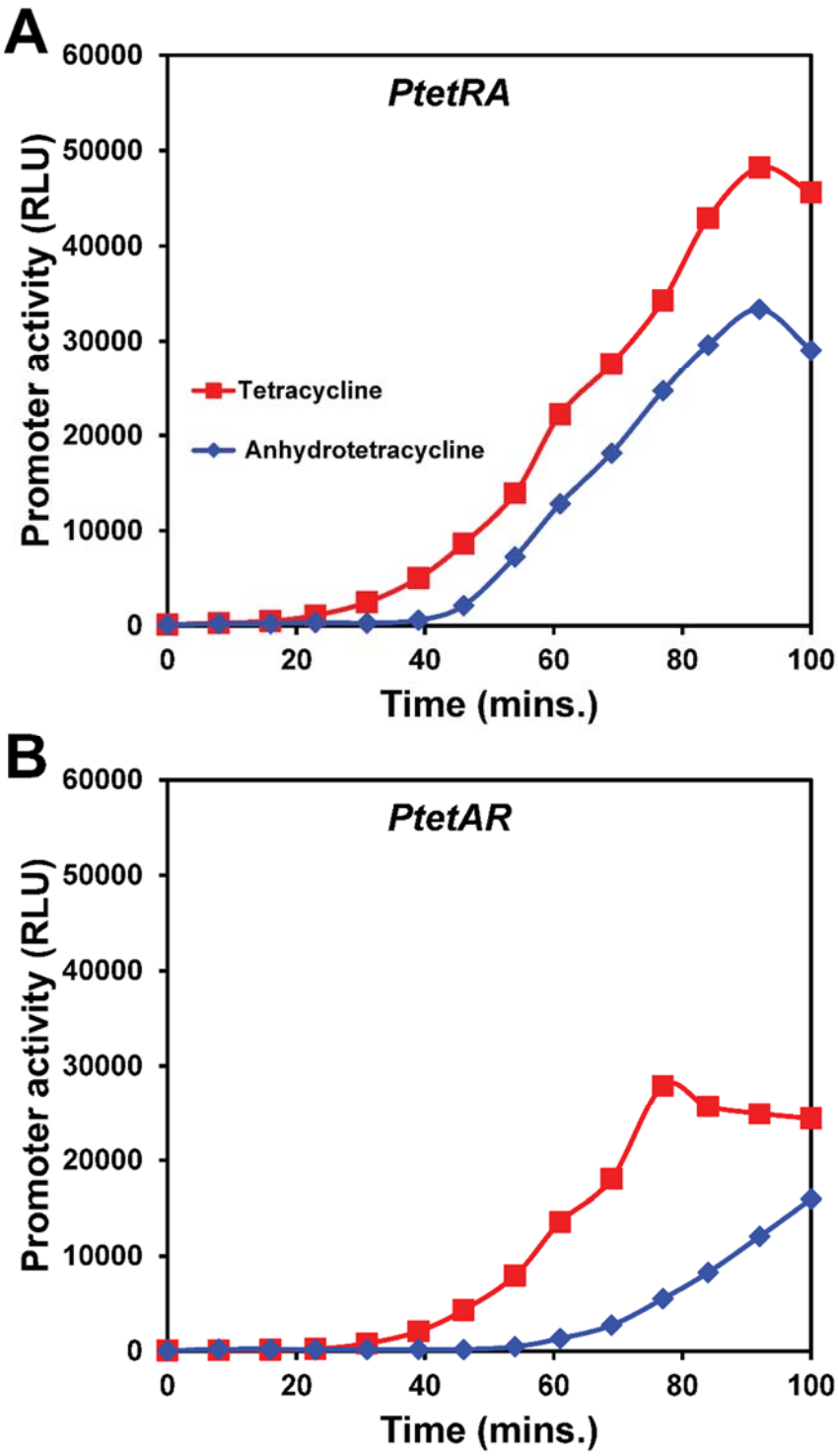


Figure 23. Efficiency comparison between tetracycline and anhydrotetracycline as inducer of flagellar gene expression where flhDc is expressed from either P_{tetRA} or P_{tetAR}. Tetracycline shows a more effective impact on flagellar gene expression than anhydrotetracycline in spite of promoter type. Experiment represents a minimal of three independent repeats. Strains used in this experiment where: P_{tetRA} = TPA3967 and P_{tetAR} = TPA3803.

4.2.4 Comparison Of Flagellar Foci Using Tetracycline And Anhydrotetracycline Induction Of The Flagellar System

To count flagellar numbers per cell previous studies have exploited a strategy based on a flagellar protein being tagged with green fluorescent protein (GFP) and examined under the fluorescent microscope (Aldridge *et al.*, 2006a). Aldridge *et al* (2006) focused on FliM-GFP fluorescent foci as a foundation of counting flagellar numbers per cell. The maximum activity of flagellar gene expression is during the late stages of exponential growth phase (Saini *et al.*, 2010). Flagellar numbers were determined by counting flagellar using MicrobeTracker program to automatically assess florescent foci (FliM-GFP) instead of manually counting (Sliusarenko *et al.*, 2011; Sim *et al.*, 2017). This section computes the number of flagellar foci for the two strains P_{tetRA}*::flhDC* and P_{tetAR}*::flhDC* induced with tetracycline and anhydrotetracycline.

The experiment used three concentrations of tetracycline (0.025, 0.25 and 2.5 µg/ml) in comparison for the two different promoters (figure 24). The flagellar foci average for P_{tetAR} was between 1 foci / cell for 0.025 µg/ml and 2 foci /cell for 2.5 µg/ml. While, the average of flagellar foci for P_{tetRA} was between 8-10 foci / cell at all concentrations. These data for tetracycline show that flagellar foci change relatively little with a gradual increase in tetracycline, regardless of which promoter was used. However, there is a significant difference in the average of flagellar foci when P_{tetRA} is compared to P_{tetAR}. This can be explained by P_{tetRA} being stronger than P_{tetAR}. In the other words, there was a direct correlation between the strength of the tetracycline system and flagellar foci per cell. However, this does not correlate to motility and flagellar gene expression.

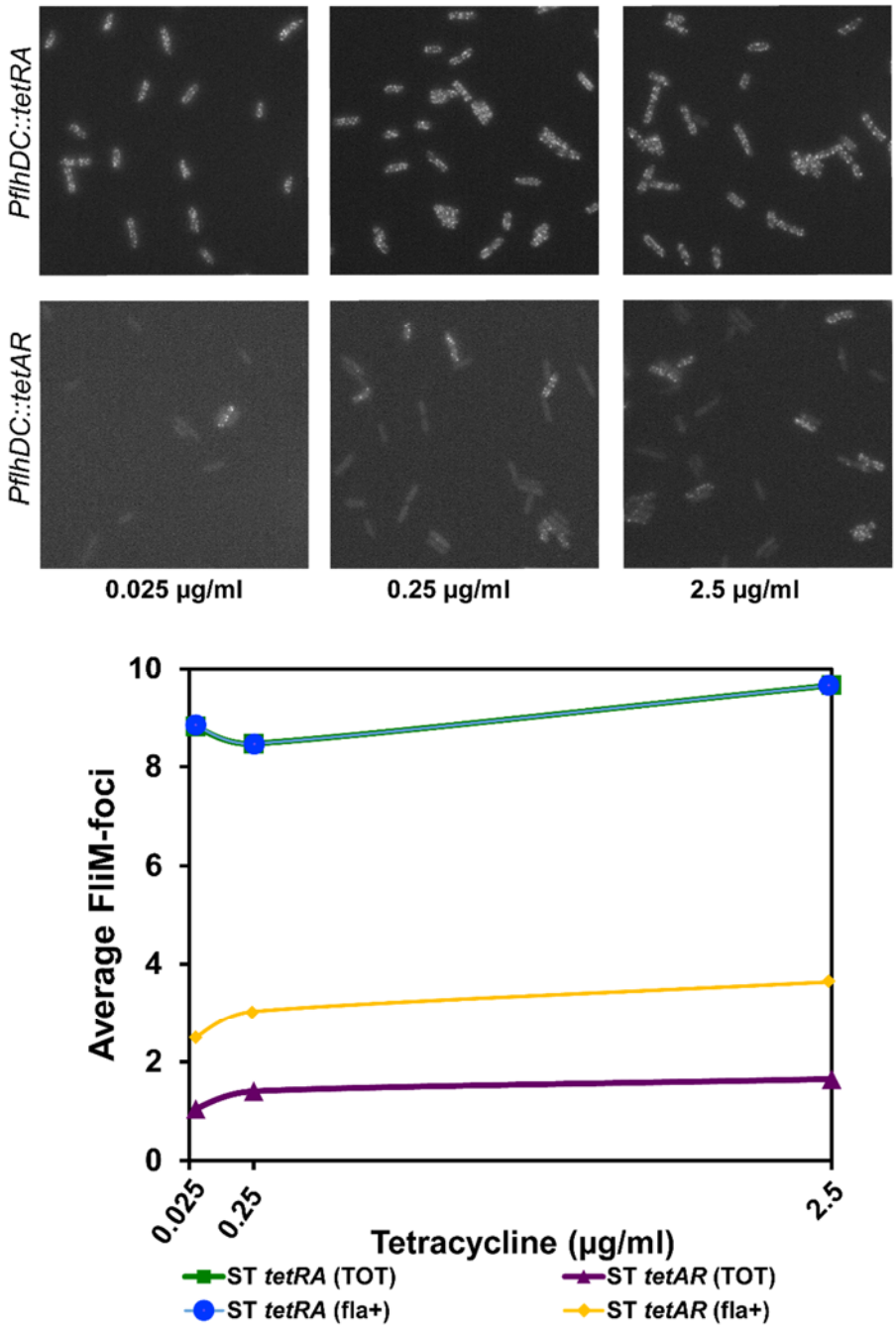


Figure 24. The impact on flagella numbers using different concentrations of tetracycline for both P_{tetRA} and P_{tetAR} driven systems. The curves indicate the average flagellar foci per cell. Blue line: Average foci for P_{tetRA} when considering only cells with foci (fla+) (8-10 flagellar foci); Green line: average foci per cell using the total population; Yellow line: fla+ average foci for the P_{tetAR} strain; Purple line: Average foci per cell when consdieirng the total population for the P_{tetAR} driven system. Note that for P_{tetAR} the heterogeneity in the population impacts the average foci per cell, while for P_{tetRA} the majority of cells possess at least one foci. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3959 and P_{flhDC}*::tetAR* =TPA3789.

With respect to anhydrotetracycline induction, the flagellar foci per cell average was again between 7-10 foci/cell for 5, 10 and 25 ng/ml in the case of P_{tetRA}. However, the average of flagellar decreased radically in P_{tetAR}. This time, however, the increase was gradual from 1 foci/cell at 5 ng/ml to 3 foci/cell for 25 ng/ml (figure 25).

Interestingly, the calculated averages took in to consideration the total population. When flagellar foci were quantified only in fla+ cells for P_{tetAR} strains it was clear that for tetracycline and anhydrotetracycline induction flagellar foci per cell was between 1 and 4 foci per cell, which reflected the images captured for the analysis (Figures 24 and 25). This therefore suggests that the sensitivity of transcriptional changes via P_{tetAR} on *flhDC* expression using these two inducers can influence the output of the flagellar system.

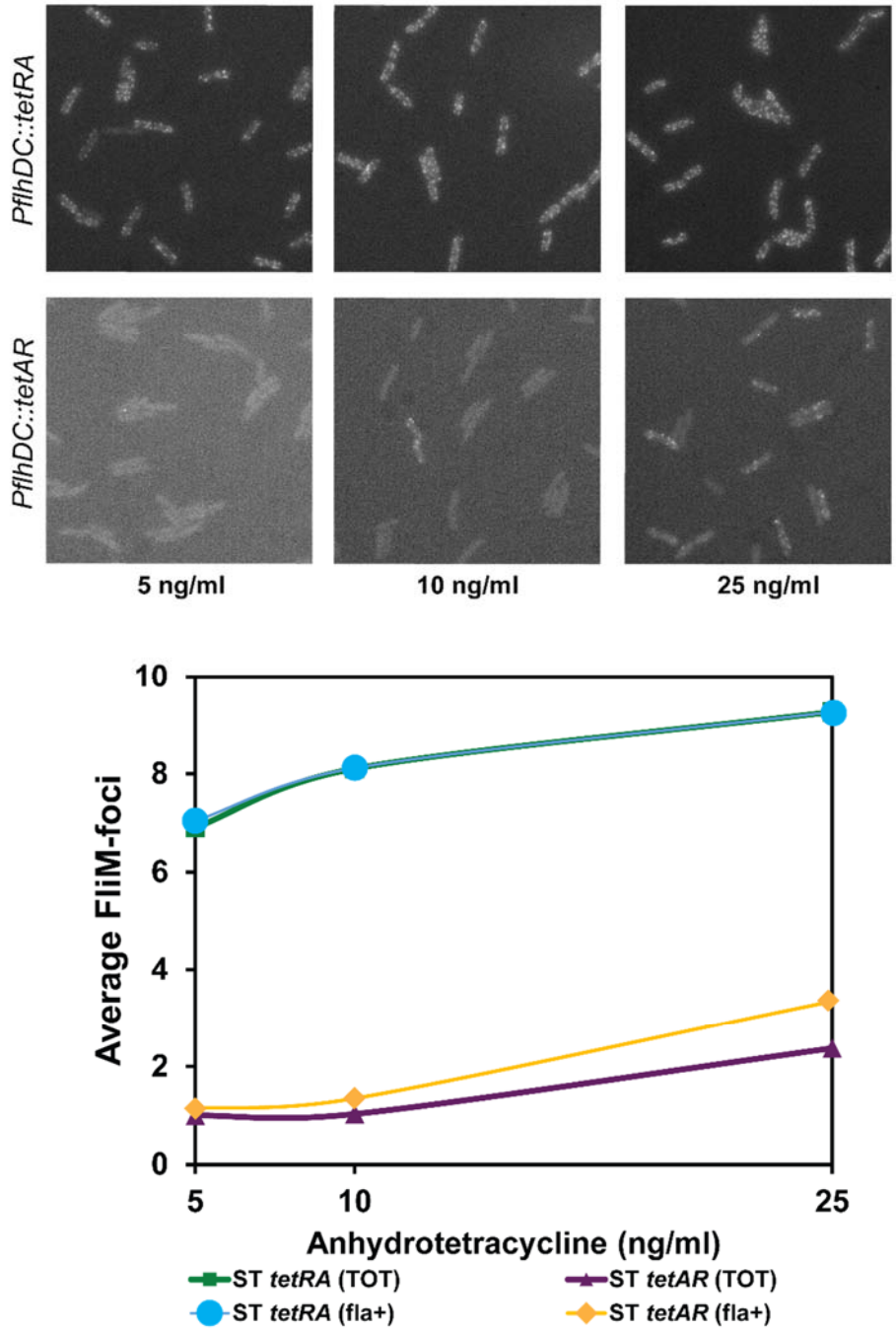


Figure 25. The impact on flagella numbers in terms different concentration of anhydrotetracycline by using both promoters *tetRA* and *tetAR*. The curves indicate the average flagellar foci per cell. Blue line: Average foci for P_{tetRA} when considering only cells with foci (fla+) (8-10 flagellar foci); Green line: average foci per cell using the total population; Yellow line: fla+ average foci for the P_{tetAR} strain; Purple line: Average foci per cell when consdieirng the total population for the P_{tetAR} driven system. Like for tetracylince induction the impact of P_{tetAR} expression is eveident in average foci per cell counts when comparing the total population to just fla+ cells. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment where: P_{flhDC}*::tetRA* = TPA3959 and P_{flhDC}*::tetAR* =TPA3789.

4.3 Determination Of Flagellar Gene Expression In *Salmonella enterica* Serovars Identifies Species Wide Differences In Flagellar Gene Regulation

The biosynthesis and function of the flagellar system demand the expression of almost 60 genes that are organised across 17 operons, which provides a framework for regulation of flagellar assembly (Macnab, 1996). The regulation of these genes is coordinated by different responses such as environmental signals. Even strains within the same species may have special cues which eventually contribute to the extent of flagellar assembly and movement. We wanted to inspect flagellar gene regulation across serovars to define comprehensively the motility phenotype and flagellar gene expression.

4.3.1 Comparing Motility Phenotype across Salmonella serovars

The motility assay is always the initial step used to appreciate motility phenotypes. Several studies have compared the swimming motility phenotypes for strains of specific *S. enterica* serovars. For example, they have considered bacterial movement with strains of serovar Typhimurium based on flagellin (FliC) classification (Martins *et al.*, 2013; Bogomolnaya *et al.*, 2014). A subset of *S. enterica* serovars were quantified for motility using semisolid agar 0.3% in comparison to serovar Typhimurium strains (figure 26).

We first looked at the *S. enterica* serovars under control of the native flagellar promoter P_{flhDC}. Typhimurium strain LT2 was considered the control which all others were compared to. The Typhimurium strains 14028s, SJW1103, Typhimurium and ST4/74 had a similar motility phenotype in comparison with LT2. However, the motility of SL1344 was dramatically decreased ($P<0.001$, figure 26). In contrast, the movement of Java was also reduced compared to the control ($P<0.001$, figure 26).

In terms of P_{tetAR} control, 14028s, SJW1103, SL1344, ST4/74, Typhimurium and Javiana all had a statistically significant increase compared to LT2 ($P<0.05$, figure 27). However, Berta and Zanzibar were comparable to LT2 ($P=0.14$). Interestingly, Java had a consistent response for P_{tetAR} compared to P_{flhDC} driven expression, showing a reduced swim compared to LT2-P_{tetAR} ($P<0.01$, figure 27).

This investigation showed the diversity and robustness of the movement phenotype for each serovar. Interestingly, SL1344 strain behaved differently from other serovars especially with respect to P_{tetAR}. The movement of SL1344 was clearly increased compared to the native promoter. In general, even though these strains were motile, the main interest was to ask how do these strains behave with respect to changes in flagellar gene expression and the temporal activation of their flagellar system (section 4.4).

4.3.2 Flagellar Gene Expression Levels Between Salmonella Serovars

Flagellar gene expression in *Salmonella* has been frequently monitored via the activation and magnitude of the flagellar promoter activities in Typhimurium (Brown *et al.*, 2008). Therefore, the nine strains/serovars were tested in comparison to *Salmonella* Typhimurium strain LT2. The strains were investigated for the activity of the class 2 (*flgA)* and class 3 (*fliC*) flagellar promoters. This would lead to an understanding of the kinetics of flagellar assembly according to the chronology and magnitude of flagellar gene expression. A micro-plate assay was used for detection of P_{fliC} and P_{flgA} activity via the expression of the *luxABCDE* operon that leads to bioluminescence (Goodier and Ahmer, 2001; Brown *et al.*, 2008) (figure 28). The orginal source of the reporter promoters was the strains 14028s, as previously mentioned (Goodier and Ahmer, 2001). Phylogentic analysis (see section 4.x) argues that this will provide a robust output as the flagellar promoters show a significant level

of identity. P_{flgA} expression comes on at 30 min (figure 28A), while P_{fliC} expression is detected at 60 min (figure 28B). This implies that the timing of *flgA* and *fliC* gene expression does not change. This data particularly correlates to *Salmonella* strains used previously (Brown *et al.*, 2008). However, the magnitude of *flgA* and *fliC* gene expression compared to the control varies across all strains tested (figure 28). For example, the expression of P_{flgA} for SWJ1103 was at an intermediate value of 25000 RLU compared to P_{fliC} that possessed the highest activity (40000 RLU) of all strains tested (figure 28). Conversely, the control LT2 strain possessed high expression of P_{flgA} and intermediate P_{fliC} activity. Berta and Zanzibar also behaved differently exhibiting the same level P_{fliC} activity but greater variation for P_{flgA} . Interestingly, Java exhibited a reciprocal and significant reduction in the magnitude of flagellar gene expression even though it is motile (figure 26 and 27).

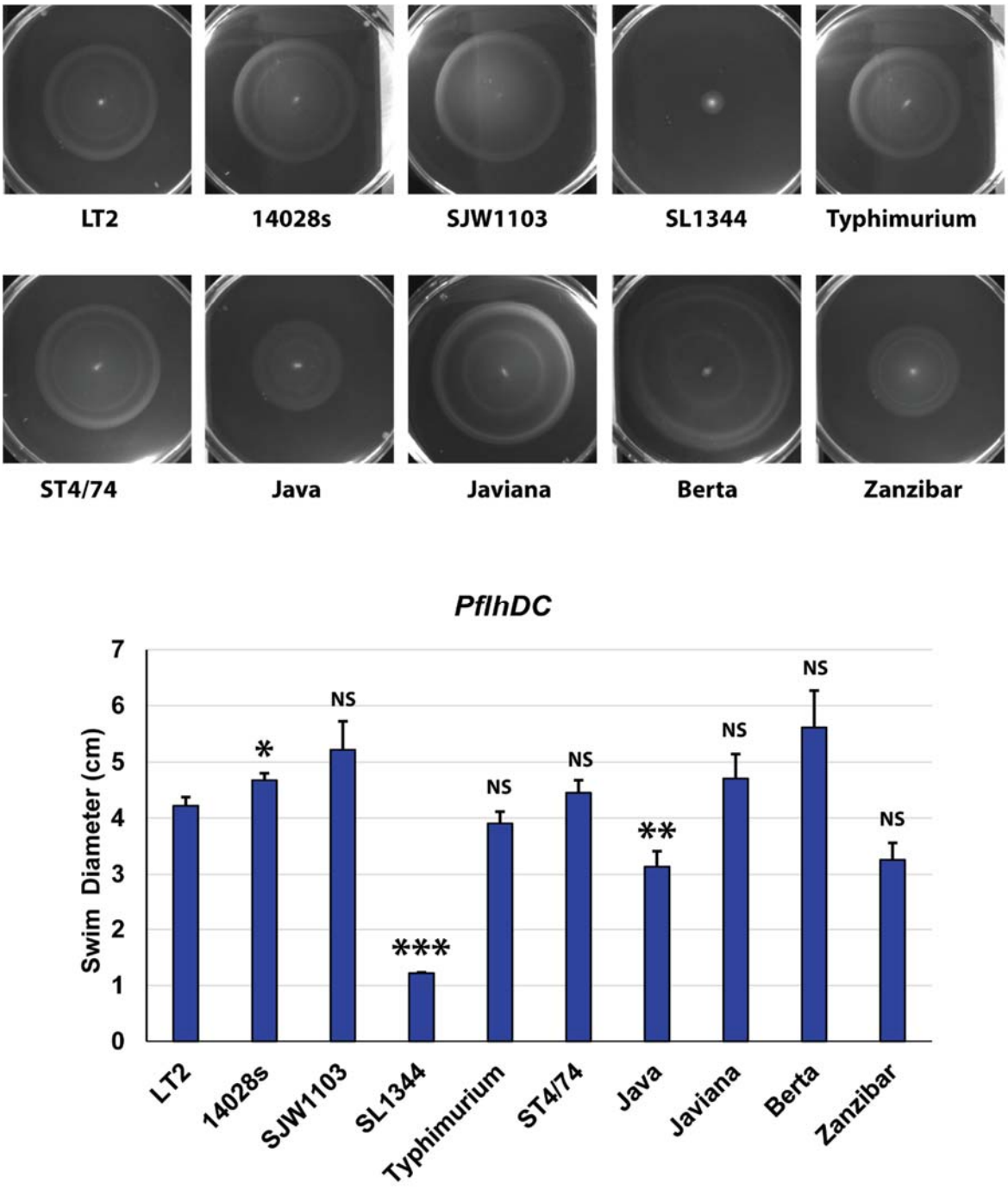


Figure 26. The quantification of motility among *Salmonella* serovars under control of P_{flhDC}. The motility phenotype behaved similar in SJW1103, Typhimurium, ST4/74, Javiana and Berta compared to LT2. For SL1344 the movement was dramatically decreased. In contrast, Java and Zanzibar were slightly reduced. Quantification based on three repeats. * = P<0.05; ** = P<0.01; *** = P<0.001; NS = Not significant (P>0.05). Strains used in this experiment where: LT2 = TPA1, 14028s = TPA277, SJW1103 = TPA788, Typhimurium = TPA2735, ST4/74 = TPA3690, Java = TPA2734, Javiana = TPA2739, Berta = TPA2740 and Zanzibar = TPA2741.

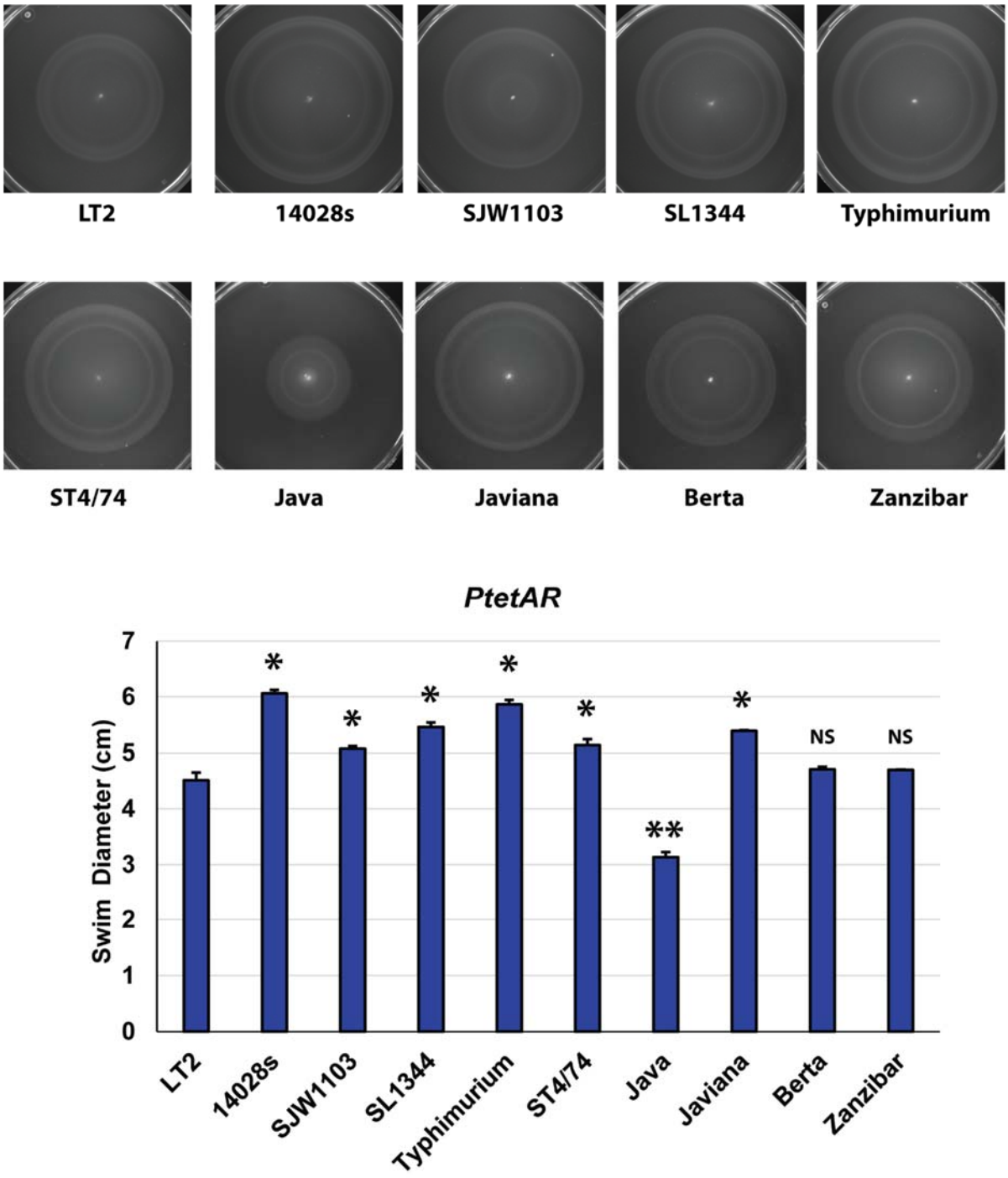


Figure 27. The quantification of motility among *Salmonella* serovars under control of P_{tetAR}. Significant changes compared to LT2 are shown using statistical annotation. Quantification is based on three repeats. * = P<0.05; ** = P<0.01; NS = Not significant (P>0.05). Strains used in this experiment where: LT2 = TPA3789, 14028s = TPA3790, SJW1103 = TPA3791, Typhimurium = TPA3794, ST4/74 = TPA3798, Java = TPA3793, Javiana = TPA3795, Berta = TPA3796 and Zanzibar = TPA3797.

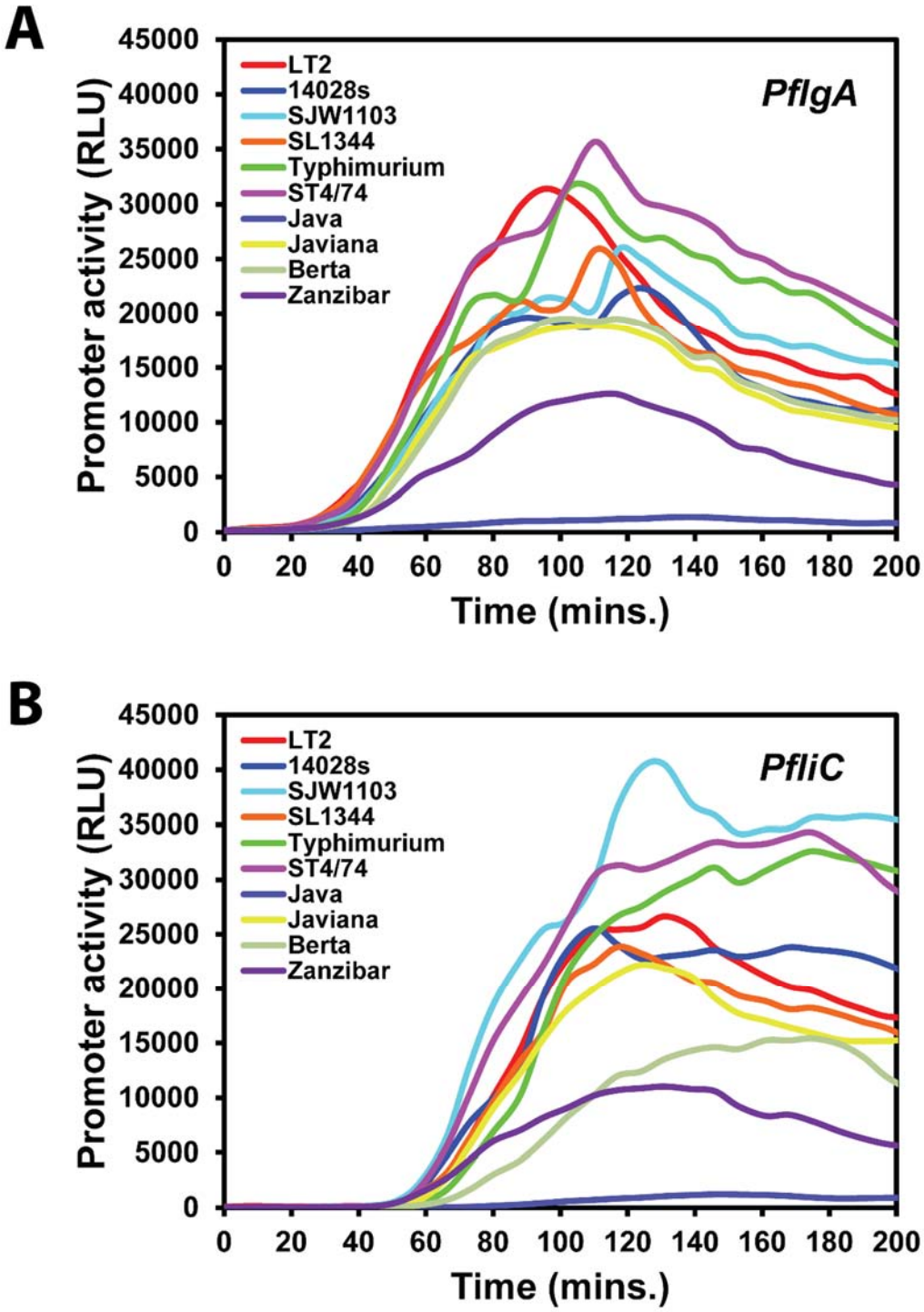


Figure 28. Comparison of flagellar gene expression (P_{flgA}, P_{fliC}) across of *Salmonella* serovars. A positive response with respect to levels of expression was showed for all serovars compared to LT2 except Java. Interestingly, Java has very low gene expression, but is still motile. In contrast, for flagellar gene expression in all other serovars P_{flgA} and P_{fliC} were expressed at 30, 60 mins respectively. The chronological expression was not changed when compared to Brown *et al* (2008). Experiment represents a minimal of three independent repeats (n=3). (A) pRG51 was transformed into the strains used in Figure 28 for P_{flgA} detection generating the following strains LT2 = TPA3804, 14028s = TPA3806, SJW1103 = TPA3810, Typhimurium = TPA3816, ST4/74 = TPA3819, Java = TPA3830, Javiana = TPA3833, Berta = TPA3836 and Zanzibar = TPA3839. (B): pRG38 was transformed into the above strains for P_{flhDC} detection.

4.4 Investigation With Other *Salmonella* Serovars Isolated From Around The World And From Different Organisms

4.4.1 Correlation Between Motility Phenotype and P_{flhDC} *Expression*

For this section, a wider range of *S.enterica* serovars, fifteen in total, were used in order to increase our appreciation of flagellar regulation and expression in *Salmonella* as a species compared to LT2, considered as the control. The serovars were examined for motility under control of the native *flhDC* promoter (figure 29A). All *S.enterica* serovars were motile (figure 29), except for Gallinarum which is a known non motile serovar (Hossain *et al.*, 2006). Gallinarum, therefore, acts as a negative control for this study. The average diameter of Lexington, Montevideo, Limete and Abony were not significantly different when compared to LT2 (figure 29). However, for Panama, Indiana and Vinohrady, the average diameter was greater than LT2. For all other serovars an obvious drop in motility was observed (figure 29A).

The transcription of P_{flhDC} was assessed using the plasmid pRG38 (*P_{flhDC}::luxCDABE)* in order to appreciate *flhDC* gene expression levels across the *Salmonella* serovars. The P_{flhDC} activity for Othmarschen, Emek, Alachua, Senftenberg, and Simsbury were between 10000-12000 RLU. However, Lexington, Panama, Indiana, Montevideo, Limete and Vinohrady were behaved like LT2. Abony and Vilvoorde were increased, although no statistical significance compared to LT2 was observed. Interestingly, Vilvoorde exhibited a similar phenotype to Haifa. Both serovars have reduced motility but comparable P_{flhDC} activity to LT2. Finally, Gallinarum did not express *flhDC*, consistent with the non-motile phenotype (figure 29B). This data provides evidence with respect to each *S. enterica* serovar and their capability to move with the magnitude of *flhDC* gene expression.

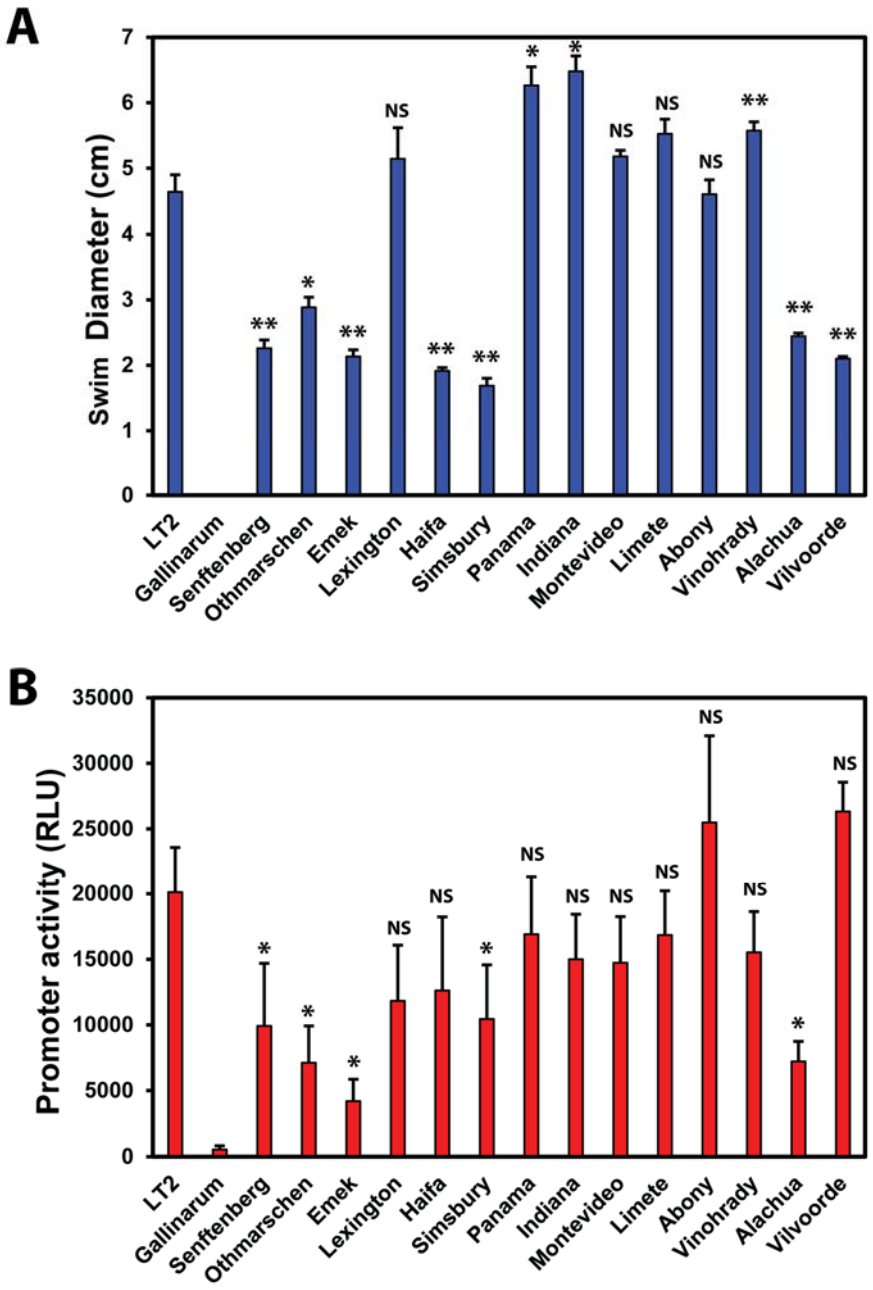


Figure 29. (A) The average swim diameter for *Salmonella* serovars under control of P_{flhDC} compared to LT2. (B) The magnitude of *flhDC* gene expression in the serovars tested. *flhDC* gene expression levels fluctuate between *Salmonella* serovars some such as Haifa and Vilvoorde exhibit normal *flhDC* expression but lower motility. Quantification based on three repeats. * = P<0.05; ** = P<0.01; NS = Not significant (P>0.05). Strains used in this experiment were, (A): LT2 = TPA1, Gallinarum = TPA4273, Senftenberg= TPA4272, Othmarschen = TPA4274, Emek = TPA4275, Lexington = TPA4276, Haifa = TPA4277, Simsbury = TPA4278, Panama = TPA4279, Indiana =TPA4280, Montevideo = TPA4281, Limete = TPA4284, Abony = TPA4285, Vinohrady = TPA4286, Alachua = TPA4287 and Vilvoorde = TPA4288. (B): pRG38 was transformed into the above strains for P_{flhDC} detection.

4.4.2 Impact of tetAR and tetRA Promoters On Motility Phenotype And Flagellar Gene Expression

The Fifteen *S.enterica* serovars were investigated further with respect to motility and gene expression under the control of P_{tetRA} and P_{tetAR}. For P_{tetAR}*::flhDC* expression, the motility pattern of the majority of the *Salmonella* serovars followed a similar pattern to P_{flhDC} analysis (figure 29 and 30). Two key differences were observed for Alachua and Vilvoorde that showed low motility for P_{flhDC} expression. However, for P_{flhDC}*::tetAR* motility swims were comparable to LT2 (figure 30A).

The activity of P_{flgA} and P_{fliC} showed consistent magnitudes across the majority of serovars. One difference was seen for Haifa and Emek that had increased P_{fliC} activity compared to their expression of P_{flgA} (figure 30B, C). This is consistent to the analysis of the original strains tested where P_{flgA} and P_{fliC} activity did not always correlate (figure 28). Interestingly, two phenotypes were observed with respect to P_{flgA} and P_{fliC} activity. Five serovars had activity similar to LT2 for P_{flgA} . In contrast, the rest had very low activity even though all strains showed robust motility phenotypes (figure 30). This suggests that flagellar gene expression and motility phenotype may not always correlate.

Using P_{tetRA}, with respect to motility, the swim diameter of all *S. enterica* serovars were altered producing a much more uniform response. One exception was Emek that had a comparable swim diameter of 6.5 cm to LT2 while all others were reduced. With respect to flagellar gene expression, the majority of serovars expressed flagellar genes stronger compared to P_{tetAR} data (figure 31). Senftenberg, Haifa and Montevideo were significantly increased between 45000-60000 RLU in particular compared to LT2. In contrast, Emek and Abony serovars were decreased which was unexpected especially when compared to the motility phenotype, P_{flhDC} and P_{tetAR} data (figures 29 to 31).

Taken together, it is clear there are cases that exist where little correlation between flagellar gene expression, swim diameter and responses to changes in *flhDC* expression amongst the serovars tested. This suggests potential differential regulation of the flagellar systems across serovars. A plausible working hypothesis that explains the observed changes is explored in chapter 5.

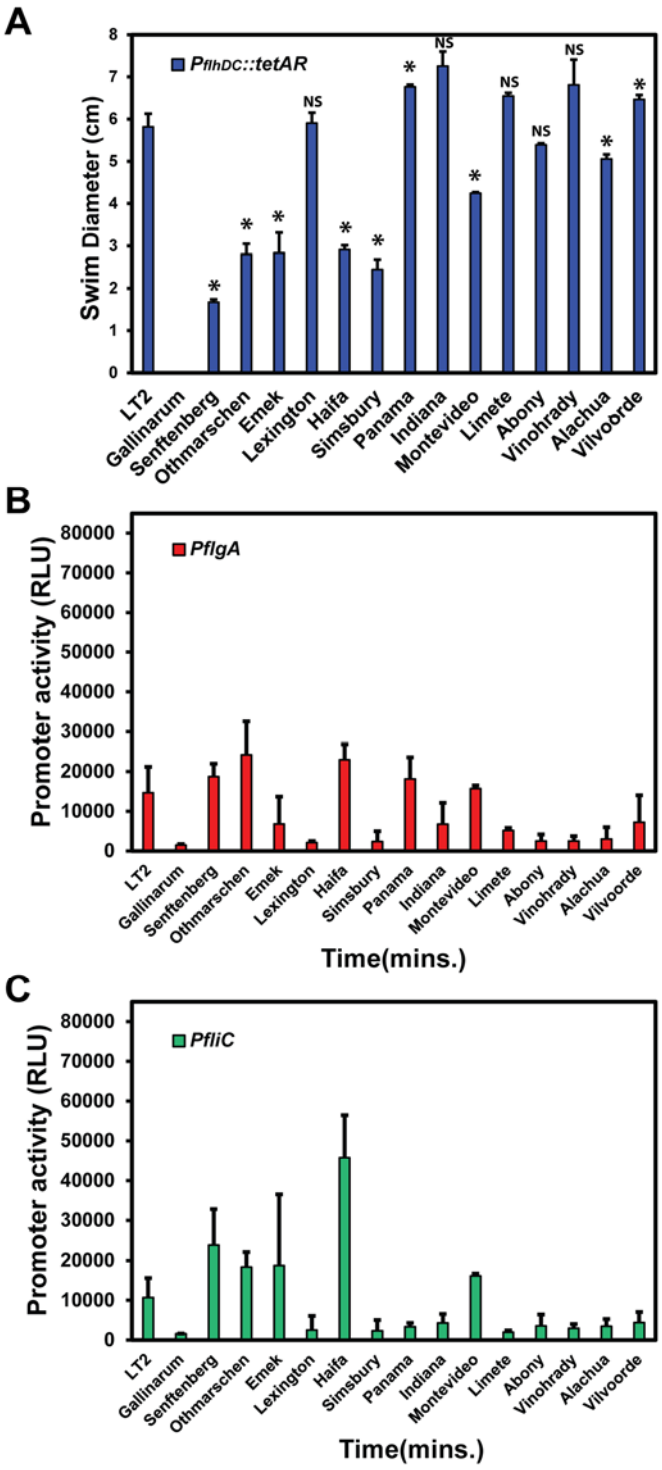


Figure 30. (A) Swimming motility analysed on 0.3% agar for *Salmonella* serovars under control of P_{tetAR}. Maximal P_{flgA} (B) and P_{fliC} (C) activity under control *tetAR* promoter. Quantification based on three repeats. * = P, 0.05; NS = Not significant (P>0.05). Strains used in this experiment were, (A): LT2 = TPA3789, Gallinarum = TPA4327, Senftenberg= TPA4326, Othmarschen = TPA4328, Emek = TPA4329, Lexington = TPA4330, Haifa = TPA4331, Simsbury = TPA4332, Panama = TPA4333, Indiana =TPA4334, Montevideo = TPA4335, Limete = TPA4336, Abony = TPA4337, Vinohrady = TPA4338, Alachua = TPA4339 and Vilvoorde = TPA4340. (B): pRG51 was transformed into the above strains and for P_{flgA} detection. (C): pRG39 was transformed into the above strains and for P_{fliC} detection.

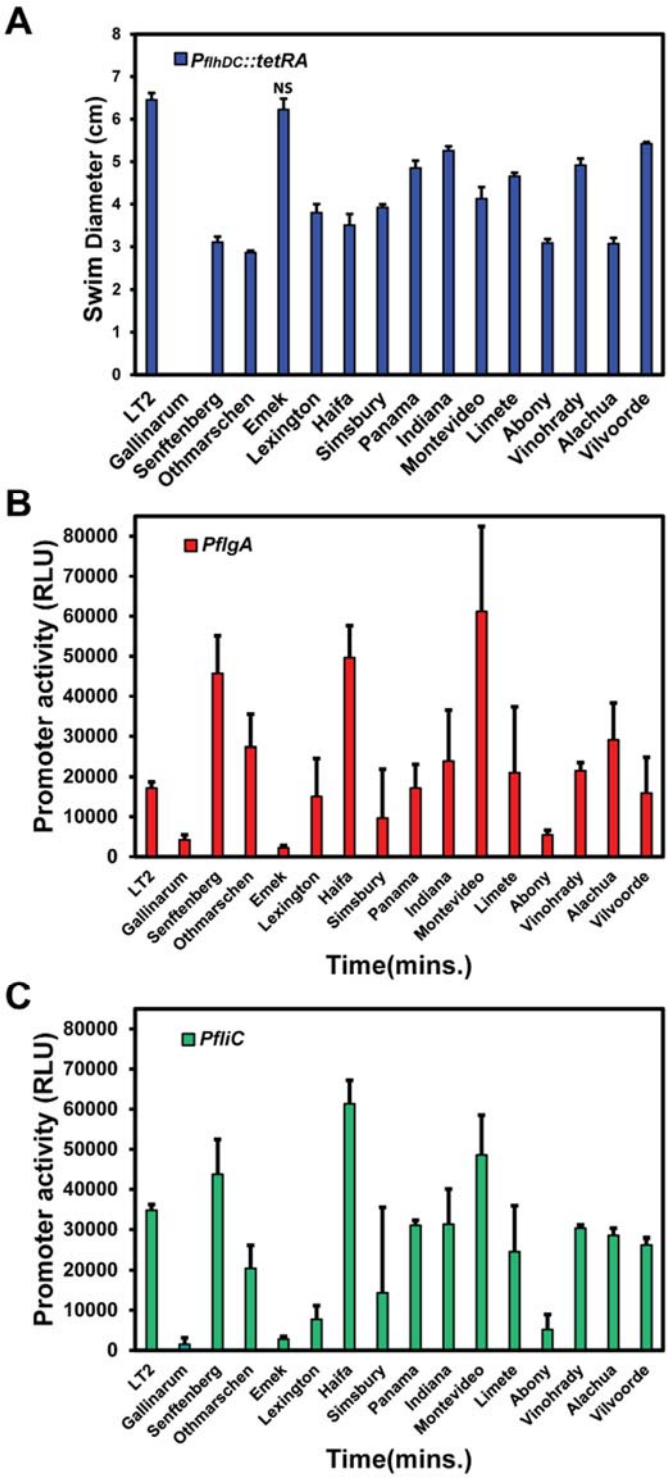


Figure 31. (A) Swimming motility analysed on 0.3% agar for *Salmonella* serovars under control of P_{tetRA}. Maximal P_{flgA} (B) and P_{fliC} (C) activity under control *tetRA* promoter. Quantification based on three repeats. NS = Not significant (P>0.05). Strains used in this experiment were, (A): LT2 = TPA3959, Gallinarum = TPA4342, Senftenberg= TPA4341, Othmarschen = TPA4343, Emek = TPA4344, Lexington = TPA4345, Haifa = TPA4346, Simsbury = TPA4347, Panama = TPA4348, Indiana =TPA4349, Montevideo = TPA4350, Limete = TPA4351, Abony = TPA4352, Vinohrady = TPA4353, Alachua = TPA4354 and Vilvoorde = TPA4355. (B): pRG51 was transformed into the above strains and for P_{flgA} detection. (C): pRG39 was transformed into the above strains and for P_{fliC} detection.

4.4.3 Activation Of Flagellar Gene Expression In Selected Serovars

Using a selection of serovars we find that dependent on the transcription of *flhDC* a diverse range of responses is observed when comparing motility, P_{flgA} and P_{fliC} activity. A key aspect of the flagellar system is its temporal activation. We were therefore interested in how different serovars respond to activation of the flagellar system. The observations to this point suggested very little change in timing but greater changes in the magnitude of gene expression would be detected.

Eight *S. enterica* serovars were chosen for analysis (figure 32). The expression of P_{flgA} and P_{fliC} activation were dramatically different in Emek, Java, Lexington and Abony (figure 32). This pattern was most obvious in Java when compared to the control LT2 (figure 32F). The activity of P_{flgA} and P_{fliC} were radically decreased even though this strain is still motile (figure 26 and 27). Meanwhile, flagellar gene expression for Indiana, Vinohrady and Alachua had profiles comparable to LT2 although clear differences were still evident (figure 32A to D). In the case of P_{tetAR} expression in Indiana, Vinohrady and Alachua all had lowered activity (figure 32). Overall, it is apparent from the results presented that the magnitude of P_{flgA} and P_{fliC} does not always correlate with the P_{tetAR} or P_{tetRA} driven activation. We have noticed for some serovars motility is comparable to LT2, but illogically they were not expressing flagellar gene expression consistently with respect to their motile phenotype. For example, Emek, Lexington, Java and Abony consistently exhibit low expression. Another example is Indiana that P_{tetAR} dynamics suggests correct timing but lower activity of P_{flgA} / P_{flic}. In contrast motility of Indiana is robust across all strains.

