PHENOTYPIC AND GENOTYPIC EFFECTS OF FLHC MEDIATED GENE REGULATION IN *ESCHERICHIA COLI* O157:H7

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Title

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REGULATION IN ESCHERICHIA COLI O157:H7

By

PREETI SULE

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

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ABSTRACT

Sule, Preeti; PhD.; Department of Veterinary and Microbiological Sciences; College of Agriculture, Food Systems and Natural Resources; North Dakota State University; May 2011. Phenotypic and Genotypic Effects of FlhC Mediated Gene Regulation in *Escherichia coli* O157: H7. Major Professor: Dr. Birgit Prüß.

Escherichia coli (E. coli) O157:H7, a pathogen belonging to the enterohemorrhagic group of *E. coli,* has long been a concern to human health. The pathogen causes a myriad of symptoms in humans, ranging from diarrhea and malaise to renal failure. Human infection with the spread of the pathogen is mainly attributed to consumption of contaminated food material such as meat. Decontamination of meat via sprays have to date been the most commonly practiced method to reduce contamination, which now has little relevance in the face of developing resistance by the pathogen. In the following study we investigated FlhC mediated gene regulation in *E. coli* O157:H7 on the surface of meat, in an attempt to recognize FlhC regulated targets, which may ultimately serve as targets for the development of novel decontaminating sprays.

Microarray experiments were conducted to compare gene expression levels between a parental *E. coli* O157:H7 strain and its isogenic *flhC* mutant, both grown on meat. Putative FlhC targets were then grouped based on their function. Real-time PCR experiment was done to confirm the regulation. Additionally, experiments were done to investigate the phenotypic effects of the regulation. To test the effect of FlhC on biofilm formation, an ATP based assay was first developed in *E. coli* K-12, which has been detailed in the following dissertation. This assay was used to quantify biofilm biomass in *E. coli* O157.

Microarray experiments revealed 287 genes as being down regulated by FlhC. These genes belonged to functions relating to cell division, metabolism, biofilm formation and pathogenicity. Real-time PCR confirmed the regulation of 87% of the tested genes. An additional 13 genes were tested with real-time PCR. These belonged to the same functional groups, but were either not spotted on the microarray chips or had missing data points and were hence not included in the analysis. All 13 of these genes appeared to be regulated by FlhC. The phenotypic experiments performed elucidated that the FlhC mutants divided to 20 times higher cell densities, formed five times more biofilm biomass and were twice as pathogenic in a chicken embryo lethality assay, when compared to the parental strain.

The following dissertation also reports the development of a combination assay for the quantification of biofilm that takes advantage of the previously mentioned ATP assay and the PhenotypeMicroarray ™ (PM) system. The assay was developed using the parental *E. coli* strain AJW678 and later applied to its isogenic *flhD* mutant to elaborate on the differences in nutritional requirements between the two strains during biofilm formation. Metabolic modeling and statistical testing was also applied to the data obtained. This assay will be used in the future to study biofilm formation by the parental strain *E. coli* O157:H7 strain and its isogenic FlhC mutants on single carbon sources, hence identifying potential metabolites which differentially support biofilm formation in the parental and the mutant strain.

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DEDICATION

This dissertation is dedicated to:

My parents, Sanat and Krishna Singh, thank you for being there with me at every step and dreaming so high for me.

My advisor Dr. Birgit Prüß, I would not have been able to finish my dissertation without you. Thank you for all you have done. My parents dreamt high for me, but you made me realize I could make them into reality.

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RATIONALE

FlhD/C protein sits at the top of the gene regulatory hierarchy of flagellar system in *Escherichia coli* K-12. It is indispensable for flagella formation and hence locomotion in the bacteria. Apart from its role in locomotion, FlhD/C is also involved in regulating various crucial cellular processes like cell division, metabolism, etc. in many bacterial systems. FlhD/C in turn can be regulated by varying the acetyl-phosphate level in the cell, hence presenting a unique signaling system where FlhD/C is the intermediate molecule, sensing environmental cues and relegating effects to downstream cellular processes.

This dissertation investigates the possibility of such a signaling pathway in the human pathogen *E. coli* O157:H7, which would provide researchers with valuable information regarding the ongoing gene regulation in the pathogen. Finally, such knowledge will prove crucial in devising new and effective sprays that will target cellular processes via its effect on the FlhD/C molecule.

TABLE OF CONTENTS

ABSTRACT iii
ACKNOWLEDGEMENTS v
DEDICATION vii
RATIONALE viii
LIST OF TABLES xii
LIST OF FIGURES xiii
GENERAL INTRODUCTION AND SPECIFIC AIMS 1
LITERATURE REVIEW 4
REFERENCES 12

PAPER 1. REGULATION OF VIRULENCE GENES IN *ESCHERICHIA COLI* O157:H7 19

ABSTRACT 20

INTRODUCTION 21

REFERENCES 36

PAPER 2. FLHC REGULATES CELL DIVISION, BIOFILM FORMATION AND VIRULENCE IN *ESCHERICHIA COLI* O157:H7 GROWN ON MEAT 53

ABSTRACT 54

INTRODUCTION 55

MATERIALS AND METHODS 59

RESULTS 71

DISCUSSION 83

FUTURE DIRECTIONS 89

ACKNOWLEDGEMENTS 90

REFERENCES 91

PAPER 3. A COMBINATION OF ASSAYS REVEALS BIOMASS DIFFERENCES IN BIOFILMS FORMED BY *ESCHERICHIA COLI* MUTANTS 101

ABSTRACT 102

INTRODUCTION 103

MATERIALS AND METHODS 105

RESULTS 109

DISCUSSION 113

ACKNOWLEDGEMENTS 114

REFERENCES 115

PAPER 4. A COMBINATION OF PHENOTYPE MICROARRAY™ TECHNOLOGY WITH THE ATP ASSAY DETERMINES THE NUTRITIONAL DEPENDENCE OF *ESCHERICHIA COLI* BIOFILM BIOMASS 119

ABSTRACT 120

INTRODUCTION 125

MATERIALS AND METHODS 133

RESULTS 139

DISCUSSION 146

CONCLUSION 152

ACKNOWLEDGEMENTS 153

REFERENCES 154

GENERAL DISCUSSION 165
REFERENCES 169

LIST OF TABLES

Table **Page**

1: Strains and plasmids used in this study 59

2: Primer sequences of primers used in real-time PCR 66

3: Sample enumeration of meat flora, with and without inoculation....................... 72

4: Genes representing the functional groups included in the study....................... 75

5: Comparison of different quantitative biofilm assays....................................... 129

6: Bacterial strains used for this study.. 133

7: Biofilm amounts on carbon sources which formed their own Duncan's grouping for the parent strain and structurally related carbon sources............ 143

8: Biofilm amounts on carbon sources which formed their own Duncan's grouping for the *flhD* strain and structurally related carbon sources 145

LIST OF FIGURES

Figure **Page**

1: Phases in biofilm formation, with time course progression from left to right....... 5

2: Diagrammatic representation of the ATP assay. 9

3: Time course of biofilm formation, indicating the global regulators that contribute to each phase. 11

4: Arrangement of the LEE pathogenicity island. 26

5: Regulatory pathways for *E. coli* O157:H7 involving the LEE pathogenicity island..................................... 28

6: Diagrammatic representation of the protocol followed to obtain microarray data. 60

7: Real-time PCR. 77

8: Cell division and biofilm formation..................................... 80

9: Scanning electron micrographs of biofilms..................................... 81

10: Lethality in the ELA. 82

11: Working model for the regulation of the FlhD and FlhC target genes............. 85

12: Biofilm estimation by ATP and CV assays. 110

13: Representative electron micrographs of biofilms formed by each of the strains at amagnification of 6,500 ×. 112

14: Time course of biofilm formation. 126

15: Work flow for the determination of biofilm amounts on PM plates with the ATP assay..................................... 135

16: Electron micrographs at 3,000 fold magnification for the AJW678 parent strain, and its isogenic mutants in *flhD*, *fliA*, *fimA*, and *fimH*. 140

17: Biofilm formation in the parent strain and the *flhD* mutant were compared using a *t*-test. 141

18: Metabolic pathways from the top biofilm producing carbon sources
for both *E. coli* strains, feeding into the Embden-Meyerhof pathway. 146

19: Metabolic pathways from the top biofilm producing carbon sources
for both strains to the production of acetate.. 147

20: Diagrammatic representation of the study principal. 166

GENERAL INTRODUCTION AND SPECIFIC AIMS

The following thesis aims at elucidating FlhC mediated genetic regulation in *Escherichia coli* O157:H7 on meat. *E. coli* O157:H7 is an eminent problem in the food industry and causes approximately 60, 000 infections annually (CDC). Various sprays have been used to control the spread of the bacteria, but evolving resistance to these sprays has consistently added to the intensity of the problem. The following study was based on the observation that FlhD/C regulated essential processes such as cell division etc in *E. coli* K-12, such regulation in *E. coli* O157:H7 would essentially open avenues for metabolic control of these cellular processes via specific environmental cues affecting FlhC.The following section shall outline the logical sequence of the chapters presented in the thesis and the Specific Aims of the study.

Specific aim 1: Establishing FlhC mediated regulation on the surface of meat and testing the phenotypic effect of this regulation

This aim will address the working hypothesis that FlhC regulates essential cellular processes in *E. coli* O157:H7 on surface of meat.

Specific Aim 1 is addressed in the **first chapter** of the thesis, and reports regulation of cell division, biofilm, and virulence related genes in *E. coli* O157:H7. The phenotypic effect of this regulation was also established and is reported in the chapter.

Specific aim 2: Development of an ATP based assay for the quantification of biofilm biomass

This Specific Aim addresses the working hypothesis that cells involved in biofilm formation can be estimated based on the ATP content of the biomass involved.

Specific Aim 2 is addressed in the **second chapter** of the thesis and was designated to further investigate the effect of FlhC on biofilm formation. The assay was developed on the *E. coli* K-12 strain AJW678, a good biofilm former, and later used for the poor biofilm forming *E. coli* O157:H7.

Specific aim 3: Development of a combination assay involving the ATP assay and PhenotypeMicroarray™ technology

This Specific Aim addresses the working hypothesis that environmental factors, specifically nutrients play an important role in biofilm development.

Dissertation organization

The thesis has been organized to provide a comprehensive understanding to the readers regarding the gene regulation in *E. coli* O157:H7 and the advances in control measures. The Literature review comprises of a published book chapter and additional relevant information.

The subsequent chapters are in the form of published manuscript and effectively cover the experimental procedures and related outcomes. Discussion at

the end of the dissertation is a general discussion that aims at providing a general view of the research motive.

LITERATURE REVIEW

Biofilm formation

Biofilms are sessile communities of cells, the formation of which was believed to occur in temporal phases (39). The initial phase of reversible attachment of the bacteria is mediated by flagella, followed by irreversible attachment mediated by type I fimbriae or curli. The irreversible attachment to a surface is then followed by biofilm maturation, which involves the development of extracellular matrix, termed as extracellular polysaccharide layer (EPS) in which the bacterial cells are embedded. The capability to secrete the EPS layer differs from one bacterial species to the other. Pathogens such as *Streptococcus pneumonia* have been shown to produce a wide variety of such matrix, which considerably aid in their pathogenesis (48). At later stages certain cells break free from the biofilm setup, which constitutes the dispersal phase (Fig. 1).

Mature biofilms also include interstitial empty spaces, which serve as channels for transport of nutrients and water throughout the otherwise immobile biofilm (46). These channels aid the exchange of metabolic waste and hence are essential structural or morphological components of a mature biofilm (14).

Recent advances in research on biofilm, however, challenge this existing dogma of temporal phases and relate formation of biofilm to metabolic changes ongoing in the bacterial cell. Furthermore, research suggests that biofilm related metabolic changes are manifested in response to the bacterial environment. Each

bacterium hence undergoes a different biofilm specific regulatory pathway upon exposure to environmental cues (32).

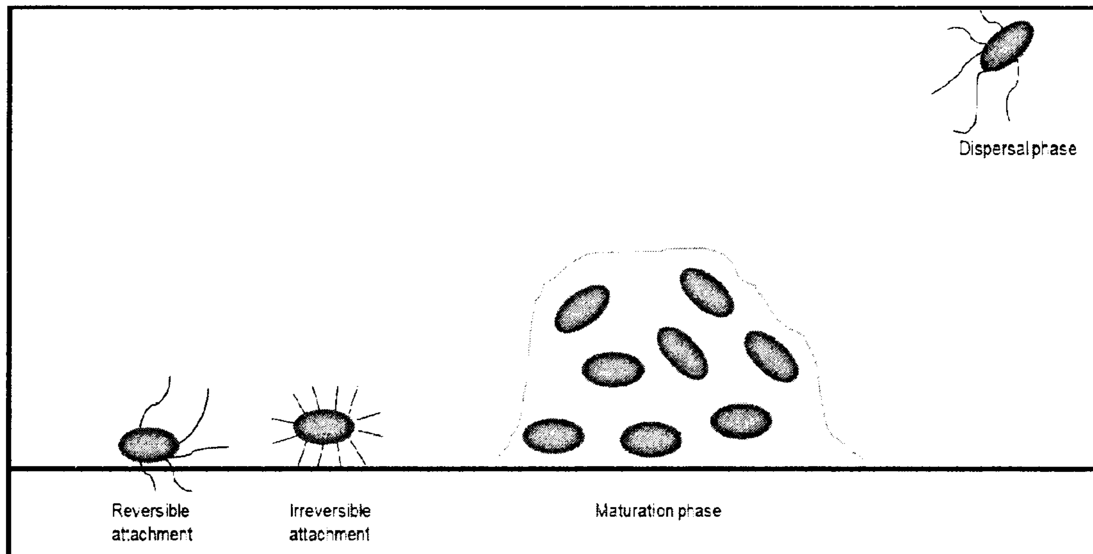

Figure 1: Phases in biofilm formation, with time course progression from left to right.

Biofilm associated problems

The formation of biofilms has often caused severe problems in many natural (19, 35), clinical (34) and industrial settings (11, 52). On the other hand, benefits of bacterial and multispecies biofilms are emerging in areas of bio-fuel production, waste water treatment and bioremediation of oil spills (3, 51). Despite the industrial advantages of bacterial biofilms, biofilms in clinical environments typically increase patient mortality and make treatment challenging.

Biofilms serve as a protective niche for the bacteria involved. There have been numerous studies indicating the benefits conferred by the EPS of a mature biofilm. The benefits range from protection against osmotic shock and UV radiation to

desiccation (5, 18, 20). Resistance to antibiotics is increasingly becoming common and can be explained on several bases. Studies have suggested that certain biofilms, like those formed by *Klebsiella* and *Pseudomonas aeruginosa* prevent penetration of disinfecting agents such as chlorine, which is regularly used for disinfection (15). Researchers have also demonstrated that pathogens such as *P. aeruginosa* and *Staphylococcus epidermidis* also exhibit similar traits of resistance via non penetration of antibiotics (16, 24). However the same does not hold true for all biofilms, suggesting the role of other key mechanisms that make biofilms more antibiotic resistant.

It has been reported that under the conditions found inside a biofilm, a general stress response is initiated. The stress response sigma factor RpoS, which was initially determined to be expressed in stationary phase, was later discovered to be induced in biofilms, owing to the high cell density in such environments. The induced RpoS in turn regulates a myriad of genes enabling the biofilms to tide over various hazardous conditions (21). Recent studies on *E. coli* have suggested that *rpoS* mutants lack the ability to form robust biofilms, further supporting the proposed reasoning (1). The OmpF and OmpC membrane proteins have also been indicated to aid resistance against β-lactams antibiotics (25). Despite these advances in research and understanding of antimicrobial resistance in biofilms, the exact mechanism still needs to be elucidated. The heterogeneity in environmental biofilms furthermore adds to the complexity of the situation and should be taken into account when dealing with cases of recurrent infection (30).

In the light of these findings and the progress in biofilms related research, two major questions still remain unanswered. The first being spatial arrangement of proteins involved in biofilms formation and the second being the effect of the environment on the formation of biofilms. To answer these questions, the requirement of a reliable quantitative biofilm assay is becoming increasingly important.

Current techniques used to quantify biofilms

Researchers have been using various techniques to quantify biofilms. The most widely used method is the crystal violet assay. The assay, though suitable for high throughput screening, stains the EPS matrix and is non-specific, hence does not differentiate between live and dead bacteria (27, 37, 42). Other, highly specific proteins that bind to selective carbohydrates have also been used to quantify biofilms. Examples are WGA (wheat germ agglutinin) and SBA (soybean agglutinin) which selectively bind and form complexes with lipo-oligosaccharides and colanic acid (12, 44). These assays detect live and dead cells alike. Further testing regarding their usefulness in high throughput settings is however needed. Additional assays that are based on the energy content of the bacterial cells are also available. One of these is based upon tetrazolium dyes, such as XTT (4-nitro-5-sulpfophenyl-5[(phenylamino) carbonyll]-2-H-terazolium hydroxide). The assay works on the principal that XTT is reduced by NADH in the cell to an orange colored water soluble formazon derivative, the optical density of which can be measured (13). Consequently, XTT dyes live cells only. It may be used for high throughput screening. Lastly, the ATP assay instrumental in quantifying live cells

involved in biofilms formation and also has the potential to be used in high-throughput environments. ATP has been used previously to measure biomass by various researchers, since its concentration remains fairly constant across many growth conditions (33, 49). Both, the XTT and the ATP assay do not directly quantify biofilms, but are considered indicative of bacterial biomass.

ATP assay for biofilm quantification

The ATP assay that was developed for the quantification of biofilm associated biomass during my masters takes advantage of the BacTiter Glo™ system from Promega (Madison, WI). The assay system utilizes beetle luciferin and a recombinant firefly luciferase. The luciferin reacts with ATP in the presence of metal ions (such as magnesium) and produces oxyluciferin and light. The light is detected by a luminometer. The whole reaction is catalyzed by the recombinant luciferase which is included in the system and provided by the manufacturer. The assay, when adapted to *E. coli,* established that a two fold increase in bioluminescence did indeed relate to a two- fold increase in the ATP concentration and a two fold increase in the bacterial cell (48). The protocol for the ATP assay developed by Sule et al (48) has been outlined in Fig. 2. In the Results section, I will present data, where I continued and refined the development of the assay and used it to test eight isogenic strains of *E. coli* for their biofilm amounts, comparing them to electron micrographs obtained from the same strains.

Phenotype Microarray™ system

As mentioned earlier, the evidence that environment plays a vital role in biofilm development is increasingly becoming more evident. The Phenotype Microarray technology (PM) used in this study provides an excellent source of single nutrients for testing their effect on biofilm formation. The plates consist of single nutrients dried down onto the base of 96 well plates. A total of 20 such plates are available and are designated PM 1-20. The PM1 plate alone allows testing of a total of 95

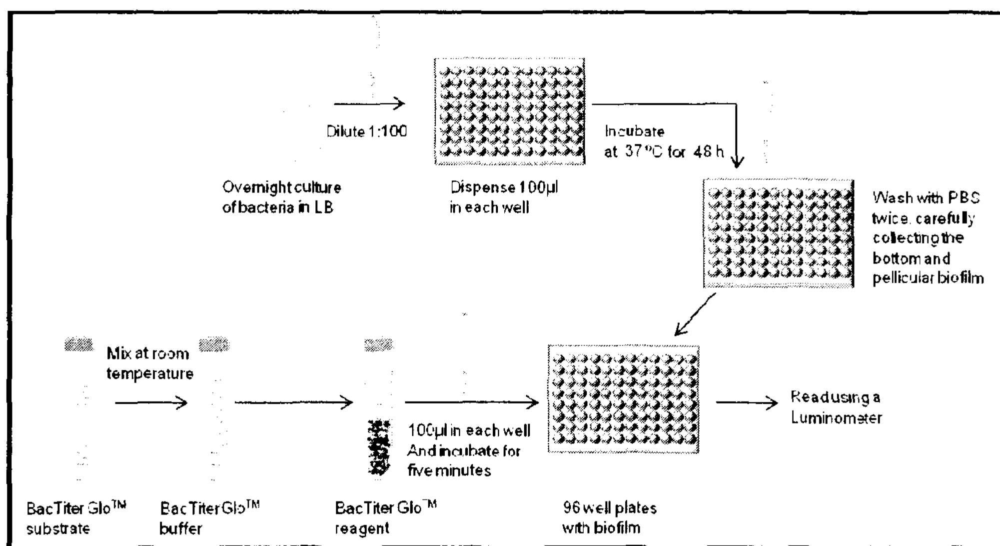

Figure 2: Diagrammatic representation of the ATP assay.

carbon sources, one well is kept empty on each of the plates, designed to serve as a negative control. The PM system is designed to study changes in bacterial characteristics based on phenotypic changes (9).The PM system has since been used for monitoring bacterial growth in numerous previous studies (6, 17, 31). Its application in measuring bacterial stress response to factors such as alkalinity (45) and identification of bacteria have also been reported (2). The application of the

PM plates to assessing biofilms formation is relatively recent and not well characterized (10). The following thesis will report the use of the PM technology to study the nutritional requirements of *E. coli* during biofilm formation.

Genes involved in biofilm formation

Studies have shown that biofilm development is often cued by cell to cell signaling, known as quorum sensing (28, 29, 41), as detailed in Part 1 of this Literature Review. An additional gene regulatory mechanism in bacteria is mediated by two-component signaling. *E. coli* contains 37 of such two-component systems (2CSTS), co-ordinately regulating the expression of hundreds of genes. A 2CSTS generally consists of a sensor kinase and a response regulator. 2CSTSs are very instrumental in sensing environmental changes (22, 43). A recently published review article (38) summarized a network in *E. coli* K-12 that included the flagellar master regulator FlhD/FlhC and a number of 2CSTSs. A total of 16 global regulators regulated the expression of several hundreds of genes, many of them encoding biofilm associated cell surface organelles. Among the 2CSTSsthat were included in the network are the osmoregulator EnvZ/OmprR and the colanic acid regulator RcsCDB. The EnvZ/OmpR system regulates the synthesis of flagella (40), type I fimbriae (36) and curli (26), RcsCDB regulates biofilm by serving as an activator of the extracellular matrix component colanic acid (23). The hypothesis that was deduced from the review article is summarized in Fig. 3.

