GENE EXPRESSION AND EVOLUTION IN *ESCHERICHIA COLI* BIOFILM

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Priyankar Samanta

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Birgit M. Pruess
Chair

Dr. Michael J. Christoffers

Dr. Penelope Gibbs

Dr. Margaret L. Khaitsa

Approved:

11-6-2014
Date

Dr. Charlene Wolf-Hall
Department Chair

ABSTRACT

Biofilms can be defined as a complex aggregation of bacterial communities that involves many gene regulatory mechanisms, as well as evolutionary processes to increase biodiversity. Specific Aim 1 used a gene regulation approach to identity novel targets for the development of biofilm prevention and treatment techniques. The goal was to determine genes that get expressed early in biofilm development (prevention targets) and genes that get expressed late and in the outer layer of the biofilm (treatment targets). Biofilm formation is regulated by numerous regulators, including the two-component osmoregulator system EnvZ/OmpR, the colanic acid activator *rcsCDB* and the global regulator FlhD/FlhC. In this study, we determined the temporal and spatial expression of *flhD, ompR* and *rcsB* in *E. coli* k-12 AJW678 biofilm, as well as the gene expression of *flhD* in isogenic *ompR* and *rcsB* mutants. Results indicated that *flhD* was expressed early, and in the outer layer of the mature biofilm. We concluded that FlhD/FlhC would be the first target for novel prevention and treatment technique.

One mechanism to increase biodiversity in biofilm is the insertion of transposon elements, which was investigated as Specific Aim 2. Insertion of IS elements into the *flhD* promoter resulted in increased motility in numerous *E. coli* K-12 strains has been shown in previous study. In this study, we recovered isolates from biofilm, where IS1 had inserted in the *flhD* promoter further downstream than in previously described strains. These isolates showed reduced motility. We also wanted to determine the effect of an IS element insertion on regulation of *flhD* expression by OmpR and RcsB in biofilm. Temporal and spatial gene expression of three different GFP-tagged *flhD* promoters was measured. The results indicated that IS*5* insertion in the *flhD* promoter

at the published hotspot did not have any effect on regulation of *flhD* expression by OmpR and RcsB in biofilm.

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DEDICATION

This dissertation is dedicated to:

My parents, Mr. S K Samanta and Mrs. S Samanta, for being supportive with all my decisions. My uncle, Mr. D D Nayak, for guiding me through my right career path. And my advisor, Dr. B Prüß, for being the best mentor.

PREFACE

The FlhD/FlhC complex is a flagellar and global regulator. It is also involved in regulating various cellular processes including cell division and metabolism in many bacterial systems. The expression of *flhD* can be regulated by various two-component signal transduction systems (2CSTSs), such as the colanic acid activator RcsCDB and the osmoregulator EnvZ/OmpR. FlhD/FlhC, RcsCDB and EnvZ/OmpR are part of a transcriptional network, which regulates all the biofilm-associated cell surface organelles. Furthermore, the expression level of *flhD* is also controlled by the insertion of IS elements into the *flhD* promoter.

The gene expression of *flhD, ompR* and *rcsB* has previously been studied in planktonic bacterial culture. As a first Specific Aim, we studied the temporal and the spatial gene expression of these three genes in biofilm. To identity novel targets for the development of biofilm prevention and treatment techniques, our long-term goal is to determine genes that get expressed early in biofilm development (prevention targets) and genes that get expressed late and in the outer layer of the biofilm (treatment targets). As a second Specific Aim, we investigated the effect of IS element insertion in the *flhD* promoter region on *flhD* gene regulation by OmpR and RcsB.

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GENERAL INTRODUCTION AND SPECIFIC AIMS

Biofilms are highly abundant in nature; almost 90 percent of bacteria establish themselves in any environment by producing biofilms. Biofilms have a profound impact in many natural, clinical, and industrial settings. In order to produce a functioning biofilm, the bacteria have to synthesize a number of surface organelles (flagella, fimbria, curli) in the correct order and at the correct time. These surface organelles are regulated by the global regulator FlhD/FlhC and several two-component systems, including EnvZ/OmpR and RcsCDB. Understanding the temporal and spatial regulation of these genes will help us to understand how the biofilm forms, with the ultimate goal to influence it's formation by external signals. The following section outlines the logical sequence of the chapters presented in the thesis and the Specific Aims of the study.

Specific Aim 1: Study the temporal and spatial gene expression of *flhD, ompR,* and *rcsB*

This aim will address the working hypothesis that FlhD/FlhC could be used as a target for the development of novel biofilm treatment and prevention options. Two-component systems, EnvZ/OmpR and RcsCDB would serve as two mechanisms to control the target. Specific Aim 1 is addressed in the **first chapter** of the dissertation.

Specific Aim 2: Study the effect of IS element insertion in the *flhD* promoter on the regulation network of *flhD, ompR* and *rcsB*

This Specific Aim addresses the working hypothesis that the high motility in *E. coli* strains that carry an IS element in the -99 to -96 region (or further upstream) of the

flhD promoter might be due to a relieving of transcriptional repression by OmpR and RcsB. Specific Aim 2 is addressed in the **second chapter** of the dissertation.

Dissertation organization

The thesis has been organized to provide a comprehensive understanding regarding the gene regulation network of *flhD, ompR and rcsB,* as well as the impact that IS element insertion has on this regulation.

- The Literature review comprises of one published review article and additional relevant information. Paragraphs that are taken from the published article are indicated **[Lynnes, Prüß & Samanta, 2013].**
- The subsequent chapters constitute one published [**Samanta, Clark, Knutson, Horne & Prüß, 2013**] and one submitted [**Samanta, Sayler, Horne & Prüß, 2014**] manuscript and effectively cover the experimental procedures and related outcomes that constitute Specific Aims I and II, respectively. Each chapter contains the submitted version of the paper. For chapter 1, an addendum 1 gives details of the methods that were not covered in the paper, and an addendum 2 addresses some of the reviewer's concerns. For chapter 1, addendum 1 provides the sequence alignments for some of the *flhD* promoters.
- The Discussion at the end of the dissertation is a general discussion that aims at providing a general view of the future perspective and outlook.

LITERATURE REVIEW

Biofilm formation

Bacterial biofilms are defined as sessile communities of bacteria that form on air-liquid or liquid-solid interfaces. Bacteria are embedded in an extrapolymeric matrix that they produce and are characterized by various phenotypes that differ from planktonic bacteria. The formation of biofilm is a very complex developmental process (Figure 1) that requires a number of different cell surface organelles, some of which actively contribute to the disease progression. Any one of these organelles is characteristic of a distinct phase of biofilm development (for a review on the phases of biofilm formation, please see Sauer et al., 2002).

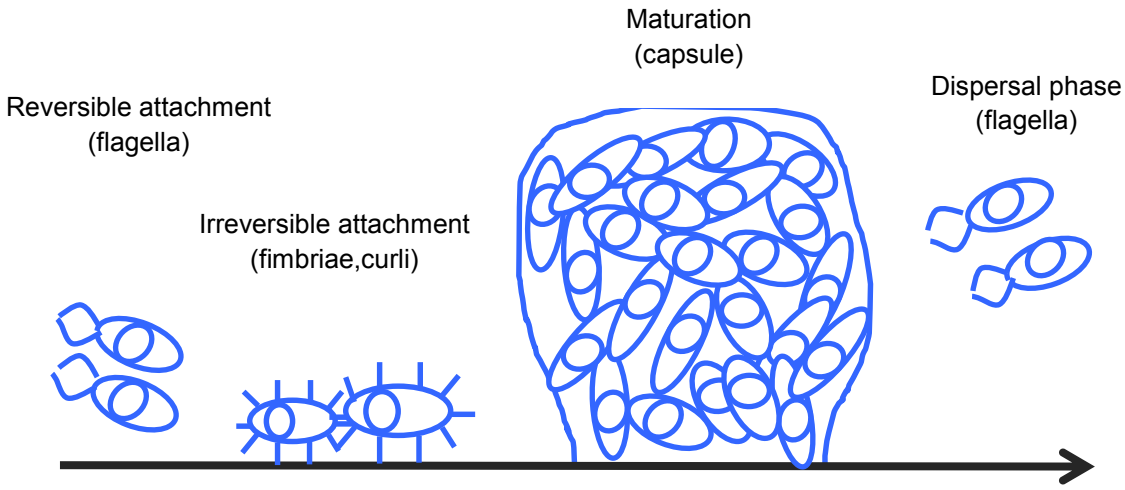

Figure 1: Phases in biofilm formation and time course progression.

Phase I constitutes the reversible attachment phase that is mediated by flagella when planktonic bacteria loosely attach to a surface. Phase II is the irreversible attachment when bacteria stick to the surface by fimbriae, pili, or curli. Phase III

constitutes the maturation phase which is characterized by an extracellular polysaccharide (EPS) layer that the bacteria themselves produce. This EPS layer gives the bacterial community the shape and a strong adherence power to attach to the surface and to each other. This layer also protects the bacterial community from external challenges such as antibiotics or the human immune system. Phase IV is the dispersal phase. At this phase newly divided bacteria leave the biofilm and bacteria will again require the synthesis of flagella, as bacteria will leave the microcolony. In cases of infectious disease, these dispersed bacteria can spread the infection and can serve as a constant reservoir of bacteria that keep feeding the infection. Altogether, the timely co-ordinated synthesis of all these organelles requires tight control over the expression of the genes that encode the respective components.

Biofilm and bacterial infections [Lynnes, Prüß & Samanta, 2013]

The Centers for Disease Control and prevention (CDC) and National Institutes of Health (NIH) estimate that 60% to 80% of all human bacterial infections involve biofilm. Many of the infectious diseases that *E. coli* is associated with and that are typically attributed to specific pathotypes of *E. coli* are worsened by the formation of biofilm. Table 1 summarizes examples of these pathotypes and the biofilm-associated diseases that they can cause. UPEC stands for Uropathogenic *E. coli*, a pathotype that causes infections of the urinary tract, often acquired in a hospital in connection with long-term catheterization. The adherence of UPEC to the host cells is mediated by short adhesive fibers, such as curli (Wu et al., 2012) and type I fimbriae (Mulvey et al., 1998). Intriguingly, UPEC can even form intracellular biofilm-like structures in the host cell

cytosol which increase the persistence of the chronic illness (Goller and Seed, 2010; Anderson et al., 2003). EaggEC are Enteroaggregative *E. coli* that are adhering particularly tightly to one another, in part by means of AafA fimbriae which have been targeted by nitazoxanide to inhibit biofilm formation and hemagglutinin production (Shamir et al., 2010). STEC is a classification that combines all *E. coli* that are capable of Shiga Toxin production. Typically, the STEC genome includes the LEE pathogenicity island (Locus of Enterocyte Effacement). An interesting STEC variant are LEE-negative *E. coli* that can cause hemolytic uremic syndrome by means of the Sab autotransporter which contributes to biofilm formation and adherence (Herold, Paton, and Paton, 2009). As a final example, a number of interesting variants of *E. coli* have recently been identified in periprosthetic joint infection. These were all deficient in typical characteristics of *E. coli*, such as the production of ß-galactosidase, flagella, indole, and the resistance towards aminoglycosides (Sendi et al., 2010).

Table 1: Biofilm associated infectious diseases caused by *E. coli*.

***E. coli* pathotype**	**Disease**	**Major virulence factors**	**Reference**
Uropathogenic *E. coli* (UPEC)	Urinary tract infections	Curli, type I fimbriae, K-capsule	(Wu et al., 2012; Goller and Seed, 2010; Mulvey et al., 1998)
Enteroaggregative *E. coli* (EaggEC)	Persistent diarrhea, malnutrition	Fimbrial adhesins	(Shamir et al., 2010)
LEE-negative shiga toxin producing *E. coli* (STEC) O113:H21	Hemolytic uremic syndrome	Sab autotransporter	(Herold, Paton, and Paton, 2009)
Variants of *E. coli* that are ß-gal negative, non-motile, and aminoglycoside resistant	Prosthetic joint infection		(Sendi et al., 2010)

Gene regulation in biofilm [Lynnes, Prüß & Samanta, 2013]

Two-component signal transduction systems permit bacteria to respond to the signals from the environment (for reviews on two-component signaling, please see Galperin, 2004; Kenney, 2002; Parkinson, 1993; West and Stock, 2001). The two components that form these systems are a sensor kinase and a response regulator. The environmental signal is translated into a phosphotransfer reaction from the sensor kinase to the response regulator, and the phosphorylation status of the response regulator determines its transcriptional activity. In the Gram-negative *E. coli*, a total of thirty-seven 2CSTS control many metabolic phenotypes in response to a diversity of signals from the environment (Zhou et al., 2003). Pathogenic strains of *E. coli,* which often have up to 1,000 genes in excess of non-pathogenic *E. coli* harbor additional 2CSTS that regulate their virulence genes (Tobe, 2008). Altogether, 2CSTS exist in most bacteria, but not in higher eukaryotes (e.g. humans), making 2CSTS suitable for the development of novel prevention and treatment techniques for biofilm-associated infectious diseases in humans, animals, and plants.

A 2CSTS that was investigated early and contributed to defining this system as a new paradigm of gene regulation is the EnvZ/OmpR system. This 2CSTS regulates the relative expression of the outer membrane porins OmpF and OmpC in response to changes in osmolarity (Igo, Slauch, and Silhavy, 1990). We used this system to explain the concept of 2CSTS. EnvZ constitutes the histidine kinase (Forst et al., 1987), which is membrane bound and acts as the osmolarity sensor. In response to increases in

osmolarity, autophosphorylation occurs at a conserved histidine within the transmitter domain of EnvZ. The OmpR response regulator (Hall and Silhavy, 1981) receives the phosphate from EnvZ at a conserved aspartate within its N-terminal receiver domain, causing a conformational change at the C-terminus. Through differential affinities to the OmpR-P binding sites on the *ompF* and *ompC* promoters, low levels of phosphorylated OmpR favor the expression of *ompF*, whereas high levels of phospho-OmpR favor *ompC* expression. As one example of a slightly different type of 2CSTS, the more complex colanic activator RcsCDB consists of three proteins (Gottesman, Trisler, and Torres-Cabassa, 1985) and has two each of the receiver and transmitter domains.

2CSTS systems that affect biofilm formation include the colanic acid activator system RcsCDB (Gottesman, Trisler, and Torres-Cabassa, 1985) and the osmoregulation system EnvZ/OmpR (Prigent-Combaret et al., 2001). In a previous review article (Prüß et al., 2006), our lab summarized a partial network of transcriptional regulation that affected all the biofilm-associated cell surface organelles and contained 16 regulators and several hundreds of regulated genes. FlhD/FlhC was the center of this gene regulation network. FlhD/FlhC was initially described as the flagellar master regulator (Bartlett, Frantz, and Matsumura, 1988) and later recognized as a global regulator of *E. coli* (Prüß et al., 2001;Prüß et al., 2003). In a later study (Denton et al., 2008) we summarized that certain two-component systems such as EnvZ/OmpR and RcsCDB hava an impact on biofilm formation by responding the environmental stress to downstream regulators, such as FlhD/FlhC. The gene intoructions of the flagella regulator FlhD/FlhC, EnvZ/OmpR and RcsCDB, which are critical to the production of biofilm-associated cell surface organelles (Figure 2).

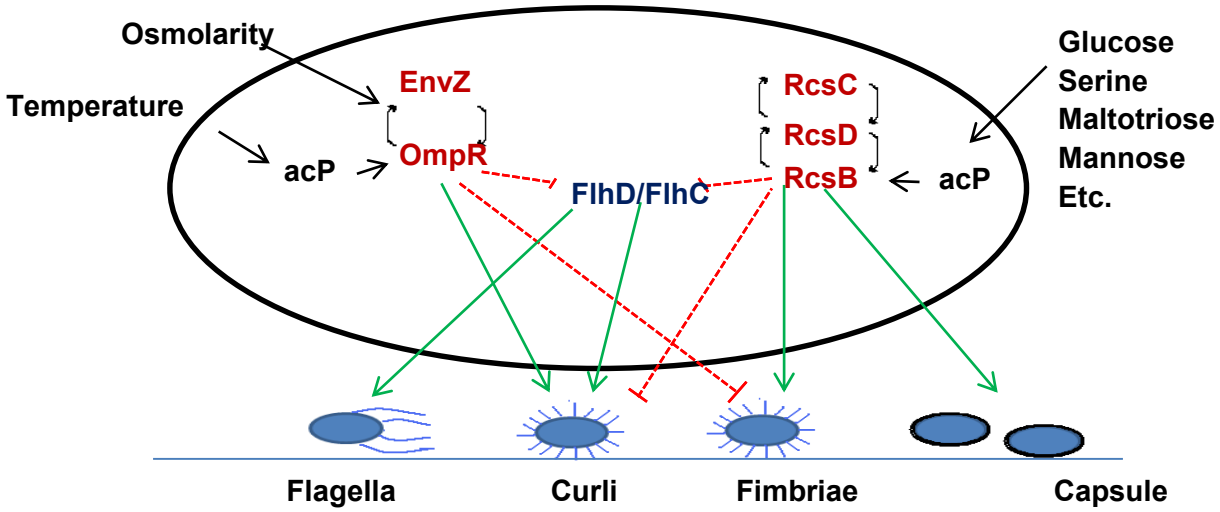

Figure 2: Gene interaction of flagella regulator FlhD/FlhC and the 2CSTSs EnvZ/OmpR and RcsCDB.

In Specific Aim I, we investigated the temporal and spatial expression of *flhD*, *ompR*, and *rcsB* using flow cell and fluorescence microscopy. The hypothesis for the temporal gene expression study (see Figure 3) is that the expression of FlhD/FlhC will be highest at the very early phase (reversible attachment) of biofilm formation, as well as the very late phase (dispersal phase). Being a positive regulator of curli, we expected the expression of OmpR to be highest at the irreversible attachment stage. RcsB-P activates the production of colanic acid, one of the capsule molecules whose synthesis is expected highest at maturation. The hypothesis of the spatial gene expression study is that the expression of *flhD* will be highest at the outermost edge of the microcolonies and at the surface of the colony where the bacteria disperse (Jackson et al., 2002). OmpR-P affects fimbriae and curli and could be highest at the bottom of the colony, where the colony attaches to the surface.

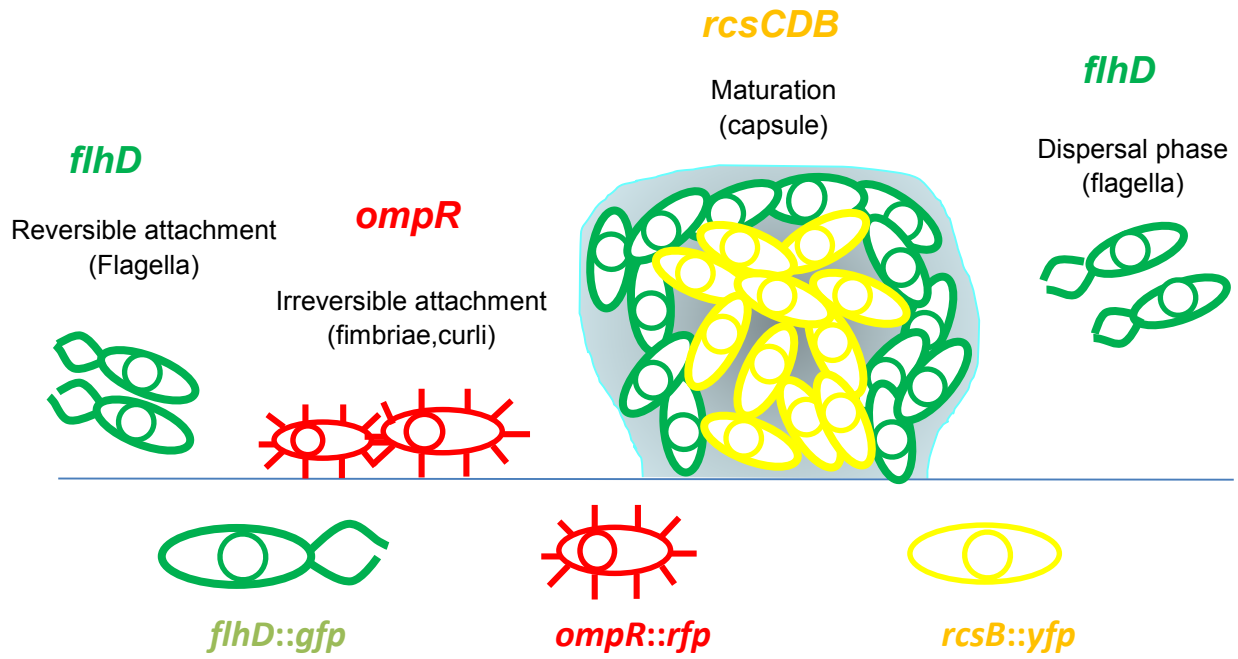

Figure 3: Temporal and spatial gene expression of *flhD, ompR* and *rcsB*.
As a translational aspect of the work, we propose that genes expressed early in biofilm development can be used as prevention targets and that genes expressed late and in the outer layer of the biofilm could be used as treatment targets for biofilm-related diseases. Our hypothesis was that FlhD/FlhC would be the first target for both the development of novel prevention and treatment techniques and that RcsB and OmpR would be two mechanisms to control this target gene.

Effects of IS element insertion in the *flhD* promoter on gene expression

The formation of biofilm involves a vast amount of gene regulation, while also promotes the evolution of biodiversity (Boles and Singh, 2008; Boles and Singh, 2008). In addition to gene expression, evolution of biodiversity was studied extensively in *E. coli* biofilms. Using a stochastic population model, it was shown that during the formation of biofilm *E. coli* undergoes dramatic diversification (Ponciano et al., 2009). This behavior of bacteria is also described as the GASP phenotype (growth advantage in stationary phase) of planktonic bacteria (Kraigsley and Finkel, 2009). A previous study shows that *E. coli* harvested from 22-day-old biofilm exhibited a competitive

advantage over bacteria that were isolated from a younger biofilm. As a conclusion, it was postulated that there may be evolutionary pressure for bacteria to disperse from late stage biofilms (McDougald et al., 2012).

One mechanism that enables bacteria to adapt to diverse ecological niches is the insertion of IS elements (Gaffe et al., 2011). A previous study showed that IS5 insertion into the *flhD* promoter of a poorly motile *E. coli* K-12 strain, BW25311, increased both motility and the bacteria's ability to form biofilm (Wang and Wood, 2011). The IS*5* inserted into a 4-bp target site (5'-TTAA-3') at 96–99 bp upstream of the transcription start of *flhD.* This IS*5* insertion also caused a 2.7-fold increase in the expression of *flhD* and a 7-fold increase in the swarm rate of the non-motile *E. coli* K-12 strain MG1655 Fnr- (Barker, Prüß, and Matsumura, 2004). Another study (Lee and Park, 2013) showed that insertion of IS*5* at -315 bp or -166 bp and IS*1* at -303 bp upstream of the transcriptional start for *flhD* increased the motility in the non-motile MG1655 strain.

Specific Aim II of this study is to address whether it is possible to obtain IS element insertions in the *flhD* promoter that will reduce motility under conditions where motility is a disadvantage. From a previous studies we determined that flagella regulator *flhD* plays an important role in biofilm formation (Samanta et al., 2013). Gaffe at al demonstrated that one evolutionary mechanism that enables bacteria to adapt to diverse ecological niches is the insertion of IS elements (Gaffe et al., 2011). Therefore, the insertion of an IS elements in the *flhD* promoter is an evolutionary event, but then it has an effect on gene regulation of *flhD*, which could directly affect biofilm formation. So, we had two hypotheses in this part of the study. We hypothesized that i) biofilm contains niches where motility would be a disadvantage and ii) insertion of an IS

element will increase *flhD* expression and motility by relieving the effects of some negative regulators like *ompR* and *rcsB*.