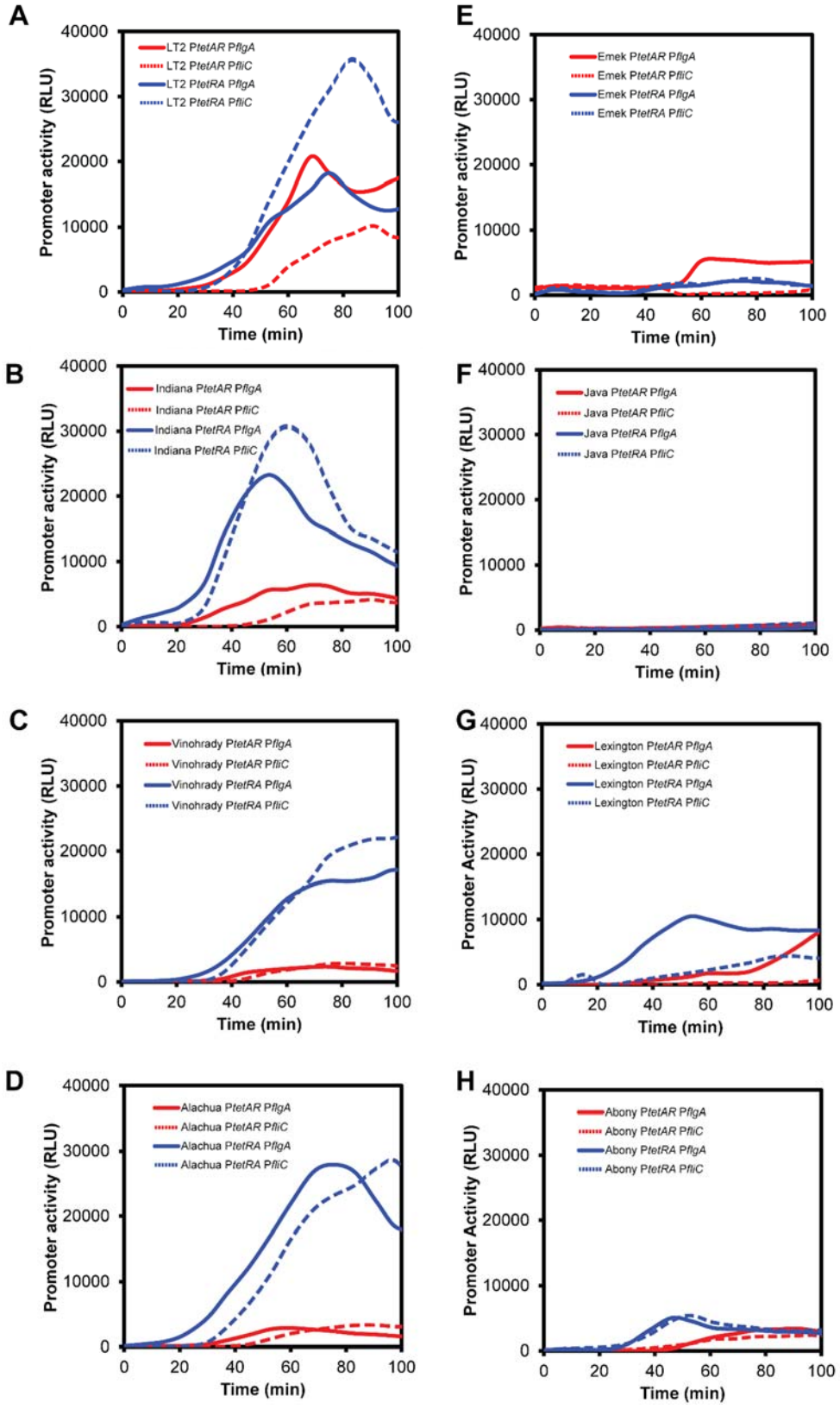


Figure 32. Flagellar genes expression (P_{flgA} , P_{fliC}) profiles of *Salmonella* serovars under control of P_{tetAR} and P_{tetRA}. The promoter activity for Emek, Lexington, Java and Abony were lower compared to LT2. While, Indiana, Vinohrady and Alachua expressed flagellar genes comparable to LT2 in terms of P_{tetRA} but for P_{tetAR} they were all exhibited reduced activity. Data represents the average activity of a minimal of 3 independent repeats. Strains used are those generated for figures 31 and 32.

4.5 Phylogenetic tree among *Salmonella* serovars based on flagellar promoters

The genus *Salmonella* has two species *S. enterica* and *S. bongori*. However, *S. enterica* is known to be a complex species which can be phylogenetically organized into 3 clades A1, A2 and B. In order to understand the basis of *Salmonella* evolution and adaptation especially based on the observed response of the flagellar system across serovars of *S. enterica* we performed a phylogenetic analysis. We exploited recent developments in sequencing technologies to assist us in obtaining sequence data for examples of each serovar from the database Enterobase, except serovar Simsbury (https://enterobase.warwick.ac.uk/). The aim was to use sequenced data available to gain insight into genetic similarity rather than sequence all strains directly.

Timme *et al*. (2013) have reported and compared whole genome sequences between an extensive range of the *Salmonella* serovars (Timme *et al.*, 2013). The analysis for this section focused on the DNA sequences used to type *Salmonella* strains via multi-locus sequence typing (MLST) (Achtman *et al.*, 2012b), the $P_{flhDC,}$ P_{flgAB} and P_{fliC} regions. Our results suggest some serovars differ with respect to flagellar gene expression and motility phenotypes. The data argues for these differences to be based on transcription which requires DNA:Protein interactions. This means it is more logical to focus on DNA phylogenetics rather than look at protein sequences. Furthermore, MLST analysis focusses strictly on variation in DNA sequences of specific housekeeping genes.

MLST sequences were concatenated and used to generate a *S. enterica* phylogenetic tree (figure 33A). This tree shows that our choice of strains reflected the A and B clades of *S. enterica.* Comparing the MLST tree in terms of P_{flhDC} sequence analysis surprisingly differentiated the clade A / B structure better than MLST (figure

33B). Using Timme *et al* (2013) as a foundation to declare clade members P_{flhDC} analysis identified four serovars from A1 and clade B respectively, compared to 10 serovars in clade A2. However, MLST analysis only split clade B from A. A matrix analysis of the two trees showed that the sequence variation between serovars was between 98 and 100% identity. This was irrespective of the P_{flhDC} DNA fragment being 836 bp long compared to a concatenated MLST sequence of 3336 bp long.

Mouslim and Hughes have shown that transcription of P_{flhDC} in *S. enterica* strain LT2 has 6 putative -10 regions based on primer extension mapping or RNAseq analysis (Mouslim and Hughes, 2014). This study has further shown that of the six -10 regions only that of P1 and P5 are the major contirbutors to P_{flhDC} transcription. Analysis of the phylogenetic data identifies the majority of changes that influence the phylogeny of P_{flhDC} are within the vicinity of P5 or sit within the LrhA binding site where P3 and P6 are located (figure 34). Interestingly, all of the identified variation are single base changes, for example 9 serovars have a 'G' instead of an 'A' at base -117 from the *flhD* ATG. However, based on the phylogeny and data presented in figures 30 to 33 these base changes do not correlate with a specific response of the flagellar systems that can be explained by a change in *flhDC* transcription.

In contrast, P_{flgAB} sequence analysis created a tree where most serovars are organized into two main groups exhibiting 100% identity irrespective of the clade source (figure 35). The P_{flgAB} region is 162 base pairs long and identity analysis showed greater conservation across serovars between 99 and 100% identity. The significant grouping of Alachua to Zanzibar, for example, differs from the rest by a T to C change at position 109 in the sequence analysed. This base change sits in the spacer region between the -35 and -10 regions of the P_{flgA} promoter used for gene expression studies. In comparison to dynamic data the low activity serovars: Emek, Java, Lexington and Abony did not cluster phylogenetically for P_{flgAB}. Interestingly,

these four serovars do exhibit some relationship when considering the MLST tree (figure 33A). However, P_{flhDC} analysis splits these four away from each other (figure 33B). Importantly, even though there is one base change that leads to the P_{flgAB} phylogeny the $FlhD_4C_2$ binding site is strictly conserved across the serovars, argueing that irrespective of the source of the P_{flgA} promoter binding will be identical. This argues that variation in activity is the result of upstream regulation via either *flhDC* transcription, translation or post-translational regulation of $FlhD_4C_2$ activity.

In P_{fliC} sequence analysis, a similar trend as seen for P_{flgAB} was observed (figure 36). The majority of the *Salmonella* serovars grouped in two 100% identical groups. These groupings, however, did not strictly correlate to P_{flgAB} which may reflect the ability of *S. enterica* to vary *fliC* sequences due to immune pressure in the host. Interestingly, for P_{flgAB} clade B serovars Panama, Montevideo and Othmarschen did cluster but for P_{fliC} analysis these three integrated across the other groups (figure 36). Further analysis showed that the class III promoter and the *fliC* untranslated region were strictly conserved across all serovars and the phylogenetic architecture is driven by single nucleotide polymorphisms upstream of the promoter region (data not shown). These changes however could potentially impact *fliD* transcription as they all sit within a region where the P_{fliD} class II and class III promoters would be.

By investigation the phylogeny of P_{flhDC}, P_{flgAB} and P_{fliC}, we recognized that the master regulator of flagellar systems produced a triple grouping that reflected the clade structure (A1, A2 and B2). However, it was very difficult to correlate the expression and motility data to specific clade groupings. The closest evidence for a relationship being the possible MLST cluster of the four low activity serovars Emek, Java, Lexington and Abony.

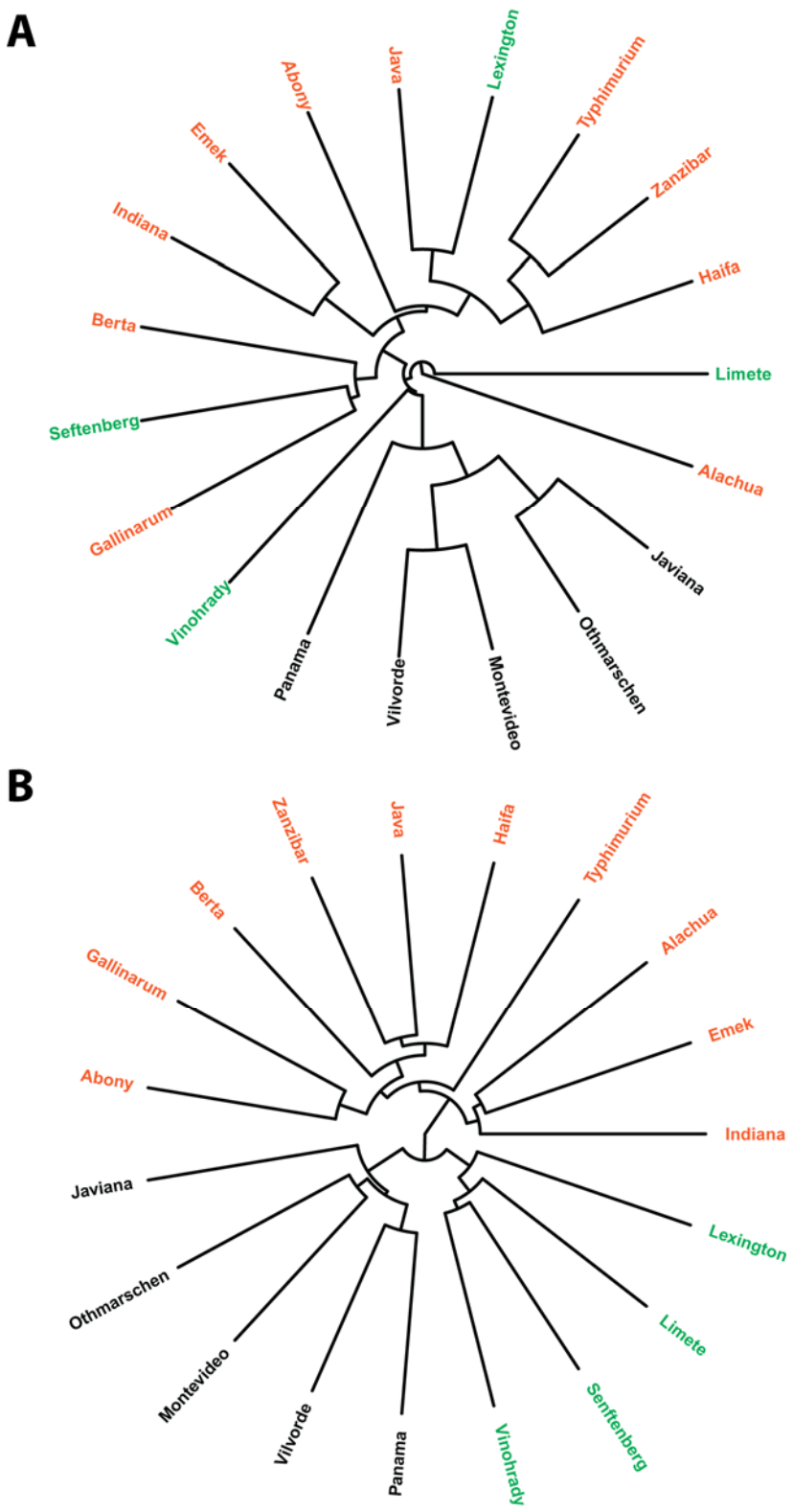


Figure 33. Phylogenetic comparison of MLST (A) sequences and P_{flhDC} (B) from serovars used in this chapter. The colouring of the serovar names is based on the clade foundation declared by Timme *et al* (2013). Red: Clade A2; Green: Clade A1; and Black: Clade B.

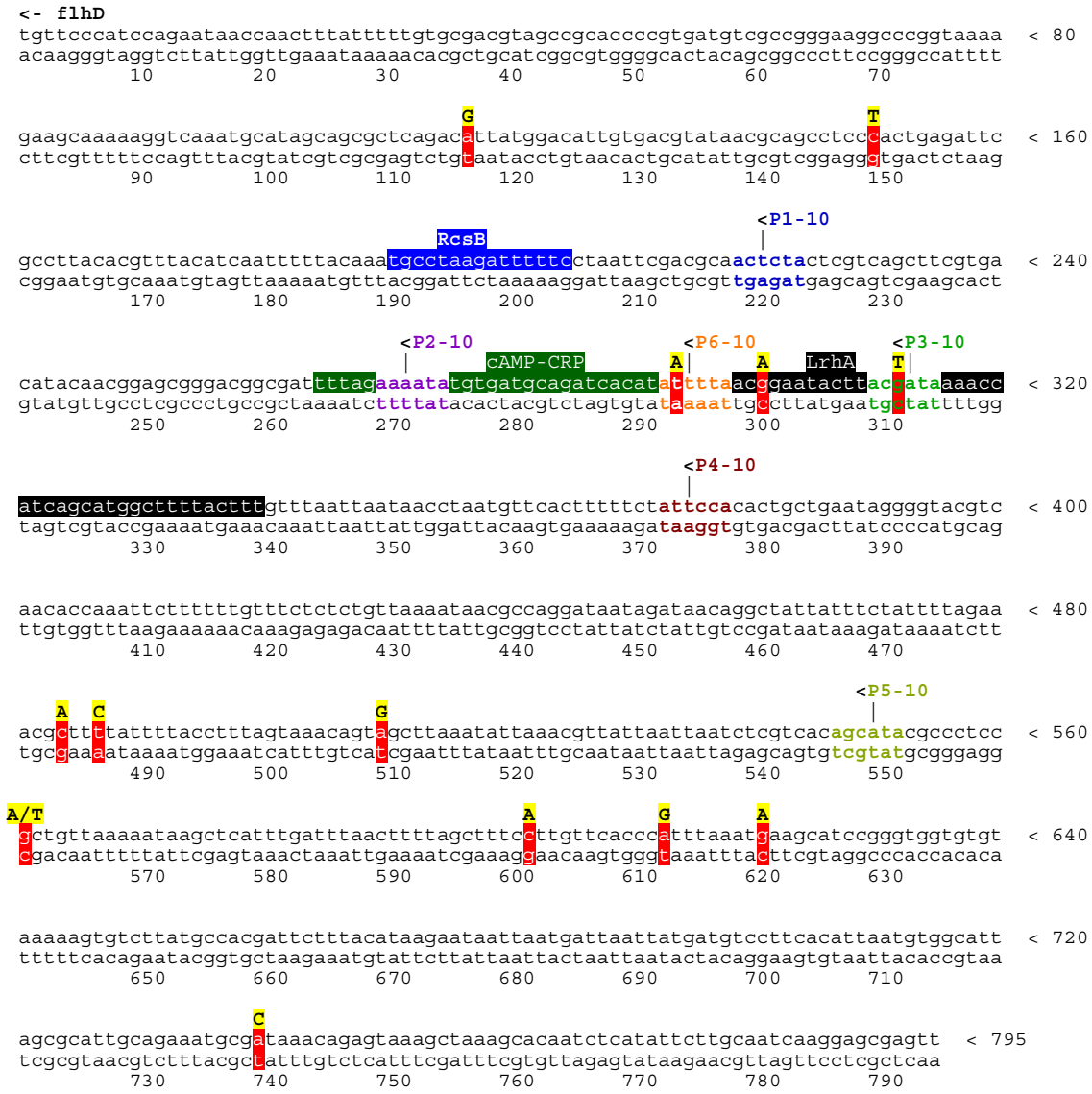


Figure 34. *S. enterica* P_{flhDC} region of serovar Typhimurium strain LT2. Sequence is annotated based on the promoter -10 regions defined by Mouslim and Hughes (2014) Overlaid on the sequence is then the bases that showed variation across the serovars used in Figure 33B. The majority of changes are upstream of the second key promoter P5. Three changes sit across P6 and P3 and it cannot be ruled out that these changes may impact the utilisation of these promoters or the binding affinity of the regulatory LrhA. Importantly the regions surrounding P1 and P5 are strictly conserved across all serovars. The binding sites for the regulators RcsB, cAMP-CRP and LrhA are shown as highlighted text in the upper strand.

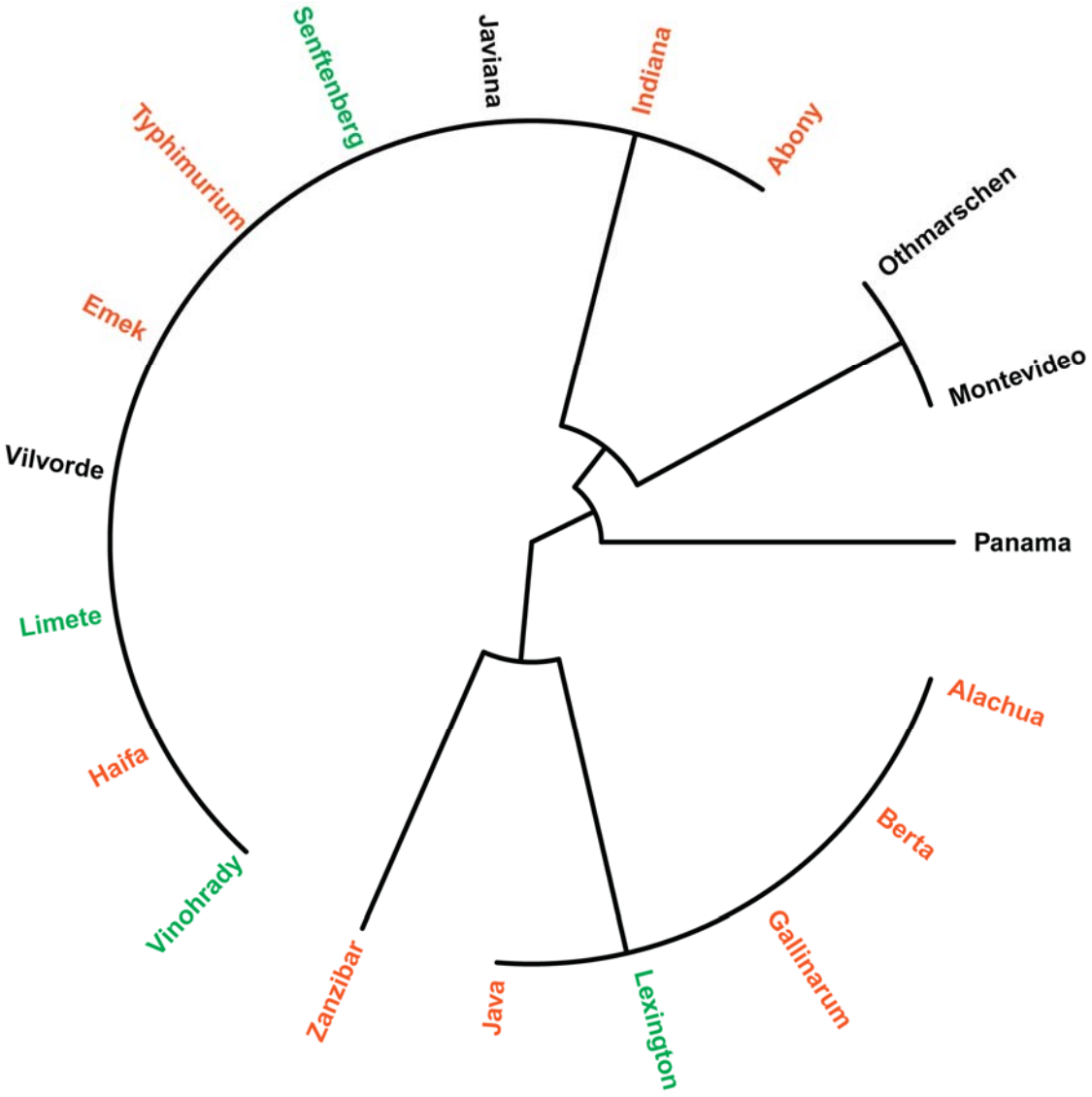


Figure 35. Phylogenetic tree designed from P_{flgAB} sequences of the derived from genomic DNA sequences from examples of the serovars used in this chapter. The colouring of the serovar names is based on the clade foundation declared by Timme *et al* (2013). Red: Clade A2; Green: Clade A1; and Black: Clade B.

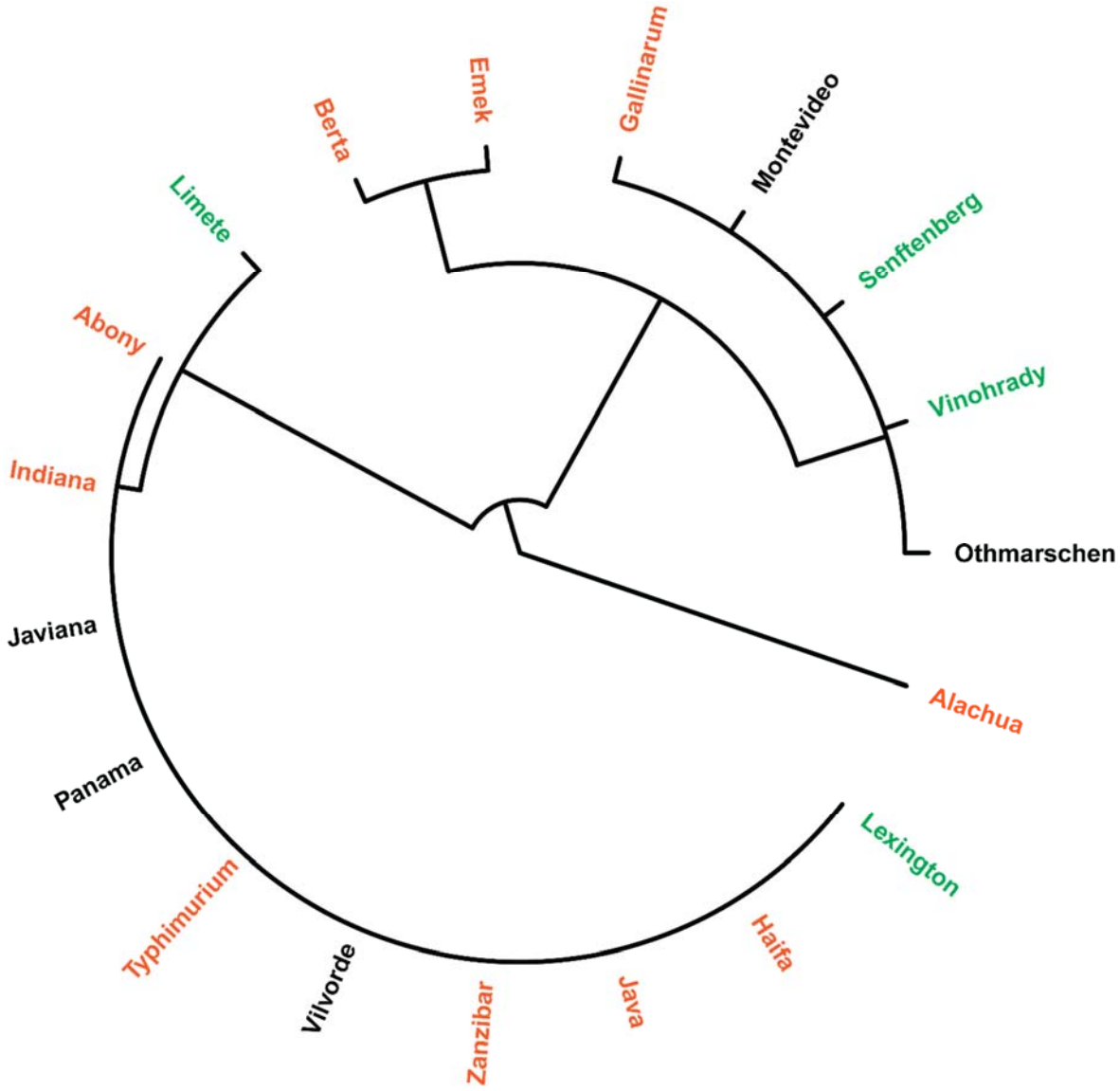


Figure 36. Phylogenetic tree designed from P_{fliC} sequences of the derived from genomic DNA sequences from examples of the serovars used in this chapter. The colouring of the serovar names is based on the clade foundation declared by Timme *et al* (2013). Red: Clade A2; Green: Clade A1; and Black: Clade B.

4.6 Summary

This chapter has revealed the robustness of the inducible tetracycline system and its impact on flagellar gene expression and functional output as motility. Focussing on anhydrotetracycline and tetracycline activity, tetracycline was more efficient as an inducer. Different experiments were performed in order to emphasis the similarity in antibiotic induction. The data suggested that although both P_{tetRA} and P_{tetRA} could drive motility when care was taken to drive *flhDC* expression a difference between expression, motility and flagellar foci was observed.

Further investigation of this chapter focused on the activation of flagellar gene expression in different *Salmonella* serovars. The data has provided a real visibility about the variation of the magnitude in flagellar gene expression across serovars. Furthermore, like careful expression of *flhDC* using the tetracycline system, we could identify serovars where the motility phenotype did not always correlate with flagellar gene expression. The majority of *Salmonella* serovars behaved similarly with respect to motility output. Unexpected results, for example, for Java could also not be explained via a phylogenetic analysis of MLST data versus P_{flhDC}, P_{flgAB} and P_{fliC} evolution. However, the phylogenetic analysis did suggest that the key source of serovar variation would poetnitally be changes to the DNA sequence within the P_{flhDC} region. This analysis identified 3 base changes thatdictated the phylogeny structure that sat within the biding site of the regulator LrhA and overlapped two minor promoter -10 regions (P3 and P6). Importantly the data suggests the two major promoters of P1 and P5 are conserved across all serovars. This all suggests that a different level of flagellar regulation exists. For example, the observed response of Java could be due to post-transcriptional regulation such as protein degradation after translation or regulation of translation itself. The *flhDC* transcript does encode a

significant untranslated region that could be subject to regulation at the RNA level via small regulatory RNAs or the global regulator Hfq (Sittka *et al.*, 2008). An alternative explanation could be the presence of motile and non-motile cells generating what is known as population heterogeneity. These two plausible reasons for our observations are to be investigated in chapter 5.

Chapter Five: Population Heterogeneity Underpins Motility Robustness across *Salmonella enterica* serovars

5.1 Introduction

In the previous chapter we defined the flagellar gene expression with respect P_{flgA} and P_{fliC} activity for twenty-three strains representing nineteen *Salmonella* serovars. Most serovars had a strong correlation between the magnitude of flagellar gene expression and motility. However, Java, for example, had a normal motility phenotype although it exhibited a significantly lower magnitude and temporal pattern of flagellar gene expression when compared to LT2. As Java is still motile this leads to the question: what is the difference in *flhDC* regulation leading to the reduced gene expression activity in Java? This chapter focuses on this question regarding the mysteries of the behaviour associated with Java. Following investigation of Java, other serovars were included identifying a potential species wide regulatory mechanism of flagellar gene expression.

The first part of this chapter focuses on the hypothesis that in Java $FlhD_4C_2$ activity was strongly influenced by post-transcriptional regulation, such as protein degradation. The proteins FlhD and FlhC exhibit strict conservation across S. enterica, reflected by the conservation of class II $FlhD_4C_2$-dependent promoters (figure 35). Using the tetracycline system, the differences between LT2 and Java flagellar gene expression were investigated in detail. The $FlhD_4C_2$ protein complex in Java was then over-expressed using pSE-*flhDC* and P_{flgA} and P_{fliC} activity measured. To rule out artifacts leading to the observed Java related phenotypes, and across *S. enterica* serovars, reporter plasmid copy number was quantified.

The Java data suggested that protein stability was a key factor in the response we observed. We know that protein stability, via the action of ClpXP and YdiV, drives a level of heterogeneity in the Typhimrium flagellar system (Koirala *et al.*, 2014b). Therefore, the hypothesis that cell heterogeneity played a role in associated

differences across different serovars with respect to flagella gene expression was investigated. This data suggests that Java, and other serovars, exhibit significant heterogeneity while maintaining a motile phenotype.

5.2 Quantification Of Motility for Java Serovar Under-control Tetracycline Inducible System *tetRA* & *tetAR*

Here the objective was to compare the flagellar system from Java under control of P_{flhDC}, P_{tetAR} and P_{tetRA}. The aim of this experiment was to increase the transcriptional output of *flhDC* and ultimately induce a positive response of flagellar gene expression. Motility assays were performed for Java and LT2 using the tetracycline system for expression (figure 37). The average diameter of swimming zones for Java in both tetracycline derivatives were comparable to P_{flhDC} driven expression (figure 26). While, the average swim diameter of LT2 under control P_{tetRA} appears greater than P_{tetAR} control there is no statistical difference (P=0.3) (figure 37 and mentioned in chapter 4). This was surprising as in spite of P_{tetRA} being stronger than P_{tetAR}, Java was still less motile compared to LT2 (P=0.001). This suggests that Java has decreased $FlhD_4C_2$ activity.

5.3 Transcription Activity of P_{flhDC} For Java.

In chapter 4 we quantified P_{flhDC} activity for serovars using plasmid pRG38. pRG38 has P_{flhDC} from the Typhimurium strain 14028s driving transcription of the luciferase operon. The maximum P_{flhDC} activity for Java was dramatically decreased compared to LT2 (figure 38). A logical conclusion for this result suggests that Java has both altered *flhDC* transcription and lower $FlhD_4C_2$ activity. The phylogenetic analysis of P_{flhDC} placed Java close to Typhimurium with having only 2 nucleotide

changes compared to that of Typhimurium (figures 34 and 35). However, the drop in P_{flhDC} activity does not correlate well with *flhDC* tetracycline dependent expression in these strains. One explanation focuses on the impact of several proteins that act as anti - $FlhD_4C_2$ factors. Might it be that these proteins have affected directly or indirectly *flhDC* transcription and activity in Java (Yamamoto and Kutsukake, 2006a)? The changes identified in the P_{flhDC} DNA sequence for Java compared to Typhimurium can also not be ruled out as a reason for the low activity. However, further assessment identifies other serovars with the same changes but comparable activities to Typhimurium.

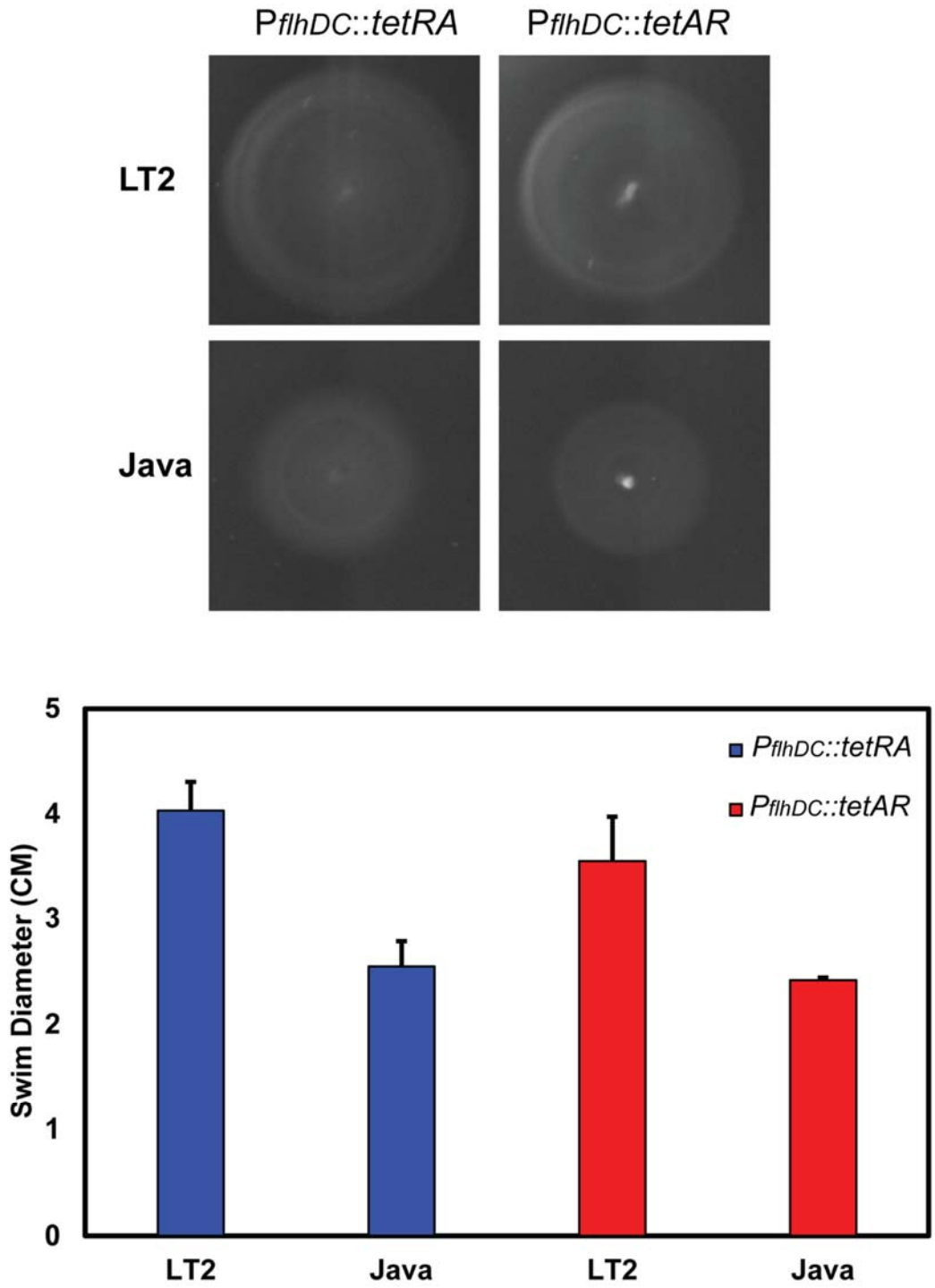


Figure 37. Data shows the average swim diameter of Java and LT2 *Salmonella* serovars under control of P_{tetRA} and P_{tetAR}. There were no significant differences between Java strains under control of the different promoters. However, there was a significant increase in the swim diameter for LT2 comapred to Java. This represents 3 biological replicates. Strains used in this experiment where, LT2 P_{flhDC}::*tetAR* = TPA3789, LT2 P_{flhDC}::*tetRA* = TPA3959, Java P_{flhDC}::tetAR = TPA3793 and Java P_{flhDC}::*tetRA* = TPA3963.

5.4 Titration Of Flagellar Gene Expression in Java

We asked: what is the response of P_{flgA} and P_{fliC} in Java during titration of the tetracycline system to drive *flhDC* expression? To compare Java and LT2 *flhDC* under the control of P_{tetRA} induction was used. The decision to do this related to P_{tetRA} being the stronger of the two promoters, potentially driving greater levels of *flhDC* transcription. Anhydrotetracycline concentrations were used from 100 ng/ml down to 1 ng/ml. Focusing first on P_{flgA}, the percentage of relative activity for Java increased slightly reaching a maximum of 40% of LT2 activity with 100ng/ml anhydrotetracycline (figure 39A). In contrast, LT2-P_{flgA} activity reached a plateau at 80-95% relative activity for concentrations greater than 2.5 ng/ml (figure 39A). However, at the same concentration of anhydrotetracycline the relative activity of Java- P_{flgA} was approximately 5 %.

Interestingly, even though Java- P_{flgA} activity was low, anhydrotetracycline at 100ng/ml showed that temporal activation was comparable to LT2 (figure 39B and C). Consistently, 100ng/ml anhydrotetracycline drove measurable P_{fliC} gene expression for Java reaching a relative maximum of 20% (figure 39D). Conversely, the P_{fliC} flagellar gene expression for LT2 reached a maximum activity of 90% between 25-50 ng/ml. Furthermore, temporal activation was again comparable for both serovars when considering P_{fliC} activity (figure 39E and F). Taken together, in spite of the ability to titrate the tetracycline system, flagellar gene expression for Java was still relatively low even with higher concentrations of anhydrotetracycline when was compared to LT2. These data are consistent with the hypothesis that $FlhD_4C_2$ from Java has low activity. We can rule out protein sequence differences as we know that FlhD and FlhC show extensive conservation across *S. enterica* (data not shown).

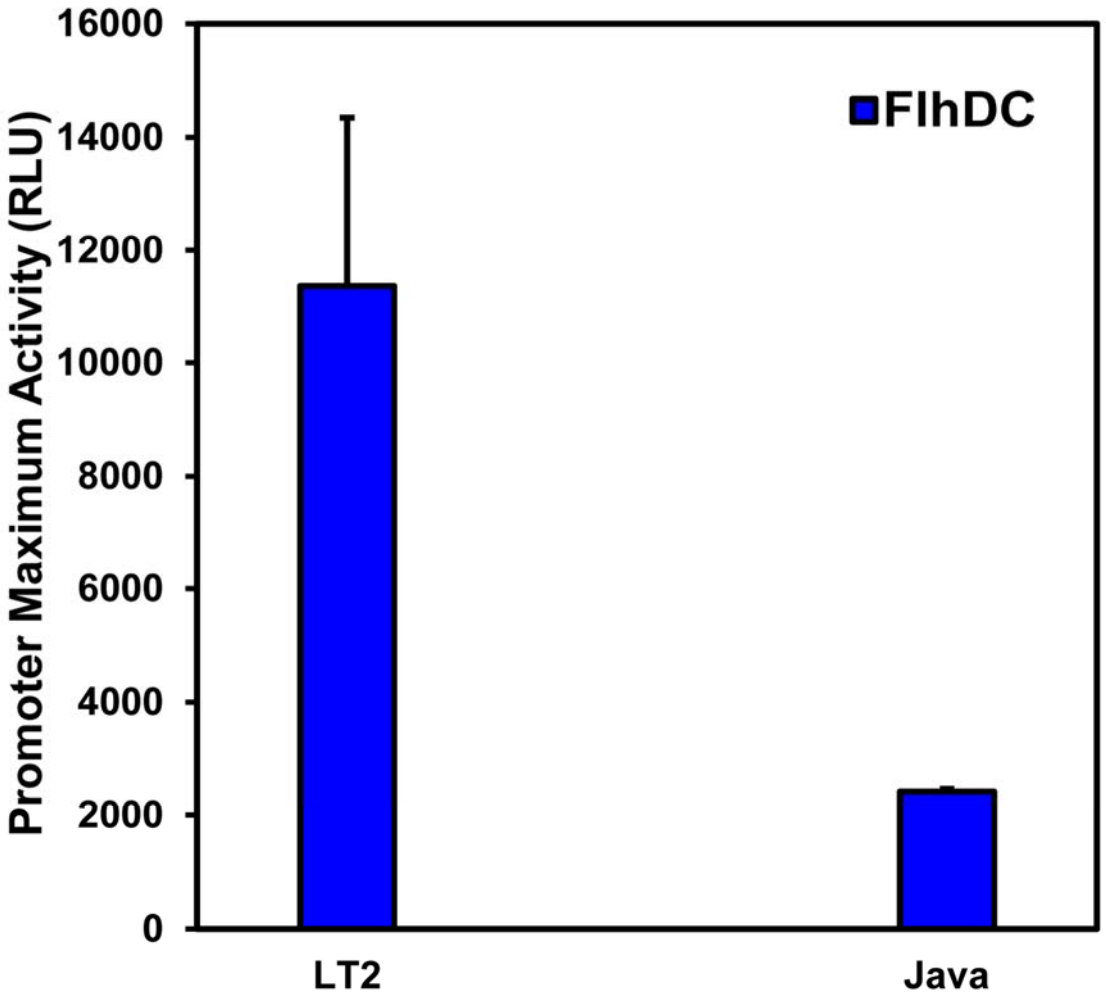


Figure 38. Comparison of P_{flhDC} transcriptional activity for Java and LT2 serovars. The maximum activity for the transcription of the flagellar master regulator operon was dramatically decreased for Java. This experiment was repeated biologically three times with Standard Deviation error bars displayed for comparison. Strains used in this experiment were, LT2 =TPA4219 and Java = TPA4221 transformed with the plasmid pRG38 for P_{flhDC} detection

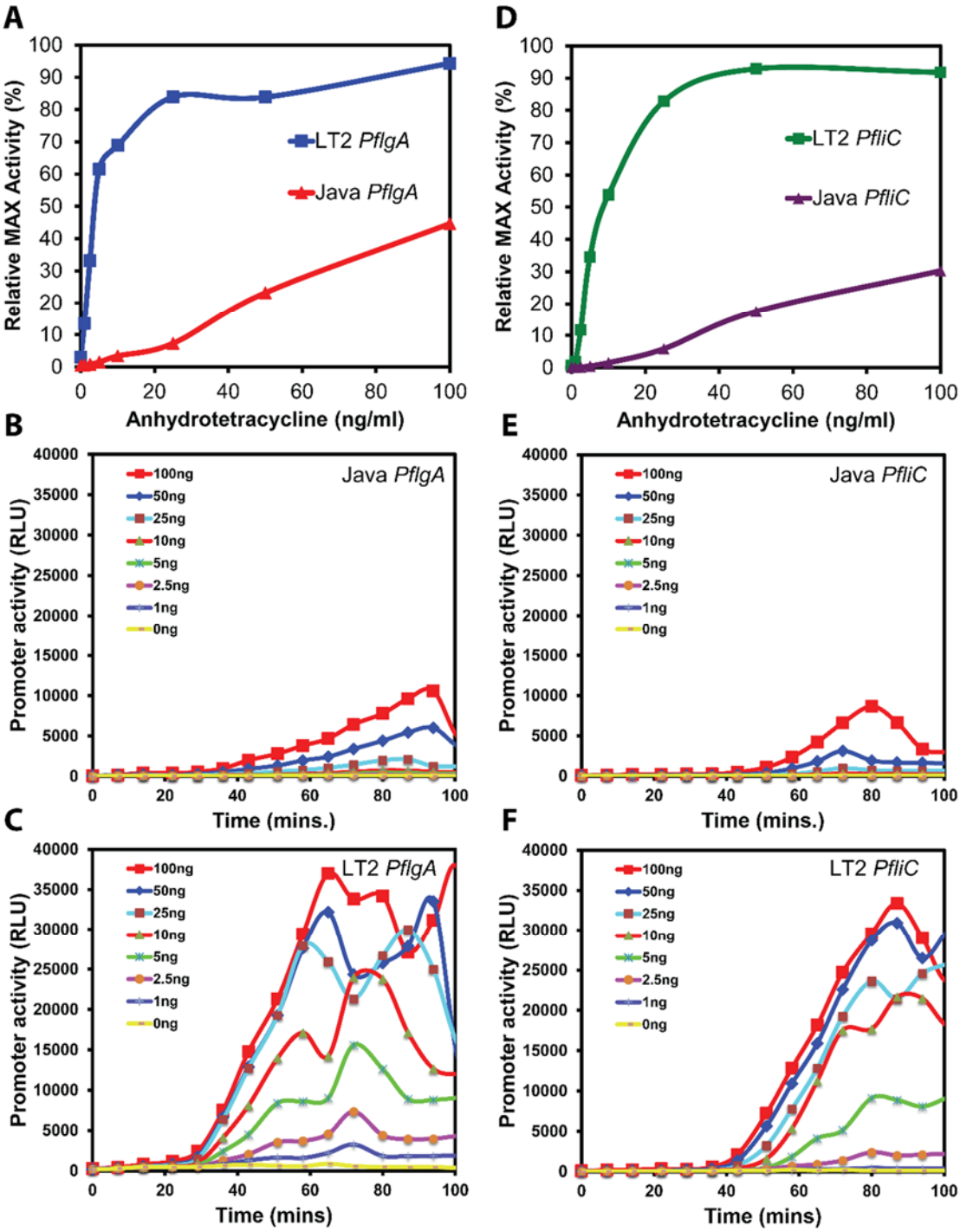


Figure 39. Titration of *flhDC* transcription monitored by flagellar gene expression from P_{flgA} and P_{fliC} in Java and LT2 serovars by using different concentrations of anhydrotetracycline. A) Relative MAX activity of P_{flgA} plotted against anhydrotetracycline concentration. Analysis shows that at all concentrations Java has reduced P_{flgA} activity. B) Temporal activity of P_{flgA} with increasing concentrations of anhydrotetracycline. C) comparative induction data for LT2. Compared to (B) the temporal activation of Java and LT2 was very similar, while the magnitude was significantly different. D) Titration plot for relative MAX activity of P_{fliC} E) Temproal induction of P_{fliC} in Java. F) Temporal induction of P_{fliC} in LT2. Here again temporal activiation in both strains reflects the P_{flgA} data, while LT2 has stronger magnitude of expression. Experimental data represents a minimal of three independent repeats (n=3). Strains used in this experiment were, (A to C) LT2 *flgA* = TPA3968 and Java *flgA* = TPA 3974. (D to F) LT2 *fliC* = TPA3967 and Java *fliC* = TPA 3973.

5.5 Effects Of FlhDC Overexpression In Java Serovar On Class II And Class III Flagellar Genes Expression

As changing *flhDC* expression did not significantly impact flagellar output, we asked whether overexpressing the flagellar master operon from LT2 via plasmid based expression using pSE-*flhDC* would increase flagellar gene expression activity. pSE-*flhDC,* containing *flhDC* from LT2*,* was introduced into Java and LT2. Flagellar gene expression was determined based on the activity of P_{flgA} and P_{fliC} (Brown ***et al.***, 2008). As a control, the *flhDC* operon was also deleted from Java and LT2 to eliminate *flhDC* expression from the chromosome. All strains were compared to *flhDC*$^{+}$ Java and LT2 wild type (figure 40).

For Java a slight increase in flagellar gene expression was observed in particular compared to Java wild type (figure 40A). Consistently the Δ*flhDC* Java mutant showed no activity. LT2, as expected, exhibited a much stronger response. However, in LT2 Δ*flhDC* pSE-*flhDC* could only compliment the *flhDC* deletion, not increase promoter activity further. Our data therefore suggested that expression of *flhDC* from pSE backbone was not strictly the overexpression we had assumed as promoter activity was not increased in either LT2 pSE-*flhDC* or Δ*flhDC* pSE-*flhDC* compared to LT2 without pSE-*flhDC*. We can conclude that expression of *flhDC* from a plasmid in Java leads to reduced activity compared to LT2. However, expression of *flhDC* from pSE-*flhDC* in Java is much more efficient than in LT2 as unlike LT2 we observed a 3 to 4-fold increase in flagellar gene expression when pSE-*flhDC* was present. This is further evidence that $FlhD_4C_2$ activity is repressed in Java compared to LT2 and is consistent with data in chapter 4 where we show that low transcription of *flhDC* can lead to significant changes in the output of the flagellar system. What this data is unable to explain is why when flagellar gene expression is low does a serovar like Java possess a robust motility phenotype in motility assays?

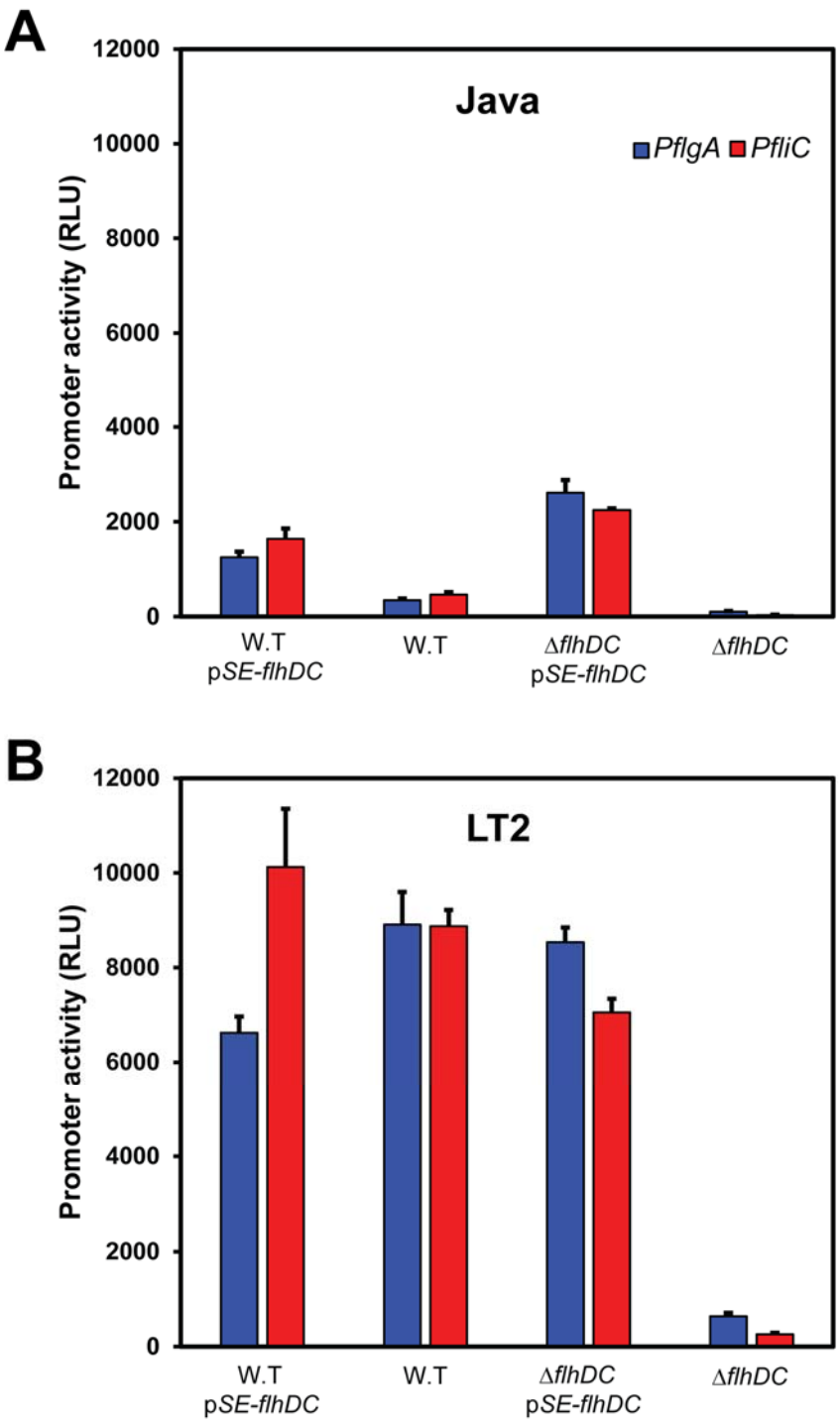


Figure 40. Characteristics of flagellar genes expression activity after plasmid based *flhDC* expression in (A) Java compared to (B) LT2. Expression of *flhDC* on a plasmid imporved flagellar gene expression in Java, although still not to the levels measured for LT2. Having pSE-*flhDC* as the only source of *flhDC* did not improve or alter the observed response. Importantly, pSE-*flhDC* in both Java and LT2 could compliment the Δ*flhDC* mutant. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, (A P*flgA*) W.T p*SE-flhDC* = TPA4265, W.T = TPA 3974, Δ-*flhDC* p*SE-flhDC* = TPA4268 and Δ-*flhDC* = TPA 3974. (A P*fliC*) W.Tp*SE-flhDC* = TPA4264, W.T = TPA3973, Δ-*flhDC* p*SE-flhDC* = TPA4267 and Δ-*flhDC* = TPA 3973. (B P*flgA*) W.Tp*SE-flhDC* = TPA4227, W.T = TPA3968, Δ-*flhDC* p*SE-flhDC* = TPA4232 and Δ-*flhDC* = TPA4255. (B P*fliC*) W.Tp*SE-flhDC* = TPA4226, W.T = TPA3967, Δ-*flhDC* p*SE-flhDC* = TPA4232 and Δ-*flhDC* = TPA4254.

5.6 Quantifying Plasmids Reporter Copy Number for P_{flgA} and P_{fliC} via qPCR In Selected serovars

A quantitative real time PCR, based on SYBR Green detection, was established to validate the ratio between copy numbers of the luminescence reporter plasmids P_{flgA} and P_{fliC} compared to the chromosome. Our method was based on previous experimental designs (Lee *et al.*, 2006). The aim of this experiment was to measure plasmid copy number to rule out any changes in *S. enterica* serovars. This was an important control especially for serovars such as Java that possess very low expression for flagellar genes but still are motile. Differences in numbers of the reporter plasmid among *S. enterica* serovars will affect the magnitude of flagellar gene expression, leading to a false impression of expression, especially as these reporters are very sensitive to low levels of expression (Hakkila *et al.*, 2002).