The role of flagella and motility in biofilm formation has been very controversial. In agreement with the positive role of flagella in the manifestation of

reversible attachment (39), a recent study with *Pseudomonas aeruginosa* suggests that mutations in either MotAB or MotCD, the two flagellar stators, inhibit biofilm

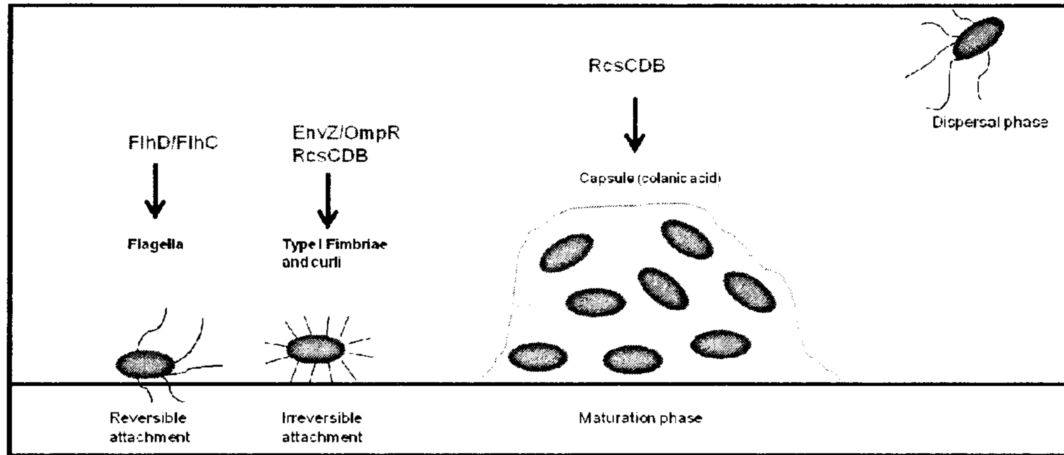

Figure 3: Time course of biofilm formation, indicating the global regulators that contribute to each phase.

formation with no evident decline of motility (50). In conflict with this positive role, researchers have reported that in other systems, such as *Flavobacterium pshychrophylum*, mutations that inhibit gliding motility enhance the biofilm forming capability of the organism (4). Similarly, in *Bacillus subtilis* motility was detrimental to biofilm formation. The EspE operon that was required for biofilm formation on *B. subtilis*, was instrumental in shutting down the motility of the organism (8). Amidst such conflicting reports, the exact role of motility remains unclear and evidently needs further investigation. In addition, establishing a role for 2CSTS in biofilm formation will open avenues for the development of novel biofilm prevention and treatment options through inhibition of two-component signaling pathways

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PAPER 1. REGULATION OF VIRULENCE GENES IN *ESCHERICHIA COLI* O157:H7

Preeti Sule and Birgit M. Prüß. 2009. Recent Res. Devel. Microbiology, 11:43-59.

This review article was written by Preeti Sule under the guidance of Birgit M. Prüß and is a compilation of current literature pertaining to the subject.

ABSTRACT

Escherichia coli O157:H7 belongs to the attaching effacing class of enterohemorrhagic *E. coli.* In the recent past, several cases of meat and vegetable related outbreaks have been reported, amounting to serious concern with respect to food safety and food handling. The organism is known to have a thousand genes in excess of the *E. coli* K-12 laboratory strains, most of the virulence genes are arranged to form a pathogenicity island in *E. coli* O157:H7 as in many other bacteria. The virulence of the bacteria is highly regulated, involving genes present on the island, as well as elsewhere in the genome.

INTRODUCTION

In the following chapter, *E. coli* O157:H7 will be discussed as a pathogen in terms of the diseases caused by it, its genetic makeup, and the intense regulation of virulence genes.

E. coli O157:H7, a pathogen

Effect on human host

Both enteropathogenic (EPEC) and enterohemorhagic *E. coli* (EHEC) are known to cause substantial morbidity and mortality in humans (17,45). The organism is known to infect the elderly and children at higher rates attributable to low immunity levels. The presence of the organism in cattle is generally asymptomatic (65), but in humans it is known to cause bloody diarrhea, often leading to hemolytic uremic syndrome (HUS). HUS is the common cause of acute renal failure in children and has a mortality rate of 3- 5%. The hallmark symptoms of HUS are often summarized as being a triad of microangiopathic anemia, thrombocytopenia and acute renal failure. Death due to HUS is mostly related to severe extra renal disease often involving the central nervous system (79).

The association between shiga toxin produced by *E. coli* O157:H7 and HUS were made much later as compared to the first reported case in 1955 (29). Shiga toxin produced by the organism causes local tissue damage in the host intestine. This damage then leads to absorption of the toxin into the blood stream leading to micro vascular endothelial injury, defining the basic pathophysiology of HUS (62). Predisposition to HUS can be explained in terms of use of antimotility drugs or

antibiotics (83,101) nausea, vomiting, bloody diarrhea, elevated leukocyte counts in the blood, extremes of ages and female gender.

Cattle and other wild animals such as deer have been reported as carriers or reservoirs of the bacteria (5,11). The pathogen is detected using multiplex PCR with primers directed towards the *eaeA* gene encoding intimin, shiga like *toxin I* gene (*stxI*), shiga like *toxin II* genes (*stxII*), and a segment of the 60 MDa plasmid (27). Other techniques such as pulse field gel electrophoresis (PFGE) and antimicrobial resistance have been used for the detection of the bacteria (71).

Outbreaks

There have been several reported outbreaks of *E. coli* O157:H7, most of them associated with the consumption of contaminated beef or meat products. The earliest recorded outbreak in the United States was in 1982 in Michigan. 47 people were taken ill following the consumption of hamburgers at a fast food restaurant (74,95). I the same year, an outbreak at a nursing home in Canada (Ontario) reported one death out of 31 infected cases registered. The cause was suspected to be contaminated hamburgers but could not be confirmed (CDC 1983) (42).

In 1984, a hamburger related outbreak was reported in Nebraska claiming four lives (42,77). Similar outbreaks in the preceding years were reported in states of Washington, Wisconsin, and Minnesota, affecting the general public and bringing into limelight a pathogen that was previously less known (10,61). Minor outbreaks related to consumption of contaminated meat products have since been reported almost every year. The mortality rate, however, has been low except for one case

in 1985 where 17 people died after consumption of contaminated sandwiches at a nursing home in Canada. Another outbreak of a greater magnitude was reported in 1993 where more than 500 people were taken ill after consumption of hamburgers at a fast food restaurant in as many as four states (Idaho, Nevada, Washington, and California) (9). Most of the major outbreaks have been traced to the consumption of contaminated meat products, though the geographical locations of the outbreaks have been widespread.

Ground beef has been reported as the most frequently identified vehicle for *E. coli* O157:H7 spread and infection, though other products have also been a source of concern. Reports suggest that from year 1982-2002, 34 % of the produce related outbreaks were due to contaminated lettuce, 8 % were due to apple cider, 16 % due to contaminated salad, 11 % due to coleslaw and melons, 8 % due to sprouts and about 3 % due to grapes (72). Transmission of the pathogen via other sources such as dairy products and fecal oral route has been reported but are rare.

The path to pathogenicity

The above classical incidences raise the question, as to what distinguishes *E. coli* O157:H7 from other *E. coli* strains.

Genomics and evolution of *E. coli* O157:H7

The bacterium belongs to a group of closely related pathogens that comprise what is termed as a clone complex. The members of the clone complex are genetically similar to each other differing in only minor aspects. Whittam showed

that *E. coli* O157:H7 isolated from separated outbreaks in North America were closely related to each other, sharing an average 90% of their alleles (96). In similar studies performed by the same group, 194 isolates of *E. coli* O157 were examined. The results strongly indicated that the *E. coli* O157:H7 lineage was not recently derived from the already existing O157 serogroup (97). It was later shown that the 43.358 bp sequence of the LEE EDL933, an enterohemorrhagic *E. coli* O157:H7 serovar isolated from contaminated hamburger patties, not only had prophage related genes but was also similar to EPEC O55:H7 (64). Studies done in the same year by Feng and coworkers showed that a common ancestral strain gave rise to EPEC O55:H7, which over the course of time became modified to *E. coli* O157:H7 as we know it today (25).

Lateral gene transfer is believed to be an important instrument in the evolution of *E. coli* O157:H7. Strain specific clusters of diverse sizes were found in the organism. These clusters included about 1387 new genes. Both the *E. coli* K-12 strain MG1655 and the *E. coli* O157:H7 isolate EDL933 share a common backbone except for a 422 kb inversion spanning the replication terminus. Homology is further interrupted by EDL933 specific O islands and MG1655 specific K islands. 26% of the EDL933 genes (1387/5416) lie completely within the O island, 40% of which can be assigned a function and 33% are phage related and could be the remains of phage genome. 528 of the MG1655 genes (528/4405) are not found in EDL933. There are about 100 examples of the O-islands and the K-islands being present at the same location with respect to the conserved backbone (64). The presence of a promiscuous sequence of about 2.9 kb has been observed

in the intergenic region between the *mutS* and the *rpoS* gene. At the same locus in *Shigella dysenteriae*1, a sequence identical to *E. coli* O157:H7 was found. A high polymorphic rate at this region strongly indicates the presence of horizontal transfer events in the course of evolution (98).

The pathogenicity island

The mechanism of pathogenicity in *E. coli* O157:H7 has not yet been completely understood but is believed to be related to the cluster of genes specific to the organism, and likely acquired by horizontal gene transfer. The organism causes disease by its ability to attach and adhere to the host cell membrane and subsequently colonizes the large intestine; this process is often referred to as the attaching effacing (AE) mechanism. The formation of such lesions follows three basic steps, localized adherence, signal transduction and intimate adherence. Following the intimate adherence of the bacteria to the intestine, effacement of the underlying microvilli occurs, leading to the accumulation of filamentous actin in the adjacent cytoplasm (19). The genes responsible for the formation of such lesions are located on the locus of enterocyte effacement (LEE) island. The LEE island is a 43 kb fragment situated on the chromosome with a G+C content lower than the rest of the genome (99). The LEE region is divided into 5 segments (Fig.4) (22,58), LEE5 contains the *eaeA* gene that encodes a 94-97 kDa outer membrane protein known as intimin. Intimin is important for attaching and effacing property of the pathogen. The *eaeA* gene has been cloned and sequenced. The *eaeA* gene of the enteropathogenic *E. coli* is found to be 97% homologous to EHEC gene at the 5' end and only about 59% homologous at the 3' end (52). Though the EPEC and

EHEC intimin itself is 49% similar (99), EPEC unlike EHEC is known to form lesions in the small as well as in the large intestine.

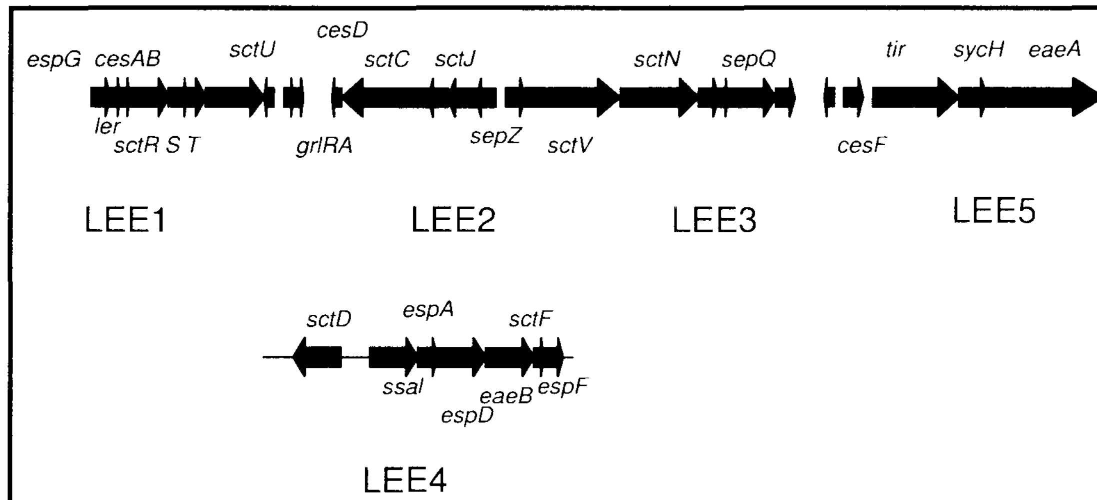

Figure 4: Arrangement of the LEE pathogenicity island. The LEE pathogenicity island has five segments, designated as LEE 1-5. LEE4 is located adjacent to LEE5.

LEE5 also contains the *tir* gene that is responsible for the formation of a 90 kDa eukaryotic cytoskeletal protein. The protein is capable of phosphorylating tyrosine residues in response to infection and can serve as a receptor for the intimin protein (86). Tir is initially produced by the bacterial cell as a 78 kDa unphosphorylated protein; the unphosphorylated protein is translocated to the host cell where it is later phosphorylated.

The type III secretion system (TTSS) that is responsible for the export of Tir is encoded by the third segment, LEE3. The type III secretion system is induced upon contact of the bacteria with the host cell and is involved in the delivery of the virulence factors directly into the eukaryotic host. The organism uses the type III secretion system to secrete several proteins that are responsible for signal

transduction during the AE lesion formation (48). Apart from the LEE regions, the EHEC plasmid encodes certain virulence factors like the RTX cytotoxin-hemolysin (Hly) and the auto transporter toxin Esp (13). These factors aid the virulence potential of the bacteria.

Regulation of the virulence genes

Previously, we summarized a network of regulation derived from microarray and other experimental data (68) for *E. coli* K-12 strains. The presence of an additional 1000 genes in *E. coli* O157:H7 (64) add regulatory pathways to the previously network centered around the pathogenicity island. Fig. 5 summarizes the additional regulatory pathways that will be summarized in this chapter.

Genes of the LEE pathogenicity island are regulated by internal regulators that are encoded by the genes of the island, as well as external regulators. Many of these regulators also affect flagellar gene expression.

The Ler regulator

The *LEE* pathogenicity island encodes for various regulators that affect the virulence of the organism. The gene for one such global virulence regulator, *ler,* is present on the *LEE1* region of the pathogenicity island (21). Ler is a 15 kDa protein and is the central, most important regulator involved in the positive regulation of the *LEE* genes. (14,21,28,58). Ler belongs to the HNS family of protein and has a DNA binding domain at the carboxy terminal, exhibiting high amino acid similarity

to the HNS like proteins. Interestingly, Ler is also regulated by HNS.

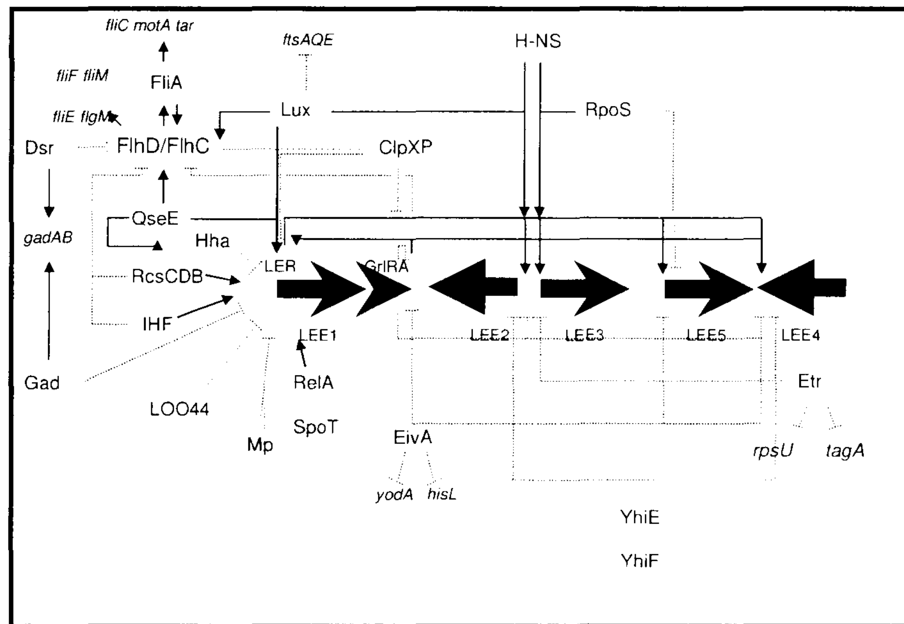

Figure 5: Regulatory pathways for *E. coli* O157:H7 involving the LEE pathogenicity island. Ler is a part of a Per-mediated regulatory cascade that upregulates *LEE2*, *LEE3* and *LEE4* (21). Hha acts as a negative regulator of Ler (81). HNS acts in an antagonistic pattern as compared to Ler (91). The GrlRA regulatory system controls both the flagellar and *LEE* encoded genes (40). Internal regulators on the *LEE* pathogenicity island include EivF and EtrA that exert a negative effect on the transcription of genes in the *LEE* pathogenicity island (100). ClpXP protease controls the expression of the type III secretion system that is located in the LEE pathogenicity island (41,90). The quorum sensing system also affects the expression of the LEE Pathogenicity island, QseA directly activates the expression of the *LEE1* and *LEE2* regions (82). Other regulators such as QseBC and QseEF are also involved in the expression of the flagellar operon, as well as the *LEE* pathogenicity island (44).

The HNS protein itself has been previously established as a global regulator (4,46,66). It is a pleiotropic transcription factor that affects almost 5% of the genes in the *E. coli* genome (4,37). HNS is now known to effect the expression of various virulence genes in a myriad of different organisms (23,24,75). HNS repression of the *LEE1* and *LEE2* genes is overcome by Ler, via simple displacement of the protein from its bound sites on the DNA (93). In certain other genes, the two

regulators Ler and HNS exhibit a different pattern of regulation. One such marked difference can be observed in the case of long polar fimbriae in *E. coli* O157:H7, where Ler and HNS interaction has experimentally proven to be of inhibition rather than displacement. As another example, binding of Ler to the promoter of the *lpf* gene inhibits the binding of the repressor HNS, causing a sustained expression of the same (91). Ler was also found to elevate repression of *LEE*5 by an unknown repressor, which was later characterized as HNS (33). Recent studies have shown that HNS exerts its effect on the transcription of Ler indirectly by repressing the sigma factor RpoD which replaces the sigma factor RpoS in the core RNA polymerase (3,12).

Ler has also been known to be negatively regulated by Hha. Hha is a negative regulator of α-hemolysin in pathogenic *E. coli* (60) and has been recently implicated in indirect repression of *esp* genes located in the *LEE4* region of EHEC (81). The exact mechanism of Hha repression of Ler, an activator of *esp* transcription is not known, it is believed that multimers of Hha bind to the *ler* promoter in a cooperative manner. Regulatory proteins of this nature have an N-terminal oligomerization domain that bind regulatory sequences to affect the downstream genes at the transcriptional level (35,53).

Another important regulator of Ler is the stress activated sigma factor RpoS, which is believed to work in consortium with a non-coding RNA, DsrA. DsrA has been previously shown to modulate the expression of both, HNS and RpoS regulatory proteins in a contradictory manner (50,54). In *E. coli* O157:H7, the effect of RpoS in the regulation has been established (47). The DsrA/RpoS system

activates the *LEE* genes in a Ler dependent manner and its effect is totally abolished in Ler mutants, as is the effect of the inhibitory, antagonistic HNS/GrlA system in the absence of the protein Ler (47). It was later established that the effect of DsrA/Rpos on the Ler protein is at the transcriptional level; hence in the presence of abundant DsrA, an increase in the level of functional *ler* transcripts were observed. RpoS is also a regulator of flagellar genes and shall be discussed later in the chapter.

The LEE internal regulators: GrlA/GrlR system

GrlA is a recent addition to the list of positive regulators of the *ler* gene (18,41). The presence of Ler is critical and essential for the expression of *grlA* which is located on the LEE Pathogenicity island between LEE1 and LEE2 (22). In the absence of Ler, GrlA expression completely ceases. In the presence of Ler, the GrlA protein is found in abundance and plays an important role in the regulation of *LEE* genes by reversing the HNS mediated repression of the alternative sigma factor RpoD, which is then used for transcription of the *LEE* genes including the *ler* operon (47). Both the DsrA/RpoS system and the HNS/GrlA system have however been found to act independently of each other. GrlA and GrlR are transcribed from the same open reading frame and are the positive and negative regulators, respectively, of *ler* gene transcription (6,51). The negative role of GrlR in the transcription of Ler is dependent on its interaction with GrlA (44). In the absence of GrlR, GrlA up regulates the *LEE* genes via Ler. In consistence with this mode of interaction, the *LEE* gene induction in stationary phase (73) is facilitated by the phase dependant regulation of GrlR. Hence, in stationary phase the level of GrlR

decreases and GrlA is available for induction of the *LEE* genes (42). Please note that the *grlR* and the *grlA* genes have been alternatively known as *LOO44* and *LOO43* respectively.

EivF and EtrA

Type III secretion is used by EHEC like many other pathogenic bacteria to introduce bacterial effectors into the host cell in order to overcome the protective signaling pathways of the host (43). A well characterized TTSS is essential for the formation of attaching effacing lesions and bacterial pathogenesis (55,56), and the components of this system are located on the LEE pathogenicity island. The procurement of this system by the bacteria can be largely attributed to horizontal gene transfer (34,36,63). Apart from the TTSS found on the pathogenicity island, another cluster of type III secretion system related cryptic genes have been found elsewhere in the genome and is exclusively called ETT2 (36,63,73). Mutational inhibition of two of the ETT2 genes, namely *etrA* and *eivF*, greatly increases the expression of *LEE* genes and improves the adhesion and colonization properties of the pathogen to the human intestinal cells. Additionally, the effect of the ETT2 regulators is suggested to influence *LEE* genes indirectly via its effect on GrlA and GrlR (100). Variation in the ETT2 profile may account for the difference in LEE mediated protein secretion and hence strain to strain variation in the regulation of the LEE pathogenicity island (20,57).

LEE regulators affecting flagellar genes: A link between pathogenesis and motility

Co-regulation between motility and virulence has been observed for many bacteria such as *Proteus mirabilis* (1,2), *Serratia liquifaciens* (30) *Y. enterocolitica* (80,92) and *E. coli* (38). Another such example of simultaneous regulation is seen in *E. coli* O157:H7 flagella and LEE gene expression.

Quorum sensing

Several regulators of the quorum sensing system co-regulate flagellar and LEE genes. Quorum sensing is the response of bacteria to population fluctuations, which is brought about by changes in gene expression (59). This cell to cell signaling is brought about by specific signaling molecules known as autoinducers. The LuxS and the Qse quorum sensing systems exert an effect on the LEE genes and flagellar genes simultaneously. This segment of the chapter elucidates these regulations, and since most of the quorum sensing systems are two-component systems, we also discuss the role of RcsCDB, another such system involving phosphorelay, in the regulation of LEE genes.

The Lux system: The world of autoinducers

Bacterial communication via autoinducers is a well-established mechanism of cross talk, not only at an intraspecies level but also at an interspecies level (87). Both the Gram- negative and Gram-positive bacteria are known to produce auto inducers. Gram-negative bacteria produce two types of autoinducers, the first is acyl-homoserine lactone (AHL), generally referred to as autoinducer 1 (AI-1) (15).

The second is uncharacterized and is referred to as AI-2 (7,8). AI-2 is the choice of molecule when the bacteria intent to access not only its own population but also other bacterial populations in a given environment (78). The gene for the AI-2 synthetase was cloned, sequenced and named *luxS* (88). In a later study, it was confirmed that at least 404 genes were regulated by LuxS in enteric bacteria. These genes involved the motility genes such as *motA*, flagellar genes such as *fliA* and *fliF*, cell division genes such as *ftsAQ*, and 19 transcriptional regulators. The cell division rate of the *flhD* mutant was shown to be 5 times higher than the wild-type (WT) cells (67,70). This effect on cell division may be due to the effect of FlhD on *cadA*, an acid response gene (69).