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PAPER 1: OMPR AND RCSB ABOLISH TEMPORAL AND SPATIAL CHANGES IN EXPRESSION OF *FLHD* IN *ESCHERICHIA COLI* BIOFILM[1]

Abstract

Biofilms are communities of bacteria that are characterized by specific phenotypes, including an increased resistance towards anti-microbials and the host immune system. This calls for the development of novel biofilm prevention and treatment options to combat infectious disease. In *Escherichia coli*, numerous global regulators have been implicated in the control of biofilm-associated cell surface organelles. These include the flagellar regulator FlhD/FlhC, the osmoregulator EnvZ/OmpR, and the colanic acid activator RcsCDB. Using flow cell technology and fluorescence microscopy, we determined the temporal expression from *flhD*::*gfp*, *ompR*::*gfp*, and *rcsB*::*gfp* in *E. coli* biofilm. Additionally, the impact of the negative regulation of *flhD* by OmpR and RcsB. Spatial gene expression was investigated from *flhD*::*gfp*.

[1] **Priyankar Samanta,** Emily R. Clark, Katie Knutson, Shelley M. Horne, and Birgit M. Prüß. 2013. BMC Microbiol. 13:182

Most of the work, including the writing of the first draft of the manuscript, was done by Priyankar Samanta. Emily Clark and Katie Knutson each cloned one of the plasmids. Below is the first draft that was written by Priyankar Samanta and submitted for publication. Detailed information about the cloning of plasmid pP71 is presented as addendum 1. Comments by the reviewers are addressed in addendum 2 to this chapter.

The temporal gene expression profile for *flhD* yielded an early peak at 12 h, a minimum of expression at 35 h, and a second increase in expression towards 51 h of biofilm development. This was the inverse of the *ompR* profile which showed a peak at 35 h. Consistent with this, a mutation in *ompR* abolished time dependence of *flhD* expression after the initial time period of 12 h. Intriguingly, *rcsB* expression did not correlate inversely with *flhD* expression, yet a mutation in *rcsB* abolished time dependence of *flhD* expression as well. Spatially, expression of *flhD* was highest in the outermost layer of the biofilm in the parent strain. In *ompR* and *rcsB* mutants, *flhD* was expressed throughout the biofilm. Additionally, biofilms by mutants in *ompR* and *rcsB* contained less biomass and *ompR* mutant bacteria were longer than parental bacteria.

We believe that FlhD/FlhC may be our first target for the development of novel biofilm prevention (early expression) and treatment (expression in the outermost layer of late biofilm) techniques. Negative regulation of *flhD* expression by numerous response regulators offers ample opportunity at controlling biofilm amounts through FlhD/FlhC.

Background

Bacterial biofilms are defined as sessile communities of bacteria that form on air-liquid or liquid-solid interfaces, or even intracellularly (Goller and Seed, 2010). Due to their high resistance to any attempts of removing them, biofilms have a profound impact in many clinical settings, including catheter-associated urinary tract infections (Saint and Chenoweth, 2003), periodontitis (Schaudinn et al., 2009), and otitis (Hoa et al., 2009), as well as *Pseudomonas aeruginosa* infections of cystic fibrosis patients (Bjarnsholt et al., 2009). Much research has been done on disease mechanisms relating to the biofilm

lifestyle. Yet, many of the early studies do not consider that growth conditions for the bacteria differ across the biofilm and also change with time. As one example, bacteria residing within fully matured biofilm have limited access to nutrients and oxygen, but are also well protected from anti-microbials, as well as the host immune system. In contrast, bacteria that grow at the surface of the three-dimensional structure or are still in the early phases of biofilm formation would have better access to nutrients and oxygen, but are also more exposed to anti-microbials. Some temporal studies of gene expression in biofilms were done years ago (Domka et al., 2007). Spatial studies have been done more recently. These were facilitated by advances in microscopy techniques, as well as the development of fluorescent probes (Klausen et al., 2003; Pamp, Sternberg, and Tolker-Nielsen, 2009; Villena et al., 2010).

Fusions of gene promoters to the structural genes of fluorescence proteins were used to study heterogeneity in biofilms of multiple bacterial species. This was done to measure: i) spatial gene regulation in biofilm of *Bacillus subtilis* (McLoon et al., 2011), ii) real-time spatial gene expression in *Geobacter sulfurreducens* electricity-producing biofilm (Franks, Glaven, and Lovley, 2012), iii) quantitative gene expression in biofilm of *Salmonella* (Grantcharova et al., 2010), iv) single-cell gene expression in *B. subtilis* biofilm (Garcia-Betancur et al., 2012), and v) the effect of inhibitors on *Pseudomonas aeruginosa* biofilm (Jakobsen et al., 2011). To reduce complexity and facilitate genetics experiments, flow cell technology was developed to grow the biofilm (Branda et al., 2005; Pamp, Sternberg, and Tolker-Nielsen, 2009). This technology allows the biofilm to form under continuous hydrodynamic conditions at a controlled and reproducible flow rate. In this study, we used promoter fusions to green fluorescence protein (GFP), flow

cell biofilms, and fluorescence microscopy to measure temporal and spatial expression of selected biofilm-associated genes in *Escherichia coli* biofilms.

The genetic system used for the present study consists of the flagellar (Bartlett, Frantz, and Matsumura, 1988) and global regulator (Prüß, Markovic, and Matsumura, 1997; Prüß et al., 2001; Prüß et al., 2003) complex $FlhD_4/FlhC_2$ (Wang et al., 2006) and the two-component systems for osmoregulation EnvZ/OmpR (Mizuno et al., 1988) and colanic acid activation RcsCDB (Gottesman, Trisler, and Torres-Cabassa, 1985). These three regulatory systems are part of a partial transcriptional network that was summarized several years ago (Prüß et al., 2006), centered around FlhD/FlhC, and regulated all the biofilm-associated cell surface organelles. In particular, OmpR and RcsB in their phosphorylated form are inhibitors of *flhD* expression (Shin and Park, 1995). RcsB is also a regulator of type I fimbriae (Schwan et al., 2007), both OmpR and RcsB control expression of many other genes (Hagiwara et al., 2003; Oshima et al., 2002). In planktonic *E. coli*, growth-phase-dependent expression of *flhD* required OmpR. Additionally, *flhD* expression in the *ompR* mutant was much higher (Prüß, 1998). This was also true for *flhD* expression and swarming of *Xenorhabdus nematophila* (Kim et al., 2003).

With this study, we wanted to accomplished three goals: i) provide proof of concept that the study of temporal and spatial expression of biofilm-associated genes can lead to the identification of novel targets for the development of biofilm prevention (gene is expressed early in biofilm development) and treatment (gene is expressed late and at the edge of the biofilm) techniques; ii) attempt to identify FlhD/FlhC as the first of such targets, because it is a transmitter between numerous environmental conditions

and many cellular responses, and iii) establish OmpR and RcsB as part of a control mechanism that increases *flhD* expression and reduces biofilm amounts. The distinction of early and late biofilm genes enabled us to identify marker genes for the different phases of biofilm development. Finally, an unintentional observation of increased cell length in the *ompR* mutant connected back to previous studies on the effect of FlhD and/or FlhC on the cell division rate (Prüß and Matsumura, 1996; Prüß, Markovic, and Matsumura, 1997; Prüß, 1998; Sule et al., 2011).

Results

Temporal gene expression of *flhD*, *ompR*, and *rcsB* in *E. coli* biofilm

Expression of *flhD* peaked at 12 h and increased towards 51 h of biofilm formation

Fluorescence microscopy images were produced from flow-cell-grown biofilm of the parent strain AJW678 that contained the *flhD*::*gfp* fusion plasmid, called pPS71. Fluorescence signals obtained from these biofilms were highest at 12 h, lowest at 35 h, and then increased again towards 51 h of biofilm formation. This was seen in all four time series of images that had been taken from four independently formed biofilms (Figure 4). Occasionally, we observed high signals in individual bacteria of the 3 h sample, but the number of bacteria on the slides was not indicative of a biofilm at that point in time. The time plot of expression that was obtained from total pixel numbers of the images with Image Pro software showed a peak at 12 h with 17,500 pixels (Figure 5). Total pixels were lowest at 35 h and increased again towards 51 h. We also noticed a small single point peak at 3 h, which is in agreement with the occasional high fluorescence of small numbers of bacteria that was visualized on the images (Figure 4).

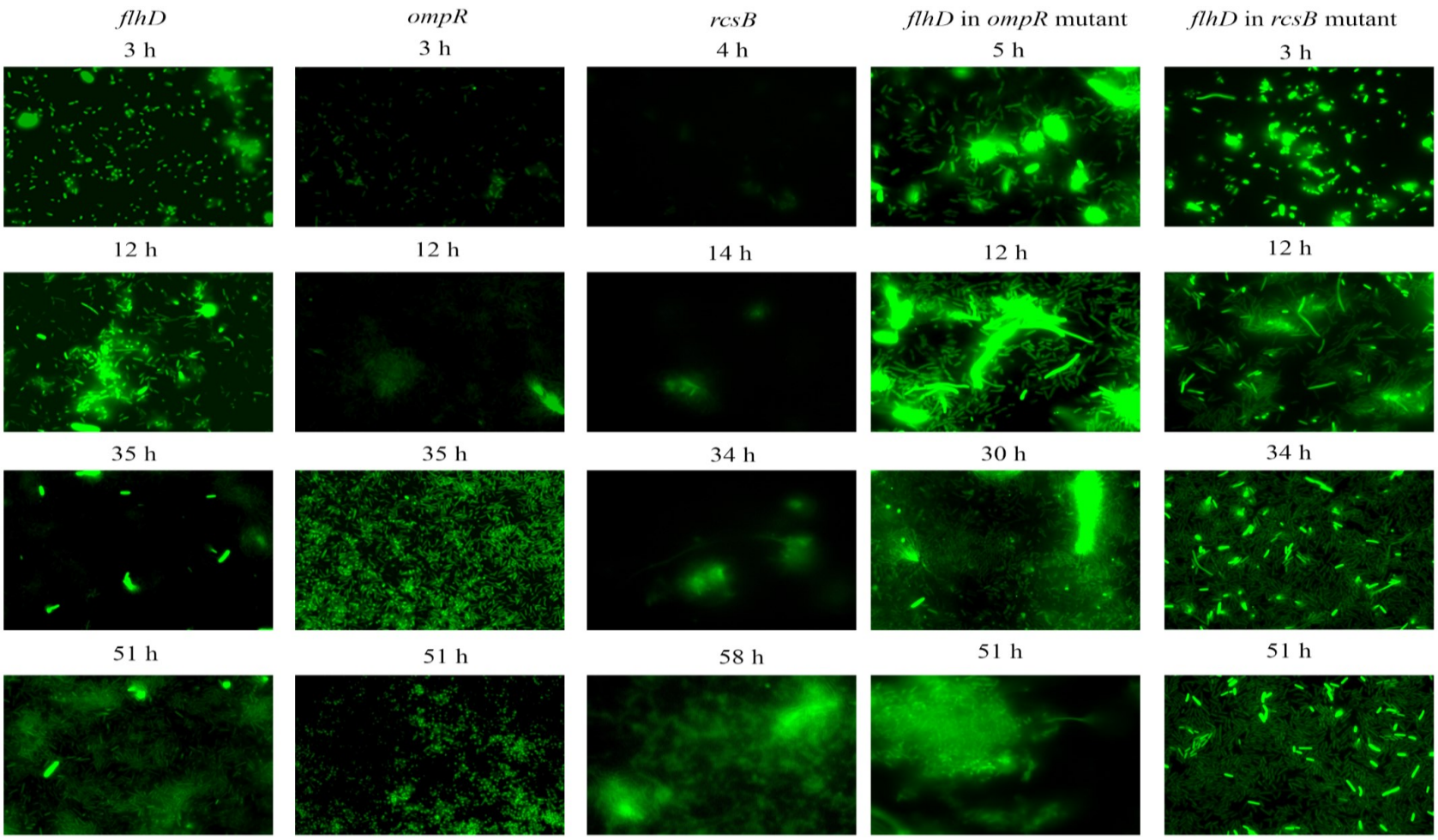

Figure 4: Fluorescent images of *flhD::gfp, ompR::gfp, rcsB::gfp* in AJW678 and *flhD* in BP1531 (*ompR::Tn10*) and BP1531 (*rcsB::Tn5*).

Biofilms of BP1470, BP1432, BP1462, BP1531, and BP1532 were grown in flow cells and subjected to fluorescence microscopy. Four time points were selected for each strain, these are printed on top of the respective images. Promoter names are printed at the very top of each column. Images were taken at 1,000-fold magnification.

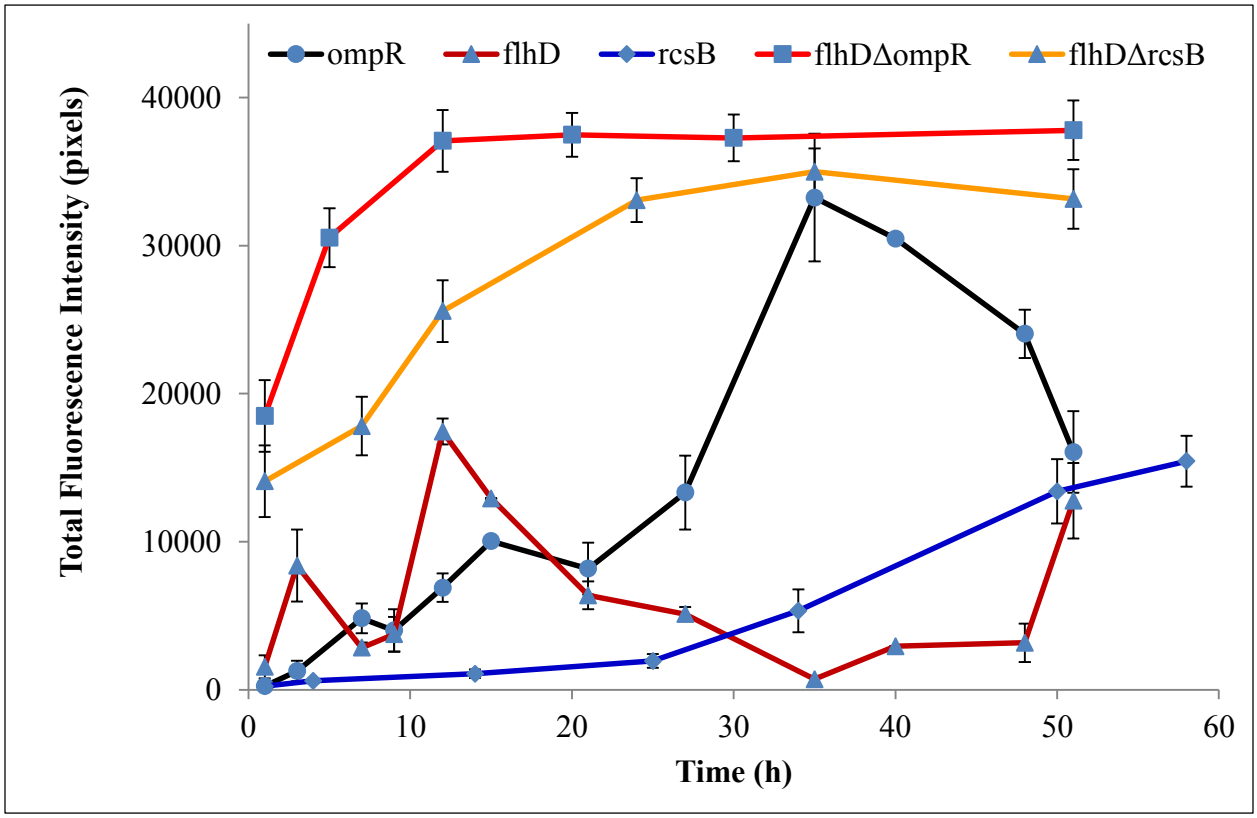

Figure 5: Temporal expression of *flhD, ompR, rcsB* in AJW678 and *flhD* in the *ompR* and *rcsB* mutant strains.
Total pixel numbers were calculated from the images, along with averages and standard deviations. The x-axis indicates the time (hours) of biofilm formation. The y-axis indicates the total fluorescence intensity of the different strains at different time points, measured by Image-Pro Plus software. The dark red, black, and blue lines are showing the gene expression profile of BP1470 (AJW678 *flhD::gfp*), BP1432 (AJW678 *ompR::gfp*), and BP1462 (AJW678 *rcsB::gfp*), respectively. The red line is the temporal expression profile of BP1531 (*flhD::gfp ompR::Tn10*), and the orange line that of BP1532 (*flhD::gfp rcsB::Tn5*).

The temporal expression of *ompR*, but not *rcsB*, correlated inversely with that of *flhD*

Expression of the negative regulator of *flhD* expression, OmpR, exhibited a temporal profile that was almost the inverse of *flhD* expression (Figure 4, second column from the left and Figure 5, black line, blue circles). Pixel values were below 10,000 for the first 20 h of biofilm formation and really only started to increase after 20

h. At 35 h, the total pixel values peaked around 35,000 pixels. Expression of another negative regulator of *flhD* expression, RcsB, did not correlate with the temporal expression profile for *flhD* (Figure 4, center column and Figure 5, blue line, blue diamonds). Initially, the fluorescence signal from the *rcsB*::*gfp* plasmid-containing strain was very weak, but increased steadily after this point in time to 14,000 pixels at 58 h.

Mutations in *ompR* and *rcsB* abolished temporal differences in *flhD* expression

The fluorescence signals from *flhD*::*gfp* in the *ompR* and *rcsB* mutant strains were significantly higher than those from the other strains. Note that the images shown in Fig. 4 and the temporal expression profiles were taken at 10% of the available excitation light, as opposed to 90% for the previous strains. Even at this low level of excitation, signals from *flhD*::*gfp* in the mutant strains were higher than in the isogenic parent strain. Expression of *flhD* in the *ompR* mutant increased over the first 12 h and reached a steady state level after that (Fig. 5, red line, blue squares). Expression of *flhD* in the *rcsB* mutant increased more slowly than in the *ompR* mutant, but was reasonably growth-phase independent after 20 h as well (Fig. 5, orange line, blue triangles).

Spatial gene expression of *flhD, ompR, and rcsB* in *E. coli* biofilm

Expression of *flhD* is highest at the top layer of the biofilm

From the temporal gene expression experiment, we knew that the highest expression of *flhD* was at 12 h and 51 h of biofilm formation. As a consequence, we performed the spatial gene expression experiment for *flhD* at those two time points (Figure 6).

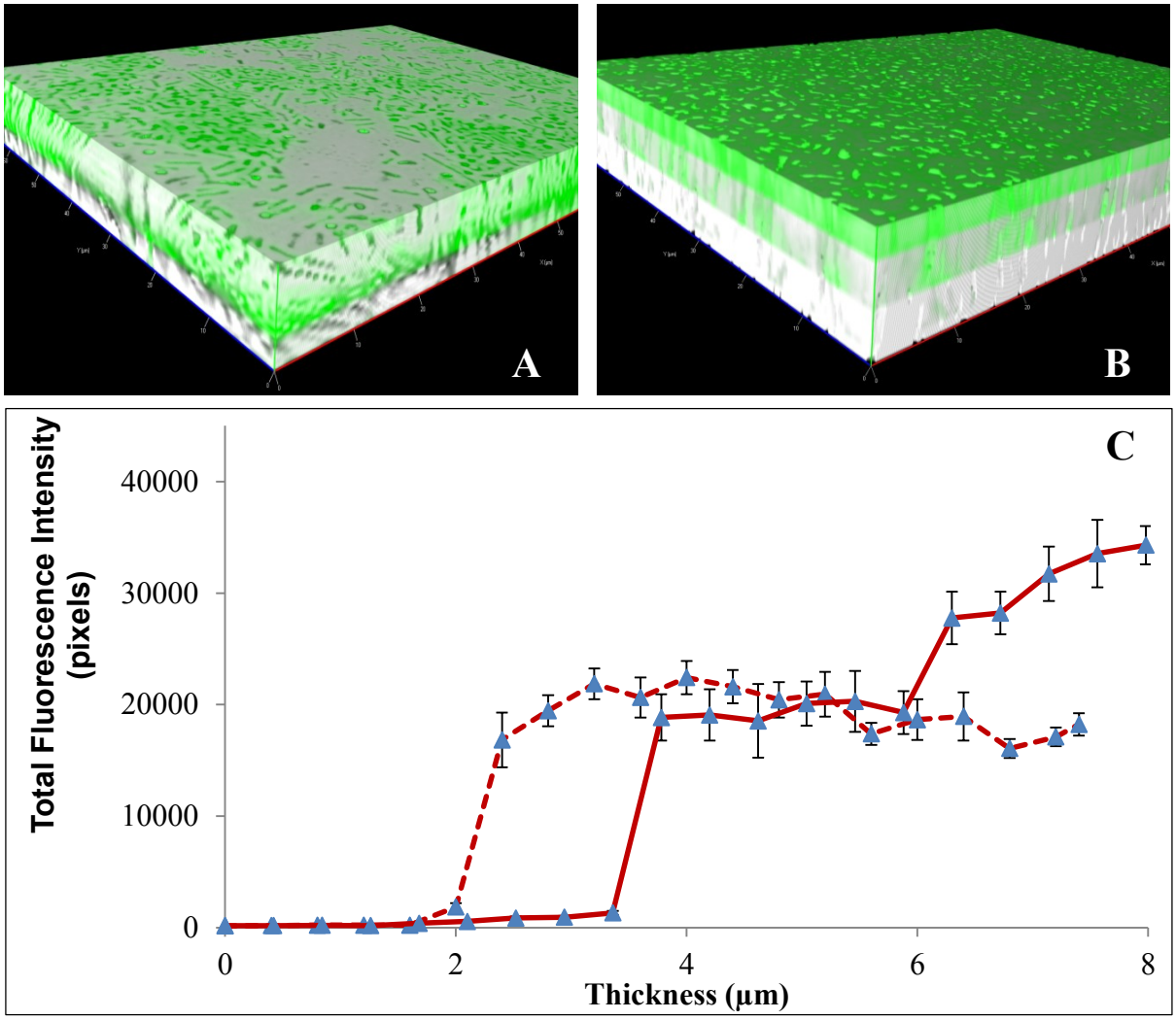

Figure 6: Spatial gene expression of *flhD*.
A and B are the 3D images constructed from the z-stacking images (bright field and fluorescence) at 12 hours (A) and 51 hours (B), using BP1470 (AJW678 pPS71). C is the graphical representation of the spatial gene expression of *flhD* at 12 hours (dashed line) and 51 hours (solid line) of biofilm formation.

In both the 12 and 51 h biofilms, the expression of *flhD* was highest at the outer layer of the biofilms (Fig. 6). Total pixel values from the individual images of the *z*-stacks showed that at 12 h, there was little or no expression of *flhD* within the first 2 µm from the surface that the biofilm had formed on. Expression increased rapidly at 2 µm to approximately 20,000 pixels. In 51 h biofilms, there were three distinct intensity levels.

Until 3 μm, the expression of *flhD* was very low; but at 3.5 μm, the expression jumped to 20,000 pixels and maintained this level until 6 μm; and across the upper 2 μm of our biofilm, *flhD* expression increased to approximately 30,000 pixels.

Expression of *ompR* is highest at the bottom of the biofilm

Spatial gene expression experiment for *ompR* at 34 h is the time point of maximum expression in the temporal experiment (Figure 7). Fluorescence signals from BP1432 were highest at the very bottom layer of the biofilm and could only be visualized in the bottom-to-top version of the 3D image (Figure 7A and Figure 7B). Total pixel values were highest at approximately 28,000 at the very bottom layer and decreased steadily towards the top (Figure 7C). Intriguingly, expression of *ompR* was inversely correlated to the distance from the surface that the biofilm had grown on.