Five *S. enterica* serovars were examined using two chromosomal target genes and two reference genes on the vector backbone of the reporter plasmid pSB401. A chromosome and plasmid dilution series were used as standard curves to define a middle point dilution for test experiments. The plasmid-genome ratio for P_{flgA} and P_{fliC} plasmids exhibited no significant differences between *S. enterica* serovars (figure 41A and 42A). In the other words, the copy numbers of the P_{flgA} and P_{fliC} reporter plasmids have similar ratios in all *S. enterica* serovars tested (LT2, Indiana, Vinohrady, Java and Lexington). Statistical analysis using Tukey's method of comparison defined no significant difference between data sets (figure 41B and 42B). Taken together, we established an assay that compared different serovars with LT2 in order to emphasise no variation in copy number of the plasmid reporter system. Consequently, this data argues that there are biologically relevant explanations behind the low level of flagellar gene expression observed in Java and other serovars compared to LT2.

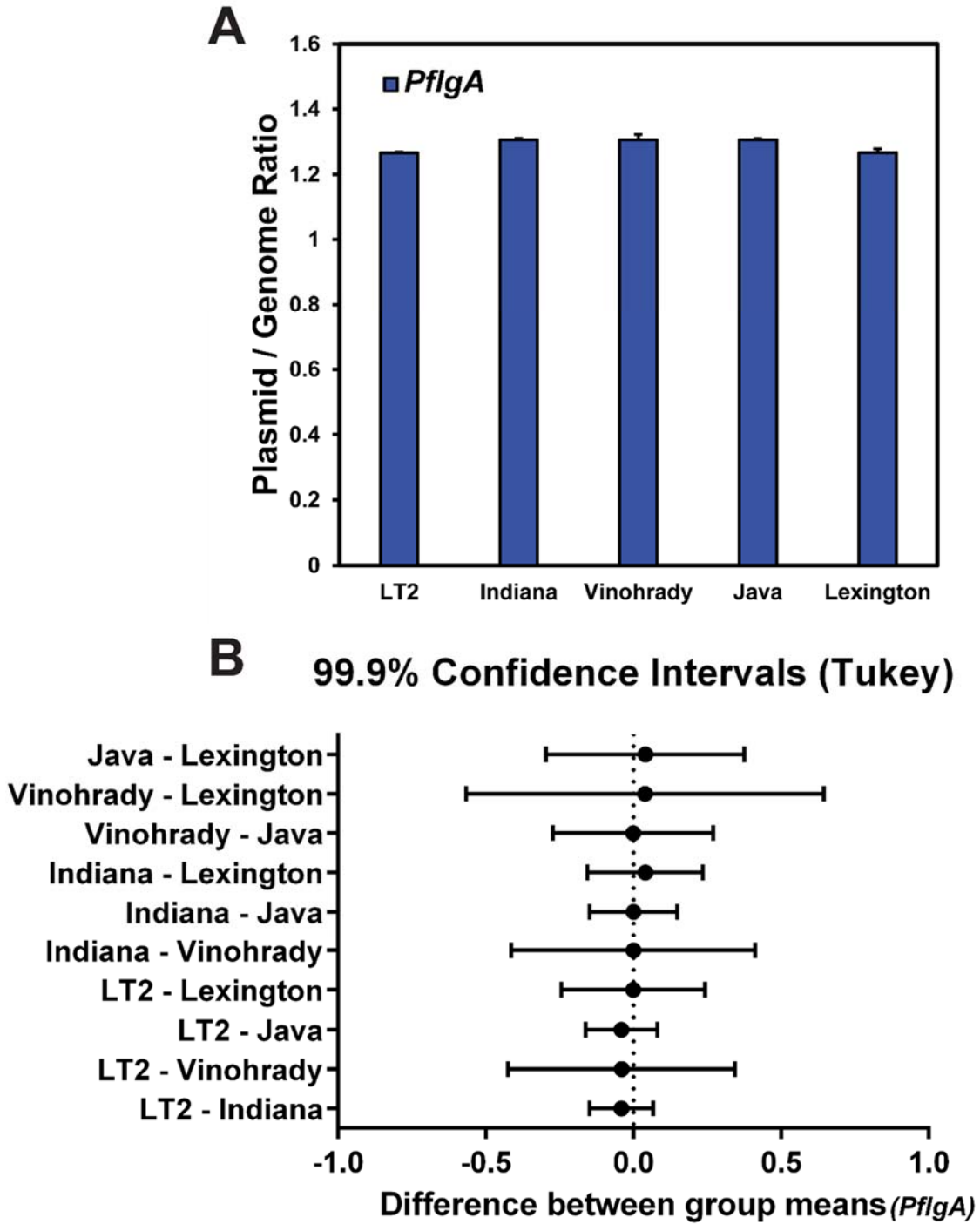


Figure 41. Evaluation of the ratio between the copy number of the reporter plasmid harboring P_{flgA} compared to a genomic target using real-time PCR target. A) The calculated ratio of report plasmid to genomic DNA content for the indicated serovars. There were no significant differences between between *Salmonella* serovars. B) Statistical analysis data (Tukey method) emphasized the robustness of plasmid copy number across *Salmonella* serovars when compared to LT2 (p. 0.01). Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, LT2 = TPA3968, Lexington = TPA4415, Indiana =TPA4441, Vinohrady = TPA4486 and Java = TPA3974.

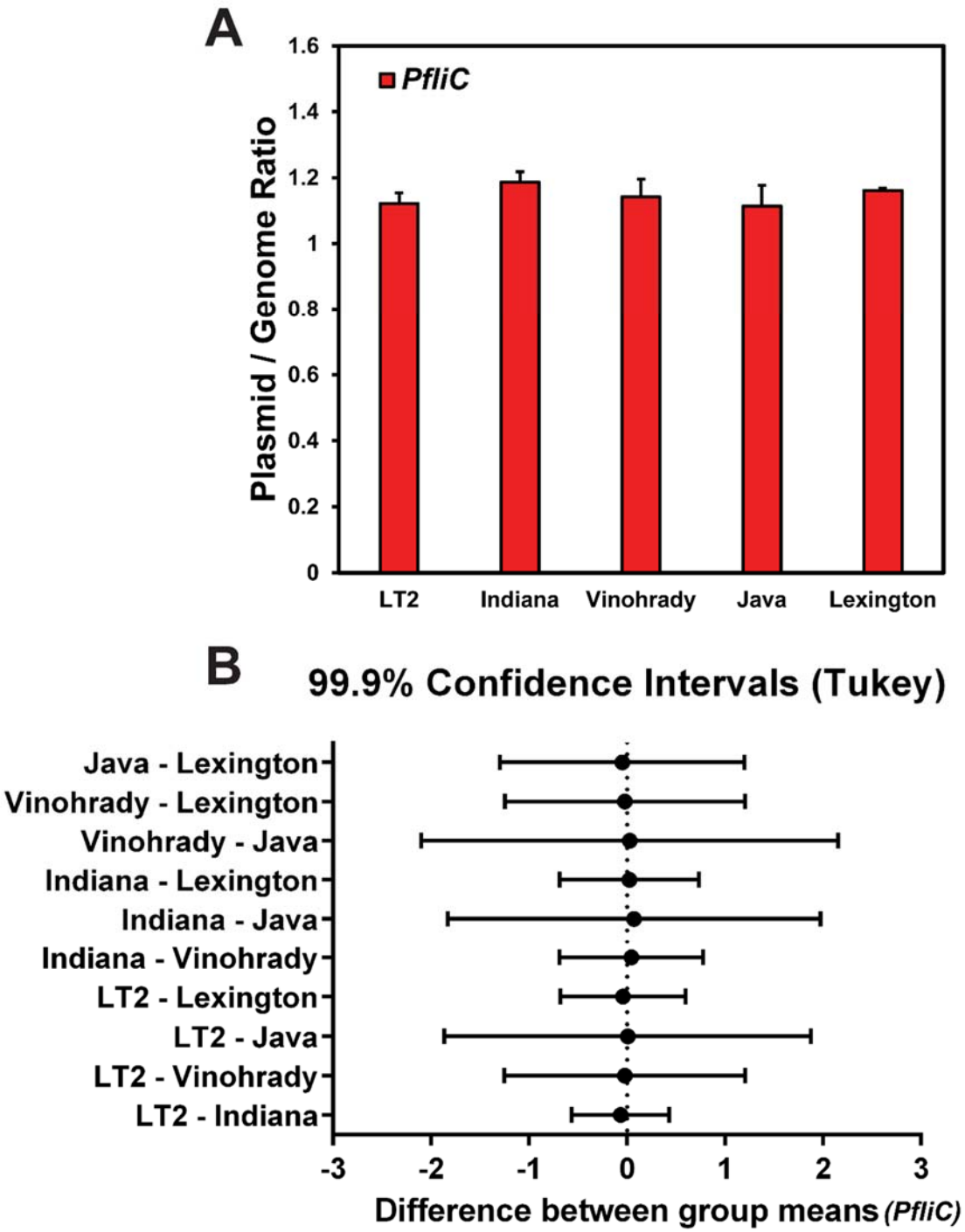


Figure 42. Evaluation of the ratio between the copy number of the reporter plasmid harboring P_{fliC} compared to a genomic target using real-time PCR target. A) The calculated ratio of report plasmid to genomic DNA content for the indicated serovars. There were no significant differences between between *Salmonella* serovars. B) Statistical analysis data (Tukey method) emphasized the robustness of plasmid copy number across *Salmonella* serovars when compared to LT2 (p. 0.01). Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment where, LT2 = TPA3967, Lexington = TPA4414, Indiana =TPA4440, Vinohrady = TPA4485 and Java = TPA 3973.

5.7 Comparison The Phenotypic Heterogeneity Of The *Salmonella* Serovars Based On The Flagellum, Class II And Class III Proteins Synthesis

In order to ascertain why Java and some other *Salmonella* serovars have a low-output with respect to flagellar gene expression meanwhile still exhibiting robust motile phenotype, we proposed that phenotypic heterogeneity was a key player. Heterogeneity would generate a subpopulation of motile cells with typical flagellar gene expression. However, in a population assay we measure the whole population. To investigate heterogeneous flagellar gene expression, P_{flgA} and P_{motA} GFP reporter plasmids were used to measure transcription per cell. Fluorescence microscopy images were analysed using the Microbetracker program to differentiate between cells which are flagella-ON from flagella-OFF. Six *S.enterica* serovars were tested in comparison to LT2. Serovar choice was based on the temporal dynamics observed in figure 32 taking three examples of each subset of strains to compare to LT2. In terms of P_{flgA}, phenotypic heterogeneity between serovars was significant (figure 43). For example in Java, Lexington and Alaucha a noticeable decline in P_{flgA} transcription among the population is evident when compared to LT2 (figure 43). This is seen by the strong clustering of the data at the bottom of the distribution plots. In comparison for LT2 the distribution of the data produced a larger and stronger cloud (figure 43). In contrast, Emek, Indiana and Vinohrady increased the response of P_{flgA} in comparison to LT2. Importantly these three serovars exhibited a much stronger split between cells with P_{flgA} activity versus cells with no activity. The strongest examples being Emek and Indiana.

A similar response was measured for P_{motA}, an alternative class 3 promoter (figure 44). All serovars exhibited a stronger split between P_{motA} active cells than seen for LT2. The data strongly suggests that the behaviour of Java and other

serovars is the result of population heterogeneity. The nature of phenotypic heterogeneity creates a subpopulation of cells among *Salmonella* serovars that are motile. This observation argues that motility agar should be considered as a positive selection for motile sub-populations.

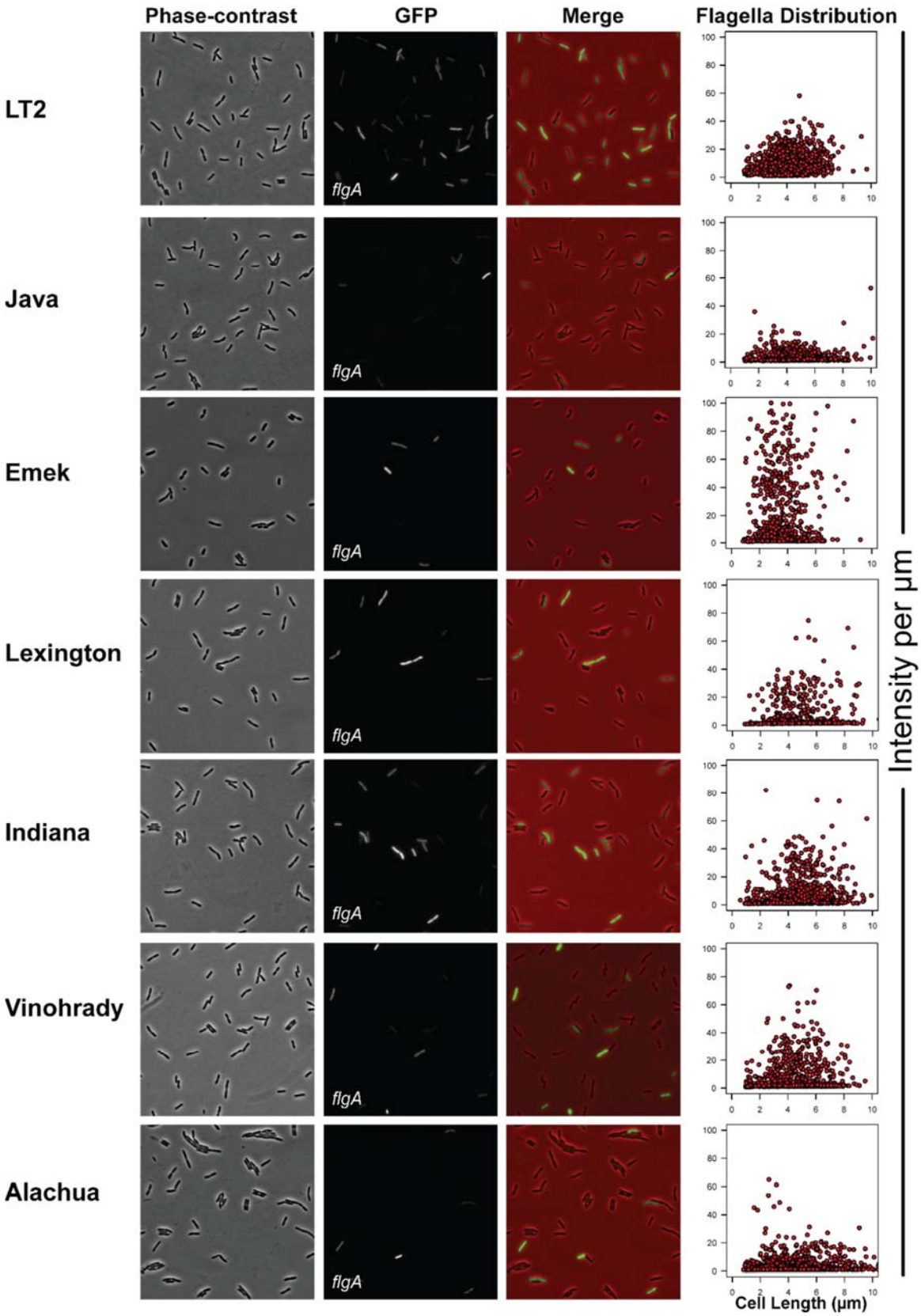


Figure 43. Comparison of population heterogeneity between *Salmonella* serovars, based on a P_{flgA} -GFP transcriptional fusion. The activity of P_{flgA} was significantly changed in Java, Emek, Lexington and Alaucha when compared to LT2. Experiment represents the total cell count derived from 5 fields of view from three biological independent repeats (n=3). Strains used in this experiment were, LT2= TPA5135, Java= TPA5129, Emek=TPA5130, Lexington =TPA5131, Indiana = TPA5132, Vinohrady = TPA5133 and Alchua =TPA5134.

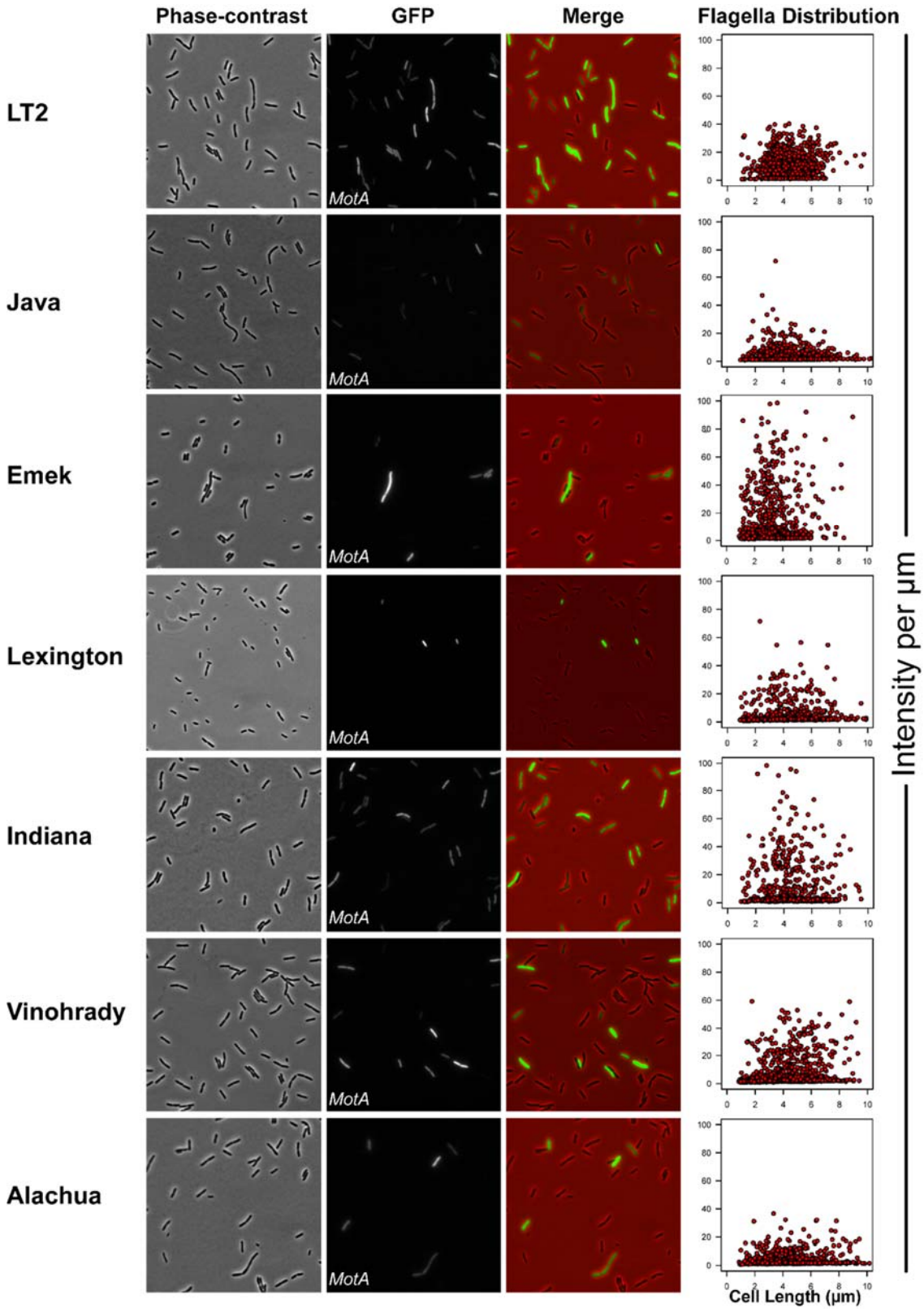


Figure 44. Comparison of population heterogeneity between *Salmonella* serovars, based on a P_{motA}-GFP transcriptional fusion. The activity of P_{motA} was significantly changed in all serovars when compared to LT2. Importantly the data for P_{motA} correlates to P_{flgA} activity in these populations. Experiment represents the total cell count derived from 5 fields of view from three biological independent repeats (n=3). Strains used in this experiment were,, LT2= TPA5128, Java= TPA5122, Emek=TPA5123, Lexignton =TPA5124, Indiana = TPA5125, Vinohrady = TPA5126 and Alchua =TPA5127.

5.8 Summary

This chapter has revealed that most *S.enterica* serovars which are consistently motile exhibit several factors that impact the activity of the underpinning flagellar system. In the previous chapter we observed Java had a low magnitude of flagellar gene expression but was still motile. In this chapter we investigated why Java behaved like this. We found that *flhDC*-Java was lower than *flhDC*-LT2 activity. However, in motility assays, Java with two different promoters (P_{tetRA} and P_{tetAR}) driving *flhDC* transcription were still decreased compared to LT2. Titration of *flhDC* expression in Java still exhibited a significant decrease when compared to LT2. However, high concentrations of inducer did identify correct temporal activation of the flagellar system. Furthermore, attempts to increase *flhDC* transcription activity by using an overexpression plasmid, unfortunately, did not improve Java's flagellar gene expression.

We questioned whether the reporter plasmid copy number was responsible for giving us the low signal for flagellar gene expression, as a reason for the Java phenotype. Real-time PCR suggested that the plasmid reporter in Java, LT2 and other serovars were comparable. This result has a wider implication in supporting the use of reporter plasmids to measure flagellar gene expression.

Finally, the last experiment was able to provide some evidence to the mystery of Java's behavior. Exploring phenotypic heterogeneity provided evidence to suggest a variation in subpopulations of *S. enterica* serovars expressing flagella. We conclude that the underpinning regulation of flagellar system across serovars leads to population heterogeneity with respect to motility. Our data suggests that motility in agar possibly selects for propagation of the motile population providing a biased opinion of a motility phenotype.

Chapter Six: Synthetically Engineering $FlhD_4C_2$ from *Escherichia coli* RP437 into *Salmonella enterica* and its Impact on Motility Phenotype and Flagellar Gene Expression

6.1 Introduction

In chapter six we focus our research to expanding up on a previous project (Sim, 2014), where flagellar assembly was compared to the growth rate between two different *Enterobacteriaceae* species *E. coli* and *S. enterica*. The high similarity of the flagellar systems for *E. coli* and *S. enterica* represents a crucial point of correlation. For example in FlhD and FlhC only sixteen amino acids are unique to the indivdual proteins (figure 45). Previous data suggests that both *E. coli* and *S. enterica* respond similarly to growth conditions controlled using a chemostat (Sim *et al.*, 2017). However, when at a fast growth rate, *S.enterica* generates a subpopulation of non‐motile cells while *E.coli* exhibits a homogeneous population (figure 46). The hypothesis to be tested argues that the difference between FlhD and FlhC in these two species is key to the responses observed with respect growth rate (Sim *et al.*, 2017). To test this hypothesis, we will generate a *S. enterica* system driven by *flhDC-E. coli* from the *flhDC* chromosomal locus, so that we can measure the impact of physiological signals on flagellar gene expression.

We quantified the motility phenotype of the *S.enterica* $flhDC_{(ec)}$ strain compared to wild type ($flhDC_{(st)}$) by using the tools and assays introduced during chapters 4 and 5. We determined flagellar gene expression to look at the magnitude of P_{flgA} and P_{fliC} activity. We investigated the impact of growth rate on flagellar abundance. The tetracycline system was used to titrate transcription controlling the levels of flagellar gene expression. Studying the response to $flhDC_{(ec)}$ switch led to the creation of two further *S. enterica* strains replacing just *flhD* or *flhC* separately from *E.coli*. Through a comparison of all four strains we show a key difference between FlhD and FlhC activity during flagellar gene expression.

Figure 45. Amino acid sequence comparison for FlhD and FlhC-*S.enterica* (LT2) versus FlhD and FlhC-*E.coli* (RP437). There is, respectively, 94.7 and 96.9% sequencing identity between the FlhD and FlhC homologues from *S. enterica* and *E. coli*. This high level of identity equates to 6 non-conserved amino acid substitutions in the FlhD homologues and 10 in FlhC.

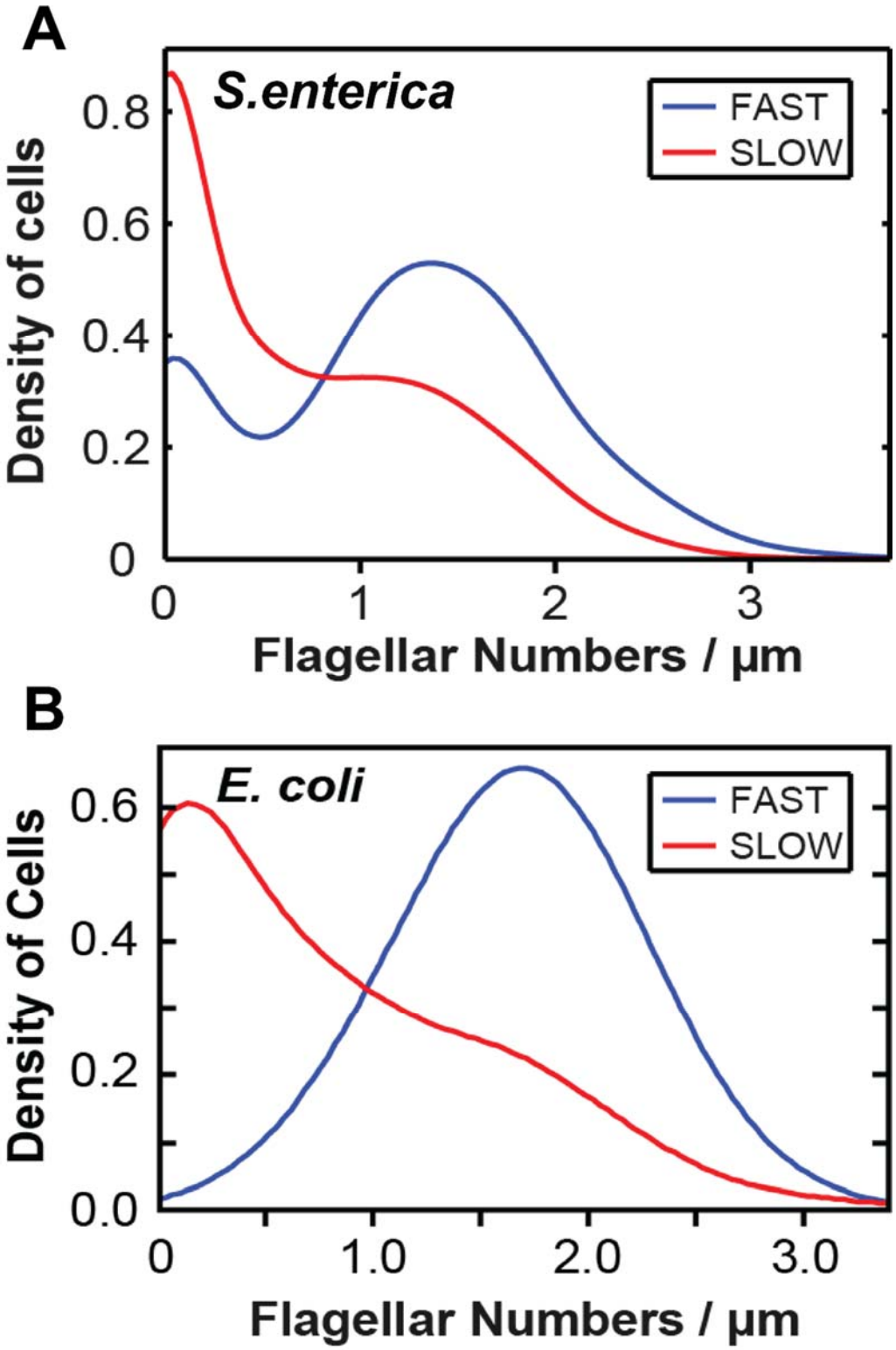


Figure 46. The abundance of flagellar numbers per cell for *S. enterica* (A) versus *E.coli* (B) in terms of two growth conditions fast (blue) and slow (red). In the slow growth condition *S. enterica* and *E. coli* had similar response of reduced flagellar per cell. In contrast, in the fast growth conditions for *S. enterica* approximately 15 % of the cells were Fla^-, while 100% of the *E. coli* population produced flagella. These results are adapted from Sim (2014) and Sim *et al* (2017).

6.2 Swapping *flhDC* in *Salmonella enterica* (LT2) With *flhDC* From *E. coli* (RP437)

We swapped *flhDC* from *E.coli* into the *flhDC S.enterica* loci by manipulating the *Salmonella* genome following methods described previously (Blank *et al.*, 2011). This allows for the precise deletion of $flhDC_{(ST)}$ from the genome, and swapping it with $flhDC_{(ec)}$ from strain RP437, using λ Red recombination (Datsenko and Wanner, 2000). All mutants strains were checked by sequencing to ensure that the *flhDC-E.coli* replacement was correct. We then examined the strains comparing to wild type $flhDC_{(ST)}$.

6.2.1 Quantification Of Motility Phenotype

The motility phenotype was measured exploiting three different types of promoters (P_{flhDC}, P_{tetRA} and P_{tetAR}) to assess the motility of $flhDC_{(ec)}$ in comparison to wild-type. In terms of the native promoter, the average swim diameter was not significantly different from wild type (P = 0.780). For P_{tetRA}, a slight but not significant increase compared to wild-type was observed (P = 0.610). However, for P_{tetAR}, the swim diameter was lower than wild-type but still not significant (P = 0.266) (figure 47). Overall, the motility phenotype for the new strain was comparable to wild-type regardless of which kind of the promoter was used.

In contrast, when $flhDC_{(ec)}$ was compared across the different promoters (P_{flhDC}, P_{tetRA} and P_{tetAR}), the average swim diameter for P_{tetAR} was significantly decreased compared to P_{flhDC} and P_{tetRA} ($P<0.05$). No significant difference for P_{tetRA} versus P_{flhDC} were observed. An implication of these findings is that the motility phenotype of the $flhDC_{(ec)}$ strain behaved almost exactly as wild-type (LT2) in spite of the flagellum system being under control of a different master regulator (figure 47). This is similar to what we previously observed for a number of serovars in chapter 4.

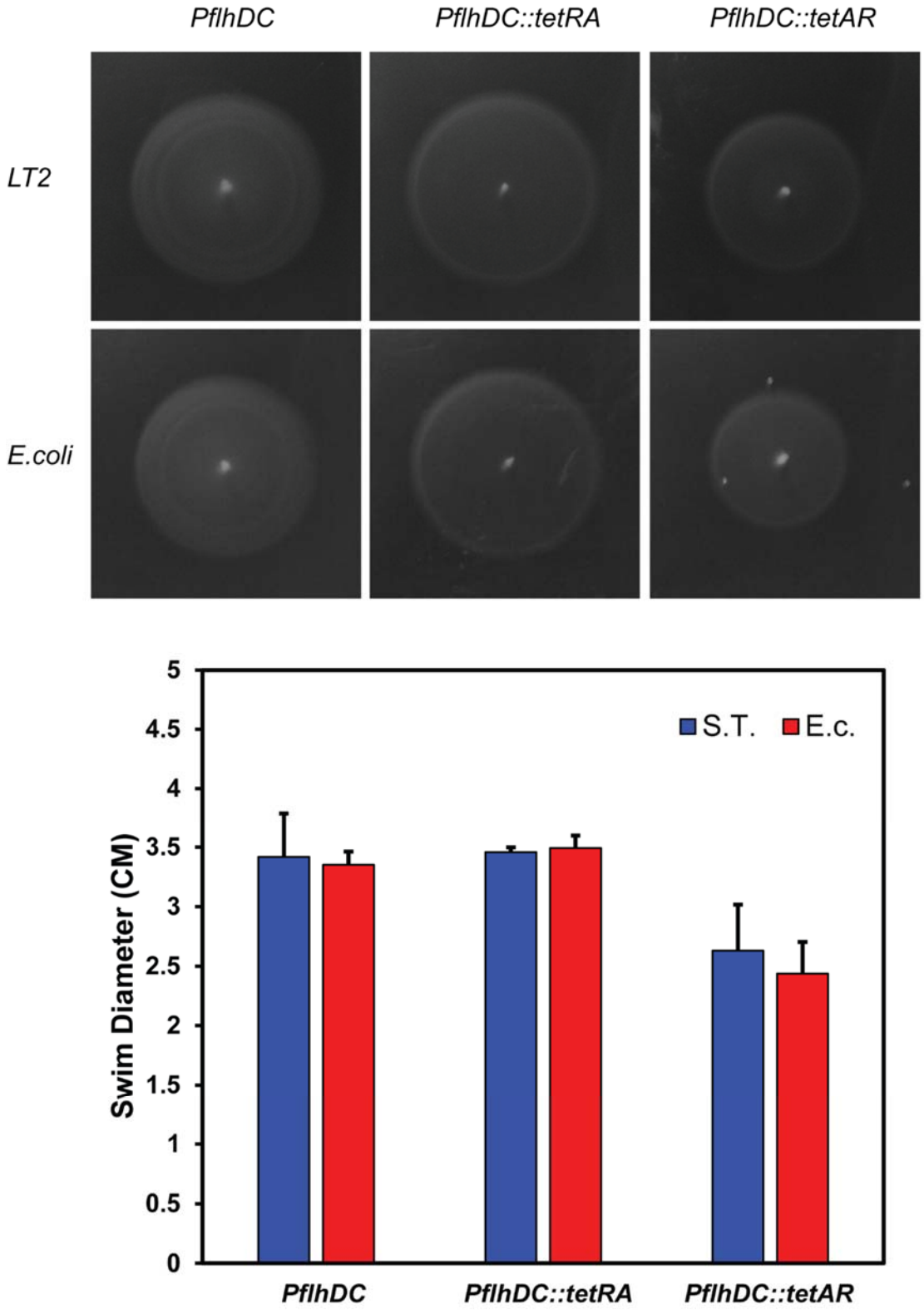


Figure 47. Quantification of motility for different strains: ST is *S. enterica* (wild-type) and EC is *S. enterica* with *flhDC* from *E.coli.* This experiment was repeated in triplicate and statistical significance is mentioned in the main text where appropriate. Strains used in this experiment were, LT2 (also defined as S.T.) P_{flhDC} = TPA1107, LT2 P*flhDC*::*tetRA* = TPA4028, LT2 *PflhDC*::*tetAR* = TPA4029, E. coli (also defined as E.C.) P_{flhDC} = TPA3997, E. coli P*flhDC::tetRA* = TPA4022 and E. coli P*flhDC::tetAR* = TPA4096.

6.2.2 Impact of Growth Rate Control On Flagellar Abundance

Sim *et al* (2017) and during the thesis of Sim (2014) a correlation between flagellar numbers and the growth-rate of the bacteria depending on the growth conditions used was identified (Sim *et al.*, 2017). The prime aim for the creation of *flhDC*$_{(ec)}$ was to compare flagellar numbers per cell in different growth environments to *flhDC*$_{(ST)}$. This was achieved using a FliM-GFP reporter fusion as a biosensor for flagellar production. Each FliM-GFP foci in the bacterial cell can be used as a proxy for a single flagellum using fluorescence microscopy (Aldridge *et al.*, 2006a; Sim *et al.*, 2017). This allowed the numbers of flagella per cell to be quantified using the MicrobeTracker (Sliusarenko *et al.*, 2011).

The flagellar numbers of *flhDC*$_{(ec)}$ versus *flhDC*$_{(ST)}$ were measured during fast and slow batch growth conditions. The work of Sim (2014) exploited chemostat growth conditions, however, both this study and the work of Sim are based on the growth control in *S. enterica* from Aldridge *et al* (2010). These two studies show that the methods used are comparable. For the fast growth condition, the percentage distribution of flagellar foci per cell for *flhDC*$_{(ec)}$ was not significantly different compared to *flhDC*$_{(ST)}$ (figure 48A). The range of flagellar per cell for both strains was 0-15 foci. Conversely, with respect to slow growth conditions, the distribution of flagellar foci per cell was between 0-6 foci per cell (figure 48B).

There was no impact on effectiveness in terms of fast and slow growth conditions on the distribution of flagellar numbers per cell in spite of the genetic differences. This is in agreement with the strains motility phenotype. Sim *et al* (2017) has shown that in fast growing conditions 100% of the population is motile. However, unlike in *E.coli*, *flhDC*$_{(ec)}$ in *S. enterica* produced a proportion of cells with no flagellar at the fast growth rate (figure 48A). This suggests the control leading to this Fla^{-}

population can be derived from *flhDC* transcription control, leading to some cells not expressing the *flhDC* operon. Alternatively, the regulatory input that leads to this sub population of non-motile cells is unable to differentiate between the *S. enterica* and *E. coli* $FlhD_4C_2$ complex.

6.2.3 Determination Class II and Class III Flagellar Gene Expression

Flagellar gene expression has been extensively studied in chapter 4 and chapter 5 exploiting the tetracycline system to activate flagellar gene expression. To measure promoter activity, we used a reporter plasmid encoding the luciferase operon (*luxCDABE*) originally from *Photorhabdus luminescens* (Winson *et al.*, 1998). The *luxCDABE* operon was transcribed from flagellar class II or class III promoters derived form the *S. enterica* serovar Typhimurium strain 14028s. Phylogenetic analysis validated this choice as the P_{flgAB} $FlhD_4C_2$ binding was conserved across all serovars as was the σ^{28} promoter sequence of P_{fliC}.

In this section, we have measured the dynamics of flagellar gene expression for P_{flgA} *and* $P_{fliC,}$ comparing the activity for $flhDC_{(ST)}$ and $flhDC_{(ec)}$ (figure 49). The activities in P_{flgA} and P_{fliC} in $flhDC_{(ec)}$ were less efficient than $flhDC_{(ST)}$, most notably for P_{tetAR} driven expression of *flhDC* (figure 49B). However, the induction time for $flhDC_{(ST)}$ and $flhDC_{(ec)}$ were similar. Furthermore, for P_{tetRA}, induction and magnitude were significantly faster and higher when compared to P_{tetAR}. We attribute this to the promoter activity (mentioned chapter 4). Consistently, differences in activity are clearer when using P_{tetAR} as was seen for the comparison of serovar dynamics.

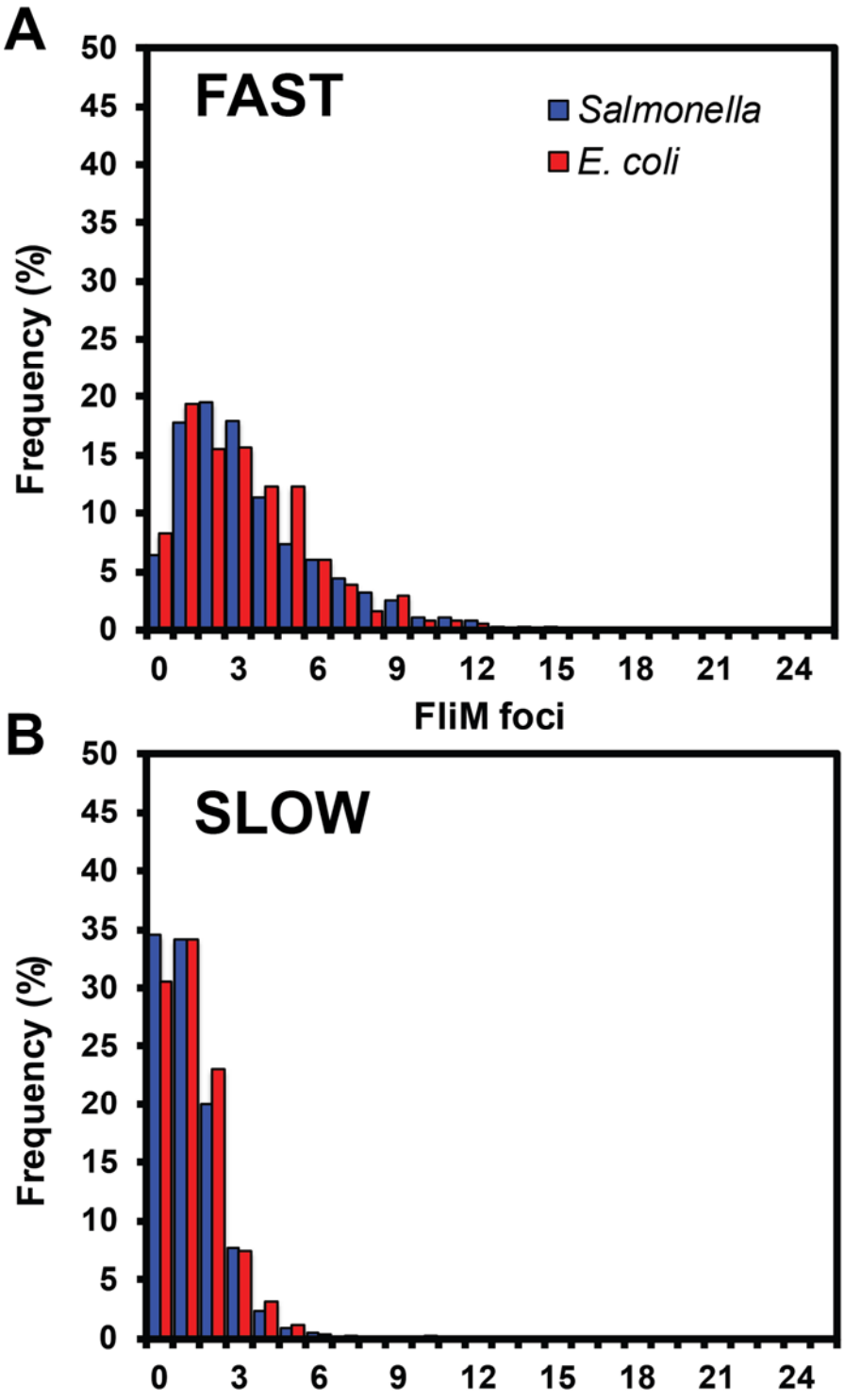


Figure 48. The distribution of FliM–GFP foci per cell in ST (wild type: *Salmonella enterica*) versus EC (*flhDC*$_{(ec)}$) in two different conditions. (A) Fast Growth: MinE media 0.2% Glucose with 3g/L Yeast Extract; (B) Slow Growth: MinE media 0.2% Glucose without Yeast Extract (Aldridge *et al.*, 2010). Experiment represents total cell populations derived from a minimal of five independent repeats (n=5). Strains used in this experiment were *Salmonella* = TPA 1107 and *E. coli* = TPA 3997.

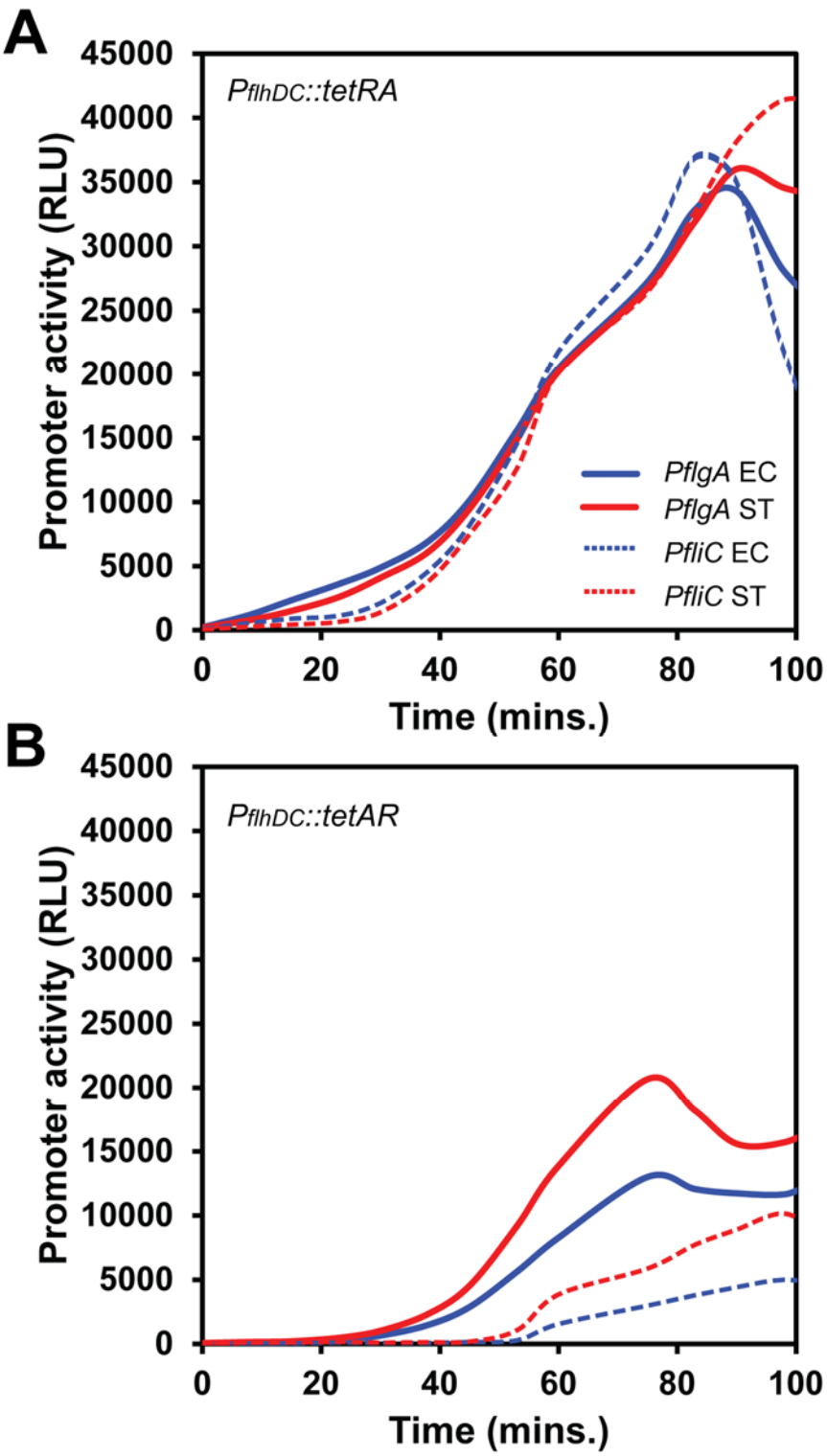


Figure 49. Kinetic comparison of the flagellar gene expression for P_{flgA} (solid) and P_{fliC} (dashed) between ST (*flhDC*$_{(ST)}$) and EC (*flhDC*$_{(ec)}$) under control of tetracycline inducible promoters. (A) P_{tetRA} and (B) P_{tetAR}. Data represents the average activity calculated from a minimum of three independent repeats. Strains used in this experiment were (A) P_{flhDC}::*tetRA*/P_{flgA}EC= TPA4032, P_{flhDC}::*tetRA*/ P_{flgA} ST= TPA4050, P_{flhDC}::*tetRA*/P_{fliC}EC= TPA4031 and P_{flhDC}::*tetRA*/P_{fliC}ST= TPA4049. (B) P_{flhDC}::*tetAR*/ P_{flgA}EC= TPA4099, P_{flhDC}::*tetAR*/P P_{flgA} ST= TPA4053, P_{flhDC}::*tetAR*/P_{fliC}EC= TPA4098 and P_{flhDC}::*tetAR*/P_{fliC}ST= TPA4052.

6.2.4 The impact of Titrating flhDC transcription on Flagellar Gene Expression and flagellar numbers

Motility and induction experiments have shown how comparable *flhDC* from *E.coli* and *S. enterica* are in relation to driving the *S. enterica* flagellar system. In chapter 4 and 5, we have used titration of P_{tetRA} and P_{tetAR} to investigate the impact of *flhDC* transcription. This identified an interesting disparity between flagellar gene expression and flagellar numbers. Therefore, we asked whether titration experiments would show a similar or a different response when comparing $flhDC_{(ec)}$ output to $flhDC_{(ST)}$.

In terms flagellar gene expression titration, the highest expression for $flhDC_{(ec)}$ was at 50 ng/ml with respect to $P_{tetRA,}$ while for $flhDC_{(ST)}$ it was 10 ng/ml for both P_{flgA} and P_{fliC} activity (figure 50A). In contrast, for flagellar numbers an increased average of flagellar foci was seen for $flhDC_{(ec)}$ over $flhDC_{(ST)}$ with 9 foci per cell at 25 ng/ml anhydrotetracycline concentration (figure 50C). However, for $P_{tetAR,}$ the peak activity of flagellar gene expression for $flhDC_{(ec)}$ was 15000 RLU at 100 ng/ml anhydrotetracycline, while the same level of activity for $flhDC_{(ST)}$ was reached between 25 and 50 ng/ml (figure 50B). This is a 2-fold reduction in activity requiring significantly higher inducer concentrations. However, this equated to a 5-fold difference in the average flagella foci per cell when similar anhydrotetracycline concentrations were tested (figure 50D).

To conclude, the activity of flagellar gene expression and flagellar numbers per cell in titration assays show that $flhDC_{(ec)}$ and $flhDC_{(ST)}$ were clearly comparable, regardless which promoters were used to drive *flhDC i* (figure 50). This emphasises the difference but not the reciprocal changes in output because the efficiency of *flhDC* transcription is key in maintaining flagellar gene expression at a rate that sustains optimal flagellation.

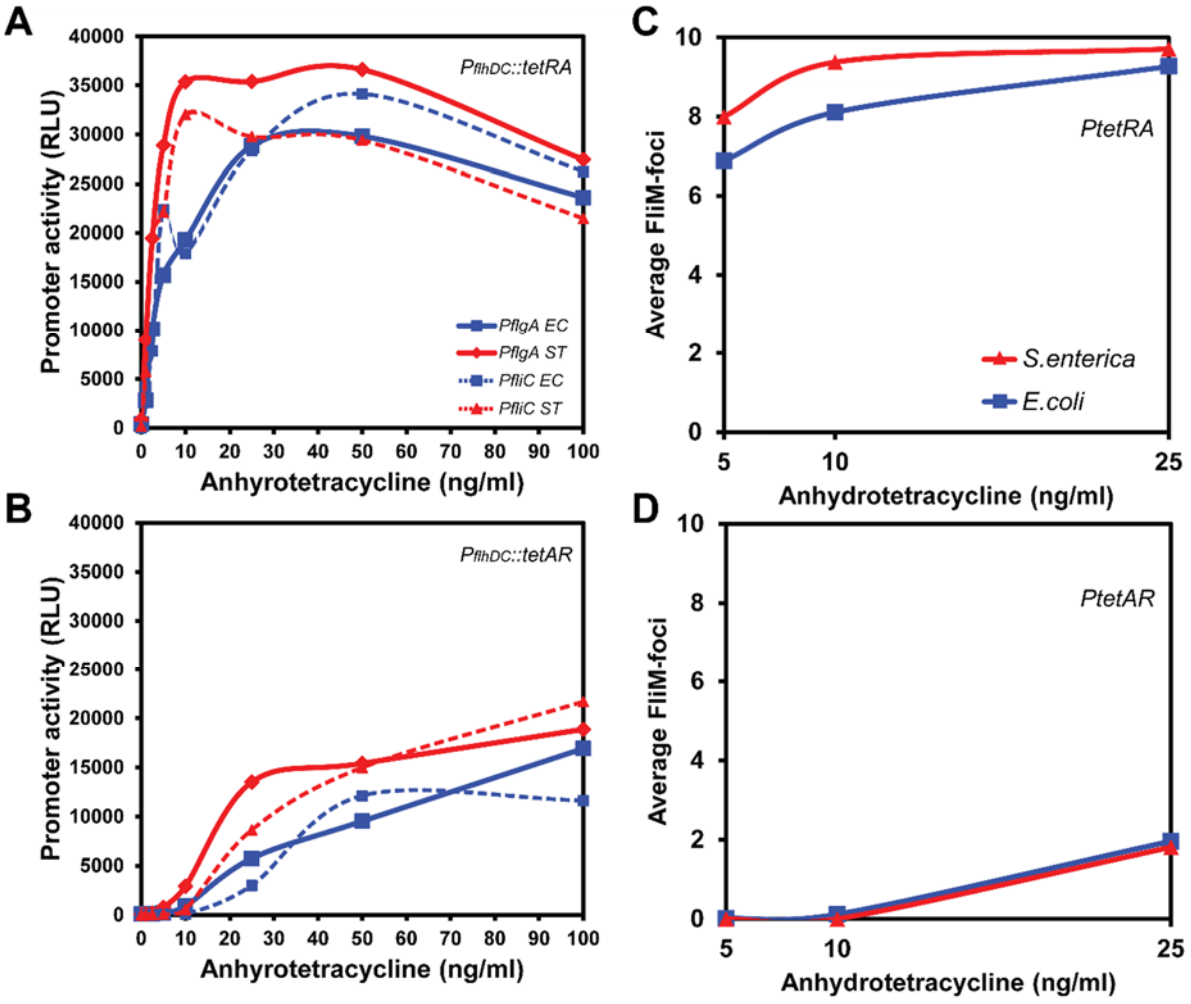


Figure 50. Titration of flagellar gene expression comparing *flhDC*$_{(ec)}$ and *flhDC*$_{(ST)}$ activity when expression is driven by P$_{tetRA}$ (A) or P$_{tetAR}$ (B). ST: *flhDC*$_{(ST)}$; EC: *flhDC*$_{(ec)}$. (C and D) The impact on flagellar numbers comparing *flhDC*$_{(ec)}$ and *flhDC*$_{(ST)}$ activity in terms of different concentrations for anhydrotetracycline. Average flagellar numbers were comparable for the two *flhDC* variants for each promoter variant. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment where (A and C) P$_{flhDC}$::*tetRA*/P$_{flgA}$EC= TPA4032, P$_{flhDC}$::*tetRA*/P$_{flgA}$ ST= TPA4050, P$_{flhDC}$::*tetRA*/P$_{fliC}$EC= TPA4031 and P$_{flhDC}$::*tetRA*/P$_{fliC}$ST= TPA4049. (B and D) P$_{flhDC}$::*tetAR*/P$_{flgA}$EC= TPA4099, P$_{flhDC}$::*tetAR*/P$_{flgA}$ST= TPA4053, P$_{flhDC}$::*tetAR*/P$_{fliC}$EC= TPA4098 and P$_{flhDC}$::*tetAR*/P$_{fliC}$ST= TPA4052.

