One-step inactivation of *rpoS* to investigate its role on *Escherichia coli* O157:H7 biofilm formation and survival

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ABSTRACT OF THE THESIS

Homologous linear recombination using the recombinogenic capabilities of λ phage *red* genes *exo*, *bet* and *gam*, has been used increasingly to generate targeted gene replacements in Gram-negative bacteria. While λ Red recombination has been widely applied in nonpathogenic strains of *Escherichia coli*, the system has been employed to a lesser extent in pathogenic strains of *E. coli*, and the frequency of these successes is rarely reported. In this thesis, λ Red recombination was used to generate a disruption of *rpoS* in pathogenic *E. coli* O157:H7. Four gene cassettes carrying either a green fluorescent protein – kanamycin resistant gene cassette (*gfp-aph*(3')I) or a gentamycin resistance determinant (*acc*C1, herein *gm*) flanked by 40 or 500 bp of *rpoS* homology were tested for their ability to facilitate gene replacement using this system. A singular success resulted from these efforts using *gm* flanked by 500 bp homologous to *rpoS* in H32-*gfp*, a strain of *E. coli* O157:H7 H32 labeled with *gfp* gene through random insertion of a Tn5 transposon. The frequency of this success was 80 gene disruptions per 10^8 cells surviving transformation (cell survivors), with an efficiency of $4x10^2$ gene replacements per μg of linear DNA (1.97×10^{11} linear cassette copies). The frequency and efficiency of the gene replacement event, coupled with the non-reproducible nature of the *rpoS* disruption suggests that the activity of the λ Red system is hindered in *E. coli* O157:H7.

The stationary phase sigma factor, RpoS, has been implicated both in the survival of planktonic *E. coli* during periods of stress and in biofilm formation. The effect of *rpoS* deletion on *E. coli* O157:H7 biofilm formation and survival was examined through the establishment of biofilms on glass coverslips and subsequent examination through confocal laser scanning microscopy and viable cell counts, respectively. The influence of *rpoS* on biofilm formation was found to be nutrient-dependent, where *rpoS* mutants displayed a negligible reduction in biofilm formation relative to wild-type strains in nutrient-rich media (TSB and $1/5^{th}$ strength TSB), but enhanced

biofilm formation in nutrient-poor media (MSM + 0.04% glucose). The *rpoS* mutants exhibited 3.5 to 7-fold greater biovolume relative to H32 and H32-*gfp*, respectively, and 7-fold greater substratum coverage relative to both H32 and H32-*gfp*. Biofilm formation in both the wild-type and *rpoS*-knockout (H32-*gfp*Δ*rpoS*::*gm*) strains was found to be negatively correlated with nutrient availability, where all strains displayed enhanced biofilm formation in MSM + 0.04% glucose.

Over a 13-day period of starvation in deionized water, the presence or absence of *rpoS* did not have an apparent influence in the survival of *E. coli* O157:H7 biofilms, whereas *rpoS* enhanced the overall survival of *E. coli* O157:H7 under these conditions when both attached and detached biofilm cells were considered. The *rpoS*-knockout also exhibited decreased numbers of detached viable cells, suggesting that *rpoS* may enhance detachment, survival of detached cells, or both. H32-*gfp* biofilm cells exhibited decreased survival and increased biofilm detachment relative to H32, suggesting that the *gfp*-labeling of H32 influenced survival and detachment of this strain. The transposon insertion site was identified as *uhpC*, a regulatory protein in sugar phosphate transport. UhpC is therefore implicated in the detachment and subsequent survival of *E coli* cells under conditions of starvation and osmotic stress.

The findings of this thesis suggest that while Lambda Red recombination does not represent an ideal means of generating gene disruptions in pathogenic *E. coli*, the generation of these targeted manipulations is invaluable to the elucidation of gene functions such as *rpoS*. While the full role of *rpoS* has not been revealed, it is evident that *rpoS* plays an important role in biofilm formation and in the survival of *E. coli* O157:H7. The influence of culture media on the impact of *rpoS*- on biofilm formation highlights the importance of consistency between investigations and helps to reconcile the contradictory findings in the literature.

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CHAPTER 1 – LITERATURE REVIEW

1.0 INTRODUCTION

1.0.1 *Escherichia coli* O157:H7

In 1982, Michigan and Oregon states were afflicted by outbreaks of an unknown, atypical gastrointestinal disease. Illness was characterized by severe abdominal cramping, watery diarrhea which progressed into profuse hemorrhagic colitis (bloody diarrhea), and an occasional low-grade fever. The duration of sickness ranged from three to over seven days, the median of which was not impacted by treatment with antimicrobials. Forty-seven cases were identified in the outbreaks, 33 of which resulted in hospitalization (Riley *et al.* 1983).

The causative agent of the aforementioned outbreaks was identified as *Escherichia coli* O157:H7, a novel serotype of *Escherichia coli*. *E. coli* is a facultatively anaerobic, Gram-negative, rod-shaped bacterium with an approximate length of 1-2 microns and a diameter of 0.5-1.0 microns (Trun and Trempy 2005). *E. coli* is part of the normal intestinal flora of warm-blooded animals, and as such has an optimal growth temperature of 37°C. Although most strains of *E. coli* are harmless, some strains possess virulence factors such as enterotoxins, flagella (motility), lipopolysaccharides, surface structures for adhesion, and capsules. Such strains are capable of causing diarrheal diseases, urinary tract infections, septicemia, meningitis, and various nosocomial infections. Pathogenic strains of *E. coli* are subdivided into serotypes based on their capsular (K), flagellar (H), and lipopolysaccharide or somatic (O) antigens (Holt *et al.* 1994).

The identification of O157:H7 in the 1982 outbreaks represented the first presentation of this *E. coli* serotype, with the exception of a single sporadic case of hemorrhagic colitis in 1975. Consumption of undercooked hamburger patties from a fast-food restaurant chain was implicated as the source of the *E. coli* outbreaks in Michigan and Oregon (Riley *et al.* 1983). Since then, *E. coli*

O157:H7 has been identified as a leading cause of foodborne illness, largely associated with the consumption of undercooked ground beef (Doyle 1991). Unpasteurized milk and milk products, unpasteurized apple juice and cider, roast beef, salami, raw fruits and vegetables, and contaminated water have also been identified as sources of *E. coli* O157:H7 infection (Besser *et al.* 1999; Ostroff *et al.* 1989). Contaminated water represents a particular concern since the median size of waterborne outbreaks (26 cases) has been shown to be much larger than the median case size of all other types of outbreaks (8 cases; Rangel *et al.* 2005). Numerous waterborne outbreaks of *E. coli* O157:H7 have been reported, including cases associated with swimming in contaminated bodies of water, and consumption of contaminated drinking water (Ackman *et al.* 1997; Olsen *et al.* 2002; Rangel *et al.* 2005; Swerdlow *et al.* 1992). The contamination of municipal drinking water with *E. coli* O157:H7 presents a significant risk of morbidity and mortality, given the potential to infect a great number of people (Hrudey *et al.* 2003).