Spatial expression of *rcsB* depends on the biofilm phase

From the temporal gene expression study, the expression of *rcsB* increased over time, starting at 25 h. We selected 33 h and 62 h as time points for the spatial study. The 3D reconstruction of the biofilm revealed that expression of *rcsB* was limited to the top of the biofilm at 33 h (Figure 8A) and to the bottom at 62 h (Figure 8B and 8C). At 33 h, total pixel values increased in several steps from the bottom of the biofilm to the top until they reached approximately 24,000 pixels (Figure 8D). At 62 h, total pixel values were highest at approximately 28,000 at the very bottom and decreased steadily across the 8 μm towards the top (Figure 8D).

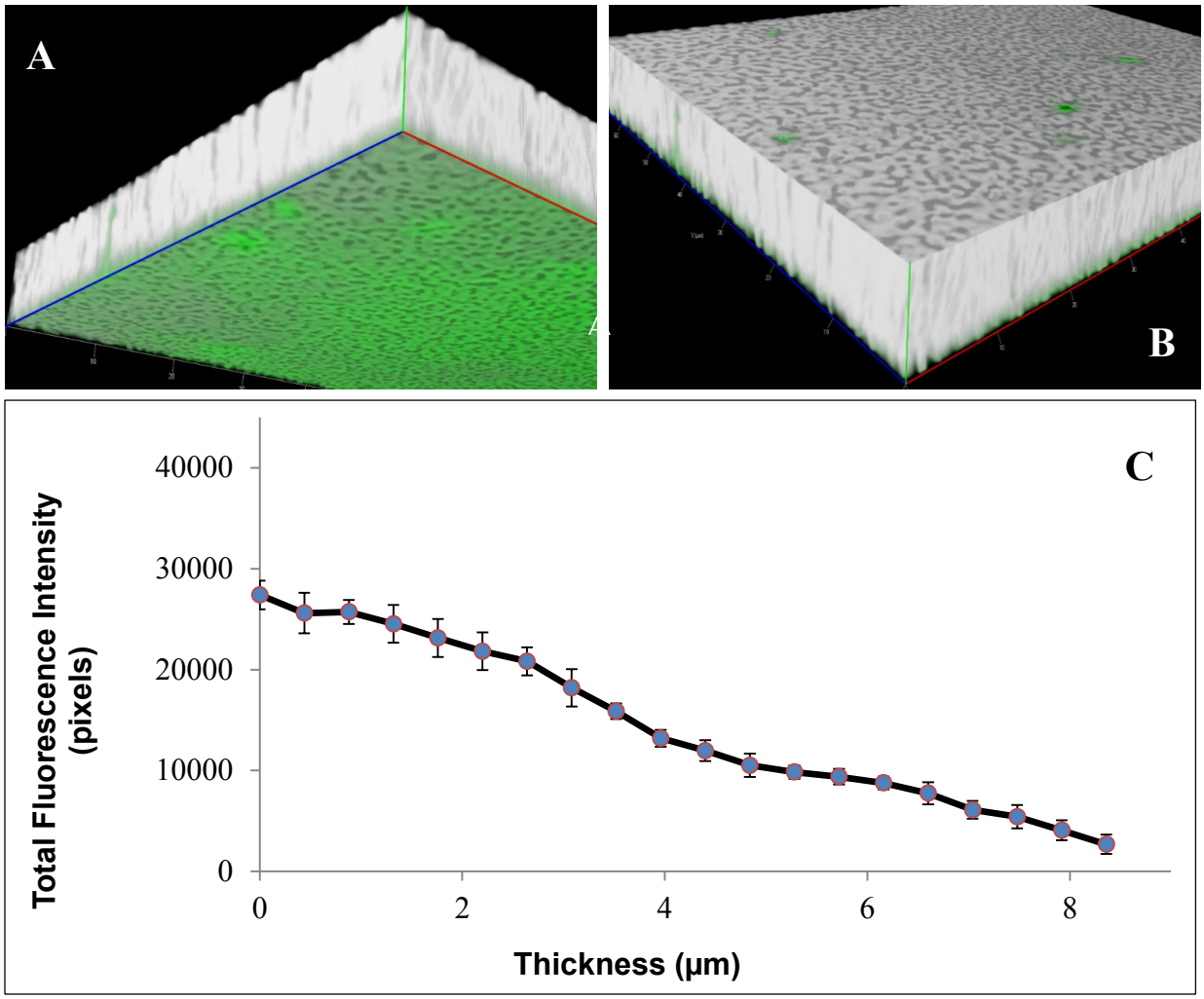

Figure 7: Spatial gene expression of *ompR*.
A and B are the 3D images constructed from the z-stacking images (bright field and fluorescence) from bottom view (A) and top view (B). C is the graphical representation of the spatial gene expression of *ompR* at 34 h of biofilm formation.

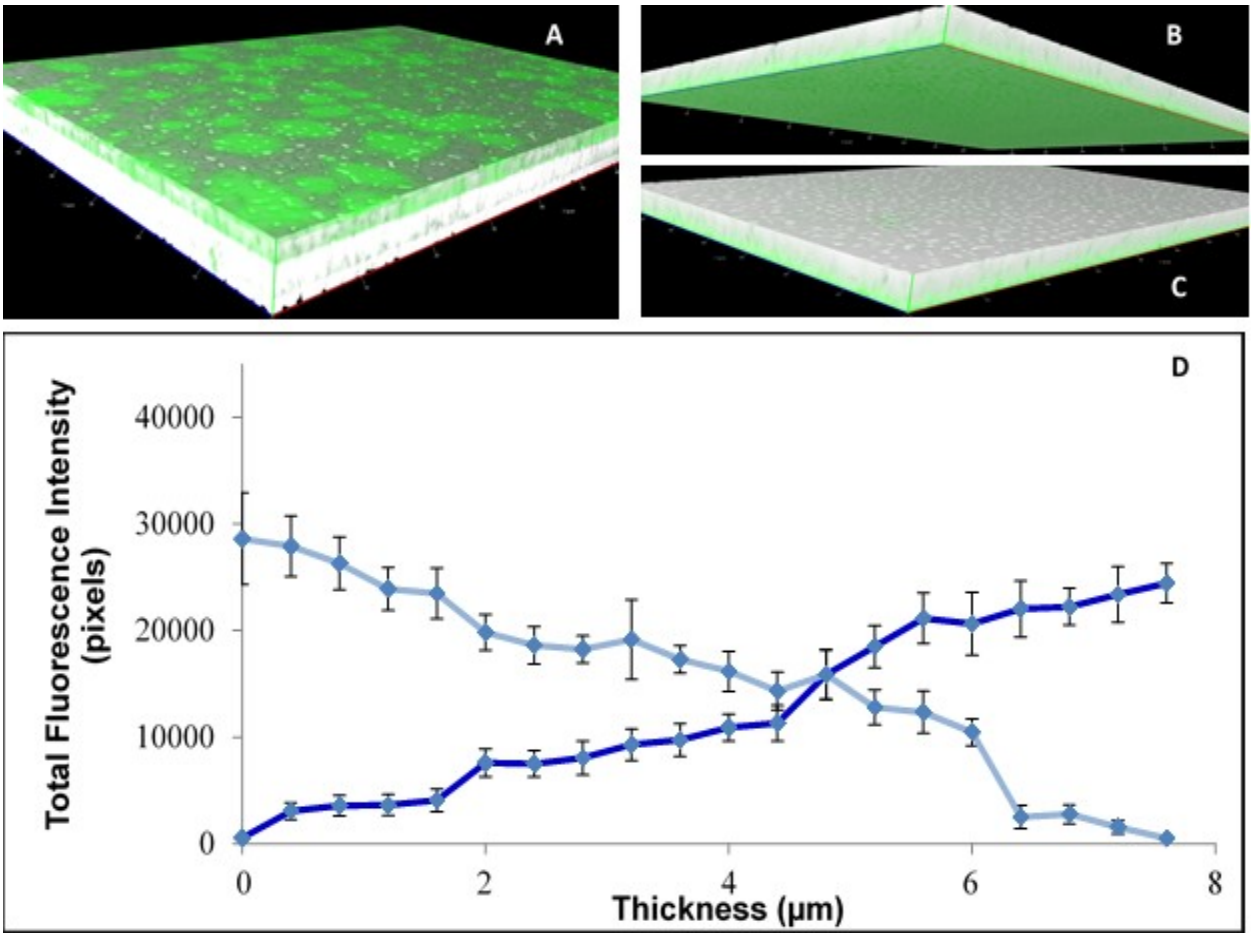

Figure 8: Spatial gene expression of *rcsB.*
A, B and C are 3D images constructed from z-stacking images of the 33 h biofilm top view (A), and 62 h biofilm bottom (B) and top views (C). D is the graphical representation of the spatial gene expression of rcsB at 33 h (dark blue) and 62 h (light blue) of biofilm formation.

Expression of *flhD* in the *ompR* and *rcsB* mutant strains is high across the entire biofilm

The temporal gene expression study determined that the expression of *flhD* in the *ompR* and *rcsB* mutant strains was constitutively high throughout the experiment after a primary increase during the initial time period of biofilm formation. As time points for the spatial experiment, we selected 33 h for the *ompR* mutant (Figure 9A) and 51 h for the *rcsB* m utant (Figure 9B).

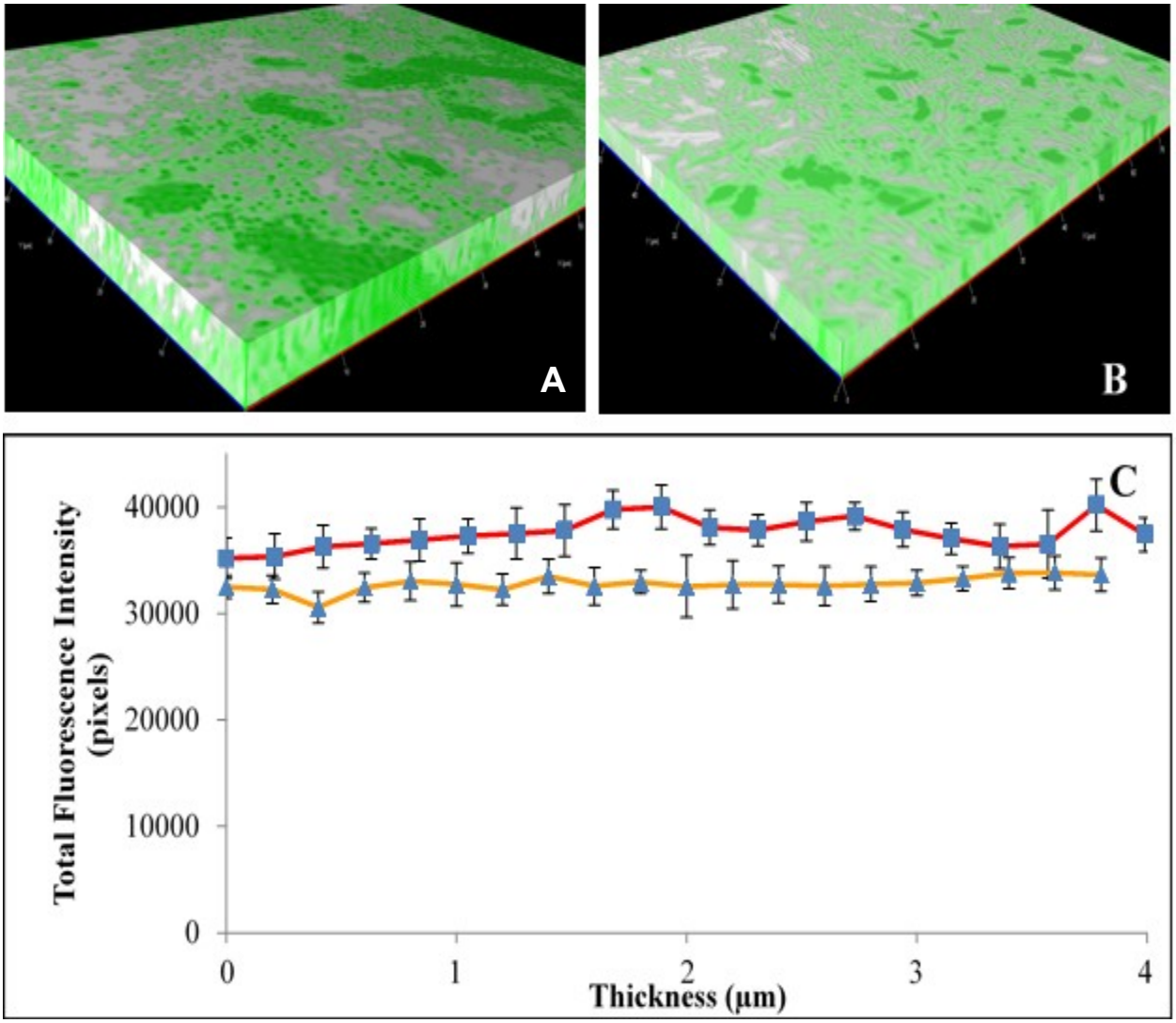

Figure 9: Spatial gene expression of *flhD* in the *ompR* and *rcsB* mutant strains. A is the 3D image of the 33 h biofilm from BP1531 (*ompR::Tn10* pPS71), B is the respective image from the 51 h biofilm from BP1532 (*rcsB::Tn5* pKK12). C is the graphical representation of the spatial gene expression of *flhD* in the *ompR* mutant (red line) and the *rcsB* mutant (orange line) at the time points represented in A and B.

As explained for the temporal experiment, the images were taken at 10% of the available excitation light. Interestingly, expression of *flhD* in both mutants was high across all layers of the biofilm. Total pixel values were at 30,000 to 40,000 pixels all across both biofilms (Figure 9C). By all appearances, both OmpR and RcsB abolished spatial differences in *flhD* expression together with temporal ones.

Mutations in *ompR* and *rcsB* reduced biofilm biomass

The 3D reconstructions of the biofilms showed that the biofilm from the *ompR* and *rcsB* mutants was much thinner than that of the parent strain. The mutant biofilms were no more than 4 μm, as opposed to >8 μm for biofilm from the parent strain (notice x-axis of Figure 9C versus that of Figure 6C). Due to optical limitations of the 100x lens used for these experiments, we could not quantify thickness of the parental biofilm with fluorescence microscopy beyond 8 μm. To quantify biofilm biomass, the crystal violet (CV) assay was performed with parent bacteria, and *ompR* and *rcsB* mutants. The parent strain produced 2 to 2.5 times more biofilm-associated biomass than either of the two mutants (Figure 10). This difference from the parent was seen at all-time points.

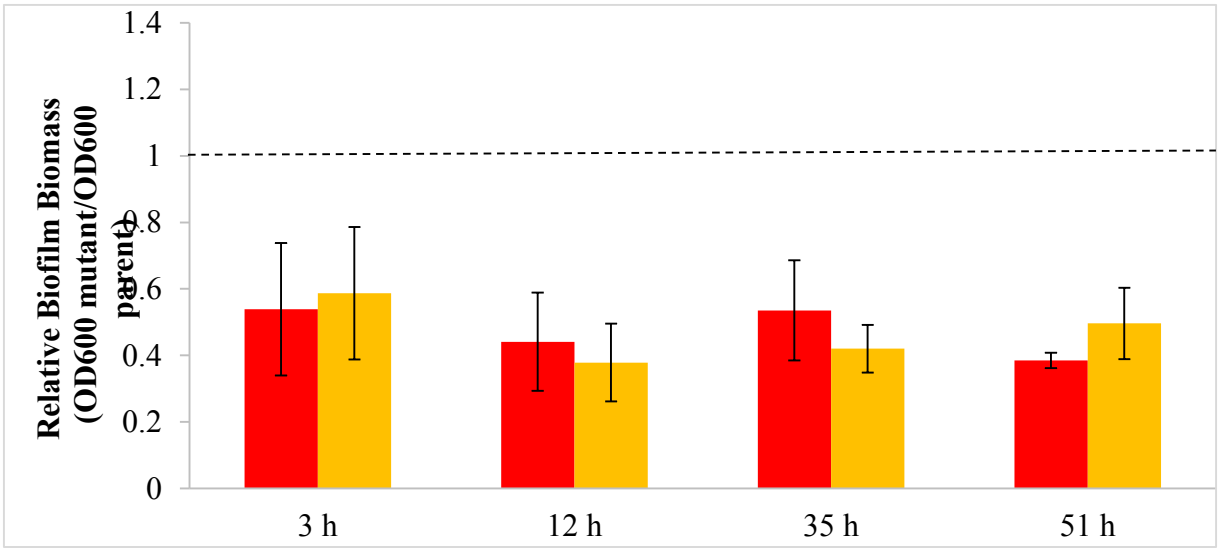

Figure 10: CV assay to quantify the biofilm amounts of the *ompR* and *rcsB* mutants in comparison to the parent strain.
The biofilm biomass was determined for BP1470 (*AJW678* pPS71), BP1531 (*ompR::Tn10* pPS71) and BP1532 (*rcsB::Tn5* pKK12). This was done at four different time points, which are indicated on the x-axis. The parent strain BP1470 was used as a reference strain and set at 1. The red bars are the biomass of BP1531, and the orange bars that of BP1532, both in comparison to the reference strain. Averages and standard deviations were calculated across three replicate experiments. The dashed line indicates the biomass of the reference strain.

An increase in cell length accompanied the reduced biofilm thickness of the *ompR* mutant

An observation that was made outside of the objectives for this study but in agreement with previous studies (Prüß and Matsumura, 1996; Sule et al., 2011) related to the increased cell length of bacteria that expressed excessive amounts of *flhD*. In the AJW678 parent strain, we observed the occasional occurrence of long and highly motile bacteria (snake cells) on top of the biofilm. These produced very bright fluorescence signals, which is indicative of high expression from the *flhD* promoter (Figure 11A). The effect was dramatically enhanced in the *ompR* mutant where we observed a much larger number of snake cells (Figure 11B). The longest mutant bacteria were also longer than those bacteria that were found in the biofilm of the parental strain. The quantitative measurements of bacteria from the top images of the two biofilms revealed that the majority of the parental bacteria were between 1 to 5 μm in length, while the longest bacteria that we could find were 14 μm (Figure 11C). In contrast, *ompR* mutants could be up to 35 μm long. While we did not quantify length of bacteria for the *rcsB* mutant, there was no visible difference from the length of the parental bacteria (data not shown).

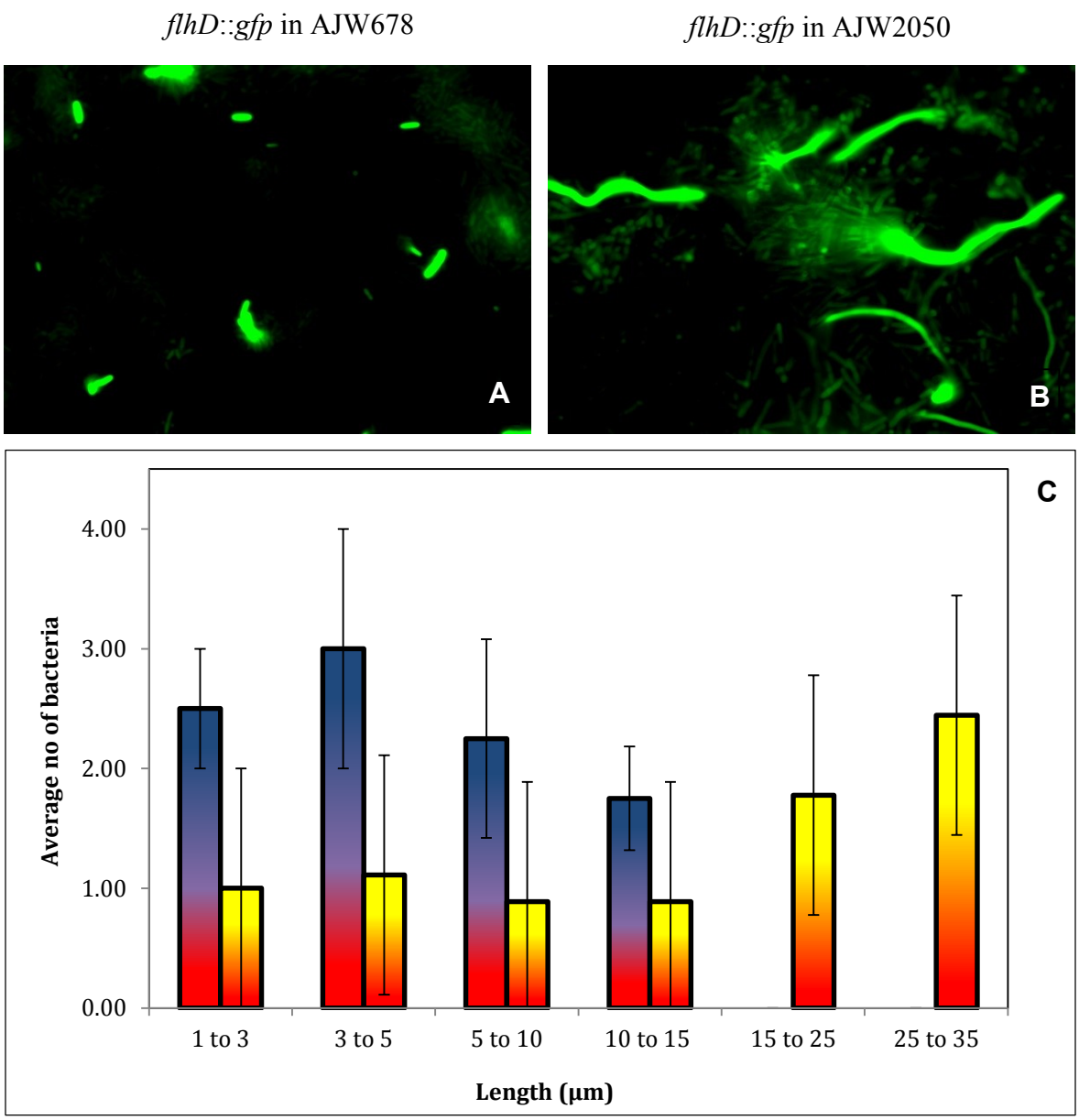

Figure 11: Lengths of *E. coli* parental strain and *ompR* mutants.
A is one representative image for parent strain BP1470, as is B for the *ompR* mutant strain BP1531. Both images were taken at the very top of the biofilm. C is the length profile of the bacterial populations, determined from 38 bacteria for the parent strain and 73 for the mutant. Percentages were determined across the measured population of bacteria. Dark red cross-hatched bars represent the parent strain, and red hatched bars represent the *ompR* mutant.

Discussion

In the Introduction, we hypothesised that a biofilm prevention target would be characterized by its expression early in biofilm development. This was the case for *flhD* whose expression peaked at 12 h. A biofilm treatment target was postulated to be characterized by expression late in biofilm development and at the outermost edge of the biofilm. This, too, was true for FlhD/FlhC. Expression of *flhD* increased again towards 51 h, and the highest expression of *flhD* was in the outer layer of the biofilm. Based upon these results, we come to the conclusion that the flagella master regulator complex FlhD/FlhC may be our first target for both biofilm prevention and treatment techniques. This would fulfill our first two goals: i) provide proof of concept that our approach can identify targets for biofilm prevention and treatment techniques and ii) establish FlhD/FlhC as the first such target. In fulfillment of the final goal of this study, we identified one mechanism to control *flhD* expression levels, as well as biofilm amounts and cell division. Mutations in both *ompR* and *rcsB* increased *flhD* expression to the point where temporal and spatial differences in expression were abolished. These expression increases where paralleled by decreases in biofilm amounts, relative to the parent strain. Lastly, increased *flhD* expression levels and decreased biofilm amounts of the *ompR* mutant were accompanied by an increased cell length.