6.3 Swapping *flhD* or *flhC* in *Salmonella enterica* (LT2) With *flhD or flhC* from *E. coli* (RP437)

At the top of the flagellar gene hierarchy is the *flhDC* operon. It consists of the two genes *flhD* and *flhC* (Bartlett *et al.*, 1988). FlhD and FlhC act together as the flagellar transcriptional activator (Kutsukake, 1997). At this point, all analysis has used a clean *flhDC* swap. Therefore, we inquired what happens with respect to motility and flagellar gene expression if substitution of either *flhD* or *flhC* from *E. coli* separately into *S. enterica* was performed. The two *S. enterica* strains created *flhD-S.enterica* with *flhC-E.coli (flhD(s)flhC(e)*) and *flhD-E.coli flhC-S.enterica (flhD(e)flhC(s))* were generated using the scarless mutagenesis technique (figure 51) (Blank *et al.*, 2011). In order to ensure the proper gene recombination, we analysed *flhDC* by sequencing to confirm 100% correct sequence integration.

6.3.1 Phenotypic Motility For flhD or flhC from E. coli RP437

We have investigated the strains *flhD(s)flhC(e)* and *flhD(e)flhC(s)* under control of the *Salmonella* P_{flhDC} and the tetracycline-inducible promoter system (figure 52). The single gene switch strains were compared to $flhDC_{(ec)}$ and $flhDC_{(ST)}$, through testing for motility with and without tetracycline. The results reveal, the average swim diameters varied significantly. For example, for P_{flhDC} dependent expression in terms of *flhD(e)flhC(s)* a significant increase compared to wild-type and $flhDC_{(ec)}$ was observed (P = 0.01). In contrast, for *flhD(s)flhC(e)*, the average swim diameter was dramatically decreased from the control (P = 0.001). For P_{tetRA}*, flhDC expression*, motility increased in all strains. However, *flhD(s)flhC(e)* was still noticeably impaired for motility.

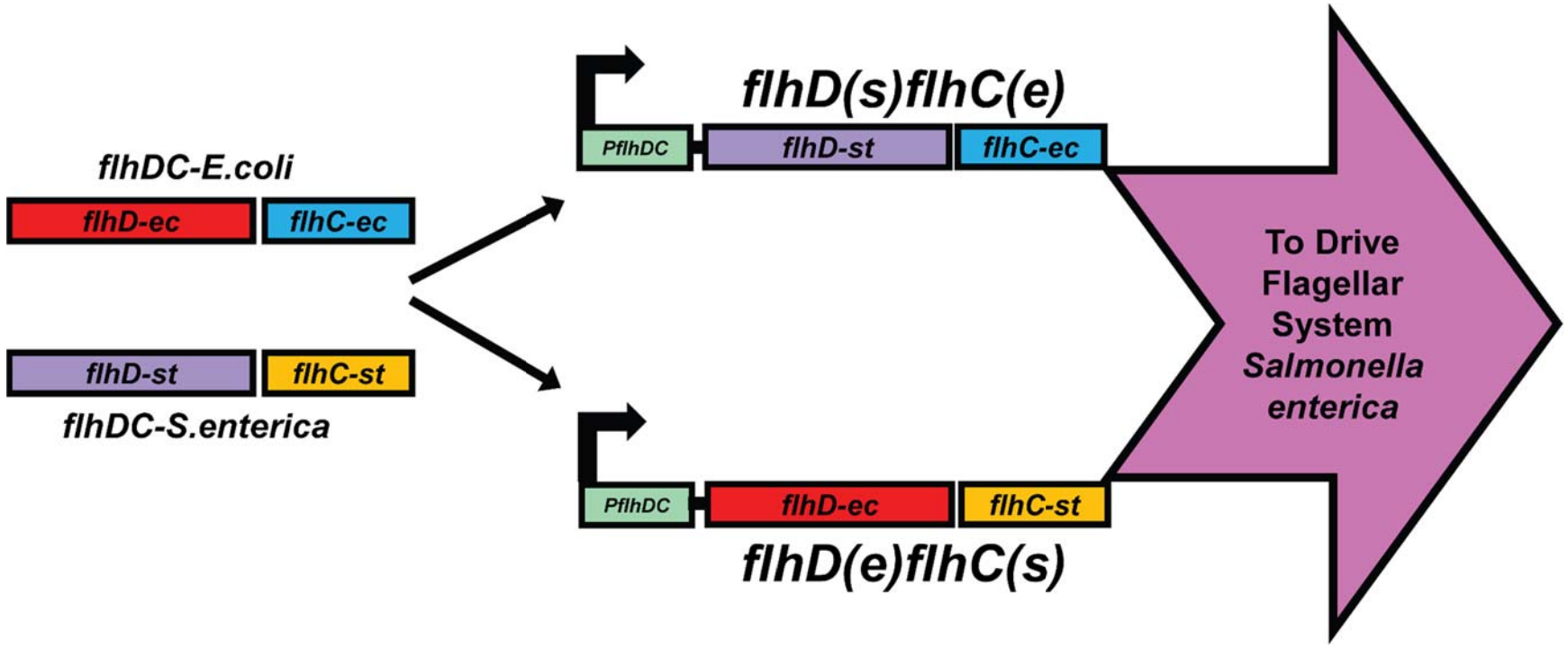
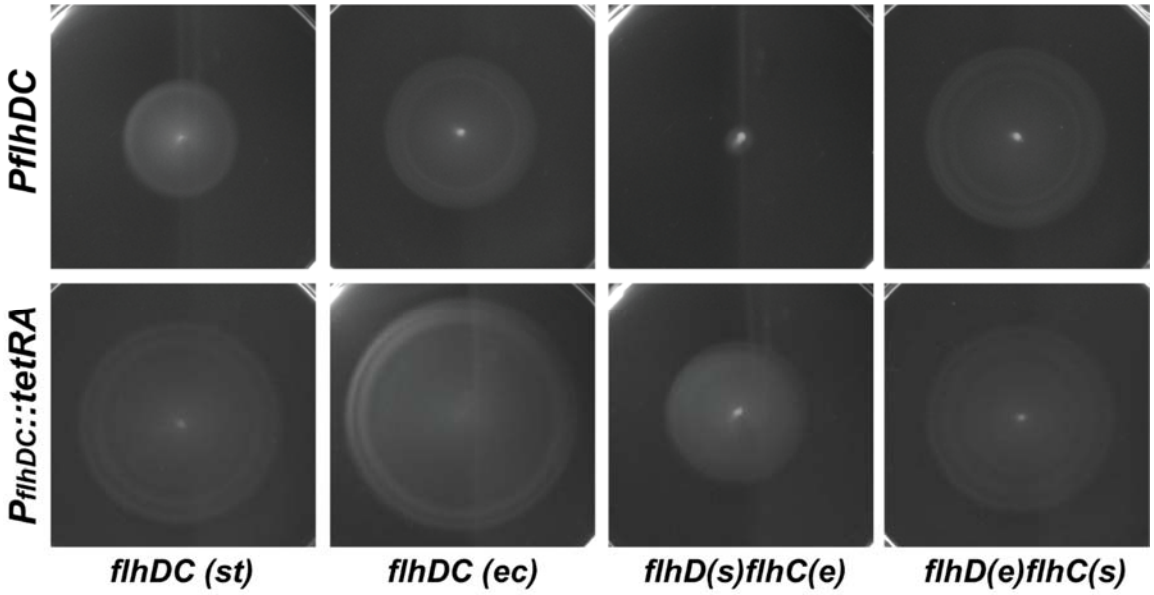


Figure 51 The *flhD(s)flhC(e) and flhD(e)flhC(s)* constructs were designed from *flhDC-E.coli* and *flhDC-S.enterica.* They were replaced into *S. enterica* (LT2) to look at the motility and flagellar gene expression output.

The generation of the combination complexes *flhD(e)flhC(s)* and *flhD(s)flhC(e)* altered the motility phenotype of *S. enterica* in an unexpected manner. It had been assumed that based on the $flhDC_{(ec)}$ and $flhDC_{(ST)}$ data that a comparable output would have been detected. Altering the operon structure of *flhDC* may have been on eexplanation for the observed outcome of these two single replacements. Especially as the strongest negative phenotype was for the construct replacing the second gene in the operon, *flhC*. Further investigation was required to define the mechanistic source of the observed reduction in motility relating to FlhC*(e)*.

6.3.2 Determination Of Class II & Class III Flagellar Gene Expression

At this point, we have compared flagellar gene expression for P_{flgA} and P_{fliC} in all strains of *S. enterica*: *flhD(s)flhC(e), flhD(e)flhC(s)* and *flhDC-E.coli(ec)* compared to wild-type. Astonishingly, we observed a stronger maximum increase for flagellar gene expression in the *flhD(e)flhC(s)* than $flhDC_{(ec)}$ when compared to wild-type (figure 53). However, *flhD(s)flhC(e)* exhibited a sharp decline in flagellar genes expression, consistent with its motility phenotype. Furthermore, with respect to P_{fliC} *flhD(s)flhC(e)* possessed a notably stronger reduced activity compared to P_{flgA} (figure 53B). These data indicate that introducing *flhD* from *E.coli* potentially increases flagellar gene expression and motility. In contrast, when introducing *flhC* from *E.coli* decreases P_{fliC} activity and negatively affects the phenotypic motility output. This leads to the conclusion that the *flhD(s)flhC(e)* combination is unable to drive the whole flagellar system efficiently. In contrast, the *flhD(e)flhC(s)* combination had a harmonious effect on the whole flagellar system of *S. enterica* producing a more robust output.



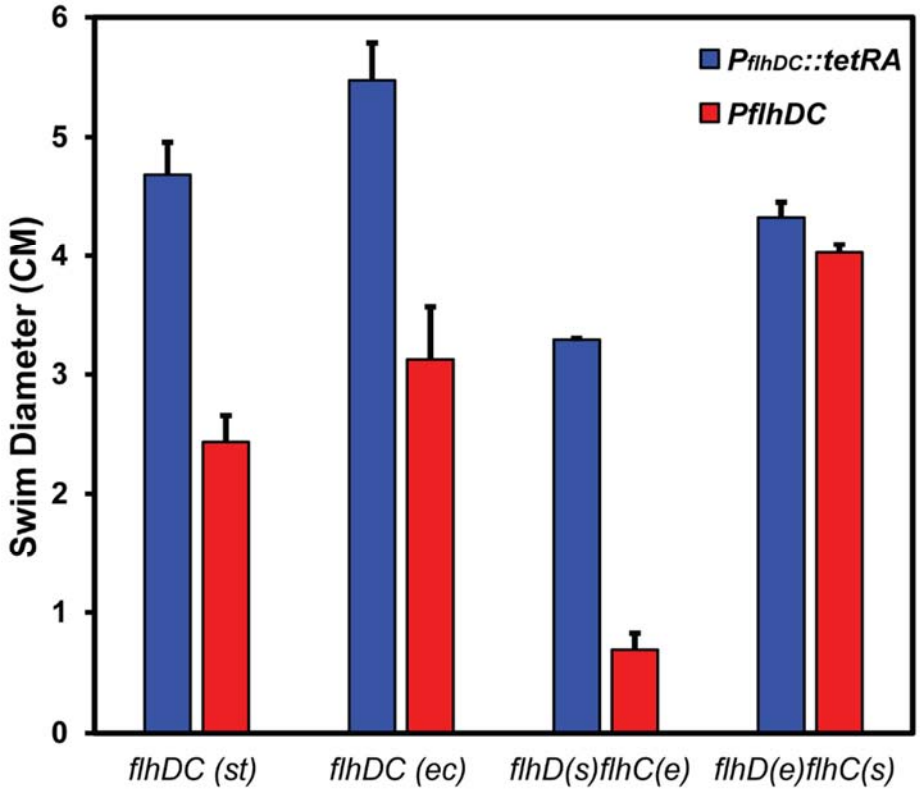


Figure 52. The comparison of the average swim diameter among *S. enterica* (st), *S. enterica* with *flhDC E.coli* (ec), *flhD(s)flhC(e)* and *flhD(e)flhC(s).* In terms of *PflhDC*, there was a dramatic drop for *flhD(s)flhC(e)* when compared to wild-type (st) while, *flhD(e)flhC(s)* was slightly increased. However, in P_{tetRA}, there is a significant variation between strains and still *flhD(s)flhC(e)* is less efficient that wild-type(st). Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment where, *flhDC(st)* = TPA1107, S.TP*flhDC*::*tetRA* = TPA4028, E.CP$_{flhDC}$ = TPA3997, E.CP*flhDC::tetRA* = TPA4022, *flhD(s)flhC(e)*P$_{flhDC}$ = TPA4128, *flhD(s)flhC(e)*P*flhDC*::tetRA= TPA4193, *flhD(e)flhC(s)*P$_{flhDC}$ = TPA4135 and *flhD(e)flhC(s)*P*flhDC*::tetRA= TPA4194.

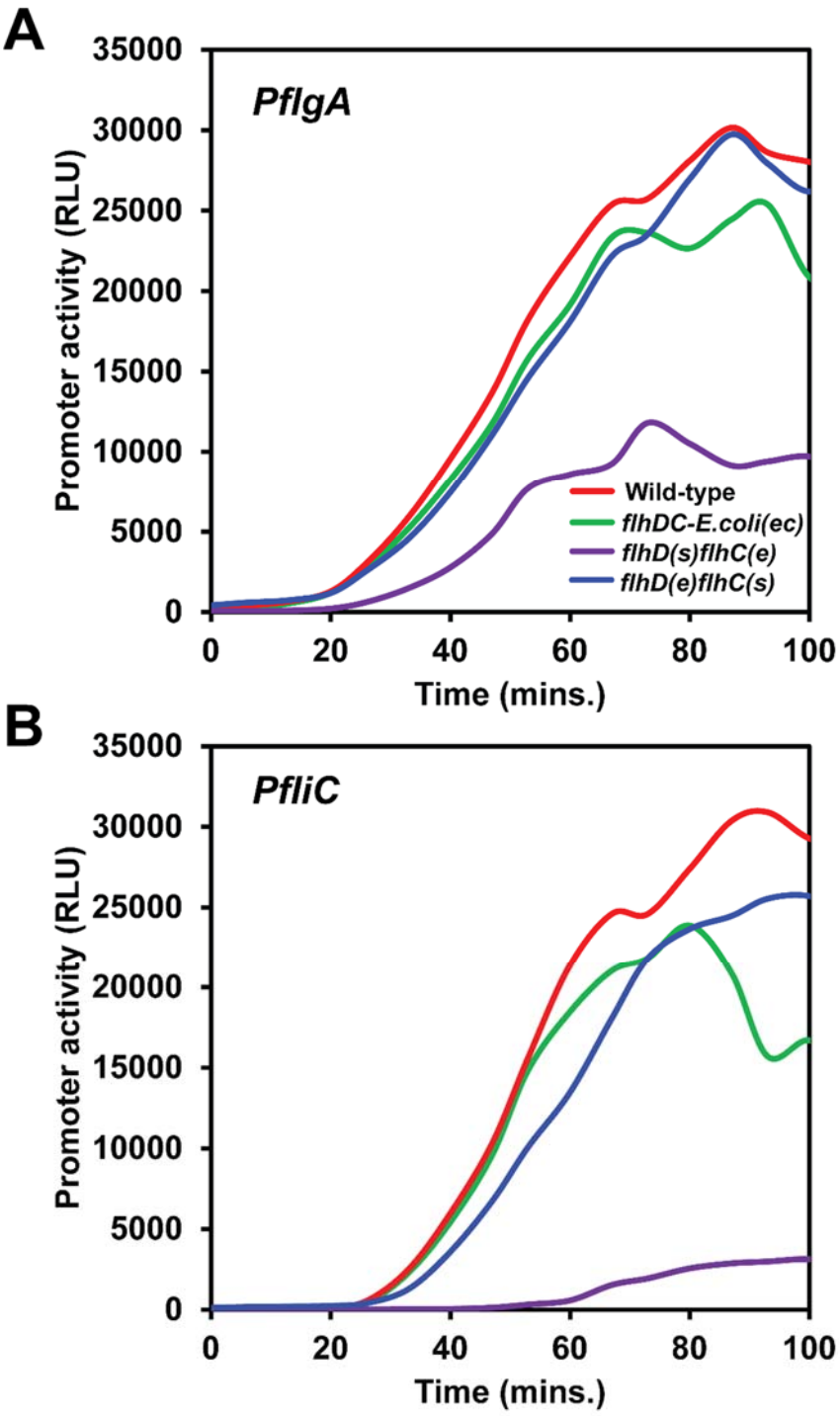


Figure 53. Kinetic comparison of class II (P_{flgA}) (A) and class III (P_{fliC}) (B) flagellar gene expression for *flhD(s)flhC(e), flhD(e)flhC(s), flhDC (ec)* compared to wild type. Reporter plasmids pRG51 and pRG39 were used to measure flagellar gen expression as in previous experiments. The data is consistent with the motility phenotypes. Interestingly for *flhD(s)flhC(e)* a further drop in P_{fliC} activity was measured in comparison to P_{flgA} activity. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were (A) P_{flgA} Wlid Type= TPA4050, P_{flgA} *flhDC(ec)*= TPA4032, P_{flgA} *flhD(s)flhC(e)*= TPA4215 and P_{flgA} *flhD(e)flhC(s)*= TPA4218. (B) P_{fliC} Wild Type= TPA4049, P_{fliC} *flhDC(ec)*= TPA4031, P_{fliC} *flhD(s)flhC(e)*= TPA4214 and P_{fliC} *flhD(e)flhC(s)*= TPA4217.

6.3.3 Titration Of Class II & Class III Flagellar Genes Expression

In order to manipulate the levels of flagellar gene expression all strains were tested using titration of P_{tetRA} activity using different concentrations of anhydrotetracycline. Interestingly, *flhD(e)flhC(s),* exhibited the highest relative activity for P_{flgA} (figure 54A). In contrast, the maximum activity of P_{fliC} expression was comparable for *flhD(e)flhC(s)* and $flhDC_{(ST)}$ (figure 54B).

With respect to *flhD(s)flhC(e)* strain, the P_{flgA} expression reached a peak at 100 ng/ml, but still was lower than the control at 60% maximum expression. This response is similar to the profile for Java, further supporting the proposed argument that *flhDC* in Java is less active and suggesting reduced activity for the *flhD(s)flhC(e)* combination. In contrast, however, very little P_{fliC} expression was observed for *flhD(s)flhC(e)* with a dramatic decrease (10%) even at 100ng/ml inducer compared to the controls (figure 55B).

Collectively, the ascending concentrations of anhydrotetracycline impacted flagellar gene expression magnitude in all strains especially in *flhD(s)flhC(e)*. This experiment has observed that generally, *flhD* and *flhC* from *E. coli* individually generate different outputs with respect to flagellar gene expression when combined with *flhD* or *flhC* from *S. enterica*. These differences are observed even though these proteins are over 94 % identical.

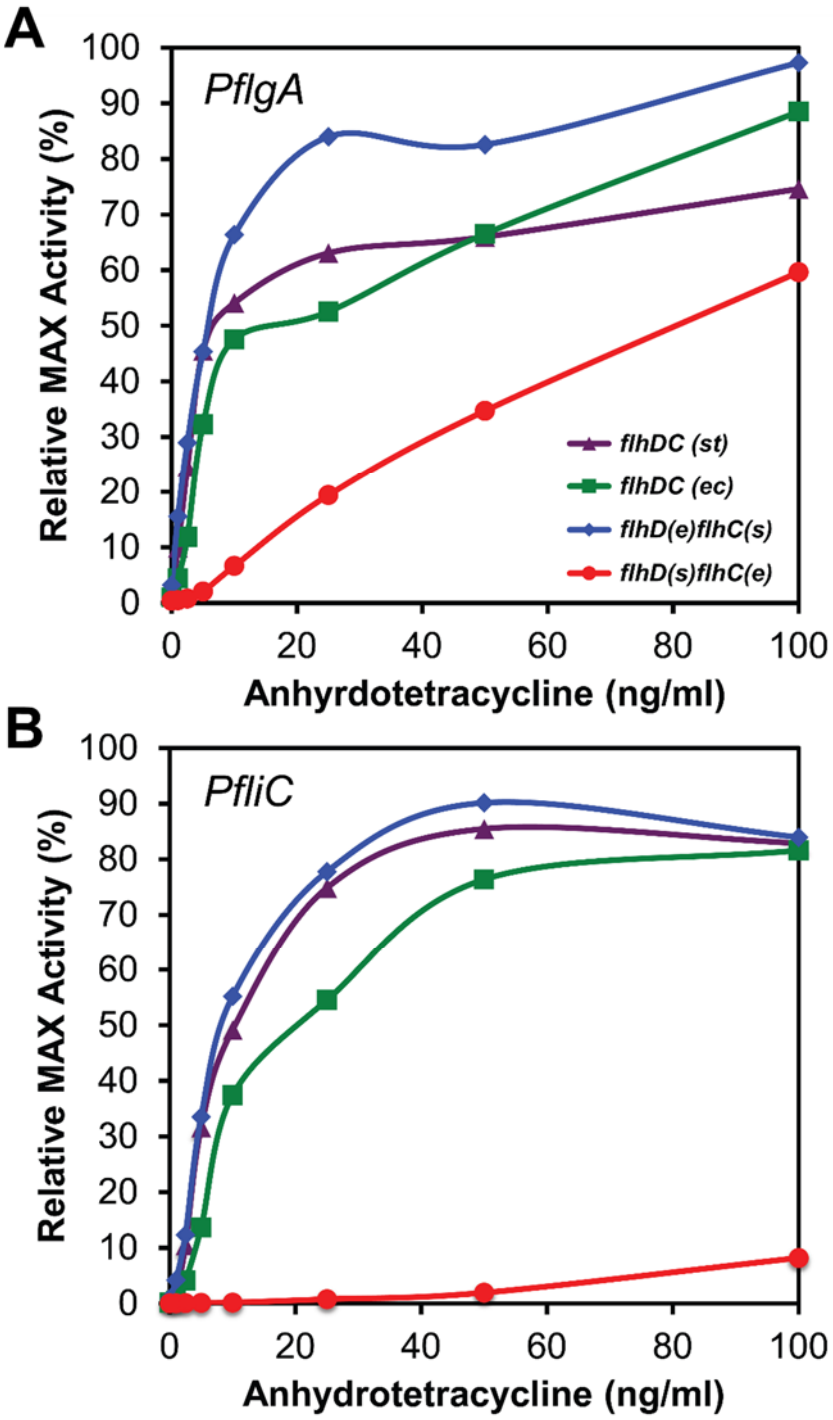


Figure 54. Titrated levels of flagellar genes expression for (A) P_{flgA} and (B) P_{fliC} between *flhD(s)flhC(e), flhD(e)flhC(s), flhDC*$_{(ec)}$ compared to wild-type. Surprisingly, the strongest response was associated with *flhD(e)flhC(s)* for both P_{flgA} and P_{fliC}. Interestingly, a stronger reduction in P_{fliC} was observed for *flhD(s)flhC(e)* that would have been predicted. Experiment represent a minimal of three independent repeats (n=3). Strains used in this experiment were (A) *flhDC(st)* = TPA4050, *flhDC(ec)*= TPA4032, *flhD(s)flhC(e)*= TPA4215 and *flhD(e)flhC(s)*= TPA4218. (B) *flhDC(st)* = TPA4049, *flhDC(ec)*= TPA4031, *flhD(s)flhC(e)*= TPA4214 and *flhD(e)flhC(s)*= TPA4217.

6.3.4 Impact Of Promoters Source On Flagella Abundance Output.

The FliM protein was visualized under the microscope exploiting the FliM-GFP reporter fusion (Aldridge *et al.*, 2006a). All strains were screened under the fluorescent microscope at mid log phase (OD_{600} = 0.6). All pictures were analysed for flagellar foci per cell using MicrobeTracker. With regards to the *flhDC* promoter, the proportion of the population possessing flagellar foci for *flhD(s)flhC(e)* strain was clearly decreased in particular compared to other strains, where they were similar (figure 55). In contrast, with respect to P_{tetRA}, all strains and wild-type expressed FliM-GFP foci fivefold higher than P_{flhDC} strains (figure 55B). Astonishingly, flagellar abundance of all strains regardless of the promoter were relatively comparable with the average motility diameter (figure 52).

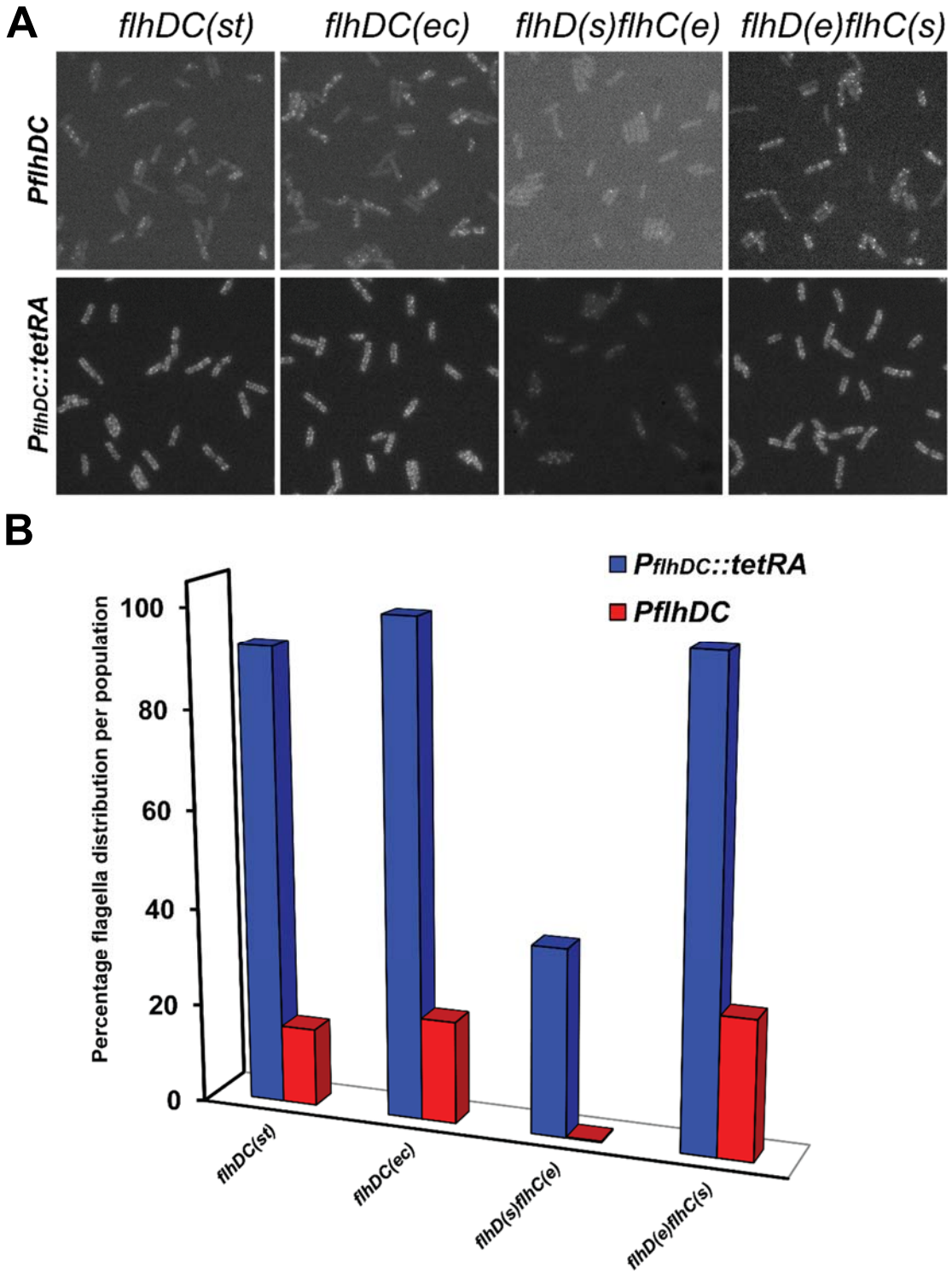


Figure 55. Comparison of *flhD(s)flhC(e), flhD(e)flhC(s), flhDC(ec)* and wild-type based on (A) the number of FilM-GFP foci per cell and (B) the flagellated population. There were similar responses for *flhD(e)flhC(s), flhDC*$_{(ec)}$ and wild-type in terms of P$_{flhDC}$ and P$_{tetRA}$. While, for *flhD(s)flhC(e)* was significantly decreased in respect of P$_{flhDC}$ but, *tetRA* promoter did increase the percentage of cells that had flagellar foci. Note: the data was presented as a 3D bar chart to display and compare results of the *flhD(s)flhC(e)* strain. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, S.T P$_{flhDC}$ = TPA1107, S. P$_{flhDC}$ ::*tetRA* = TPA4028, E.C P$_{flhDC}$ = TPA3997, E.C P$_{flhDC}$ *::tetRA* = TPA4022, *flhD(s)flhC(e)* P$_{flhDC}$ = TPA4128, *flhD(s)flhC(e)* P$_{flhDC}$ ::tetRA= TPA4193, *flhD(e)flhC(s)* P$_{flhDC}$ = TPA4135 and *flhD(e)flhC(s)* P$_{flhDC}$ ::tetRA= TPA4194.

6.4 Summary

In this chapter, we posed a question regarding the changing behaviours of the S. enterica flagellar system when driven by $FlhD_4C_2$ from *E. coli* versus *S. enterica*. The intent was to measure the impact of this genetic modification had upon flagellated cellular population present under fast growth control. Where up to 15% of the *S. enterica* population did not have a flagellum, previous data suggests *E.coli* produces are more homogenous response. As *E. coli* and *S. enterica* are very similar genetically, we asked what will happen in *S.enterica* if *flhDC* from *E.coli* replaced the *S.enterica* coding regions*?*

These genetic manipulations have allowed for a comparison of the motility phenotypes and flagellar gene expression between differing *flhD* and *flhC* combinations. The motility phenotypes were not significantly different for $flhDC_{(ec)}$ versus wild-type regardless of the power of promoter driving *flhDC* expression. Measuring flagellar gene expression showed a slight difference, especially compared to wild-type if *flhDC* transcription was low, via P_{tetAR} driven trasncription. However, the number of flagellar per cell in fast and slow growth control were comparable. Importantly we still observed a fraction of cells producing no flagella. This suggested that the control of the flagellar system leading to a marked non-motile sub-population was not strictly dependent on the activity of the $FlhD_4C_2$ complex. We know that the activity of this complex is dictacted by transcriptional, translationa dnpost-transcriptional regulatory inputs. For example, there is strong evidence that protein stability and its regulation via YdiV/ClpXP help drive population heterogeneity in the *S. enterica* system (Koirala *et al.*, 2014). The data presented here argues, however, that the generation for the non-motile subpopulation is via a system that is unable to differentiate the source of *flhDC*. Even though protein stability is a feasible argument

the data was unable to delineate whether the regulation leading to this response was via transcription of *flhDC*, a second input pathway that would not directly differentiate between *flhDC* from *E. coli* versus *S. enterica*.

With respect to motility, the *flhC(e)* strain exhibited a dramatic change. Flagellar gene expression for all strains also reflected the motility phenotype. The *flhD(e)* behaved like wild-type for P_{flgA} or P_{fliC} expression, while, *flhC(e)* was remarkably decreased especially for P_{fliC} expression.

Taken together, we were surprised especially when comparing the motility phenotype, flagellar number and flagellar gene expression for all strains with wild-type. Having observed these difference between the strains activity, we expect that the FlhD*(e)* has interacted properly with FlhC*(s)* and produced a robust $FlhD_4C_2$ complex with the capability to drive the flagellar system in the right way. In contrast, for FlhC*(e)* we predict has not interacted well with FlhD*(s)* and the consequence produces an unstable complex or a complex with reduced $FlhD_4C_2$ activity both models, however, impact negatively on the flagellar system output. One explanation for the observed reduction in activity of this one complex will be investigated in chapter 7. Namely the direct ability of $FlhD_4C_2$ combinations to recognise a *S. enterica* class II $FlhD_4C_2$ DNA binding site.

The operon structure and its impact on translation of *flhDC* cannot be ruled out as a potential source of the reduced activity of the *flhD(s)flhC(e)* combination. For example, this specific construct has had the second gene of the operon replaced. It is plausible that this destablises the *flhDC* transcript, while the *flhD* switch has stabilised the RNA. Testing RNA stability of the constructs was beyond the scope of the project, however, these are feasible additional experiments to compliment the flagellar system output assays performed. Furthermore, complimenting RNA experiments with

the protein work to be presented in chapter 7 would provide a fuller picture of the impact of the genetic manipulations.

Chapter Seven: Protein-Protein Interaction

7.1 Introduction

In this chapter, we focus on how the FlhD and FlhC proteins from *E. coli* and *S. enterica* interact. This study follows the hypothesis generated in chapter six arguing that the low activity observed for *flhD(s)flhC(e)* is a result of an unstable $FlhD_4C_2$ complex being formed. The hypothesis is derived from the motility phenotype and flagellar gene expression output driven by *flhD(s)flhC(e)* in comparison to the combinations: $flhD_{(s)}flhC_{(e)}$, $flhDC_{(ec)}$ and $flhDC_{(st)}$. Furthermore, the data with respect to motility and flagellar gene expression for serovars such as Java agrees with the *flhD(s)flhC(e)* exhibiting low activity.

In order to determine *in vitro* $FlhD_4C_2$ combinations functionality, we cloned the four *flhDC* operons into the expression plasmid pET-28a. This allowed us to overexpress the $FlhD_4C_2$ complexes, purify them and after assess complex isolation and function (figure 56). Apart from His-tag based isolation of complexes, complex integrity and function was assessed using a second purification method. Heparin column purification was used to mimic the structure of DNA allowing functional complex isolation. An electrophoretic mobility shift assay (EMSA) was used to evaluate $FlhD_4C_2$ functionality using the *flgAB* promoter region after obtaining each pure complex. This chapter will provide strong supporting evidence for our hypothesis that FlhD and FlhC from *E. coli* have different interaction properties when combined with FlhD or FlhC from *S. enterica*. Furthermore, DNA binding affinities suggest a difference between *E. coli* and *S. enterica* correlates with the source of FlhC and flagellar gene expression profiles.

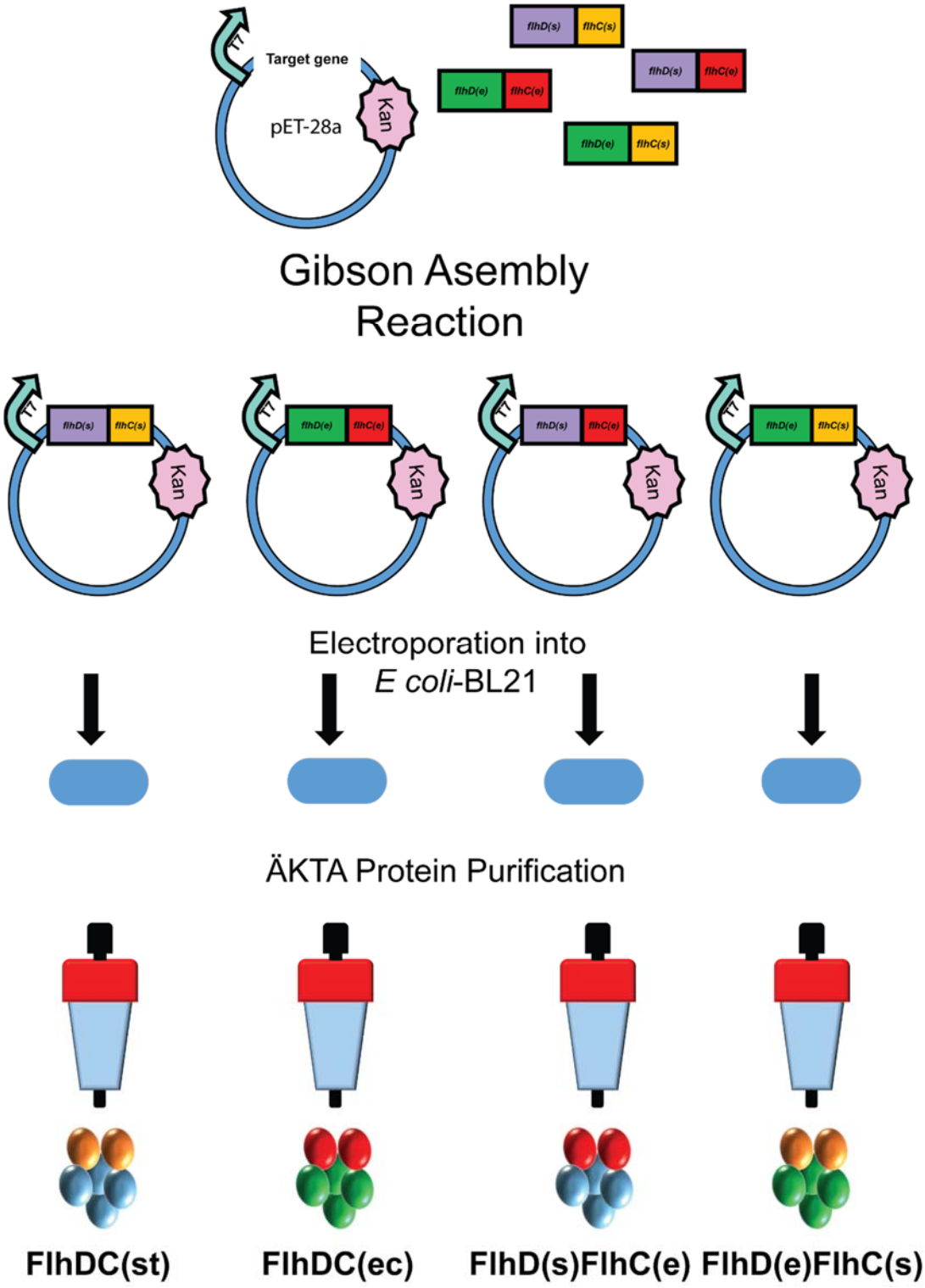


Figure 56. Schematic diagram for the stages of purification of $FlhD_4C_2$ complexes. The process starts with cloning the *flhDC* operons into pET28a. Transformation into BL21 allowed for overexpression and processing to get the pure protein at the last stage by using ÄKTA protein purification systems. Strains used in this experiment were, FlhDC(st) = TPA640, FlhDC(ec) = TPA4594, FlhD(s)FlhC(e)=TPA4592 and FlhD(e)FlhC(s)=TPA4593.

7.2 Over-Expression and Protein Purification

7.2.1 Biochemical description of Protein-Protein Interactions

We inserted *flhD(s)flhC(e)*, *flhD(e)flhC(s)*, *flhDC*$_{(ec)}$ and *flhDC*$_{(st)}$ DNA fragments into pET28a plasmid using Gibson assembly. All constructs were confirmed for the precise insertion by restriction digest and sequencing analysis. The diagnostic digests verified the *flhDC* fragment inserted into the the pET28a plasmid using BamHI and SacI enzymes, which have restriction sites outside the *flhDC* fragment (figure 57).

All plasmids were electroporated into the protein expression *E. coli* strain BL21. All strains were grown in LB media with IPTG induction. Growth and expression was monitored using samples at 90 and 180 minutes to identify the ideal time, which gave high levels of FlhD and FlhC expression (figure 58). All inductions were compared to the strains left un-induced as a negative control (figure 58). On induction, the FlhD and FlhC proteins were produced at 90 minutes after IPTG add induction and reached suitable levels of expression at 3 hours. Each of the *flhDC*$_{(st)}$*, flhDC*$_{(ec)}$ and *flhD(e)flhC(s)* cells produced identifiable bands for FlhC (22kDa) and FlhD (13kDa) respectively (figure 58). However, for *flhD(s)flhC(e)* only the FlhD protein was observed while, the FlhC protein was not detected by SDS-PAGE-gel. In contrast, the control samples did not produce bands of similar sizes to the induction samples (figure 58).

These findings suggest that *flhDC*$_{(st)}$*, flhDC*$_{(ec)}$ and *flhD(e)flhC(s)* are being expressed effeciently and these proteins exhibited similar predicted sizes as indicated in previous studies (Campos and Matsumura, 2001). In contrast, the *flhD(s)flhC(e)* construct is either not being expressed correctly or FlhC is degraded compared to the other constructs. FlhD and FlhC are targets of rapid protein

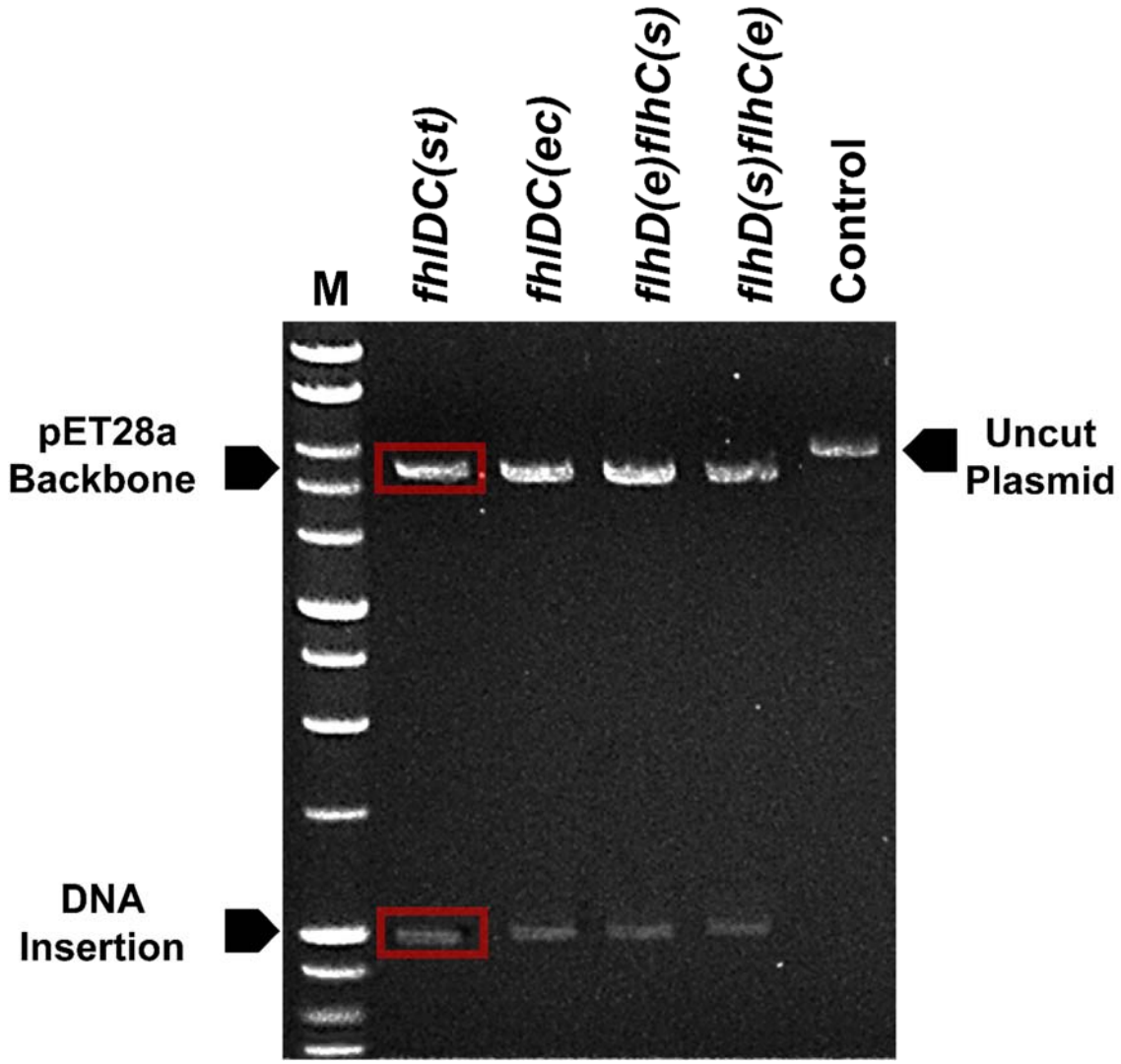


Figure 57. The Restriction digestion gel image includes a1kb DNA ladder (lane1). The digested pET28plasmid has total backbone size of 5.3kb with 0.9kb each of *flhDC*$_{(st)}$, *flhDC*$_{(ec)}$, *flhD(e)flhC(s)* and *flhD(s)flhC(e)* (lane 2 to 5 respectively). The plasmid was digested with 2 specific enzymes (BamHI and SacI). Lane 6 comprising of uncut plasmid (pET28a with *flhDC* fragment) representative intact plasmid.

degradation (Tomoyasu *et al.*, 2003). This data, although suggesting a weak interaction, cannot rule out that FlhC expression is causing the observed phenotype in *S. enterica.* It was decided to continue with purification as a means of defining which of these possibilities is the stronger explanation for reduced FlhD(s)FlhC(e) activity.

7.2.2 Purification Of FlhDC$_{(st)}$, FlhDC$_{(ec)}$, FlhD(s)FlhC(e) and FlhD(e)FlhC(s)

The *flhDC*$_{(st)}$*, flhDC*$_{(ec)}$*, flhD(s)flhC(e)* and *flhD(e)flhC(s*) constructs were tagged with N-terminal His-x6 protein sequence to *flhD* allowing for Nickel affinity (Hi-Trap) purification (Aldridge *et al.*, 2010). The Hi-Trap column has an ability to bind directly with the histidine (x6) protein sequence, trapping any tagged protein. The $FlhD_4C_2$ complexes were purified and eluted in 2 ml fractions. Elution profiles were recorded using absorbance at 280 nm (figure 59 to 62). In terms of *flhDC*$_{(st)}$*,* fractions 18-27 were the highest absorbance peak and compared to clear protein bands when loaded on Tricine SDS-PAGE-gels (figure 59). However, for *flhDC*$_{(ec)}$ and *flhD(e)flhC(s*), the highest absorbance peak shifted to fractions 15-20 confirmed by FlhD and FlhC visualisation on the Tricine SDS-PAGE-gel (figures 60 and 61). In contrast, *flhD(s)flhC(e)* eluted between fractions 20-24 comparable to Tricine SDS-PAGE-gel analysis (figure 62). On normalising to protein concentration the differences between purified complexes were compared to two concentrations (figure 63). For *flhD(s)flhC(e)* production, FlhC was observed but at reduced levels compared to the other three complexes. This suggests the *flhD(s)flhC(e)* does produce the $FlhD_4C_2$ complex but FlhC levels are much lower. This assay cannot differentiate between low expression and complex stability.

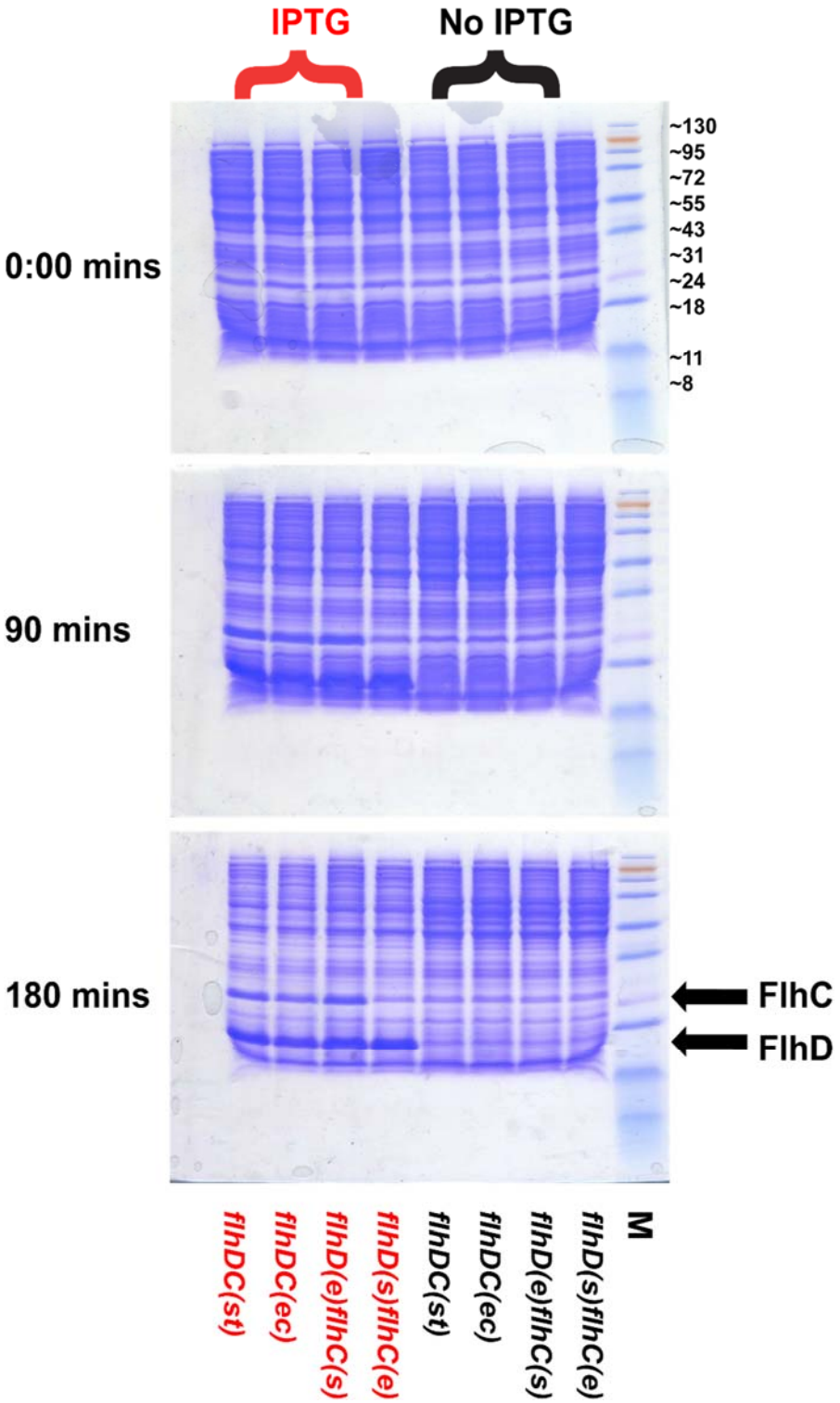


Figure 58. Expression test for the four-*flhDC* operons. Total protein samples were separated by SDS-PAGE-gel and stained with Coomassie brilliant blue. Cells lysates for each operon *flhDC*$_{(st)}$, *flhDC*$_{(ec)}$, *flhD(e)flhC(s)* and *flhD(s)flhC(e)* after adding IPTG inducer and sampling at three times 0, 90 and 180 minutes respectively. The same cells were used without adding the IPTG inducer and considered as a negative control. M: protein marker. Strains used in this experiment were, *flhDC*(st) = TPA640, *flhDC*(ec) = TPA4594, *flhD*(s)*flhC*(e)=TPA4592 and *flhD*(e)*flhC*(s)=TPA4593.