Quorum sensing is known to regulate the TTSS and protein secretion in EHEC. The components of the TTSS are homologus to the basal body components of the flagella and hence co-regulation of both the systems is observed and has been previously demonstrated in *Salmonella* and other pathogenic bacterial species (31). Quorum sensing directly induces transcription from the *LEE1* and the *LEE2* promoters. Indirect activation of LEE3 and the *tir* operon via *ler* is also observed (84).

The Qse genes, their role in quorum sensing

Two Qse systems are important in the regulation of motility, as well as virulence in *E. coli* O157:H7. The first system involves the regulator QseA which is a member of the LysR family and directly exerts its effect by activating *ler* (85). Apart from its direct effect on the *LEE1* genes (82), QseA autoregulates itself and

also activates *grlRA* in a Ler independent fashion. GrlRA then regulates the transcription of *LEE1* and other *LEE* operons (76). Recent studies have shown that although QseA can directly activate transcription of *LEE1*, its effect on upstream sequences of *LEE1* highlight the involvement of one or more uncharacterized factors.

The second system is a couple of two-component systems, comprising of QseBC and QseEF. QseF and QseB are response regulators and QseC and Qse E are sensor kinases. The QseBC system is mainly involved in regulation of the flagellar system and its effect on other genes, such as *LEE* genes has not been elucidated (85). QseEF is activated by auto inducers and regulate the LEE genes (16). Additionally the QseEF activates QseA which then exerts its effect on the *LEE* genes as mentioned earlier.

The RcsBCD system

The Rcs system is a his-asp phosphorelay that is similar to two-component systems that have been shown to regulate virulence in diverse bacteria (32,49) through various mechanisms such as the induction of capsule synthesis that is involved in the formation of biofilms. Additionally, RcsCDB is a global regulator, regulating about 5% of the *E. coli* genome (39,94). In EHEC over expressing Rcs CBD increased the production of *espB*. Experiments in which *LEE* genes were over expressed in RcsB mutants suggest that RcsB is an inhibitor of *LEE* genes expression. Hence, the RcsB Protein is projected as both, a positive and negative regulatory protein depending on other factors. GrvA mediates the positive effect.

The negative effect is mediated by PchA, which in itself is a positive reguator of Ler (89). The Rcs system on the other hand has also been shown to negatively regulate the FlhD/FlhC master regulator and is an excellent example of co-regulation of both the LEE and flagellar genes in EHEC (26,89).

In conclusion, though the direct interaction of flagellar and virulence genes has not been established, the presence of myriad common regulators cannot be neglected. Future research in this direction shall help in understanding the role of motility in virulence of *E. coli* O157:H7, thereby enabling the public health professional to enforce effective preventive measures to restrict the spread of the organism.

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PAPER 2. FLHC REGULATES CELL DIVISION, BIOFILM FORMATION AND VIRULENCE IN *ESCHERICHIA COLI* O157:H7 GROWN ON MEAT

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ABSTRACT

In an attempt to understand the continuous problems that *Escherichia coli* O157:H7 causes as a food pathogen, this study assessed global gene regulation in bacteria growing on meat. Since FlhD/FlhC of *E. coli* K-12 laboratory strains was previously established as a major control point in transducing signals from the environment to a number of cellular processes, this study compared the expression pattern of an *E. coli* O157:H7 parent strain to its isogenic *flhC* mutant. This was done in bacteria that had been grown on the surface of meat. Microarray experiments revealed 287 putative targets of FlhC. Real-time PCR with 15 representative genes confirmed 87% of these as FlhC regulated. An additional 13 genes whose gene products belonged to similar functional categories were tested with real-time PCR and also appeared to be regulated by FlhC. The twenty six FlhC regulated genes are representatives of cellular functions, such as central metabolism, cell division, biofilm formation, and pathogenicity. Physiological experiments were performed and confirmed that FlhC reduced the cell division rate, the amount of biofilm biomass, and pathogenicity in a chicken embryo lethality model. Altogether, this study has provided valuable insight into the complex regulatory network of the pathogen that enables its survival under various environmental conditions. This information may be used to develop strategies that could be used to reduce the number or pathogenicity of *E. coli* O157:H7 on meat by interfering with the signal transduction pathways.

INTRODUCTION

Escherichia coli O157:H7, belonging to the enterohemorrhagic group of *E. coli* (EHEC), causes nearly 70,000 cases of human infection annually in the US (31). Symptoms of infection range from bloody diarrhea to urogenital infections and can involve renal failure and meningitis. The severe forms of the disease result from the bacteria's capability to produce Shiga toxin (51). Infection in cattle is usually asymptomatic, but cattle can serve as a primary reservoir for humans (22). Transmission occurs via consumption of contaminated meat or meat products, drinking contaminated water, or personal contact (16, 32, 52).

Protection of consumers from infection by *E. coli* O157:H7 through meat occurs at varies stages. At the production end, spraying the carcasses with a variety of chemicals, predominantly acids, is partially successful (5). At the consumer end, the Food Safety and Inspection Service (FSIS) recommend storage of meat at low temperature and thorough cooking before consumption. As a consequence of these measures, the number and frequency of outbreaks and meat recalls has declined steadily between 1994-2005 (FSIS). Still, recalls and outbreaks occur and *E. coli* O157:H7 remains a safety hazard in the kitchen (1, 2).

Predominant amongst the reasons for the incomplete effectiveness of the acid sprays is the fact that bacteria develop resistance (11) against any such antibacterial substances that aim at reducing their growth rate. In the case of acid resistance, this is particularly troublesome because bacteria now find it easier to survive? the acid barrier of the stomach (31, 44). As a novel research direction, impacting the pathways that bacteria use to transmit signals from the environment

to the regulation of gene expression and, consequently, certain cellular processes may be less likely to induce resistance.

As one example of such a signal transduction pathway, early research by our lab has shown that extracellular serine, added to *E. coli* K-12 grown into mid-exponential phase on mixed amino acids, simultaneously increased the cell division rate and decreased the synthesis of flagella (38). The signaling transduction cascade involved the formation of acetyl phosphate (39), the phosphorylation of the response regulator of the osmoregulation two-component system, OmpR (35), and the inhibition of *flhDC* expression by phosphorylated OmpR (47). The *flhDC* operon encodes the FlhD/FlhC transcriptional activator complex that was initially described as a flagella regulator (4). Later research demonstrated that FlhD/FlhC was actually a global regulator, regulating a myriad of cellular processes in *E. coli* K-12 (36, 41), including cell division (37, 38). Gene regulation studies suggested a role for FlhD/FlhC in regulation of anaerobic respiration and sugar acid degradation via the methyl accepting chemotactic protein Aer (41). Later on, phenotypic studies established an effect of Aer on colonization of the mouse intestine (20).

In continuation of the gene regulation studies, Prüß and coworkers summarized a network of transcriptional regulation, centered around FlhD/FlhC (40). The network included many genes encoding ceil surface organelles that are characteristic of the different stages of biofilm formation, as well as their regulators. The study hypothesized the importance of the master regulator FlhD/FlhC in yet another important aspect of bacterial survival, biofilm formation. The effect of

components of the network on biofilm formation was confirmed by a high-throughput study, investigating the environmental and genetic factors that determine biofilm amounts (42).

In an attempt to investigate similar regulatory networks in pathogenic *E. coli* O157:H7, literature pertaining to individual regulation studies was reviewed and a network was constructed (49). Two very intense centers of regulation were found. The first of these was the locus of Enterocyte Effacement (LEE), a chromosomally located pathogenicity island in *E. coli* O157:H7. The locus comprises many genes involved in attachment, type III secretion, and the genes encoding intimin (*eaeA*) and its trans-located receptor (*tir*) (34). The second center of intense regulation was observed around the *flhDC* operon. The network, however, lacked any indication of a regulatory connection between the LEE pathogenicity genes and FlhD/FlhC.

Understanding the importance of FlhD/FlhC as an integrator between environmental signals and cellular responses, this study investigated gene regulation by FlhD/FlhC in *E. coli* O157:H7 on meat. We carried out a series of microarray experiments, followed by real-time PCR analysis and comparison of the expression level of genes in a *flhC* mutant with its isogenic *E. coli* O157:H7 parent. This was done in *E. coli* growing on the surface of meat over a time period of 10 days at a temperature of 10°C. This study provided the first evidence that FlhC regulates genes in *E. coli* O157:H7, grown on meat. The regulated genes that will be discussed in this study include genes of cell division, biofilm formation, and the LEE pathogenicity island. Phenotypes relating to cell division, biofilm formation,

and pathogenicity were consistent with gene regulation. The implications of these findings in the context of the development of antimicrobials will be discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and storage conditions

Bacterial strains used in the study are listed in Table 1. *E. coli* O157:H7 and its isogenic mutant *flhC* (14) were kindly provided by Scott A. Minnich (University of Idaho, Moscow ID). Resistance towards streptomycin sulphate (1000 µg/ml; Sigma-Aldrich, MO, USA) was introduced into the parental strain (PS1) and the *flhC* mutant (PS2), using a previously established technique (6). PS1 was also made resistant towards nalidixic acid (Sigma-Aldrich) at a concentration of 50 µg/ml (PS1).

Designation	Relevant Genotype	Reference
Strains		
ATCC 43894	*E. coli* O157:H7	American Type Culture Collection (ATCC)(14)
E. coli O157:H$^-$	*flhC*	(12)
PS1	*E. coli* O157:H7 str^R nal^R	This study
PS2	*E. coli* O157:H str^R	This study
PS3	PS2 pXL26	This study
Plasmids		
pXL26	pT7-7 *flhC*	(30)

Table 1: Strains and plasmids used in this study. The designation H$^-$ refers to the lack of the H7 flagellin due to the *flhC* mutation, which renders the entire flagellar master regulator complex, FlhD/FlhC, dysfunctional.

PS2 was transformed with the FlhC expressing plasmid pXL26 (30) to yield PS3.

All strains were stored as freezer stocks at -80°C in 8 % dimethysulfoxide (DMSO).

The cultures were plated onto Luria Bertani plates (LB; 1% tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar) prior to use, and incubated overnight at 37°C.

Comparing gene expression between the parental strain and the *flhC* mutant on the surface of meat

The combined experimental design of the microarray and real-time PCR experiments is outlined in Fig. 6, the following sections describe the individual steps (1 through 10) of the procedure.

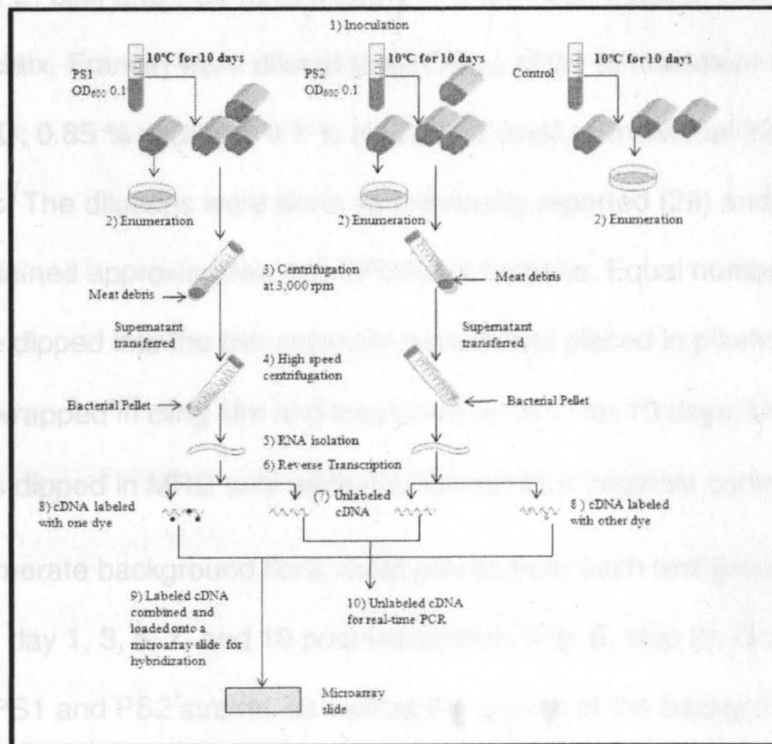


Figure 6: Diagrammatic representation of the protocol followed to obtain microarray data.

Preparation and inoculation of the meat pieces

Beef strip loin was purchased from a local supplier and transported to the lab within an hour of purchase. The meat was processed by trimming excess fat from the surface and surface sterilized by dipping in boiling water for 30s. The sterilized meat was re-trimmed aseptically to remove the outer layer. The meat was then cut into uniform pieces of about 10 cm length, 1 cm width, and 1 cm thickness.

For the inoculation of the meat pieces (Fig. 6, step 1), overnight cultures of PS1 (parent *E. coli*) and PS2 (*flhC* mutant) in brain heart infusion broth (BHI; BD, LePont de claix, France) were diluted to an OD_{600} of 0.1 in maximum recovery diluent (MRD; 0.85 % peptone, 0.1 % NaCl) and used as individual inocula for the meat pieces. The dilutions were done as previously reported (29) and the final inocula contained approximately 10^6 CFU/ml of bacteria. Equal number of meat pieces were dipped into the two separate inocula and placed in plastic trays. The trays were wrapped in cling film and incubated at 10°C for 10 days. Un-inoculated meat pieces dipped in MRD only were maintained as a negative control.

To enumerate background flora, meat pieces from each test group were sampled on day 1, 3, 5, 7, and 10 post-inoculation (Fig. 6, step 2). Growth of the inoculated PS1 and PS2 strains, as well as the growth of the background flora that is characteristic for beef was determined. Sampling involved homogenization of 20 g of meat sample in 80 ml of MRD, using Stomacher® 400 circulator (Seward, NY, USA), followed by serial dilution and plating onto different media plates for enumeration. PS1 and PS2 numbers on the meat pieces were established by plating onto Sorbitol MacConkey Agar (SMAC; BD), to which streptomycin was

added at a concentration of 1000 μg/ml. The population of *Enterobacteriaceae* on meat was established using Violet Red Bile Glucose Agar (VRBGA; OXOID, Hampshire, UK), *Brocothrix* by using Streptomycin-Thallous Acetate-Actidione agar (STAA; OXOID), heterofermentative *Lactobacillus* by using All Purpose Tween Agar (APT; BD), and *Pseudomonas* by using *PSeudomonas* agar (PS; OXOID). Additionally, the total bacterial count was determined, using standard Plate Count Agar (PCA; BD).

RNA isolation and cDNA synthesis

The meat pieces inoculated with PS1 and PS2 were homogenized separately after an incubation period of ten days as described above. The eluents from the homogenized samples were collected in sterile bottles. Stop solution (5 % phenol in ethanol) was added at a ratio of 1:10, to inhibit further bacterial growth and degradation of mRNA. The sample was then pelleted at 3,000 rpm to sediment the remaining meat debris (Fig. 6, step 3). The resultant supernatant was subjected to high speed centrifugation to pellet bacterial cells (Fig. 6, step 4). The bacterial pellets hence obtained from the two inocula were marked, flash frozen over a mixture of dry ice and acetone, and stored at -80°C for a maximum of 10 days. The pellets were thawed as required for RNA isolation.

RNA for microarray experiments was isolated from pellets of three independently grown bacterial cultures per strain (Fig. 6, step 5). For the first real-time PCR experiment, the RNA samples were used that were produced for the microarray experiment. One additional RNA sample was produced from each strain to complete the real-time PCR data set. Isolation of RNA was done using the

previously established hot phenol-sodium dodecyl sulfate method (12). Briefly, one phenol, one phenol/chloroform and one chloroform extraction was performed, followed by isopropyl alcohol precipitation. RNA was treated with DNaseI twice and passed through RNeasy mini columns (Qiagen, Los Angeles, MD, USA) for a final clean-up. RNA was reverse transcribed to cDNA (Fig. 6, step 6), using a previously validated protocol by Patrick O. Brown (http://www.cmgm.Stanford.edu/pbrown/protocol/aadUTPCoupling Procedure.html). For real-time PCR, cDNA was prepared using unlabeled dNTPs (Fig. 6, step7). cDNA was stored at -20°C. For the microarray experiment, RNA was reverse transcribed using amino-allyl labeled dUTP (Fig. 6, step 8), followed by labeling with Alexa Fluor™ 555 (Invitrogen, CA, USA) or Alexa Fluor™647. For each experiment, the cDNA samples from the two different bacterial strains were labeled with different dyes. The labeled cDNA samples were vacuum dried individually in a Vacufuge™ (Eppendorf, NY, USA) and stored at -20°C.

Microarray experiment

The *E. coli* O157 OciChip™ from Ocimum Biosolutions (Hyderabad, AP, India) was used. The chip consists of 6,336 spots and covers the genomes of three strains, *E. coli* K-12 MG1655, *E. coli* O157:H7 EDL933, and *E. coli* O157:H7 VT2-Sakai. Each spot is comprised of a 50 mer oligonucleotide, which is representative of one protein coding open reading frame. The three strains used for the design of the microarray chips share a common backbone of roughly 3,500 genes. The array hence includes these common genes, as well as genes that are unique to each

strain. As one example, the genomes of the two *E. coli* O157:H7 strains contain the LEE pathogenicity island.

Prior to the hybridization, two differentially labeled cDNA samples (derived from the two bacterial strains) were re-suspended separately in 60 μl of the hybridization buffer (Ocimum Biosolutions) and preheated at 42°C for 10 min. The two suspensions were then combined to form the hybridization mixture (Fig. 6, step 9). The hybridization mixture was heated for 3 min at 95 °C, cooled on ice, and briefly centrifuged. This mixture was then loaded onto the slides in a Hyb4 hybridization station (Genomic Solutions, Ann Arbor, MI, USA). After hybridization at 42 ºC overnight, the slides were washed for 5 min each in wash buffer 1(2x SSC, 0.1 % SDS) and wash buffer 2 (1x SSC), and 1 min in wash buffer 3 (0.1x SSC). A total of five such hybridizations were performed from the three independently grown bacterial cultures. All slides were scanned with a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA) at 635 nm and 532 nm. The voltage of the photomultiplier tubes was adjusted such that maximal signal could be obtained with minimal loss due to bleaching of the dye.

Analysis of the individual slides was done using the GenePix Pro 6.0 software package from Axon (Molecular Devices). Median intensities were used after background subtraction. Pixel values for the parental strain were divided by those for the mutant to obtain expression ratios. Data were imported into Microsoft Excel. Ratio based normalization was performed across the five arrays, using the average expression ratios for each array. We considered genes expressed higher in the parent strain than in the mutant when the expression ratio was higher than 2

and expressed higher in the mutant than in the parent strain when the expression ratio was lower than 0.5. This is consistent with current literature (3, 26). Genes that were lacking more than two data points out of the five replicate experiments were excluded from the analysis. Statistical testing was completed using the *z*-test (http://www.helios.bto.ed.ac.uk/bto/statistics/tress4a.html) that evaluated whether the means of the expression ratios is statistically different from a set number. In our case, this set number is 1, indicating genes that are not regulated. Genes that had a *p*-value of 0.05 or less were considered putative targets of FlhC and further investigated using real-time PCR. The complete list of putative targets will be deposited on Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/). Table 1 lists the selection of putative target genes that are discussed in this study.

Real-time PCR

Unlabelled cDNA samples from PS1 and PS2 grown on meat were used for this part of the experiment and tested in three replicate reactions (Fig. 6, step 10). The primer couples to quantify the selected genes were designed using BeaconDesign 3.0 (Premier Biosoft International, CA, USA) and are listed in Table 2.

	Gene	Forward Primer	Reverse Primer
1	*mdh*	5'-GGATGAAAGTCGCAGTCCTC-3'	5'-TGATTTTCACACGCAGTAGGGA-3'
2	*cyoA*	5'-GTAATTCTGCGCTGTTAGATCC-3'	5'-GTTTCATTGGGTAATTCAAGC-3'
3	*sdhA*	5'-GAACCGCTGGAACAATAATC-3'	5'-CTCGCTGGAAGTGTCATC-3'
4	*ompF*	5'-TCTACCTGGCAGCGAACTAC-3'	5'-ATCAACATCACCGATACCTTCTAC-3'

5	*ompC*	5`-TTGGCGGATCTATCACTTATG-3`	5`-GAGTTGCGTTGTAGGTCTG-3`
6	*oppB*	5`-CGGGCGGTGGCTGGAATG-3`	5`-TGGCGATATAGGCGAGTGACAAC-3`
7	*oppC*	5`-TGGATATGGCTCGTATTGTG-3`	5`-CCAGAAGTGATGCGTAGAC-3`
8	*ftsY*	5`-AAGCGGCGGAAGAAGAAG-3`	5`-CGGAGGAACCACCATCAC-3`
9	*ftsZ*	5`-GACCTGCGTCTGGATGAG-3`	5`-CTGCTGAACCTGCTTATTGG-3`
10	*ftsK*	5`-TTGCTCCACAGTCTACATAC-3`	5`-CGCTTCTCTTCAACTTCTTC-3`
11	*minC*	5`-CCGAATTGATTGCCGATGG-3`	5`-TTCACCTGCGATGGACAC-3`
12	*clpX*	5`-ACCACCTGGACGATTACG-3`	5`-ACCGATCAGCAGAATGTTAC-3`
13	*tir*	5`-GCACCTCCATTACCTTCAC-3`	5`-CGCCAGAATCAGCCATAG-3`
14	*escN*	5`-GCTACTAACTTGCGGTATTGG-3`	5`-AGCGTGGATTGAGGTAAGAG-3`
15	*eaeA*	5`-ACGGTAATAAGAAGTCCAGTGAAC-3`	5`-CAACAGGCGGCGAGTCTC-3`
16	*escV*	5`- CGCCATTACGAGCATTAGTG-3`	5`-CTGTATTGTCTGTCGGTGATG-3`
17	*escC*	5`-GCGGAACATTGCTGAGAGTG-3`	5`-CGATTGATTGCCATCCTGAATATC-3`
18	*escQ*	5`-TGCCGTTGTTGCCAGTTATC-3`	5`-CGAATACCATGACCGCTGAC-3`
19	*escR*	5`-TTGTTGGTATTGGTACTTCATTCC-3` ´	5`-CGGTATAATTGATTGGTTCCTGAG-3`
20	*grlR*	5`-TTATTAGCAATGAAGACTCCTGTG-3`	5`-GTTTAGCACCGAGGGAATTTC-3`
21	*grlA*	5`-CCTTGAGGCGGTTCCGATAG-3`	5`-GTCCCACAATACCATTACCAACTC-3`
22	*ler*	5`GGATATACTAATGTGCCTGATG-3`	5`-TCTTCATTCCATTCAACAGTG-3`
23`	*cesA*	5`-CGCCGAATTTGATGTCGTAAAAG-3`	5`-TGCTGGACTCAGTGTCTCTATTAG-3`
24	*espZ*	5`-TCGCACCGTTAGAGTTATAGC-3` ´	5`-CGCCAACCGCAGTAAGAG-3`
25	*rcsF*	5`-CGATACATACAGCCTGACGATAG-3`	5`-ACTCTCCGCCGAGCATTC-3`
26	*rcsB*	5`-TTGAAGACTCTACAGCACTGATC-3`	5`-TCCAATACCGCACTAAGAATCG-3`
27	*wcaB*	5`-CGTCAATATGGGTCAAAGAATAAC-3`	5`-TCCTGGTTGGTGTGGTAATC-3`
28	*wcaD*	5`-ATTGATGATTGCCTTAGGGATTTG-3`	5`-CGTAATATCTTGCGATGGTTGTG-3`

Table 2: Primer sequences of primers used in real-time PCR.Gene sequences were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Primers were designed with BeaconDesign 3.0 (Premier Biosoft International, CA, USA).