The expression profiles of *flhD, ompR*, and *rcsB* can be related to biofilm phases

Originally described in *Pseudomonas aeruginosa,* it is now widely accepted that biofilm development in many bacteria involves reversible attachment, irreversible attachment, maturation, and dispersion (Sauer et al., 2002). These phases are

characterized by cell surface organelles such as flagella, type I fimbriae and curli, as well as numerous exopolysaccharides. The following three paragraphs relate the temporal expression profiles of *flhD* (positive regulator of flagella), *ompR* (negative regulator of flagella and positive regulator of curli), and *rcsB* (negative regulator of flagella and positive regulator of type I fimbriae and colanic acid capsule) to current literature on biofilm developmental phases. According to our previous review (Prüß et al., 2006), the hypothesis for the temporal expression profiles was that *flhD* expression may peak during reversible attachment, *ompR* expression during irreversible attachment, and *rcsB* expression may increase towards maturation.

A recent review article summarized the regulation of motility during biofilm formation (Guttenplan and Kearns, 2013). The authors believe that flagella are important in the motility-to-biofilm transition in a way that inhibition of motility encourages biofilm formation by means of several functional (e.g. YcgR) and regulatory (e.g. RcsB) mechanisms (Ko and Park, 2000; Kaiser et al., 2010; Gottesman, Trisler, and Torres-Cabassa, 1985). Our temporal expression profile of *flhD* is partially in agreement with this postulate. We saw a peak in fluorescence at 12 hours (Figure 5), which may resemble reversible attachment, and a time period of low *flhD* expression around 34 h, possibly resembling irreversible attachment. However, expression of *flhD* increased again towards 51 h (Figure 5). This late increase is not necessarily in agreement with current biofilm models. However, Guttenplan and Kearns leave room for flagella regulators that may still be discovered (Guttenplan and Kearns, 2013). Also, the role for flagella in dispersal is controversial.

The hypothesis (Prüß et al., 2006) that *ompR* expression may be highest during irreversible attachment was built upon the fact that phospho-OmpR was a negative regulator of *flhD* expression (Shin and Park, 1995) and a positive regulator of curli (Oshima et al., 2002; Jubelin et al., 2005). Our temporal expression profile of *ompR* is in support this hypothesis. The peak for *ompR* was at 34 h, where *flhD* expression was minimal (Figure 5). The production of curli has previously been recognized as a control mechanism for biofilm formation (Gerstel and Romling, 2003), an adherence tool to human uroepithelical cells (Kikuchi et al., 2005), and part of the motility-to-biofilm transition. CsgD contributes to this transition by activating the expression of curli and inhibiting flagella biosynthesis (Ogasawara, Yamamoto, and Ishihama, 2011). The expression peak of the positive curli regulator, OmpR, at 34 h could be our marker for irreversible attachment.

Maturation of a biofilm typically requires the synthesis of an exopolysaccharide capsule that serves as a 'glue' to keep the microcolony together and contributes to adherence to the surface. This capsule can consist of many different substances, among them the K-capsule polysaccharide that is a contributor to the intracellular lifestyle of uropathogenic *E. coli* (Goller and Seed, 2010) and colanic acid, which has been recognized early as an important factor in forming the three dimensional structures that constitute the biofilm (Danese, Pratt, and Kolter, 2000). The phosphorelay system RcsCDB is an activator of colanic acid production (Stout and Gottesman, 1990), while also activating the synthesis of type I fimbriae (Schwan et al., 2007). These multiple functions of RcsB may explain the slow and steady increase of *rcsB* expression during biofilm formation (Figure 5) that cannot be correlated with a single phase of biofilm

development. With the exception of the late increase in *flhD* expression, our temporal expression profiles are in agreement with our hypothesis from the review article (Prüß et al., 2006), as well as current literature.

Regulation of *flhD* by multiple response regulators offers an opportunity to control biofilm amounts and cell division

Since the goal of our research was to modulate signal transduction pathways and reduce biofilm amounts, the next step after the identification of FlhD/FlhC as our first target would be the attempt to modulate *flhD* expression levels, ultimately causing a reduction in biofilm amounts and possibly other bacterial phenotypes, such as cell division.

The expression of *flhD* is regulated by many environmental and genetic factors. Environmental factors include temperature (Shi et al., 1992), osmolarity (Shin and Park, 1995), and the nutritional state of the cell (Prüß et al., 2010). Genetic factors are similarly diverse and include the Catabolite Repressor Protein (CRP) and the nucleoid-associated protein H-NS (Soutourina et al., 1999), the transcriptional regulator LrhA (Lehnen et al., 2002), the LysR family protein HdfR (Ko and Park, 2000), and the insertion of IS elements into the *flhD* promoter (Lee and Park, 2013; Wang and Wood, 2011; Barker, Prüß, and Matsumura, 2004). Post-transcriptional regulation involves the carbon storage regulator CsrA (Wei et al., 2001) and a negative regulator of cell motility, YdiV (Li et al., 2012). At the transcriptional level, regulation of *flhD* expression can be accomplished by several of the response regulators of two-component systems, such as RcsB (Francez-Charlot et al., 2003), OmpR (Shin and Park, 1995), and QseC

(Sperandio, Torres, and Kaper, 2002). Knock-out mutations in *rcsB* and *ompR* yielded an impressive increase in *flhD* expression in the *ompR* and *rcsB* mutants (Figs. 5 and 9). Additionally, expression of *flhD* was no longer dependent upon the biofilm phase, after the biofilm had formed (Figure 5), or the location of the individual bacterium within the biofilm (Figure 9). The temporal expression profile of *flhD* in the *ompR* mutant is similar to the one that was observed previously in planktonic bacteria (Prüß, 1998). However, in planktonic bacteria, we never observed more than 2 or 3 fold increases in *flhD* expression in the *ompR* mutant, relative to its isogenic parent. Considering that we had to decrease the excitation light for the fluorescence microscopy from 90% to 10% of the available light, it is obvious that the difference in *flhD* expression in the parent strain and the *ompR* and *rcsB* mutants was much larger than 2 or 3 fold.

Intriguingly, the *ompR* and *rcsB* mutants are also our first two examples of controlling biofilm amounts and cell division by modulating the expression levels of FlhD/FlhC. In addition to exhibiting increased expression levels of *flhD*, the mutants also produced reduced biofilm amounts (Figure 10). This observation provides confidence in our conclusion that impacting the signal transduction cascade, consisting of multiple two-component response regulators and FlhD/FlhC, can be used to control biofilm amounts. While cell division was not among the objectives of this study, we noticed that the *ompR* mutant also had a cell division defect in addition to the reduced ability to form biofilm (Figure 9). Bacterial cells of the *ompR* mutant strain were much longer than those of the parent strain. We cannot explain why the *rcsB* mutant did not exhibit this phenotype, but have previously seen a similar increase in cell size and decrease in cell number in the *ompR* mutant in planktonic bacteria (Prüß, 1998).

Since the number of two-component systems in *E. coli* are many (Oshima et al., 2002) and response regulators respond to a diversity of environmental signals, the two-component signal transduction mechanism offers ample opportunity at controlling bacterial phenotypes and behaviors by deliberately changing the bacterial environment. In particular, varying the nutrient source for the bacteria has proven successful in controlling biofilm amounts, as well as the cell division rate. As an example for biofilm control, the two-component system for C4-dicarboxylic acid metabolism, DcuS/DcuR (Golby et al., 1999), was found to be required for biofilm formation in the presence of fumarate, malate, and succinate (Prüß et al., 2010). Likewise, we were able to increase the cell division rate by continuous additions of serine to the bacterial growth medium, the signal transduction cascade involved phosphorylation of OmpR by acetyl phosphate and inhibition of *flhD* expression (Prüß and Matsumura, 1996). In *E. coli* O157:H7, the bacterial carbon source ß-phenylethylamine was able to reduce biofilm amounts on plastic and bacterial cell counts in liquid beef broth medium (Lynnes *et al*., submitted to Meat Science). Bacterial cell counts were reduced when bacteria were grown on the surface of beef meat.

Conclusions and Outlook

The goal of this study was to show that the study of temporal and spatial gene expression can lead to the identification of targets for the development of novel biofilm prevention and treatment options. We propose FlhD/FlhC as the first of such targets. Our intention is to identify more of these targets using the temporal/spatial gene expression approach on a selection of biofilm-associated genes. With respect to

FlhD/FlhC, we demostrated that a gene that is highly regulated by so many environmental and genetic factors is ideally suited to be controlled by deliberate changes to the environment, through a signal transduction cascade that may involve additional two-component response regulators beyond the two that were tested in this study, ultimately impacting biofilm amounts and possibly cell division.

Methods

Bacterial strains, plasmids, and growth conditions

All the bacterial strains and plasmids that were used for this study are listed in Table 2. Throughout the study, we used the *E. coli* K-12 strain AJW678 as a parental strain because it is a good biofilm former (Kumari et al., 2000) and wild-type for the biogenesis of flagella and type I fimbriae and curli. AJW678 is lacking the IS element (Prüß et al., 2010) in the *flhD* promoter that makes bacteria highly motile. MC1000 is another K-12 strain (Anonymous, 2010; Casadaban and Cohen, 1980). It contains an IS5 in the *flhD* promoter (Barker, Prüß, and Matsumura, 2004) and is highly motile, but produces much reduced biofilm amounts. To assure maximal expression of *flhD*, we used this promoter to construct the *flhD*::*gfp* fusion plasmid pPS71.

AJW2050 is an *ompR* mutant strain due to the insertion of a *Tn*10 transposon, AJW2143 is an *rcsB* mutant strain due to *Tn*5 insertion. AJW678, AJW2050, and AJW2143 were used in several of our previous studies (Prüß et al., 2010; Sule et al., 2009). Plasmids pPS71 (*flhD::gfp*), pKK12 (pPS71 cm^R) and pEC2 (*rcsB::gfp*) were constructed for this study. The *ompR::gfp* plasmid was obtained from the Open Biosystems promoter collection (Zaslaver et al., 2006) (Thermo, Huntsville, AL).

Table 2: Bacterial strains and plasmids.

Strains	Relevant genotypes	Reference
AJW678	*thi-1 thr-1(am) leuB6 metF159(Am) rpsL136 ΔlaxX74*	(Kumari et al., 2000)
AJW2050	AJW678 *ompR::Tn10*	(Prüß et al., 2010)
AJW2143	AJW678 *rcsB::Tn 5*	(Fredericks et al., 2006)
MC1000	F-, *araD*139 Δ(*araAB leu*)7696 Δ(*lacX74*) *galU galK strA prsL thi*	(Casadaban and Cohen, 1980)
BP1470	AJW678 pPS71	This study
BP1531	AJW2050 pPS71	This study
BP1532	AJW2143 pKK12	This study
BP1432	AJW678 *ompR::gfp*	This study
BP1462	AJW678 pEC2	This study
Plasmids		
pPS71	pUA66 *flhD::gfp*	This study
pKK12	pPS71 cm^R	This study
pOmpR::*gfp*	pUA66 *ompR::gfp*	(Zaslaver et al., 2006)
pEC2	pAcGFP *rcsB::gfp*	This study

The *Tn*10 and *Tn*5 transposons confer resistance towards tetracycline and kanamycin, respectively. Δ constitutes a deletion of the respective gene. cm^R indicates chloramphenicol resistance. *gfp* encodes green fluorescence protein.

Cloning of *flhD::gfp* (pPS71), pPS71 Cm^R (pKK12) and *rcsB::gfp* (pEC2) plasmids

pPS71: To construct the *flhD*::*gfp* containing plasmid, the *flhD* promoter region that starts 1,419 bp upstream of the +1 transcriptional start site and ends 502 bp downstream of the +1 was amplified from MC1000, using 5'-TCCTCGAGTGACTGTGCGCAACATCCCATT-3' as forward primer and 5'-AGGTACCTGCCAGCTTAACCATTTGCGGA-3' as reverse primer. This promoter fragment contains the IS5 that increases *flhD* expression and is located at -1,294 bp to -94 bp (Barker, Prüß, and Matsumura, 2004), making the fragment 1,921 bp in length. The forward and reverse primers were designed with XhoI and BamHI restriction enzyme recognition sites at the 5' ends. The *flhD* promoter fragment was then double-digested with XhoI and BamHI. The vector pUA66 (Open Biosystems, Huntsville, AL), containing *gfpmut2* as a reporter gene and a kanamycin resistance cassette, was also digested with these enzymes. To reduce re-ligation of the plasmid, digested pUA66 vector was treated with Calf Intestinal Alkaline Phosphatase (CIAP, Promega, Madison WI) that removes the 5' phosphate. The double-digested *flhD* promoter region was ligated into the digested and CIAP-treated pUA66 vector. Competent JM109 cells (Promega, Madison WI) were transformed with the resulting plasmid pPS71. The insertion was confirmed by restriction digest and sequencing. Ultimately, pPS71 was transformed into chemically competent AJW678 and AJW2050.

pKK12: To permit the transformation of mutants that exhibited resistance towards kanamycin, the kanamycin resistance of pPS71 was changed to chloramphenicol resistance. pPS71 was digested with EagI to remove 280 bp from pPS71. This removed region started upstream of the *flhD* promoter, extended into the kanamycin resistance

gene, and caused inactivation of kanamycin resistance. The digested plasmid was blunt ended with Klenow (Promega, Madison WI), and treated with CIAP. pHP45Ω-Cm was the source of the chloramphenical resistance gene cassette (Fellay, Frey, and Krisch, 1987) and was digested with EcoRI and blunt ended with Klenow. The CIAP-treated pPS71 and pHP45Ω-Cm DNA fragments were ligated. Competent JM109 were transformed with the resulting plasmid pKK12, transformants being resistant to chloramphenicol but not to kanamycin. Competent AJW2143 (*rcsB*::*Kn*) were then transformed with pKK12.

pEC2: To construct this plasmid, the *rcsB* promoter region that starts 100 bp upstream of its +1 transcriptional start site and ends 50 bp downstream was PCR-amplified from AJW678, using 5'-GAGAGATCTGCAACCTGTATCACACCCGATGAAAG-3' as forward primer and 5'-GCAAAGCTTCGGATGGTCATCGGCAATAATTACG-3' as reverse primer. The PCR-amplified region was then cleaned up and ligated into pGEM-T Easy (Promega, Madison WI). Successful ligations were identified by the white color of the transformed colonies. Plasmids were digested using the HindIII and BglII restriction sites that had been added to the 5' ends of the primers. The promoterless pAcGFP1-1 encodes the green fluorescent protein AcGFP1, a derivative of AcGFP from *Aequorea coerulescens,* and has a kanamycin resistance gene (Clontech, Mountain View, CA). This plasmid was also double-digested with the same enzymes. The digested *rcsB* promoter region was ligated into the digested pAcGFP1-1 vector. Competent JM109 cells were transformed with the resulting plasmid pEC2. The insertion region was confirmed by restriction

digest and sequencing. Ultimately, pEC2 was transformed into chemically competent AJW678.

Bacterial strains were stored at -80˚C in 10% dimethyl sulfoxide (DMSO). Before use, the bacterial strains were streaked onto LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar plates and incubated overnight at 37˚C. From the plates, cultures were inoculated into liquid tryptone broth (TB, 1% tryptone, 0.5% NaCl) and grown overnight at 37˚C. For bacterial strains containing pPS71, 25 µg/ml of kanamycin were added to the bacterial growth medium. For pEC2, 50 µg/ml of kanamycin were added. For pKK12, 50 µg/ml of chloramphenicol were added.

Temporal and spatial expression of *flhD*, *ompR*, and *rcsB*

E. coli strains were grown in TB overnight at 37°C. One ml of each culture was injected into one channel of a 3-channel flow cell (Stovall, Greensboro NC) with a syringe as described (Pamp, Sternberg, and Tolker-Nielsen, 2009). The flow cell was incubated at room temperature for 1 h without any media flow. After that, TB was pumped by an Isma Tec Low Flow High Accuracy Peristaltic Pump (Stovall) into the flow cell at 1 ml/min, equaling 0.33 ml/min per channel. For temporal expression experiments, the flow cell was disconnected after a maximum of 62 h. For spatial expression experiments, the flow cell was disconnected at time points of interest. Each of the investigated bacterial strains was processed at least three times for both temporal and spatial experiments. The flow cell system was kept free of air bubbles by the bubble trap that is part of the Stovall system.

We used an Axio Observer Z2 upright fluorescence microscope with ApoTome2 from Zeiss (Germany) to detect the fluorescence signals coming from the promoter::*gfp* fusion plasmid containing strains. The Zeiss Axio Observer Z2 microscope is one of the high-end technologies from Zeiss to get the brightest and the clearest image from a weak signal. For the temporal experiment, fluorescence images were taken at 1 h, 3 h, 7 h, 9 h, 12 h, 14 h, 21 h, 27 h, 35 h, 48 h, and 51 h for BP1470 (*flhD*::*gfp*) and BP1432 (*ompR*::*gfp*); 1 h, 4 h, 14 h, 25 h, 34 h, 50 h, and 58 h were selected as time points for BP1462 (*rcsB*::*gfp*); 1 h, 5 h, 12 h, 20 h, 30 h and 51 h were chosen for BP1531 (*ompR*::*Tn*10 *flhD*::*gfp*); and 1 h, 7 h, 12 h, 22 h, 34 h, and 51 h were selected for BP1532 (*rcsB*::*Tn*5, *flhD*::*gfp*). For the spatial experiments, *z*-stacking images were taken at 12 h and 51 h for biofilms of BP1470; 34 h for biofilms of BP1432; 33 h and 62 h for biofilms of BP1462; 33 h for biofilms of BP1531; and 51 h for biofilms of BP1532. This was done separately for fluorescence and bright field. For both temporal and spatial experiments, images were taken at 1,000 X magnification using a 100 x/1.46 Oil α-Plan-apochromatic objective. This objective at this high magnification can only take *z*-stacks across 8 μm. Even though some of our biofilms were 15 to 20 μm thick, we selected areas of the biofilm that were consistent with the limitation of the objective.

The intensities of the fluorescence signals of *flhD* from the *ompR* and *rcsB* mutant strains turned out to be much higher than those from the parental strain. For this reason, we performed microscopy for BP1531 and BP1532 at 10% of the available excitation light. For BP1470, BP1432, and BP1462, we used 90% of the available excitation light.

For temporal and spatial gene expression experiments, we used Image-Pro Plus software to determine the total intensity of the fluorescence signals across each image. Next, we determined the average and standard deviation across all 9 images (3 images per biological replicate) for BP1531, BP1532, and BP1462 and across the 4 images (1 image from each biological replicate) for BP1470 and BP1432 that were obtained for each time point. Finally, the average total intensity was plotted against time for the temporal experiment or distance from the surface for the spatial experiment.

For spatial gene expression experiments, 3D reconstructions of the biofilms were done from the *z*-stacks with AxioVision v-4.7.1 software from Zeiss, using both fluorescence and bright field images. This software was also used to determine the lengths of bacterial cells from the parental strain and the *ompR* mutant from the outermost top images. Length of the bacteria was categorized: 1-3 μm, 3-5 μm, 5-10 μm, 10-15 μm, 15-25 μm, and 25-35 μm. The total number of bacteria that fall into each category was determined across the replicate images (four images for BP1470 (parent strain) and nine images for BP1531 (*ompR* mutant)). The number of bacteria in each length category was divided by the total number of bacteria that were measured. Results are expressed as percentage.

Crystal violet assay to determine biofilm biomass

Biofilm of BP1470, BP1531, and BP1532 were grown in individual wells of a 24-well plate in TB for 3 h, 12 h, 35 h, and 51 h at room temperature. Liquid bacterial growth medium was removed and biofilms were washed twice with phosphate buffered saline (PBS). Biofilms were stained with crystal violet (CV) as described (O'Toole et al.,

1999; Pratt and Kolter, 1998; Stafslien et al., 2007; Stafslien et al., 2006). The OD_{600} of the extracted CV was determined from a 1:10 dilution with a Synergy H1 plate reader from BioTek (Winooski, VT). Relative biomass was determined by dividing the OD_{600} for each mutant strain by the parental strain. Averages and standard deviations were determined across the three replicate experiments.

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Addendum 1; Detailed information about the cloning of plasmid pPS71

Cloning of pPS71

The following Figure 12 is the graphical representation of the cloning of pPS71.

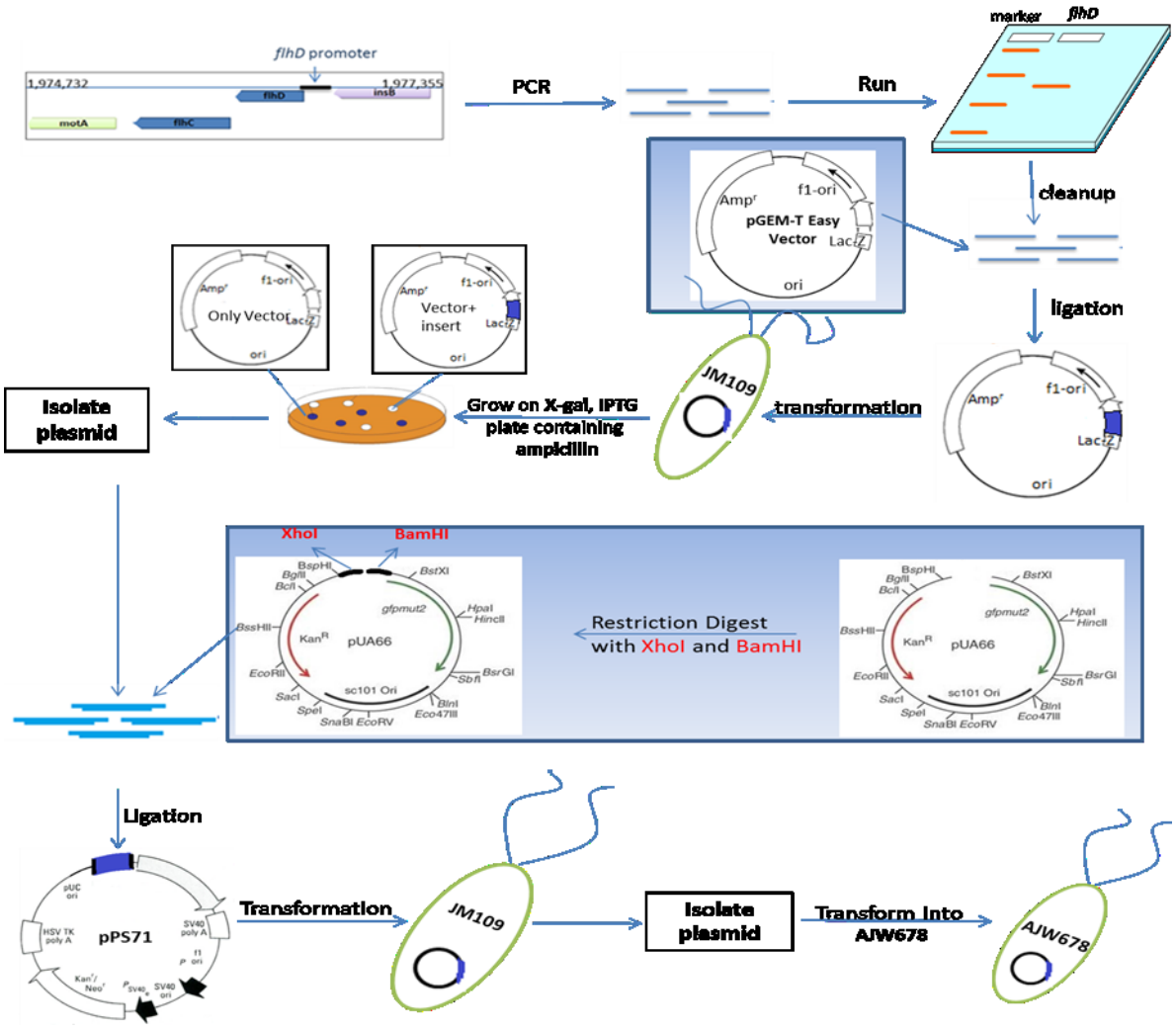

Figure 12: Cloning of pPS71.
flhD promoter regions were cloned into pUA66. Promoter regions were first ligated into pGEM-T Easy vector and transformed into JM109. The resulting plasmids were digested with XhoI and BamHI and ligated into pPA66 plasmids that had been digested with the same enzymes. First JM109 and then AJW678 competent cells were transformed with the promoter fused pUA66.