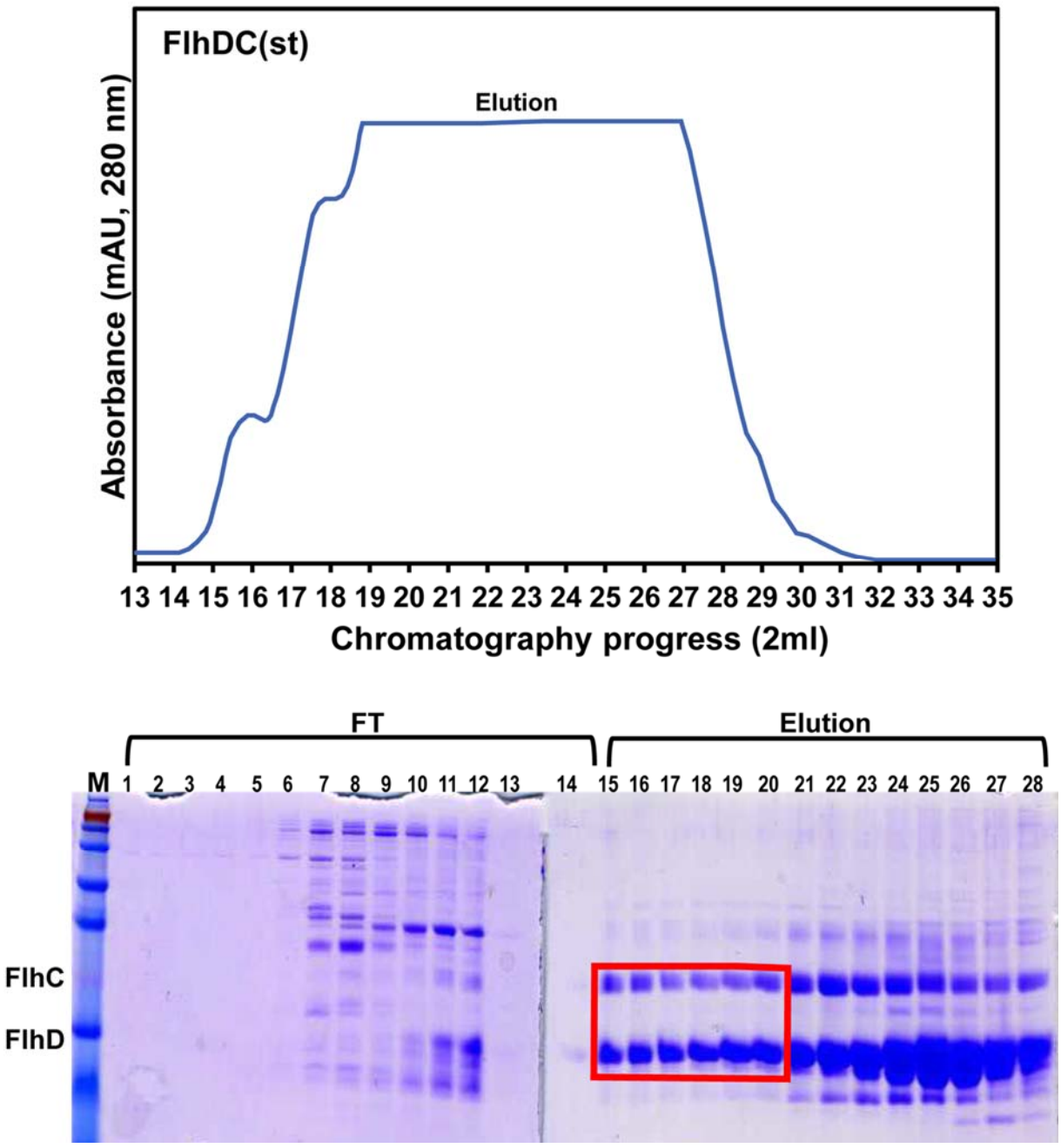


Figure 59. Purification of FlhC and FlhD from *flhDC*$_{(st)}$ using Nickel affinity column chromatography (Hi-Trap). Fractions 18-27 showed the highest peak at the absorbance λ=280 nm. All fractions were loaded on the Tricine SDS-PAGE gel (12 %) and visualized. M: protein markers. FT: flow-through. Experiment represents a minimal of three independent repeats (n=3). The strain used in this experiment was, FlhDC(st) = TPA640.

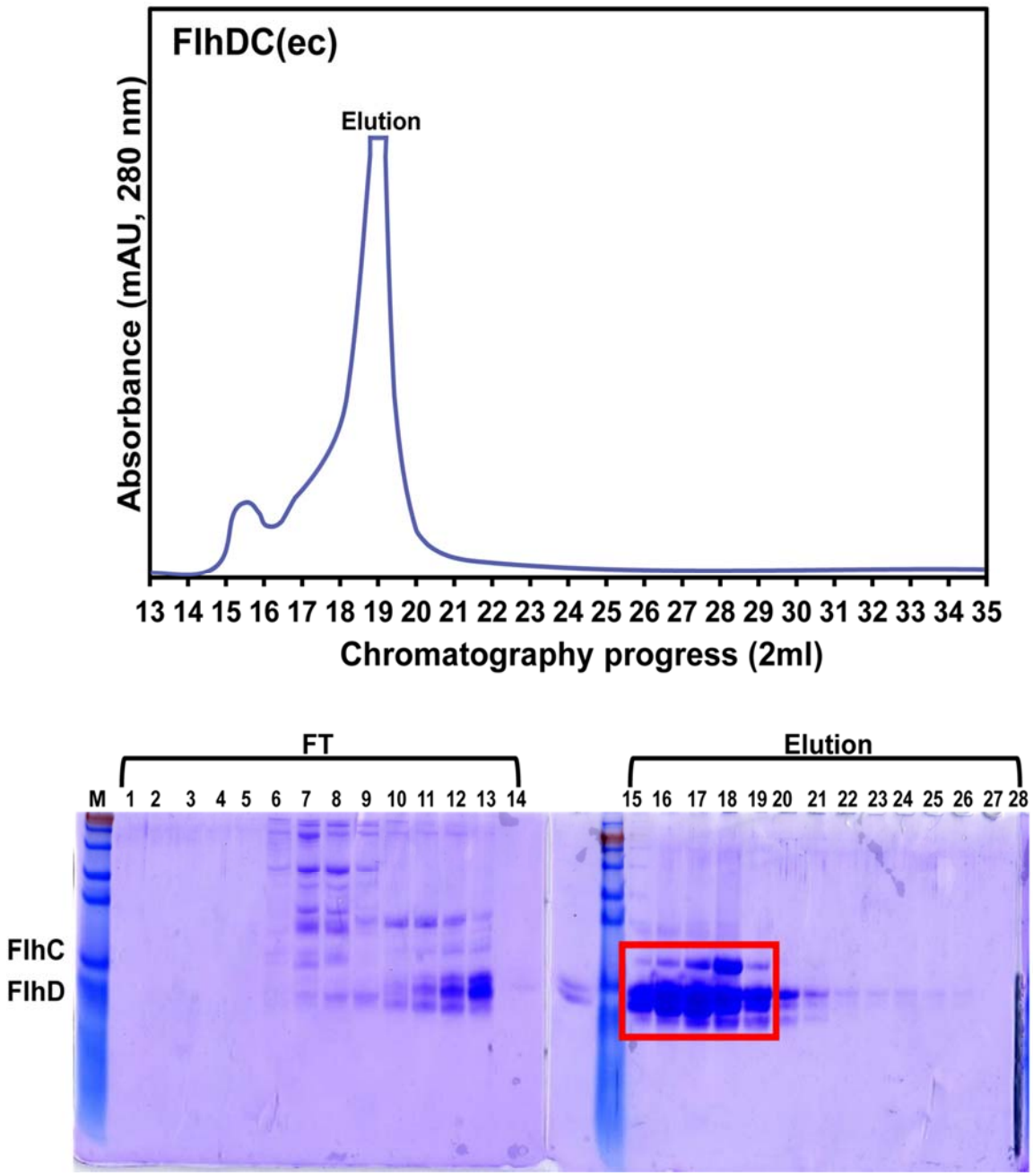


Figure 60. Purification of FlhC and FlhD from *flhDC*$_{(ec)}$ using Nickel affinity column chromatography (Hi-Trap). Fractions 15-19 showed the highest peak at the absorbance λ=280 nm. All fractions were loaded on the Tricine SDS-PAGE gel (12 %) and visualized. M: protein markers. FT: flow-through. The strain used in this experiment was, FlhDC(ec) = TPA4594

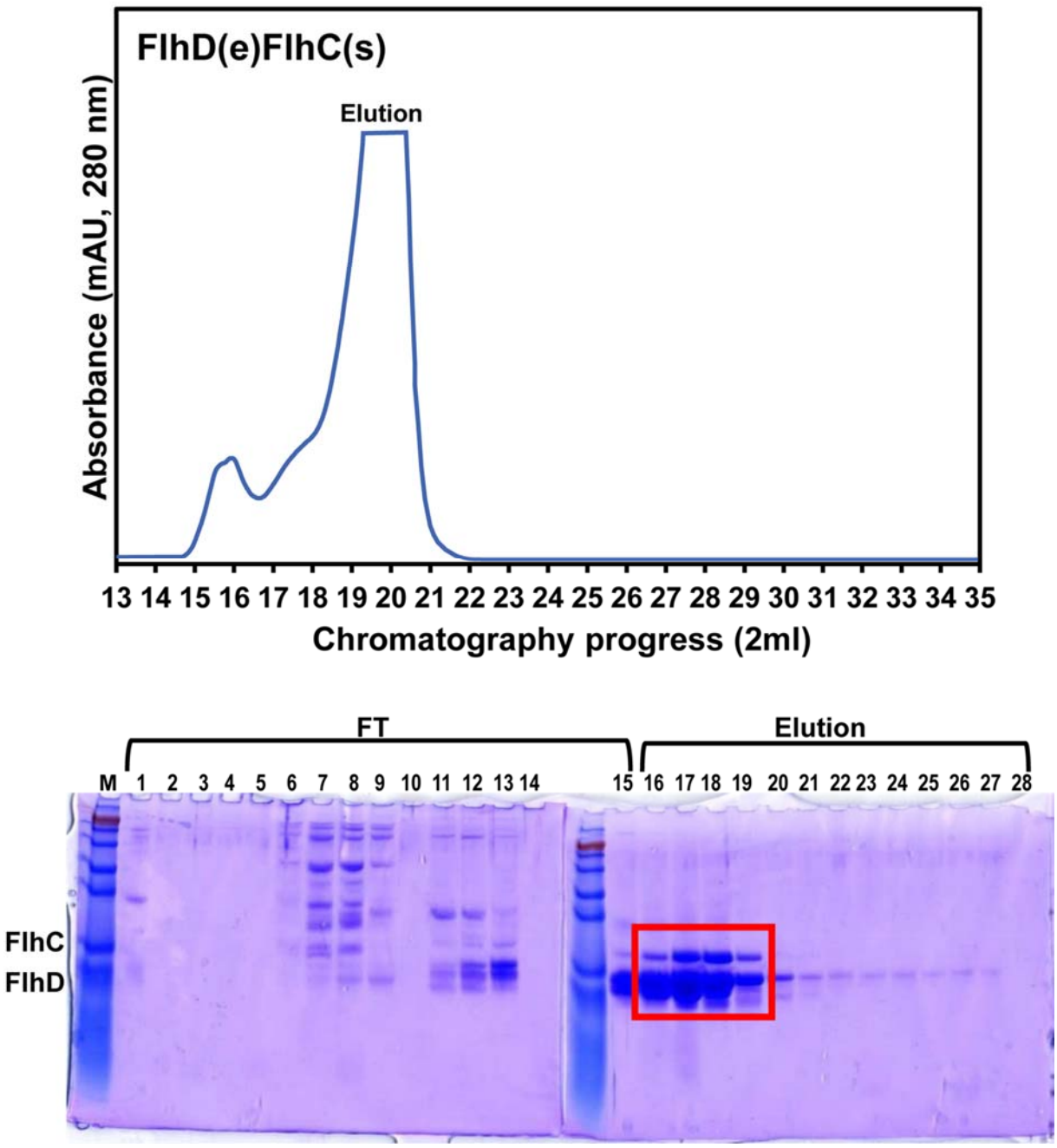


Figure 61. Purification of FlhC and FlhD from *flhD(e)flhC(s)* using Nickel affinity column chromatography (Hi-Trap). Fractions 16-19 showed the highest peak at the absorbance λ=280 nm. All fractions were loaded on the Tricine SDS-PAGE gel (12 %) and visualized. M: protein markers. FT: flow-through. Elution section indicates the fractions comprising of FlhC (22kDa) and FlhD(13kDa). Experiment represents a minimal of three independent repeats (n=3). The strain used in this experiment was, FlhD(e)FlhC(s) = TPA4593.

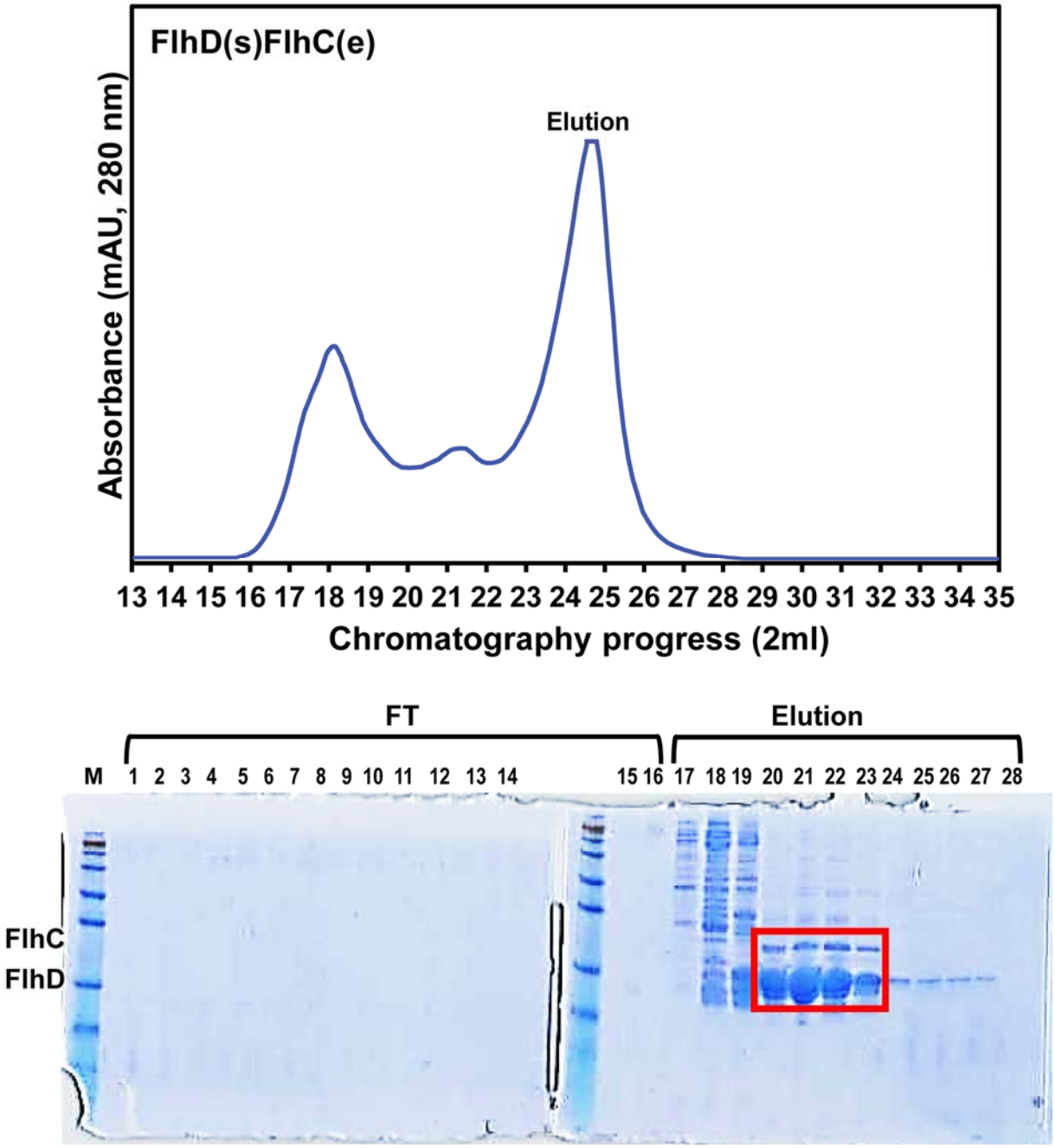


Figure 62. Purification of FlhC and FlhD from *flhD(s)flhC(e)* using Nickel affinity column chromatography (Hi-Trap). Fractions 20-24 showed the highest peak at the absorbance λ=280 nm. All fractions were loaded on the Tricine SDS-PAGE gel (12 %) and visualized. M: protein markers. FT: flow-through. Elution section indicates the fractions comprising of FlhC (22kDa) and FlhD(13kDa). Experiment represents a minimal of three independent repeats (n=3). The strain used in this experiment was, FlhD(s)FlhC(e) = TPA4592

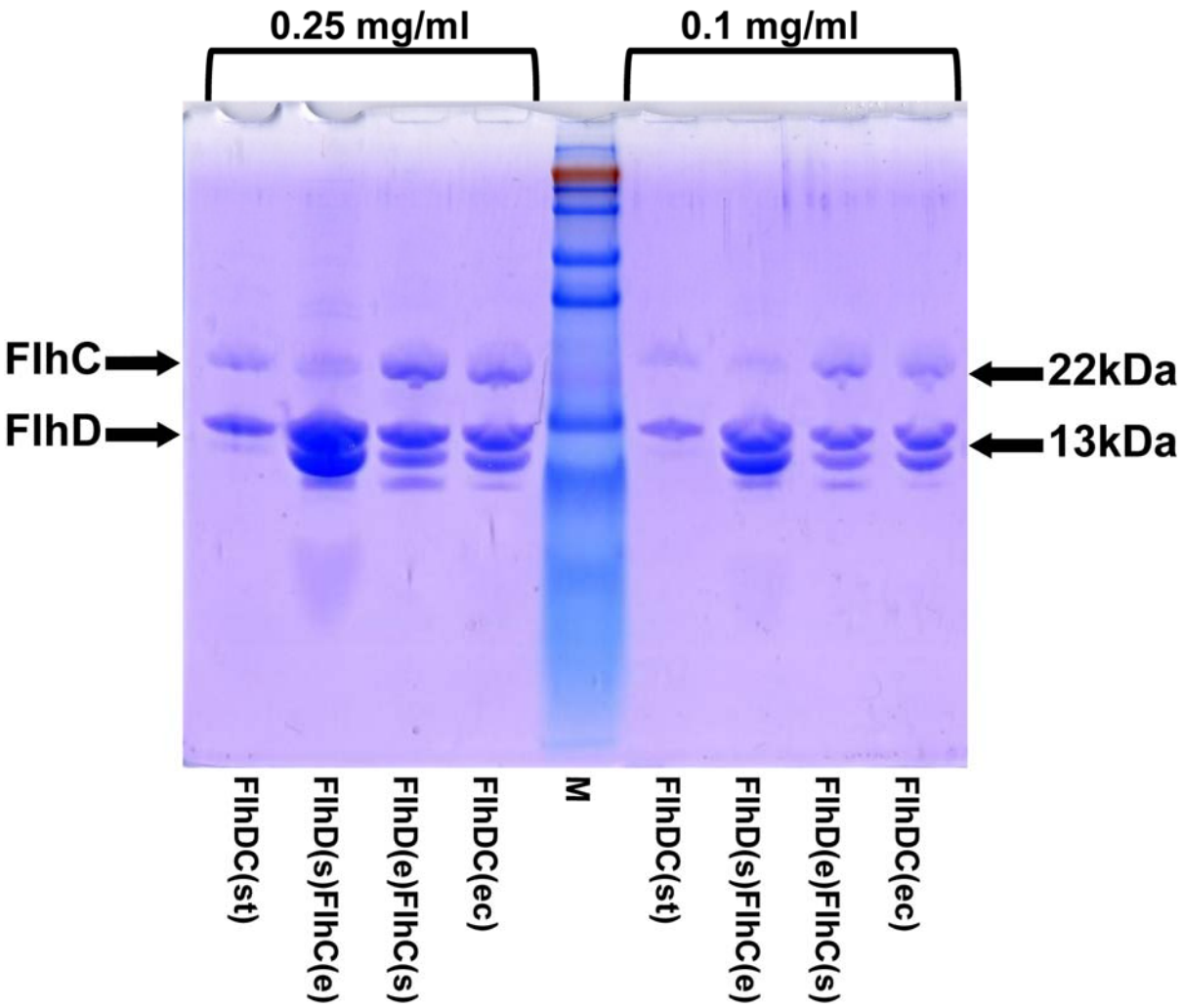


Figure 63. Comparison of normalized protein samples for complexes: $FlhDC_{(st)}$, $FlhDC_{(ec)}$, FlhD(e)FlhC(s) and FlhD(s)flhC(e) by using two concentrations 0.25 and 0.1 mg/ml. The protein signal for FlhC (22 kDa) and FlhD (13 kDa) are higher at 0.25mg/ml concentration than in 0.1 mg/ml. M: protein marker to identify the precise molecular weight of samples. Strains used in this experiment were, FlhDC(st) = TPA640, FlhDC(ec) = TPA4594, FlhD(s)FlhC(e)=TPA4592 and FlhD(e)FlhC(s)=TPA4593.

7.3 Examination Binding Activity For The $FlhD_4C_2$ Mutants Protein

7.3.1 Protein-DNA binding assay by using Heparin column

In order to screen $FlhD_4C_2$ functional activity through the ability to bind DNA, we used Heparin affinity chromatography column purification (Liu and Matsumura, 1994; Aldridge *et al.*, 2010). Heparin has for some time been considered as a DNA substitute, having a high-affinity to DNA binding proteins. Therefore, the mechanism of DNA interacting proteins with heparin gives us an indication about the functionality of the protein or complex via its ability to be captured in-vitro (Pooncharoen, 2013).

We eluted *flhDC*$_{(st)}$*, flhDc*$_{(ec)}$*, flhD(e)flhC(s)* and *flhD(s)flhC(e)* samples through ÄKTA based Heparin purification. The results were visualized on SDS-PAGE-gel using Coomassie brilliant blue stain. With respect to *flhDC*$_{(st)}$*, flhDc*$_{(ec)}$ and *flhD(e)flhC(s)*, all three complexes possessed a significantly similar Heparin elution pattern leading to the isolation of $FlhD_4C_2$ complexes (figures 64 to 66). However, for the *flhD(s)flhC(e)* sample, the Heparin column failed to bind significant quantities of the $FlhD_4C_2$ complex and as a consequence the FlhD and FlhC bands were not clear compared to other 3 combinations (figure 67).

Comparison of Histidine versus Heparin elution further exemplifies the characteristics of the $FlhD_4C_2$ complexes especially for *flhD(s)flhC(e)* (figure 68). Normalisation of protein concentration provided evidence to suggest weak or no isolation of the FlhD(s)FlhC(e) complex using Heparin purification. This suggests our hypothesis is feasible as the complex can be purified, at a low yield, using Histidine (x6) purification. In contrast, $FlhD_4C_2$ purified from *flhDC*$_{(ec)}$ and *flhD(e)flhC(s)* using Heparin showed strong recovery of FlhD and FlhC (figure 68).

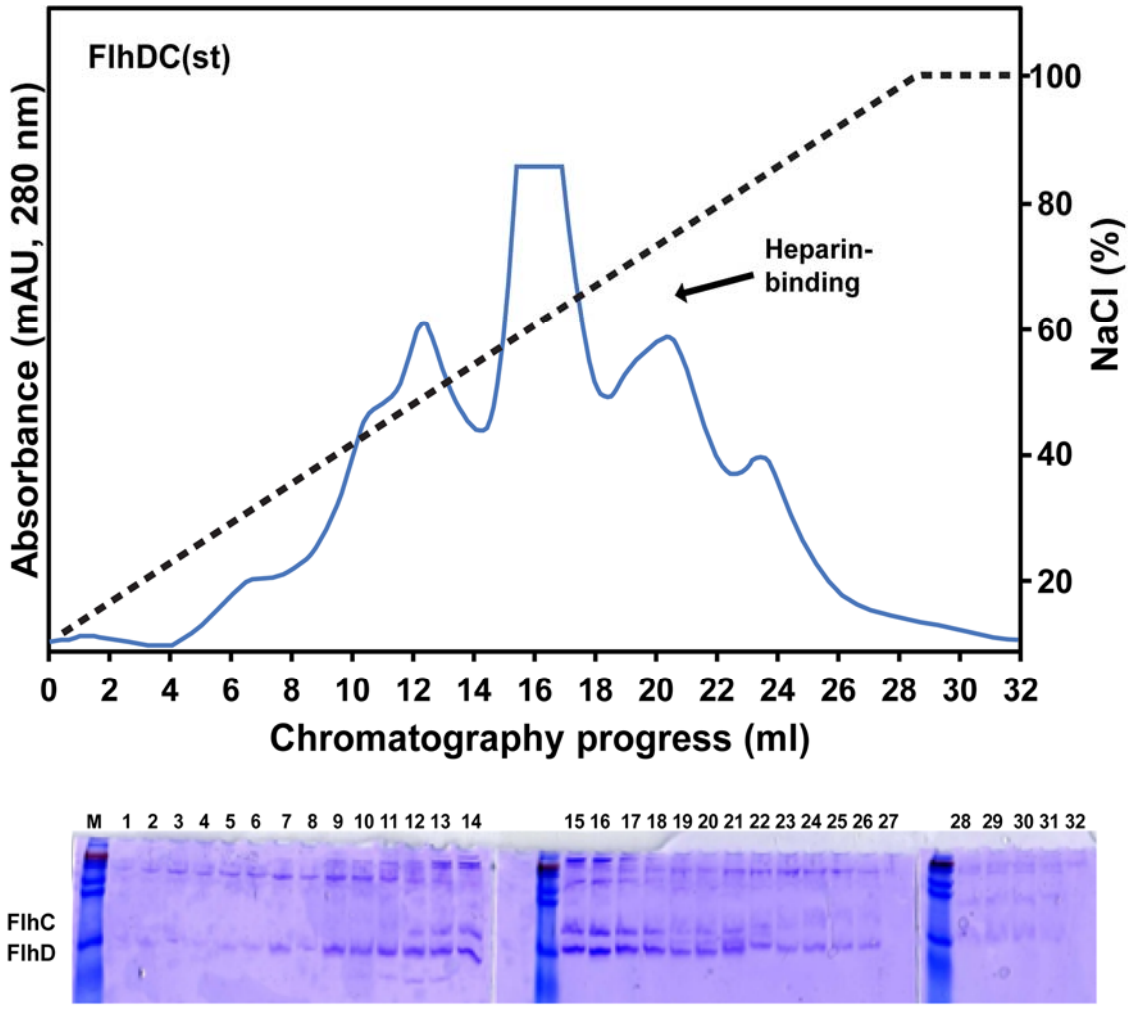


Figure 64. ÄKTA purification using Heparin to assess the DNA binding properties of the $FlhD_4C_2$ complex for *flhDC*$_{(st)}$. Fractions 1 to 32 were eluted with a linear NaCl gradient (0 -100%). The FlhC and FlhD proteins were identified in fractions 12-22 by SDS-PAGE-gel (12%). The dashed lines represents the concentration gradient of NaCl (%). The absorbance was monitored at λ=280 nm. M: protein markers. The strain used in this experiment was, FlhDC(st) = TPA640

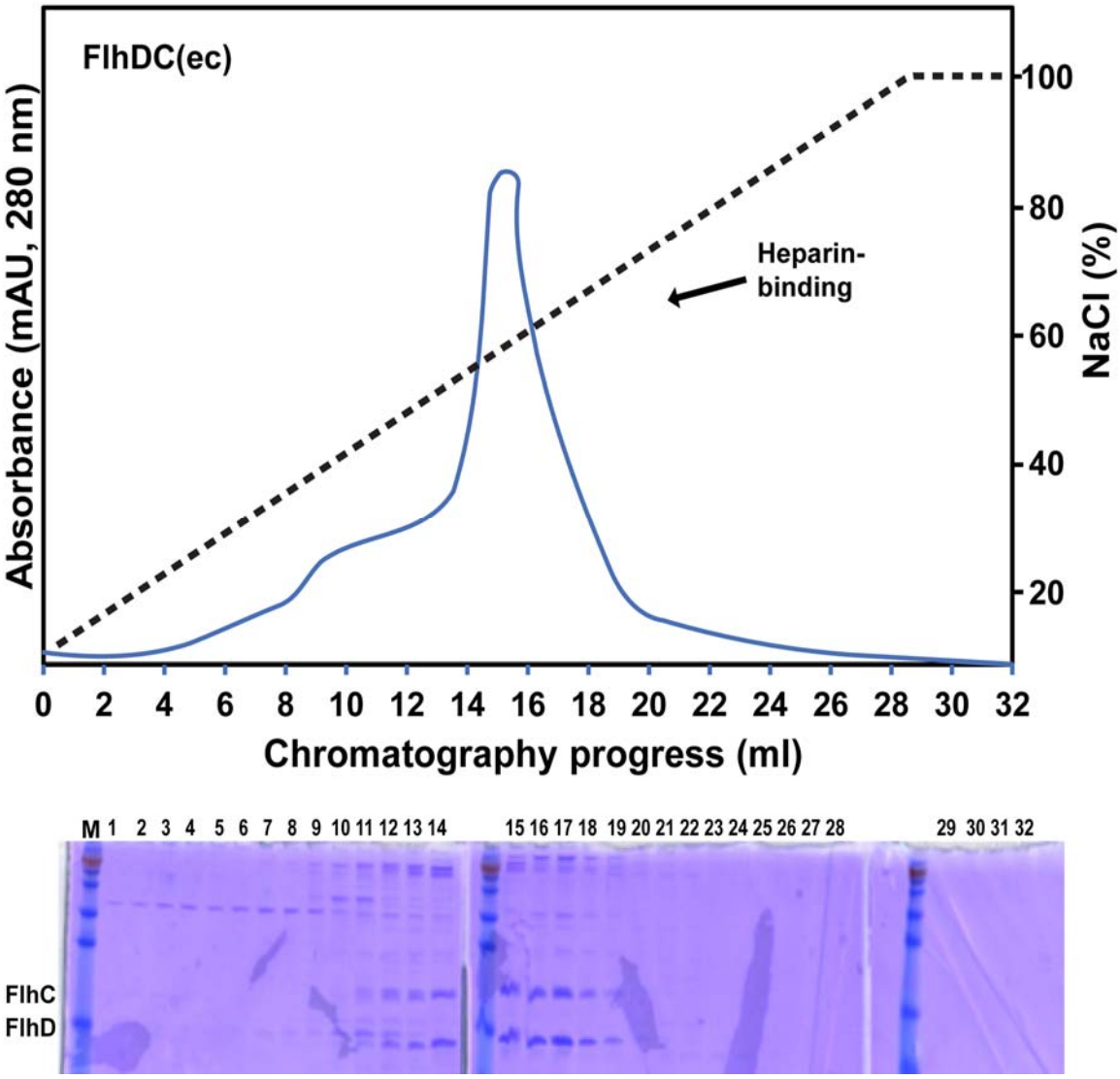


Figure 65. ÄKTA purification using Heparin to assess the DNA binding properties of the $FlhD_4C_2$ complex for *flhDC*$_{(ec)}$. Fractions 1 to 32 were eluted with a linear NaCl gradient (0-100%). The FlhC and FlhD proteins were identified in fractions 9-19 by SDS-PAGE-gel (12% with a peak at fraction 15). The dashed lines represents the concentration gradient of NaCl (%). The absorbance was monitored at λ=280 nm. M: protein markers. The strain used in this experiment was, FlhDC(ec) = TPA4594

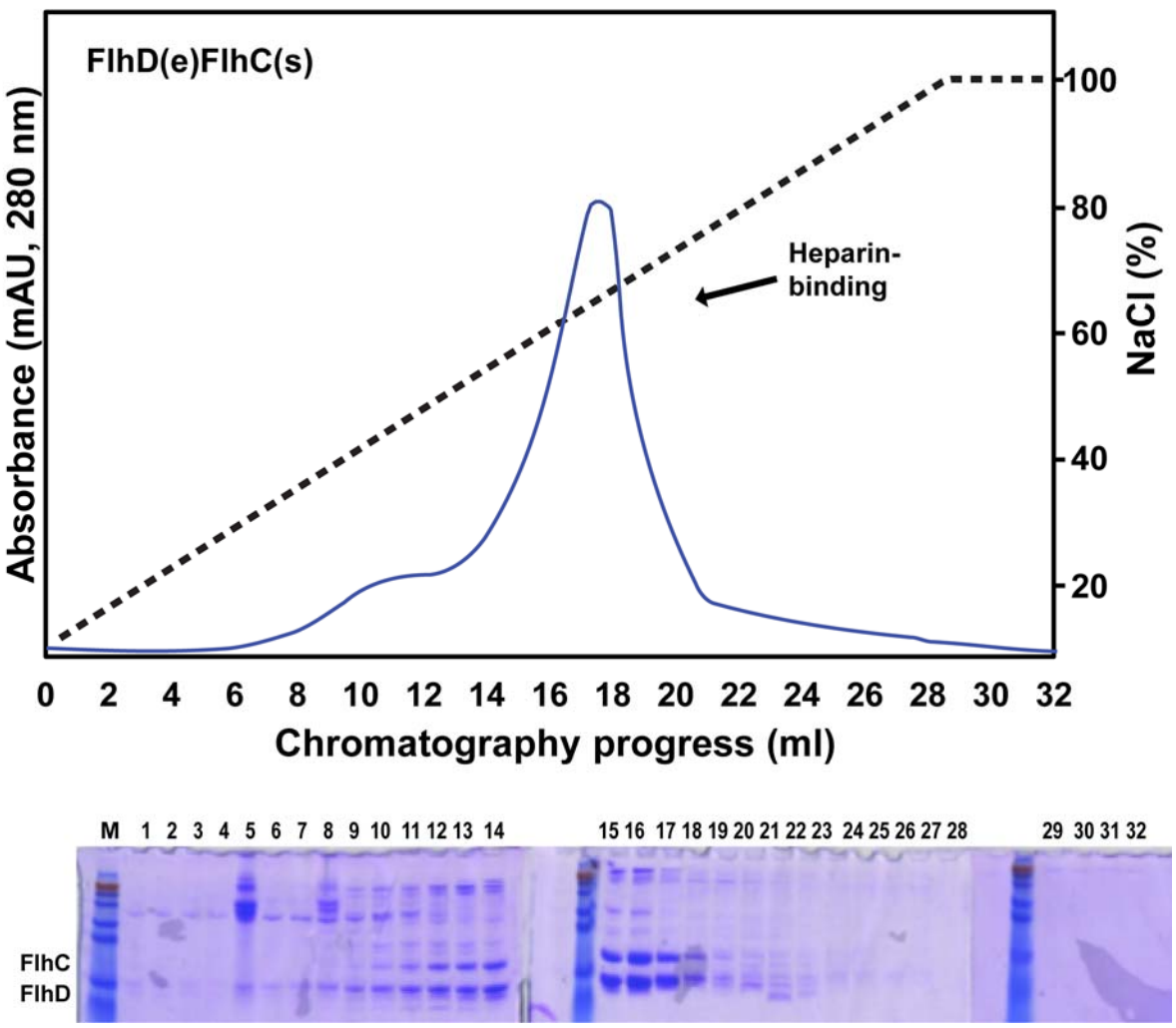


Figure 66. ÄKTA purification using Heparin to assess the DNA binding properties of the $FlhD_4C_2$ complex for FlhD(e)FlhC(s). Fractions 1 to 32 were eluted with a linear NaCl gradient (0-100%). The FlhC and FlhD proteins were identified in fractions 10-19 by SDS-PAGE-gel (12%) peaking between fraction 15 and 16. The dashed lines represents the concentration gradient of NaCl (%). The absorbance was monitored at λ=280 nm. M: protein markers. The strain used in this experiment was, FlhD(e)FlhC(s) = TPA4593.

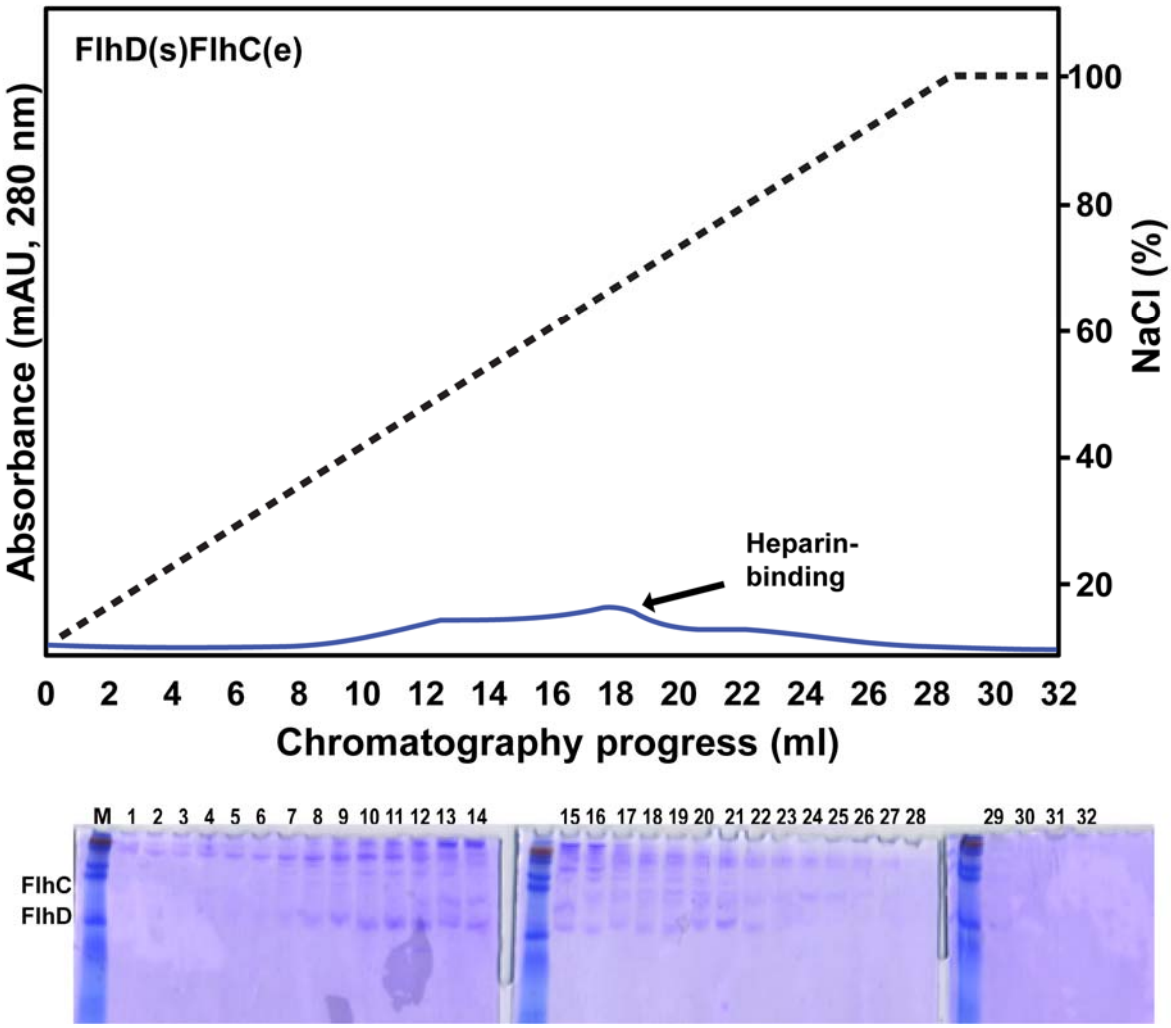


Figure 67. ÄKTA purification using Heparin to assess the DNA binding properties of the $FlhD_4C_2$ complex for FlhD(s)FlhC(e). Fractions 1 to 32 were eluted with a linear NaCl gradient (0-100%). Significant quantities compared to other combinations of the FlhC and FlhD proteins were not identified in any fraction by SDS-PAGE-gel (12%). Based on the profiles in figures 63 to 64 and the ABS280 profile a peak of protein should be identifiable in fractions 15 to 18, but this was not visible. The dashed lines represent the concentration gradient of NaCl (%). The absorbance was monitored at λ=280 nm. M: protein markers. The strain used in this experiment was, FlhD(s)FlhC(e) = TPA4592

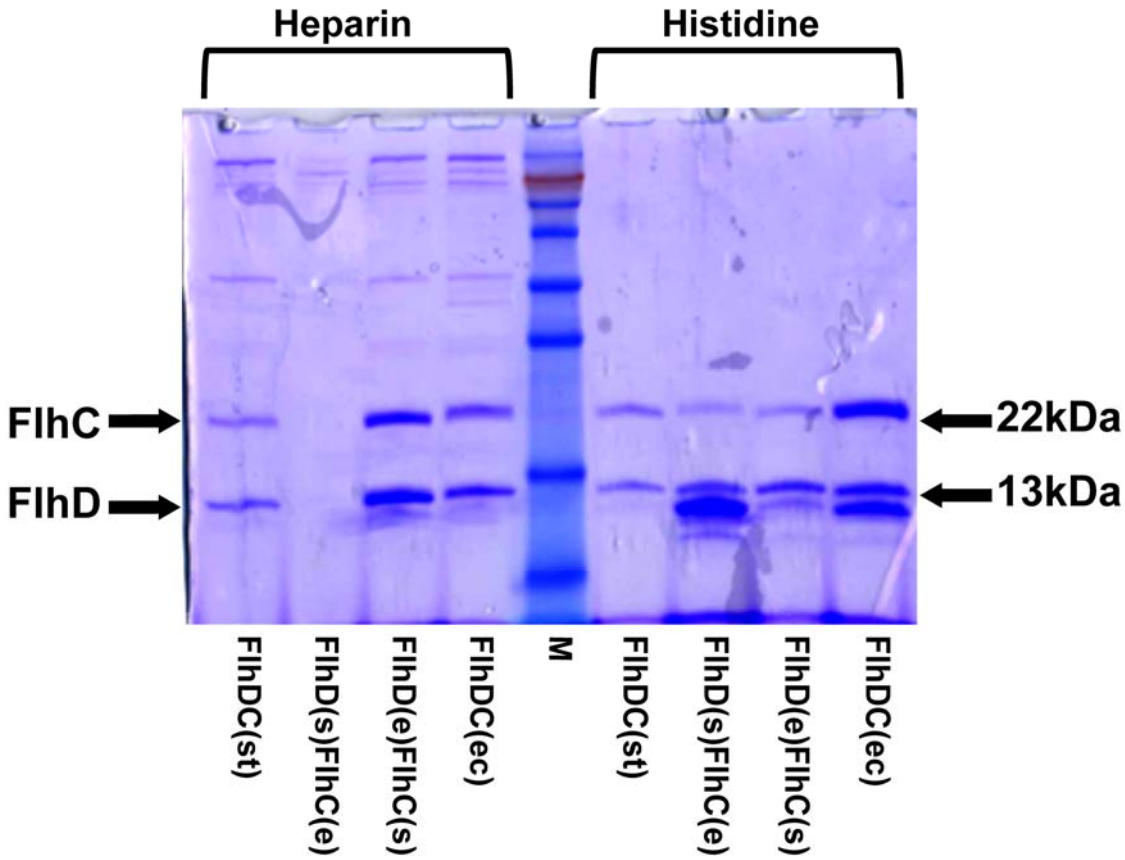


Figure 68. SDS-PAGE-gel comparing the two isolation methods for FlhDC purification. In terms of Heparin purification, $FlhDC_{(st)}$, $FlhDC_{(ec)}$ and FlhD(e)FlhC(s) were clearly identified. In contrast, the FlhD and FlhC proteins bands for all combinations, including FlhD(s)FlhC(e), were identifiable with respect to His-trap purification. M: protein markers. Strains used in this experiment to purify the complexes were, FlhDC(st) = TPA640, FlhDC(ec) = TPA4594, FlhD(s)FlhC(e)=TPA4592 and FlhD(e)FlhC(s)=TPA4593.

7.3.2 Electrophoretic Mobility Shift Assay (ESMA)

In order to test the functionality of the isolated complexes, an electrophoretic mobility shift assay was used to estimate the activity of the $FlhD_4C_2$ complexes ability to recognize the *flgAB S. enterica* promoter region. The $FlhD_4C_2$ complexes were mixed with P_{flgAB}-DNA at varying concentrations of protein (100 to 700 nM) (Wang *et al.*, 2006). A substantial change in the shift of $FlhD_4C_2$-DNA (P_{flgAB}) complexes using $FlhDC_{(st)}$ was observed (figure 69). $FlhDC_{(ec)}$ affinity to P_{flgAB} exhibited an altered pattern compared to $FlhDC_{(st)}$ (figure 69). Surprisingly, the FlhD(e)FlhC(s) exhibited no significant difference and possessed an almost identical binding pattern in comparison with $FlhDC_{(st)}$ (figure 69A and B). In contrast, the FlhD(s)FlhC(e) complex bound P_{flgAB} the weakest, based on unbound DNA remaining (figure 69A).

When quantified using the unbound DNA band intensity as 100% an interesting profile of complex activity was observed (figure 69B). It was noted that the amount of unbound DNA remaining for $FlhDC_{(ec)}$ and FlhD(s)FlhC(e) were very similar. In contrast, both complexes with FlhC(s) also exhibited a similar binding profile (figure 69B). This is consistent with chapter 6 *in-vivo* analysis that the *E. coli* derived complexes have lower flagellar gene expression (figure 53). Our data also argues that it is the stability or availability of the FlhD(s)FlhC(e) complex that drives its observed *in-vivo* phenotype. This statement is derived from the observation quantification of FlhD(s)FlhC(e) complex binding DNA was comparable to the $FlhDC_{(ec)}$ complex.

In this investigation the aim was to assess the different $FlhD_4C_2$ complexes for DNA-binding activity using the P_{flgAB} promoter region. Interestingly, the $FlhDC_{(ec)}$ protein has a different affinity to P_{flgAB} from $FlhDC_{(st)}$. However, the FlhD(e)FlhC(s) protein has a similar activity compared to $FlhDC_{(st)}$ to interact with P_{flgAB}. Consistently,

the FlhD(s)FlhC(e) complex, once isolated, interacts with P_{flgAB} in a similar to $FlhDC_{(ec)}$. However, it is clear that the EMSA profile suggests the FlhD(s)FlhC(e) complex exhibits a weaker interaction with DNA (figure 69A). We conclude that *in vivo* a combination of factors defining the output efficiency of FlhD(s)FlhC(e) plays a role in the measured reduction in the output of the flagellar system driven by this cross-species hybrid complex.

7.4 Summary

The results displayed in this chapter characterize the nature of $FlhD_4C_2$ DNA binding activity compared to the wild-type $FlhDC_{(st)}$ complex. We succeeded in the purification of all four FlhD and FlhC complexes. In general, the complex yield reflected the *in-vivo* data with *flhD(s)flhC(e)* being the hardest to isolate. In order to confirm this, we used Heparin purification of $FlhD_4C_2$ complexes. Predictably, all $FlhD_4C_2$ complexes were trapped by Heparin except the FlhD(s)FlhC(e) complex. We confirmed the DNA activity further by using an Electrophoretic Mobility Shift Assay.

These data potentially strengthen the model based on the phenotypic characterization of the *in vivo* activity of each complex in chapter 6. The data would suggest that the weak response of the flagellar genes expression and phenotypic motility output for *flhD(s)flhC(e)* is a combination of its weaker ability to bind DNA and an instable complex. However, there are alternative explanations for the low abundance of FlhC during the purification process that cannot be ruled out as playing a role in defining the functionality of this complex. Firstly there is the translation of the *flhD(s)flhC(e)* operon in pET28a. Ways to control for this would be to clone the flhDC operons into alternative expression vectors generating a C-terminal HISx6tag on flhC. However, previous work has shown that this is an inefficient way to isolate the

complex with the N-terminal Hisx6 tagging of FlhD being the better option. An alternative approach would be to isolate each protein individually and reconsititute the complexes before EMSA analysis. However, this would overcome a translational impact and focus once more of the activity of the complex formed. The data presented in this chapter and in chapter 6 are consistent that, even if multiple factors lead to lower availability of FlhD(s)FlhC(e) complex, the overall outcome is reduced flagellar gene expression and motility phenotype.

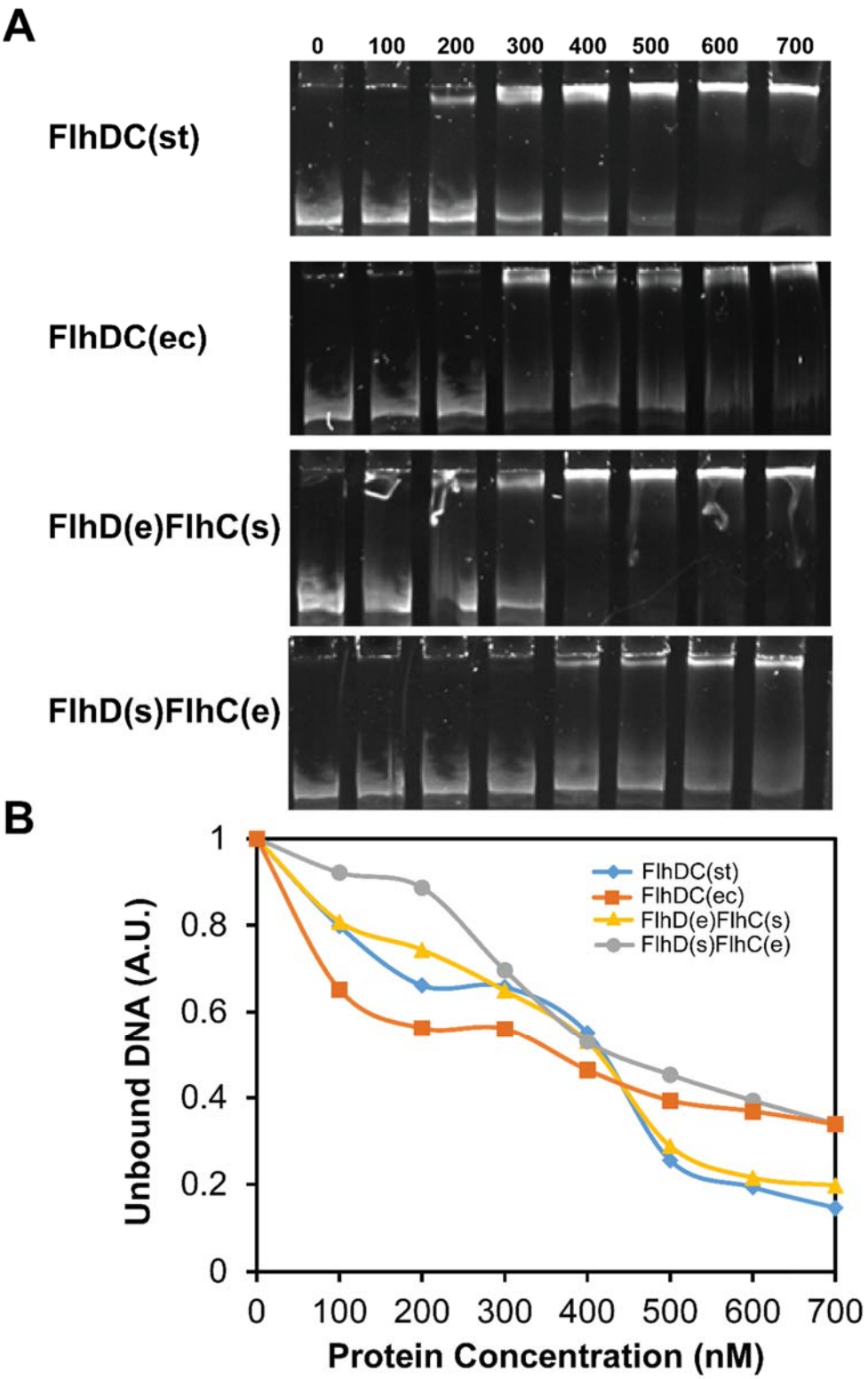


Figure 69. An Electrophoretic Mobility Shift Assay (ESMA) was carried out by titration of the various $FlhD_4C_2$ complexes at concentrations ranging from 100, to 700 nM. The ability to shift the P_{flgAB} DNA fragment was visualised using conventional DNA staining techniques. (A) Representative EMSA gels for each complex. (B) Average quantification of three independent repeats where the unbound DNA fragment intensity was calculated using ImageJ. Strains used in this experiment were, FlhDC(st) = TPA640, FlhDC(ec) = TPA4594, FlhD(s)FlhC(e)=TPA4592 and FlhD(e)FlhC(s)=TPA4593.