Since its initial identification, severe complications of *E. coli* O157:H7 have also been identified, namely hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Griffin and Tauxe 1991). Both of the aforementioned syndromes can be attributed to the production of bacteriophage-encoded verotoxins by *E. coli* O157:H7. The toxins produced by enterohemorrhagic *E. coli* strains, including serotype O157:H7, are referred to as Shiga-like toxins (SLT) I and II, indicative of their suspected phage-mediated transfer from *Shigella dysenteriae* (O'Brien and Holmes 1987). While *E. coli* serotype O157:H7 represents the prototypic and most frequently recovered EHEC strain, and other disease-causing, SLT-carrying serotypes include O26:H11, O111:H8, O113:H21, O145:NM, O5:NM, and O128:NM (Ojeda *et al.* 1995). Receptors capable of binding SLTs are found in vascular endothelial cells, contributing to intravascular platelet clumping. Platelet clumping within the microcirculation leads to thrombocytopenia, erythrocyte fragmentation, and organ dysfunction - often with central nervous system and renal involvement ((TTP) Lammle *et al.* 2005). Receptors for SLTs also occur in high concentration in

human renal tissue, contributing to acute kidney failure, destruction of erythrocytes, and thrombocytopenia (HUS). Incidentally, *E. coli* O157:H7 represents the leading cause of HUS, a syndrome with fatality rate of 10 percent when treated and a long-term morbidity rate of 30 percent (Karmali 1989; Lammle *et al.* 2005). Approximately 2-7 percent of *E. coli* O157:H7 cases develop serious sequelae such HUS (Griffin and Tauxe 1991), which in turn is a leading cause of acute renal failure in children (Karmali 1989).

In addition to concern over the increasingly diverse range of sources for *E. coli* O157:H7 infection and the potential for the development of serious sequelae beyond hemorrhagic colitis, *E. coli* O157:H7 also demonstrates a proclivity to developing serious symptomatic infections in those individuals at the extremes of age (Su and Brandt 1995). Consequently, outbreak fatality rates have been reported as high as 35% in institutions such as nursing homes (Carter *et al.* 1987). Furthermore, as of 2004, verotoxigenic *E. coli* was the third most frequently isolated foodborne pathogen in Canada, behind *Campylobacter* sp. and *Salmonella* sp. (Anonymous 2005). As such, *E. coli* O157:H7 represents a formidable foodborne and waterborne pathogen, and further efforts in elucidating the means it employs to survive in environment are warranted. This literature review will discuss the knowledge and identify the shortcomings within the current research on *E. coli* O157:H7 with respect to four areas of investigation: the ability of *E. coli* to form biofilms and the culture conditions influencing the development of these biofilms, the differential gene expression of *E. coli* between the sessile (biofilm) and free-living (planktonic) states, the role of the stationary phase sigma factor gene (*rpoS*) in *E. coli* biofilm formation, and the application of traditional and novel genetic engineering techniques to *E. coli* O157:H7 to elucidate the role of genes such as *rpoS*.

1.1 BIOFILM FORMATION IN *E. COLI*

1.1.1 Introduction to biofilms

Despite the preponderance of studies investigating bacteria in a pure-culture, free-living, single-cell state, bacteria in natural systems rarely exist in this simplified view. The pure-culture, planktonic paradigm has generated a wealth of studies on microbial physiology and genetics, leading to the isolation and identification of numerous pathogens, the development of antibiotics and vaccines, and has alluded to the complexity that follows powerful phenotypic plasticity. Since the early twentieth century, it has become increasingly obvious that bacteria, particularly in aquatic systems, are not only capable of adhering to surfaces, but that a substrate-attached, commensal existence may be the lifestyle preference for bacteria under many environmental conditions (Geesey *et al.* 1977; Henrici 1933; Zobell 1943).

Bacteria adhered to surfaces often exist in complex community organizations which are enclosed in a protective extracellular matrix (ECM), collectively referred to as a biofilm (Costerton *et al.* 1995). Exopolysaccharides represent the bulk of the ECM of bacterial biofilms, but nucleic acids, proteins, and other secreted and captured substances also assemble to collectively confer a measure of shelter to cells within the biofilm (Davey and O'Toole 2000). Consequently, bacteria in biofilms have a demonstrable resistance, relative to their planktonic counterparts, to an extensive list of assaults including antibiotics (Marrie *et al.* 1982; Nickel *et al.* 1985a; Nickel *et al.* 1985b), biocides such as chlorine (De Beer *et al.* 1994), host defense strategies (Jensen *et al.* 1990), and desiccation (Costerton *et al.* 1987). Resistances of biofilm cells are thought to develop through any or all of the following: poor penetration of compounds through the ECM, slow growth due to nutrient limitations within the depths of the biofilm, phenotypic heterogeneity of the biofilm community, cross-protection conferred by a general stress response, or production of highly resistant spore-like persister cells (For review see Mah and O'Toole 2001; Stewart and Costerton 2001; Stewart 2002). Regardless of the mechanism, the resistance of biofilm cells to eradication by

antibiotics, host defenses and biocides represents an area of significant concern and cost in medicine, industry, food processing, and water distribution (Costerton *et al.* 1987; Costerton *et al.* 1995; Costerton *et al.* 1999; LeChevallier *et al.* 1987; Wong 1998).

1.1.2 *E. coli* K-12 biofilm formation

Pseudomonas aeruginosa represents the most frequently studied bacterium in the context of biofilms, and consequently serves as the leading model organism for biofilm formation and structure (O'Toole *et al.* 2000). The generalized process of biofilm formation, as developed through work with *P. aeruginosa*, typically involves five stages (Costerton *et al.* 1987; Costerton *et al.* 1995; O'Toole *et al.* 2000):

i) Initial transitory association of bacterial cells with a solute-coated (conditioned) surface, through which some cells develop a reversible attachment.

ii) Irreversible attachment involving a subset of cells from step (i), facilitated by the adhesive exopolysaccharide matrix.

iii) Development of small clusters of cells, known as microcolonies, through the division of bacteria with favourable positions within the biofilm.

iv) Formation of mature biofilms through further division of biofilm phase cells and recruitment of planktonic phase cells, giving rise to complex mushroom-shaped colonies separated by a primitive circulatory system-like network of water channels.

v) Detachment of biofilm cells from the mature biofilm, leading to dispersal and further colonization.