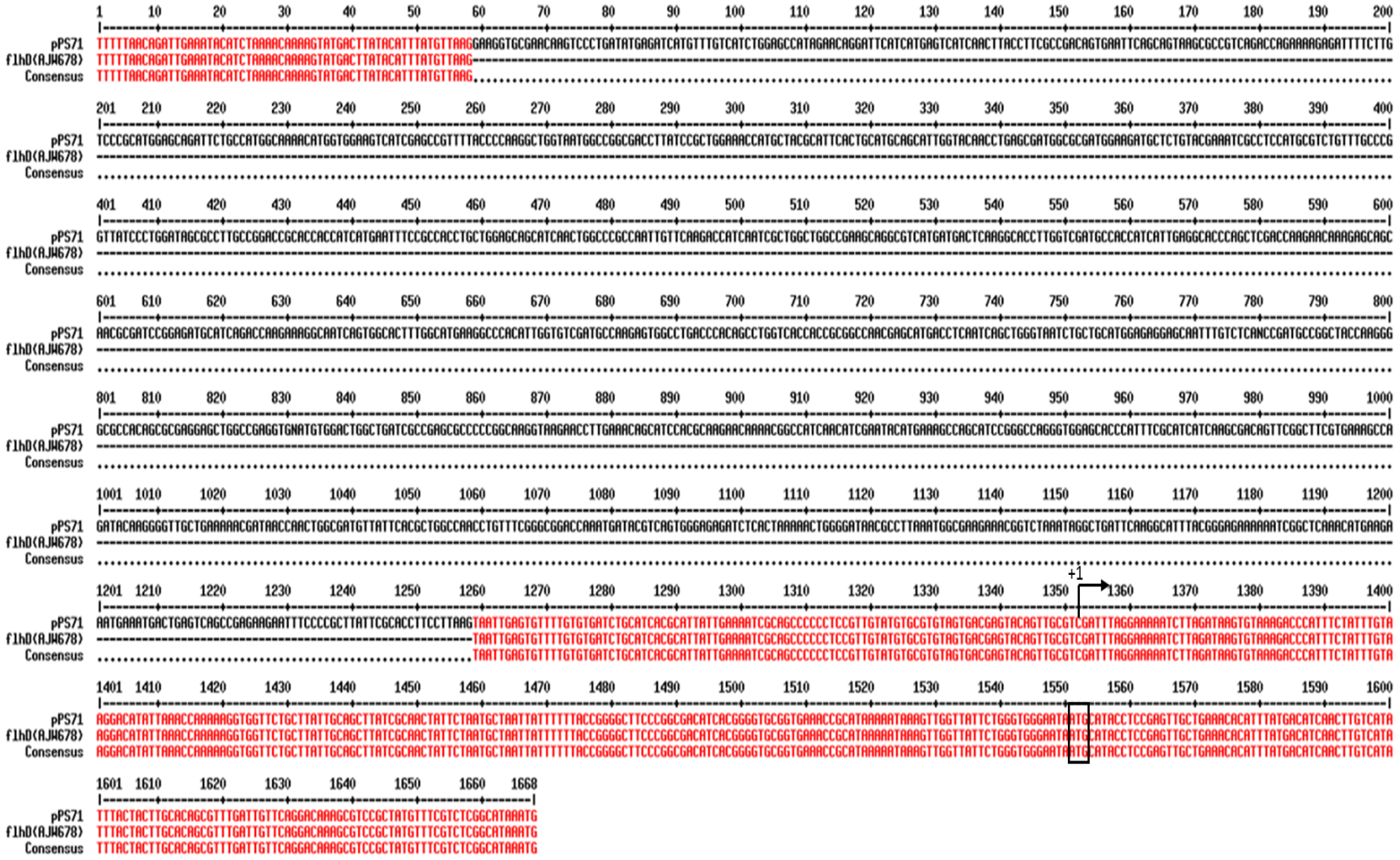

Figure 13: Sequence alignment of the cloned *flhD* promoter of pPS71 and *flhD* promoter of AJW678.
The +1 transcriptional start site and the ATG start codon are indicated. The IS5 element of cloned *flhD* promoter of pPS71 is shown in the black colored sequences.

Sequencing alignment result of *flhD* promoter of pPS71

The *flhD* promoter sequences from AJW678 and *flhD* promoter sequences pPS71 plasmids are compared in Figure 13. The top and bottom alignment clearly shows the difference between those two promoter regions, due to the 1200 bp IS5 element in the *flhD* promoter of pPS71. The IS5 element started at -1294 bp and ended at -94 bp from the +1 transcriptional start sites.

<u>Addendum 2; response to reviewer's concerns</u>

1. Reviewers requested a statistical analysis of the temporal gene expression data that we did with the Loess procedure. The statistical analysis yielded confidence bands for the *ompR* and *rcsB* mutant strains that did not overlap with that of the parent (Figure 14). This indicates that there is indeed a statistically significant difference between the parent strain and either of the two mutants.

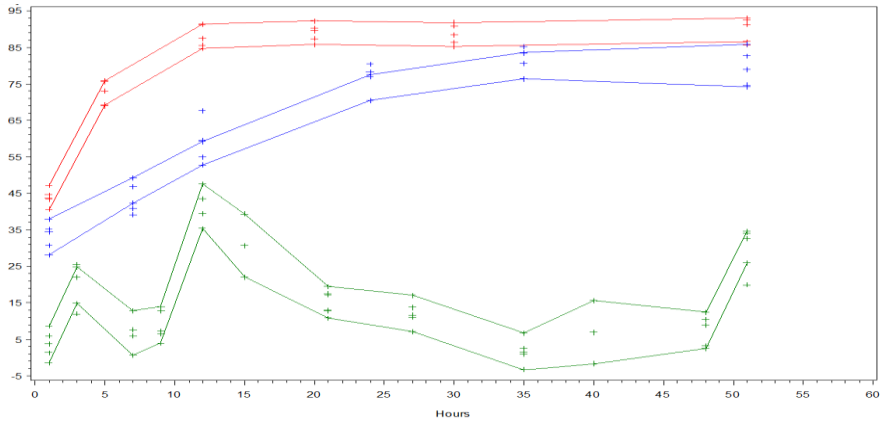

Figure 14: Statistical analysis of temporal expression of *flhD, ompR* and *rcsB* in AJW678 and *flhD* in the *ompR* and *rcsB* mutant strains.
Upper and lower lines of each colors indicate the highest and the lowest level of the total fluorescence intensity. The dark red, black, and blue lines show the gene expression profile of *flhD, ompR* and *rcsB*, respectively.

2. Reviewers also requested a housekeeping gene for the temporal and spatial gene expression. We used *aceK*, which encodes isocitrate dehydrogenase. This enzyme catalyzes the oxidative decarboxylation of isocitrate to produce α-ketoglutarate and CO_2 in the tricarboxylic acid cycle. The temporal gene expression of *aceK* was done for up to 58 h and the spatial gene expression was measured on 58 h of biofilms (Figure 15). The spatial expression of aceK was done at 34 h (Figure 16).

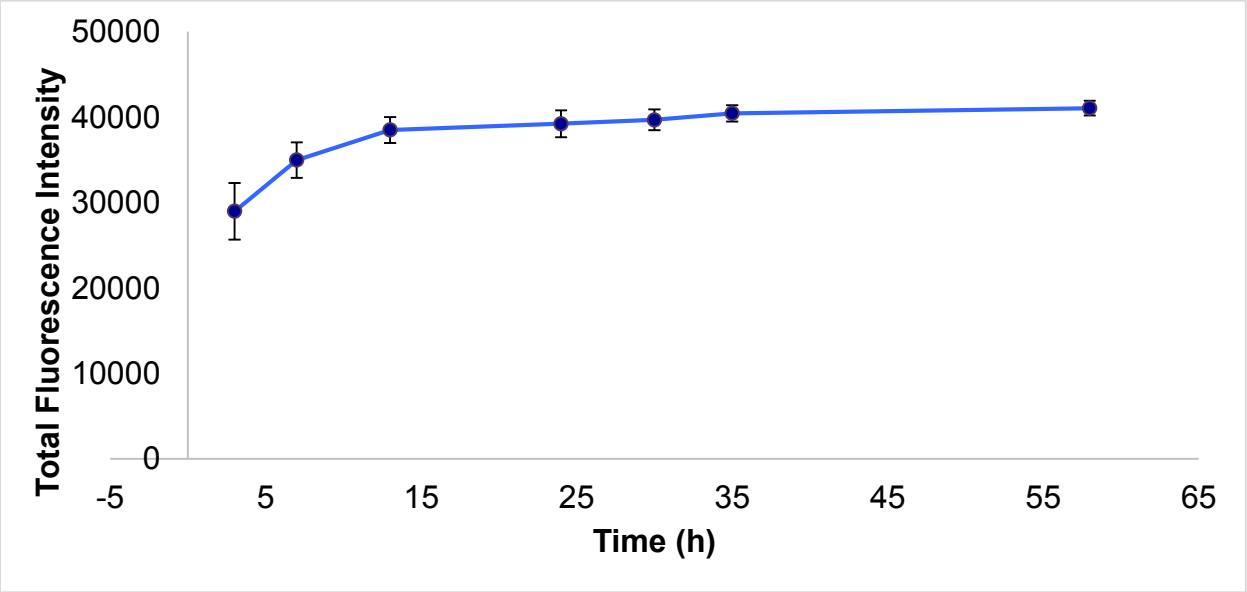

Figure 15: Temporal gene expression of *aceK*.

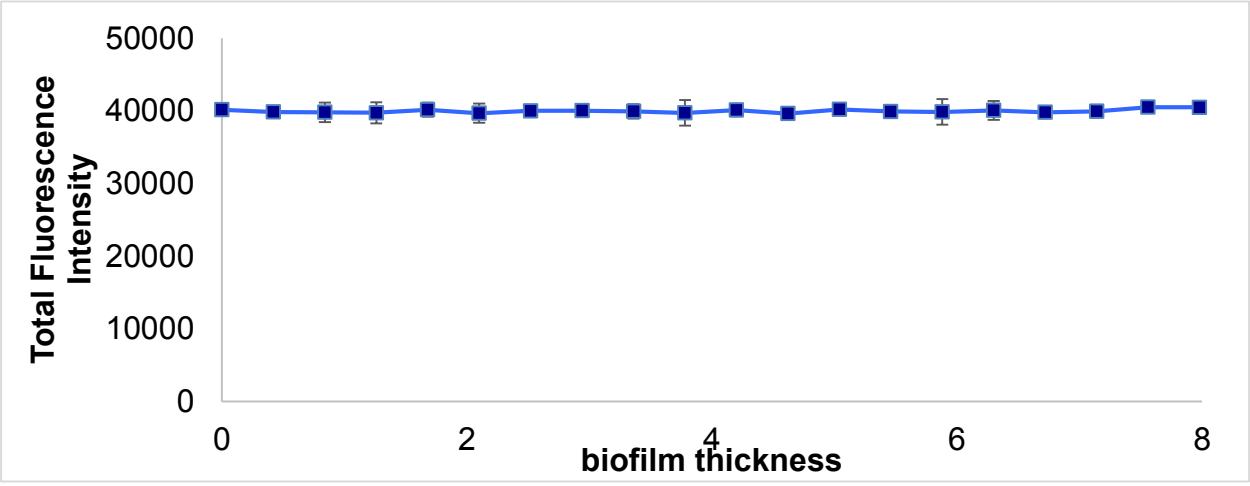

Figure 16: Spatial gene expression of *aceK* at 34 h.

PAPER 2: IS ELEMENT INSERTION PLAYS AN IMPORTANT ROLE IN THE REGULATION OF *E. COLI FLHD* GENE EXPRESSION AND EVOLUTION OF THE BIOFILM[2]

Abstract

Biofilm is an aggregation of bacterial communities that respond to changes in the environment by means of evolutionary adaptation, as well as changes in gene expression. Both evolution and gene expression regulation have been studied on FlhD/FlhC, the master regulator of flagella expression and global regulator of many metabolic genes. It is possible that IS element insertion into the *flhD* promoter is a major adaptive mechanism with regard to both evolution and gene expression.

We recovered colonies from mature biofilms, formed by a highly motile, poor biofilm forming *E. coli*. A total of 85 isolates were recovered that had little or no motility. Twenty-seven of the non-motile isolates contained insertions of IS1 in their *flhD* operon. All the IS elements had inserted in close proximity to the translational start of FlhD or within the open reading frame of FlhC.

[2] **Priyankar Samanta,** Joseph Sayler, Shelley M. Horne, and Birgit M. Prüß* 2014

Most of the work, including the writing of the first draft of the manuscript was done by Priyankar Samanta. Joseph Sayler isolated the colonies, Shelley Horne performed PCR reactions. Below is the first draft that was written by Priyankar Samanta and submitted for publication. Addendum 1 provides the sequence alignments for some of the *flhD* promoters.

Using flow cell techniques, *gfp* reporter gene fusions, and fluorescence microscopy, we determined the temporal and spatial expression of several different *flhD* promoter regions that contained IS elements. The expression pattern from the *flhD1* promoter (no IS) was similar to the previously published pattern with the *flhD3* promoter that contained an IS5 at -99 to -96 upstream of the transcriptional start of *flhD*. Expression of *flhD1* was increased in knock-out mutants of OmpR or RcsB and also highest at the outermost edge of the biofilm. There was little or no expression from the *flhD4* promoter that contained an IS1 element at 5 bp upstream of the start codon for FlhD in addition to the IS5 at -99 to -96 from the transcriptional start.

We conclude that insertion of an IS element into the *flhD* promoter can have positive or negative effects on *flhD* expression, depending on the position of the IS insertion. We believe that we were able to recover non-motile isolates because the biofilm contains niches where motility may be a disadvantage. These niches may be located at the bottom of the surface, where we could not detect any *flhD* expression.

Keywords: *Escherichia coli*, biofilm, IS element insertion, two-component response regulators

Background

Escherichia coli FlhD/FlhC is the master regulator of the flagella transcriptional hierarchy (Silverman and Simon, 1973; Bartlett, Frantz, and Matsumura, 1988) and a global regulator of many metabolic genes (Prüß *et al.*, 2003; Prüß *et al.*, 2001). The role of FlhD/FlhC in the formation of biofilm, a complex community of bacteria that forms on surfaces, is controversial. Research by our own lab is in agreement with the idea that whether FlhD/FlhC constitutes an advantage or a disadvantage for biofilm-bound

bacteria may depend on the time point of biofilm formation, as well as the location of the individual bacterium within the biofilm (Samanta *et al.*, 2013).

FlhD/FlhC is tightly controlled in response to many signals from the environment. This is accomplished in part by means of two-component signal transduction systems (2CSTSs) which also provide a link between signaling and central metabolism in the form of acetyl phosphate (Wolfe *et al.*, 2003) or acetyl-CoA (Thao *et al.*, 2010). Examples of 2CSTSs that either activate or inhibit the expression of FlhD/FlhC are the osmoregulator EnvZ/OmpR (Shin and Park, 1995), the colanic acid activator RcsCDB (Francez-Charlot *et al.*, 2003), and the quorum sensing system QseB/QseC (Sperandio, Torres, and Kaper, 2002). These three 2CSTSs also have functions in *E. coli* biofilms (Prigent-Combaret et al., 2001; Gottesman, Trisler, and Torres-Cabassa, 1985; Kostakioti et al., 2009). Additional regulators that control the expression of FlhD/FlhC include the anaerobic regulator LrhA (Lehnen *et al.*, 2002), the DNA folding protein H-NS (Bertin *et al.*, 1994), the catabolite repressor protein CRP (Soutourina *et al.*, 1999), multiple heat shock proteins (Shi *et al.*, 1992; Mizushima *et al.*, 1994), the anti-FlhD/FlhC factor YdiV (Li *et al.*, 2012), Hha (Sharma and Bearson, 2013), and even a small RNA (Thomason *et al.*, 2012). Our previous research focused on a triangle of regulation, consisting of OmpR and RcsB, both inhibiting the expression of FlhD/FlhC in their phosphorylated form by binding to specific sequences within the *flhD* promoter (Prüß and Wolfe, 1994; Samanta *et al.*, 2013; Prüß *et al.*, 2010).

While regulation of gene expression is undeniably important for bacteria that are associated with a biofilm, the many different niches that constitute the biofilm also give ample opportunity for evolution to act on the bacteria (Saint-Ruf *et al.*, 2014). Some of

this adaptation involves the *flhD* promoter. As one example, an insertion of IS5 into the *flhD* promoter of the originally non-motile version of the *E. coli* K-12 strain MG1655 Fnr- occurred on motility plates, causing a 2.7 fold increase in the expression of *flhD* and a 7 fold increase in the swarm rate (Barker, Prüß, and Matsumura, 2004). Since the IS element had inserted into the binding sites for the *flhD* inhibitors OmpR and LrhA, Barker and coworkers hypothesized that the increase in *flhD* expression might be due to a relieving of transcriptional repression by those two regulators. Wang and Wood determined that IS5 element insertion into the *flhD* promoter of BW25311 increased both motility and the bacteria's ability to form biofilm (Wang and Wood, 2011). In both these previous reports (Barker, Prüß, and Matsumura, 2004; Wang and Wood, 2011), IS5 inserted into the same 4-bp target site (5'-TTAA-3') at 96–99 bp upstream of the transcription start of *flhD,* causing a strain with originally poor motility to become more motile. A third group led by C. Park in South Korea found IS elements further upstream of the transcriptional start for *flhD* (Lee and Park, 2013) after selecting for motility in a way similar to Barker et al. (Barker, Prüß, and Matsumura, 2004). The precise insertion spots were at -315 for an IS5, -303 for an IS1, and -166 for another IS5 (Lee and Park, 2013). This was done with the non-motile version of MG1655.

The above-described insertions of IS elements in the *flhD* promoters of different genetic *E. coli* backgrounds have in common that they cause an increase in the expression of FlhD/FlhC and motility. However, they were also selected on motility plates, where an increase in motility constitutes a definite advantage. The Wood group determined that their IS5 insertion also occurred in biofilm, creating biodiversity within the biofilm. They concluded that the bacteria may be able to sense whether motility is

an advantage or a disadvantage before they undergo mutagenesis, calling this process 'Quasi-Lamarckian' (Wang and Wood, 2011).

This raises the intriguing question whether it might be possible to obtain IS element insertions in the *flhD* promoter that will reduce motility under conditions where motility is a disadvantage. In addition to this question, we have two hypotheses. The first one is on evolution, where we hypothesize that the biofilm may contain such motility counter-selecting niches. The second hypothesis is on gene expression and builds upon the hypothesis from Barker et al. (Barker, Prüß, and Matsumura, 2004) that the IS elements increased *flhD* expression and motility by relieving the effect of some of the negative regulators. As two examples, our study will test the effect of IS element insertion on regulation of *flhD* expression by OmpR and RcsB.

The question whether it is possible for IS elements in the *flhD* promoter to reduce motility was answered by the isolation of 62 non-motile isolates from MC1000 biofilm. This is also in support of our first hypothesis that the biofilm may contain niches where motility is a disadvantage. With the second hypothesis in mind, we constructed fusions of two different *flhD* promoters to the open reading frame of green fluorescence protein (*gfp)*. These are the *flhD1* promoter from AJW678 that does not contain an IS element and the *flhD4* promoter from JS58 which contains the IS5 from its parent MC1000 and an additional IS1 element at -5 bp to the transcriptional start. JS58 is one of the 62 non-motile isolates from MC1000 biofilm. Gene expression from *flhD1* and *flhD4* was determined in biofilm of the parental AJW678 and isogenic *ompR* and *rcsB* mutants. The Discussion compares these gene expression patterns with the previously published gene expression pattern of the *flhD3* promoter from MC1000.

Results

Recovery of isolates from MC1000 biofilm

Individual isolates were recovered from biofilms of the *E. coli* K-12 strain MC1000 after 7 days and 14 days. Of the 368 colonies that were recovered after 7 days, 4 were non-motile and 9 exhibited partial motility. After 14 days of incubation, 58 of the 1,217 isolates were non-motile, and an additional 14 were partially motile. Altogether, we recovered 85 reduced-motility (62 non-motile plus 23 partially motile) colonies from the highly motile MC1000 strain. These isolates were designated JS17 through JS101 and maintained as freezer stock at -80^{o}C. Table 3 summarizes the results from all experiments that were performed with these isolates.

IS1 elements in the *flhD* operon were discovered in 27 of 62 non-motile isolates

Complementation with the *flhD*-expressing plasmid pXL27 was performed to test for the presence of mutations in the *flhD* operon (Table 1, pXL27 compl. column). This was done with all non-motile isolates. Of the 62 isolates tested, 54 complemented to full MC1000 motility. This indicates the presence of mutations within the *flhD* operon. Interestingly, 8 non-motile isolates only complemented to partial MC1000 motility. These may have acquired mutations in flagellar genes other than *flhD* and were not further characterized as part of this study.

PCR1 and PCR2 (Figure 17; Table 3, entire PCR1/PCR2 block of columns; (Barker, Prüß, and Matsumura, 2004) were performed with all partially motile and non-motile isolates. All partially motile isolates yielded PCR products that were identical in length to those of the MC1000 parent strain.

Table 3: Summary of phenotypes for colonies that were recovered from biofilms of MC1000.

Incubation	# of col.	Motility[1]	Colony designations[2]	pXL27 compl.[3]	PCR1/PCR2[4]				PCR3[5]
					Partially Motile	Non-Motile			
						Category I	Category II	Category III	
7d	368	4 NM (1%)	JS28- JS31	4 col. PM		3 col. with parental PCR fragments	1 col. with mutation in primer binding site		ND
		9 PM (2.4%)	JS19-JS27	ND	9 col. with parental PCR fragments				ND
14 d	1,217	58 NM (4.8%)	JS18, JS42-79, JS82-JS100	54 col. FM 4 col. PM		21 col. with parental PCR fragments	8 col. with mutations in primer binding site	29 col. with 800 bp extended PCR fragments	5 col. Rev orient, 22 col. Fwd orient, 2 col. no PCR3
		14 PM (1.1%)	S17, JS32-JS41, JS80, JS81, JS101	ND	14 col. with parental PCR fragments				ND

[1]Motility was determined on motility plates: NM, the colony was completely non-motile; PM, the colony exhibited partial motility relative to its parent strain. [2]All 85 colonies that exhibited a motility phenotype that was different from the respective parent were given JS designations. [3] Non-motile colonies from MC1000 biofilms were tested for complementation with pXL27: FM, at least 7 of 8 transformants exhibited the full motility of the parent; PM, fewer than 7 of 8 transformants were fully motile or all colonies were partially motile. ND, not determined. [4]All 85 colonies were subjected to PCR1 and PCR2 to test for insertions/deletions within the *flhD* operon. [5]The 29 colonies from category III were subjected to PCR3 to identify IS1 insertions.

The non-motile isolates were divided into three categories. Category I contained 24 isolates that produced PCR products that were of identical length as those produced by MC1000. Category II contained 9 isolates that failed to produce a PCR product in one of the two reactions. Category III consisted of 29 isolates that produced PCR products that were larger than those of MC1000 in both reactions. These category III isolates were investigated further.

Among the 29 category III isolates, 27 produced PCR fragments of 2 and 3.3 kb in PCR1 and PCR2, respectively. Examples of this group of isolates are JS44, JS58, and JS90 (Figure 17, Panel B). This combination of PCR products is indicative of an insertion of approximately 800 bp within the part of the *flhD* operon that gets amplified by PCR1. The remaining two isolates in this category, JS43 and JS70, produced PCR products that were even larger than those of JS44 (Fig 17, Panel B).