Chapter Eight: The Influence of FliT, ClpP, YdiV and FliZ Regulators on $FlhD_4C_2$ Activity

8.1 Introduction

In chapter seven we investigated the activity of $FlhD_4C_2$ combinations *in vitro* compared to wild-type $FlhDC_{(st)}$. The biochemical analysis correlated to the observe in vivo phenotypes of the different complexes. Introducing just *flhC* led to a dramatic drop in $FlhD_4C_2$ activity that correlated to low yield during purification and changes in P_{flgAB} DNA binding assays. However, the binding profile matched that of FlhDC(ec), arguing that FlhC from *E. coli* recognises DNA independent of FlhD. Importantly, we know that the $FlhD_4C_2$ complex is tightly regulated. It was therefore of interest to ask: how do the combination complexes react to the key regulators of $FlhD_4C_2$ activity? To develop this question, we assayed the $FlhD_4C_2$ activity in deletion mutants of *fliT, fliZ, clpP* and *ydiV,* all four of which have significant impact on the flagellar master regulator activity. Each protein has a specific function toward $FlhD_4C_2$, for ClpP and YdiV, these two proteins act as negative regulators by repressing $FlhD_4C_2$ activity via protein degradation (Takaya *et al.*, 2012). FliT is also a negative regulator of $FlhD_4C_2$ by reducing the availability of free $FlhD_4C_2$ complexes to bind class 2 promoters by disrupting the complex (Aldridge *et al.*, 2010). In contrast, FliZ is a positive regulator of $FlhD_4C_2$ activity via regulating YdiV expression and activity (Kutsukake *et al.*, 1999) (figure 70). Using all the tools available to assess the expression, assembly and output of the flagellar system we have assessed the impact of deletion mutants in each of these regulatory genes.

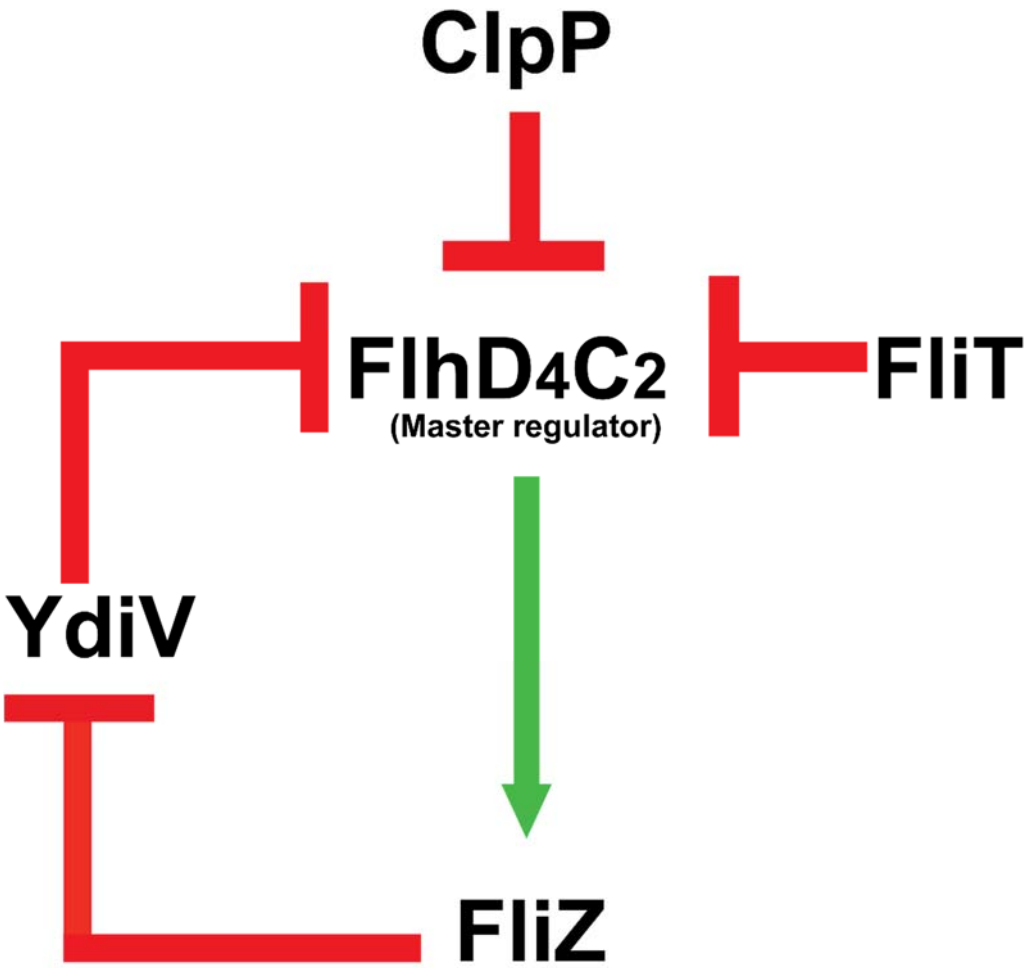


Figure 70. System regulatory network that modulates flagellar gene expression in *S. enterica* via $FlhD_4C_2$ activity. FliZ regulatory protein is helping to sustain $FlhD_4C_2$ activity, acting as a negative regulator of YdiV which in turn acts as a negative regulator of $FlhD_4C_2$. FliT has two functions: the T3S chaperone of FliD and repression of $FlhD_4C_2$ (Soutourina and Bertin, 2003). Finally, ClpP is a negative regulator of $FlhD_4C_2$ activity reducing the master protein concentrations via protein degradation (Smith and Hoover, 2009).

8.2 Effects the Motility Output Each Of Δ*fliT*, Δ*clpP*, Δ*ydiV* and Δ*fliZ* On $FlhD_4C_2$

There are many proteins that intervene with $FlhD_4C_2$ activity and therefore will affect the motility output. What happens, however, if those proteins are missing in the $FlhD_4C_2$ combination strains: *flhDC*$_{(ec)}$, *flhD(e)flhC(s*) and *flhD(s)flhC(e)*? To answer this question, we deleted *ydiV, clpP, fliT* and *fliZ* genes individually in all *S. enterica flhDC* strains and Wild-type. Motility assays were used to investigate motility output quantifying swim diameter for each mutant strain and compared to the parental strains (figures 71-73).

For *flhDC*$_{(ec)}$, the average swim diameter for Δ*clpP* and Δ*ydiV* were significantly increased when compared to the parental strain (P-value = 0.038 and 0.003) (figure 71). The swim diameter for Δ*fliZ* was decreased, consistent with FliZ positively improving $FlhD_4C_2$ activity. Surprisingly, the swim diameter in Δ*fliT* was obviously decreased when compared to wild-type and the *flhDC*$_{(st)}$ Δ*fliT* strain (P-value = 0.025). Motility is supposed to be increased as FliT acts as a repressor of $FlhD_4C_2$ activity. This is seen when deleting *fliT* in the $FlhD_4C_{2(st)}$ strain as an increase in motility (Aldridge *et al.*, 2010) (figure 71).

With respect to *flhD(e)flhC(s*) strains, the average of the swim diameter in Δ*clpP* and Δ*ydiV* were also markedly increased showing a similar response of Δ*clpP* and Δ*ydiV* mutants in *flhDC*$_{(st)}$ and the *flhDC*$_{(ec)}$ strains (figure 72). *flhD(e)flhC(s*) Δ*fliT* possessed a slight decrease in its motility phenotype when compared to other strains. Furthermore, the *flhD(e)flhC(s*) Δ*fliZ* strain also behaved in a similar manner exhibiting a swim diameter that was clearly decreased compared to the intact *fliZ*$^{+}$ strain (figure 72). Interestingly, with regard to *flhD(s)flhC(e)* strains, the average swim diameter increased in the Δ*fliT*, Δ*clpP* and Δ*ydiV* mutants (P-value <0.05) when compared to the parental strains (figure 73). However, this improvement in the

motility is still significantly lower than observed for the corresponding *flhDC*$_{(st)}$ strains (figure 73). This data is consistent with our conclusions from chapter 7 that the *flhD(s)flhC(e)* generates an active but inefficient $FlhD_4C_2$ complex. As removing levels of negative regulation improve motility with this *flhDC* combination.

The results presented in these assays gave us a brief picture of the impact of the regulatory mutants of $FlhD_4C_2$. The results of all strains in terms of Δ*clpP*, Δ*ydiV* and Δ*fliZ* were as expected based on the function of each protein interaction with the $FlhD_4C_2$ complex. Unexpectedly, the motility for *flhDC*$_{(ec)}$ Δ*fliT* and *flhD(e)flhC(s)* Δ*fliT* strains were significantly decreased. This is in contrast to how FliT acts on $FlhD_4C_{2(st)}$ and suggests that instead of working in a negative manner, FliT behaves as a positive regulator. Noticeably this is just when *flhD* from *E. coli* is present. Astonishingly, even though the swim diameter in *flhD(s)flhC(e)* was dramatically decreased compared to *flhDC*$_{(st)}$, the results of Δ*fliT*, Δ*clpP*, Δ*ydiV* and Δ*fliZ* were relatively identical to the expected out comes. Thus, the FliT, ClpP, YdiV and FliZ proteins can differentiate between the source of FlhD and FlhC.

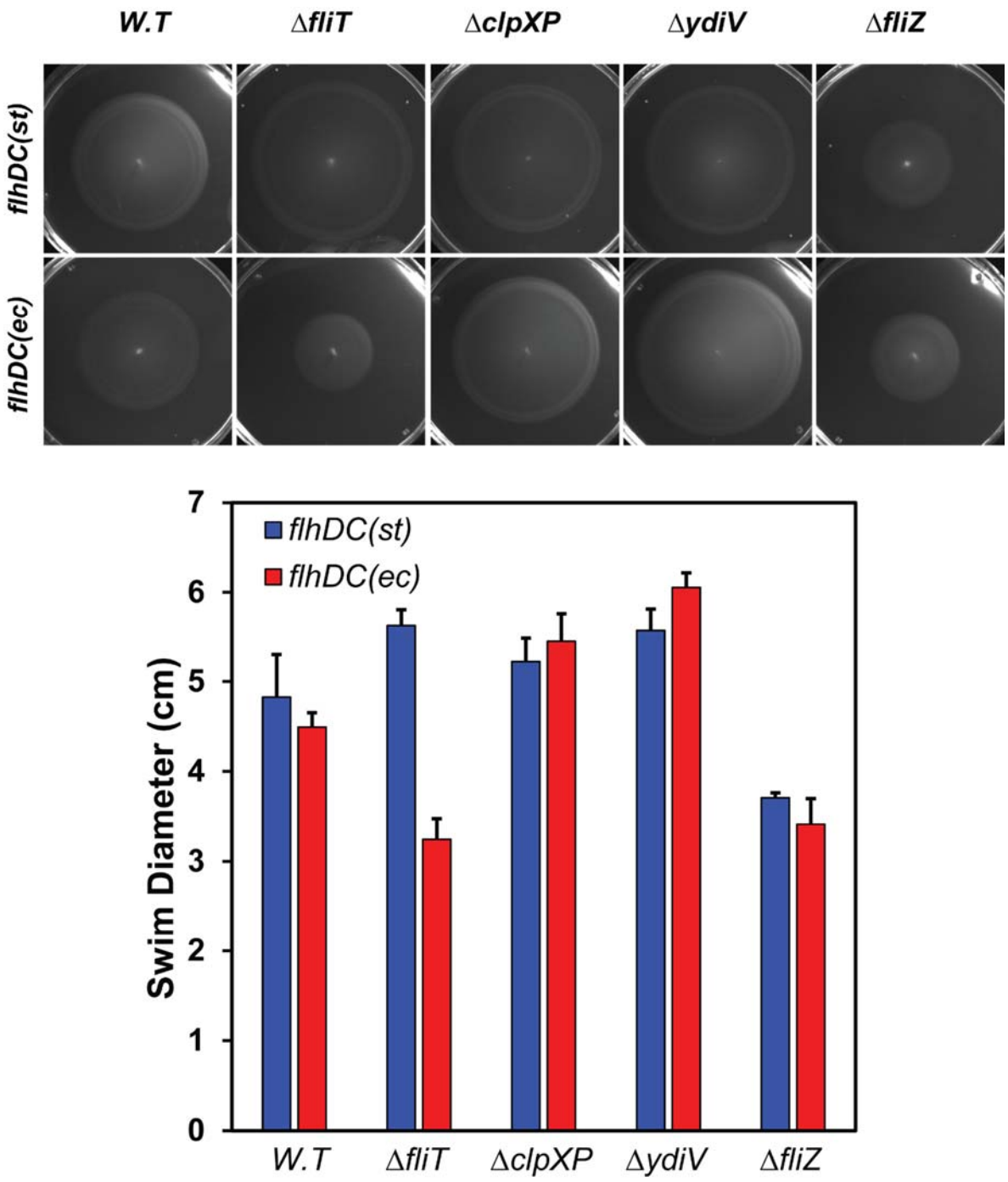


Figure 71. Comparison of the motility phenotypes between *flhDC*$_{(ec)}$ and *flhDC*$_{(st)}$ in with respect to FliT, ClpP, YdiV and FliZ regulation. The motility assay was performed on the 0.3% agar and incubated at 37 ^{o}C between 6-8 hours. Error bars refer to the calculated standard deviations. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, *flhDC*(st) W.T= TPA1107, *flhDC*(st) Δ*fliT* = TPA20, *flhDC*(st) Δ*clpP* = TPA2546, *flhDC*(st) Δ*ydiV* = TPA3356, *flhDC*(st) Δ*fliZ* = TPA3369, *flhDC*(ec) W.T= TPA3997, *flhDC*(ec) Δ*fliT* = TPA4576, *flhDC*(ec) Δ*clpP* = TPA4579, *flhDC*(ec) Δ*ydiV* = TPA4582 and *flhDC*(ec) Δ*fliZ* = TPA4585.

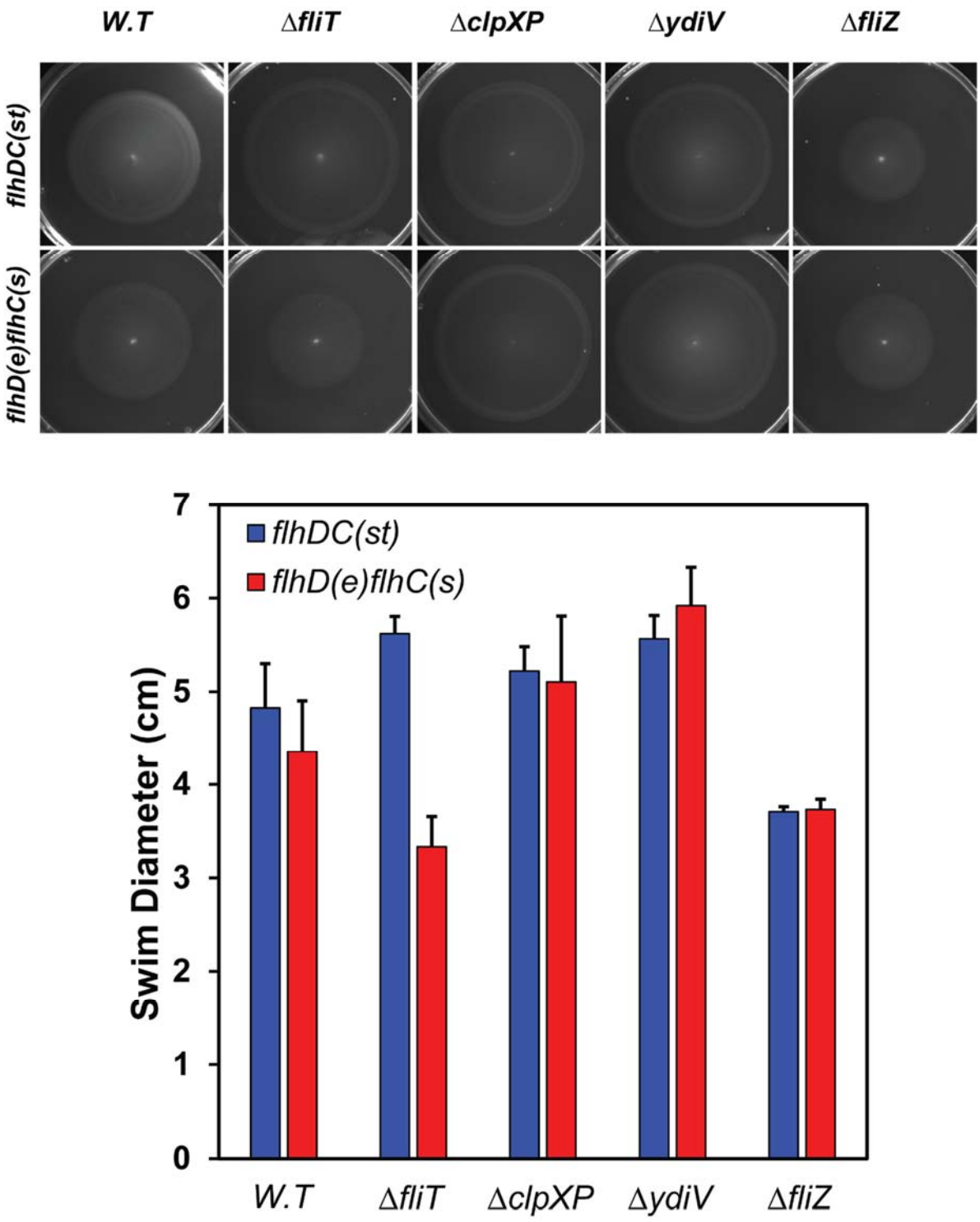


Figure 72. Comparison of the motility phenotypes between *flhD(e)flhC(s)* and $flhDC_{(st)}$ with respect to FliT, ClpP, YdiV and FliZ regulation. The motility assay was performed on 0.3% agar and incubated at 37°C for between 6-8 hours. Error bars refer to the calculated standard deviations. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, *flhDC*(st) W.T= TPA1107, *flhDC*(st) Δ*fliT* = TPA20, *flhDC*(st) Δ*clpP* = TPA2546, *flhDC*(st) Δ*ydiV* = TPA3356, *flhDC*(st) Δ*fliZ* = TPA3369, *flhD(e)flhC*(s) W.T= TPA4135, *flhD(e)flhC(s)* Δ*fliT* = TPA4575, *flhD(e)flhC(s)* Δ*clpP* = TPA4578, *flhD(e)flhC(s)* Δ*ydiV* = TPA4581 and *flhD(e)flhC(s)* Δ*fliZ* = TPA4584.

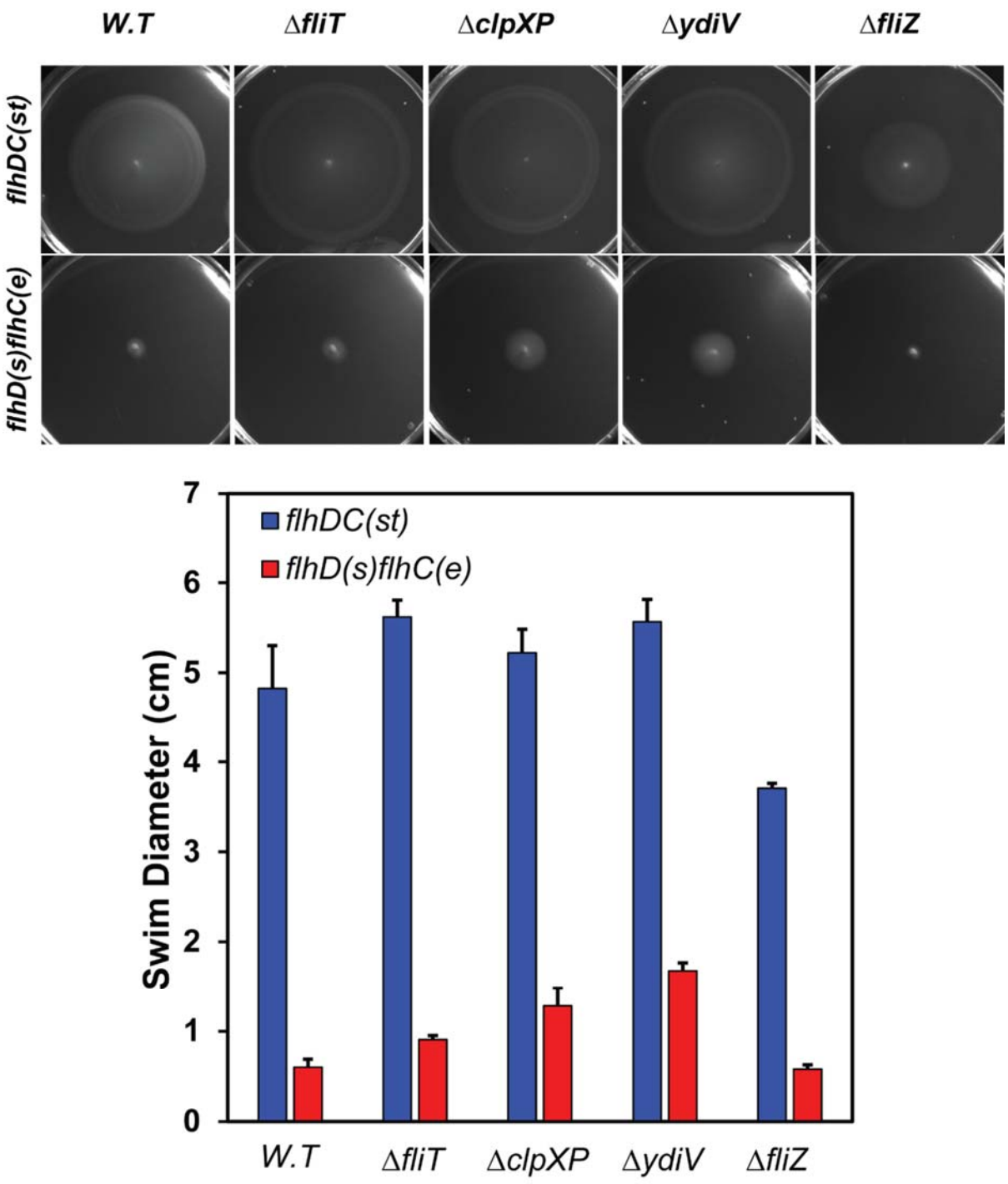


Figure 73. Comparison of the motility phenotypes between the *flhD(s)flhC(e* and *flhDC*$_{(st)}$ with respect to FliT, ClpP, YdiV and FliZ regulation. The motility assay was performed on 0.3% agar and incubated at 37^{o}C for between 6-8 hours. Error bars refer to the calculated standard deviations. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment were, *flhDC*(st) W.T= TPA1107, *flhDC*(st) Δ*fliT* = TPA20, *flhDC*(st) Δ*clpP* = TPA2546, *flhDC*(st) Δ*ydiV* = TPA3356, *flhDC*(st) Δ*fliZ* = TPA3369, *flhD(s)flhC*(e) W.T= TPA4128, *flhD(s)flhC*(e) Δ*fliT* = TPA4574, *flhD(s)flhC*(e) Δ*clpP* = TPA4577, *flhD(s)flhC*(e) Δ*ydiV* = TPA4580 and *flhD(s)flhC*(e) Δ*fliZ* = TPA4583.

8.3 Determination The Flagellar Gene Expression Class II and Class III

Motility is only one way to measure the impact on $FlhD_4C_2$ activity. Therefore, flagellar gene expression (P_{flgA} / P_{fliC}) for the mutants and *flhDC* combinations was quantified. All strains were compared to LT2 (figure 74 to 77). With respect to LT2-Δ*fliT*, Δ*clpP* and Δ*ydiV,* flagellar gene expression was clearly increased. Consistently, flagellar gene expression in LT2-Δ*fliZ* was decreased (figure 74). In terms of $flhDC_{(ec)}$ strains, the flagellar genes magnitude in Δ*fliT* and Δ*fliZ* were reduced in comparison the magnitude of Δ*clpP* and Δ*ydiV* (figure 75). For *flhD(e)flhC(s)*, flagellar gene expression was twofold increased in terms of Δ*clpP* and Δ*ydiV* (figure 76). For *flhD(e)flhC(s)* Δ*fliT* the motility phenotype was reflected in reduced expression. Figure 77 shows the flagellar gene expression for *flhD(s)flhC(e)* strains, Consistently, Δ*fliT*, Δ*clpP* and Δ*ydiV* exhibited a rise in gene expression compared to *flhD(s)flhC(e)*. In contrast, Δ*fliZ,* like its motility phenotype produced an expected decrease in flagellar gene expression.

When comparing the percentage of maximum activity for flagellar gene expression the bar chart gave us an overview of the impact of each regulatory mutant (figure 78). In particular, for strains that possess *flhD* from *E.coli* when missing the FliT protein, the maximum activities were significantly decreased compared to the strains that have *flhD* from *S.enterica* (figure 78). These findings indicate the impact of losing ClpP, YdiV, FliZ and FliT regulation upon $FlhD_4C_2$ and its *E. coli* / *S. enterica* combinations. Interestingly, even though FliT is a negative regulator of $FlhD_4C_{2(st)}$, the $FlhD_4C_{2(ec)}$ complex and the FlhD(e)FlhC(s) complex did not react to the loss of FliT. Previous studies have shown FliT interacts with FlhC (Kutsukake *et al.*, 1999; Imada *et al.*, 2010). However, our data suggests a key role for FlhD in FliT

regulation, as the two complexes that do not respond to Δ*fliT* both include FlhD from *E.coli.*

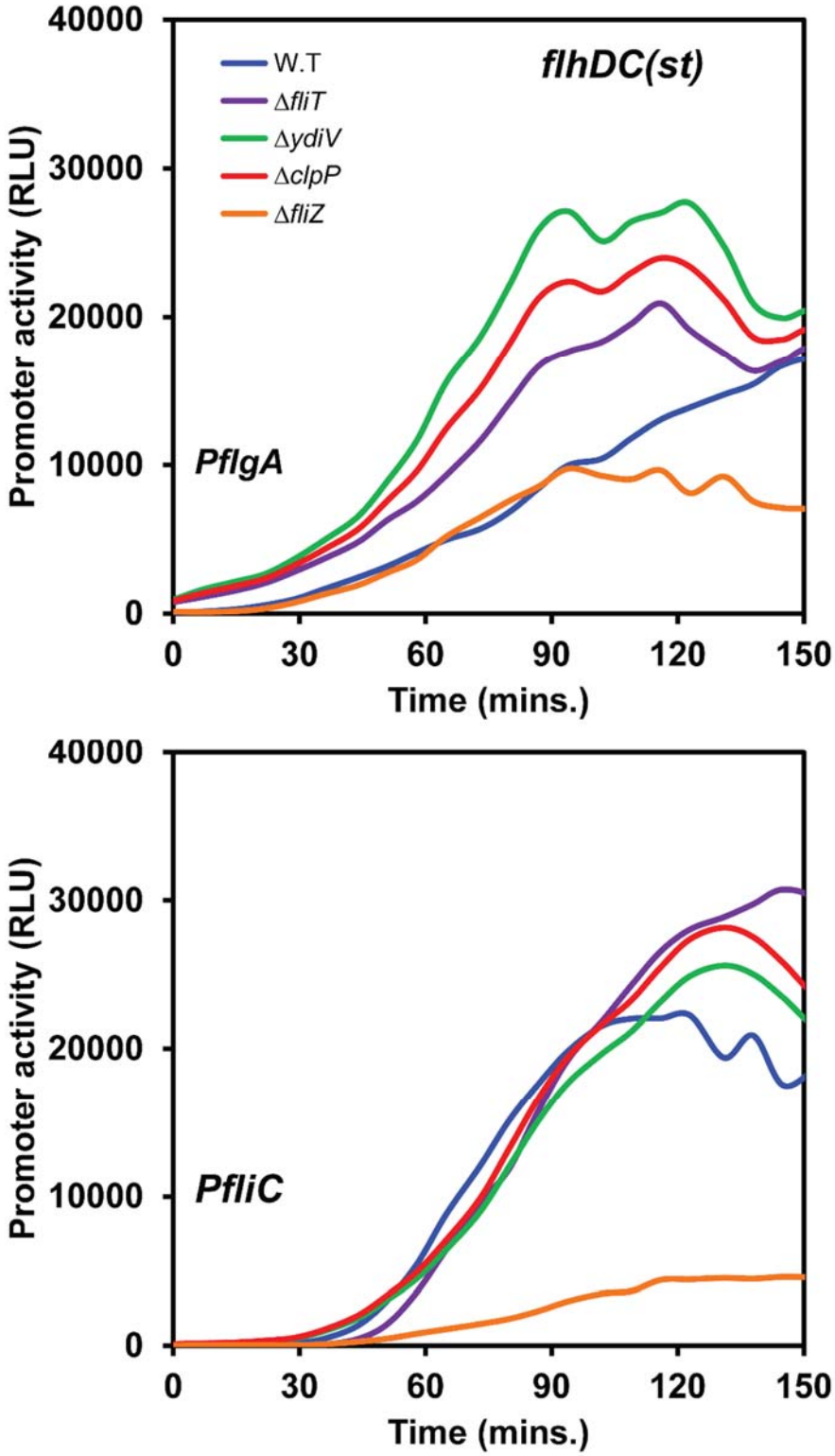


Figure 74. Kinetic comparison of the flagellar gene expression in terms class II (P_{flgA}) and class III (P_{fliC}) in *flhDC*$_{(st)}$ (Δ*fliT*, Δ*clpP,* Δ*ydiV* and Δ*fliT*). All mutant strains under control tetracycline inducible system (*tetRA* promoter), the differences of flagellar gene expression magnitudes based on the mechanism of action of each regulator which consequences directly affected in FlhD4C2 activity. In terms of Δ*fliT*, Δ*clpP* and Δ*ydiV* the flagellar gene expression were significantly increased from wild-type, while, Δ*fliZ* was dramatically dropped. Experiment represents a minimal of three independent repeats (n=3). Strains used in this experiment where, (A) P_{flgA}*flhDC*(st) W.T= TPA4050, P_{flgA}*flhDC*(st) Δ*fliT* = TPA4662, P_{flgA}*flhDC*(st) Δ*clpP* = TPA4711, P_{flgA}*flhDC*(st) Δ*ydiV* = TPA4666, P_{flgA}*flhDC*(st) Δ*fliZ* = TPA4647. pRG51 was transformed into the above strains and for P_{flgA} detection. (B) P_{fliC}*flhDC*(st) W.T= TPA4049, P_{fliC}*flhDC*(st) Δ*fliT* = TPA4661, P_{fliC}*flhDC*(st) Δ*clpP* = TPA4710, P_{fliC}*flhDC*(st) Δ*ydiV* = TPA4665 and P_{fliC}*flhDC*(st) Δ*fliZ* = TPA4646. pRG39 was transformed into the above strains and for P_{fliC} detection.

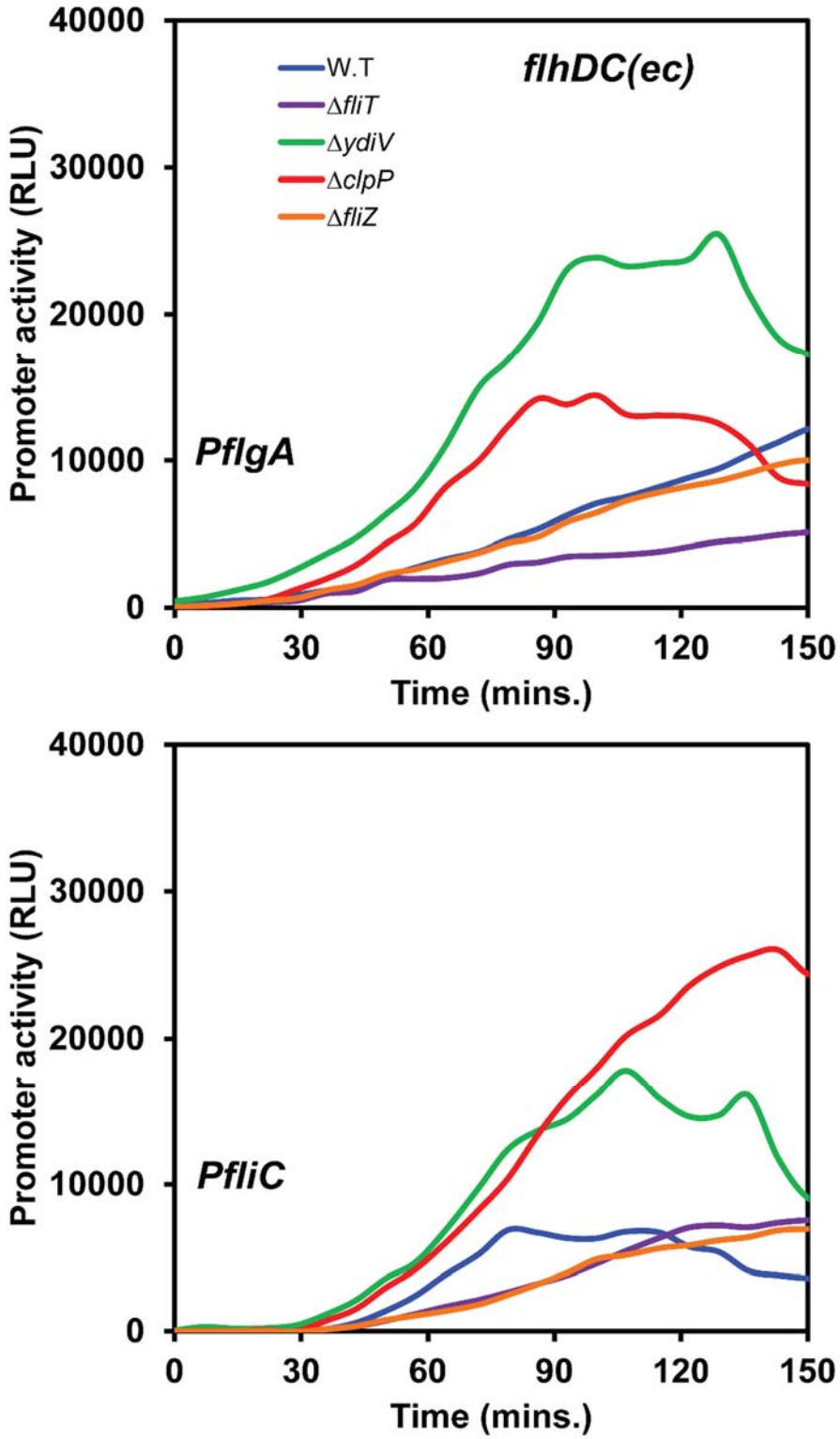


Figure 75. Kinetic comparison of flagellar gene expression for P_{flgA} and P_{fliC} in *flhDC(ec)* Δ*fliT*, Δ*clpP,* Δ*ydiV* and Δ*fliT* mutants. All mutant strains are under control of the P_{tetRA} tetracycline inducible system. Experimental data represents a minimal of three independent repeats. Strains used in this experiment were, (A) *flhDC*(ec) W.T= TPA4032, *flhDC*(ec) Δ*fliT* = TPA4639, *flhDC*(ec) Δ*clpP* = TPA4715, *flhDC*(ec) Δ*ydiV* = TPA4660, *flhDC*(ec) Δ*fliZ* = TPA4645. pRG51 was transformed into the above strains for P_{flgA} detection. (B) *flhDC*(ec) W.T= TPA4031, *flhDC*(ec) Δ*fliT* = TPA4638, *flhDC*(ec) Δ*clpP* = TPA4714, *flhDC*(ec) Δ*ydiV* = TPA4659 and *flhDC*(ec) Δ*fliZ* = TPA4644. pRG39 was transformed into the above strains for P_{fliC} detection.

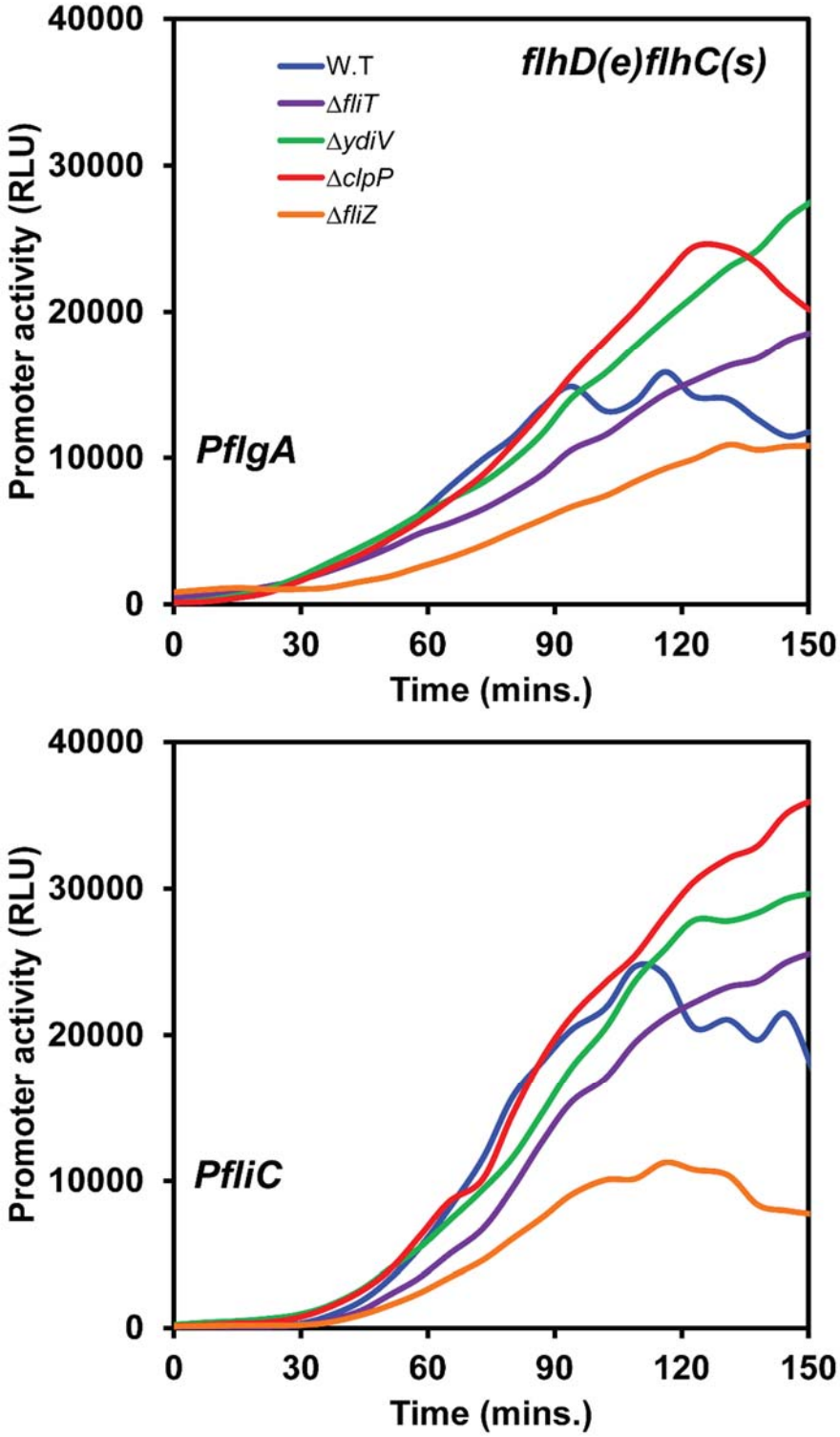


Figure 76. Kinetic comparison of flagellar gene expression for P_{flgA} and P_{fliC} in *flhD(e)flhC(s)* Δ*fliT*, Δ*clpP,* Δ*ydiV* and Δ*fliT* mutants. All mutant strains are under control of the P_{tetRA} tetracycline inducible system. Experimental data represents a minimal of three independent repeats. Strains used in this experiment were, (A) pRG51 was transformed into the following strains for P_{flgA} detection: *flhD(e)C*(s) W.T= TPA4218, *flhD(e)C*(s) Δ*fliT* = TPA4637, *flhD(e)C*(s) Δ*clpP* = TPA4717, *flhD(e)C*(s) Δ*ydiV* = TPA4658, *flhD(e)C*(s) Δ*fliZ* = TPA4643. (B) pRG39 was transformed into the following strains for P_{fliC} detection: *flhD(e)C*(s) W.T= TPA4217, *flhD(e)C*(s) Δ*fliT* = TPA4636, *flhD(e)C*(s) Δ*clpP* = TPA4716, *flhD(e)C*(s) Δ*ydiV* = TPA4657 and *flhD(e)C*(s) Δ*fliZ* = TPA4642.

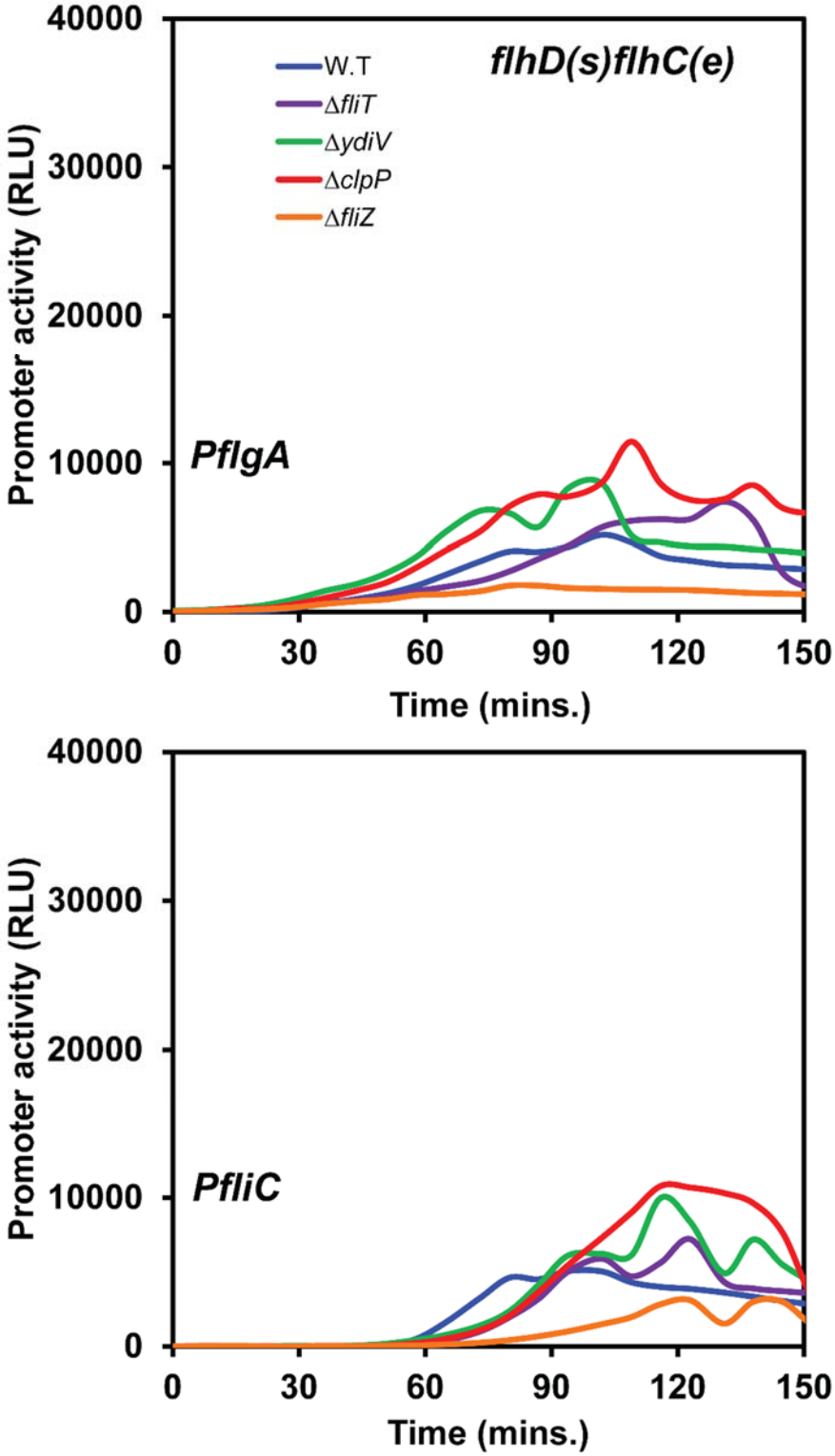


Figure 77. Kinetic comparison of flagellar gene expression for P_{flgA} and P_{fliC} in *flhD(s)flhC(e)* Δ*fliT*, Δ*clpP,* Δ*ydiV* and Δ*fliT* mutants. All mutant strains are under control of the P_{tetRA} tetracycline inducible system. Experimental data represents a minimal of three independent repeats. Strains used in this experiment were, (A) *flhD(s)C*(e) W.T= TPA4215, *flhD(s)C*(e) Δ*fliT* = TPA4635, *flhD(s)C*(e) Δ*clpP* = TPA4713, *flhD(s)C*(e) Δ*ydiV* = TPA4656, *flhD(s)C*(e) Δ*fliZ* = TPA464. pRG51 was transformed into the above strains for P_{flgA} detection. (B) *flhD(s)C*(e) W.T= TPA4214, *flhD(s)C*(e) Δ*fliT* = TPA4634, *flhD(s)C*(e) Δ*clpP* = TPA4712, *flhD(s)C*(e) Δ*ydiV* = TPA4655 and *flhD(s)C*(e) Δ*fliZ* = TPA4640. pRG39 was transformed into the above strains and for P_{fliC} detection.

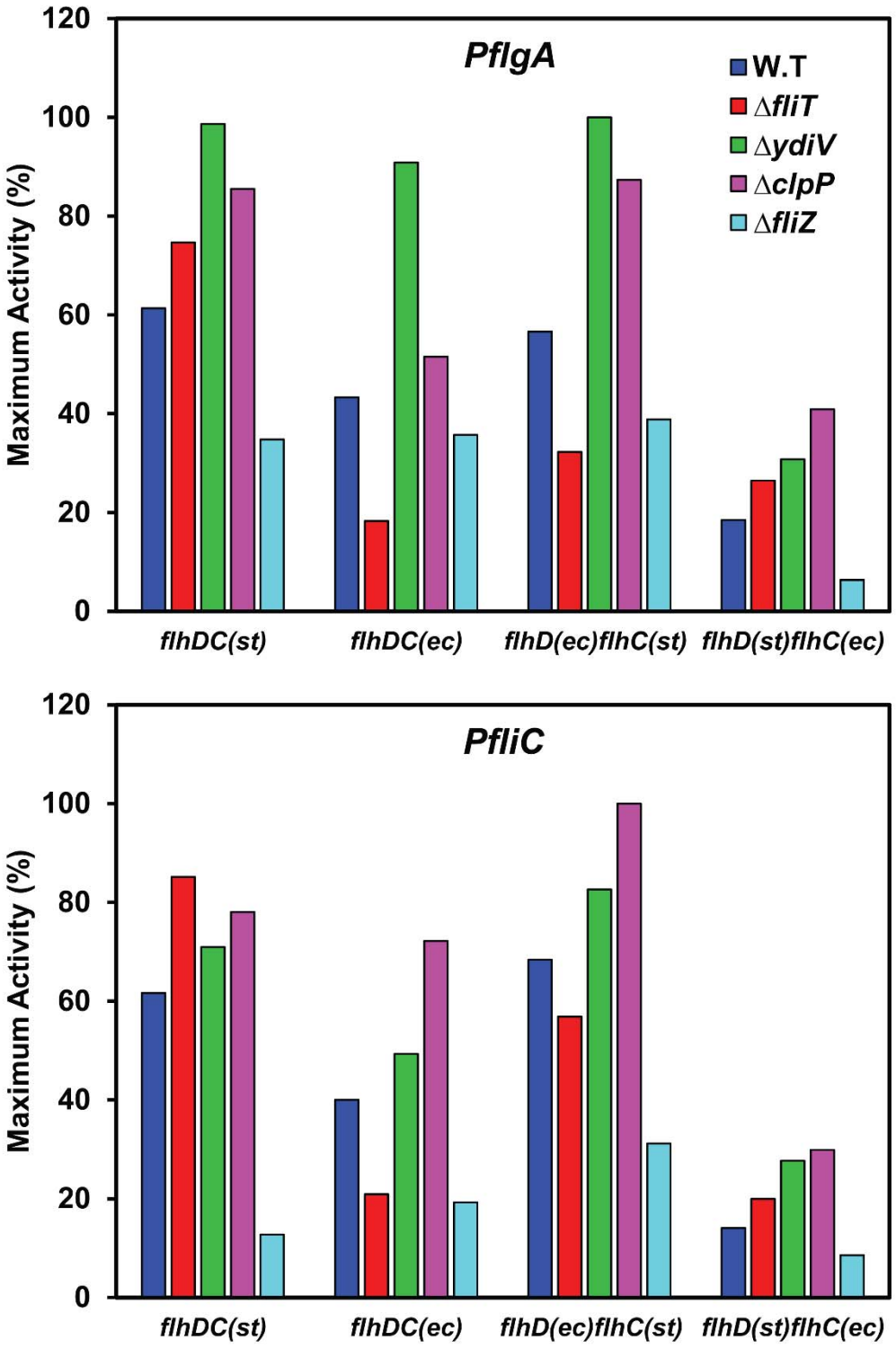


Figure 78. Percentage of maximum activity for P_{flgA} and P_{fliC} flagellar gene expression between $FlhD_4C_2$ complex combinations and Δ*fliT*, Δ*ydiV*, Δ*clpP* and Δ*fliZ* mutants. The profiles of response for $flhDC_{(st)}$ and *flhD(s)flhC(e)* were similar, even though the later had reduced $FlhD_4C_2$ activity due to the biochemical properties of the complex. In contrast, a surprising but consistent result based on motility assays was observed for the Δ*fliT* mutant in $flhDC_{(ec)}$ and *flhD(e)flhC(s)* strains. Here the magnitude of actvity were decreased compared to the parental strains. Experiment represents a minimal of three independent repeats. Strains and dat used for this analysis is derived from Figures 74 to 77.

8.4 Impact Growth Rate Control of Flagellar Abundance at Fast and Slow Growth Conditions

We examined all *flhDC*$_{(st)}$, *flhDC*$_{(ec)}$, *flhD(e)flhC(s*) and *flhD(s)flhC(e)* strains with and without the regulators FliT, ClpXP, YdiV and FliZ by using a high nutrient media (MinE 0.2% Glucose with 3g/L Yeast Extract) to mimic fast growth and low nutrient media (MinE 0.2% Glucose with without Yeast Extract) for slow growth (Aldridge *et al.*, 2010). An alternative method to appreciate the impact on flagellar foci is to plot cell length against flagellar (FliM-GFP) foci (figures 79 to 83). This comparative method also allows for the identification of *flhDC* combinations versus regulatory mutants that do not behave as expected. For example, analysis of *flhD(s)flhC(e)* in comparison to *flhDC*$_{(st)}$ exemplifies the dramatic drop in motility and flagellar gene expression for *flhD(s)flhC(e)* (figure 79 to 83). In contrast, the flagellar foci per cell in *flhDC*$_{(ec)}$, *flhD(e)flhC(s*) strains are comparable to *flhDC*$_{(st)}$ in both growth conditions (figure 79).

Deletion of *fliT* altered the distribution of flagellar per cell in *flhDC*$_{(ec)}$, *flhD(e)flhC(s*) (figure 80). This is seen for *flhDC*$_{(ec)}$ having lines closer together for both growth conditions compared to *flhDC*$_{(st)}$ Δ*fliT* and *flhDC*$_{(st)}$ *fliT*$^{+}$ (figures 79A, 80 A and 80C). However, for *flhD(e)flhC(s*) the impact of Δ*fliT* is only noticeable at the fast growth condition (figure80D).

In terms of deletion the *clpP* and *ydiV*, the distribution of the flagellar per cell for all mutants strains (*flhDC*$_{(st)}$, *flhDC*$_{(ec)}$, *flhD(e)flhC(s*) and *flhD(s)flhC(e)*) were noticeably changed in fast and slow growth conditions (figure 81, 82). However, it is still possible to differentiate *flhDC*$_{(st)}$ from the other combinations with respect to Δ*ydiV*. *flhDC*$_{(st)}$ exhibited a similar slope using this plot method with respect to Δ*ydiV*. In contrast, while slow growth flagellar foci were increased for *flhDC*$_{(ec)}$ and *flhD(e)flhC(s)* a clear difference is seen for fast and slow growth. Interestingly this is

not apparent in the mutant of the target for YdiV regulation, ClpP. Once more these observations associate with the presence of FlhD from *E. coli* further strengthening the argument for a key regulatory role of this complex subunit.

Finally, with regard to deletion of *fliZ*, the patterns produced for *flhDC*$_{(st)}$, *flhDC*$_{(ec)}$ and *flhD(e)flhC(s)* strains are consistent with FliZ acting as a positive regulator of the system with a noticeable drop in foci numbers in fast growth conditions (figure 83).

Taken together, these results are consistent with the motility and flagellar gene expression data. However, here the analysis of flagellar foci in different growth conditions highlights the impact of either FlhD or FlhC in the regulation of the $FlhD_4C_2$ activity by the four key regulators. With respect to flagellar gene expression in relation to FliT regulation, assaying flagellar foci further predicts a role for FlhD. This time, however, the impact is when YdiV regulation is lost. Suggesting, a change in degradation kinetics.