Despite performing a fundamental role in the study of microbial genetics, studies of *E. coli* biofilm structure have lagged behind that of *P. aeruginosa* (O'Toole *et al.* 2000; Wood *et al.* 2006). This delay in *E. coli* biofilm investigations can be partially credited to the challenges faced in the *in vitro* development of mature *E. coli* K-12 biofilms. Various authors have found certain *E. coli* K-12

strains incapable of forming mature biofilms, as described for *Pseudomonas* sp., without the use of conjugative plasmids to mediate cell-to-cell interaction (Ghigo 2001; Reisner *et al.* 2003). Furthermore, the formation of biofilms by *E. coli* K-12 has been found to be largely dependent on physiochemical conditions such as oxygen availability (Bjergbæk *et al.* 2006; Colon-Gonzalez *et al.* 2004) and media used in cultivation (Bjergbæk *et al.* 2006), as well as genetic factors such as strain variation (Bjergbæk *et al.* 2006; Reisner *et al.* 2006). Nevertheless, some authors report strains of *E. coli* capable of forming biofilms comparable to those of *P. aeruginosa*, both in developmental stages and in overall architecture (Pratt and Kolter 1998; Vidal *et al.* 1998; Wood *et al.* 2006).

Despite the initial challenges in cultivating *E. coli* K-12 biofilms, this organism has gradually become a secondary model organism for biofilm studies, particularly with respect to the cell surface structures involved in biofilm initiation and maturation. In a summary of recent research, Van Houdt and Michiels (2005) identified the main cell surface structures investigated with respect to biofilm formation in *E. coli*. Flagella have been implicated during the biofilm formation stages of transitory contact, reversible attachment, microcolony formation, and dispersal, allowing cells to overcome repulsive forces at the abiotic surface, contribute to vertical expansion of the biofilm, and escape the biofilm to colonize new areas, respectively (Jackson *et al.* 2002; Pratt and Kolter 1998; Wood *et al.* 2006). Type I fimbriae and curli (both adhesive cell structures), as well as exopolysaccharides, have shown involvement during irreversible attachment by mediating cell-to-cell and cell-to-surface interactions (Pratt and Kolter 1998). Curli, Antigen 43 (involved in autoaggregation), and exopolysaccharides may be involved during the formation of microcolonies, (Danese *et al.* 2000a; Danese *et al.* 2000b; Prigent-Combaret *et al.* 2000; Ren *et al.* 2004), whereas curli, conjugative pili and exopolysaccharides likely contribute to the overall interactions necessary to develop the complex architecture of mature biofilms (Ghigo 2001; Prigent-Combaret *et al.* 2000; Reisner *et al.* 2003). Although a detailed discussion of each of these cell surface structures is

outside the scope of this review, some of these structures have been further implicated during the investigation of global gene expression in *E. coli* biofilms.

While *P. aeruginosa* still remains at the forefront of investigations of biofilm architecture, *E. coli*, as the model of microbial genetics, is proving to be a valuable organism for investigating the genes and genetic mechanisms involved in biofilm formation. It should be noted, however, that even as *E. coli* has increasingly established a prominent role in the *in vitro* study of biofilms, the vast majority of these investigations have been confined to the study of *E. coli* K-12 in rich media (O'Toole *et al.* 2000; Reisner *et al.* 2006; Van Houdt and Michiels 2005). K-12 is a highly domesticated, nonpathogenic, laboratory strain of *E. coli* that differs significantly from pathogenic *E. coli* both in genetics and in ecophysiology. K-12 was originally isolated from the feces of a healthy human in 1922, and has been extensively cultivated to the point that its O surface antigens have been lost (Lederberg 2004). *E. coli* O157:H7 shares 4.1Mb of its genome with K-12, however it also possesses 1.4Mb of O157:H7 specific sequences that code for at least 131 proteins unique to this strain (Hayashi *et al.* 2001). Though the insights provided into biofilm formation through the study of K-12 are invaluable, they cannot adequately predict the phenotypes and molecular mechanisms involved in biofilm formation by pathogenic *E. coli*. Consequently, further elucidation into the processes involved in *E. coli* O157:H7 biofilm formation is required.

1.1.3 Culture conditions and *E. coli* O157:H7 biofilms

The ability of *E. coli* O157:H7 to interact with abiotic surfaces was first reported by Dewanti and Wong in 1995. The authors of this study cultivated *E. coli* O157:H7 biofilms on stainless steel chips in a variety of media including trypticase soy broth (TSB), 1/5th strength TSB, and minimal salts media (MSM) supplemented with 0.04% glucose. Unlike most other bacterial species which transition from planktonic growth to biofilm growth in response to rich media (O'Toole *et al.* 2000), *E. coli* O157:H7 exhibited poor biofilm formation in rich media (TSB). Visualization of the TSB grown biofilms by scanning electron microscopy (SEM) revealed only single, sparse cells adhered to

the stainless steel chips and limited ECM production. Furthermore, the biofilms grown in TSB detached readily from the substrate when agitated in buffer. Conversely, biofilms grown in MSM with 0.04% glucose appeared thicker than those in TSB, and demonstrated both extensive ECM production and reduced detachment when compared to their TSB grown counterparts (Dewanti and Wong 1995).

The importance of culture conditions on biofilm formation was further illustrated in a 2007 study investigating the morphology and surface properties of *E. coli* O157:H7 biofilms grown in low and high nutrient media, respectively (Oh *et al.* 2007). In this study, biofilms were grown on glass coverslips and visualized using atomic force microscopy (AFM). AFM is not only capable of generating high resolution images with sub-micrometre level detail, but can also measure the adhesion force of the sample. Through the use of AFM, the authors were able to demonstrate that *E. coli* O157:H7 is capable of forming biofilms on glass surfaces in a nutrient-dependent fashion. Under low nutrient conditions, *E. coli* O157:H7 was shown to establish thicker, more adhesive biofilms with significantly more ECM, more quickly than *E. coli* grown in biofilms grown in rich media (Oh *et al.* 2007).