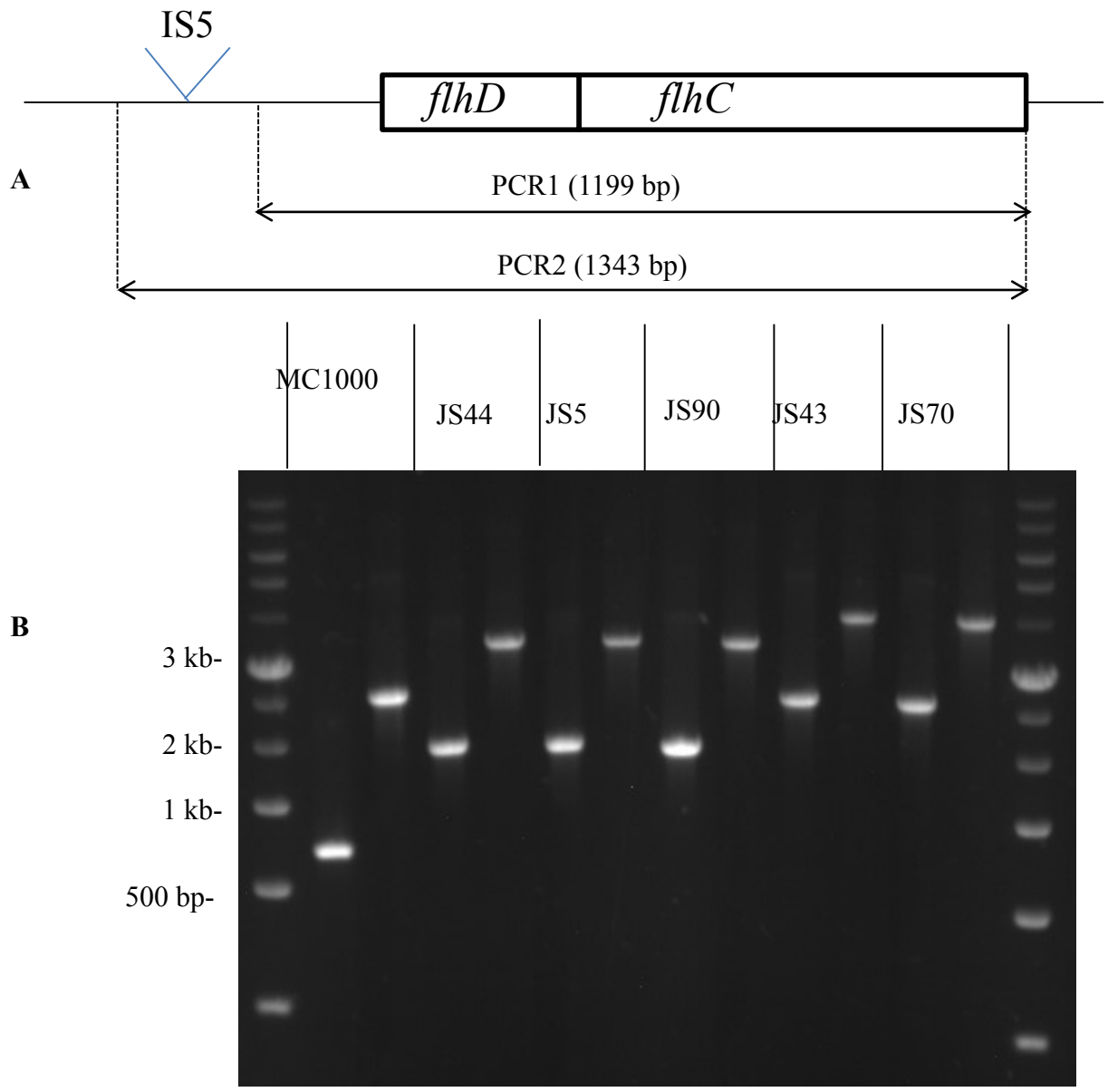

Figure 17: PCR1 and PCR2.
Panel A details the flhD operon and the two PCR reactions (Barker, Prüß, and Matsumura, 2004). PCR1 is expected to yield a 1,199 bp product, and PCR2 is expected to yield a 1,344 bp product in the absence of insertions or deletions. FP1 stands for Forward Primer for PCR1, FP2 stands for Forward Primer for PCR2, and RP stands for Reverse Primer for both PCR1 and PCR2. Panel B contains the 1 kb ladder (Amresco, Solon OH) and 12 lanes of PCR products: lanes 1 & 2, MC1000; lanes 3 & 4, JS44; lanes 5 & 6, JS58; lanes 7 & 8, JS90; lanes 9 & 10, JS43; lanes 11 & 12, JS70. Odd numbered lanes show products of PCR1, and even numbered lanes show those of PCR2.

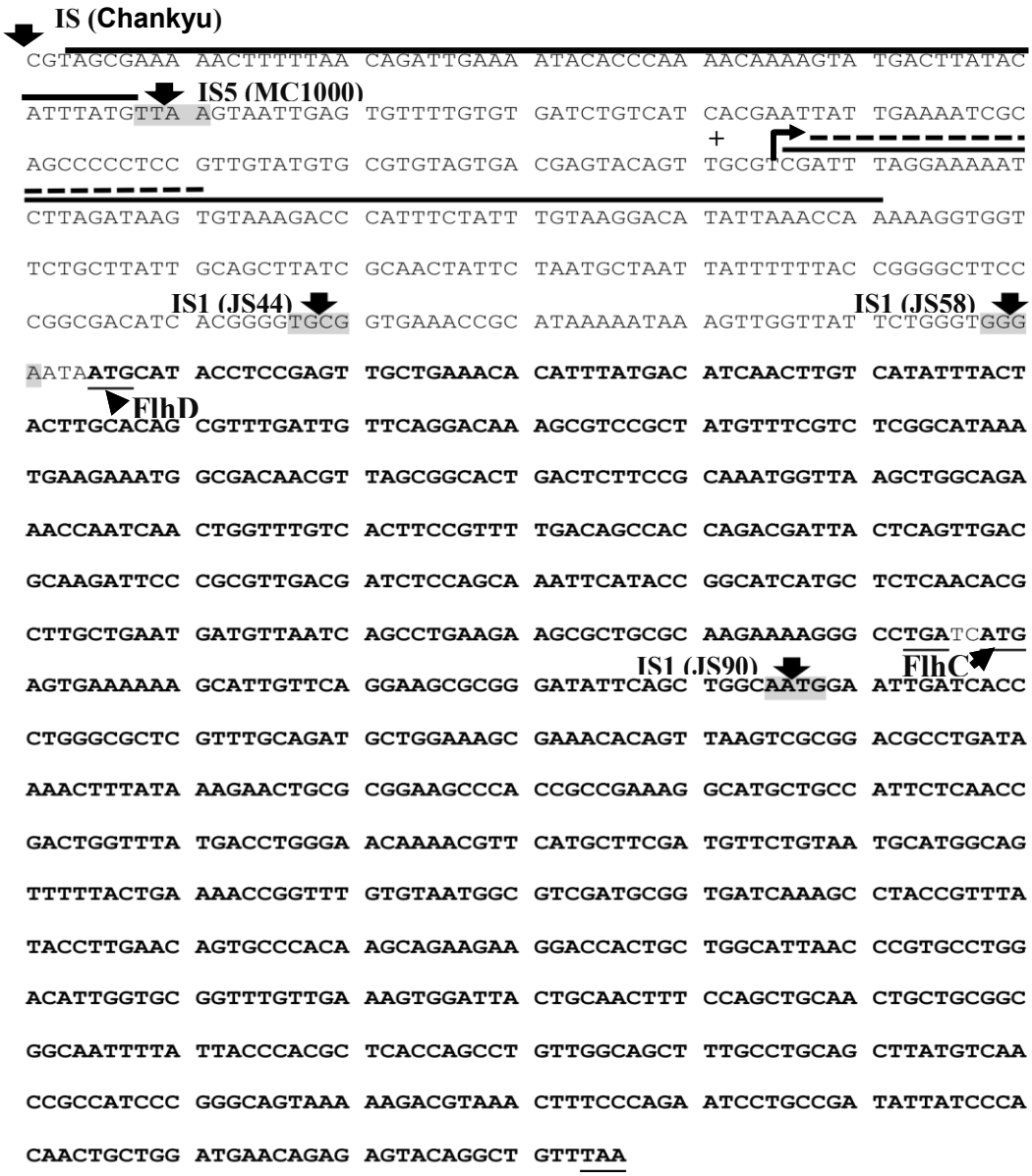

Figure 18: Sequence analysis of the *flhD* operons of MC1000, JS44, JS58, and JS90.

Open reading frames for FlhD and FlhC are marked in bold, and start and stop codons are underlined. The horizontal arrow marks the transcriptional start. Insertion sites of IS elements are highlighted in grey for IS5 from MC1000 (TTAA), IS1 from JS44 (TGCG), IS1 from JS58 (GGGA), and IS1 from JS90 (AATG). Vertical arrows mark the precise insertion sites. The IS5 that was described by C. Park has its insertion spot right before the start of the presented sequence (Lee and Park, 2013). Solid lines above the sequence indicate the two binding sites for OmpR-P. The dashed line above the sequence marks the binding site for RcsAB.

The *flhD* operons of JS44, JS58, and JS90 were sequenced. JS44 contained an IS1 element 46 bp upstream of the ATG start codon of FlhD (Figure 18). This IS1 is in the reverse orientation (Figure 19). JS58 contained an IS1 element at 5 bp upstream of the ATG start codon of FlhD (Figure 18). This IS1 is also in the reverse orientation (Figure 19). This IS1 is flanked by a string of base pairs (AATAATG) on the upstream site which constitutes a duplication of the 7 bp downstream of the IS1. This duplication does not interrupt the ATG start codon of FlhD or its open reading frame. JS90 contained an IS1 in the open reading frame for FlhC, 49 bp downstream of the ATG (Figure 18). This IS1 is in the forward orientation (Figure 19).

To determine the presence of IS1 elements in the remaining 27 non-motile isolates from category III, we performed PCR3 (Table 3, PCR3 column). We found three more isolates (JS61, JS75, and JS92) that had IS1 in the reverse orientation and at the approximate same position as JS44 and JS58. 21 isolates had IS1 in the forward orientation. Examples for these two groups of insertions are included in Figure 19. JS51 was the only isolate in the third group and had the IS1 inserted close to the JS44/JS58 insertion spot, but in the forward orientation. The two isolates that had produced PCR products larger than 2 and 3.3 kb in PCR1 and PCR2, JS43 and JS70, failed to produce a PCR3 product.

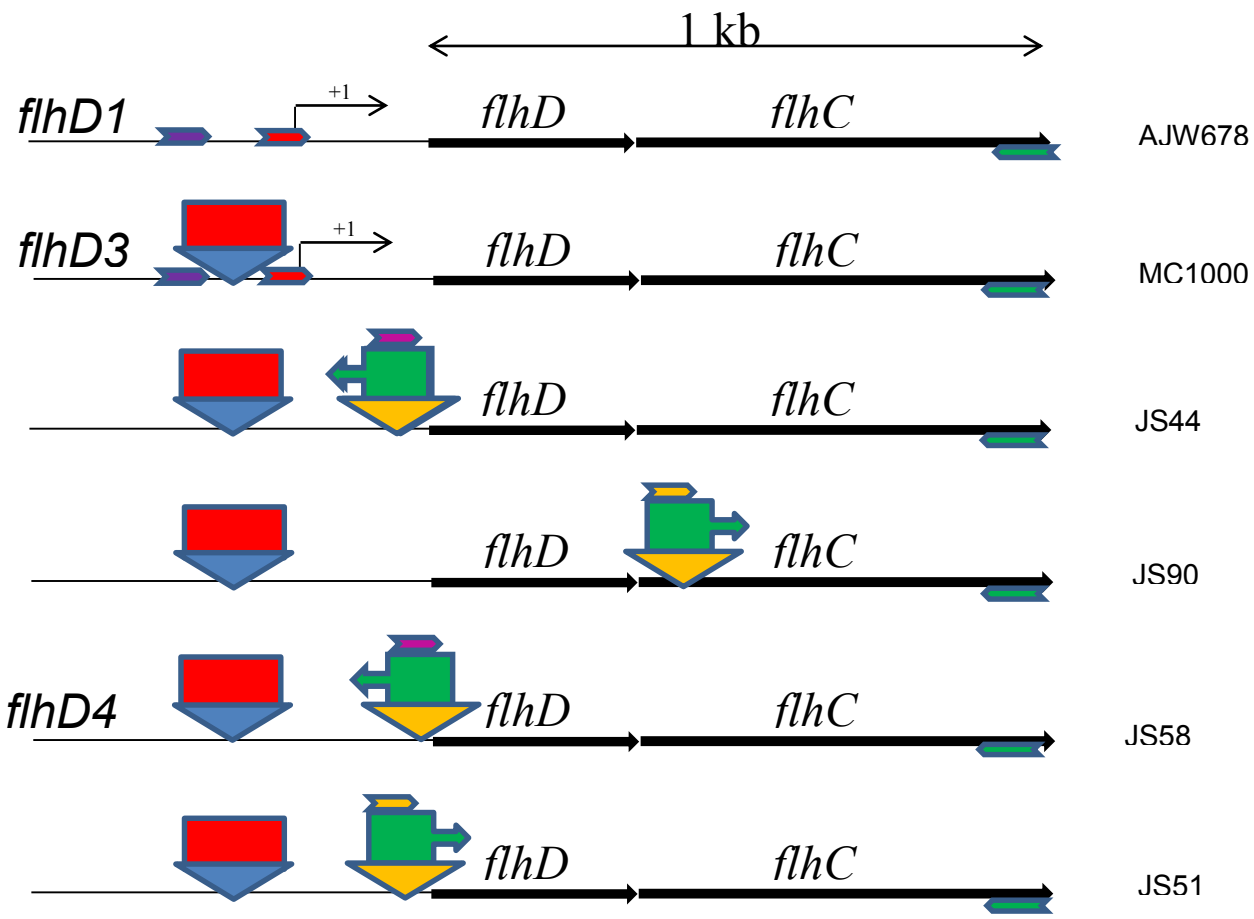

Figure 19: Outcome of PCR3.
IS5 elements, IS1 elements, and primers are indicated as follows:

IS5 element of MC1000 (1,195 bp)

IS1 elements of non-motile MC1000 isolates (768 bp)

Reverse primer for all PCR reactions

Forward primer 3A (indicative of an IS1 element in negative orientation)

Forward primer 3B (indicative of an IS1 element in the positive orientation)

Forward primer 1 (indicated only for AJW678 and MC1000)

Forward primer 2 (indicated only for AJW678 and MC1000)

Transcriptional start.

Gene expression from *flhD1* was higher in *ompR* and *rcsB* mutants than in the parent strain

To test whether mutations in *ompR* and/or *rcsB* would increase expression from the *flhD1* promoter (no IS), we performed fluorescence microscopy on flow-cell-grown biofilms of BP1506 (AJW678 *flhD1*::*gfp*), BP1507 (AJW678 *ompR*::*Tn10 flhD1*::*gfp*), and BP1510 (AJW678 *rcsB*::*Tn5 flhD1*::*gfp*). The images for all these strains are presented in Figure 20, and the quantitative analysis of the images is explained below.

For the *flhD1* promoter, temporal gene expression in the parental strain (BP1506) peaked at 12 h and increased again towards 52 h (Figure 21A, blue line). In the *ompR* and *rcsB* mutant strains (BP1507 and BP1510), expression from the *flhD1* promoter was constitutively high throughout the experiment after a primary increase during the initial time period of biofilm formation (Figure 21A, red and black lines). As a consequence, the spatial gene expression experiment for *flhD1* in all three strains was performed on 52 h biofilms.

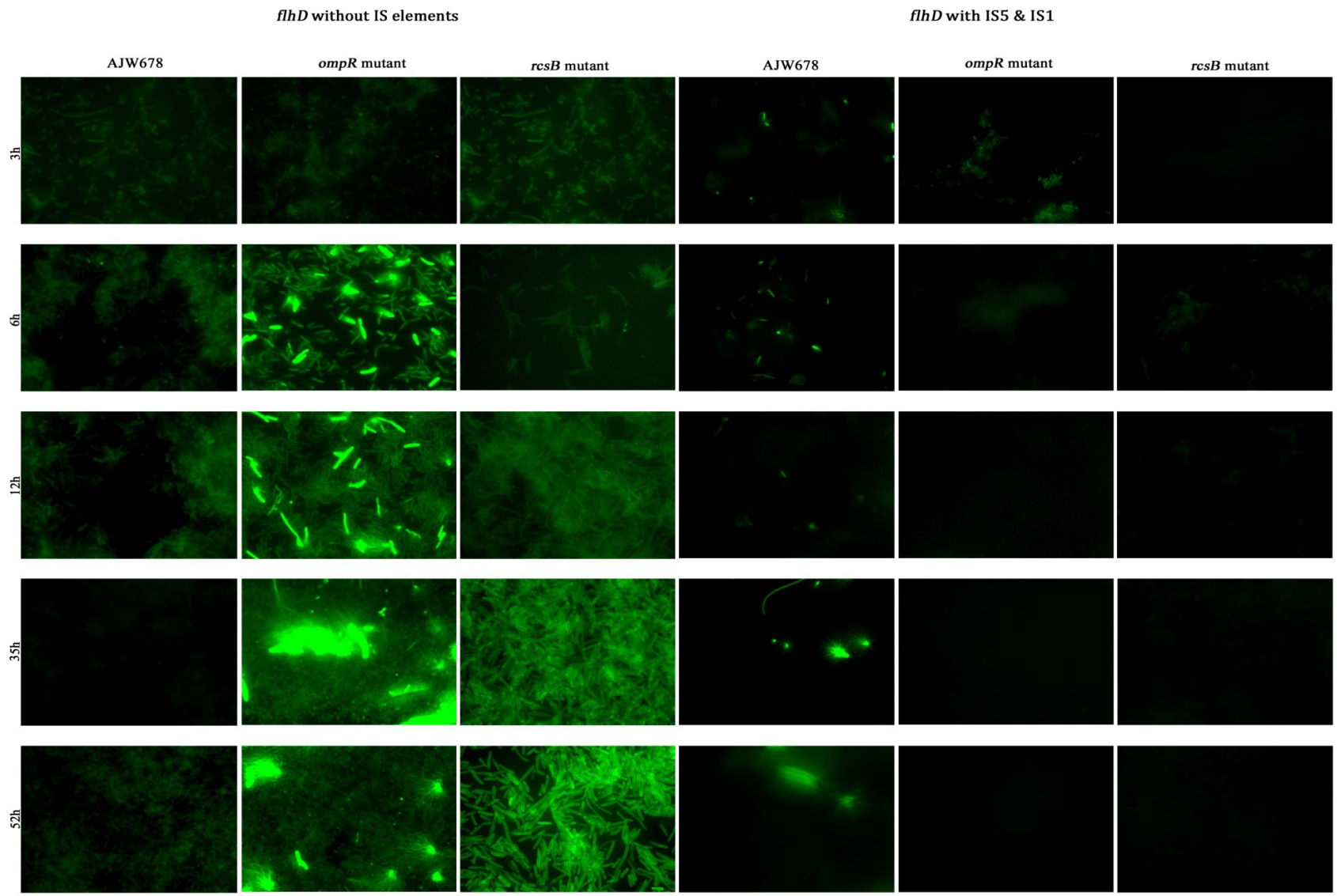

Figure 20: Fluorescence images of *flhD1::gfp (left), and flhD4::gfp (right)* in AJW678 and *ompR* and *rcsB* mutants. Biofilms were grown in flow cells and subjected to fluorescence microscopy. Images were taken at 1,000x magnification.

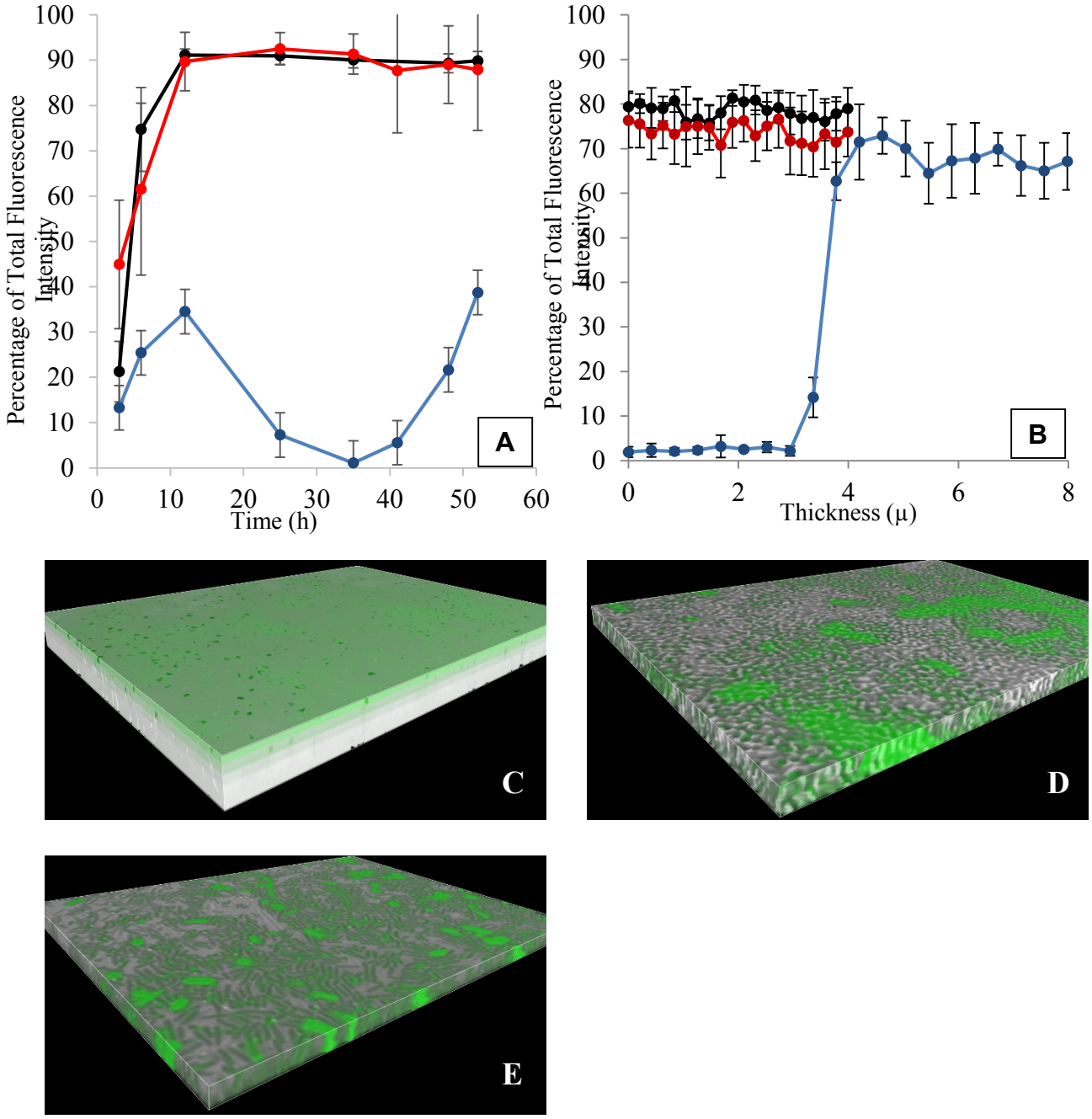

Figure 21: Temporal and spatial gene expression of *flhD1* in the parent strain and isogenic *ompR* and *rcsB* mutant strains.
A and B are the quantitative representation of the temporal and spatial gene expression of *flhD1*, respectively. Blue lines indicate *flhD1* expression in AJW678. The black and red lines represent *flhD1* expression in ompR and rcsB mutants, respectively. C, D and E are the 3D images constructed from the z-stacked images at 51 hours for BP1506, BP1507 and BP1510, respectively.