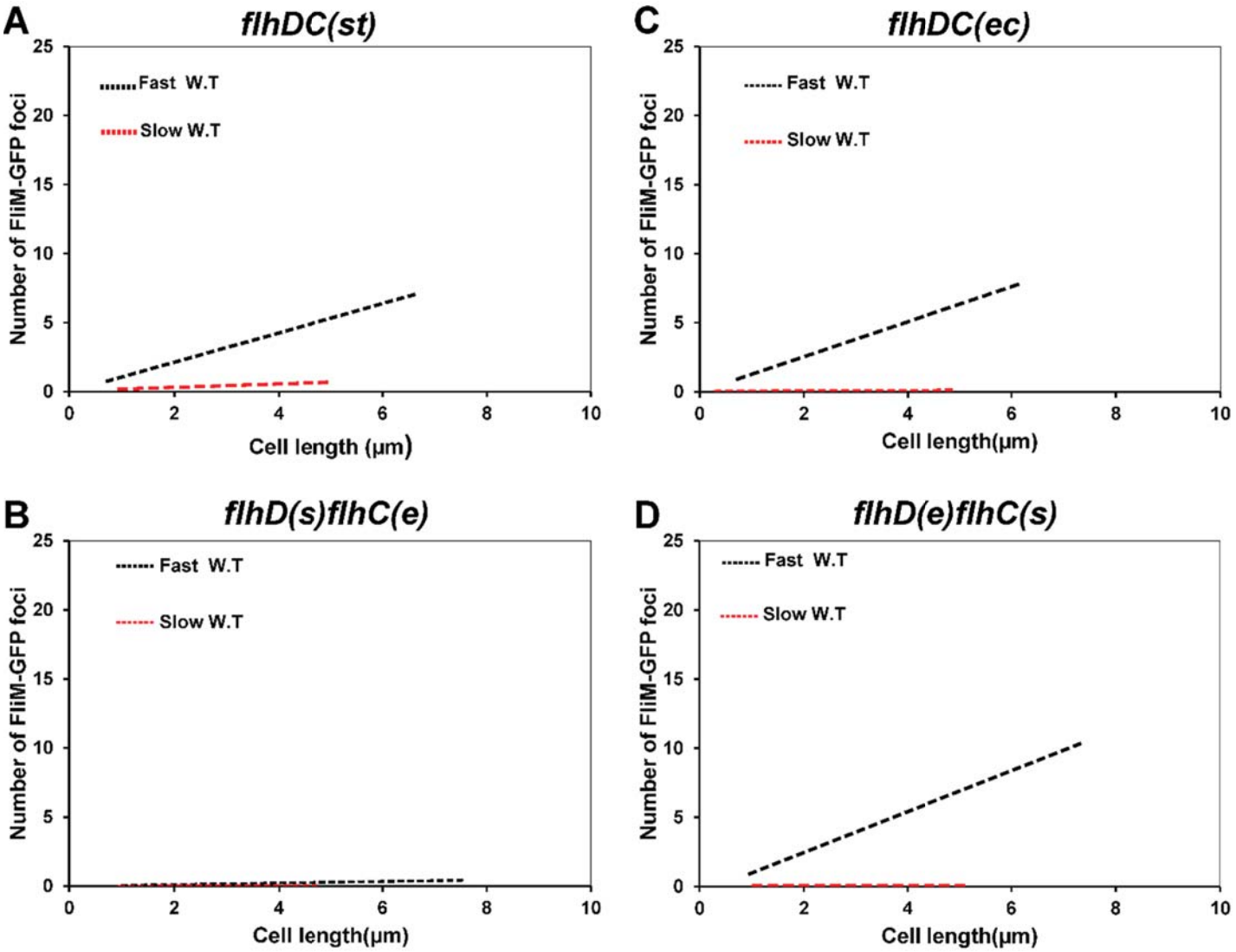


Figure 79. The distribution of FliM–GFP foci per cell plotted against cell length (µm) in strains with intact regulation. (A) *flhDC*$_{(st)}$; (B) *flhD(s)flhC(e);* (C) *flhDC*$_{(ec)}$; (D) *flhD(e)flhC(s*) and) in different conditions Fast growth (black lines): MinE media 0.2% glucose with 3g/L yeast extract and Slow growth (red lines): MinE media 0.2% glucose without Yeast Extract. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment were, *flhDC*(st) W.T= TPA1107, *flhDC*(ec) W.T= TPA3997, *flhD(e)flhC*(s) W.T= TPA4135, *flhD(s)flhC*(e) W.T= TPA4128.

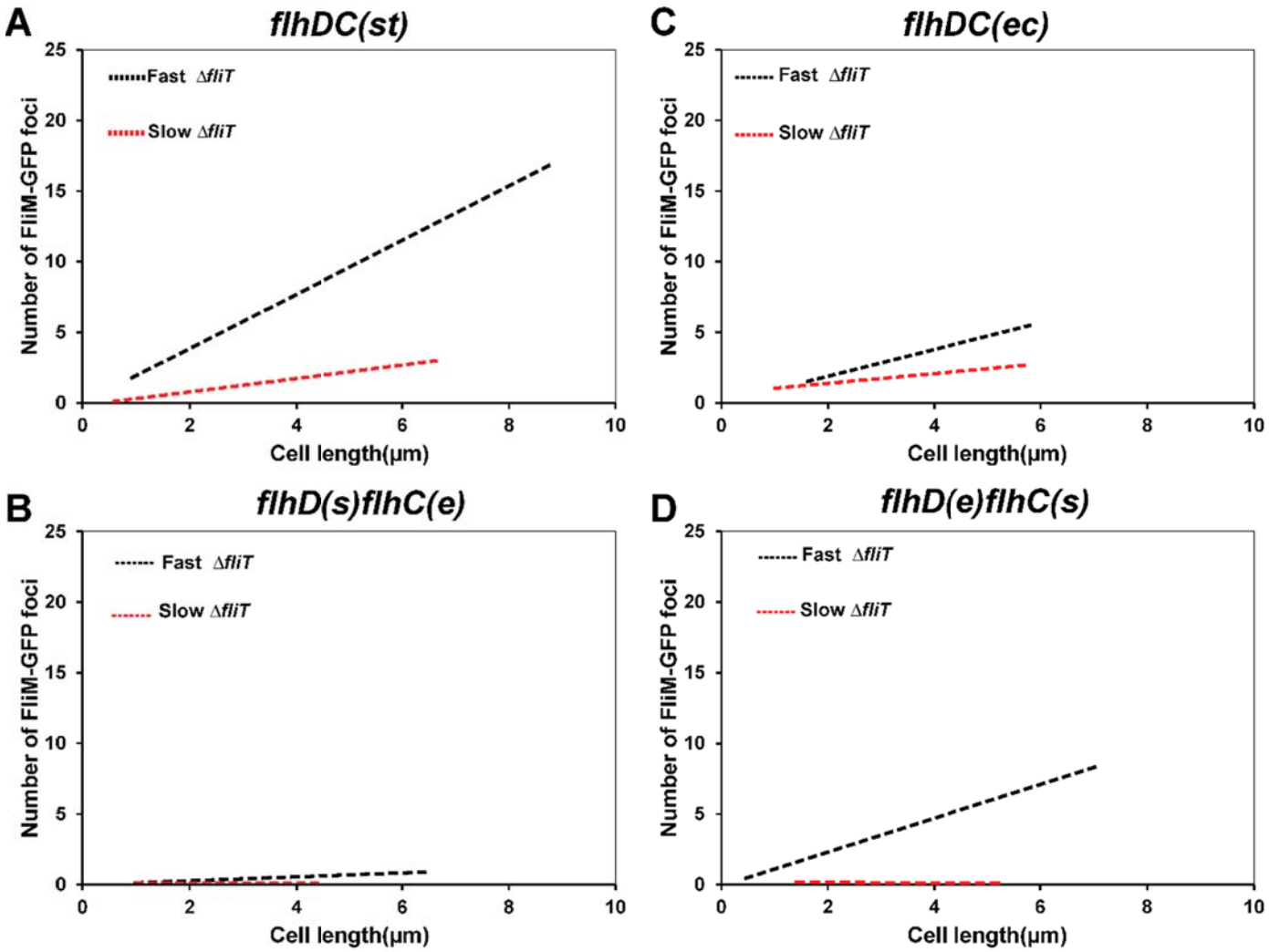


Figure 80. The distribution of FliM–GFP foci per cell plotted against cell length (μm) in Δ*fliT*. (A) *flhDC*$_{(st)}$; (B) *flhD(s)flhC(e);* (C) *flhDC*$_{(ec)}$; (D) *flhD(e)flhC(s)* and) in different conditions Fast growth (black lines): MinE media 0.2% glucose with 3g/L yeast extract and Slow growth (red lines): MinE media 0.2% glucose without yeast Extract. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment were, *flhDC*(st) Δ*fliT* = TPA20, *flhDC*(ec) Δ*fliT* = TPA4576, *flhD(e)flhC*(s) Δ*fliT* = TPA4575, *flhD(s)flhC*(e) Δ*fliT* = TPA4574.

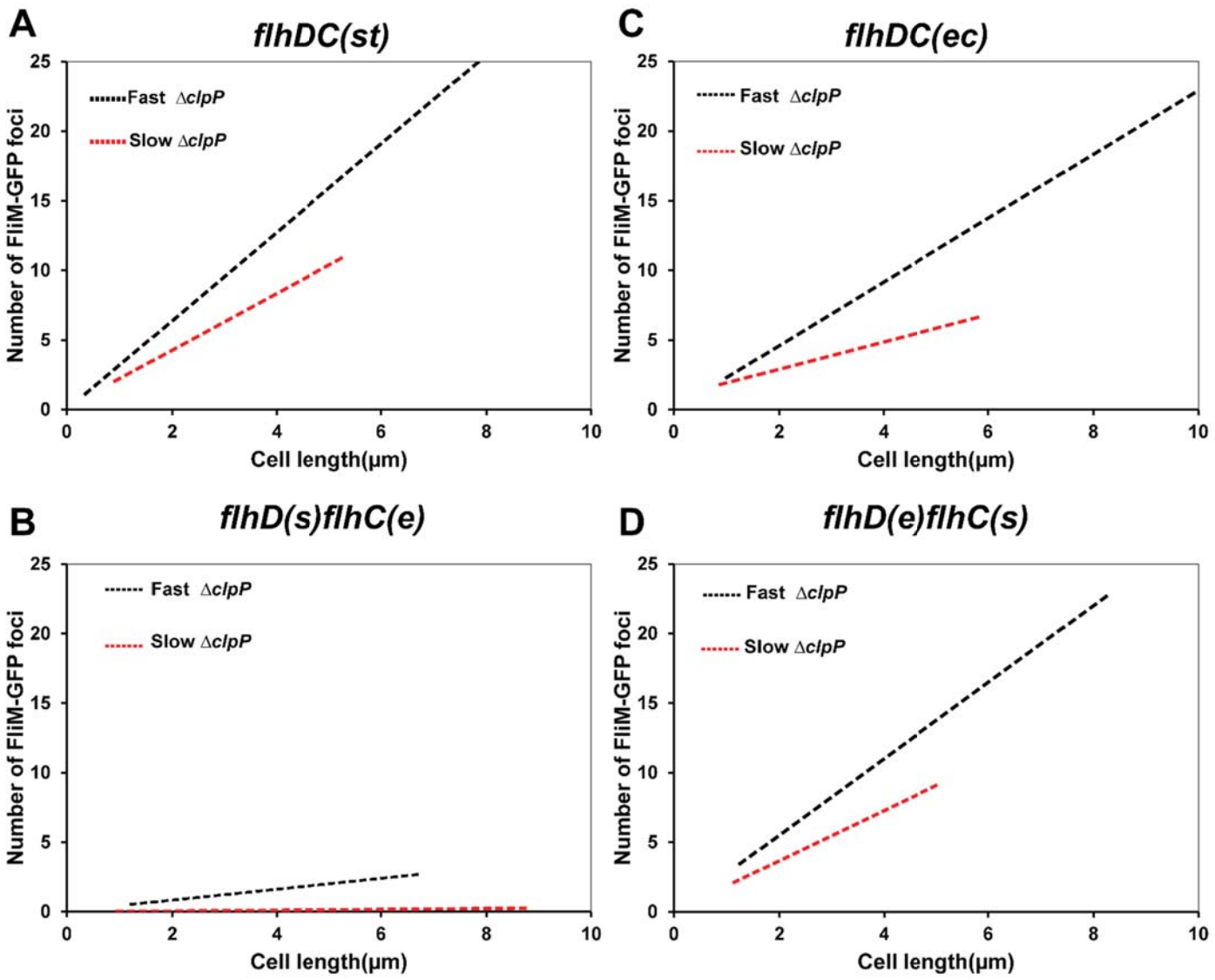


Figure 81. The distribution of FliM–GFP foci per cell plotted against cell length (μm) in Δ*clpP* strains. (A) *flhDC*$_{(st)}$; (B) *flhD(s)flhC(e);* (C) *flhDC*$_{(ec)}$; (D) *flhD(e)flhC(s*) and) in different conditions Fast growth (black lines): MinE media 0.2% glucose with 3g/L yeast extract and Slow growth (red lines): MinE media 0.2% glucose without yeast extract. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment were, *flhDC*(st) Δ*clpP* = TPA2546, *flhDC*(ec) Δ*clpP* = TPA4579, *flhD(e)flhC*(s) Δ*clpP* = TPA4578, *flhD(s)flhC*(e) Δ*clpP* = TPA4577.

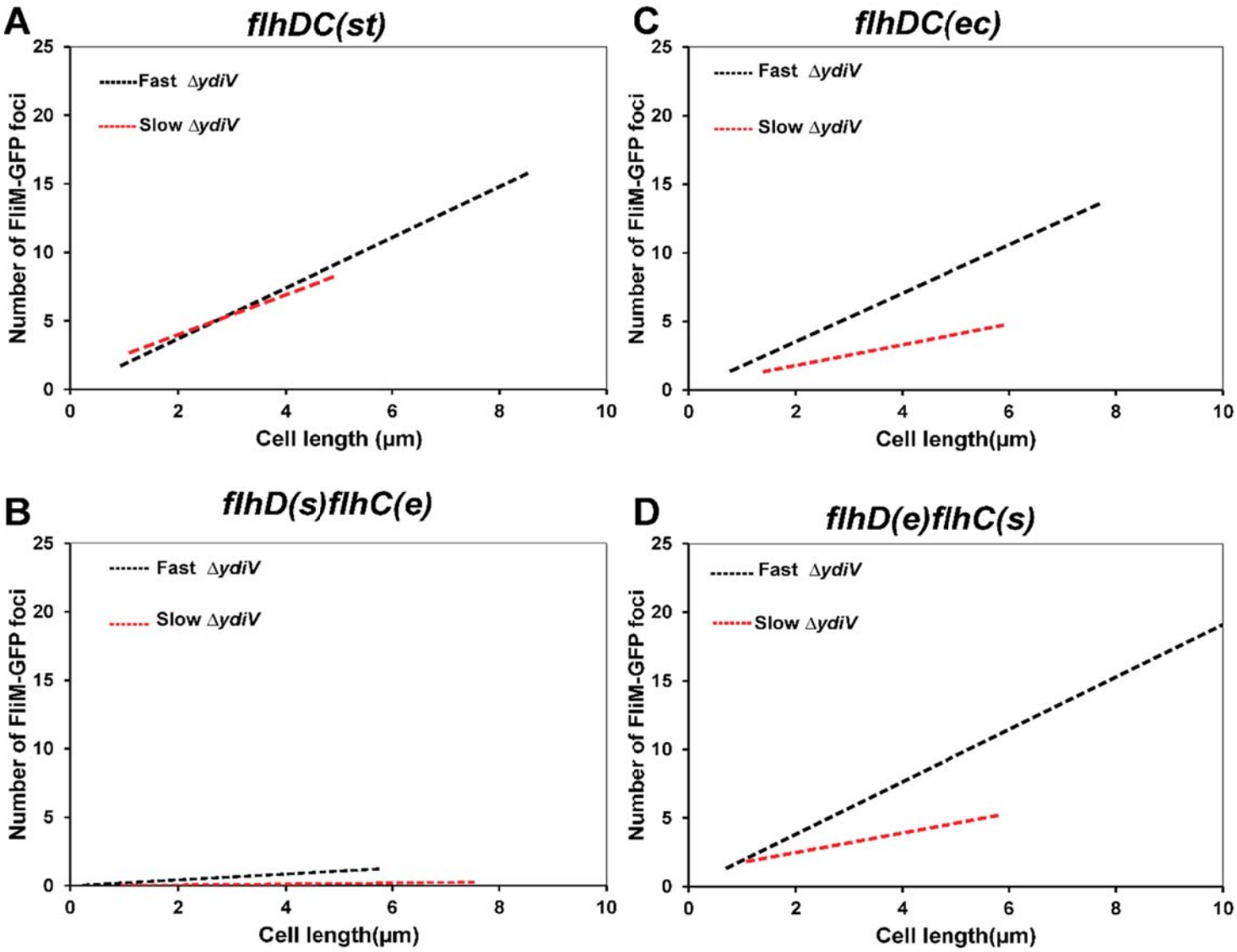


Figure 82. The distribution of FliM–GFP foci per cell plotted against cell length (μm) in Δ*ydiV* strains. (A) *flhDC*$_{(st)}$; (B) *flhD(s)flhC(e);* (C) *flhDC*$_{(ec)}$; (D) *flhD(e)flhC(s*) and) in different conditions Fast growth (black lines): MinE media 0.2% glucose with 3g/L yeast extract and Slow growth (red lines): MinE media 0.2% glucose without yeast extract. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment were, *flhDC*(st) Δ*ydiV* = TPA3356, *flhDC*(ec) Δ *ydiV* = TPA4582, *flhD(e)flhC*(s) Δ*ydiV* = TPA4581, *flhD(s)flhC*(e) Δ *ydiV* = TPA4580.

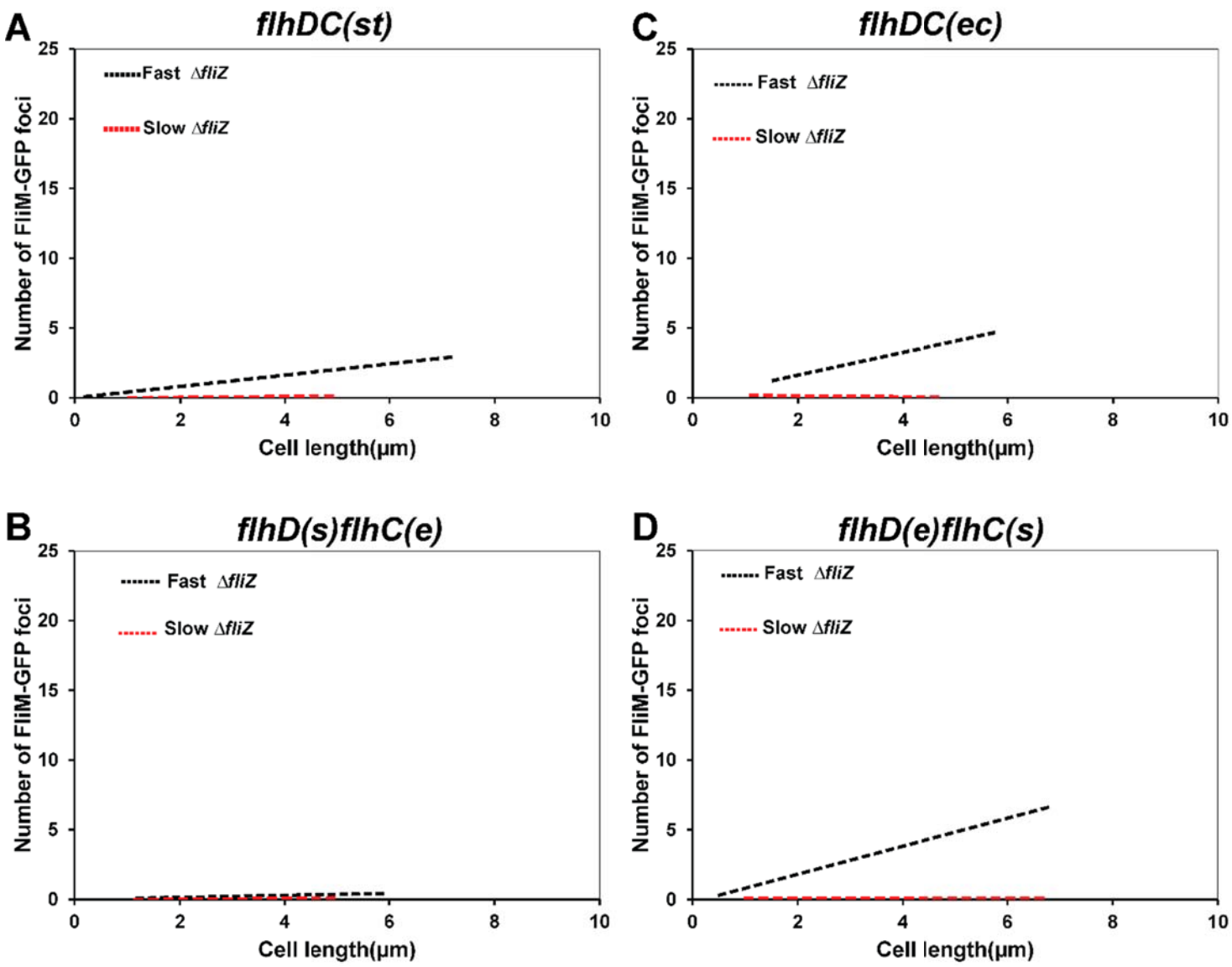


Figure 83. The distribution of FliM–GFP foci per cell plotted against cell length (μm) in Δ*fliZ* strains. (A) *flhDC*$_{(st)}$; (B) *flhD(s)flhC(e);* (C) *flhDC*$_{(ec)}$; (D) *flhD(e)flhC*(s) and) in different conditions Fast growth (black lines): MinE media 0.2% glucose with 3g/L yeast extract and Slow growth (red lines): MinE media 0.2% glucose without yeast extract. Experiment represents a minimal of five independent repeats (n=5). Strains used in this experiment were, *flhDC*(st) Δ*fliZ* = TPA3369, *flhDC*(ec) Δ *fliZ*= TPA4585, *flhD(e)flhC*(s) Δ*fliZ* = TPA4584, *flhD(s)flhC*(e) Δ*fliZ*= TPA4583.

8.5 Summary

In this chapter, we focussed on the impact on $FlhD_4C_2$ activity by the regulators, FliT, ClpP, YdiV and FliZ. This analysis used all standard techniques which have been utilized to assess the flagellar systems response to changes. There was a strong correlation among the flagellar output via quantification of phenotypic motility, determination of the flagellar gene expression and distribution of the flagellar foci in the population. Although a unpredictable result associated with Δ*fliT* for $flhDC_{(ec)}$ and *flhD(e)flhC(s*) was observed that led us to argue that FliT regulation was altered when FlhD from *E. coli* was present. Furthermore, assessment of the regulatory impact on flagellar foci again highlighted a role for FlhD. Flagellar foci changes correlated for FliT but we also observed a change in response when assessing Δ*ydiV* impact. Here potentially the strongest phenotype was observed when comparing the response of $flhDC_{(st)}$ to the two *flhD*(e) combinations. These observations are consistent with the knowledge that YdiV interacts with $FlhD_4C_2$ via contact with FlhD (Wada *et al.*, 2011).

Chapter Nine: General Discussion

9.1 Introduction

In this study, we have made a comprehensive survey among *Salmonella* serovars, originally isolated from around the world and from different hosts, with respect to flagellar system output. Secondly, we have tested the hypothesis that an observed growth control of flagellar abundance in *S.enterica* (LT2) is due to the genetic nature of *flhDC* by replacing these two key flagellar genes with their *E. coli* (RP437) homologues. This analysis has been underpinned by a set of experimental tools that allow the measurement of motility, flagellar gene expression dynamics, and flagellar abundance.

A significant finding from both sides of this study has been the impact the rate of *flhDC* transcription has upon the flagellar system. We found that a motile phenotype does not always correlate to the assumed level of flagellar gene expression and flagellar numbers seen for domesticated strains such as our control strain *S. enterica* serovar Typhimurium LT2. An important aspect of our experimental design that allowed us to come to this conclusion has been the exploitation of the tetracycline system to either induce or titrate *flhDC* expression.

9.2 Dynamics of Flagellar Regulation Controlled by the Tetracycline Inducible System

The first task of this study was to determine the ideal conditions for usage of the tetracycline system. This included exploring which orientation of the *tetRA* system was our baseline to control the flagellar system for the rest of this project. It has been previously demonstrated that the tetracycline system output is dependent on the orientation of the two divergent promoters P_{tetR} and P_{tetA} (Meier *et al.*, 1988). We compared P_{tetAR}, P_{tetRA}, and P_{tetRA} from within the transposon Tn10 derivative T-POP via flagellar output. The P_{tetRA} promoter was the ideal orientation to drive the flagellar

system strongly when compared to P_{tetAR}. Surprisingly, we found P_{tetAR}, and P_{tetRA}-T-POP promoters possessed a similar flagellar output response. This suggests that although T-POP was designed to allow transcription to exit the transposon, the additional sequences that allow T-POP to move, via transposition, has a negative impact on P_{tetRA} to transcribe downstream genes. These findings and conclusions therefore led us to focus on P_{tetRA} and P_{tetAR} usage taken out of the context of T-POP to use in our study of the flagellar system. Using both promoters allowed for a degree of flexibility in *flhDC* expression while investigating different aspects of the project.

9.3 Comparison Between Tetracycline and Anhydrotetracycline as an Inducer.

Anhydrotetracycline is a derivative of tetracycline that possesses no antibiotic activity. It was constructed to be employed when desiring tetracycline-controlled gene expression in the molecular biology field (Berens, 2003). In our project, we compared the activities of the two inducers with respect to flagellar gene expression. Tetracycline was significantly better, possessing a stronger activity than anhydrotetracycline with respect to flagellar output (chapter 4 figure 23). These results contradict the original findings that anhydrotetracycline is a better inducer being 50- to 100-fold more effective than tetracycline (Moyed *et al.*, 1983; Smith and Hoover, 2009). However, it is important to recognise that we have used anhydrotetracycline in the ng/ml range while we used µg/ml for tetracycline. Interestingly, increasing the concentration of anhyrdotetracycline above 25 ng/ml did not improve flagellar gene expression and was comparable to the concentration range used for tetracycline. Our conclusions, however, are derived from the level of maximal activity we achieve with tetracycline compared to anhydrotetracycline for the flagellar system. Therefore, we argue that as an inducer, tetracycline generates a more robust induction while similar results can be achieved with very low

concentrations of its derivative anhydrotetracycline. As a result, we used both inducers at times in our studies taking in to account the differences of their induction profiles and the tetracycline promoter driving *flhDC* expression.

9.4 Heterogeneity of The Flagellar System Output within *Salmonella* serovars

S. enterica serovars were determined to exhibit variation in their motility phenotype and flagellar gene expression. Motility assays are considered the primary evaluation of motility in the flagellar field. We went further and examined flagellar gene expression for each tier of the flagellar transcriptional hierarchy assessing P_{flhDC}, P_{flgA} and P_{fliC} activity. With respect to the timing of P_{flgA} and P_{fliC} activation on induction using the tetracycline system, the period of expression for all serovars was between 30 – 60 mins. These timings are comparable to previous studies such as Brown *et al* (2008). In contrast, serovars exhibited a greater fluctuation in the magnitudes of expression. Furthermore, some serovars showed mid-range P_{flgA} activity but high P_{fliC} activity while others the opposite.

We had assumed, based on the literature, that as the transcription of flagellar genes is hierarchical that an increase in P_{flgA} would lead to an increase in P_{fliC}. This assumption has been proved wrong by our results. What could be the reason behind this? Considering the flagellar system specifically, one explanation could be genetic variation amongst the multiple flagellar specific regulatory components, including the promoter regions that drive flagellar gene expression. However, phylogenetic analysis of the promoter regions for P_{flhDC}, P_{flgAB} and P_{fliC} did not identify a specific group of serovars with a specific flagellar gene expression profile or motility phenotype. The closest correlation was when considering the phylogeny derived from concatenated MLST sequences where four serovars with low P_{flgA} and P_{fliC} activity may cluster (Chapter 4 figures 33, 34, 35 and 36). This cluster is lost for P_{flhDC} and

the P_{flgAB} and P_{fliC} promoters exhibited too high a degree of conservation to differentiate specific serovars. Therefore, we conclude that external regulatory inputs dictate the variation we have observed within the flagellar systems of the *S. enterica* serovars tested. This conclusion is supported by the variation identified across the P_{flhDC} promoter region. Transcription of *flhDC in S. enterica* is complex with 6 potential -10 regions existing that have accompanying evidence that they are utilised by RNA polymerase and s70 to activate transcription (Mouslim and Hughes, 2014). However, Mouslim and Hughes (2014) have shown in vivo that only 2 of the six promoters drive *flhDC* transcription. Interestingly, some variation is located around the binding sites of two known transcriptional regulators that impacts P1 actvity (Mouslim and Hughes, 2014). This suggests that changes in flagellar output across serovars is predominately dependent on integration of regulatory signals that impact *flhDC* transcription. The use of P_{tetRA} and P_{tetAR} to drive *flhDC* transcription supports this conclusion as we did measure changes in some serovars but not all.

Surprisingly, the serovar Java behaved very differently from others as there was a little to no detectable activity for P_{flgA} and P_{fliC}, but Java was motile. At this point, we asked why Java behaved like this, and are there any other serovars that behaved like Java? Indeed, we found Emek, Abony and Lexington to behave similarly to Java. Replacing P_{flhDC} with P_{tetRA} did not improve flagellar gene expression and motility. However, titration using anhyrdotetracycline did achieve a detectable level of activity in Java at high concentrations. In doing so we observed that once more timing of activation was not effected just magnitude. Using plasmid based expression of *flhDC* could also improve flagellar gene expression but not to levels comparable with LT2. This supports the argument for *flhDC* transcription playing a significant role in dictating flagellar output. It did not, however, completely explain the Java motility phenotype when considering motility versus flagellar gene expression.

Koirala *et al* (2014) demonstrated that the serovar Typhimurium flagellar system is bistable generating population heterogeneity. A conclusion based on Java and other serovars was that $FlhD_4C_2$ activity was in some ways being repressed in these serovars. An outcome of reduced $FlhD_4C_2$ activity is that a greater degree of heterogeneity occurs. Therefore, we tested the hypothesis that the measured flagellar output was a result of population heterogeneity. Indeed, our data analysis suggested that heterogeneity varies significantly between the *Salmonella* serovars especially for Java, Emek, and Lexington.

So why then are these serovars producing good swims on motility agar? We argue that motility agar is a positive selection for the motile subpopulation. Either by selection or a genetic switch when we observe motility in agar we are potentially seeing cells derived from the initial motile population. This explanation and conclusion is consistent with population heterogeneity being the output of a strong regulatory network driving $FlhD_4C_2$ activity (Koirala *et al.*, 2014b). In this study the mechanistic source of the heterogeneity was not investigated. Koirala *et al* (2014) has shown the impact of YdiV regulation on heterogeneity. It is feasible to argue that YdiV regulation will differ across serovars leading to the diversity we observed in our experiments. However, it is also plausible that the mechanistic source of heterogeneity comes from an alternative regulatory input. For example, in Java specifically, the inability to significantly raise flagellar output by expressing *flhDC* from a plasmid or the P_{tetRA} system argues for post-transcriptional regulation, such as lead by YdiV. However, it is feasible to argue that translational control also plays a role in driving the population heterogeneity and variation in flagellar gene expression noted in this study. One source of translational regulation is via small regulatory non-coding RNA molecules (Kröger *et al.*, 2012; Westermann *et al.*, 2016). Importantly, transcription from the mapped P1 and P5 promoters in the P_{flhDC} region generate long

untranslated regions that are a feature of systems regulated by small RNA molecules at the translational level. However, the P_{tetRA} data in Java and other serovars removes these untranslated regions without significantly improving flagellar output. Therefore, we conclude that translational regulation is a feasible source of the observed variation, however, the data also strongly argues for post-transcriptional regulation playing a major role in the system.

9.5 The Outcome of Replacing *flhDC* in *S. enterica* with their *E. coli* Homologues.

This study elucidates how different *flhDC* combinations, when considering the *S. enterica* and *E. coli* homologues, operate to drive motility in *S.enterica* Typhimurium. A previous thesis project has shown that *E. coli* and *S. enterica* respond to growth conditions in a similar manner. However, one noticeable difference was the presence of non-motile cells when *S. enterica* was grown at a fast growth rate, while *E. coli* was 100% motile. One hypothesis drawn from these growth experiments was that the genetic variation between *flhDC* is a driver to the observed response for *E. coli* and *S. enterica*. Therefore, this study planned to replace *flhDC* from *E. coli* in *S. enterica* and ask: do we lose the non-motile cells at fast growth? When replaced $FlhDC_{(ec)}$ supported motility allowing us to conclude that the system was complimented. This then enabled us to investigate the initial question where we demonstrated that $FlhDC_{(ec)}$ did not impact the growth rate response of *S. enterica*. This argues that the generation of a subpopulation of cells at a fast growth rate in *S. enterica* is dictated by factors other than $FlhD_4C_2$ itself. Evidence from previous studies, and the arm of this study that focussed on serovar variation, argues that a significant impact on population heterogeneity is not via the nature of the $FlhD_4C_2$ complex but the level of activity the intercellular pool of this complex has in each cell.

Purposefully, we examined the flagellar system output with our complete toolkit of standard assays for motility, flagellar gene expression and flagellar numbers for the *flhDC* switched strain. To further emphasise the role of *flhDC*, we replaced *flhD* and *flhC* individually. On examining the flagellar system output the consequence of altering the source and format of the $FlhD_4C_2$ complex generated a series of interesting observations. Surprisingly, the data argues that the hybrid FlhD(e)FlhC(s) complex exhibited a more robust phenotype. In contrast FlhD(s)FlhC(e), was a very inefficient complex showing low output in all assays. Surprisingly, although $FlhDC_{(ec)}$ drove motility when measuring flagellar gene expression a noticeable difference was evident in comparison to the other complexes tested. Indeed, one conclusion from this analysis was the importance of FlhD to influence flagellar system output. Furthermore, a comparison of the data sets to the serovar data argued that the FlhD(s)FlhC(e) complex had low activity either by its reduced ability to form a complex or to bind DNA.

In chapter 7 we purified the four complexes to explore the biochemical explanation of the FlhD(s)FlhC(e) complex activity. Our results provided on insight in to the functionality of the protein-protein interactions and also the ability of the complexes to binding DNA. Importantly, FlhC(e) was purified with FlhD(s) but only in detectable amounts when using His-tag purification. Using Heparin to mimic DNA led to only FlhD(s) being isolated from this complex. In comparison the other three complexes were all efficiently purified using the heparin method. The purification data could not rule out the possibility that the *flhD*(s)*flhC*(e) operon was not being transcribed or translated efficiently. The net outcome, however, is that this weak complex can be formed, can activate the flagellar system but the interactions between FlhD and FlhC are weak. A weakness of our analysis is the difficulties associated with detecting FlhD and FlhC *in vivo* using immunoblot technology. Antibodies to both FlhD and

FlhC exist and have a high level of specificity for both proteins as determined against purified complexes(Pooncharoen, 2013). However, Claret and Hughes (2000) have shown that FlhD and FlhC are rapidly turned over in vivo with a half-life of approximately 2 mins. The result of this is that the detectable levels of FlhD and FlhC in *S. enterica* are kept very low, meaning that detection in lysates of *S. enterica* samples is very difficult (Pooncharoen, 2013). Some studies have worked around this by using C-terminal protein tags to detect these proteins, however, this has the net effect of stabilising the proteins and altering flagellar output itself (Saini *et al.*, 2008). If these technical and biological aspect were overcome, then it was a feasible option for further study to correlate the findings of this study with *in vivo* protein level detection to determine the impact of the genetic manipulations made on FlhD and FlhC protein levels. Furthermore, this analysis could be widened to encompass the serovar experiments to assess FlhD and FlhC availability in serovars and whether this would also aid our understanding of the variation we observed across the *S. enterica* species.

We used the ESMA assay to look at the ability of DNA:complex formation. All complexes were able to shift P_{flgAB} DNA in a predictable fashion. For FlhD(s)FlhC(e) complex this did require high concentrations of the protein complex. Previous work on the $FlhD_4C_2$ complex argued that FlhC was the DNA binding protein (Claret and Hughes, 2000a). Our data was consistent with this previous conclusion as quantification of the DNA binding activity differentiated between which FlhC protein was present. Interestingly, this was also seen for FlhD(s)FlhC(e) arguing further that the low efficiency of this complex is not its ability to bind DNA but the efficency of complex formation, leading to apparent low activity.

9.6 Regulating the Activity of $FlhD_4C_2$ Complex Combinations.

$FlhD_4C_2$ activity is tightly regulated by four factors ClpP, YdiV, FliZ and FliT. ClpP and YdiV regulate the protein stability of FlhD and FlhC. FliZ regulates $FlhD_4C_2$ indirectly in *S. enterica* regulating *ydiV* expression, while FliT interacts directly with $FlhD_4C_2$ to disrupt the complex reducing its availability to bind DNA. Surprisingly, Δ*fliT* strains behaved differently with respect to if *E. coli* or *S. enterica flhDC* was present. We found that Δ*fliT* impact on $FlhDC_{(ec)}$ suggested that FliT may positively regulate $FlhD_4C_2$ activity if FlhD or both FlhD and FlhC from *E.coli* were present. This is entirely different from how FliT negatively regulates the *S. enterica* FlhDC complex by reducing its concentration via disrupting the complex (Yamamoto and Kutsukake, 2006a; Aldridge *et al.*, 2010). Furthermore, the *S. enterica* data has clearly demonstrated that FliT interacts with FlhC, while our interpretation of our data argues the case for FlhD playing a regulatory role in dictating FliT impact on *E. coli* containing combinations. This is strong evidence that *E. coli* perceives FliT differently even though the two flagellar systems show significant similarity at the protein level of FlhD, FlhC and FliT.

In contrast, our results that related to Δ*clpP,* Δ*ydiV* and Δ*fliZ* were more in agreement with them acting in a consistent manner irrespective of the *flhDC* source. Interestingly, one observation argued a consistency with the FliT data with respect to a response associated with YdiV. Once more when FlhD(e) was present flagellar foci could differentiate between the source of FlhD. YdiV does however interact with FlhD, while FliT interacts with FlhC. Furthermore, Wada *et al* (2011), have shown that while *ydiV* is expressed in *S. enterica* expression and potentially the mode of action in *E coli* is different (Wada *et al.*, 2012). This argues that FlhD(e) may have evolved in the absence of YdiV regulation. This argues that for both species although regulation of $FlhD_4C_2$ activity is shared there are some aspects of the interactions

between these known regulators that differ leading to clear changes in the downstream response of the flagellar system.

9.7 Conclusions

In conclusion, this project has provided an insight in to the fine details of how the species *E. coli* and *S. enterica* control $FlhD_4C_2$ activity. We have based our findings of the response to changes in the genetic makeup of *flhDC* in *S. enterica*. Our data, however, is consistent with other studies arguing a case for the observed details to hold when considering flagellar gene regulation in *E. coli* as a species. We recognise that it would now be of interest to potentially confirm some of our findings, especially detailing around the role of FlhD, by exploring the response of the *E. coli* flagellar system to similar or more specific genetic manipulations. In general, this project has provided the following points to note:

- *Salmonella enterica* serovars conserve the timing of flagellar gene activation but magnitude of expression varies.
- Data from Java suggests post-transcriptional regulation is key to the observed variation.
- There is evidence that the potent nature of phenotypic heterogeneity dictates the output of the flagellar system across the species *S. enterica*.
- Transcriptional activity/efficiency of *flhDC* is a key player in dictating a response to signals with respect to flagellar abundance in both *S. enterica* and *E. coli.*
- $FlhD_4C_2$ -*E.coli* can sustain motility in *S.enterica,* but has an altered activity as a transcriptional activator.

- Our data suggests that FlhC is a key driver in $FlhD_4C_2$-dependent promoter selectivity. In contrast, FlhD plays a key role in $FlhD_4C_2$ activity
- The regulation of $FlhD_4C_2$ activity by FliT differs in *E. coli* versus *S. enterica.*

Based on these observations and the conclusions drawn from our data we argue the importance in our current research climate to take into consideration the species not only the strains we regularly work with. The diversity we observe in just a small subset of *S. enteria* serovars that describe neatly this species has made us reconsider a number of assumptions we make about the regulation of the flagellar system. This study has made us aware that this variation impacts how the flagellar system is exploited by specific strains and potentially its role during environmental and host-bacterial interactions.

Chapter Ten: Appendix

10.1 Bacterial growth media

All bacterial growth media suspended in Milli-Q water and send to an autoclave centre for sterilization. Filtered antibiotics and growth complements were added after the temperature media was reduced less than 40 ^{o}C.

Table 6: Luria-Bertani (LB) broth media

Luria-Bertani (LB) broth media / 1 litre		
Tryptone	10 g	Bacto
Yeast Extract	5 g	Bacto
Sodium Chloride NaCl	5 g	Melford
Water	Up to one litre	

Table 7: Luria-Bertani (LB) agar

Luria-Bertani (LB) agar / 1 litre		
Tryptone	10 g	Bacto
Yeast Extract	5 g	Bacto
Sodium Chloride NaCl	5 g	Melford
Agar	15 g	Bacto
Water	Up to one litre	

Table 8: Motility agar

Motility agar / 1 litre		
Tryptone	10 g	Bacto
Sodium Chloride NaCl	5 g	Melford
Agar	3 g	Bacto
Water	Up to one litre	

Table 9: Minimal media 3gram

Minimal media 3gram / 1 litre		
2x Minimal E salts	50 ml	Bacto
Bacto Yeast Extract 25g/L	12ml	Bacto
50% Glucose	0.4 ml	Sigma-Aldrich
Water	Up to one litre	

Table 10: Minimal media 1gram

Minimal media 1gram / 1 litre		
2x Minimal E salts	50 ml	Bacto
Bacto Yeast Extract 25g/L	4 ml	Bacto
50% Glucose	0.4 ml	Sigma-Aldrich
Water	Up to one litre	

Table 11: Minimal media 0.2gram

Minimal media 0.2gram / 1 litre		
2x Minimal E salts	50 ml	Bacto
Bacto Yeast Extract 25g/L	0.8ml	Bacto
50% Glucose	0.4 ml	Sigma-Aldrich
Water	Up to one litre	

Table 12: Minimal media 0.04gram

Minimal media 0.04gram / 1 litre		
2x Minimal E salts	50 ml	Bacto
Bacto Yeast Extract 25g/L	0.16 ml	Bacto
50% Glucose	0.4 ml	Sigma-Aldrich
Water	Up to one litre	

Table 13: P22 phage Buffer

P22 phage Buffer	
LB liquid medium	100 ml
50x Minimal E salts	2 ml
20 % glucose	1 ml

Table 14: Green agar

Green agar / 1 litre		
D-Glucose	7.4 g	Sigma-Aldrich
Tryptone	7.8 g	Bacto
Yeast-Extract	1 g	Bacto
Sodium Chloride NaCl	5 g	Melford
Agar	15 g	Bacto
Methyl Blue	0.07 g	Sigma-Aldrich
Alizarin Yellow	0.6 g	Sigma-Aldrich
Water	Up to one litre	

10.2 Agarose Gel Electrophoresis

Table 15: 10 x DNA Loading Buffer

10 x DNA Loading Buffer		
Tris Acetate	200 mM	Sigma-Aldrich
EDTA (PH 8.0)	5 mM	Sigma-Aldrich
Glycerol	50%	Sigma-Aldrich
Bromophenol Blue	0.1%	BIO-RAD
Xylene Cyanole FF	0.1%	Sigma-Aldrich
Orang G	0.2%	Sigma-Aldrich
Sterile Water	Up to 50 ml	

Table 16: Agarose Gel 1%

Agarose Gel 1%		
Agarose	4 g	Peqlab
DNA Nancy-520 stain	9µl	Sigma-Aldrich
TAE buffer 1X	Up to 400 ml	Sigma-Aldrich

10.3 Tricine SDS-polyacrylamide Gel Electrophoresis

Table 17: Tricine Gel Buffer pH=8.45

Table 15: Tricine Gel Buffer pH=8.45		
Tris Base	3M	Peqlab
Tris.Hcl	3M	Sigma-Aldrich
Sodium Dodecyl Sulphate SDS	0.6g	Sigma-Aldrich
Water	Up to 200 ml	

Table 18: SDS gel (Separating Gel12%)

SDS gel (Separating Gel12%)		
Separating Acrylamide (49.5 % - 16.5 % T 3 % C)	3.6 ml	Severn Biotech LTD
Ammonium Persulphate APS (10 %)	75 µl	Sigma-Aldrich
Tetramethylethylenediamine TEMED	7.5 µl	BDH
Glycerol (50 %)	5 ml	Sigma-Aldrich
Tricine Gel Buffer	5 ml	
Sterile water	1.4 ml	

Table 19: SDS gel (Stacking Gel 3.96%)

SDS gel (Stacking Gel 3.96%)		
Stacking Acrylamide (49.5 %-4 % T 3 % C)	1 ml	Severn Biotech LTD
Tricine Gel Buffer	3.1 ml	Sigma-Aldrich
Ammonium Persulphate APS (10 %)	100µl	Sigma-Aldrich
Tetramethylethylenediamine TEMED	10 µl	BDH
Sterile water	8.4 ml	

Table 20: Anode Running Buffer X10 pH=8.9

Anode Running Buffer X10 pH=8.9		
Tris Base	2 M	Sigma-Aldrich
Tris-Hcl	38.4g	Sigma-Aldrich
Sterile water	100 ml of buffer Up to one litre	

Table 21: Cathode Running Buffer X10 pH=8.25

Cathode Running Buffer X10 pH=8.25		
Tris Base	1 M	Sigma-Aldrich
Tricine	1 M	Sigma-Aldrich
SDS	100ml 10 %	Sigma-Aldrich
Sterile water	Up to one litre	

Table 22: Coomassie Blue Stain

Coomassie Blue Stain		
Ethanol	50 %	University Stores
Acetic Acid	5 %	VWR
Coomassie Blue	0.02 %	Fluka
Sterile water	Up to 1 litre	

Table 23: Destaining Solution

Destaining Solution		
Ethanol	50 %	University Stores
Acetic Acid	5 %	VWR
Sterile water	Up to 1 litre	

10.4 Protein Purification

Table 24: His-Chelating Loading Buffer pH=7.5

His-Chelating Loading Buffer pH=7.5		
Hepes	50mM	Sigma-Aldrich
Sodium Chloride NaCl	150mM	Melford
Imidazole	20mM	Sigma-Aldrich
2-Mercaptoethanol	2mM	VWR
Sterile water	Up to 1 litre	

Table 25: His-Chelating Elute Buffer pH=7.5

His-Chelating Elute Buffer pH=7.5		
Hepes	50mM	Sigma-Aldrich
Sodium Chloride NaCl	150mM	Melford
Imidazole	1M	Sigma-Aldrich
2-Mercaptoethanol	2mM	VWR
Sterile water	Up to 1 litre	

Table 26: Stripping Buffer pH=7.4

Stripping Buffer pH=7.4		
Sodium Phosphate	20 mM	Sigma-Aldrich
Sodium Chloride NaCl	500mM	Melford

Ethylenediaminetetraacetic EDTA	50 mM	Sigma-Aldrich
Sterile water	Up to 1 litre	

Table 27: Nickel solution

Nickel solution		
NiCl2	0.1M	Sigma-Aldrich
Sterile water	Up to one litre	

Table 28: Heparin Loading Buffer pH=7.9

Heparin Loading Buffer pH=7.9		
Tris (Trizma Base)	10mM	Sigma-Aldrich
Sterile water	Up to one litre	

Table 29: Heparin elution Buffer pH=7.9

Heparin elution Buffer pH=7.9		
Tris (Trizma Base)	10mM	Sigma-Aldrich
Sodium Chloride NaCl	1M	Melford
Sterile water	Up to 1 litre	

Table 30: Changing buffer pH=7.9

Changing buffer pH=7.9		
Tris Base	10 mM	Sigma-Aldrich
Sodium Chloride NaCl	300mM	Melford
Dithiothreitol DTT	1 mM	Melford
Sterile water	Up to 1 litre	

Table 31: TBE buffer X10

TBE buffer X10		
Tris Base	89 mM	Sigma-Aldrich
Boric acid	89mM	Sigma-Aldrich
0.5M EDTA(pH8.0)	40ml	Sigma-Aldrich
Sterile water	Up to 1 litre	

Table 32: ESMA gel

ESMA gel		
40% Acrylamide	2.5ml	Merck
Tetramethylethylenediamine TEMED	13 µl	BDH
Ammonium Persulphate APS (10%)	150 µl	Sigma-Aldrich
10X TBE buffer	750µl	
Sterile water	11.69 ml	

10.5 Table Of Plasmids

Table 33: Plasmid Feature(s)

Plasmid name	plasmid feature(s)	Reference
pKD46	λRed-expression under control of arabinose-inducible promoter, temperature-sensitive, Ampr	(Datsenko and Wanner 2000)
pWRG99	pKD46 with *I-SceI* endonuclease under control of tetracycline-inducible promoter (P_{tetA}), temperature-sensitive, orientation 2 (5'-.3': *I-SceI-tetR*), Ampr	(Blank, Hensel *et al*. 2011)
pWRG100	*I-SceI* recognition site, Cmr	(Blank, Hensel *et al*. 2011)
pKD3	Red template plasmid, suicide vector (ori R6K), Ampr , Cm	Barry L. Wanner
pKD4	Red template plasmid, suicide vector (ori R6K), Ampr , kan	Barry L. Wanner
pACYC184-*motA*	pRG19::FCF/ P(*flhDC*)5451::Tn10dTc[del-25]	Aldridge's lab
pACYC184-*flgA*	pRG39::FCF/ P(*flhDC*)5451::Tn10dTc[del-25]	Aldridge's lab
pACYC184-*fliC*	pRG51::FCF/ P(*flhDC*)5451::Tn10dTc[del-25]	Aldridge's lab
pACYC184-*flhD*	pRG38::FCF/ (P_{flhD}-*luxCDABE* TcR)	Aldridge's lab
pSE380	trc_promoter, lac operator, superlinker multiple cloning site, AMP resistance gene.	Invitrogen
pSE380-*flhDC3*	pSE380-flhDC3 Δ*flhDC*7011::FCF	Aldridge's lab
pET28a mod	Bacterial expression vector with T7lac promoter, adds N-terminal His tag, thrombin cleavage site	Novagen
pET28a-*flhDsCe*	pET28a-*flhD*(*S. enterica*)-*flhC*(E.coli437)	This study
pET28a-*flhDCe*	pET28a-*flhDC*(*E.coli* 437)	This study
pET28a-*flhDeCs*	pET28a-*flhD*(*E.coli* 437)-*flhC*(*S.enterica*)	This study
pET28a-*flhDCs*	pET28a-*flhDC*(*S.enterica*)	This study
pCP20	FLP, chloramphenicol and ampicillin resistant genes, and temperature sensitive replication.	(Cherepanov &Wackernagel,(1995)

10.6 Oligonucleotides

Table 34: Primers sequences

Primer Number	Primer Name	Primer Sequence (5' to 3')
981	pTetR_flhD_F(LT2)	aactcgctccttgattgcaagaatatgagattgtgctttaCTAAGCACTTGTCTCCTG
982	pTetA_flhD_R (LT2)	caactttatttttgtgcgacgtagccgcaccccgtgatgtTTAAGACCCACTTTCACA
1083	oAJ1 pWRG100_F	tgtcatatttactccttgcacagcgtttgatcgtccaggacaaagCGCCTTACGCCCCGCCCTGC
1084	oAJ2 pWRG100_R	cagcatctcgggaaagtttacgtctttttactgcgcgggatggcgCTAGACTATATTACCCTGTT
1085	oAJ3 flhD-e.coli F	acatcacggggtgcggctacgtcgcacaaaaataaagttggttattctggatgcatacctCCGAGTTGCTG
1086	oAJ4 flhC e.coli R	gcagcggtaatgacttaccgctgctggagtgtttgtccacaccgtttCGGTTAAACAGCCTGTACTCTCTG
471	flhD-42FEco	gcggaattcGGGTGCGGCTACGTCGCAC
467	flhC+616REco	gcggaattcCGCTGCTGGAGTGTTTGTCC
1166	WRG_flhD+6F	tgcggctacgtcgcacaaaaataaagttggttattctggatgggaCGCCTTACGCCCCGCCCTGC
1167	WRG_flhC+579R	gacttaccgctgctggagtgtttgtccacaccgtttcggttaaacCTAGACTATATTACCCTGTT
1168	flhD-125F	cgtcacaatgtccataatgtc
1169	flhC+744R	ccgataaccaccaggtaacc
405	tetA+3ndeIR	gcgcatatgacttttctctatcactgatag
4	flgN+532R	gagtttgttcgccggacg
5	flgM-82F	gattttgtcgcggctgcc
25	fliA-118F	ggcgctacaggttacataag
26	fliA+765R	tagtctatacgttgtgcggc
1179	PflhDC-786_tetR	aactcgctccttgattgcaagaatatgagattgtgctttattaagacccactttcaca