The real-time PCR was performed using iQ ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA). The reaction mixture contained 10-100 ng cDNA

from one of the two strains, 50 mM KCl, 20 mM Tris-HCl pH 8.4, 0.625 U iTaq DNA polymerase, 0.2 mM of each dNTP, 3 mM MgCl2, SYBR Green I, 10 nM fluorescein and 100 nM of each primer. The PCR reaction was performed in an iCyclerQ™ (BioRad), using the following cycling conditions: 95ºC for 3 min, followed by 50 cycles of 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min. The reactions were concluded at 72°C for 10 min.

Melting curves for each reaction were obtained and yielded single peaks for all primer couples. The absence of genomic DNA was determined by subjecting the original RNA samples to the above described procedure. Additionally, dilutions of cDNA samples were analyzed as controls. For all samples, a 2 fold dilution yielded a difference in the threshold cycle (Tc) of 1.

To normalize real-time PCR data, various techniques are routinely employed (56). For our experimental purposes, we used the $2^{-\Delta\Delta Tc}$ technique after normalization with two housekeeping genes. The genes *ompX* and *gapA* were selected as housekeeping genes after screening a total of five genes for the apparent lack of regulation by FlhC under the conditions of our experiment. The average difference in Tc was determined for *ompX* and *gapA* (ΔTc house). Differential threshold crossings (ΔTc gene) were calculated for each tested gene by subtracting the Tc of the mutant strain from that of the parent. ΔTc gene values were normalized by subtracting ΔTc house from ΔTc gene to yield ΔΔTc. Expression ratios were then calculated as $2^{-\Delta\Delta Tc}$. This was done for each individual replicate. For each gene, the average of the expression ratios was determined across the replicate experiments. The standard error is indicated in Figure 7.

Comparing phenotypes between the parental strain and the *flhC* mutant

Biofilm and cell division

The estimations of biofilm amounts and bacterial cell numbers (indicative of cell division) were done in a combined experiment on 96 well plates. Three independent overnight cultures (biological replicates) of PS1 (parent), PS2 (*flhC* mutant) and PS3 (PS2 transformed with FlhC expressing plasmid pXL26) were diluted 1:100 in beef broth (0.3 % beef extract, 0.5 % peptone). 100 µl of the diluted cultures were dispensed into each well of a 96 well plate in such a way that five technical replicates of each of the three biological replicates were achieved. The plate was incubated at 10 ºC for 10 days. On day 10, the supernatant from each well was carefully aspirated, serially diluted, and plated on SMAC-strep (BD) agar plates to obtain cell counts. Cell counts were reported as CFU/ml. The biofilms that formed at the bottom of the wells were then quantified, using the previously established ATP assay (48). Briefly, biofilms were washed twice with phosphate buffered saline (PBS) in order to remove any residual media components. The biofilms were air dried and quantified using 100 µl of BacTiter Glo™ reagent (Promega, WI, USA). The biofilms were incubated with the reagent for 10 min at room temperature and the bioluminescence was recorded with a TD 20/20 luminometer (Turner Design, CA, USA). The bioluminescence was reported as relative lux units (RLU).

Electron microscopy of biofilms

Biofilms were processed for scanning electron microscopy as described (48). Biofilms from PS1 and PS2 were grown for 10 days at 10°C in beef broth separately on glass slips (Assistant, Niderau, Germany), air dried and fixed in glutaraldehyde. The fixed biofilm was subjected to graded alcohol dehydration and coated with gold-palladium. Images were obtained using JEOL JSM-6490LV (JOEL ltd, Tokya, Japan) scanning electron microscope at 1,000-x magnification.

Chicken embryo lethality assay (ELA)

Overnight cultures of PS1 and PS2 grown in beef broth were centrifuged and washed twice with PBS. The bacterial pellets were re-suspended in PBS and diluted to a range of 100-300 CFU/ ml. 100 µl of this dilution was used as inoculum.

Pathogen free chicken embryos (Sunrise Farms, Inc, NY, USA) were incubated in a Sportsman incubator, model # 1502 (G. Q. F. MFG. CO, GA, USA) at 37 ºC for twelve days. The humidity level of the incubator was maintained at 86 %. On the twelfth day, the allantoic fluids of the embryonated eggs were inoculated with the bacterial suspensions, each containing 10 to 30 bacteria. Each strain was tested in four independent experiments in test groups of fifteen embryos, totaling 60 embryos per bacterial strain. The previously described (18, 50) avian *E. coli* strains V1 and A4 served as positive and negative controls, respectively. Additional negative control groups of either un-inoculated embryos or embryos inoculated with PBS were also maintained. All embryos were candled once a day

for three consecutive days and the number of dead embryos on each day was recorded. The lethality was reported as the percentage of dead embryos across the total of 60 embryos. This was done for each strain and the un-inoculated and PBS inoculated controls.

Due to the discontinuous nature of percentage data and the fact that it is not normally distributed, percent lethality was subjected to logistic regression analysis. Odds were calculated for experimental strains as (p/ (1-p)) with p being the percentage lethality determined by ELA. Odds ratios across multiple experiments were determined, using the PS1 strain as baseline reference strain for the logistic regression. Lethality by the tested strains was also compared using the Duncan's Multiple Group Comparison, a test that determines the statistical significance of differences in lethality between the test groups. All statistical analysis was performed on SAS v 9.1 (SAS Institute Inc., NC, USA).

RESULTS

FlhC mediated gene regulation in *E. coli* O157:H7 growing on the surface of meat was investigated using microarray experiments. The putative target genes were confirmed using real-time PCR. Additional, functionally related genes were also tested. Three different phenotypes were determined and correlated with the observed gene regulation. The following sections report the experimental and statistical results of the outlined experimental procedures.

Determination of bacterial flora on meat

Residual bacterial spoilage flora on surface sterilized meat was determined prior to the microarray experiments. The ability of our inocula (PS1, PS2) to grow within this bacterial community was investigated in an independent experiment. Meat pieces that were either un-inoculated or inoculated with PS1 or PS2 and incubated at 10 °C for 10 days (Fig. 6, step 1) were homogenized in a stomacher, serially diluted and plated onto different selective media plates for enumeration (Fig. 6, step 2). The un-inoculated control sample was monitored for background flora, as well as *E. coli* O157:H7 contamination. The PS1 and PS2 inoculated meat samples were tested for *E. coli* O157:H7 only. Sample enumerations generated from one of the two replicate experiments are depicted in Table 3.

The un-inoculated control sample had a total bacterial count of 8.7×10^{10} CFU/gm. The bacterial counts obtained from the selective media (1.25×10^{10} for *Brocothrix*, 5×10^{10} for heterofermentative *Lactobacillus*, 1.25×10^{10} for *Pseudomonas*) added up to approximately this number. The total bacterial count of

the meat sample inoculated with PS1 was 1.25×10^{11} CFU/gm, meat inoculated with PS2 had a total bacterial count of 1.37×10^{11} CFU/gm. Adding the background flora count obtained from the control group (8.7×10^{10} CFU/gm) to the PS1 (1.25×10^{10} CFU/gm) and PS2 (5×10^{10} CFU/gm) count yielded the approximate total bacterial count for the respective group.

Tested bacterial species	Inoculum		Control
	PS1 (parent)	(PS2) *flhC*	
Naturally occurring bacteria on beef			
Total bacterial count [1]	1.25×10^{11}	1.37×10^{11}	8.7×10^{10}
Enterobacteriaceae [2]	ND	ND	0
Brocothrix [3]	ND	ND	1.25×10^{10}
Heterofermentative *Lactobacillus* [4]	ND	ND	5×10^{10}
Pseudomonas [5]	ND	ND	1.25×10^{10}
Inoculated bacteria			
E. coli O157:H7 [6]	1.25×10^{10}	5×10^{10}	0

Table 3: Sample enumeration of meat flora, with and without inoculation.[1] The total number of bacteria was determined with the standard Plate Count Agar (PCA).[2] Enumeration of *Enterobacteriaceae* was done using the Violet Red Bile Glucose Agar (VRBGA).[3] Enumeration of *Brocothrix* was done using the Streptomycin-Thallous Acetate-Actidione Agar (STAA) (17).[4] Heterofermentative *Lactobacillus* were enumerated using the All Purpose Tween agar (APT) (15).[5] *Pseudomonas* enumerations were done using the PSeudomonas agar (PS).[6] *E. coli* O157:H7 was enumerated on Sorbitol MacConkey agar (SMAC). ND: Not done

In *E. coli* O157:H7 growing on the surface of meat, FlhC regulates genes responsible for cell division, biofilm formation and pathogenicity

The previous experiment had established that our inocula (PS1 and PS2) were able to grow on meat and represented 10 to 50% of the total bacterial flora. Determination of genes that were regulated by FlhC in *E. coli* O157:H7 growing on

meat was done next. The following section outlines the results obtained from the microarray experiments and the real-time PCR.

The microarray experiment hypothesized 287 genes as regulated by FlhC

The microarray experiment was done to determine FlhC targets in *E. coli* O157:H7, when bacteria grow on the surface of meat. PS1 and PS2 were grown on meat as described in Fig. 1, mRNA and differentially labeled cDNA samples were obtained as described in Materials and Methods. mRNA levels for 287 genes were higher in the *flhC* mutant (PS2) than in the parental strain (PS1). The regulated genes were then divided into functional groups, based on information provided by the manufacturer of the microarray chips. This information is summarized in the online supplement. 287 genes were found to be regulated. 125 genes belonged to central metabolism, 11 genes were virulence and LEE pathogenicity related, and seven genes were related to cell division. Additionally, 34 of the regulated genes encoded transporters. Several biofilm related genes were also identified as a putative target of FlhC. Table 4 lists a selection of relevant genes belonging to six of the functional groups. Representative genes from each of these groups were selected and subjected to real-time PCR analysis for confirmation.

Real-time PCR confirmed that 87 % of the selected genes were regulated by FlhC

Real-time PCR was carried out to confirm putative FlhC targets obtained from the microarray experiments. A total of 15 representative genes were selected from

the functional groups listed in Table 4. The genes included the metabolic genes *mdh*, *cyoA* and *sdhA*, transporter genes *ompF*, *ompC* and *oppB*, cell division genes *ftsY*, *ftsZ*, *ftsK* and *minC*, LEE pathogenicity genes *tir* and *escN*, biofilm related genes *rcsF* and *wcaB,* and a protease encoding gene *clpX*. The results from the real-time PCR are represented in Fig.7A. Of the 15 tested genes, 13 (87 %) could be confirmed as regulated by FlhC. The regulation of *cyoA* and *sdhA* was border line, using 2 fold as a cutoff for regulated genes. Interestingly, the two genes (*tir* and *escN*) that are involved in virulence and located on the LEE pathogenicity island were the most highly regulated genes (10 to 30 fold). Genes from the remaining functional groups were between 2 and 5 fold inhibited by FlhC.

ORF[1]	Gene	Protein function[2]	Expression ratio[3]	Z-test (*p* values)[4]
Metabolism (125 genes total)				
b3236	*mdh**	Malate dehydrogenase	0.15	5.5×10^{-10}
ECS0753	*sucC*	Succinate dehydrogenase	0.17	2.6×10^{-5}
Z0877	*sdhA**	Succinate dehydrogenase	0.28	7.7×10^{-4}
b0430	*cyoA**	Cytrochrome o-oxidase	0.25	0.0032
b0432	*cyoC*	Cytrochrome o-oxidase	0.14	1.27×10^{-4}
Transport (34 genes total)				
Z1276	*ompF**	Outer membrane protein F	0.47	0.0098
Z3473	*ompC**	Outer membrane protein C	0.13	2.74×10^{-5}
ECS1744	*oppB**	Oligopeptide permease	0.38	0.0009
Z3570	*hisQ*	Histidine permease	0.39	0.0187
ECS5330	*osmY*	Hyperosmotically inducible periplasmic protein	0.20	6.1×10^{-4}

b4123	*dcuB*	Anaerobic dicarboxylate transporter	0.29	0.0548
Cell Division (7 genes total)				
b0039	*ftsW*	Membrane protein involved in shape determination	0.085	1.93×10^{-9}
ECS0975	*ftsK**	Cell division	0.14	8.06×10^{-9}
Z0105	*ftsZ**	Tubulin like GTP binding protein	0.43	0.0032
Z4333	*ftsY**	Cell division membrane protein	0.33	1.6×10^{-4}
b0924	*mukB*	Kinesin like cell division protein	0.093	7.7×10^{-7}
Z5336	*minC**	Inhibits *ftsZ* ring formation	0.30	0.057
ECS1670	*chpB*	Probable growth inhibitor	0.40	0.0062
LEE Pathogenicity Island (11 genes total)				
Z5119	*escN**	Type III secretion system	0.35	0.0047
Z5112	*tir**	Translocated intimin receptor	0.10	4.03×10^{-9}
Biofilm (2 genes total)				
b0196	*rcsF**	Regulator in colanic acid synthesis	0.38	0.0025
b2058	*wcaB**	Putative transferase	0.44	0.0087
Miscellaneous				
b0438	*clpX**	ATP-dependent specificity component of ClpX/ClpP complex	0.23	6.17×10^{-5}

Table 4: Genes representing the functional groups included in the study.[1]Open reading frames (ORF) are taken from *E. coli* K-12 MG1655 (7), *E. coli* O157:H7 EDL933 (34), and *E. coli* O157:H7 Sakai (19).[2]Protein functions were determined with the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg).[3] The expression ratio was determined, dividing pixel values of the parent strain by those of the *flhC* mutant for each experiment. The average over 5 experiments is presented.[4] z-tests was performed on the expression ratios obtained and ratios having p-value of 0.05 or less were selected. The asterisks behind gene designations represent those genes that were selected for real-time PCR.

Based on this observation, 13 additional genes were selected for real-time PCR analysis. These genes had not been determined as regulated by FlhC in the microarray experiment, but were either from the same operon or had functional similarity to the 15 genes that were previously tested (Fig. 7A). Included in this experiment were the transporter gene *oppC*, genes of the LEE pathogenicity island *ler*, *cesA*, *escR*, *grlR*, *grlA*, *escC* , *escV*, *eaeA*, *escQ* and *espZ*, and the biofilm related genes *rcsB* and *wcaD*.

The results from the real-time PCR with these additional genes are represented in Fig. 7B. All of the additionally tested genes were determined as being regulated by FlhC. Within the functional groups, regulation was similar to Fig. 7A. The LEE genes were highly regulated (10 to 30 fold), the two biofilm genes showed a borderline regulation of approximately 2 fold. It was concluded that FlhC may regulate crucial processes in *E. coli* O157:H7, such as cell division, pathogenicity, and biofilm formation, when the organism was grown on meat.

A comparison of phenotypes between the parental strain and the *flhC* mutant indicates differences in cell division, biofilm formation and lethality

The microarray and the real-time PCR indicated genes involved in cell division, biofilm formation and pathogenicity of *E. coli* O157:H7 as being targets of FlhC regulation. The following experiments were done to investigate the effect of the above confirmed regulation at the phenotypic level.

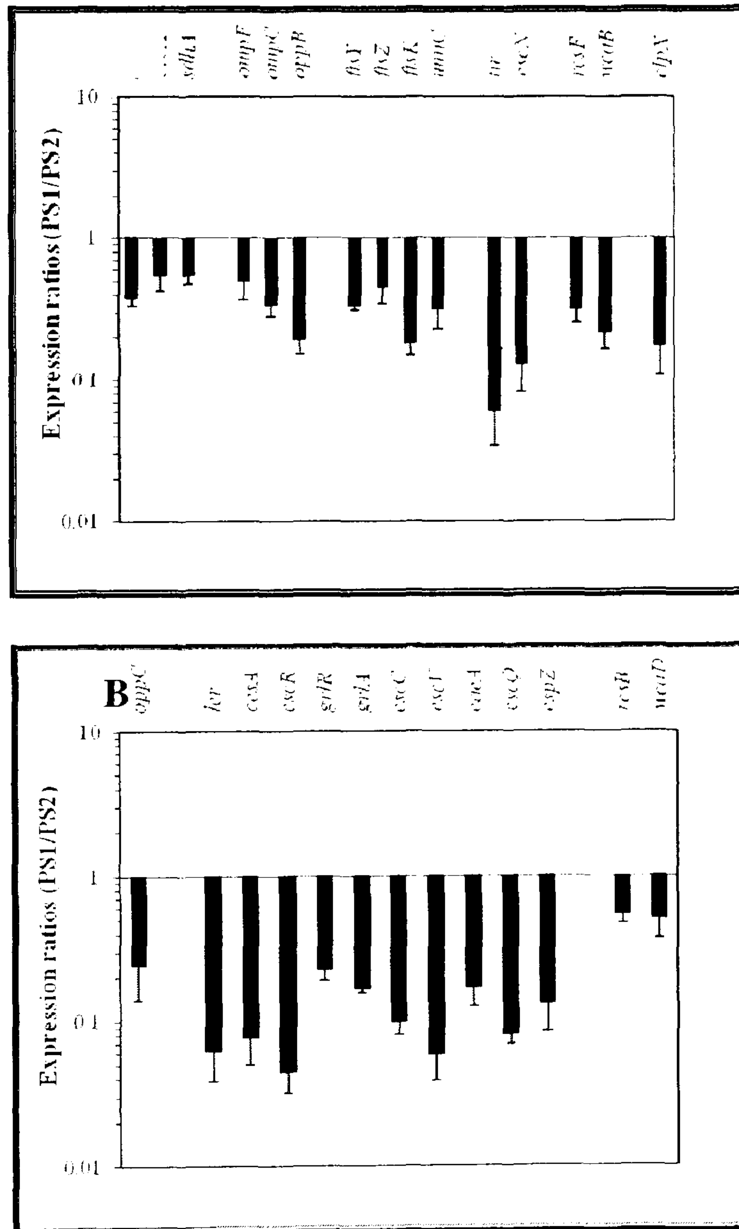


Figure 7: Real-time PCR. The regulation by FlhC of selected genes was tested with real-time PCR. The experiment was done three times each with cDNA from two independently grown bacterial cultures. The average was determined across all six data points. Standard errors are indicated.Panel A contains the genes that were hypothesized as regulated by FlhD in the microarray experiment. Fifteen of the genes that were found to be regulated by FlhC in the microarray experiment were subjected to real-time PCR. The different functional groups are separated by a blank on the x-axis. Panel B contains additional genes of transport, the LEE pathogenicity island, and biofilm formation. These were selected based on their location and functional similarity to the genes in Panel A.

Comparing cell division and biofilm formation between the parental strain and the *flhC* mutant

Overnight cultures of PS1, PS2 and PS3 were grown in 96 well plates for 10 days as described earlier. Bacterial cell numbers and biofilm amounts were determined in each well. The results for the cell count experiment are represented in Fig. 8A. At day ten, PS2 had divided to cell densities that were 20 times higher than the parental PS1 strain, indicating an inhibitory effect of FlhC on the cell division rate. Complementing PS2 with pXL26 (PS3) restored the phenotype of the mutant to almost that of the parental strain.

After removal of the supernatants from each well, the biofilm that was formed at the bottom of the each well was carefully processed and subjected to ATP assay. The ATP assay is an indirect measure of biomass and indicative of the number of cells that form the biofilm. The results for this experiment are represented in Fig.8B. At day 10, PS2 had formed approximately 5 times more biofilm than the PS1 strain, implying that a loss in FlhC was beneficial for biofilm amounts. Furthermore, PS2 complemented with pXL26 (PS3), showed about a five fold reduction in the ability of PS2 to form biofilm, restoring the phenotype of the *flhC* mutant to that of the parental strain. The electron micrographs of the biofilm formed by PS1 and PS2 (Fig. 9) at day 10 showed visual differences in the number of bacterial cells that had attached to the surface of the glass slips, corroborating the quantitative differences observed.

Comparing lethality of PS1 and PS2 with the ELA

The ELA assay was performed with PS1 and PS2 strains to investigate the phenotypic effect of FlhC mediated regulation of the LEE pathogenicity genes. The assay involved inoculation of 10-30 CFU of each bacterial strain into the allantoic fluid of embryonated eggs. Avian *E. coli* strains V1 and A4 were used as positive and negative controls, respectively. Additionally, embryos were inoculated with PBS and un-inoculated embryos and maintained as negative controls. The eggs were candled daily for 3 consecutive days post- inoculation and the number of dead embryos was noted. Fig 10 represents the percentage of dead embryos for all the tested strains, as well as the un-inoculated and PBS controls on day three.73 % of the embryos inoculated with PS2 were reported dead by the end of day three, exhibiting a similar lethality as that of the positive control strain V1 (78 %).

In agreement with this, Duncan's multiple group comparison sorted these two test groups into one group A (Fig. 10). The PS1 strain was less lethal as compared to PS2 and V1, and only 35 % of the embryos that had been inoculated with PS1 were reported dead. This strain formed group B in the Duncan's test. The odds ratio between PS1 and PS2 was 5.107, indicating that an embryo was roughly 5 times more likely to die if inoculated with the *flhC* mutant strain than with the parental *E. coli*. This lethality was comparable to the negative control strain A4 (23 %), though the two strains each formed their own Duncan's group (B and C, respectively). There was no lethality in the un-inoculated embryos and one embryo inoculated with PBS was reported dead.