In order to account for the impact of genetic diversity and environmental conditions on the formation of *E. coli* biofilms, Reisner *et al.* (2006) compared the biofilm formation of 331 clinical isolates that had been minimally cultured in a laboratory (undomesticated) with the biofilms formed by common nonpathogenic and pathogenic laboratory strains. The clinical isolates represented nonpathogenic collections of *E. coli* associated with normal intestinal flora, as well as pathogenic collections associated with diarrhea, bacteremia, and urinary tract infections. Since pathogenicity has been linked to increased biofilm formation, as demonstrated with *Enterococcus faecalis* and infective endocarditis (Mohamed *et al.* 2004), it was hypothesized that the source of the *E. coli* might be a predictor of biofilm-forming capabilities (Reisner *et al.* 2006). Considerable variation existed not only in the biofilm-forming capabilities of the clinical isolates, but also in the

biofilm-forming capabilities of a given strain in different media. Surprisingly, no correlation was found between specific source groups and their ability to form biofilms. There was, however, a strong correlation between culture media and biofilm growth. Consistent with the findings of Dewanti and Wong (1995) and Oh *et al.* (2007), it was shown that biofilm formation of *E. coli* on a polystyrene substrate was enhanced by the utilization of low nutrient media relative to high nutrient media. The above findings highlight the importance of media selection in biofilm studies, since *E. coli* isolates, regardless of source, respond very differently to varied environmental situations. Furthermore, the dangers of using the biofilm-forming capabilities of laboratory type strains to predict that of undomesticated isolates was implied (Reisner *et al.* 2006).

The demonstrated ability of *E. coli* O157:H7 to form biofilms on surfaces commonly found in food processing environments, such as stainless steel, glass, and polystyrene (Dewanti and Wong 1995; Oh *et al.* 2007; Reisner *et al.* 2006; Ryu and Beuchat 2005; Ryu *et al.* 2004), suggests an area of concern for food safety. The propensity of *E. coli* O157:H7 establish biofilms under low nutrient conditions, and the increased resistance these biofilm cells to chlorine relative to their planktonic counterparts (Ryu and Beuchat 2005), also implies a potentially troublesome situation for water distribution. In fact, the presence of pathogenic *E. coli* in mixed culture biofilms of drinking water sources and distribution systems has been demonstrated (Banning *et al.* 2003; Fass *et al.* 1996). These issues advocate for the specific study of the environmental factors and molecular mechanisms governing the formation of pathogenic *E. coli* biofilms.

1.1.4 Gene expression in *E. coli* biofilms

The development of DNA microarray technology in conjunction with the availability of complete genome sequences has allowed for the genome-wide comparison of gene expression in *E. coli* under differing conditions (Sauer 2003). To date, a number of studies have been completed using DNA microarrays to evaluate gene expression of nonpathogenic *E. coli* in planktonic and biofilm states. Although a wealth of information has been gleaned from these genetic studies, gene

expression profiles for *E. coli* in planktonic and biofilm states remain highly variable between studies, with no consistent profile being generated for biofilms.

The first study to suggest fundamentally different gene expression profiles between planktonic and biofilm states, and consequently to precipitate the study of biofilm genetics, was conducted on *E. coli* K-12 using random insertion mutagenesis (Prigent-Combaret *et al.* 1999). In this study, mutagenesis was performed using a bacteriophage, Mu dX, carrying a promoterless *lacZ* reporter gene. Differences in gene expression between planktonic and biofilm states were assessed, revealing that 38% of *E. coli* genes were differentially transcribed between the two states. Notably, genes for colanic acid production (an exopolysaccharide), transport of the glycine betaine – an osmoprotectant (Perroud and Le Rudulier 1985), and a high-affinity nickel transport system were induced, whereas flagellar genes and an unidentified putative protein were reduced. Enhanced colanic acid production in the biofilm state stresses the importance of exopolysaccharides in the formation of the biofilm ECM, a finding consistent with data from earlier studies (Allison and Sutherland 1987; Costerton and Irvin 1981). The study also revealed that biofilm cells experience higher density, higher osmolarity, and lower oxygen conditions than planktonic cells, all physiochemical factors the authors hypothesize may contribute to the differential gene expression in the biofilm state (Prigent-Combaret *et al.* 1999).

In the first study to employ DNA microarray technology to investigate *E. coli* K-12 biofilm formation, Schembri *et al.* (2003) compared the genes expressed by biofilm cells to those expressed by planktonic cells in the both exponential and stationary phases of growth. Mature biofilms were grown on glass coverslips in a flow cell and harvested after 42 hours. All 4290 predicted open reading frames (ORFs) of the *E. coli* K-12 genome were probed (Blattner *et al.* 1997), revealing 206 (4.8%) which were up-regulated and 27 (0.63%) which were down-regulated in biofilm cells relative to exponential phase planktonic cells. Relative to stationary phase planktonic cells, 389 genes (9.07%) were up-regulated and 192 (4.48%) genes were down-regulated in biofilm cells.

Thus, depending on which planktonic state was used as a reference, 5-10% of the *E. coli* ORFs had altered expression between the biofilm state and one of the planktonic states. Only 79 genes (1.84%) had altered expression when both planktonic states were employed as a reference (Schembri *et al.* 2003). These finding suggest that although a number of genes may be involved in the transition from the planktonic to biofilm state, only a limited number of genes are unique to the formation and maintenance of biofilms.

Generally, genes with altered expressions in the aforementioned study could be assembled into six distinct categories: energy metabolism, catabolism of carbon compounds, proteins with unknown functions, undefined enzymes, transport system and binding proteins, and putative transport proteins (Schembri *et al.* 2003). A number of genes found differentially expressed in the earlier mutagenesis study of *E. coli* K-12 (Prigent-Combaret *et al.* 1999) fall within these broad categories, however the latter study by Schembri *et al.* (2003) reports far fewer genes with significantly different expression (38% versus a generous 5-10%). Notable genes with altered expressions in the biofilm state include genes for cell surface attachment structures such as type I fimbriae (for adhesion) and Antigen 43 (for autoaggregation). As previously discussed, these cell structures are implicated in the attachment of bacterial cells to the substratum during initial colonization and microcolony formation, respectively. Furthermore, a number of genes regulated by the stationary phase sigma factor, RpoS, were differentially expressed including those usually exercised in response to stresses such as starvation, osmotic shock, cold shock, and nutrient limitation (Schembri *et al.* 2003). Of the 65 genes reported to be regulated by the stationary phase sigma factor gene, *rpoS* (Loewen *et al.* 1998), 30 (46%) were found to have altered expression during biofilm growth. This data supports the earlier finding that biofilm cells experience higher cell density, as well as greater osmotic and oxidative stress than free-living cells (Prigent-Combaret *et al.* 1999). Though *rpoS* has been implicated in the genetic control of biofilm formation, the exact

role it plays is unknown. As such, elucidating the relationship between *rpoS* and biofilms represents a fundamental area of research, one that will be further discussed in this report.