At 52 h, the expression of *flhD1* was highest at the outer layer of the AJW678 biofilms (Figure 21B, blue line and Figure 21C). The total pixel values from the individual images of the *z*-stacks showed that until 3 μm from the surface, the expression of *flhD1* was very low; then the expression jumped to approximately 70% of the total area of the images at the top layers of biofilms. Expression of *flhD1* in both mutants was high across all bacteria that were part of this biofilm, and fluorescence was around 75 to 80 % coverage across the entire biofilm of both mutants (Figure 21B, D, and E). The 3D reconstructions of the biofilms also showed that the biofilm from both mutants was no more than 4 μm, as opposed to ~8 μm for the parent strain.

Gene expression from *flhD4* was low in all tested strains

To test whether mutations in *ompR* and/or *rcsB* would increase expression from the *flhD4* promoter, the temporal gene expression experiment was performed with the *flhD4* promoter that contained both the IS5 that comes from the parent strain and the additional IS1 that JS58 had acquired during the experiment (strains BP1503, BP1504, and BP1513). Expression was very low throughout the 52 h of the experiment in the parental strain (Figure 22). Expression from this *flhD* promoter was not increased in the *ompR* and/or *rcsB* mutants. In all three strains, the fluorescence was less than 5% coverage across the entire biofilm throughout the 52 h flow cell experiment. As a consequence, we did not perform the spatial experiment. Differences in biofilm thickness between the parent and the two mutant strains were similar to those observed with the *flhD1* promoter.

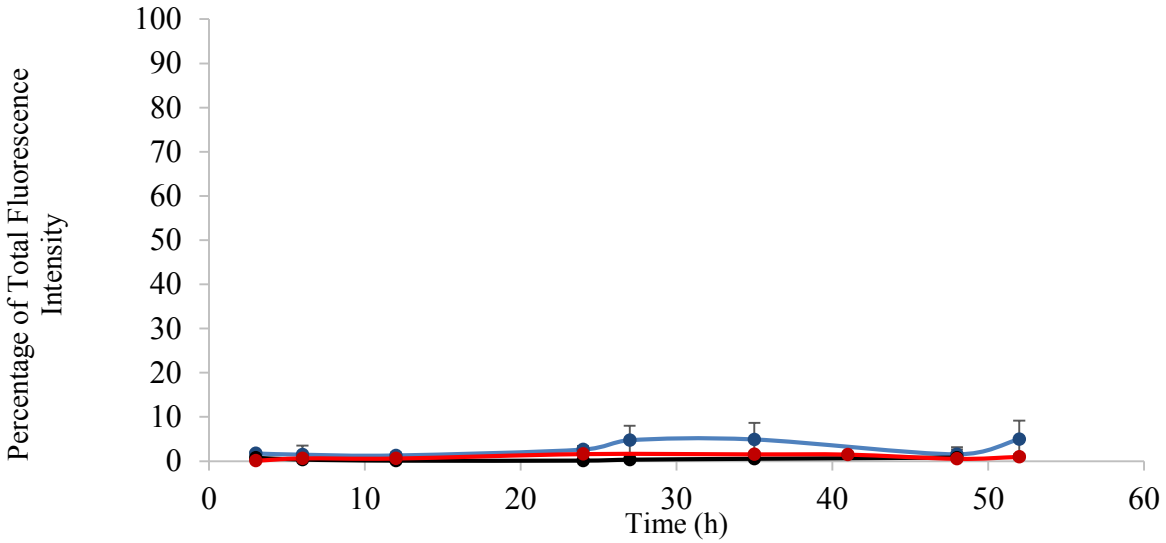

Figure 22: Temporal gene expression of *flhD4* in the parent strain and isogenic *ompR* and *rcsB* mutant strains.
This is the quantification of temporal gene expression of *flhD4*. Blue lines represent *flhD4* expression in AJW678. The black and red lines represent *flhD4* expression in the *ompR* and *rcsB* mutants, respectively.

A double mutant in *ompR and rcsB* produced much reduced biofilm amounts

Since single mutations in both *ompR* and *rcsB* showed highly increased *flhD1* expression, accompanied by reduced biofilm amounts, we wanted to test the effect of an *ompR rcsB* double mutant (BP1515). This was done with the temporal gene expression experiment, where expression was measured from the *flhD1* promoter (no IS). The double mutant produced almost no biofilm in the flow cell, so we were unable to measure expression from *flhD1*.

To confirm the inability of the *ompR rcsB* double mutant to form a biofilm, we quantified amounts of a static biofilm (Figure 23). The CV assay was performed with biofilm formed by the parental strain, the *ompR* mutant, the *rcsB* mutant, and the *ompR rcsB* double mutant (BP1506, BP1507, BP1510 and BP1515, respectively). Both single

mutant strains produced lower amounts of biofilm than their isogenic parental strain (60 - 70% of parental strain). The double-mutant strain produced only about 25% biofilm biomass compared to the parent on static biofilm.

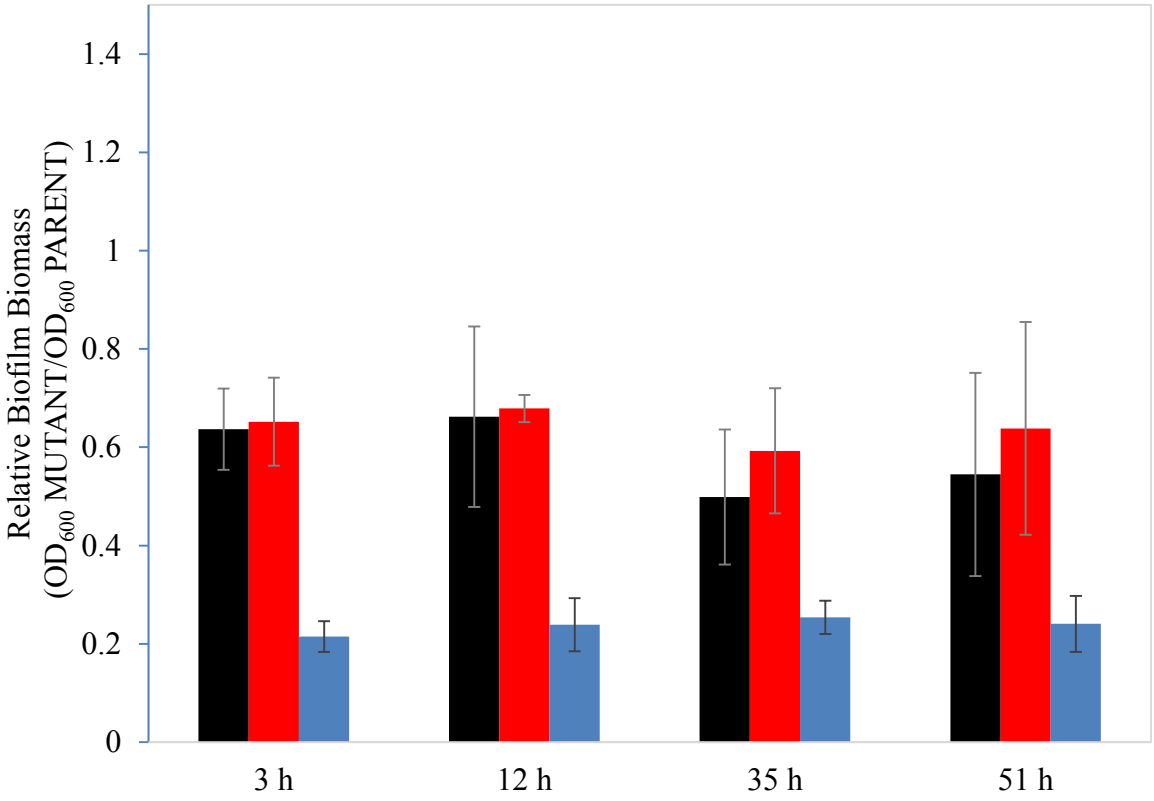

Figure 23: Biofilm amounts in single and double mutants of *ompR* and *rcsB*.
The biofilm biomasses of the *ompR* mutant (BP1507), the *rcsB* mutant (BP1510) and *ompR-rcsB* double-mutant strains (BP1515) were measured and compared to the parental strain (BP1506) at four different time points. The black bars are the relative biomass of BP1507, the red bars are the relative biomass of BP1510, and the blue bars are the relative biomass of BP1515. To determine relative biomass, the OD_{600} value for each individual strain was divided by those of the parent. The experiment was performed 3 times, and both average and standard deviations are presented.

Discussion

This study was started with a question and two hypotheses. The question was whether it is possible to obtain insertions of IS elements in the *flhD* promoter that render the strain non-motile under conditions where motility might be a disadvantage. By all appearances, this is the case. We isolated a total of 62 non-motile colonies from *E. coli* MC1000 biofilm. The fact that the mutations fall into different categories indicates that they are not all derived from the same mutational event, besides the fact that all were not from the same biofilm. Specifically, JS44 has an IS1 at +160 from the transcriptional start and 46 bp upstream of the ATG for the FlhD open reading frame. The IS1 in JS58 is even closer to the ATG at 5 bp upstream. Both these IS1 elements are within the un-translated region of the mRNA transcript, where they might interfere with the binding of the ribosome. The IS1 element in JS90 is located within the open reading frame of FlhC, rendering the strain non-motile due to the truncation of FlhC. Altogether, these three insertions are further downstream in the *flhD* operon than those previously described (Barker, Prüß, and Matsumura, 2004; Wang and Wood, 2011; Lee and Park, 2013). By all appearances, the precise insertion spot of the IS element determines whether the IS has a positive or negative effect on *flhD* expression, flagella synthesis, and motility.

The first hypothesis of this study was that a mature biofilm contains many different ecological niches. In some of these niches, motility may be a disadvantage. The fact that we were able to recover 62 non-motile colonies from *E. coli* biofilm is in agreement with this hypothesis. Furthermore, the percentage of non-motile isolates (relative to all tested colonies from that sample) was higher for 14-day-old biofilm (4.8%)

than for 7-day-old biofilm (1%). While the sample size may not have been high enough for proper statistical analysis, this is some evidence that there may be increasing selective pressure towards non-motility on a small fraction of the bacteria as the biofilm matures.

These observations feed into ongoing discussions whether motility constitutes an advantage or disadvantage during biofilm formation. Flagella are the attachment tool that permits reversible attachment, the first phase of biofilm formation (Sauer *et al.*, 2002). During irreversible attachment, the second phase of biofilm development, flagella are believed to be absent. During maturation, there may be a need for some flagellation, as flagella may be key architectural elements to construct and reinforce the biofilm superstructure (Serra *et al.*, 2013). In agreement with this, flagella and motility are considered a requirement for biofilm formation by many bacteria, including *Pseudomonas aeruginosa* (Shrout *et al.*, 2011; Toutain *et al.*, 2007), *Aeromonas* spp. (Kirov, Castrisios, and Shaw, 2004), and *Vibrio parahaemolyticus* (Enos-Berlage *et al.*, 2005). In contradiction, research by our own lab indicates that FlhD/FlhC may constitute a disadvantage during *Escherichia coli* biofilm formation under certain conditions. First, the highly motile MC1000 is a poor former of biofilm (Prüß *et al.*, 2010), whereas AJW678 is less motile than MC1000, but forms ample amounts of biofilm (Wolfe *et al.*, 2003). Second, a *flhC* mutation enabled a strain of *E. coli* O157:H7 to form biofilm, whereas the motile parent strain was almost unable to produce biofilm (Sule *et al.*, 2011). This is a clear contradiction to a study of seven non-O157:H7 shiga toxin-producing strains where five of the strains that did not form a biofilm were also non-motile (Chen *et al.*, 2013).

Environmental conditions that have been investigated in a previous study (Prüß *et al.*, 2010) can't be held responsible for the entirety of these apparent contradictions. We believe that the question whether motility is an advantage or a disadvantage for biofilm-bound bacteria may depend on the degree of maturation of the biofilm and the location of the individual bacterium within the biofilm. This belief was also the reason for the previous study (Samanta *et al.*, 2013) on temporal and spatial gene expression, where we used the *flhD3* promoter from MC1000 that contains the IS5 at -99 to -96. Temporal expression of *flhD3* exhibited a similar profile to the one that was described in this study for the *flhD1* promoter (no IS) from AJW678, namely a peak at 12 h and a second increase towards 52 h. This is in agreement with the idea that flagella are needed for reversible attachment (12 h peak), not needed for irreversible attachment (25 to 45 h), and then contribute to the architecture of the mature biofilm (52 h increase). Also, expression from both *flhD3* and *flhD1* was spatially dependent in a way that only bacteria at the outermost layer of the biofilm expressed FlhD/FlhC. Altogether, data presented in this study are in agreement with a hypothesis where motility can be both an advantage and a disadvantage and that the biofilm may be best served by a mixture of both motile and non-motile bacteria.

The purpose of the temporal and spatial *flhD* expression experiment was to address our second hypothesis. Based upon previous studies (Barker, Prüß, and Matsumura, 2004; Wang and Wood, 2011; Lee and Park, 2013), we postulated that the high motility in *E. coli* strains that carry an IS element at -99 to -96 region (or further upstream) from the transcriptional start of the *flhD* operon might be due to a relieving of transcriptional repression by one or several of the negative regulators that bind in that

region. If this hypothesis was true, *flhD* expression should be higher from the IS5-containing *flhD3* promoter (Samanta *et al.*, 2013) than for the *flhD1* promoter that does not contain an IS element (Figures 22 and 23). Also, knock-out mutations in *ompR* or *rcsB* should increase *flhD* expression to the same level, regardless of the presence or absence of the IS5.

However, the expression patterns for the two promoters looked rather similar in the parent strain, with expression from *flhD3* being only slightly higher than from *flhD1*. Knock-out mutations in either *ompR* or *rcsB* increased expression from both promoters. By first view, the increase seemed rather similar. However, the fluorescence data for the *flhD1* experiment (this study) were taken at 90% of the available excitation light for all three strains. In contrast, *flhD3* data in the previous study were obtained at 90% for the parent and 10% for the two mutants. This was done because of the large fluorescence signals obtained from the *flhD3* promoter in the mutants. This indicates that the expression increase caused by the *ompR* and *rcsB* mutations was larger for *flhD3* than for *flhD1*. It appears as though regulation of *flhD* by OmpR and RcsB is stronger in the presence of the IS5 element than in its absence, which is not in agreement with our hypothesis. It is possible that the IS5 increases *flhD* expression by a mechanism other than relieving repression by OmpR and RcsB. It is also possible that the biofilm environment, where many bacteria are in a dormant state (Kwan *et al.*, 2013), is sufficiently different from the planktonic state that regulation is mechanistically different.

The final *flhD* promoter that was investigated in this study was the *flhD4* promoter from JS58 that contained an IS1 inserted right before the translational start of FlhD in addition to the IS5 at -99 to -96 from MC1000. This promoter was unable to

demonstrate any detectable expression and we believe that this IS might interfere with ribosome binding due to its close proximity to the start of the open reading frame.

Conclusions and Outlook

The biofilm environment constitutes many environmentally different ecological niches, which expose biofilm-associated bacteria to evolutionary events. In addition, their genes undergo precise regulation of expression. This study attempted to combine investigations of these two important mechanisms of adaptation, evolution and gene expression. We were able to recover non-motile isolates from mature biofilms that contained IS elements in their *flhD* promoters further downstream than all previously reported IS elements that were found in *flhD* promoters (Barker, Prüß, and Matsumura, 2004; Wang and Wood, 2011; Lee and Park, 2013). Our non-motile isolates may have evolved in niches where motility may be a disadvantage, whereas the hyper-motility isolates from T. Wood's group (Wang and Wood, 2011) may have been from niches where motility was an advantage. This is in agreement with our observations from the gene expression experiments, where *flhD* expression was highest at the outermost edge of the biofilm and also increased towards maturation.

Methods

Bacterial strains

Bacterial strains used for this study are listed in Table 4. MC1000 is a highly motile (5.9 mm/h on motility plates (Prüß *et al.*, 2010)) *E. coli* K-12 strain. The high motility is due to the presence of an IS5 element in its *flhD* promoter (Barker, Prüß, and Matsumura, 2004). The strain forms biofilm poorly (Prüß *et al.*, 2010). All JS17-101 isolates (Table 3) were recovered as non-motile or poorly-motile colonies from MC1000 biofilm. AJW678 is a good biofilm-forming *E. coli* K-12 strain (Kumari *et al.*, 2000), but lacking an IS element in its *flhD* promoter makes the strain less motile than MC1000 (1.9 mm/h on motility plates Prüß *et al.*, 2010). AJW2050 contains an *ompR::Tn10* (Wolfe *et al.*, 2003) and AJW2143 an *rcsB::Tn5* (Wolfe *et al.*, 2003). BP1240 contains both *ompR*::Tn*10* and *rcsB*::Tn5 and was produced in this study by means of P1 transduction, using AJW2050 as the donor and AJW2143 as the recipient. All bacterial strains were maintained as freezer stocks at -80^{o}C and streaked onto Luria Bertani (LB; 1% tryptone, 0.5% NaCl, 0.5% yeast extract) agar plates prior to the experiment.

Biofilm formation and colony recovery

Biofilms were grown from MC1000 in separate wells of a 6-well plate in LB at 32^{o}C. After 7 and 14 days, liquid growth media was removed and biofilms were washed twice with PBS. Bacteria were suspended in PBS, serially diluted, and plated on LB agar plates. Several hundred isolated colonies were picked from these plates for each strain and screened for motility. These are referred to as isolates throughout this study.

Table 4: Bacterial Strains.

Bacterial Strains		
AJW678	*thi-1 thr-1(am) leuB6 metF159(Am) rpsL136 ΔlaxX74*	(Kumari *et al.*, 2000)
MC1000	F-, *araD*139 Δ(*araAB leu*)7696 Δ(*lacX74*) *galU galK strA prsL thi*	(Casadaban and Cohen, 1980)
JS17-101	derived from the parental MC1000	This study
AJW2050	AJW678 *ompR::Tn10*	(Fredericks *et al.*, 2006)
AJW2143	AJW678 *rcsB::Tn 5*	(Fredericks *et al.*, 2006)
BP1240	AJW678 *ompR::Tn10 rcsB::Tn 5*	This study
BP1506	AJW678 pPS72 (*flhD1*::*gfp*) kn^R	This study
BP1507	AJW2050 pPS72 (*flhD1*::*gfp*) kn^R	This study
BP1510	AJW2143 pPS75 (*flhD1*::*gfp*) tc^R	This study
BP1503	AJW678 pPS74 (*flhD4*::*gfp*) kn^R	This study
BP1504	AJW2050 pPS74 (*flhD4*::*gfp*) kn^R	This study
BP1513	AJW2143 pPS76 (*flhD4*::*gfp*) tc^R	This study
BP1515	BP1240 pPS77 (*flhD1*::*gfp*) cm^R	This study
Plasmids		
pPS72	pUA66 *flhD1* no IS*::gfp* kn^R	This study
pPS74	pUA66 *flhD4* with IS1+IS5*::gfp* kn^R	This study
pPS75	pPS72 tc^R	This study
pPS76	pPS74 tc^R	This study
pPS77	pPS72 cm^R	This study

Determination of motility phenotypes

Motility testing was done on semi-solid agar plates made from tryptone broth (TB; 1% tryptone, 0.5% NaCl) and 0.3% agar (Wolfe and Berg, 1989). Plates were incubated for 4-8 h at 32^{o}C in a humid environment. The diameters of the swarm rings were compared between the MC1000 and its derivative isolates. Isolates that had a different motility phenotype than MC1000 were maintained as freezer stocks and are designated JS17 through JS101. Motility phenotypes of JS17 through JS101 were confirmed by testing the respective colony on motility plates two more times.

Complementation of the motility phenotype with pXL27

All non-motile isolates were transformed with plasmid pXL27 expressing *flhD* and *flhC* (Liu and Matsumura, 1994) to test for complementation of the motility phenotype. Plasmid pXL27 was moved into the respective bacterial isolates via chemical transformation, taking advantage of the penicillin resistance gene on the plasmid as a selective marker. For each non-motile colony, 8 independent transformants were tested on motility plates.

Determination of mutations within the *flhD* operon

Two PCR reactions (PCR1 and PCR2) were performed that were originally designed to detect insertions of IS elements within the *flhD* promoter (Barker, Prüß, and Matsumura, 2004), but could also be used to detect insertions and deletions within the entire *flhD* operon.

The PCR1 fragment (Fig. 17, Panel A) starts downstream of the published (Barker, Prüß, and Matsumura, 2004; Wang and Wood, 2011) hotspot for IS1 and IS5 in the *flhD* promoter and ends at the 3'-end of the *flhC* open reading frame. This fragment is expected to be 1,199 bp in length in the absence of insertions. The PCR2 fragment (Fig. 17, Panel A) starts upstream of the hotspot. PCR2 is expected to yield a 1,343 bp fragment in the absence of IS elements. PCR1 is done with forward primer 1, and PCR2 with forward primer 2 (Table 5). Both reactions use the same reverse primer. A PCR2 fragment that is longer than 1,343 bp, together with a PCR1 fragment of 1,199 bp length, indicates an insertion between the forward primer 1 and the forward primer 2 binding sites. That could be within the published hotspot. Should both PCR fragments be longer than 1,343 bp and 1,199 bp, this would be indicative of an insertion either within the open reading frames for FlhD and FlhC or in the promoter downstream of the forward primer 1. Among the isolates that fall into this category, two isolates (JS44 and JS90) were sequenced using the forward primers 1 and 2 and the reverse primer. With the remaining isolates of this group, PCR3 was performed to test for the presence, approximate location, and orientation of IS1 elements. We used forward primer 3A and 3B together with the reverse primer to detect the two different possible orientations of the IS1. The presence of a PCR product by forward primer 3A is indicative of IS1 in forward orientation. A PCR product yielded by forward primer 3B is indicative of IS1 in reverse orientation. The length of the respective PCR fragment is indicative of the distance between binding sites for the forward and reverse primers.

Table 5: List of primers.