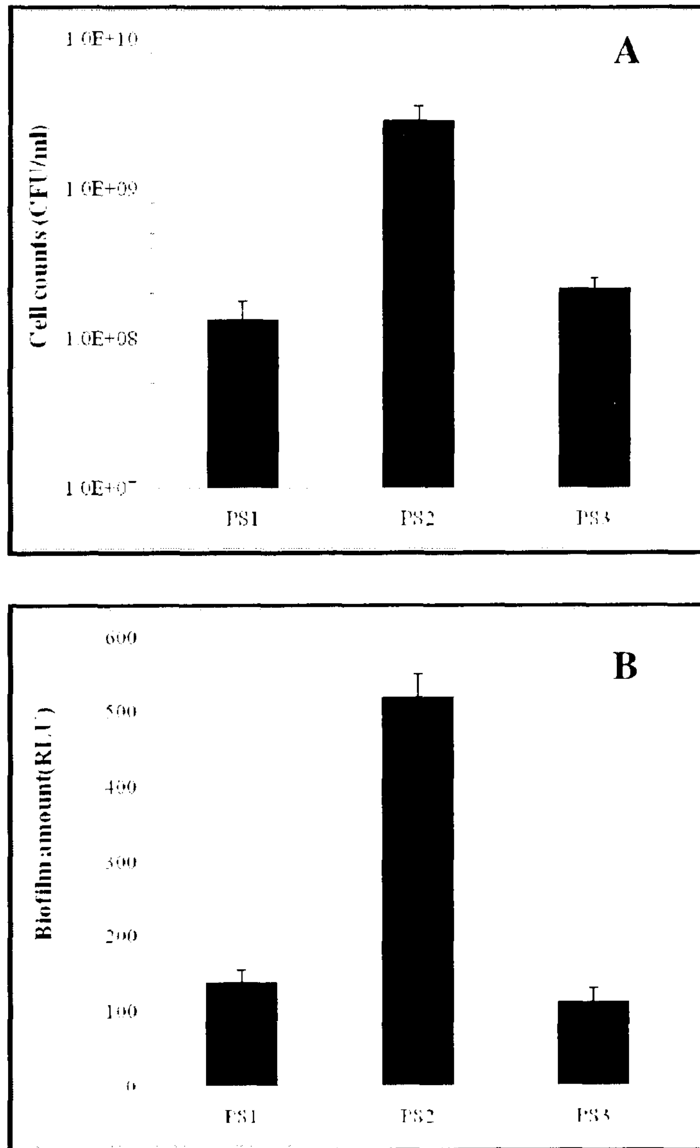
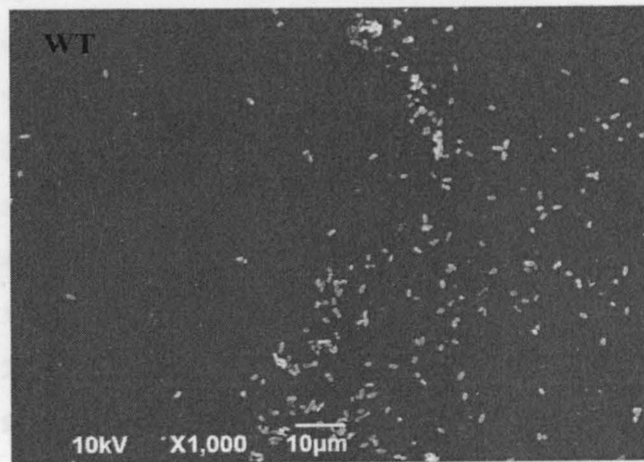


Figure 8: Cell division and biofilm formation. PS1, PS2 and PS3 were grown in beef broth on 96 well microtiter plates at 10°C for 10 days. The growth medium was carefully aspirated; serial dilutions were plated on SMAC-strep agar plates to obtain cell counts. The cell counts were indicative of cell division and were reported as CFU/ml (Panel A). Biofilms were washed twice with PBS and quantified using the ATP assay. Biofilm amounts are given as RLU (Panel B). The experiment was done in five replicates from each of three independently grown bacterial cultures.



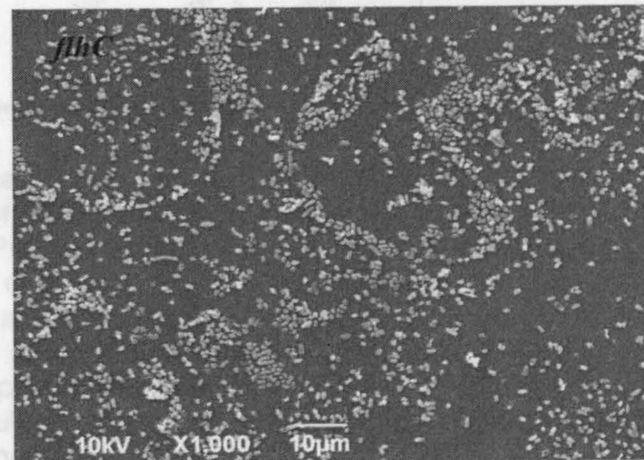


Figure 9: Scanning electron micrographs of biofilms. Biofilms formed by PS1 (Panel A) and PS2 (Panel B) on separate glass slides after 10 days of incubation at 10ºC. Micrographs were taken at a magnification of 1,000 x. One representative image is presented per strain.

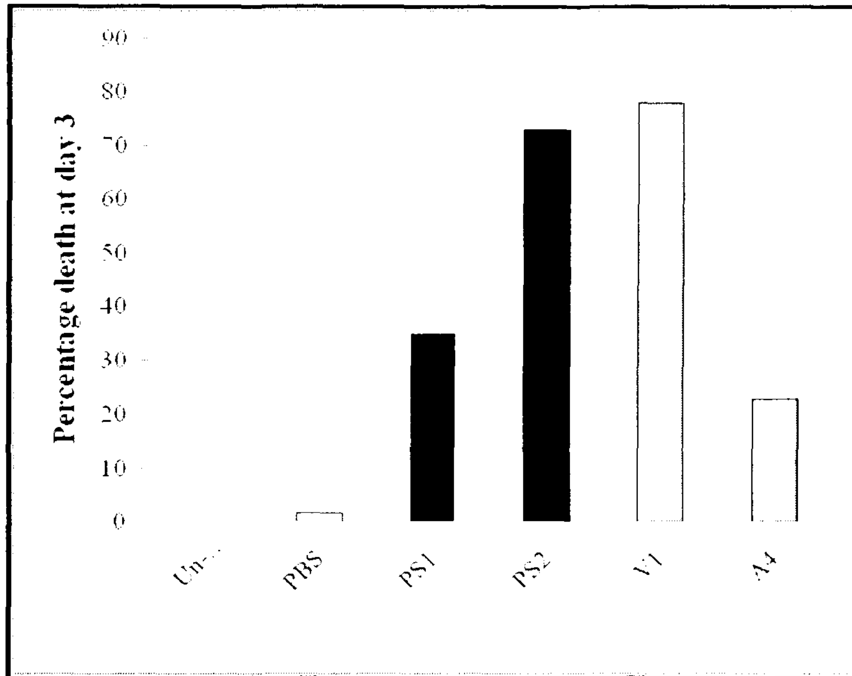


Figure 10: Lethality in the ELA. Test groups of 15 embryos were inoculated with 10-30 bacteria of PS1, PS2, V1, and A4. Additional test groups included un-inoculated and PBS inoculated embryos. The experiment was done four times. The percentage of dead embryos at day 3 post-inoculation was determined for each test group and calculated over all 60 embryos. Negative controls are printed in white bars, *E. coli* O157:H7 strains in black bars, and avian *E. coli* control strains in grey bars. Capital letters within the bars indicate the grouping that resulted from the Duncan's multiple range test, which was done for embryos only if they were inoculated with bacterial strains.

DISCUSSION

In the above described experiments, the effect of FlhC, one subunit of the global regulator complex FlhD/FlhC, on the expression level of various genes in *E. coli* O157:H7 was investigated in bacteria that had been grown on the surface of meat. The hypothesis was that FlhD/FlhC may play a crucial role in regulating the genes of the LEE pathogenicity island. In addition to the genes in the LEE pathogenicity island, we observed many other genes as regulated by FlhC. These genes were grouped into related cellular functions and shall be discussed in the following sections.

FlhC negatively regulates cell division in *E. coli* O157:H7

In the current study, it was demonstrated that FlhC negatively regulated seven cell division genes when *E. coli* O157:H7 was grown on meat at 10°C. The real-time experiments confirmed the regulation that was observed by microarray analysis and in phenotypic experiments carried out subsequently, it was observed that the *flhC* mutant divided to 20 times higher cell numbers as compared to its parental strain. The *flhC* phenotype could be restored back to the parental phenotype by complementing with the FlhC expressing plasmid pXL26.

Earlier studies by Prüß and coworkers had shown a similar effect on the cell division rate in several *E. coli* K-12 strains (38). However, this study was exclusively phenotypic and the corresponding regulation of the cell division genes was never demonstrated. Intriguingly, regulation of the cell division rate in the *E. coli* K-12 strains appeared to be mediated by the other subunit of the FlhD/FlhC

complex, FlhD. The disparity between *E. coli* K-12 and *E. coli* O157:H7 regarding regulation (or lack thereof) by FlhC may be attributed to the different functional properties of the FlhD and FlhC subunits within the heterohexameric (54) complex. The FlhC subunit is capable of binding to DNA alone, while FlhD can only bind to DNA in the presence of FlhC (13). However, binding of FlhC alone does not activate transcription, binding of FlhD to FlhC is a required step for transcription activation (10). Fig. 11 sums up a working model of regulation by FlhD and/or FlhC that includes potential substitute regulators that may bind within the same region of the promoter as FlhD/FlhC. The model is in agreement with the observation that the four promoters that were identified as regulated by FlhD and not FlhC in *E. coli* K-12 (41) were subject to complex regulation by numerous global regulators and the fact that *E. coli* O157:H7 has about a 1,000 genes in excess of the K-12 lab strains (19). We believe that regulation of the FlhD and/or FlhC regulated promoters may be determined by the ratio of multiple competing regulators, each of whose expression is in itself affected by numerous environmental conditions. In *E. coli* O157:H7, the study was restricted to a *flhC* mutant only. The possibility that expression of the FlhC regulated genes is affected by FlhD also cannot be excluded and warrants further study.

FlhC negatively regulates biofilm formation in *E. coli* O157:H7

Microarray and real-time PCR data demonstrated that FlhC down-regulates the expression of several biofilm related genes, such as *wcaB, wcaD, rcsF* and *rcsB*. In agreement with this, *flhC* mutants formed higher amounts of biofilm than the parent *E. coli* strain. Introduction of *flhC* into the mutant strain with plasmid

pXL26 resulted in a complemented strain with a phenotype similar to its parental type.

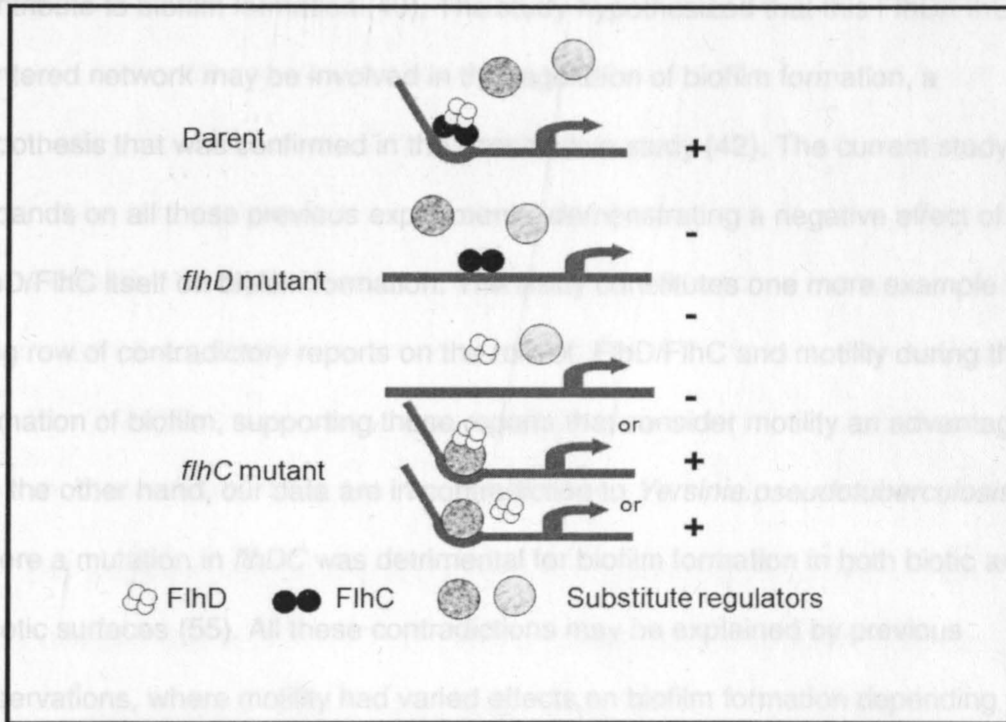


Figure 11: Working model for the regulation of the FlhD and FlhC target genes. In the parent strain, transcription would be activated by the $FlhD_4/FlhC_2$ complex. In the *flhD* mutant, FlhC would bind, without being able to activate transcription. In the flhC mutant, there could be several different scenarios. In the first scenario, FlhD alone would not bind, but neither would any of the other regulators. As a consequence, there would be no transcription and these targets would appear as regulated by FlhD and FlhC. In the second and third scenario, a substitute regulator may bind to the FlhD/FlhC binding site. With our without the help of FlhD, this would activate transcription. These targets would appear as regulated by FlhD and not by FlhC.

The increased biofilm amount of the *flhC* mutant was supported by electron microscopy (Fig. 9). Altogether, this study indicates that FlhC may be an inhibitor of biofilm formation, at least under the conditions of our experiment, and that was long-term growth at low temperature on the surface of meat.

A previous paper from the Prüß lab had summarized a network of regulation in *E. coli* K-12 that included the FlhD/FlhC complex and many genes that contribute to biofilm formation (40). The study hypothesized that this FlhD/FlhC centered network may be involved in the regulation of biofilm formation, a hypothesis that was confirmed in the consecutive study (42). The current study expands on all those previous experiments, demonstrating a negative effect of FlhD/FlhC itself on biofilm formation. The study constitutes one more example in a long row of contradictory reports on the role of FlhD/FlhC and motility during the formation of biofilm, supporting those reports that consider motility an advantage. On the other hand, our data are in contradiction to *Yersinia pseudotuberculosis*, where a mutation in *flhDC* was detrimental for biofilm formation in both biotic and abiotic surfaces (55). All these contradictions may be explained by previous observations, where motility had varied effects on biofilm formation depending on the surface on which biofilm forms (21). The precise condition under which the biofilms form has undoubtedly an effect on the observed gene regulation under each of these conditions. It is also possible that flagella constitute an advantage early during biofilm development and a disadvantage at later time points. This hypothesis would be in agreement with our previous study (42) and the current one, where the bacteria were collected from the meat samples after 10 days of incubation.

FlhC negatively regulates pathogenicity in *E. coli* O157:H7

The relationship between motility and pathogenicity has long been investigated in various bacterial species and led to contradictory results. While

flagella and motility have certainly been recognized as a virulence factor in many bacterial species, there are also examples where a non-motile species of a genus is more pathogenic than its motile counterpart. One such example is the genus *Yersinia*, where the non-motile *Yersinia pestis* is one of the most lethal bacteria on earth, while *Yersinia enterocolitica* is only a moderate pathogen. At a molecular level, three non-flagellar genes, *yplA* (46), *inv* (58), and *virF* (8), were described as part of the flagella regulon and activated by the sigma factor of the regulon, FliA. As an apparent contradiction, temperature regulation of many *Y. enterocolitica* flagellar (24, 25) and late virulence genes (8) appeared to follow an inverse pattern. This was mediated by temperature dependent expression of *fliA* (50).

The current study sheds new light into this apparent controversy; it was demonstrated that *E. coli* O157:H7 FlhC negatively regulates genes belonging to the LEE pathogenicity island. These genes were, in fact, the most highly regulated genes in our experiments. In an attempt to investigate the extent of this regulation, additional genes from the LEE pathogenicity island were selected and subjected to real-time PCR analysis that had not been hypothesized as FlhC regulated in the microarray experiment. 100 % of the selected LEE genes were confirmed as being negatively regulated by FlhC, indicating the possibility of FlhC regulating the entire LEE pathogenicity island. These data are consistent with *Y. enterocolitica*, where flagella and seven plasmid-encoded virulence genes were inversely regulated by FliA (20). They are also in agreement with observations made during colonization of the intestine, where the lack of flagella was discussed as a selective advantage (28). It can be hence implied that in environments where there is abundant

nutrition available and motility would not be required to move to more nutritious environments, the bacteria may be able to afford shutting down motility and turning on genes that aid colonization and pathogenicity. The surface of meat may just constitute one such nutrient rich environment.

FUTURE DIRECTIONS

E. coli O157:H7 has been a constant concern to the food industry. Various sprays and treatment methods have been employed with partial success to actively reduce the bacterial contamination from beef carcasses and food products (9, 23, 27, 33, 43, 45, 53, 57). This study observed that three vital cellular processes, cell division, biofilm formation, and pathogenicity, all were down-regulated by FlhC. Such an observation opens endless possibilities at simultaneously limiting these three cellular processes, via decrease of FlhC expression. The vast amount of information that is available about the regulation of *flhC* expression in response to the environment will, therefore, enable new control measures to protect consumers from infection by *E. coli* O157:H7.

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PAPER 3. A COMBINATION OF ASSAYS REVEALS BIOMASS DIFFERENCES IN BIOFILMS FORMED BY *ESCHERICHIA COLI* MUTANTS

Preeti Sule,[1] Tanush Wadhawan,[1] Nathan J. Carr,[1] Shelley M. Horne,[1] Alan J. Wolfe,[2] and Birgit M. Prüß. 2009. Lett. Appl. Microbiol. 49:299-304.

The experimental work reported in this paper was done by Preeti Sule.

ABSTRACT

The aim of this study was to develop an assay system that can quantify the amount of biomass in biofilms formed by different isogenic mutants of an *Escherichia coli* K-12 strain. The reported assay, which is based on the BacTiter-Glo™ assay from Promega, uses bioluminescence to detect the intracellular concentration of ATP, which correlates with viable bacterial cell numbers. The quantitative data obtained with this ATP assay were compared to those obtained with the conventional crystal violet assay. As a qualitative control, scanning electron microscopy was performed. The ATP assay, the crystal violet assay, and scanning electron microscopy yielded similar results for six of the eight strains tested. For the remaining two strains, the images from the scanning electron microscopy confirmed the results from the ATP assay. The ATP assay, in combination with other quantitative and qualitative assays, will allow us to perform genetic studies on the regulatory network that underlies the early steps in *E. coli* biofilm formation.

Keywords: biofilm, live biomass, quantitative assay, ATP, crystal violet, flagella, type I fimbriae, extracellular matrix

INTRODUCTION

Biofilms, sessile communities of bacteria encased in a matrix, exert a profound impact in many natural (2, 11) clinical (10, 15), and industrial settings (1, 8, 28). Biofilms develop on surfaces in a series of ordered steps mediated by surface organelles (18). In *Escherichia coli*, the first step, reversible attachment, is mediated by flagella. The next step is irreversible attachment. It requires type 1 fimbriae and the loss of flagella. The third step, formation of the mature biofilm, requires production of an extracellular polysaccharide matrix, such as colanic acid (for a review, see (26)). The coordinated expression of these surface organelles requires the involvement of several transcriptional regulators including FlhD/FlhC, OmpR, and RcsB (reviewed by (14)).

Successful dissection of this complex biofilm-associated regulatory network requires a quantitative assay that can complement microscopic visualization techniques. For this purpose, many assays have been developed (for a comparison, see (13)). For example, crystal violet (CV) and Syto9 detect extracellular matrix polymers, staining live and dead cells alike, while 2,3-bis (methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) and resazurin detect only live bacteria. Because of its wide range of applicability, the CV assay has been developed for high-throughput studies (22, 23). However, since it detects matrix, the CV assay may have limitations for studies, such as our own, that focus on the involvement of flagella and type I fimbriae in biofilm formation. The XTT assay also has limitations: it requires highly

respirative bacteria (16) and has been reported to experience intra- and interspecies variability (6).

ATP concentration is relatively constant across many different growth conditions (19). For this reason, ATP-based assays are used routinely to detect bacteria. For example, ATP-based assays have been used to monitor the effect of antimicrobials on the live biomass in biofilms (9, 17, 25). More specifically, the ATP-based BacTiter-Glo™ assay has been used to measure attachment in *Pseudomonas aeruginosa* biofilms (5). In this study, we compare the quantitative data obtained with the BacTiter-Glo™ assay with data obtained with the conventional CV assay. We then evaluate the performance of both quantitative assays by comparison to scanning electron microscopy (SEM).

MATERIALS AND METHODS

Bacterial strains and growth conditions

All bacteria used in this study were isogenic derivatives of the *E. coli* strain AJW678, which is wild-type for acetate metabolism and the biosynthesis of flagella, type 1 fimbriae, and colanic acid (7). The genes tested include *flhD*(flagellar master regulator, *flhD::kn*), *fimH* (fimbriae adhesin, *fimH::kn*), *ompR*(response regulator for osmoregulation, *ompR::Tn10*), *rcsB* (response regulator for capsule synthesis, *rcsB::kn*), *rcsC* (histidine kinase/phosphatase for capsule synthesis, *rcsC::Tn5*), *ackA* (acetate kinase, *ackA::TnphoA'-2*), and *ackA pta*(acetate kinase and phosphotransacetylase, Δ(*ackA pta hisJ hisP dhu*)). Strains were maintained at −80 °C in 15% glycerol. Bacteria were plated onto Luria Bertani plates (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and incubated overnight at 34 °C. Liquid overnight cultures were prepared in tryptone broth (TB; 1% tryptone, 0.5% NaCl).

Formation of biofilms for the quantitative assays

Bacteria were diluted 1:100 from overnight cultures into 100 µl of TB dispensed into a 96 well polystyrene microtiter plate. The microtiter plate was incubated without shaking at 37 °C for the indicated number of hours. The incubation period was 38 h for the single time point experiments and 2 to 48 h for the time course experiment. During this time, most bacterial strains formed a solid biofilm at the bottom of the well. As previously described (27), some strains also

formed a pellicle that covered the entire surface of the culture. To harvest both biofilms, we removed the culture medium carefully with a pipette. Biofilms were then rinsed carefully with 100 µl of PBS. In a final step of the biofilm preparation, the excess PBS was removed and the biofilms were air dried for 10 to 15 min.

For single time point experiments, each strain was tested inthree independent experiments, each of which included twelve replicates. The mean and the standard deviation were determined across the three independent experiments. For the time course experiment, wild-type bacteria and the isogenic *ompR* mutant were tested in three independent experiments of eight replicates each. Again, the mean was presented across all three experiments. For the single time point experiment, the wild-type strain AJW678 was used as a reference.

ATP assay

100 µl of PBS was added to each well containing biofilm prepared as described above. The bioluminescence reaction was started by the addition of 100 µl of BacTiter-Glo™ reagent (Promega, Madison WI; prepared according to the manufacturer's instructions) to each well. Incubation time was 5 min at room temperature. Bioluminescence was determined in a TN20/20 luminometer (Turner Designs, Sunnyvale CA) in single tubes. To assure greater consistency in incubation times, we processed only four wells at a time.

For the time course experiment, we also determined the ATP concentration in the unattached (planktonic) bacteria present in the liquid culture medium that overlays the biofilm that forms on the bottom of the wells. 100 µl of liquid medium

containing planktonic bacteria were removed from each well prior to the quantification of the biofilms. These were mixed with 100 µl of BacTitel-Glo™ reagent, incubated for 5 min, and measured in the TN20/20 luminometer.