In the second study to be conducted on biofilm gene expression using DNA microarrays, biofilms of *E. coli* JM109 (a derivative of K-12) were grown on glass wool and analyzed after seven hours (Ren *et al.* 2004). In contrast to the study by Schembri *et al.* (2003), only 22 genes were found to be up-regulated and 201 genes were found to be down-regulated at least 2.5 times the planktonic expression level. Only four of the top 50 differentially expressed genes listed by Schembri *et al.* (2003) were also found to be differentially expressed, and none of the biofilm-specific genes were consistent between the two studies (Ren *et al.* 2004). Additionally, none of the 65 genes listed by Loewen *et al.* (1998) as being regulated by *rpoS* demonstrated significant induction, and in fact 19 *rpoS*-controlled genes were repressed, along with *rpoS* itself - which showed a reduction in excess of 2.5-fold. The authors hypothesize that the differences between DNA microarray studies can partly be attributed to variations in experimental methodology, including the age of the biofilms and growth conditions, and they emphasize the need for considering these factors when analyzing biofilm data (Ren *et al.* 2004). Despite the inconsistencies between the results of Schembri *et al.* (2003) and Ren *et al.* (2004), the role of type I fimbriae and Antigen 43 were further confirmed in this study, accentuating their likely role in biofilm formation (Ren *et al.* 2004).

The third DNA microarray study to be conducted investigating the influence of biofilm formation on *E. coli* K-12 gene expression utilized mature, eight-day old biofilms grown in a continuous flow cell system (Beloin *et al.* 2004). Late exponential and stationary phases of planktonic growth were used as the benchmark for differential gene expression, where a two-fold change in gene expression, or greater, highlighted genes of particular significance. Overall, 1.9% of the *E. coli* K-12 genes were consistently altered more than two-fold, whereas 10% of the genome was considered to have statistically significant differential expression, despite failing to meet the

two-fold threshold. In agreement with the previous gene expression studies (Prigent-Combaret *et al.* 1999; Ren *et al.* 2004; Schembri *et al.* 2003), Beloin *et al.* (2004) found differential gene expression to be concentrated in genes required for energy metabolism, catabolism of carbohydrates or carbon compounds, biogenesis of cell surface structures such as fimbriae, transport systems, and stress responses. Furthermore, several previously undefined genes shown to be necessary for mature biofilm formation were identified. In support of the study by Schembri *et al.* (2003) and in contradistinction to the study by Ren *et al.* (2004), *rpoS* was found to be upregulated in mature *E. coli* K-12 biofilms, along with various other genes associated with stationary phase growth. Despite biofilm and stationary phase planktonic cells sharing similar gene expression patterns, Beloin *et al.* (2004) identified 23 biofilm-specific genes expressed at a level two-fold higher than both planktonic states, as well as 118 other genes that were statistically overexpressed. The identification of biofilm-specific genes indicates that mature biofilm formation activates a unique transcriptional profile necessary for the phenotypic switch from the planktonic to the biofilm state.

The most recent microarray study to be completed to date (Domka *et al.* 2007), sought to reconcile the differences in time points employed by the earlier single time point studies (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003). *E. coli* K-12 biofilms were grown on glass wool and were sampled at 4, 7, 15, and 24 hours. To avoid artificial differences in expression generated by maintaining separate planktonic and biofilm cultures, both samples were taken from the same culture, as initially implemented by Ren *et al.* (2004). Gene expression was then compared across all biofilm time points, stationary time points, and between the two states at each time point. Genes considered for further analysis had significantly different expression overall between the biofilm and planktonic states (at least a 2.5-fold difference), or significantly different expression between time points in the biofilm state (at least a 5-fold difference). Overall, 7.7% more genes were up-regulated in the biofilm state, and 2.7% more genes were down-regulated in the planktonic state.

This result suggests that despite being taken from the same culture, biofilm cells were more metabolically active than the suspension cells (Domka *et al.* 2007).

In addition to genes demonstrating differential expression between biofilm and free-living bacteria, Domka *et al.* (2007) revealed a temporal influence on gene expression within biofilms. Consistent with the findings of the previous studies (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003), fimbriae were up-regulated at all time points. Colanic acid was induced, however only after 24 hours, confirming earlier reports that although required for the development of mature biofilms, it is not required during colonization (Danese *et al.* 2000a; Prigent-Combaret *et al.* 2000). Forty-two stress response genes were found to be differentially expressed, including a number regulated by *rpoS.* Predictably, many stress-related genes were expressed similarly between the cells in suspension and those in biofilms (Domka *et al.* 2007), implying that both stationary phase planktonic cells and biofilm cells are afflicted by adverse conditions, thus further implicating *rpoS* as a potential regulator of biofilm formation.

Although the inconsistencies between DNA microarray studies can be partially ascribed to differences in experimental design, much of the differential gene expression reflects the complex and dynamic nature of biofilm growth. DNA microarray technology is highly sensitive, however it only represents an instantaneous account of transitory gene expression (Sauer 2003). Domka *et al.* (2007) were able to epitomize this phenomenon by performing DNA microarrays on the same biofilm culture at numerous time points, thus revealing that numerous genes are differentially expressed within the same biofilm over time. As such, gaining coherent information from microarray studies can be challenging. DNA microarray data has failed to generate a consistent gene expression profile for *E. coli* biofilms or an accurate prediction regarding the magnitude of differential gene expression over the entire genome (estimates range from 5-38% for *E. coli* K-12). Nevertheless, microarrays have proved to be invaluable to the study of biofilms. Numerous studies have identified the same genes or set of genes as being differentially expressed in the biofilm state

relative to the planktonic state. Genes for fimbriae and Antigen 43 have consistently demonstrated up-regulation during biofilm growth (Beloin *et al.* 2004; Domka *et al.* 2007; Ren *et al.* 2004; Schembri *et al.* 2003), whereas the necessity of colanic acid in biofilm maturation has been established (Domka *et al.* 2007; Prigent-Combaret *et al.* 1999). One gene that is continually implicated in the biofilm lifestyle (Beloin *et al.* 2004; Domka *et al.* 2007; Ren *et al.* 2004; Schembri *et al.* 2003), but whose function remains elusive is *rpoS*. Both *rpoS* and numerous genes regulated by RpoS have demonstrated differential expression between the planktonic and biofilm states. Two studies demonstrated up-regulation of *rpoS* and other stress-related genes (Beloin *et al.* 2004; Schembri *et al.* 2003), another demonstrated down-regulation of *rpoS* (Ren *et al.* 2004), and still another study saw differential expression of *rpoS*-related genes, but made no specific mention of *rpoS* itself being differentially expressed (Domka *et al.* 2007). Clearly, given the similarities between stationary phase and biofilm growth (Domka *et al.* 2007) and the differential, differential expression of *rpoS* between the planktonic and biofilm states, it is likely that the stationary phase sigma factor gene plays a role in biofilm formation.