Primer Number	Primer Name	Primer Sequence (5' to 3')
1180	PflhDC-50_tetA	caactttatttttgtgcgacgtagccgcaccccgtgatgtctaagcacttgtctcctg
1182	fliC+633R	accaagaccagtagccgagg
1183	fliD+585R	gatcttcatcgtattgtcgg
1181	flgB+413R	ctgtagcacattcatcatgc
37	flgN+435ScR	ccgagctccatccggcaatgattagatag
258	fliC-247F	ggtcttgcgcgatggtac
983	pTetRA-flhD-1035chkF	agtttgttcaatcggataatccgcc
1184	RP_flhd-59FOR	Acatcacggggtgcggctacgtcgcacaaaaataaagttggttattctggatgggaacaatgcatacctc cgagttgctg
404	tetR+SpeIF	ggcactagtctaacatctcaatggctaagg
1225	WRG_flhD+R	GCGAGCTTCCTGAACAATGCTTTTTTCACTCATTATCATGCCCTT*CTAGA CTATATTACCCTGTT*
1226	WRG_flhC+F	GCTCAATGAAGTGGACGATACGGCGCGTAAGAAAAGGGCATGATA*CGC CTTACGCCCCGCCCTGC*
1227	flhD-e.coli R	TGGATATCGCGAGCTTCCTGAACAATGCTTTTTTCACTCATTATCATGCC CTTTTCTTGCGCAGCGCTTC
1228	flhC-e.coli F	CGCGTCTGCTCAATGAAGTGGACGATACGGCGCGTAAGAAAAGGGCAT GATAGAAAAAAGCATTGTTCAG
27	fliA+1043R	tatcgaaaaaatcactctgc
469	flhD-866FEco	gcggaattcGCGATAGAGACCGCTTTAGCC
531	flhDB2H(n)R	ggcgagctca
1178	RP_flhD-11_DELR	CATAAATGTGTTTCAGCAACTCGGAGGTATGCATTATTCCCACCCCTAG ACTATATTACCCTGTT
1165	RP_yecG-40_DELF	tcataacctgttccttattctgtgaacttcaggtgacattaaagcCGCCTTACGCCCCGCCCTG C
1229	RP_flgE+675_WRG_F	TACACCCAGGATAGCAGTGATCCAAACAGCATTGCGAAGACAGCGctag actatattaccctgtt
1233	RP_flgE+690_WRG_R	gatattattcgccatcgcaccatccactaatgtgccattagcattCGCCTTACGCCCCGCCCT GC
1247	RP_flgE-155CHK	gtacacaactggttgcccagccgc
1248	RP_flgE+1375CHK	aagcccttccactggcaccgcgcg
1249	flgB+104R	gtatcggcattggcgatattcgccg
23	fliT-138F	ctatatgattcgccgtttac
24	fliT+481R	taataccagtggaagtactg
533	flhDB2H(c)R	ggcggtaccTCATGCCCTTTTCTTACG
126	flhD+1FBam	ggatccATGGGAACAATGCATACATC
127	flhC+616RSc	CCGAGCTCCGCTGCTGGAGTGTTTGTCC
1268	flhDEC+1Bam	ggatccATGCATACCTCCGAGTTGCT
1291	pET28a_SacI_F	GGACAAACACTCCAGCAGCG GAGCTCCGTCGACAAGCTTG
1292	pETSacI_flhC+616R	CAAGCTTGTCGACGGAGCTC CGCTGCTGGAGTGTTTGTCC
1293	pETBam_ECflhD+1F	GCCTGGTGCCGCGCGGATCC ATGCATACCTCCGAGTTGCT
1294	pETBam_ECD_R	AGCAACTCGGAGGTATGCAT GGATCCGCGCGGCACCAGGC
1295	pETBam_STflhD+1F	GCCTGGTGCCGCGCGGATCC ATGGGAACAATGCATACATC
1296	pETBam_STD_R	GATGTATGCATTGTTCCCAT GGATCCGCGCGGCACCAGGC
753	YdiV-510chk	CCAGAATCGATAAAGATGAATTGC
754	YdiV+1221chk	CGCGCGCGTAGTGGGAATACCC
1307	LT2_clpP_P1	GTACAGCAGATTTTTTCAATTTTTATCCAGGAGACGGAAATGTCAgtgtagg ctggagctgcttc
1308	LT2_clpP_P2	AAGGATGAAGTGTATAGCGGCACACTTGCGTCCAGGGCATCAATTcatat gaatatcctccttag
1309	LT2_clpP_chkF	tgatggacaatatgcgtaacg
1310	LT2_clpP_chkR	ttgtgccgcacacgacgacgc
1327	flgA+108R	cagacgctgggaaaaccacgtggtc
1328	qPCR_aF	ctcgcctgaaagagttgacc
1329	qPCR_aR	ccgatttttcatctgggaga
1330	qPCR_bF	attaaagttcgccacggttg
1331	qPCR_bR	tcgttgaccagattgagcag
1332	pSB401_qPCR1F	caacctccccaattttctca
1333	pSB401_qPCR1R	tatgcagcagcgacataagg
1334	pSB401_qPCR2F	tctgacgctcaaatcagtgg
1335	pSB401_qPCR2R	aggcgtggaatgagacaaac

10.7 Strains Background of Genotype

Table 35: Strains Numbers

Strain number	Species	Background of Genotype	Reference
TPA1	ST	LT2 (wild-type)(from S.Roth stab)	Aldridge's Lab
TPA2	EC	DH5α (ph1-80dlacdm15 enda1 reca1 hsdr17 supe44 tth-1 gyra96 rela12 dlacu169	Aldridge's Lab
TPA11	ST	*fliY*5221::Tn10dTc	Aldridge's Lab
TPA18	EC	BW25141/pKD4	Aldridge's Lab
TPA19	ST	LT2/pkD46	Aldridge's Lab
TPA22	EC	pkD3 in *E.Coli* (strain unknown)	Aldridge's Lab
TPA323	ST	LT2/pCP20 (CmR AmpR)	Aldridge's Lab
TPA277	ST	S.enterica sv. Typhimurium 14028s (USA patho)	Aldridge's Lab
TPA788	ST	SJW1103 (Japanese Wild Type)	Nao/Tohru
TPA1147	ST	SL1344 (Berlin)	J. Vogel
TPA2734	ST	Salmonella java	J. Perry
TPA2735	ST	Salmonella typhimurium	J. Perry
TPA2737	ST	Salmonella oranienburg	J. Perry
TPA2738	ST	Salmonella tennessee	J. Perry
TPA2739	ST	Salmonella javiana	J. Perry
TPA2740	ST	Salmonella berta	J. Perry
TPA2741	ST	Salmonella zanzibar	J. Perry
TPA2742	ST	Salmonella meleagridis	J. Perry
TPA3690	ST	ST4/74(Salmonella typhimurium W.T)	Jay Hinton
TPA3789	ST	PflhDC7128::tetAR (LT2)	This study
TPA3790	ST	PflhDC7129::tetAR (14028s)	This study
TPA3791	ST	PflhDC7130::tetAR (SJW1103)	This study
TPA3792	ST	PflhDC7131::tetAR (SL1344)	This study
TPA3793	ST	PflhDC7132::tetAR (java)	This study
TPA3794	ST	PflhDC7133::tetAR (typhimurium)	This study
TPA3795	ST	PflhDC7134::tetAR (javiana)	This study
TPA3796	ST	PflhDC7135::tetAR (berta)	This study
TPA3797	ST	PflhDC7136::tetAR (zanzibar)	This study
TPA3798	ST	PflhDC7137::tetAR (ST4/74)	This study
TPA74	ST	pRG19::FCF/ P(flhDC)5451::Tn10dTc[del-25]	Aldridge's Lab
TPA86	ST	pRG39::FCF/ P(flhDC)5451::Tn10dTc[del-25]	Aldridge's Lab
TPA94	ST	pRG51::FCF/ P(flhDC)5451::Tn10dTc[del-25]	Aldridge's Lab
TPA3802	ST	pRG19::FCF / PflhDC7128::tetAR	This study
TPA3803	ST	pRG39::FCF / PflhDC7128::tetAR	This study
TPA3804	ST	pRG51::FCF / PflhDC7128::tetAR	This study
TPA3805	ST	pRG19::FCF / PflhDC7129::tetAR	This study
TPA3806	ST	pRG39::FCF / PflhDC7129::tetAR	This study
TPA3807	ST	pRG51::FCF / PflhDC7129::tetAR	This study
TPA3808	ST	pRG19::FCF / PflhDC7130::tetAR	This study
TPA3809	ST	pRG39::FCF / PflhDC7130::tetAR	This study
TPA3810	ST	pRG51::FCF / PflhDC7130::tetAR	This study
TPA3811	ST	pRG19::FCF / PflhDC7131::tetAR	This study
TPA3812	ST	pRG39::FCF / PflhDC7131::tetAR	This study
TPA3813	ST	pRG51::FCF / PflhDC7131::tetAR	This study
TPA3814	ST	pRG19::FCF / PflhDC7133::tetAR	This study
TPA3815	ST	pRG39::FCF / PflhDC7133::tetAR	This study
TPA3816	ST	pRG51::FCF / PflhDC7133::tetAR	This study
TPA3817	ST	pRG19::FCF / PflhDC7137::tetAR	This study
TPA3818	ST	pRG39::FCF / PflhDC7137::tetAR	This study
TPA3819	ST	pRG51::FCF / PflhDC7137::tetAR	This study
TPA3828	ST	pRG19::FCF / PflhDC7132::tetAR	This study
TPA3829	ST	pRG39::FCF / PflhDC7132::tetAR	This study
TPA3830	ST	pRG51::FCF / PflhDC7132::tetAR	This study
TPA3831	ST	pRG19::FCF / PflhDC7134::tetAR	This study
TPA3832	ST	pRG39::FCF / PflhDC7134::tetAR	This study
TPA3833	ST	pRG51::FCF / PflhDC7134::tetAR	This study
TPA3834	ST	pRG19::FCF / PflhDC7135::tetAR	This study
TPA3835	ST	pRG39::FCF / PflhDC7135::tetAR	This study
TPA3836	ST	pRG51::FCF / PflhDC7135::tetAR	This study
TPA3837	ST	pRG19::FCF / PflhDC7136::tetAR	This study
TPA3838	ST	pRG39::FCF / PflhDC7136::tetAR	This study
TPA3839	ST	pRG51::FCF / PflhDC7136::tetAR	This study
TPA3800	EC	pWRG100 / DH5α	K. Gerdes

Strain number	Species	Background of Genotype	Reference
TPA3426	EC	RP437	J. Armitage
TPA2390	ST	fliM5978-gfp ΔfliT::km	Aldridge's Lab
TPA3799	EC	pWRG99 / DH5a [AmpR grow at 30°C]	K. Gerdes
TPA3846	ST	pWRG99 / LT2	This study
TPA3847	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF [pWRG100 template]	This study
TPA3848	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF [pWRG100 template]	This study
TPA3855	ST	fliM5978-gfp ΔfliT::km ΔflhDC::FICF [pWRG100 template]	This study
TPA3855	ST	fliM5978-gfp ΔfliT::km ΔflhDC::FICF [pWRG100 template]	This study
TPA3856	ST	fliM5978-gfp ΔfliT::km ΔflhDC::FICF [pWRG100 template]	This study
TPA1107	ST	fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+)	Kelly
TPA3879	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [has 18aa at end of flhC = ST]2	This study
TPA3880	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [has 18aa at end of flhC = ST]3	This study
TPA3881	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [has 5aa at end of flhC = ST]4	This study
TPA3875	ST	fliM5978-gfp ΔflhDC::FICF [pWRG100 template]	This study
TPA3876	ST	fliM5978-gfp ΔflhDC::FICF [pWRG100 template]	This study
TPA3878	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [has 18aa at end of flhC = ST]1	This study
TPA3902	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF [pWRG100 template]1	This study
TPA3903	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF [pWRG100 template]4	This study
TPA3904	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF [pWRG100 template]12	This study
TPA3905	ST	fliM5978-gfp ΔflhDC::FICF [pWRG100 template]7	This study
TPA3906	ST	fliM5978-gfp ΔflhDC::FICF [pWRG100 template]12	This study
TPA3907	ST	fliM5978-gfp ΔflhDC::FICF [pWRG100 template]14	This study
TPA3935	ST	PflhDC::tetAR (java) [981/982 remakes]	This study
TPA3936	ST	PflhDC::tetAR (oranienburg)	This study
TPA3937	ST	PflhDC::tetAR (tennesse)	This study
TPA3938	ST	PflhDC::tetAR (D23_africa)	This study
TPA3874	ST	SSS18 [KanS, CmS, AmpS, tetS, D23580 variant] (D23_africa)	Aldridge's Lab
TPA3939	ST	pRG19::FCF / PflhDC::tetAR (java)	This study
TPA3940	ST	pRG39::FCF / PflhDC::tetAR (java)	This study
TPA3941	ST	pRG51::FCF / PflhDC::tetAR (java)	This study
TPA3942	ST	pRG19::FCF / PflhDC::tetAR (oranienburg)	This study
TPA3943	ST	pRG39::FCF / PflhDC::tetAR (oranienburg)	This study
TPA3944	ST	pRG51::FCF / PflhDC::tetAR (oranienburg)	This study
TPA3945	ST	pRG19::FCF / PflhDC::tetAR (tennesse)	This study
TPA3946	ST	pRG39::FCF / PflhDC::tetAR (tennesse)	This study
TPA3947	ST	pRG51::FCF / PflhDC::tetAR (tennesse)	This study
TPA3948	ST	pRG19::FCF / PflhDC::tetAR (D23_africa)	This study
TPA3949	ST	pRG39::FCF / PflhDC::tetAR (D23_africa)	This study
TPA3950	ST	pRG51::FCF / PflhDC::tetAR (D23_africa)	This study
TPA3951	ST	pRG19::FCF / PflhDC7132::tetAR (Java)	This study
TPA3952	ST	pRG39::FCF / PflhDC7132::tetAR (Java)	This study
TPA3953	ST	pRG51::FCF / PflhDC7132::tetAR (Java)	This study
TPA3954	ST	PflhDC::tetAR (melagridis)	This study
TPA3955	ST	pRG19::FCF / PflhDC::tetAR (melagridis)	This study
TPA3956	ST	pRG39::FCF / PflhDC::tetAR (melagridis)	This study
TPA3957	ST	pRG51::FCF / PflhDC::tetAR (melagridis)	This study
TPA4003	ST	fliM5978-gfp ΔflhDC::FICF / pWRG99	This study
TPA4004	ST	fliM5978-gfp ΔfliT::Km ΔflhDC::FICF / pWRG99	This study
TPA3959	ST	PflhDC::tetRA (LT2) [note tetRA spelling! PCR1179/1180]	This study
TPA3960	ST	PflhDC::tetRA (SSS18) [note tetRA spelling! PCR1179/1180]	This study
TPA3963	ST	PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]	This study
TPA3961	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCRoAJ3/oAJ4] NO RBS!	This study
TPA3962	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) [RP437 template PCRoAJ3/oAJ4] NO RBS!	This study
TPA3964	ST	fliM5978-gfp ΔPflhDC::tetRA ΔflhDC::flhDC(EC) [PCR1179/1180] NO RBS!	This study
TPA3965	ST	fliM5978-gfp ΔfliT::km ΔPflhDC::tetRA ΔflhDC::flhDC(EC) [PCR1179/1180] NO RBS!	This study
TPA3966	ST	pRG19::FCF / PflhDC::tetRA (LT2)	This study
TPA3967	ST	pRG39::FCF / PflhDC::tetRA (LT2)	This study
TPA3968	ST	pRG51::FCF / PflhDC::tetRA (LT2)	This study
TPA3969	ST	pRG19::FCF / PflhDC::tetRA (SSS18)	This study
TPA3970	ST	pRG39::FCF / PflhDC::tetRA (SSS18)	This study
TPA3971	ST	pRG51::FCF / PflhDC::tetRA (SSS18)	This study

Strain number	Species	Background of Genotype	Reference
TPA3972	ST	pRG19::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]	This study
TPA3973	ST	pRG39::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]	This study
TPA3974	ST	pRG51::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]	This study
TPA3975	ST	pRG19::FCF / fliM5978-gfp ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3976	ST	pRG39::FCF / fliM5978-gfp ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3977	ST	pRG51::FCF / fliM5978-gfp ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3978	ST	pRG19::FCF / fliM5978-gfp ΔfliT::km ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3979	ST	pRG39::FCF / fliM5978-gfp ΔfliT::km ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3980	ST	pRG51::FCF / fliM5978-gfp ΔfliT::km ΔPflhDC::tetRA ΔflhDC::flhDC(EC)	This study
TPA3981	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3982	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [18aa]	This study
TPA3983	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [5aa]	This study
TPA3984	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3985	ST	pRG19::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3986	ST	pRG39::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3987	ST	pRG51::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3988	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [18aa]	This study
TPA3989	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [18aa]	This study
TPA3990	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [18aa]	This study
TPA3991	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [5aa]	This study
TPA3992	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [5aa]	This study
TPA3993	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA [5aa]	This study
TPA3994	ST	pRG19::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3995	ST	pRG39::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3996	ST	pRG51::FCF / fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA3997	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1	This study
TPA3998	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]2	This study
TPA3999	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]3	This study
TPA4000	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC)[RP437 template PCR1184/1086]1	This study
TPA4001	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC)[RP437 template PCR1184/1086]2	This study
TPA4002	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC)[RP437 template PCR1184/1086]3	This study
TPA4022	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4023	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4024	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4025	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4026	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4027	ST	fliM5978-gfp ΔfliT::km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4028	ST	fliM5978-gfp ΔPflhDC::tetRA	This study
TPA4029	ST	fliM5978-gfp ΔPflhDC::tetAR [NOTE THE TETRA/AR Directions]	This study
TPA4030	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4031	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4032	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4033	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4034	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4035	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4036	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study

Strain number	Species	Background of Genotype	Reference
TPA4037	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4038	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4039	ST	pRG19::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4040	ST	pRG39::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4041	ST	pRG51::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4042	ST	pRG19::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4043	ST	pRG39::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4044	ST	pRG51::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4045	ST	pRG19::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4046	ST	pRG39::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4047	ST	pRG51::FCF / fliM5978-gfp ΔfliT::Km ΔflhDC::flhDC(EC) ΔPflhDC::tetRA	This study
TPA4048	ST	pRG19::FCF / fliM5978-gfp ΔPflhDC::tetRA	This study
TPA4049	ST	pRG39::FCF / fliM5978-gfp ΔPflhDC::tetRA	This study
TPA4050	ST	pRG51::FCF / fliM5978-gfp ΔPflhDC::tetRA	This study
TPA4051	ST	pRG19::FCF / fliM5978-gfp ΔPflhDC::tetAR	This study
TPA4052	ST	pRG39::FCF / fliM5978-gfp ΔPflhDC::tetAR	This study
TPA4053	ST	pRG51::FCF / fliM5978-gfp ΔPflhDC::tetAR	This study
TPA4096	ST	fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetAR	This study
TPA1690	ST	fliM5978::GFP PflhDC5451::Tn10[del-25]	Aldridge's Lab
TPA4097	ST	pRG19::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetAR	This study
TPA4098	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetAR	This study
TPA4099	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) ΔPflhDC::tetAR	This study
TPA4114	ST	fliM5978-gfp ΔflhD::FICF	This study
TPA4115	ST	fliM5978-gfp ΔflhD::FICF	This study
TPA4116	ST	fliM5978-gfp ΔflhC::FICF	This study
TPA4117	ST	fliM5978-gfp ΔflhC::FICF	This study
TPA4128	ST	fliM5978-gfp ΔflhC::flhC437	This study
TPA4129	ST	fliM5978-gfp ΔflhC::flhC437	This study
TPA4130	ST	fliM5978-gfp ΔflhC::flhC437	This study
TPA4131	ST	fliM5978-gfp ΔflhC::flhC437	This study
TPA4134	ST	fliM5978-gfp ΔflhD::flhD437	This study
TPA4135	ST	fliM5978-gfp ΔflhD::flhD437	This study
TPA4136	ST	fliM5978-gfp ΔflhD::flhD437	This study
TPA4137	ST	fliM5978-gfp ΔflhD::flhD437	This study
TPA4219	ST	LT2/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4220	ST	S.enterica sv. Typhimurium 14028s/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4221	ST	Salmonella java / pRG38(PflhD-luxCDABE TcR)	This study
TPA4193	ST	fliM5978-gfp ΔflhC::flhC437::tetRA	This study
TPA4194	ST	fliM5978-gfp ΔflhD::flhD437::tetRA	This study
TPA4213	ST	pRG19::FCF / fliM5978-gfpΔflhC::flhC437::tetRA	This study
TPA4214	ST	pRG39::FCF / fliM5978-gfpΔflhC::flhC437::tetRA	This study
TPA4215	ST	pRG51::FCF / fliM5978-gfpΔflhC::flhC437::tetRA	This study
TPA4216	ST	pRG19::FCF / fliM5978-gfpΔflhD::flhD437::tetRA	This study
TPA4217	ST	pRG39::FCF / fliM5978-gfpΔflhD::flhD437::tetRA	This study
TPA4218	ST	pRG51::FCF / fliM5978-gfpΔflhD::flhD437::tetRA	This study
TPA2456	ST	pSE-flhDC3 / ΔflhDC7011::FCF	Aldridge's Lab
TPA1835	ST	LT2 / pSE380	Aldridge's Lab
TPA4222	ST	pRG19::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE-flhDC3	This study
TPA4223	ST	pRG39::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE-flhDC3	This study
TPA4224	ST	pRG51::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE-flhDC3	This study
TPA4225	ST	pRG19::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE-flhDC3	This study
TPA4226	ST	pRG39::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE-flhDC3	This study
TPA4227	ST	pRG51::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE-flhDC3	This study
TPA4228	ST	pRG19::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE380	This study
TPA4229	ST	pRG39::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE380	This study

Strain number	Species	Background of Genotype	Reference
TPA4230	ST	pRG51::FCF / PflhDC::tetRA (java) [note tetRA spelling! PCR1179/1180]/ pSE380	This study
TPA4231	ST	pRG19::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE380	This study
TPA4232	ST	pRG39::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE380	This study
TPA4233	ST	pRG51::FCF / fliM5978-gfp ΔPflhDC::tetRA/ pSE380	This study
TPA4250	ST	pRG19::FCF / LT2 / pSE380	This study
TPA4251	ST	pRG39::FCF / LT2 / pSE380	This study
TPA4252	ST	pRG51::FCF / LT2 / pSE380	This study
TPA4253	ST	pRG19::FCF / pSE-flhDC3 / ΔflhDC7011	This study
TPA4254	ST	pRG39::FCF / pSE-flhDC3 / ΔflhDC7011	This study
TPA4255	ST	pRG51::FCF / pSE-flhDC3 / ΔflhDC7011	This study
TPA4261	ST	Salmonella java / pSE-flhDC3	This study
TPA4262	ST	Salmonella java / pSE380	This study
TPA66	ST	pRG19::FCF (CmR TcR)/LT2	Aldridge's Lab
TPA69	ST	pRG39::FCF (CmR TcR)/LT2	Aldridge's Lab
TPA71	ST	pRG51::FCF (CmR TcR)/LT2	Aldridge's Lab
TPA4269	ST	pRG19::FCF (CmR TcR)/LT2/ pSE-flhDC3	This study
TPA4270	ST	pRG39::FCF (CmR TcR)/LT2/ pSE-flhDC3	This study
TPA4271	ST	pRG51::FCF (CmR TcR)/LT2/ pSE-flhDC3	This study
TPA4263	ST	pRG19::FCF /Salmonella java / pSE-flhDC3	This study
TPA4264	ST	pRG39::FCF /Salmonella java / pSE-flhDC3	This study
TPA4265	ST	pRG51::FCF /Salmonella java / pSE-flhDC3	This study
TPA4266	ST	pRG19::FCF /Salmonella java / pSE380	This study
TPA4267	ST	pRG39::FCF /Salmonella java / pSE380	This study
TPA4268	ST	pRG51::FCF /Salmonella java / pSE380	This study
TPA4272	ST	Salmonella senftenberg	Aldridge's Lab
TPA4273	ST	Salmonella gallinarum	Aldridge's Lab
TPA4274	ST	Salmonella othmarschen	Aldridge's Lab
TPA4275	ST	Salmonella emek	Aldridge's Lab
TPA4276	ST	Salmonella lexington	Aldridge's Lab
TPA4277	ST	Salmonella haifa	Aldridge's Lab
TPA4278	ST	Salmonella simsbury	Aldridge's Lab
TPA4279	ST	Salmonella panama	Aldridge's Lab
TPA4280	ST	Salmonella indina	Aldridge's Lab
TPA4281	ST	Salmonella montevideo	Aldridge's Lab
TPA4284	ST	Salmonella limete	Aldridge's Lab
TPA4285	ST	Salmonella abony	Aldridge's Lab
TPA4286	ST	Salmonella vinhrady	Aldridge's Lab
TPA4287	ST	Salmonella alchua	Aldridge's Lab
TPA4288	ST	Salmonella vilvoorde	Aldridge's Lab
TPA4296	ST	Salmonella senftenberg/pRG38(PflhD-luxCDABE TcR)	This study
TPA4297	ST	Salmonella gallinarum/pRG38(PflhD-luxCDABE TcR)	This study
TPA4298	ST	Salmonella othmarschen /pRG38 (PflhD-luxCDABE TcR)	This study
TPA4299	ST	Salmonella emek/pRG38(PflhD-luxCDABE TcR)	This study
TPA4300	ST	Salmonella Lexington/pRG38(PflhD-luxCDABE TcR)	This study
TPA4301	ST	Salmonella Haifa/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4302	ST	Salmonella simsbury/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4303	ST	Salmonella panama/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4304	ST	Salmonella indina/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4305	ST	Salmonella montevideo/pRG38(PflhD-luxCDABE TcR)	This study
TPA4306	ST	Salmonella limete/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4307	ST	Salmonella abony/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4308	ST	Salmonella vinhrady/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4309	ST	Salmonella alchua/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4310	ST	Salmonella vilvoorde/ pRG38(PflhD-luxCDABE TcR)	This study
TPA4326	ST	Salmonella senftenberg/ PflhDC::tetAR	This study
TPA4327	ST	Salmonella gallinarum/ PflhDC::tetAR	This study
TPA4328	ST	Salmonella othmarschen / PflhDC::tetAR	This study
TPA4329	ST	Salmonella emek/ PflhDC::tetAR	This study
TPA4330	ST	Salmonella lexington/ PflhDC::tetAR	This study
TPA4331	ST	Salmonella haifa/ PflhDC::tetAR	This study
TPA4332	ST	Salmonella simsbury/ PflhDC::tetAR	This study
TPA4333	ST	Salmonella panama/ PflhDC::tetAR	This study
TPA4334	ST	Salmonella indina/ PflhDC::tetAR	This study
TPA4335	ST	Salmonella montevideo/ PflhDC::tetAR	This study
TPA4336	ST	Salmonella limete/ PflhDC::tetAR	This study
TPA4337	ST	Salmonella abony/ PflhDC::tetAR	This study
TPA4338	ST	Salmonella vinhrady/ PflhDC::tetAR	This study
TPA4339	ST	Salmonella alchua/ PflhDC::tetAR	This study

Strain number	Species	Background of Genotype	Reference
TPA4340	ST	Salmonella vilvoorde/ PflhDC::tetAR	This study
TPA4341	ST	Salmonella senftenberg/ PflhDC::tetRA	This study
TPA4342	ST	Salmonella gallinarum/ PflhDC::tetRA	This study
TPA4343	ST	Salmonella othmarschen / PflhDC::tetRA	This study
TPA4344	ST	Salmonella emek/ PflhDC::tetRA	This study
TPA4345	ST	Salmonella lexington/ PflhDC::tetRA	This study
TPA4346	ST	Salmonella haifa/ PflhDC::tetRA	This study
TPA4347	ST	Salmonella simsbury/ PflhDC::tetRA	This study
TPA4348	ST	Salmonella panama/ PflhDC::tetRA	This study
TPA4349	ST	Salmonella indina/ PflhDC::tetRA	This study
TPA4350	ST	Salmonella montevideo/ PflhDC::tetRA	This study
TPA4351	ST	Salmonella limete/ PflhDC::tetRA	This study
TPA4352	ST	Salmonella abony/ PflhDC::tetRA	This study
TPA4353	ST	Salmonella vinhrady/ PflhDC::tetRA	This study
TPA4354	ST	Salmonella alchua/ PflhDC::tetRA	This study
TPA4355	ST	Salmonella vilvoorde/ PflhDC::tetRA	This study
TPA4356	ST	pRG19::FCF / Salmonella senftenberg/ PflhDC::tetAR	This study
TPA4357	ST	pRG39::FCF / Salmonella senftenberg/ PflhDC::tetAR	This study
TPA4358	ST	pRG51::FCF /Salmonella senftenberg/ PflhDC::tetAR	This study
TPA4359	ST	pRG19::FCF / Salmonella gallinarum/ PflhDC::tetAR	This study
TPA4360	ST	pRG39::FCF / Salmonella gallinarum/ PflhDC::tetAR	This study
TPA4361	ST	pRG51::FCF /Salmonella gallinarum/ PflhDC::tetAR	This study
TPA4362	ST	pRG19::FCF / Salmonella othmarschen / PflhDC::tetAR	This study
TPA4363	ST	pRG39::FCF / Salmonella othmarschen / PflhDC::tetAR	This study
TPA4364	ST	pRG51::FCF /Salmonella othmarschen / PflhDC::tetAR	This study
TPA4365	ST	pRG19::FCF / Salmonella emek/ PflhDC::tetAR	This study
TPA4366	ST	pRG39::FCF / Salmonella emek/ PflhDC::tetAR	This study
TPA4367	ST	pRG51::FCF /Salmonella emek/ PflhDC::tetAR	This study
TPA4368	ST	pRG19::FCF /Salmonella lexington/ PflhDC::tetAR	This study
TPA4369	ST	pRG39::FCF / Salmonella lexington/ PflhDC::tetAR	This study
TPA4370	ST	pRG51::FCF /Salmonella lexington/ PflhDC::tetAR	This study
TPA4371	ST	pRG19::FCF / Salmonella haifa/ PflhDC::tetAR	This study
TPA4372	ST	pRG39::FCF / Salmonella haifa/ PflhDC::tetAR	This study
TPA4373	ST	pRG51::FCF /Salmonella haifa/ PflhDC::tetAR	This study
TPA4374	ST	pRG19::FCF / Salmonella simsbury/ PflhDC::tetAR	This study
TPA4375	ST	pRG39::FCF / Salmonella simsbury/ PflhDC::tetAR	This study
TPA4376	ST	pRG51::FCF /Salmonella simsbury/ PflhDC::tetAR	This study
TPA4401	ST	pRG19::FCF /Salmonella senftenberg/ PflhDC::tetRA	This study
TPA4402	ST	pRG39::FCF / Salmonella senftenberg/ PflhDC::tetRA	This study
TPA4403	ST	pRG51::FCF /Salmonella senftenberg/ PflhDC::tetRA	This study
TPA4404	ST	pRG19::FCF /Salmonella gallinarum/ PflhDC::tetRA	This study
TPA4405	ST	pRG39::FCF / Salmonella gallinarum/ PflhDC::tetRA	This study
TPA4406	ST	pRG51::FCF /Salmonella gallinarum/ PflhDC::tetRA	This study
TPA4407	ST	pRG19::FCF /Salmonella othmarschen / PflhDC::tetRA	This study
TPA4408	ST	pRG39::FCF / Salmonella othmarschen / PflhDC::tetRA	This study
TPA4409	ST	pRG51::FCF /Salmonella othmarschen / PflhDC::tetRA	This study
TPA4410	ST	pRG19::FCF /Salmonella emek/ PflhDC::tetRA	This study
TPA4411	ST	pRG39::FCF / Salmonella emek/ PflhDC::tetRA	This study
TPA4412	ST	pRG51::FCF /Salmonella emek/ PflhDC::tetRA	This study
TPA4413	ST	pRG19::FCF /Salmonella lexington/ PflhDC::tetRA	This study
TPA4414	ST	pRG39::FCF / Salmonella lexington/ PflhDC::tetRA	This study
TPA4415	ST	pRG51::FCF /Salmonella lexington/ PflhDC::tetRA	This study
TPA4416	ST	pRG19::FCF /Salmonella haifa/ PflhDC::tetRA	This study
TPA4417	ST	pRG39::FCF / Salmonella haifa/ PflhDC::tetRA	This study
TPA4418	ST	pRG51::FCF /Salmonella haifa/ PflhDC::tetRA	This study
TPA4419	ST	pRG19::FCF /Salmonella simsbury/ PflhDC::tetRA	This study
TPA4420	ST	pRG39::FCF / Salmonella simsbury/ PflhDC::tetRA	This study
TPA4421	ST	pRG51::FCF /Salmonella simsbury/ PflhDC::tetRA	This study
TPA4427	ST	pRG19::FCF /Salmonella indina/ PflhDC::tetAR	This study
TPA4428	ST	pRG39::FCF / Salmonella indina/ PflhDC::tetAR	This study
TPA4429	ST	pRG51::FCF /Salmonella indina/ PflhDC::tetAR	This study
TPA4430	ST	pRG19::FCF /Salmonella abony/ PflhDC::tetAR	This study
TPA4431	ST	pRG39::FCF / Salmonella abony/ PflhDC::tetAR	This study
TPA4432	ST	pRG51::FCF /Salmonella abony/ PflhDC::tetAR	This study
TPA4433	ST	pRG19::FCF /Salmonella alchua/ PflhDC::tetAR	This study
TPA4434	ST	pRG39::FCF / Salmonella alchua/ PflhDC::tetAR	This study
TPA4435	ST	pRG51::FCF /Salmonella alchua/ PflhDC::tetAR	This study
TPA4436	ST	pRG19::FCF /Salmonella vilvoorde/ PflhDC::tetAR	This study
TPA4437	ST	pRG39::FCF / Salmonella vilvoorde/ PflhDC::tetAR	This study

Strain number	Species	Background of Genotype	Reference
TPA4438	ST	pRG51::FCF /Salmonella vilvoorde/ PflhDC::tetAR	This study
TPA4439	ST	pRG19::FCF /Salmonella indina/ PflhDC::tetRA	This study
TPA4440	ST	pRG39::FCF / Salmonella indina/ PflhDC::tetRA	This study
TPA4441	ST	pRG51::FCF /Salmonella indina/ PflhDC::tetRA	This study
TPA4442	ST	pRG19::FCF /Salmonella montevideo/ PflhDC::tetRA	This study
TPA4443	ST	pRG39::FCF / Salmonella montevideo/ PflhDC::tetRA	This study
TPA4444	ST	pRG51::FCF /Salmonella montevideo/ PflhDC::tetRA	This study
TPA4445	ST	pRG19::FCF /Salmonella abony/ PflhDC::tetRA	This study
TPA4446	ST	pRG39::FCF / Salmonella abony/ PflhDC::tetRA	This study
TPA4447	ST	pRG51::FCF /Salmonella abony/ PflhDC::tetRA	This study
TPA4448	ST	pRG19::FCF /Salmonella alchua/ PflhDC::tetRA	This study
TPA4449	ST	pRG39::FCF / Salmonella alchua/ PflhDC::tetRA	This study
TPA4450	ST	pRG51::FCF /Salmonella alchua/ PflhDC::tetRA	This study
TPA4451	ST	pRG19::FCF /Salmonella vilvoorde/ PflhDC::tetRA	This study
TPA4452	ST	pRG39::FCF / Salmonella vilvoorde/ PflhDC::tetRA	This study
TPA4453	ST	pRG51::FCF /Salmonella vilvoorde/ PflhDC::tetRA	This study
TPA4466	ST	pRG19::FCF /Salmonella montevideo/ PflhDC::tetAR	This study
TPA4467	ST	pRG39::FCF / Salmonella montevideo/ PflhDC::tetAR	This study
TPA4468	ST	pRG51::FCF /Salmonella montevideo/ PflhDC::tetAR	This study
TPA4469	ST	pRG19::FCF /Salmonella limete/ PflhDC::tetAR	This study
TPA4470	ST	pRG39::FCF / Salmonella limete/ PflhDC::tetAR	This study
TPA4471	ST	pRG51::FCF /Salmonella limete/ PflhDC::tetAR	This study
TPA4472	ST	pRG19::FCF /Salmonella limete/ PflhDC::tetRA	This study
TPA4473	ST	pRG39::FCF / Salmonella limete/ PflhDC::tetRA	This study
TPA4474	ST	pRG51::FCF /Salmonella limete/ PflhDC::tetRA	This study
TPA4475	ST	pRG19::FCF /Salmonella panama/ PflhDC::tetAR	This study
TPA4476	ST	pRG39::FCF / Salmonella panama/ PflhDC::tetAR	This study
TPA4477	ST	pRG51::FCF /Salmonella panama/ PflhDC::tetAR	This study
TPA4478	ST	pRG19::FCF /Salmonella panama/ PflhDC::tetRA	This study
TPA4479	ST	pRG39::FCF / Salmonella panama/ PflhDC::tetRA	This study
TPA4480	ST	pRG51::FCF /Salmonella panama/ PflhDC::tetRA	This study
TPA4481	ST	pRG19::FCF /Salmonella vinhrady/ PflhDC::tetAR	This study
TPA4482	ST	pRG39::FCF / Salmonella vinhrady/ PflhDC::tetAR	This study
TPA4483	ST	pRG51::FCF /Salmonella vinhrady/ PflhDC::tetAR	This study
TPA4484	ST	pRG19::FCF /Salmonella vinhrady/ PflhDC::tetRA	This study
TPA4485	ST	pRG39::FCF / Salmonella vinhrady/ PflhDC::tetRA	This study
TPA4486	ST	pRG51::FCF /Salmonella vinhrady/ PflhDC::tetRA	This study
TPA1944	EC	DH5a / pBluescriptKSII	Aldridge's Lab
TPA1071	EC	DH5a / pET28aMod	Aldridge's Lab
TPA4517	ST	pRG39::FCF / Salmonella senftenberg	This study
TPA4518	ST	pRG39::FCF / Salmonella gallinarum	This study
TPA4519	ST	pRG39::FCF / Salmonella othmarschen	This study
TPA4520	ST	pRG39::FCF / Salmonella emek	This study
TPA4521	ST	pRG39::FCF / Salmonella lexington	This study
TPA4522	ST	pRG39::FCF / Salmonella haifa	This study
TPA4523	ST	pRG39::FCF / Salmonella simsbury	This study
TPA4524	ST	pRG39::FCF / Salmonella panama	This study
TPA4525	ST	pRG39::FCF / Salmonella indina	This study
TPA4526	ST	pRG39::FCF / Salmonella montevideo	This study
TPA4527	ST	pRG39::FCF / Salmonella limete	This study
TPA4528	ST	pRG39::FCF / Salmonella abony	This study
TPA4529	ST	pRG39::FCF / Salmonella vinhrady	This study
TPA4530	ST	pRG39::FCF / Salmonella alchua	This study
TPA4531	ST	pRG39::FCF / Salmonella vilvoorde	This study
TPA4532	ST	pRG51::FCF /Salmonella senftenberg	This study
TPA4533	ST	pRG51::FCF /Salmonella gallinarum	This study
TPA4534	ST	pRG51::FCF /Salmonella othmarschen	This study
TPA4535	ST	pRG51::FCF /Salmonella emek	This study
TPA4536	ST	pRG51::FCF /Salmonella lexington	This study
TPA4537	ST	pRG51::FCF /Salmonella haifa	This study
TPA4538	ST	pRG51::FCF /Salmonella simsbury	This study
TPA4539	ST	pRG51::FCF /Salmonella panama	This study
TPA4540	ST	pRG51::FCF /Salmonella indina	This study
TPA4541	ST	pRG51::FCF /Salmonella montevideo	This study
TPA4542	ST	pRG51::FCF /Salmonella limete	This study
TPA4543	ST	pRG51::FCF /Salmonella abony	This study
TPA4544	ST	pRG51::FCF /Salmonella vinhrady	This study
TPA4545	ST	pRG51::FCF /Salmonella alchua	This study
TPA4546	ST	pRG51::FCF /Salmonella vilvoorde	This study

Strain number	Species	Background of Genotype	Reference
TPA20	ST	LT2/fliT::Km	Aldridge's Lab
TPA2546	ST	LT2/ fliM::gfp ΔfliT ΔclpP::Cm	Aldridge's Lab
TPA3356	ST	LT2/ΔydiV:FCF	Aldridge's Lab
TPA3369	ST	LT2/fliM5978-gfp ΔfliZ7070::FKF	Aldridge's Lab
TPA4574	ST	fliM5978-gfp ΔflhC::flhC437/ fliT::Km	This study
TPA4575	ST	fliM5978-gfp ΔflhD::flhD437/ fliT::Km	This study
TPA4576	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ fliT::Km	This study
TPA4577	ST	fliM5978-gfp ΔflhC::flhC437/ fliM::gfp ΔfliT ΔclpP::Cm	This study
TPA4578	ST	fliM5978-gfp ΔflhD::flhD437/ fliM::gfp ΔfliT ΔclpP::Cm	This study
TPA4579	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ fliM::gfp ΔfliT ΔclpP::Cm	This study
TPA4580	ST	fliM5978-gfp ΔflhC::flhC437/ΔydiV:FCF	This study
TPA4581	ST	fliM5978-gfp ΔflhD::flhD437/ΔydiV:FCF	This study
TPA4582	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔydiV:FCF	This study
TPA4583	ST	fliM5978-gfp ΔflhC::flhC437/ΔfliZ7070::FKF	This study
TPA4584	ST	fliM5978-gfp ΔflhD::flhD437/ΔfliZ7070::FKF	This study
TPA4585	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔfliZ7070::FKF	This study
TPA4588	EC	DH5a/ pET28aMod/flhDsCe(4128)	This study
TPA4589	EC	DH5a/ pET28aMod/flhDCs(1107)	This study
TPA4590	EC	DH5a/ pET28aMod/flhDeCs(4135)	This study
TPA4591	EC	DH5a/ pET28aMod/flhDCe(3997)	This study
TPA342	EC	BL21	mark banfield
TPA4592	EC	BL21/pET28aMod/flhDsCe(4128)	This study
TPA4593	EC	BL21/pET28aMod/flhDeCs(4135)	This study
TPA4594	EC	BL21/pET28aMod/flhDCe(3997)	This study
TPA4599	ST	fliM5978-gfp ΔflhC::flhC437/ΔydiV:FCF:FRT	This study
TPA4600	ST	fliM5978-gfp ΔflhD::flhD437/ΔydiV:FCF:FRT	This study
TPA4601	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔydiV:FCF:FRT	This study
TPA4626	ST	fliM5978-gfp ΔflhC::flhC437/ fliT::Km/ PflhDC::tetRA	This study
TPA4627	ST	fliM5978-gfp ΔflhD::flhD437/ fliT::Km/ PflhDC::tetRA	This study
TPA4628	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 templatePCR1184/1086]1/fliT::Km/PflhDC::tetRA	This study
TPA4629	ST	fliM5978-gfp ΔflhC::flhC437/ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4630	ST	fliM5978-gfp ΔflhD::flhD437/ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4631	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4632	ST	LT2/fliM5978-gfp ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4650	ST	ΔydiV::FCF ΔfliA5647:FCF:FRT	This study
TPA4634	ST	pRG39::FCF / fliM5978-gfp ΔflhC::flhC437/ fliT::Km/ PflhDC::tetRA	This study
TPA4635	ST	pRG51::FCF /fliM5978-gfp ΔflhC::flhC437/ fliT::Km/ PflhDC::tetRA	This study
TPA4636	ST	pRG39::FCF / fliM5978-gfp ΔflhD::flhD437/ fliT::Km/ PflhDC::tetRA	This study
TPA4637	ST	pRG51::FCF /fliM5978-gfp ΔflhD::flhD437/ fliT::Km/ PflhDC::tetRA	This study
TPA4638	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) RP437 template PCR 1184/1086] 1/fliT::Km /PflhDC::tetRA	This study
TPA4639	ST	pRG51::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) RP437 template PCR 1184/1086] 1/fliT::Km /PflhDC::tetRA	This study
TPA4640	ST	pRG39::FCF / fliM5978 gfp ΔflhC::flhC437 /ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4641	ST	pRG51::FCF/ fliM5978 gfp ΔflhC::flhC437 /ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4642	ST	pRG39::FCF / fliM5978-gfp ΔflhD::flhD437 /ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4643	ST	pRG51::FCF / fliM5978-gfp ΔflhD::flhD437 /ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4644	ST	pRG39::FCF / fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4645	ST	pRG51::FCF /fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4646	ST	pRG39::FCF / LT2/fliM5978-gfp ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4647	ST	pRG51::FCF /LT2/fliM5978-gfp ΔfliZ7070::FKF/ PflhDC::tetRA	This study
TPA4651	ST	fliM5978-gfp ΔflhC::flhC437/ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4652	ST	fliM5978-gfp ΔflhD::flhD437/ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4653	ST	fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4654	ST	fliM5978-gfp ΔfliT::km/ PflhDC::tetRA	This study
TPA4650	ST	ΔydiV::FCF ΔfliA5647:FCF::FRT	This study
TPA4664	ST	ΔydiV::FCF ΔfliA5647:FCF::FRT/ PflhDC::tetRA	This study

Strain number	Species	Background of Genotype	Reference
TPA4655	ST	Prg39 ::FCF /fliM5978-gfp ΔflhC::flhC437 /ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4656	ST	pRG51::FCF /fliM5978-gfp ΔflhC::flhC437 /ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4657	ST	pRG39::FCF /fliM5978-gfp ΔflhD::flhD437 /ΔydiV :FCF:FRT/ PflhDC::tetRA	This study
TPA4658	ST	pRG51::FCF /fliM5978-gfp ΔflhD::flhD437 /ΔydiV :FCF:FRT/ PflhDC::tetRA	This study
TPA4659	ST	pRG39::FCF /fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4660	ST	pRG51::FCF /fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1/ΔydiV:FCF:FRT/ PflhDC::tetRA	This study
TPA4661	ST	pRG39::FCF /fliM5978gfpΔfliT::km/PflhDC::tetRA	This study
TPA4662	ST	pRG51::FCF /fliM5978gfpΔfliT::km/PflhDC::tetRA	This study
TPA4664	ST	ΔydiV::FCF ΔfliA5647:FCF::FRT/PflhDC::tetRA	This study
TPA4665	ST	pRG39::FCF /ΔydiV::FCF ΔfliA5647: FCF::FRT/ PflhDC::tetRA	This study
TPA4666	ST	pRG51::FCF /ΔydiV::FCF ΔfliA5647: FCF::FRT/ PflhDC::tetRA	This study
TPA4670	ST	ΔclpP::FCF/fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+)	This study
TPA4678	ST	ΔclpP::FCF/fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+):FCF::FRT	This study
TPA4679	ST	ΔclpP::FCF fliM5978-gfpΔflhC::flhC437FCF::FRT	This study
TPA4680	ST	ΔclpP::FCF fliM5978-gfp ΔflhDC::flhDC(EC) [RP437 template PCR1184/1086]1 FCF::FRT	This study
TPA4681	ST	ΔclpP::FCF fliM5978-gfpΔflhC::flhD437FCF::FRT	This study
TPA640	EC	pPA158 (flhDC+) / BL21	Aldridge's Lab
TPA4706	ST	ΔclpP::FCF/fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+):FCF::FRT/PflhDC::tetRA	This study
TPA4707	ST	ΔclpP::FCF fliM5978-gfpΔflhC:: flhC437FCF :: FRT / PflhDC::tetRA	This study
TPA4708	ST	ΔclpP::FCF fliM5978-gfp ΔflhDC::flhDC(EC) [RP437template PCR1184/1086]1 FCF::FRT / PflhDC::tetRA	This study
TPA4709	ST	ΔclpP::FCF fliM5978-gfpΔflhC:: flhD437FCF:: FRT/PflhDC::tetRA	This study
TPA4710	ST	pRG39::FCF/ΔclpP::FCF/fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+) :FCF::FRT / PflhDC::tetRA	This study
TPA4711	ST	pRG51::FCF /ΔclpP::FCF/fliM5978 (FliM-(GAGAGA)-GFP2+(-14bp from fliN AUG)(Mot+) :FCF::FRT / PflhDC::tetRA	This study
TPA4712	ST	pRG39::FCF /ΔclpP::FCF fliM5978-gfpΔflhC:: flhC437FCF :: FRT / PflhDC::tetRA	This study
TPA4713	ST	pRG51::FCF /ΔclpP::FCF fliM5978-gfpΔflhC:: flhC437FCF :: FRT / PflhDC::tetRA	This study
TPA4714	ST	pRG39::FCF /ΔclpP::FCF fliM5978-gfp ΔflhDC :: flhDC(EC) [RP437template PCR1184/1086]1 FCF::FRT / PflhDC::tetRA	This study
TPA4715	ST	pRG51::FCF /ΔclpP::FCF fliM5978-gfp ΔflhDC :: flhDC(EC) [RP437template PCR1184/1086]1 FCF::FRT / PflhDC::tetRA	This study
TPA4716	ST	pRG39::FCF /ΔclpP::FCF fliM5978-gfpΔflhC::flhD437FCF:: FRT/PflhDC::tetRA	This study
TPA4717	ST	pRG51::FCF /ΔclpP::FCF fliM5978-gfpΔflhC::flhD437FCF:: FRT/PflhDC::tetRA	This study
TPA4843	ST	LT2/Δ thrW locus::I-SceI cmR	This study
TPA4844	ST	java/Δ thrW locus::I-SceI cmR	This study
TPA4845	ST	indina/Δ thrW locus::I-SceI cmR	This study
TPA4846	ST	limete/Δ thrW locus::I-SceI cmR	This study
TPA4847	ST	vinhrady/Δ thrW locus::I-SceI cmR	This study
TPA4848	ST	alchua/Δ thrW locus::I-SceI cmR	This study
TPA4849	ST	LT2/Δ I-SceI::Ptr-nt kan R	This study
TPA4850	ST	java/Δ I-SceI cmR::Ptr-nt kan R	This study
TPA4851	ST	indina/Δ I-SceI cmR::Ptr-nt kan R	This study
TPA4852	ST	limete/Δ I-SceI cmR::Ptr-nt kan R	This study
TPA4853	ST	vinhrady/Δ I-SceI cmR::Ptr-nt kan R	This study
TPA4854	ST	alchua/Δ I-SceI cmR::Ptr-nt kan R	This study
TPA4855	ST	LT2/ pPROmotA	This study
TPA4856	ST	Java/ pPROmotA	This study
TPA4857	ST	Emek/ pPROmotA	This study
TPA4858	ST	Lexington/ pPROmotA	This study
TPA4859	ST	Indina / pPROmotA	This study
TPA4860	ST	Vinhrady / pPROmotA	This study
TPA4861	ST	Alachua / pPROmotA	This study
TPA4862	ST	LT2 / pPROflgB	This study
TPA4863	ST	Java / pPROflgB	This study
TPA4864	ST	Emek / pPROflgB	This study

Strain number	Species	Background of Genotype	Reference
TPA4865	ST	Lexington / pPROflgB	This study
TPA4866	ST	Indina / pPROflgB	This study
TPA4867	ST	Vinhrady / pPROflgB	This study
TPA4868	ST	Alachua / pPROflgB	This study
TPA4869	ST	LT2 / pPROflgA5	This study
TPA4870	ST	Java / pPROflgA5	This study
TPA4871	ST	Emek / pPROflgA5	This study
TPA4872	ST	Lexington / pPROflgA5	This study
TPA4873	ST	Indina / pPROflgA5	This study
TPA4874	ST	Vinhrady / pPROflgA5	This study
TPA4875	ST	Alachua / pPROflgA5	This study

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