Crystal Violet assay

Biofilms were stained with 100 µl of 0.1% CV in H_2O at room temperature for 15 min. Following incubation, the CV solution was removed and the biofilms were washed twice with PBS. To elute bound CV, 100 µl of a mixture containing 80% ethanol and 20% acetone was added to each well and the plate was incubated at room temperature for 20 min. Finally, the mixture was diluted 1:20 with 80% ethanol/20% acetone and the optical density was determined at 600 nm.

Statistical analysis of the quantitative data

For each quantitative assay, the values obtained with the eight strains were tested using Analysis of Variance (ANOVA). A *p*-value below 0.05 indicates that at least one of the strains has a mean that differs from the others. For those assays that yielded a*p*-value below 0.05, Dunnett's test was performed as a post-hoc test. The significance level alpha was set at 0.05. This test determines whether the mean of a specific strain differs from the wild-type. Statistical analyses were performed on SAS v 9.1 (SAS Institute Inc., Cary NC).

Electron microscopy

Biofilms were grown on glass cover slips of 12 mm diameter (Assistant, Germany) in 6 well plates in 4 ml of TB, inoculated 1:100 from the overnight

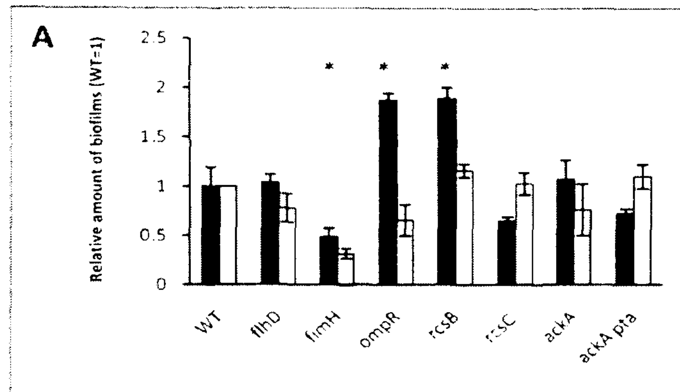
cultures. Biofilms were produced as described for the quantitative assays. The air-dried biofilms were fixed in 2 ml of 2.5% glutaraldehyde (Tousimis Research Corporation, Rockville MD) in 0.1 mol l^{-1} sodium phosphate buffer, rinsed once in the same buffer and then in deionized water. Biofilms were dehydrated, using a graded alcohol series (15 minutes each in 30%, 50%, 70%, and 90% ethanol in H_2O, twice for 15 min in 100% ethanol). Critical point drying was performed with an Autosamdri-810 critical point drier (Tousimis Research Corporation, Rockville MD) with liquid carbon dioxide as the transitional fluid. The cover slips were attached to aluminum mounts with adhesive carbon tabs or silver paint and coated with gold-palladium using a Balzers SCK 030 sputter coater (Balzers Union Ltd., Liechtenstein). Images were obtained with a JEOL JSM-6490LV scanning electron microscope (JEOL Ltd., Japan) at 3,000 ×, 6,500 ×, and 16,000 × magnification. Bacterial strains were processed two to five times; 8 to 26 images were obtained per strain. The distribution of bacteria across the cover slips was largely consistent. One representative image at 6,500 × is presented for each strain.

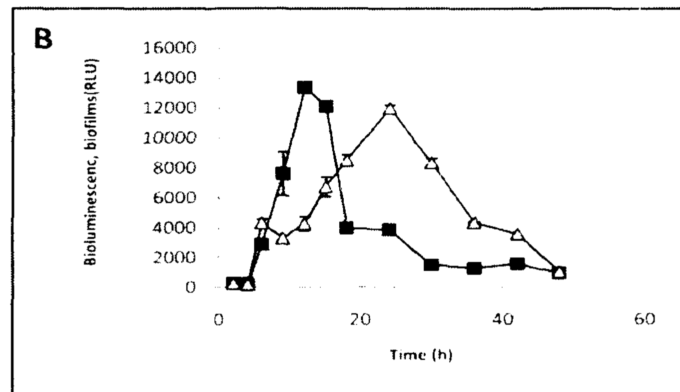
RESULTS

In a previous technical report (24). we showed that bioluminescence correlates with both ATP concentration and bacterial cell number. In this report, we compare the ATP and CV assays for their utility in quantifying biofilm biomass.

To perform this comparison, we incubated bacteria of the wild-type strain (AJW678) and its isogenic *flhD*, *fimH*, *ompR*, *rcsB*, *rcsC*, *ackA*, and *ackA pta* mutants for 38 h. The ATP assay yielded three distinct classes: a wild-type-like class (which included the *flhD*, *rcsC*, *ackA*, and *ackA pta* mutants), a reduced signal class (which included only the *fimH* mutant), and an increased signal class (which included the*rcsB* and *ompR* mutants). In contrast, the CV assay yielded only two distinct classes of strains: a wild-type-like class and a reduced signal class that consisted of the *fimH*mutant. Statistical analysis with Dunnett's test confirmed the classification for both assays. Thus, both assays indicated that the amount of biomass attached to the bottom of the wells was considerably smaller for the *fimH* mutant than for its wild-type parent. In contrast, only the ATP assay indicated that the *ompR* and *rcsB*mutants produced significantly more biofilm-associated biomass than did their wild-type parent (Fig. 12).

To validate this single time point experiment, we performed a time course experiment, limiting our study to a comparison between the biofilms formed by the*ompR* mutant and its wild-type parent (Fig.12B). Whereas the peak ATP concentration of the *ompR* mutant was approximately the same amount as that of its wild-type parent, this peak occurred 12 h later, when the wild-type concentration





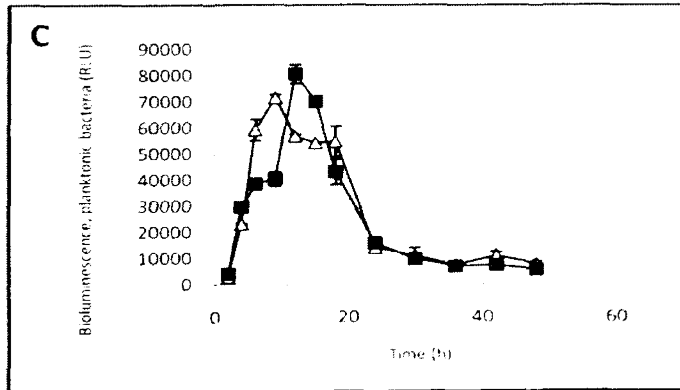


Figure 12: Biofilm estimation by ATP and CV assays. Panel A describes the results of the quantitative assays. Biofilms were formed on polystyrene plates and the biomass was determined with the ATP (black bars) and the CV (white bars) assay. Averages are presented across three independent experiments; error bars indicate the standard deviation. Asterisks above the bars indicate strains that exhibited statistically significant differences from the wild-type strain, as determined by the ATP assay (according to Dunnett's test). Panel B is the time course of biofilm formation for the wild-type strain (closed squares) and the ompR mutant (open triangle). Panel C is the time course of ATP concentration for the wild-type strain (closed squares) and the ompR mutant (open triangle).

was already in decline. Thus, at 38 h, the difference between the two strains was about two fold, which is consistent with the single time point experiment (Fig.12A).

To determine if the differences in biofilm-associated biomass could be attributed to differences in bacterial growth, we determined the ATP concentration in the planktonic bacteria present in the culture medium that overlays the biofilms. We found no significant difference in the ATP concentration (Fig.12C). Thus, the differences in biofilm associated ATP concentrations (Fig. 12B) cannot be attributed to growth rate.

To test the results of the ATP assay, we used SEM to visualize 38 h biofilms produced by all the bacterial strains (Fig.13). Consistent with both quantitative assays, fewer bacteria of the *fimH* mutant strain attached to glass. Consistent with the ATP assay (Fig. 12A), the *ompR* and *rcsB* mutants produced denser biofilms than did their wild-type parent. Consistent with both assays, the remaining strains produced an amount of biofilm quite similar to that of their wild-type parent. SEM also revealed substantial differences in the surface-associated structures exhibited by cells in biofilms formed by the different strains. Efforts to identify these structures are underway (Fig 13).

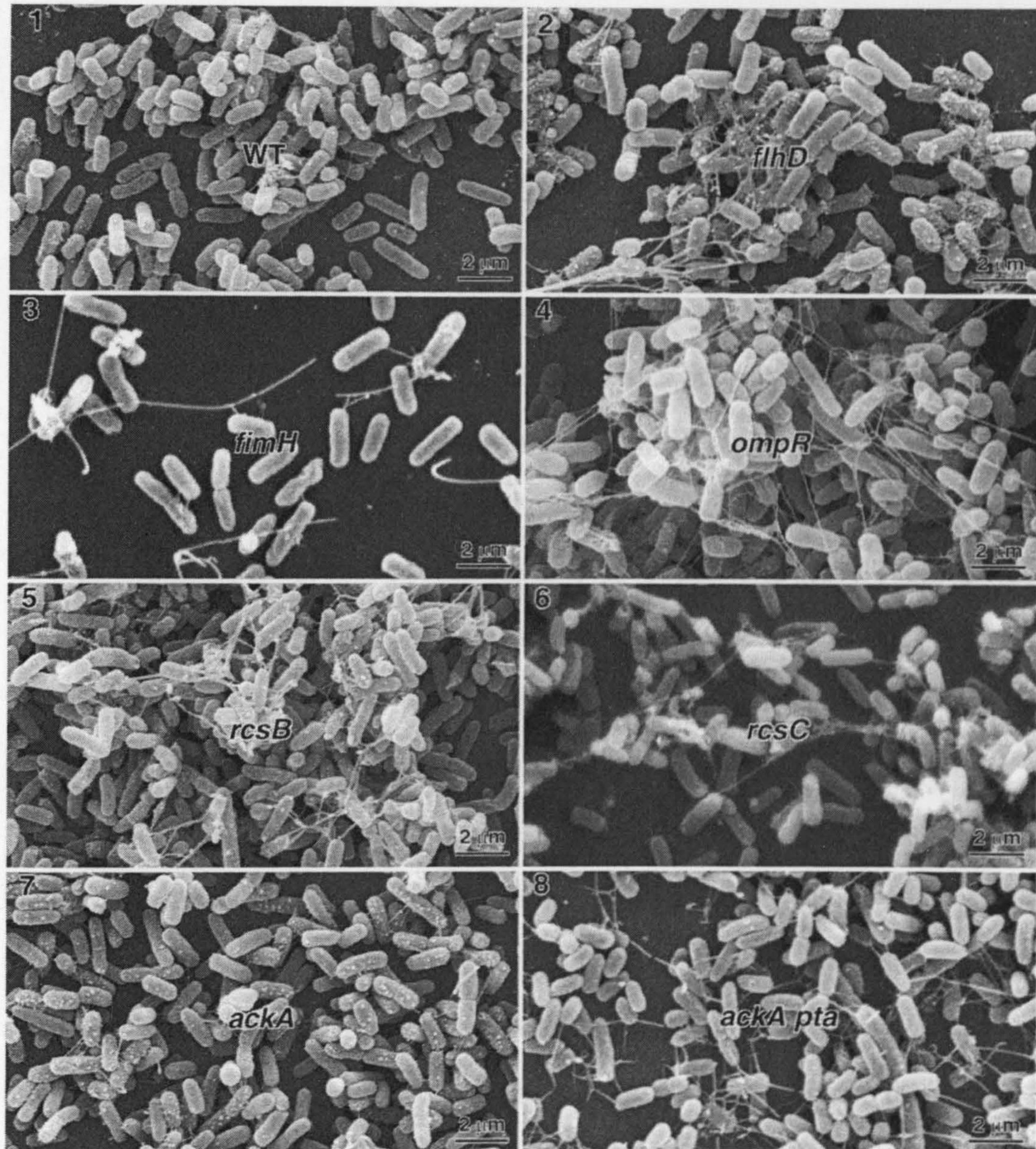


Figure 13: Representative electron micrographs of biofilms formed by each of the strains at amagnification of 6,500 ×. A bar representing 2 µm is located within each image. Strains are as follows: 1, wild-type; 2, *flhD* mutant; 3, *fimH* mutant; 4, *ompR* mutant; 5, *rcsB* mutant; 6,*rcsC* mutant; 7, *ackA* mutant; 8, *ackA pta* mutant.

DISCUSSION

In summary, the two quantitative assays were consistent with one another and the SEM for six out of the eight tested strains. Inconsistencies between the assays may have been due to the reliance of the assays on different modes of biomass determination. For example, the ATP assay detects live biomass in bacteria with low metabolic activity which makes it very suitable for physiological studies. In contrast, the CV assay detects both live and dead bacteria, as well as matrix. Thus, it may have limitations for studies that focus on the involvement of flagella or fimbriae in biofilm formation. For such studies, we recommend the ATP assay. We highly recommend including time course experiments, especially for those mutants that differ from the wild-type in the single time point experiments. SEM appears to be a good qualitative control. Taking the results from all three assays together. attachment appears to be the most critical step in the formation of biofilms. Type I fimbriae are the surface organelles that mediate irreversible attachment and the *fimH* mutant, which is unable to make the adhesive tip. had difficulties attaching to a surface and forming the biofilm. Flagella contribute to reversible attachment. This may explain why the *ompR* and *rcsB* mutants formed denser biofilms, as measured by the ATP assay and SEM. OmpR and RcsB are both two-component response regulators that, in their phosphorylated form, can bind to the *flhDC* promoter and shut down the expression of flagellar genes (3, 21). This means that mutants in *ompR* and *rcsB* exhibit increased flagellar synthesis relative to their wild-type parent (4, 12). For the *rcsB* mutant. this increase in the number of flagella appears to compensate for reduced levels of type I fimbriae (20).

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PAPER 4. A COMBINATION OF PHENOTYPE MICROARRAY™ TECHNOLOGY WITH THE ATP ASSAY DETERMINES THE NUTRITIONAL DEPENDENCE OF *ESCHERICHIA COLI* BIOFILM BIOMASS

Preeti Sule, Shelley M. Horne & Birgit M. Prüß. 2011. *In* T. E. Fatoyinbo (ed.), Remote sensing of biomass-principals and applications. InTech Publishers. Invited paper. In press.

The work reported in this paper has been done by me. The manuscript preparation has been done with the help of the co-authors.

ABSTRACT

Biofilms are defined as sessile communities of bacteria that form on surfaces and cause severe problems in many natural, clinical, and industrial settings, while being beneficial for waste water treatment and biofuel production. Identifying the precise environmental conditions that prevent or support biofilm formation, as well as understanding the regulatory pathways that signal these conditions, is a pre-requisite to both, the solving of biofilm-associated problems and the use for beneficial purposes. In a previous study (51), it was determined that nutrition ranked among the more important environmental factors affecting biofilm-associated biomass. The determination of the effect of specific nutrients will be dependent on an assay that quantifies biofilm biomass in the presence of an array of single nutrients. With this study, we will introduce an assay system that quantifies biofilm biomass formed by *Escherichia coli* mutants in the presence of single nutrients by combining the Phenotype MicroArray™ technology from BioLog (Hayward CA) with the ATP quantitative biofilm assay that was previously developed by our own lab (61), followed up by statistical analysis of the data.

The assay system was used to compare biofilm biomass formed by an *E. coli* parent strain and an isogenic mutant. A mutant was selected that i) was missing one of the cell surface organelles that are involved in biofilm formation and ii) formed a biofilm biomass, as determined with scanning electron microscopy, that was similar to the parental *E. coli* strain on complex bacterial growth media. The selected mutation is in *flhD. The flhD* operon encodes the master regulator of flagella, the organelle that mediates the first phase of biofilm formation, reversible

attachment. Mutants in type I fimbriae, which mediate the second phase of biofilm formation, irreversible attachment, did not produce enough biofilm biomass on complex growth media to warrant an extensive investigation of the effect of specific nutrients. The biological implications of the findings and the use of the assay system for high-throughput experimentation will be discussed.

The BioLog Phenotype MicroArray (PM) system has been developed for the determination of bacterial growth phenotypes (8). The system consists of 96 well plates with 95 single nutrients dried to the base of each of 95 wells (the additional well constitutes the negative control). When used with the tetrazolium dye that is provided by the manufacturer and indicative of respiration, the PM system is used to determine growth of bacterial strains on single nutrients. Since the total system consists of 30 of such plates, the user is enabled to screen growth under 3,000 conditions. With this study, we describe a protocol for the determination of biofilm biomass. We will demonstrate the usefulness and practicability of the assay with the PM1 plate that contains 95 carbon sources. The bacteria were inoculated into the wells of this plate as described by BioLog, the redox dye that is used for the determination of growth phenotypes was omitted. After 48 h of incubation at 37°C, biofilm biomass was determined with the ATP based technique. Briefly, the growth medium was removed, the biofilms were washed twice with PBS, air dried, and quantified with 100 µl BacTiter Glo™ reagent (Promega, WI). Bioluminescence was recorded with a TD 20/20 luminometer from Turner Design (Sunnyvale, CA) and reported as relative lux units (RLU).

The determination of biofilm amounts in the presence of single nutrients was performed four times for both, the parental and the mutant strain. In addition, growth on these carbon sources was determined in three independent replicate experiments, following the protocol that is described for the determination of growth phenotypes and including the redox dye (8). Carbon sources on which the respective bacterial strain grew to an average OD_{600} of 0.5 or more were selected for the statistical analysis of biofilm biomass. Data from each strain, that were normalized for experiment specific variation, were subjected to one way ANOVAs, followed up with Duncan's multiple range test. This was done with SAS software (SAS Institute Inc., 2009). The test compared the means of the amount of biofilm formed in the presence of each carbon source to all the other carbon sources within each strain. To determine differences between the two strains, we first determined the carbon sources that formed their own Duncan's grouping for the parent strain. In a second step, we looked up biofilm amounts formed by the parent strain on chemically related carbon sources. Biofilm amounts formed by the *flhD* mutant were compared to the parent strain for these carbon sources. Finally, the carbon source that yielded the largest amount of biofilm for the mutant was determined. Metabolic pathways that lead to the degradation of these carbon sources were summarized with the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg).

According to the Duncan's grouping, the two carbon sources that were the best biofilm supporters for the parent *E. coli* strain, maltotriose and maltose, formed exclusive groups for the parental strain. Without forming its own Duncan

grouping, ribose was the carbon source that supported the smallest amount of biofilm among all carbon sources tested, while still supporting growth. The parental strain also formed good amounts of biofilm on the remaining C6-sugars that were tested with the PM1 plate. The remaining C5-sugars, xylose and lyxose, did not support growth of the parental strain to the cutoff of 0.5 OD_{600}. In contrast to the parental strain, the *flhD* mutant did not grow well on C6-sugars and their oligosaccharides. Unlike the parental strain, the mutant did not grow well on ribose, but grew to the cut off of 0.5 OD_{600} on lyxose and xylose. Still, the amount of biofilm formed by this strain on C5-sugars was low (<1,000 RLU). An interesting phenomenon was observed for sugar acids. For the parent strain, sugar acids supported growth to more than 1 OD_{600}. Yet, biofilm amounts that formed in the presence of sugar acids were below 1,000 RLU. In contrast, sugar acids were found to be good supporters of biofilm for the *flhD* mutant strain (1,500 to 2,500 RLU). This was even more remarkable, considering the fact that the parental strain grew better on these sugar acids than the *flhD* mutant. The only carbon source that formed an exclusive Duncan group and was the best biofilm supporter for the *flhD* mutant (4,911 RLU) was N-acetyl-D-glucosamine.

The most striking observation is that C6-sugars support biofilm formation, whereas C5-sugars don't. While C5-sugars are degraded via the oxidative pentose phosphate cycle, C6-sugars all have pathways that feed into the Embden-Meyerhof pathway. This leads to the production of acetyl-CoA, acetyl phosphate and acetate. Of these, acetyl phosphate has previously been shown to inhibit the synthesis of flagella (56). Acetyl phosphate and acetate have been shown to

impact the formation and structure of biofilms (69, 51). Among the differences between the two tested strains, the sugar acids yielded the most interesting results. As demonstrated previously (48), the *flhD* mutant exhibited a growth deficiency on sugar acids. Yet, the mutant formed more biofilm on sugar acids than its isogenic parent. Metabolic pathways leading to the degradation of all these carbon sources will be discussed in more detail in the manuscript.

Altogether, we will present an assay that builds upon two previous assays, the PM technology and the ATP assay. Both assays have been used in much different contexts previously. However, their combination, together with the statistical analysis is novel. The combination assay enables the user to rapidly screen hundreds and thousands of single nutrients for their ability to inhibit growth and biofilm formation in one experimental setup. Integrating different mutants into the study will yield valuable insight into the regulatory mechanisms that are involved in the signaling of these nutrients. The described technique is not only cost-efficient and easy to perform, but also high-throughput in nature. It is ideally suited to provide valuable insight into the nutritional requirements that determine biofilm biomass, as well as the respective signaling pathways.

INTRODUCTION

Biofilms are defined as sessile communities of bacteria that form on surfaces and are entrapped in a matrix that they themselves produce. Biofilms cause severe problems in many natural (17, 40), clinical (39, 52), and industrial settings (11, 36, 70), while being beneficial for waste water treatment and biofuel production (66). In addition, the bioremediation of crude oil spills involves a biofilm of oil degrading microbes, potentially supplemented by marine flagellates and ciliates (21). Identifying the environmental conditions that prevent or support biofilm formation, as well as understanding the regulatory pathways that signal these conditions, is a pre-requisite to both, the solving of biofilm-associated problems and the use for beneficial purposes. In a previous study by our laboratory (51), it was determined that nutrition ranked among the more important environmental factors affecting biofilm-associated biomass in *Escherichia coli* K-12. The key to this study was a high-throughput experiment, where biofilm biomass was determined in a collection of cell surface organelle and global regulator mutants under a variety of combinations of environmental conditions. The cell surface organelles each represented a distinct phase of biofilm formation (54). Flagella are required for reversible attachment (phase I), curli or type I fimbriae are characteristic of irreversible attachment (phase II), and a polymeric capsule forms the matrix that permits the maturation of the biofilm (phase III). Eventually, flagellated bacteria are released from the biofilm (phase IV). Phases III and IV are particularly problematic for the disease progression. Bacteria that are located deep within the mature biofilm are particularly resistant to antibiotics and dispersed bacteria tend to serve

as a reservoir that continuously feed the infection. Please, see Fig. 14 for the distinction of biofilm phases.

The global regulators included in our previous study (51) are involved in the co-ordinate expression and synthesis of biofilm-associated cell surface organelles. Many of them are components of two-component systems (2CSTS), each consisting of a histidine kinase and a response regulator (for reviews on 2CSTS signaling, please, see (20, 43, 67).