1.2 STATIONARY PHASE SIGMA FACTOR, RPOS

In *Escherichia coli*, as with other eubacteria, transcription is catalyzed by the RNA polymerase core enzyme, consisting of $\alpha_2\beta'\beta\omega$ subunits, known as E (Gruber and Gross 2003; McClure 1985). Despite containing all the catalytic components necessary to conduct transcription, E lacks the abilities necessary to initiate transcription, namely to recognize promoters and promote DNA melting (Gruber and Gross 2003). These initiation functions are provided by yet another subunit, sigma (σ), which when bound to E forms the RNA polymerase holoenzyme ($E\sigma$). Binding of a given sigma factor facilitates expression of a unique subset of genes that possess a common promoter sequence recognized by σ (Gruber and Gross 2003; Helmann and Chamberlin 1988; McClure 1985). As such, transcription can be globally altered by changes in the sigma factor bound to the RNA core enzyme.

When environmental conditions are favourable and *E. coli* is growing exponentially, housekeeping genes are expressed through the binding of σ^{70} (RpoD), the principal sigma factor, to E (Gross *et al.* 1998; Helmann and Chamberlin 1988). *E. coli* also possesses six alternative sigma factors, which express specialized regulons when the cell is confronted with various different stressors or growth condition changes. Stresses that trigger altered global gene expression include starvation or stationary phase, heat shock, iron starvation, envelope damage, and nitrogen limitation and phage shock, which are countered by σ^{s}(RpoS), σ^{32} (RpoH), FecI, σ^{E} (RpoE), and σ^{N} (RpoN), respectively (Gross *et al.* 1998; Gruber and Gross 2003; Merrick 1993). Under certain environmental conditions, *E. coli* is capable of developing peritrichous flagella – a morphological change mediated by a sixth alternative sigma factor, σ^{F} ((FilA), Chilcott and Hughes 2000).

The stationary phase sigma factor (also known as the starvation sigma factor and initially designated *katF*) was first observed as a regulator of *katE*, the gene responsible for expressing hydrogen peroxidase II (HPII). *Tn10* transposon mutagenesis of *E. coli katF* produced catalase-deficient mutants, although the exact role of *katF* on *katE* was initially unclear (Loewen and Triggs 1984). Further mutagenesis studies confirmed the increased sensitivity of *katF* mutants to hydrogen peroxide, and also revealed increased sensitivity of these mutants to UV radiation in the range of 300-400 nm (Sammartano *et al.* 1986). Upon sequencing of the *katF* gene, it was shown that the *katF* bore striking similarities to the nucleotide and amino acid sequences of *rpoD*, the vegetative sigma factor of *E. coli*. Due to the similarities between *katF* and *rpoD*, as well as the apparent regulatory role of *katF* on *katE*, it was hypothesized that *katF* represented a novel sigma factor responsible for expressing genes providing protection against UV and H_2O_2 exposure (Mulvey and Loewen 1989).

Identification of *katF* as a central regulator of stationary phase, and its subsequent designation as *rpoS* (σ^{s}) for starvation or stationary phase sigma factor occurred following a study involving *lacZ* reporter gene fusions with carbon-starvation regulated genes. One fusion,

designated *csi2::lacZ* and mapped to 59 minutes on the *E. coli* chromosome was induced five-fold upon entering stationary phase, and resulted in significantly reduced survival during this phase. In addition, 16 proteins present in the wild-type *E. coli* strain were not present in *csi2::lacZ*, and the mutant displayed increased sensitivity to H_2O_2 and heat shock (Lange and Hengge-Aronis 1991a). Given that *csi2* mapped to the same location as *katF* (Loewen and Triggs 1984), was induced during stationary phase, and shared homology with *rpoD* (Mulvey and Loewen 1989), it was proposed that *csi2* and *katF* were alleles of the same major regulatory gene, *rpoS*, with the gene product σ^s (Lange and Hengge-Aronis 1991a). Lange and Hengge-Aronis (1991a) postulated that σ^s regulates the vast majority of gene expression during the transition to, and throughout, stationary phase.

1.2.1 Regulation of gene expression by RpoS

Planktonic stationary phase cells are typified by several morphological and physiological changes relative to cells in exponential phase growth (For review see Kolter *et al.* 1993). Free-living *E. coli* cells in stationary phase lose their characteristic rod-shape and acquire a spherical or ovoid appearance with a marked decrease in cell size (Lange and Hengge-Aronis 1991b). The composition of the cellular membrane becomes less permeable and more rigid, through the conversion of unsaturated fatty acids to fully saturated cyclopropane fatty acid derivatives (Cronan 1968). During starvation at low temperatures, *E. coli* forms thin, coiled, proteinaceous structures known as curli, that facilitate adhesion to surfaces (Arnqvist *et al.* 1992). Physiologically, stationary phase cells become more resistant to numerous stresses including, but not limited to, heat shock, osmotic challenge, acid shock, and oxidative stress (Arnold and Kaspar 1995; Jenkins *et al.* 1988; Jenkins *et al.* 1990). All of the aforementioned alterations to the morphology and physiology of *E. coli* cells during stationary phase, relative to exponential phase, are indicative of significant changes in global gene expression. With *rpoS* implicated as the gene responsible for expressing σ^s, and thereby regulating stationary phase gene expression, numerous studies have been conducted to clarify the role of *rpoS* on stationary phase gene expression in the planktonic state.

The role of *rpoS* in conferring resistance to a variety of stresses was emphasized during a 1991 study by McCann *et al.* Although unaware that Lange and Hengge-Aronis (1991a) had already identified *katF/rpoS* as a stationary phase sigma factor, McCann *et al.* demonstrated that a *Tn10* transposon insertion in *katF/rpoS* generated an *E. coli* mutant that lacked starvation-induced cross-protection to a variety of stresses including heat shock, osmotic shock, and oxidative damage. Furthermore, 32 proteins were shown to be absent or significantly reduced in the *rpoS* mutant, including six previously identified as Pex (post-exponential) proteins (McCann *et al.* 1991). These findings further reiterate the role of *rpoS* in conferring a plethora of resistances to planktonically grown stationary phase cells through the production unique Pex proteins.