Primer	Sequence	Purpose
PCR primers		
Forward 1	5'-CCCCCTCCGTTGTATGTGCG-3'	For PCR1 (Barker, Prüß, and Matsumura, 2004)
Forward 2	5'-CCTGTTTCATTTTTGCTTGCTAGC-3'	For PCR2 (Barker, Prüß, and Matsumura, 2004)
Reverse	5'-GGAATGTTGCGCCTCACCG-3'	For PCR1/2/3 (Barker, Prüß, and Matsumura, 2004)
Forward 3A	5'-TATGAGCCT GCTGTCACCCTTTGA-3'	For PCR3: Indicative of an IS1 element in the reverse orientation
Forward 3B	5'-TTCAGGTTATGCCGCTCAATTCGC-3'	For PCR3: Indicative of an IS1 element in the forward orientation.
Cloning primers		
flhD1 forward	5'-CTCGAGTGACTGTGCGCAACATCCCATT-3'	Amplification of *flhD* from AJW678
flhD reverse	5'-GGATCCTGCCAGCTTAACCATTTGCGGA-3'	Amplification of *flhD* from AJW678
flhD4 forward	5'-AGATCTTGACTGTGCGCAACATCCCATT-3'	Amplification of *flhD* from JS58

Cloning of *flhD1::gfp* (pPS72), *flhD4::gfp* (pPS74), pPS72 Tc^R (pPS75), pPS74 Tc^R (pPS76) and pPS72 Cm^R (pPS77) plasmids

pPS72: The flhD1 promoter region of AJW678 that starts 219 bp upstream of the +1 transcriptional start site and ends 502 bp downstream of the +1 was PCR-amplified from AJW678, using the flhD1 forward and flhD reverse primers (Table 5). The PCR-amplified region was first cloned into pGEM T-Easy (Promega, Madison, WI), then excised with XhoI and BamHI, and ligated into the respective sites of pUA66 (Open Biosystem, Huntsville, AL) that contains gfpmut2 as a reporter gene and a kanamycin resistance cassette. AJW678 and AJW2050 were transformed with the resulting plasmid, designated pPS72. The insert was confirmed by restriction digest and sequencing (Macrogen, Rockville MD).

pPS74: JS58 is one of the colonies that was recovered from 14-days-old MC1000 biofilm and contained an IS1 element in the flhD operon in addition to the IS5 that is characteristic for MC1000. The flhD4 promoter region of JS58 that starts 1,419 bp upstream of the +1 transcriptional start site and ends 1,101 bp downstream of the +1 was PCR-amplified from JS58, using flhD4 forward and flhD reverse primers. The sequence of the PCR product was confirmed (Macrogen). The PCR fragment was ligated into pGEM T-Easy, excised with ApaI, blunt ended with Klenow, digested with BamHI, and ligated into pUA66 that was treated with the same restriction enzymes. AJW678 and AJW2050 were transformed with the resulting plasmid, designated pPS74.

pPS75, pPS76, and pPS77: pPS72 and pPS74 were digested with EagI, blunt ended with Klenow, and treated with CIAP. This treatment removed 280 bp between the kanamycin cassette and the flhD promoter, abolishing kanamycin resistance. pHP45Ω-

Tc was the source of the tetracycline resistance gene cassette (Fellay, Frey, and Krisch, 1987) and was digested with EcoRI and blunt ended with Klenow. The excised tetracycline resistance cassette was ligated into pPS72 and pPS74. The resulting plasmids are designated pPS75 and pPS76, respectively. AJW2143 was transformed with either of the two plasmids.

pPS72 was digested with EagI, blunt ended with Klenow, and treated with CIAP. As a result, the kanamycin resistance was inactivated. The chloramphenicol resistance cassette was excised from pHP45Ω-Cm (Fellay, Frey, and Krisch, 1987) with EcoRI, blunt ended with Klenow, and ligated into pPS72. BP1290 was transformed with the resulting plasmid, designated pPS77.

Formation of biofilm in flow cells

Biofilms of six strains were formed in flow cells (Stovall, Greensboro NC) as previously described (Samanta *et al.*, 2013). The first set of three strains contained pPS72/pPS75, having the *flhD1* promoter (no IS) from AJW678 fused to *gfp*. These were BP1506 (parent), BP1507 (*ompR*), and BP1510 (*rcsB*). The second set of three strains contained pPS74/pPS76 that had the IS1/IS5-containing *flhD4* promoter from JS58 fused to *gfp*. These were BP1503 (parent), BP1504 (*ompR*), and BP1513 (*rcsB*).

Fluorescence microscopy

Gene expression was monitored as fluorescence from the *flhD*::*gfp* with an Axio Observer Z2 upright fluorescence microscope with ApoTome2 from Zeiss (Germany). For the temporal experiment, fluorescence images were taken at multiple time points until a maximum of 52 h. For the spatial experiments, *z*-stacking images were taken at

52 h. This was done separately for fluorescence and bright field. For both temporal and spatial experiments, images were taken at 1,000 X magnification using a 100 x/1.46 Oil α-Plan-apochromatic objective. All the experiments under the fluorescence microscopy were done at 90% of the available excitation light.

Quantifications of the fluorescence signals were done by using Image-Pro Plus software. Data are expressed as percent area of the image that produced a fluorescence signal. We also determined the average and the standard deviation across all 9 images (3 images per biological replicate) for each individual strain. Finally, the average percentage area was plotted against the time of the temporal experiments and the biofilm thickness of spatial experiments.

Determination of biofilm biomass by crystal violet assay

Biofilm of BP1503, BP1504, BP1513, and BP1515 were grown in individual wells of a 24-well plate in TB for 3 h, 12 h, 35 h, and 51 h at room temperature. After removing the liquid bacterial growth medium, biofilms were washed twice with phosphate-buffered saline (PBS). Biofilms were stained with a solution of 0.1% crystal violet (CV) in H_2O as described (O'Toole *et al.*, 1999; Pratt and Kolter, 1998; Stafslien *et al.*, 2007; Stafslien *et al.*, 2006). After 10 min, CV was removed and biofilms were washed twice with PBS. A solution of 80% ethanol and 20% acetone was used to extract the CV. The OD_{600} was determined from a 1:10 dilution with a Synergy H1 plate reader from BioTek (Winooski, VT). Relative biomass was determined by dividing the OD_{600} data for each strain by the parental strain AJW678. Averages and standard deviations were determined across the three replicate experiments.

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Addendum 1

Sequencing alignment result of the *flhD* promoter from pPS72

The *flhD* promoter sequences from AJW678 and cloned *flhD* promoter sequences pPS72 plasmids were compared in Figure 24. The top and bottom alignment clearly shows there is no difference between those two promoter regions. The ATG site is situated at +199 bp of +1 transcriptional start site. The alignment clearly indicates that there is no IS element present in the *flhD* promoter region.

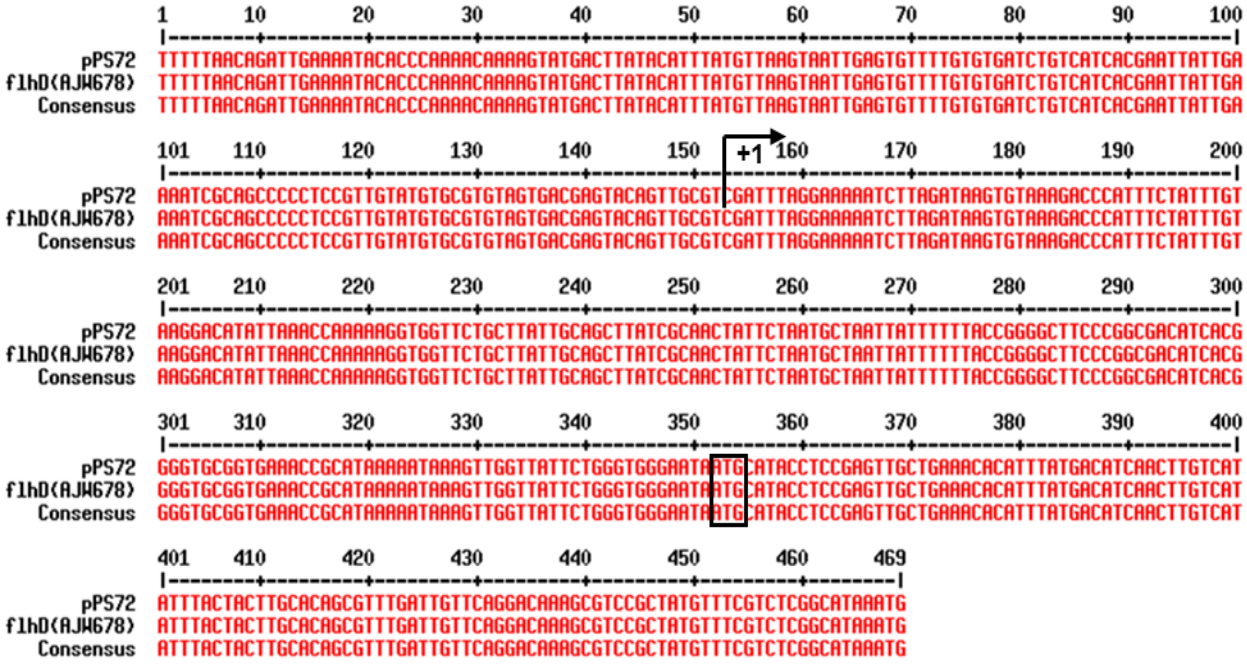

Figure 24: Sequence alignment of *flhD* promoter of pPS72 and *flhD* promoter of AJW678.
The +1 transcriptional start site and the ATG start codon are indicated.

Sequencing alignment result of the *flhD* promoter from pPS74

The *flhD* promoter sequences from AJW678 and cloned *flhD* promoter sequences pPS74 plasmids were compared. The alignment clearly shows the difference between those two promoter regions, due to the IS5 and IS1 element in the *flhD* promoter of pPS74. The IS5 element started at -1294 bp and ended at -94 bp from the +1 transcriptional start sites. The IS1 element started at +194 bp and ended at +970 bp from the +1 transcriptional start sites.

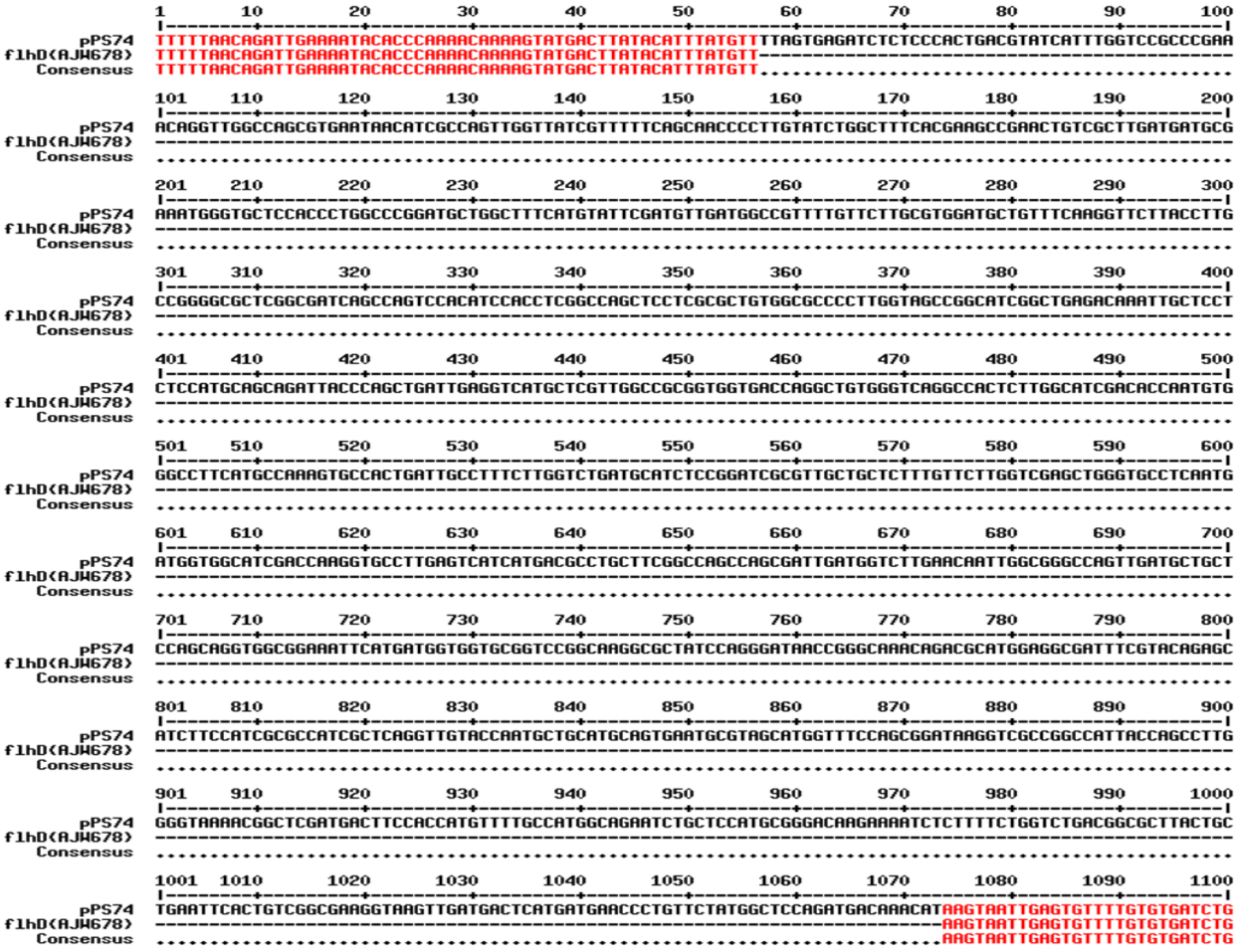

Figure 25*:* Sequence alignment of *flhD* promoter of pPS74 and *flhD* promoter of AJW678.
The +1 transcriptional start site and the ATG start codon are indicated. Both IS5 and IS1 elements are indicated as black sequence.

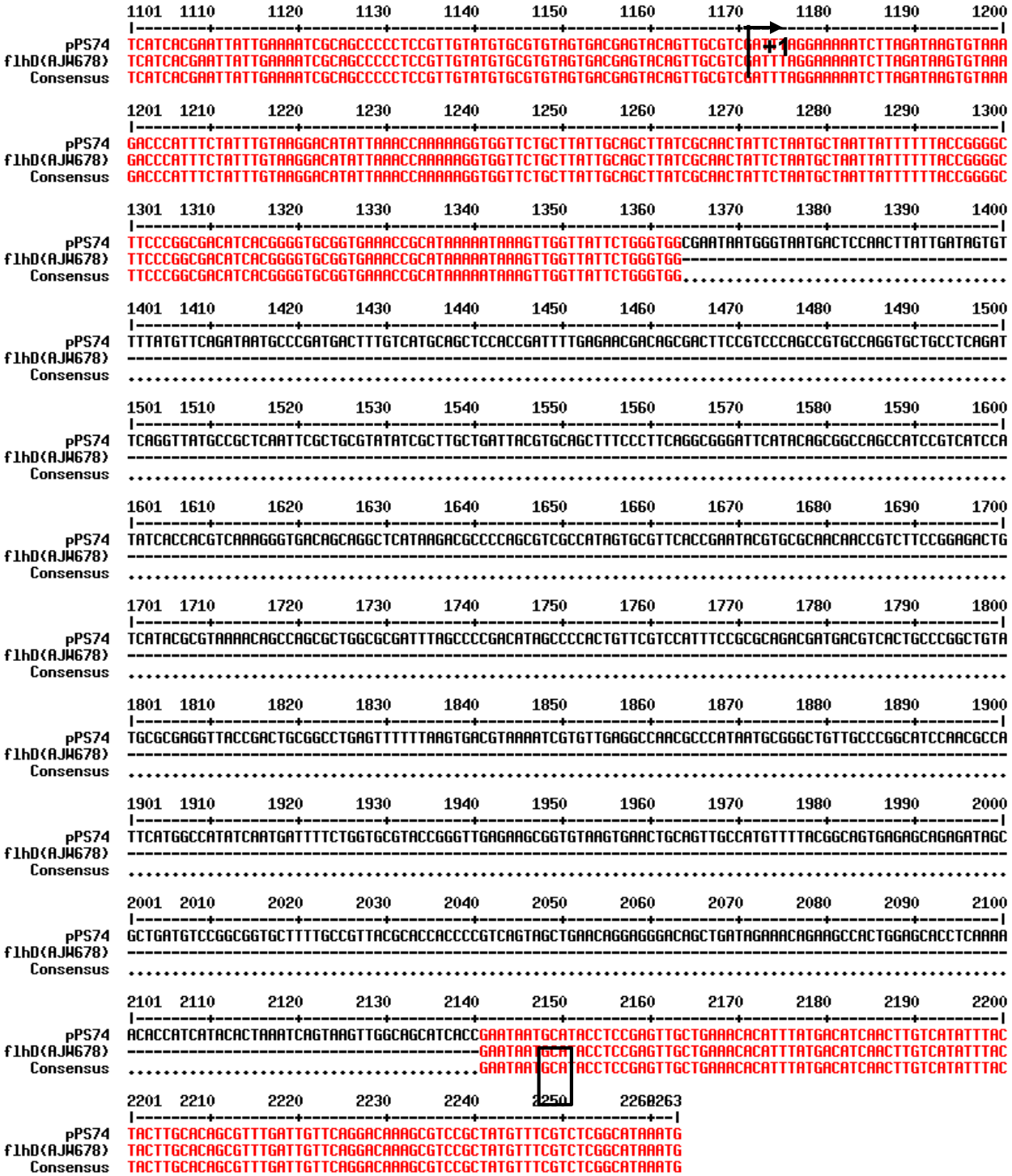

Figure 25: Sequence alignment of *flhD* promoter of pPS74 and *flhD* promoter of AJW678 (continued).

GENERAL DISCUSSION

From Specific Aim 1 of this study, we identified the flagella master regulator FlhD/FlhC as the first target for the prevention (expressed early in biofilm development) and treatment (expressed outer layer of biofilm) of biofilm-related diseases.

This research contributes to the ongoing controversy of whether flagella and motility is an advantage or disadvantage in biofilm formation. Earlier studies with various bacterial species suggested that motility has a positive influence on biofilm formation (Montie et al., 1982; de Weger et al., 1987; Grant et al., 1993; Korber, Lawrence, and Caldwell, 1994). However, these studies used genotypically uncharacterized stains as non-motile strains. Lawrence's group suggested that flagella have a direct effect on bacteria's ability to adhere to the surface (Lawrence and Neu, 1999). It has been postulated that flagella might be required for the cell to overcome the repulsive forces between the bacterium and the surface. In 1998 the O'Toole group, using an *flgK* mutant strain with incomplete flagellum synthesis, concluded that flagella are necessary for biofilm development in *P. aeruginosa* (O'Toole and Kolter, 1998). It is commonly believed that flagella contribute to reversible attachment in biofilm formation.

In contrast, several other studies indicated that motility is a disadvantage for the biofilm formation. A study showed that a mutation that inhibits gliding motility in *Flavobacterium pshychrophylum* increased biofilm formation (Alvarez et al., 2006). Similarly in *Bacillus subtilis,* the EspE operon, which is required for biofilm formation, shuts down the motility of the organism (Blair et al., 2008). Prigent-Combaret et al. showed that *E. coli* after biofilm formation down-regulates the flagellar genes and no flagella were visualized on biofilm bacteria under an electron microscope (Prigent-

Combaret et al., 1999). Our lab results from a high-throughput quantitative biofilm experiment indicated that both *fliA* and *flhD* mutants, each lacking a key regulator for flagella synthesis, resulted in a reduced biofilm formation under all growth conditions compared to parental strain (Prüß et al., 2010). Another study from our lab concluded that *flhC* negatively regulates cell division and biofilm formation of *E. coli* (Sule et al., 2011).

A temporal expression study of *flhD* in *E. coli* biofilm also provided us a clear picture about the importance of timely expression of *flhD* in biofilm formation. We postulated that timely regulation of flagella and motility is very important for biofilm formation based on the temporal gene expression pattern of *flhD*. Our data indicated that *flhD* gets expressed in the very early stage (12 h) of biofilm formation, then *flhD* expression decreases until 34 h. The gene expression pattern supports the studies which concluded that flagella contribute to reversible attachment. Our data also showed that constitutively over-expressed FlhD/FlhC from a plasmid resulted in very reduced biofilm formation relative to the WT (Samanta et al., 2013). So we hypothesized that in different scenarios *flhD* either gets constitutively over-expressed throughout biofilm formation or if *flhD* doesn't get expressed at all, biofilm formation will be hindered.

One scenario where *flhD* is repressed at all times can be created by adding β-phenylethylamine (PEA) to the growth media. As a result, PEA inhibited the first phase of biofilm development, reversible attachment. A study showed that PEA decreased *flhD* expression and biofilm amounts simultaneously (Stevenson et al., 2013). From phenotypic microarray experiments in our lab, we found that PEA and acetoacetic acid (AAA) are the two chemicals out of 95 carbon and 95 nitrogen sources tested in our lab

that can reduce biofilm formation by creating one of the above scenarios (Lynnes, Horne, and Prüß, 2013).

As a first future prospective of Specific Aim 1, we could study the effect of PEA on *flhD* expression in parental AJW678 and isogenic *ompR* and *rcsB* mutant strains, using flow cell and fluorescence microscopy. We already know that expression of *flhD* in both *ompR* and *rcsB* mutants is very high. If PEA is still able to decrease *flhD* expression in the *ompR* and/or *rcsB* mutants, this will indicate that the effect of PEA on *flhD* expression does not require OmpR and/or RcsB. If PEA is no longer able to decrease *flhD* expression, we can conclude that the effect of PEA on flhD expression requires OmpR and/or RcsB. This experiment could also be done with mutants in other two-component system response regulators to identify components of the signal transduction chain from PEA to biofilm amounts.

As a second future perspective of Specific Aim I, we could integrate PEA into coatings that can be used on medical devices to protect patients from biofilm-associated diseases. For that, we would test the effectiveness of PEA against various biofilm-forming bacteria of medical importance such as *Pseudomonas aeruginosa, Acinetobacter baumanii, Klebsiella ornithinolytica, Lactobacillus spp.* Etc. in various liquid growth media including blood, urine, and saliva.

From Specific Aim 2 of this study, we concluded that insertion of an IS1 element at -5 bp upstream of the transcription start site of *flhD* could decrease *flhD* expression and motility in an environment where motility is a disadvantage.

One past study indicated that insertion of an IS element into critical positions on the bacteria's chromosomes can serve as an adaptive mechanism and could be an

evolutionary advantage (Gaffe et al., 2011). Specific examples include IS5 insertion into a specific site of the *glpFK* promoter to reduce the inhibitory effect of GlpR (Zhang and Saier, Jr., 2011), IS1 and IS5 insertion into the *nrdAB* promoter to increase levels of ribonucleotide reductase (Feeney, Ke, and Beckwith, 2012), and insertion of IS629 into many places of the *E. coli* O157:H7 genome as a mechanism to genomically diversify this pathogen (Rump, Fischer, and Gonzalez-Escalona, 2011).

Using a stochastic population model, it was shown that *E. coli* undergoes dramatic genetic diversification when grown as a biofilm (Ponciano et al., 2009), which helps the evolution of biodiversity in biofilms. The bio-diversification in biofilms due to the increase in mutagenesis has been observed previously (Taddei et al., 1997). McDougald et al. showed that these mutagenic events permit *E. coli* biofilms to contain various micro-niches with phenotypic heterogeneity (McDougald et al., 2012). In this study, we provide one more example of IS elements creating a mutagenic event for *flhD* as an adaptive mechanism towards the evolutionary process. By inserting into the ribosome binding site of *flhD* or the open reading frame of *flhC,* IS1 elements can convert the highly motile MC1000 strain to a non-motile strain.

After reviewing the Specific Aim 2 results, we hypothesized that biofilms may need both motility and non-motility for optimal function during biofilm formation. Biofilms contain various micro-niches, and these micro-niches determine whether motility is an advantage or a disadvantage. So it is possible that for one micro-niche, motility may be a disadvantage; but at the same time for other micro-niche, motility may be very much required. To test this hypothesis, we performed a competitive experiment where biofilms were grown from a mixture of parental strain MC1000 and isogenic *flhD* mutants at a

1:1 ratio in 35 mm petri dishes over four weeks (Lynnes and Prüβ, unpublished). For the first 2-3 weeks, the biofilms contained a mixture of both strains with the *flhD* mutant being slightly more numerous than the parent most of the time. The general tendency pointed towards an increase in *flhD* mutants during week 3, reaching 100% after 4 weeks. Altogether, our preliminary data supported our hypothesis. Our result indicated that biofilms might need both parent bacteria and *flhD* mutant bacteria for optimal functionality during the first two weeks of their formation.

As a future prospective of Specific Aim 2, we propose to further study the spatial distribution of the motile and non-motile strains at various time points of biofilm formation. We would perform a competitive experiment where biofilms will be grown from a mixture of a *flhD::gfp*-containing parental strain and a *flhD::rfp*-containing isogenic *flhD* mutant at a 1:1 ratio in a flow cell. The *z*-stacking images of the biofilm will give us the ratio of motile and non-motile strains at different layers of biofilm. In this way, we can analyze the importance of the motile and non-motile strains at different time-points of biofilm formation.

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