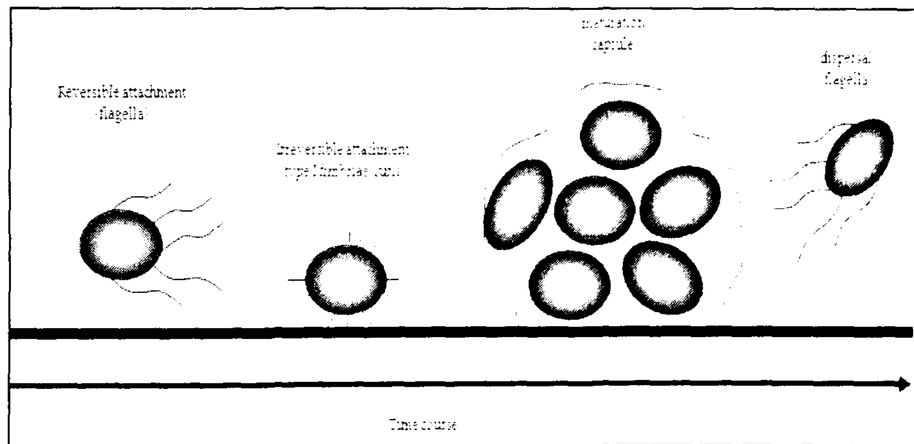

Figure 14: Time course of biofilm formation.

In response to an environmental stimulus, the sensor kinase uses ATP as a phosphodonor to auto-phosphorylate at a conserved histidine, then transferring the phosphate to the response regulator at a conserved aspartate residue. In addition, many response regulators can be phosphorylated in a kinase independent manner by the activated acetate intermediate acetyl phosphate (for a review on acetyl phosphate as a signaling molecule, please, see (68)). One 2CSTS that is involved in the formation of biofilms is EnvZ/OmpR, regulating the synthesis of flagella (56),

type I fimbriae (41), and curli (25). RcsCDB is involved in the formation of biofilms, serving as an activator of colanic acid production (22). RcsCDB constitutes a rare phosphorelay, consisting of three proteins and four signaling domains (2). Much of the effect of EnvZ/OmpR, and RcsCDB upon biofilm formation involves FlhD/FlhC (47), which was initially described as a flagella master regulator (6) and later recognized as a global regulator of bacterial gene expression (48-50).

An early review article (47) summarized the portion of the transcriptional network of regulation that centered around FlhD/FlhC. This partial network contained 16 global regulators, among them many 2CSTSs, such as EnvZ/OmpR, RcsCDB, and CpxR. The regulation of approximately 800 genes was affected by the network. Since many of these encoded components of the biofilm-associated cell surface organelles, it was hypothesized that the network may affect biofilm formation. This hypothesis was confirmed by the high-throughput study that led to the identification of nutrition as one of the more instrumental factors in determining biofilm biomass (51). The global regulators that were part of the network led to the mutant collection for the experiment. Among the tested environmental conditions were temperature, nutrition, inoculation density, and incubation time. Temperature and nutrition were more important in determining biofilm biomass than were inoculation density and incubation time. The mutant screen was consistent with the idea that acetate metabolism may act as a nutritional sensor, relaying information about the environment to the development of biofilms. This hypothesis was confirmed by SEM. A new 2CSTS, DcuS/DcuR, was identified as important in determining the amount of biofilm-associated biomass (51).

The high-throughput experiment merely determined that nutrient rich bacterial growth media are more supportive of biofilm formation than are nutrient poor media. Specific nutrients that are supportive or inhibitory to biofilm formation were not determined and are the next logical step. This will be dependent on an assay system that quantifies biofilm biomass in the presence of an array of single nutrients. With this study, we will introduce such a system that quantifies biofilm biomass formed by *Escherichia coli* mutants in the presence of single nutrients by combining the Phenotype MicroArray™ technology from BioLog (Hayward, CA) with the ATP quantitative biofilm assay that was previously developed by our own lab (61), followed up by statistical analysis of the data, and metabolic modeling.

The BioLog Phenotype MicroArray (PM) technology has been developed for the determination of bacterial growth phenotypes (7-9). The PM technology consists of 96 well plates with 95 single nutrients dried to the base of each of 95 wells (the additional well constitutes the negative control). When used with the tetrazolium dye that is provided by the manufacturer and indicative of respiration, the PM system is used to determine growth of bacterial strains on single nutrients. Since the total system consists of 20 of such plates, the user is enabled to screen growth under close to 2,000 conditions. The plates are designated PM1 through PM20, with PM1 and PM2 containing carbon sources, PM3 containing nitrogen sources, and PM4 containing sulfur and phosphorous sources. The remaining plates can be used to determine the pH range of growth or resistance to antibiotics or other harsh conditions. Liquid growth media are supplied together with the respective plates.

With respect to bacterial growth, PMs have been used in numerous previous studies (3, 14, 34, 38, 71). However, use of this technology for the investigation of biofilms has been limited (10, 19). In *E. coli*, the use of PM technology for the quantification of biofilm biomass has not been reported. In addition, both previous uses of PM technology in biofilm studies have been based on the use of the crystal violet assay for the quantification of biomass. There are, however, many more assays that have been developed for the quantification of biofilm-associated biomass, each of which serves a different purpose. The different quantitative biofilm assays are compared in Table 5.

Assay	Live/dead cells	Detected material	High-throughput suitability	Reference
Crystal violet	Live and dead cells	Exopolysaccharide	Yes	(30, 57, 58)
ATP	Live cells	Energy (ATP)	Yes	(61, 62)
XTT	Live cells	Energy (NADH)	Yes	(13)
WGA	Live and dead cells	Lipooligosaccharide	Not tested	(12, 59)
SBA	Live and dead cells	Colanic acid	Not tested	(12, 59)

Table 5: Comparison of different quantitative biofilm assays.

Crystal violet is a non-specific protein dye that stains the bacterial cells and their exopolysaccharide matrix for dead and live bacteria alike. Biofilms are

cultivated on 96 well plates and stained with 0.1% crystal violet in H_2O. In a second step, crystal violet is solubilized with a mix of ethanol and acetone (80:20) and measured spectrophotometrically (42, 45). The assay was developed as a high-throughput assay that is suitable for robotic instrumentation (30, 57, 58). ATP (adenosine triphosphate) (62, 61)and XTT (4-nitro-5-sulfophenyl-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (13) are both assays that quantify the energy metabolism of the bacteria. Therefore, only biomass of live bacteria is considered. ATP is converted by the enzyme luciferase into a bioluminescence signal, XTT is reduced by NADH to an orange colored water-soluble formazan derivative. Similar to crystal violet, fluoro-conjugated lectins quantify the biomass of live and dead bacteria alike (12). Lectins are highly-specific carbohydrate binding proteins that have been utilized to quantify different cell wall components, as well as extracellular matrix (59). Specifically, wheat germ agglutinin (WGA) and soybean agglutinin (SBA) selectively complex lipooligosaccharides and colanic acid, respectively. For our experiments, we needed an assay that quantifies biofilm biomass in live bacteria that is also suitable for high-throughput experimentation, cost effective, and rapid. The ATP assay appeared as the most suitable assay among the five compared assays (Table 5).

In the past, ATP has been used as a measure of biomass (37, 53, 63) because its concentration is relatively constant across many growth conditions (55). For the quantification of biofilms, the BacTiter Glo™ assay from Promega (Madison WI) has been used for biomass determination in *Pseudomonas aeruginosa* (26) and *E. coli* (62, 61). In *E. coli*, we established that a two fold

increase in bioluminescence did indeed relate to a two fold increase in the ATP concentration and a 2 fold increase in the number of bacteria (62). Across eight isogenic *E. coli* strains (one parent strain and seven mutants), differences in biofilm biomass that were determined with the ATP assay were paralleled by observations made with scanning electron microscopy (61).

The protocol involves the formation of the biofilms on 96 well micro titer plates, incubation at the desired temperature, and washing of the biofilms with phosphate buffered saline (PBS). Special attention is needed to distinguish the pellicle that forms at the air-liquid interface from the biofilm that forms at the bottom of the wells. In particular, the AJW678 derivatives that we are working with form a solid pellicle that covers the entire surface of the culture (69). For users who like to include the pellicle into their study, the growth medium and the PBS will be pipetted off carefully from each well. Users who wish to discard of the pellicle can flip the entire 96 well plate over and remove the liquid this way. Eventually, 100 µl of BacTiter Glo reagent are added to each well. After 5 min of incubation, bioluminescence is measured.

For this study, we will use the ATP assay to quantify biofilm biomass that forms on the PM1 plate of BioLog's PM system. The PM1 plate contains 95 single carbon sources in addition to the negative control. Besides the fact that the use of PM technology for the determination of the nutritional requirements of biofilm has not been reported in *E. coli* yet, the combination of PM technology with the ATP assay is novel. The combination of both, PM technology and ATP assay, together with the statistical analysis and metabolic modeling, enables the rapid screening of

thousands of nutrients for their ability to support or inhibit growth and biofilm formation in one experimental setup. The described technique is not only cost-efficient and easy to perform, but also high-throughput in nature, providing valuable insight into the nutritional requirements during biofilm formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study were the *E. coli* parental strain AJW678, which was characterized as an efficient biofilm former (31) and its isogenic *flhD, fliA, fimA,* and *fimH* mutants (Table 6). The *flhD* mutant was constructed by P1 transduction, using MC1000 *flhD*:kan (33) as a donor and AJW678 as a recipient. This resulted in strain BP1094. AJW2145 contained a *fliA::Tn5* insertion, AJW2063 a *fimA::Kn* mutation, and AJW2061 a *fimH::kn* mutation, all in AJW678 (69). The mutations abolish expression of FlhD/FlhC, FliA, FimA, and FimH, respectively. Mutants in *flhD* and *fliA* are non-motile, whereas mutants in *fimA* are lacking the major structural subunit and mutants in *fimH* the mannose specific adhesive tip of the type I fimbrium. Bacterial strains were stored at -80°C in 8% dimethylsulfoxide, plated onto Luria Bertani plates (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) prior to use, and incubated overnight at 37°C.

Strain	Relevant genotype	Reference
AJW678	*thi-1 thr-1*(am) *leuB6 metF159*(am) *rpsL136* Δ*lacX74*	(31)
BP1094	AJW678 *flhD::kn*	(51)
AJW2145	AJW678 *fliA::Tn5*	(69)
AJW2063	AJW678 Δ*fimA::kn*	(69)
AJW2061	AJW678 *fimH::kn*	(69)

Table 6: Bacterial strains used for this study.

Strain selection for the biofilm experiment

For this study, a mutation was needed that would abolish one of the early cell surface organelles that contribute to the biofilm, while still permitting the formation of biofilms. We performed scanning electron microscopy (SEM) to determine the ability of the five bacterial strains (parental strain, *flhD* mutant, *fliA* mutant, *fimA* mutant, *fimH* mutant) to form biofilms. Biofilms were grown for 38 h at 37°C on glass cover slips with tryptone broth (TB; 1% tryptone, 0.5% NaCl) as a growth medium. Biofilms were fixed in 2.5% glutaraldehyde and prepared for SEM as described (61). Images were obtained with a JEOL JSM-6490 LV scanning electron microscopy (SEOL Ltd., Tokyo, Japan) at 3,000 fold magnification. 10 to 15 images were obtained per bacterial strain from at least three independent biological samples. One representative image is shown per bacterial strain.

Biofilm quantification with PM technology and the ATP assay

We used the PM1 plate of the BioLog PM system that contains 95 single carbon sources. When used with the tetrazolium dye that is provided by the manufacturer and indicative of respiration (8), the PM system can be used for measuring growth of bacterial strains on single nutrients. We here describe a protocol for the determination of biofilm amounts (Fig. 15).

As recommended by the manufacturer for the determination of growth phenotypes, the bacterial cultures were streaked from LB plates onto R2A plates (to deplete nutrient stores) and incubated at 37°C for 48 hours. Bacteria were

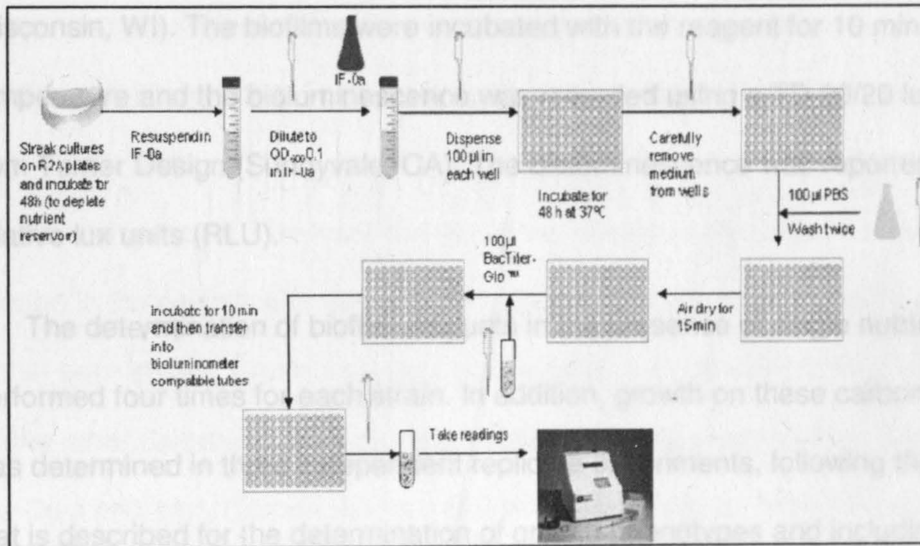

Figure 15: Work flow for the determination of biofilm amounts on PM plates with the ATP assay.

removed from the plates with a flocked swab (Copan, Murrieta CA), resuspended and then further diluted with IF-0a GN/GP Base (BioLog, Hayward CA) inoculation fluid to an optical density (OD_{600}) of 0.1. Leucine, methionine, threonine and thiamine were added at a final concentration of 20 µg/ml, the redox dye that is used for the determination of growth phenotypes was omitted for biofilm quantification. 100 µl of the inoculum was then dispensed into each of the 96 wells of the PM1 plates. The inoculated plates were wrapped with parafilm to minimize evaporation and incubated at 37°C for 48 hours. Biofilm amounts were estimated using the previously described ATP based technique (61, 62). Briefly, the growth medium was carefully aspirated out of each well, minimizing loss of biofilm at the air liquid interface. The biofilms were then washed twice with phosphate buffered saline (PBS) in order to remove any residual media components. The biofilms were air dried and quantified using 100 µl BacTiter Glo™ reagent (Promega,

Wisconsin, WI). The biofilms were incubated with the reagent for 10 min at room temperature and the bioluminescence was recorded using a TD 20/20 luminometer from Turner Design (Sunnyvale, CA). The bioluminescence was reported as relative lux units (RLU).

The determination of biofilm amounts in the presence of single nutrients was performed four times for each strain. In addition, growth on these carbon sources was determined in three independent replicate experiments, following the protocol that is described for the determination of growth phenotypes and including the redox dye (8). Carbon sources on which the respective bacterial strain grew to an average OD_{600} of 0.5 or more were selected for the statistical analysis of biofilm amounts.

Data analysis

Prior to the statistical analysis, the biofilm amounts from each strain were normalized for experiment specific variation; total bioluminescence across each plate was summed up and the fold variation of each set was calculated. Each dataset was divided by its respective fold variation to achieve normalization. The normalized data sets were subjected to two independent types of statistical analysis, all done using SAS software (SAS Institute Inc., 2009). First, we performed Student's *t*-test to determine statistically significant differences between the amounts of biofilm that were formed on a given carbon source between the two strains. Since this analysis yielded a rather large number of carbon sources, we then analyzed each strain individually and consecutively compared biofilm

amounts on individual carbon sources for specific nutrient categories of structurally related carbon sources. For this analysis, the normalized biofilm data from each strain were subjected to separate one way ANOVAs, followed up with Duncan's multiple range tests. The tests compared the means of the amount of biofilm formed in the presence of each carbon source to all the other carbon sources within each strain. Carbon sources whose mean was different from the means of all the other carbon sources with statistical significance formed their own group in the Duncan's test. Carbon sources whose mean difference from the other carbon sources was not statistically significant formed overlapping groups.

Performing Duncan's test on the parent strain, two carbon sources were identified that each formed their own group (A and B). Among the carbon sources that formed overlapping Duncan's groups, we determined those that were structurally related to group A and B carbon sources. This was done after a determination of the respective chemical structures with the Kyoto Encyclopedia of Genes and Genomes (KEGG; (27, 28). Biofilm amounts formed by the *flhD* mutant were compared to the parent strain for these carbon sources. In a second analysis, the carbon source that formed its own Duncan's group for the *flhD* mutant was determined (group A). Among the carbon sources that formed only overlapping Duncan's groups for this mutant, we identified two carbon sources that were structurally related to the group A carbon source. Biofilm amounts for these three carbon sources were compared between the two strains. For both analyses, data were summarized in a Table (7 and 8).

Metabolic modeling

Metabolic pathways that lead to the degradation of all the carbon sources that are discussed in this study were determined with KEGG. Metabolic intermediates that were common between different pathways were used to construct metabolic maps. Pathways for both strains were combined in Fig. 19.

RESULTS

Strain selection using electron micrographs

To determine the ability to form biofilm, electron microscopy was performed with the five strains that were mentioned in Materials and Methods. Fig. 16 depicts one representative illustration of the 10 to 15 images that were obtained per bacterial strain. Most of these strains formed biofilm despite mutations affecting cell surface organelles of either reversible (flagella) or irreversible (type I fimbriae) attachment. The sole exception was the *fimH* mutant which only showed a small number of scattered bacteria attached across the slide. The *fimA* mutant exhibited a large number of filamentous appendages. We are currently unable to explain these appendages.

We wanted a strain for the phenotype microarray experiment that was able to form biofilm on complex media, while lacking one of the cell surface organelles. Since the amount of biofilm formed by the *flhD* mutant was similar to that of the parental strain in the electron micrographs, the *flhD* mutant was selected for further testing using the PM1 plates. The *flhD* mutant has as an additional advantage that much of the regulation by FlhD/FlhC has been previously described. This vast amount of information will help us to analyze the complex metabolic data.

Biofilm quantification with PM technology and statistical analysis

Biofilms that formed on the PM1 plates were quantified with the ATP assay and compared between the two strains with the *t*-test. The analysis did not yield any carbon sources that supported more biofilm in the parent strain than in the mutant.

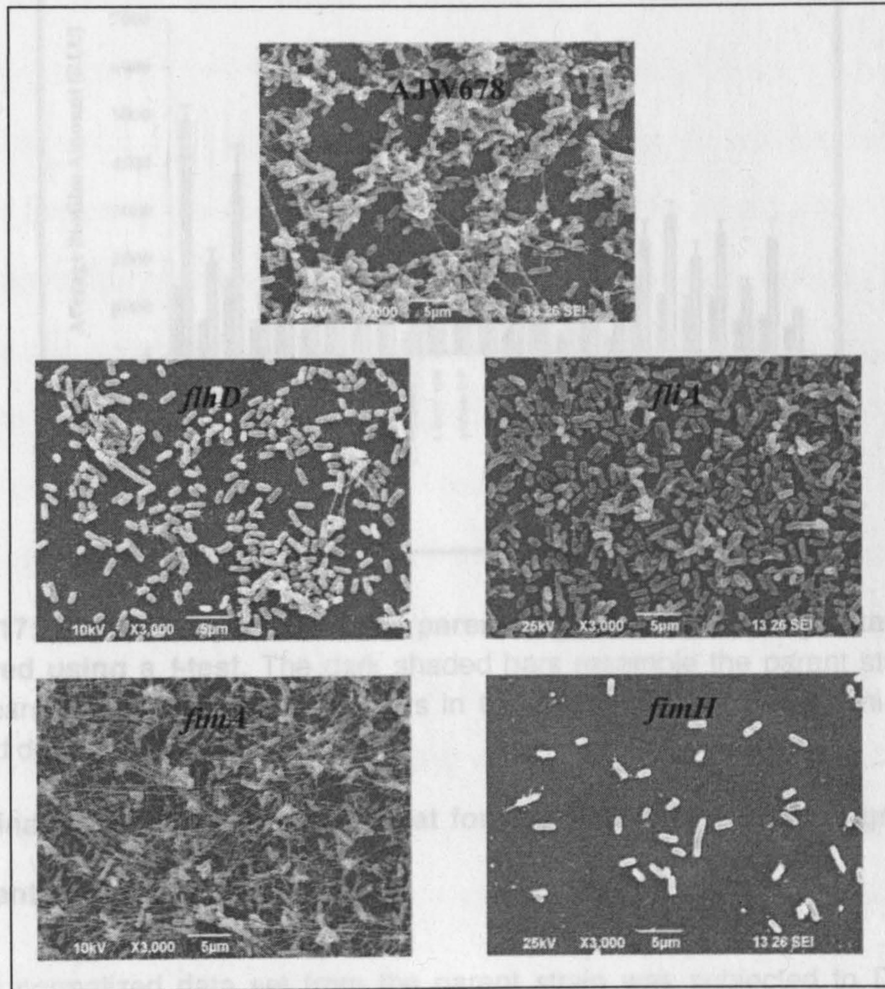

Figure 16: Electron micrographs at 3,000 fold magnification for the AJW678 parent strain, and its isogenic mutants in *flhD*, *fliA*, *fimA*, and *fimH*.

The 25 carbon sources that yielded significantly higher amounts of biofilm in the *flhD* mutant are demonstrated in Fig. 17. Since the carbon sources that supported biofilm formation by the mutant more so than by the parent are this numerous, we decided to analyze each strain statistically first and focus the comparison between the strains to specific structural categories of carbon sources. These are designated 'nutrient categories' throughout this manuscript.

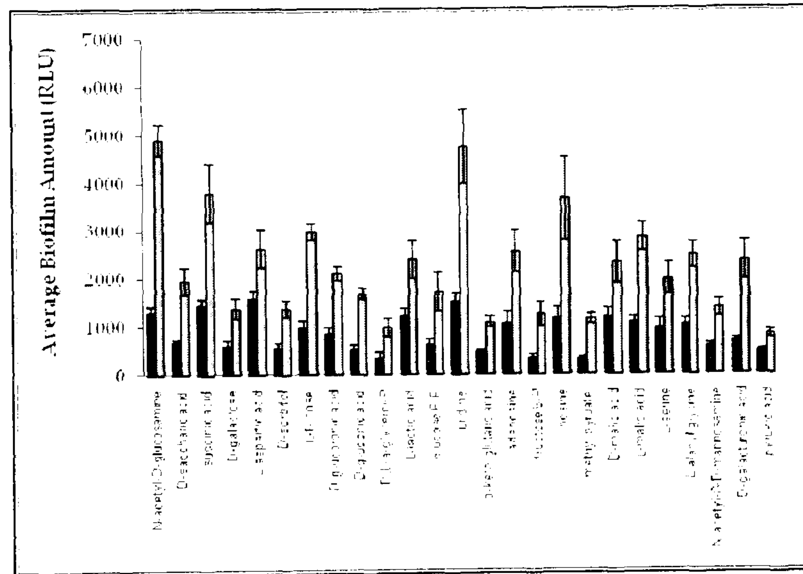

Figure 17: Biofilm formation in the parent strain and the *flhD* mutant were compared using a *t*-test. The dark shaded bars resemble the parent strain, the lighter bars the mutant. The error bars in the graph indicate plus or minus one standard deviation.