Due to the pleiotropic nature of *rpoS*$^-$ phenotypes under varying environmental conditions, and the overlap between genes induced by σ^s and other sigma factors, characterization of the regulon has proved to be challenging (Schellhorn *et al.* 1998). From the initial 16 proteins identified as being induced by σ^s (Lange and Hengge-Aronis 1991a), and the list expanded to over 50 *rpoS*-dependent genes by 1998 through the use of two-dimensional gel electrophoresis (For review and summary see Loewen and Hengge-Aronis 1994; Loewen *et al.* 1998). A later, much more substantial estimate of *rpoS*-regulated genes was achieved through the systematic disruption of *rpoS* in a bank of 5,000 mutants each with a unique *lacZ* reporter gene fusion. Comparison of β-galactosidase activity in *rpoS*$^+$ and *rpoS*$^-$ strains revealed differential expression between 2-fold and 100-fold for 105 genes. Of the 10 most highly regulated genes, four were previously identified as *rpoS*-dependent and included genes for the osmoprotectant trehalose (*otsA*), a hydrogen peroxidase (*katE*), and an osmotically-induced periplasmic protein (*osmY*) – all genes induced by various stressors (Schellhorn *et al.* 1998).

Schellhorn *et al.* (1998) also demonstrated in the previously mentioned study that although expression of some *rpoS*-dependent genes was not limited solely to stationary phase, maximal expression of most *rpoS*-dependent genes was achieved during early stationary phase. The fact that

σ^s is rapidly degraded (by an unknown mechanism), and therefore less available during exponential phase versus stationary phase (a half-life of 1.5-2 minutes versus a half-life of 25 minutes, respectively), further supports these results (Loewen and Hengge-Aronis 1994). Additionally, numerous genes were found to be growth-phase dependent, but not regulated by *rpoS*, suggesting that σ^s works in concert with other regulatory mechanisms to control stationary phase gene expression (Schellhorn *et al.* 1998). The suggestion that σ^s is not the sole regulatory control over stationary phase gene expression is consistent with the data showing that when maximally expressed, intracellular levels of σ^s only reach 30% the level of σ^{70} under the same conditions (Jishage *et al.* 1996).

Just as DNA microarray technology was used to isolate *rpoS* as a gene of interest in biofilm formation, the technology has, in turn, assisted in isolating genes regulated by *rpoS*. DNA microarrays were used to compare gene expression of *rpoS*$^+$ and *rpoS*$^-$ *E. coli* K-12 derivative strains in a study that revealed 34 genes previously unidentified as being *rpoS*-induced (Lacour and Landini 2004). Interestingly, 50% of these genes had no known function at the time of the research. Of the genes with known functions, four functional categories emerged as being significantly down-regulated in *rpoS*$^-$ strains; protein biosynthesis, uptake and storage of iron, amino acid metabolism and transport, and metabolism of carbohydrates and nucleic acids (Lacour and Landini 2004). Many of the genes expressed within these functional categories are suggestive of a nutrient-starvation coping strategy, such as iron uptake and storage proteins, which function to sequester the often limited element. Of the proteins produced during stationary phase by *rpoS*, one of particular note is indole, an extracellular signaling molecule that has been shown to regulate biofilm formation in some *E. coli* strains (Martino *et al.* 2003). As previously discussed, a correlation between *rpoS* and biofilms formation was eluded to in DNA microarray studies comparing planktonic and biofilm gene expression, respectively, however these reports were contradictory (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003).

The work by Lacour and Landini (2004) also built on previous work that sought to uncover the transcriptional controls of σ^s-regulated genes. Promoter sequence alignment suggested a conserved -10 [CTATA(A/C)T] sequence for σ^s-dependent genes (Espinosa-Urgel *et al.* 1996), which Lacour and Landini modified to [$TGN_{0-2}C(C/T)ATA(C/A)T$]. The promoter sequence was extended again in 2005 by Weber *et al.* to [KCTAYRCTTAA], where K represents T or G, Y represents T or C, and R represents A or G. The proposed promoter sequences all bear striking similarity to the -10 consensus sequence for σ^{70}-dependent genes, [TATAAT] (Lisser and Margalit 1993), evidenced by the expression of some genes by both $E\sigma^s$ and $E\sigma^{70}$ (For review see Loewen *et al.* 1998). Furthermore, no -35 consensus sequence was found for σ^s-dependent genes (Lacour and Landini 2004), suggesting that other unknown factors must mediate $E\sigma^s$ promoter binding during stationary phase. The complexity of the *rpoS* regulon is highlighted by the elusive interactions between σ^s and σ^{70}, and σ^s with other potential modulators during stationary phase gene expression.

The first study to comprehensively investigate both up-regulated and down-regulated *rpoS*-dependent genes isolated at least 130 genes were significantly up-regulated, but more surprisingly 76 genes that were significantly down-regulated by *rpoS* (Patten *et al.* 2004). DNA microarrays were used to compare gene expression between wild-type and *rpoS- E. coli* K-12 strains grown in rich media to both exponential phase and stationary phase. Of the genes that were significantly down-regulated, 15 were responsible for bacterial motility and included genes responsible for flagellar biosynthesis, and chemotaxis. Although the remaining down-regulated genes were dispersed throughout a number of inconsistent groups, a subset of *rpoS*-repressed genes were found to be involved in energy metabolism, specifically the tricarboxylic acid (TCA) cycle (Patten *et al.* 2004).

A persistent paradox of *rpoS* studies has been that under certain starvation conditions, mutations in the *rpoS* gene can actually confer an advantage. Numerous *rpoS* mutants have

demonstrated increased fitness and survival over wild-type strains in planktonic cultures incubated for a prolonged period after stationary phase growth has been achieved (Farrell and Finkel 2003; Finkel and Kolter 1999; Zambrano *et al.* 1993; Zambrano and Kolter 1996; Zinser and Kolter 1999). The fitness advantage of these *rpoS* mutants may be provided by an increased capacity to scavenge for amino acids, which are utilized by the TCA cycle as a source of energy (Finkel and Kolter 1999). In fact, *E. coli* strains with revertible *rpoS* mutations can employ succinate, a TCA cycle intermediate, as a sole source of carbon whereas wild-type *E. coli* strains do not have the same capability (Chen and Schellhorn 2003). Therefore, mutations in *rpoS* may be selected for under low nutrient conditions, thus allowing the surviving cells to more efficiently capture and utilize amino acids and other nutrients released by dead cells (Chen and Schellhorn 2003; Finkel and Kolter 1999).