Determination of carbon sources that formed their own Duncan's group for the parent strain

The normalized data set from the parent strain was subjected to Duncan's multiple range test. According to this test, the two carbon sources that were the best biofilm supporters for the parent *E. coli* strain, maltotriose and maltose, formed exclusive groups A and B. Without forming its own Duncan group, ribose was the carbon source that supported the smallest amount of biofilm among all carbon sources tested, while still supporting growth. The parent strain also formed good amounts of biofilm on the remaining C6-sugars. Interestingly, the amount of biofilm that formed on maltotriose (trisaccharide of glucose) was roughly three times the amount of biofilm that formed on glucose. The amount of biofilm that

formed on maltose (disaccharide of glucose) was about twice the amount that formed on glucose. The C5-sugars xylose and lyxose did not support growth of the parental strain to the cutoff of 0.5 OD_{600}. For all these carbon sources, biofilm amounts formed by the *flhD* mutant were compared to the parent strain (Table 7). In contrast to the parental strain, the *flhD* mutant did not grow well on C6-sugars and their oligosaccharides. Unlike the parental strain, the mutant did not grow well on ribose, but grew to the cut off of 0.5 OD_{600} on lyxose and xylose. Still, the amount of biofilm formed by this strain on C5-sugars was low (<1,000 RLU). An interesting phenomenon was observed for sugar phosphates and sugar acids. Sugar phosphates supported biofilm production by the mutant more so (>1,200 RLU) than for the parent strain (<600 RLU). Likewise, sugar acids were found to be good supporters of biofilm for the *flhD* mutant strain (1,500 to 2,500 RLU), but not for the parent (500 to 800 RLU). This was even more remarkable, considering the fact that the parental strain (OD_{600} ~ 1.0) grew better on sugar acids than the *flhD* mutant (OD_{600} of 0.2 to 0.8).

Determination of the carbon source that formed its own Duncan's group for the *flhD* mutant

The amount of biofilm formed on each carbon source by the *flhD* mutant was quantified and subjected to Duncan's multiple range test. According to the Duncan's grouping, the sole carbon source that formed its own group A for the *flhD* mutant was N-acetyl-D-glucosamine.

Nutrient category	Nutrients	AJW678	*flhD* mutant
		Biofilm Amount (RLU)	Biofilm Amount (RLU)
Trisaccharide	Maltotriose	4,935	NA*
Disaccharide	Maltose	2,928	NA*
C6-sugars	Glucose	1,615	NA*
	Fructose	1,500	NA*
	Mannose	1,745	NA*
	Rhamnose	873	NA*
C5-sugars	Ribose	147	NA*
	Lyxose	NA	650
	Xylose	NA	544
Sugar phosphates	Glucose 6-P	614	1,722
	Fructose 6-P	338	1,258
Sugar acids	D-galacturonic acid	668	2,358
	D-gluconic acid	532	1,679
	D-glucuronic acid	852	2,110

Table 7: Biofilm amounts on carbon sources which formed their own Duncan's grouping for the parent strain and structurally related carbon sources. Columns 1 and 2 indicate the nutrient categories and single carbon sources for which data are included. Columns 3 and 4 represent biofilm amounts for the parent strain and the mutant on carbon sources that permitted growth to more than 0.5 OD_{600}. NA denotes 'not applicable', where the strain grew to an OD_{600} below 0.5.

Structurally related carbon sources that were included in the PM1 plate are D-glucosaminic acid and N-acetyl-β-D-mannosamine. Biofilm amounts formed on these three carbon sources were compared between the two strains (Table 8).

On N-acetyl-D-glucosamine, the *flhD* mutant (4,900 RLU) formed a significantly larger amount of biofilm than the parent strain (1,300 RLU), while both strains grew to approximately 1 OD_{600}. On D-glucosaminic acid, the parent strain did not grow to the cutoff OD of 0.5. The *flhD* mutant grew well, but the amount of biofilm biomass was poor (~600 RLU). For N-acetyl-β-D-mannosamine, both strains grew well, the *flhD* mutant expressed more than twice the ability to form biofilm than its isogenic parent.

Metabolic modeling

Metabolic pathways were constructed for the degradation of all those carbon sources that supported amounts of biofilm larger than 1,000 RLU for one of the two tested strains. These are carbon sources of the nutrient categories C6-sugars, sugar phosphates, sugar acids, and sugar amines. C6-sugars all have pathways that feed into the Embden-Meyerhof pathway, sugar phosphates are intermediates of this pathway.

As shown in Fig. 18, mannose, fructose, and N-acetyl D-glucosamine feed into fructose 6-phosphate. Gluconate, glucuronate,galacturonate, and rhamnose feed into glyceraldehyde 3-phosphate. This leads to the production of acetyl-CoA, acetyl phosphate and acetate (Fig. 19).

Nutrient category	Nutrients	*flhD* mutant	AJW678
		Biofilm Amount (RLU)	Biofilm Amount (RLU)

Sugar amines	N-acetyl-D-glucosamine	4,911	1,285
	D-glucosaminic acid	660	NA
	N-acetyl-β-D-mannosamine	1,368	559

Table 8: Biofilm amounts on carbon sources which formed their own Duncan's grouping for the *flhD* strain and structurally related carbon sources. Columns 1 and 2 indicate the nutrient category and single carbon sources for which data are included. Columns 3 and 4 represent biofilm amounts for the *flhD* mutant and its parent strain on carbon sources that permitted growth to more than 0.5 OD_{600}. NA denotes 'not applicable', where the strain grew to an OD_{600} below 0.5.

DISCUSSION

Development of the combination assay

Altogether, we present an assay that builds upon two previous assays, the PM technology and the ATP assay. Both assays have been used in much different contexts previously. PM plates have been commonly used to discover various bacterial characteristics based on phenotypic changes (9). Studies involving PM plates include the evaluation of the alkaline stress response induced changes in the metabolism of *Desulfovibrio vulgaris* (60). PMs have also been used for the

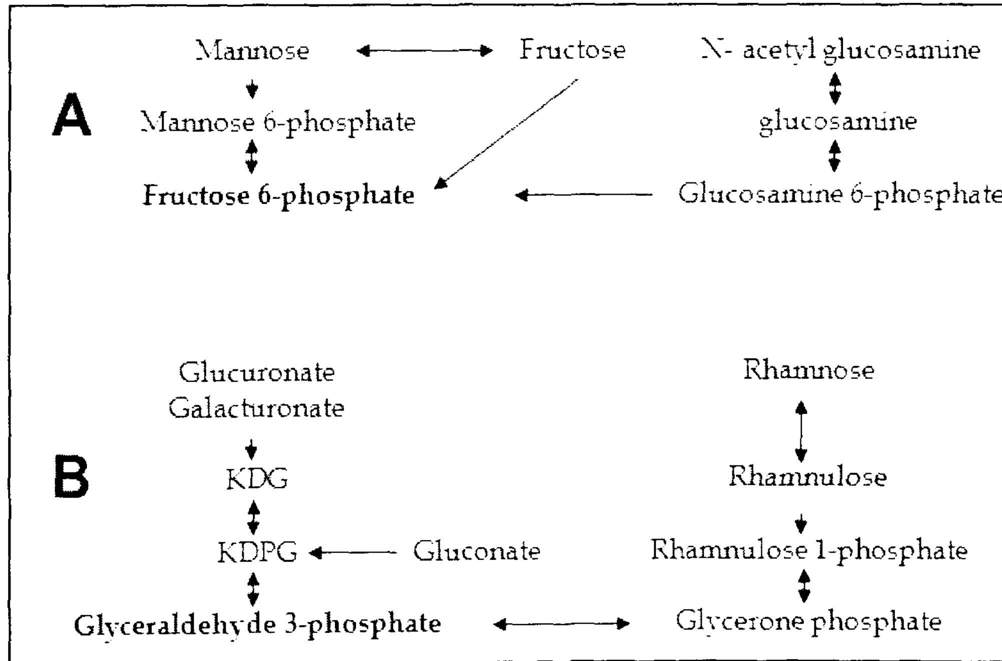

Figure 18: Metabolic pathways from the top biofilm producing carbon sources for both *E. coli* strains, feeding into the Embden-Meyerhof pathway.

identification of bacterial species (1). The use of PM technology in biofilm research is limited to a study of the ability of *E. coli* to form biofilm upon ribosomal stress (10). This study used the crystal violet assay as a tool to detect the biofilm amount.

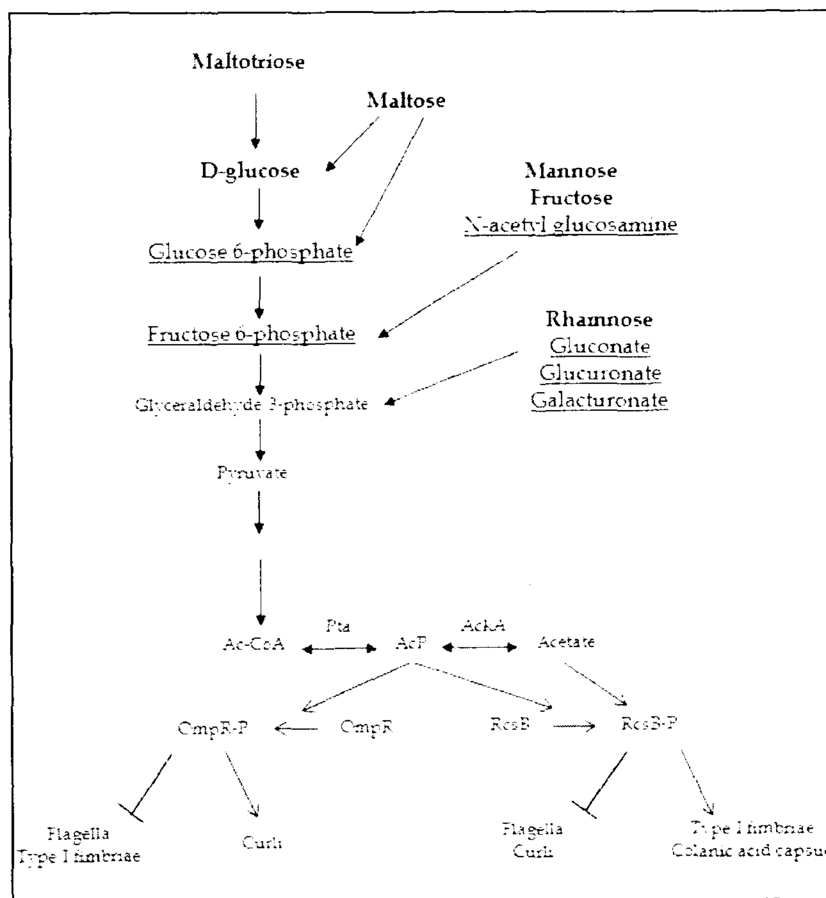

Figure 19: Metabolic pathways from the top biofilm producing carbon sources for both strains to the production of acetate. Carbon sources that are printed in bold were top biofilm supporters for the parent strain. Carbon sources that are underlined were top biofilm supporters for the *flhD* mutant. The effect of acetyl phosphate and acetate on RcsB and OmpR on the synthesis of flagella, curli, fimbriae, and capsule are indicated.

Here we report for the first time a combination of the established ATP assay along with the PM technology to assess nutritional dependence of *E. coli* during biofilm formation. Analysis of the data contains both statistics and metabolic modeling. The combination assay enables the user to rapidly screen hundreds and thousands of single nutrients for their ability to inhibit growth and biofilm formation

in one experimental setup. Integrating different mutants into the study will yield valuable insight into the regulatory mechanisms that are involved in the signaling of these nutrients. The described technique is not only cost-efficient and easy to perform, but also high-throughput in nature. It is ideally suited to provide valuable insight into the nutritional requirements that determine biofilm biomass, as well as the respective signaling pathways.

Biological analysis of the data

In the described study, we observed that the FlhD mutants made quantitatively higher amounts of biofilms on numerous carbon sources. Interestingly, the parental strain did not form higher quantities of biofilm than the mutant on any of the tested carbon sources. These observations shed light into the ongoing controversial debate, elucidating the role of motility in biofilm formation. In certain bacterial species including *Yersinia enterocolitica*, the presence of motility has been shown to be beneficial for biofilm formation (65). Several previous studies from our lab demonstrate that the absence of motility enhances the ability of *E. coli* to form substantial amounts of biofilm. As one example, strains transformed with the FlhD expressing plasmid pXL27 showed diminished biofilm forming capabilities (51). Additionally, ongoing studies carried out in the lab with *E. coli* O157:H7 and the *E. coli* K-12 strains MC1000 and AJW678 point in the same direction, exemplifying our belief that FlhD and motility are detrimental to biofilm formation for our bacterial strains and under the conditions of our experiments (unpublished data).

As a second observation, carbon sources that supported maximal biofilm formation by either strain all fed into glycolysis eventually, and produced actetate. Although the carbon sources that promoted the highest biofilm amounts were different for the two strains, they still were in the same pathway. The previous high-throughput experiment that had pointed towards nutriition as instrumental in determining biofilm associated biomass had also postulated acetate metabolism as one of the key players in biofilm formation (51). Phosphorylation of OmpR and RcsB by the activated acetate intermediate acetyl phosphate (29) and acetylation of RcsB by acetyl-CoA (64) have been described in the past. These activated 2CSTS response regulators then affect the expression level of biofilm associated cell surface organelles, such as flagella, type I fimbriae, curli, and capsule (16, 18, 41, 46, 56) (Fig. 6). The positive effect on biofilm amounts of carbon sources that lead to the production of acetate can be explained with the combined inhibitory effect of acetyl phosphate and acetyl-CoA on flagella through OmpR and RcsB and the above described disadvantage of flagella and motility during biofilm formation. We however do not state that acetate is the sole controlling mechanism as the complexity of the bacterial system cannot be explained based on a small number of signaling molecules.

The most striking observation obtained from our studies pertains to the pattern of growth and biofilm formation on sugar acids. It was observed that the FlhD mutants grew to lower optical densities on sugar acids, but formed much higher amounts of biofilm as compared to the parental strain. Previous work from the Prüß lab had shown similar defects in growth of *flhD* mutants on sugar acids (48),

biofilm formation was not tested in that study. The inverse effect of sugar acids on growth and biofilm amounts may have implications in the intestine. Mutants in *flhD* have an early disadvantage in colonization, but recover after prolonged incubation (24). They even take over the population after more than two weeks (32). The initial lack of colonization could be explained by the inability of the *flhD* mutant to degrade the numerous sugar acids present in the intestine (44). On the other hand, the ability to take over the bacterial population at a later stage may have to do with the lack of the flagellin, which is a potent cytokine inducer (35). The here discovered ability to make an increased amount of biofilm may add to the long term survival of *flhD* mutants in the intestine. Bacteria deep within the biofilm will be protected from the immune system, while metabolizing very slowly and needing much nutrition.

Among the carbon sources that were the least supportive of biofilm formation, the inability of the C5-sugars to support growth and/or biofilm formation was the most striking. Ribose supported growth by the parent strain, but yielded the lowest biofilm amount of all tested carbon sources. The *flhD* mutant did not even grow on ribose. According to Fabich and coworkers (15), ribose is not among the carbon sources that the *E. coli* K-12 strain MG1655 utilizes when bacteria colonize the intestine. Our data are consistent with this observation. Since *E. coli* O157:H7 EDL933 does actually utilize ribose in the intestine, ribose utilization may constitute a mechanism by which pathogenic *E. coli* can find a niche in the intestine to co-exist with the commensal *E. coli* strains.

The inability to grow on lyxose is also consistent with previous observations, where only a mutation in the *rha* locus enabled the bacteria to grow on lyxose via the rhamnose pathway (4). Normally, *E. coli* are unable to grow on lyxose. Most interesting is the behavior of the two strains on xylose. The parent *E. coli* strain was unable to grow on xylose. The f*lhD* mutant did grow, while producing moderately low amounts of biofilm. Co-utilization of glucose and xylose by *E. coli* strains is of upmost importance during the production of biofuels, since the fermented plant material contains both, cellulose (polymer of glucose) and hemicellulose (polymer of glucose and xylose), in addition to lignin. Much research is currently dedicated to the genetic modification of *E. coli* that enables the bacteria to utilize xylose more efficiently (5, 23). It would be interesting to see whether a mixture of our parent strain and its isogenic *flhD* mutant would be able to co-utilize glucose and xylose, particularly since the mutant produced a moderate amount of biofilm which can also be beneficial to the production of biofuels.

CONCLUSION

In summary, we developed an assay system that quantifies biofilm biomass in the presence of distinct nutrients. The assay enables the user to screen a large number of such nutrients for their effect on biofilm amounts. Examples of metabolic analysis relate back to previous literature, as well as giving raise to new hypotheses. Yielding further evidence for the previous hypothesis that acetate metabolism was important in determining biofilm amounts can serve as a positive control that the assay actually yields data of biological significance. Particularly with respect to life in the intestine and the production of biofuels, the data open new avenues of research by providing testable hypotheses. Overall, there is no limit to extensions of the assay into different bacterial species or serving the development of high-throughput data mining algorithm of the kind we developed in the past.

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GENERAL DISCUSSION

This thesis reports the phenotypic and genotypic effects of FlhC mediated gene regulation in *Escherichia coli* O157:H7 on the surface of meat. A compilation of literature in context with gene regulation in *E. coli* O157:H7 showed two intense centers of regulation (18), one around the LEE pathogenicity island and the other around the flagellar master regulator FlhD/FlhC. However, a regulation of the LEE pathogenicity island by the FlhD/FlhC molecule has not been reported. This study hence investigates the possibility of such a regulation.

E. coli O157:H7, importance as a pathogen

E. coli O157:H7 belongs to the enterohemorrhagic group of *E. coli*. The bacteria came into prominence in the 1980's when its association with contaminated hamburger patties was found to be the cause of widespread infection. Despite advances in control measures, *E. coli* O157:H7 still continues to be a cause of concern (1, 2). The use of acidic sprays has been the most commonly practiced method for removal of the bacteria from food material, however the ability of the pathogen to develop resistance against such sprays adds to the complexity of the situation (9) demanding new and effective control measures.

The FlhD/FlhC molecule: a regulator of choice

The FlhD/FlhC regulator molecule has been designated as a master regulator of flagellar genes in *E. coli* (10). It is interesting to note that such a hierarchical arrangement of the master regulator FlhD/FlhC has been observed in many

intestinal bacteria (8, 11), and a mutation in either of the two genes renders the bacteria non motile (10). Apart from regulating flagellar genes, the role of FlhD/FlhC complex as a global regulator has also been well documented (11, 12, 14). The FlhD/FlhC molecule is a hetero hexameric complex consisting of 2FlhD molecules attached to 4FlhC molecules (19). FlhD and FlhC both can separately bind to DNA molecules but with reduced affinity (5, 6). Binding of the heteromeric hexamer produces transcriptional activation, which is facilitated by a bend in the DNA that is greater than 100º (19).

Interestingly, the expression of FlhD/FlhC is regulated by a myriad of factors (17). The fact that it responds to various environmental and metabolic cues adds to the complexity of the system and its suitability for this study. As an example, the formation of acetyl phosphate (13) and subsequent phosphorylation of the osmoregulator OmpR plays a vital role in regulating the expression of the FlhD/FlhC complex (16). Based on the above existing information, Fig. 20 illustrates the principle behind this study.

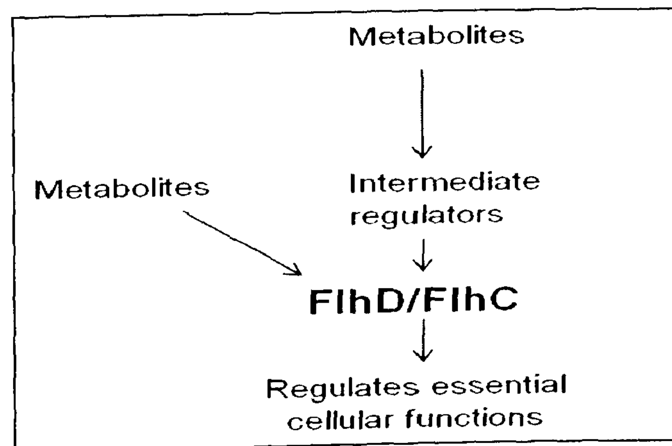

Figure 20: Diagrammatic representation of the study principal.

These metabolites (e.g. acetyl phosphate) being a part of the bacterial metabolic system, would not cause an eminent threat to bacterial survival as some of the antibacterial sprays do and hence would make development of resistance against them by the pathogenic *E. coli* O157:H7 less likely.

Study results and future directions

In the reported study, we found essential cellular functions such as cell division, virulence, metabolism and biofilm formation being down- regulated by FlhC. The phenotypic effect of the same was also confirmed. The effect of *flhC* mutation on the biofilm formation was assessed using the ATP assay that was also developed as part of this thesis. It was confirmed that the *flhC* mutant divided to 20 times higher cell densities, was twice as lethal as the parental strain and formed 5 times higher biofilm biomass as estimated by the ATP assay. These results mean that increasing the expression of FlhC would simultaneously reduce cell division, pathogenicity and biofilm forming ability of the bacteria. In the light of these findings, metabolites that would up- regulate FlhC expression would serve as effective candidates for the development of a novel spray. As an example subject to further testing, formate could serve as a suitable metabolite. Formate in the presence of acetyl-CoA is converted to pyruvate with the help of the enzyme pyruvate phosphate lyase (14). Formation of pyruvate would hence deplete the cellular pool of acety-CoA, which would eventually lead to a decrease in the amount of acetyl phosphate in the bacterial cell (4). A decreased concentration of acetyl phosphate would lead to up-regulation of FlhC (13) and hence a simultaneous down-regulation of the cellular processes it governs.

Additionally, real- time PCR experiments indicated that numerous LEE pathogenicity island genes were down- regulated by FlhC. The regulated genes included *ler*, encoding a regulator of LEE (3). This raised an interesting possibility of the entire LEE pathogenicity island being regulated by FlhC. The real-time PCR experiments however are insufficient in determining the nature of regulation and the presence of intermediate effectors cannot be ruled out. To investigate the nature of FlhC regulation with respect to the *ler* regulator, mobility shift assay could be performed. Upon binding of the regulator to the promoter of interest, a shift in the banding pattern of the promoter DNA is observed on a gel, depending on the size of the regulator. Such experiments have been often used to prove direct binding of regulatory proteins (in our case FlhC) to gene promoters (in our case the *ler* promoter) (7).

In conclusion, this thesis confirms FlhC mediated gene regulation in *E. coli* O157:H7, a pathogen that has persisted for decades as a food safety concern. The results substantiate the formulated hypothesis and indicate essential processes in the bacteria as being down regulated by FlhC, opening endless possibilities for new research, which could ultimately result in better control measures and fewer outbreaks of infection.

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