The most recent study, to date, to exhaustively investigate the role of σ^s in stationary phase gene expression, predicts that the portion of the *E. coli* genome regulated by σ^s is likely much larger than initially anticipated (Weber *et al.* 2005). Again using microarrays, but with wild-type and *rpoS*- *E. coli* strains cultivated under three different conditions; rich media, rich media with a hyperosmotic shift, and acidified rich media, 576 genes were found to be differentially expressed no less than two-fold by σ^s in at least one of the listed conditions. Of these genes, only 140 were up-regulated under all conditions, leading the authors to designate them as the core set of σ^s-dependent genes. The remaining genes were either down-regulated (95 genes) or expressed only under one of the aforementioned conditions (341 genes). Two inferences were drawn from these results; firstly, that the *rpoS* regulon is likely much larger than originally predicted, comprising approximately 10% of the *E. coli* K-12 genome, and secondly, that expression of the *rpoS* regulon is more differential than originally speculated and subject to manipulation by the environment (Weber *et al.* 2005). The finding that environmental conditions mediate expression of non-core σ^s-dependent genes is consistent with the earlier finding that some genes are expressed by both $E\sigma^s$

and $E\sigma^{70}$ (For review see Loewen *et al.* 1998). Environmental factors such as low pH or high osmolarity may directly or indirectly influence binding of $E\sigma^{s}$ and $E\sigma^{70}$, through reducing the negative supercoiling of the template which enhances $E\sigma^{s}$ binding (Ding *et al.* 1995; Kusano *et al.* 1996), or by mediating expression of other regulatory proteins (Ding *et al.* 1995; Hengge-Aronis 1999; Hengge-Aronis 2002; Kusano *et al.* 1996), respectively.

Despite the vastly complex and expansive nature of the *rpoS* regulon in *E. coli*, great strides have been taken to elucidate its regulation and expression between stationary phase and exponential phase, as well as under various environmental conditions. It is evident that σ^{s} is responsible for conferring resistance to various stresses and enhancing survival during the stationary phase growth of planktonic cells. *RpoS*-dependent genes governing the small, spherical appearance of stationary phase cells, and the generation of curli, have been identified as *bolA* and *csgA,* respectively (Lange and Hengge-Aronis 1991b; Olsen *et al.* 1993). Physiologically, genes regulated by *rpoS* are associated namely with stress responses, but also include genes for energy metabolism, protein biosynthesis, regulatory mechanisms, and various different transport systems (Lacour and Landini 2004; Weber *et al.* 2005). The rapid increase in σ^{s} upon exposure to stress, starvation, or the initiation of stationary phase growth, accounts for the characteristic cross-protection to various stressors found in free-living cells of that phase (Hengge-Aronis 2002; Klauck *et al.* 2007; Loewen *et al.* 1998). Significant insights have been made into the myriad of often redundant mechanisms governing expression of the *rpoS* regulon itself (Hengge-Aronis 2002; Klauck *et al.* 2007; Loewen *et al.* 1998). The paradoxical advantage of *rpoS* mutations under nutrient-limited conditions is reasonably accounted for by the expression of genes enhancing the scavenging abilities of the cell that typically would be repressed in *rpoS* wild-types.

In spite of the substantial inroads made in characterizing the *rpoS* regulon, there are fundamental shortcomings in the knowledge base. Studies have been limited largely to non-pathogenic *E. coli* in the planktonic state, typically under nutrient rich conditions. Few studies have

investigated the role of *rpoS* in the biofilm state, and no studies have employed the use of a pathogenic strain of *E. coli*. Thus, the role of *rpoS* in the formation and maintenance of pathogenic *E. coli* biofilms represents an area of future study, an area addressed in part by this thesis.

1.2.2 *E. coli* biofilms and RpoS

The transition of bacterial cultures into stationary phase is marked by the equilibration of cell growth and cell death. The cessation in overall growth of a stationary phase culture is due to nutrient deprivation imposed by the high cell density resulting from exponential phase growth. These starvation conditions, among other stressors, trigger a switch in global gene regulation through expression of the *rpoS* regulon by σ^s. Bacterial cells in biofilms have been shown to experience high cell density and stresses such as high osmolarity and low oxygen, relative to cells in the planktonic state, especially within the interior of the biofilm structure (Prigent-Combaret *et al.* 1999). Given the high cell density of biofilms and the corresponding potential for stress and limited nutrient situations, it is reasonable to surmise that σ^s may be influential in the biofilm lifestyle, as well. It is not surprising, therefore, that *rpoS*-dependent genes have exhibited differential expression in the biofilm state relative to the planktonic state (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003). The role of *rpoS* on biofilm formation has been investigated in a limited number of studies, and the results are conflicting.

The role of *rpoS* in the development of *E. coli* biofilms was first investigated using *E. coli* K-12 derivatives ZK126 (wild-type *rpoS*) and ZK1000 ((*rpoS*$^-$), Adams and McLean 1999). Biofilms for both strains were generated in an unspecified glucose-limited defined medium using two continuously fed biofilm devices. A chemostat supplemented with one of the K-12 strains supplied either a modified Robbins device, which uses multiple channels in parallel each containing a small plug of urinary catheter rubber as a substrate, or a glass flow cell. The rubber plugs were used to determine viable cell counts for the established biofilms, whereas the glass slides from the flow cell were visualized using confocal scanning laser microscopy (CSLM). Adams and McLean (1999)

demonstrated that statistically fewer viable biofilm cells were present after 48 hours growth in the *rpoS* mutant, relative to the wild-type. Microscopy revealed that qualitatively, the wild-type *E. coli* was better able to adhere to slides and form biofilms than the mutant. No statistically significant difference in planktonic growth was noted between the *rpoS*$^-$ mutant and the wild-type (Adams and McLean 1999).

Contrary to the findings of Adams and McLean (1999), a subsequent investigation of the comparative biofilm-forming capabilities of wild-type and *rpoS*-deficient *E. coli* K-12 found that the mutant was able to form 3-5 fold more biofilm after 48 hours (Corona-Izquierdo and Membrillo-Hernández 2002). The growth conditions for the biofilms were considerably different between studies, as the latter study cultivated biofilms in polyvinyl chloride microtitre plates in rich media (Luria-Bertani broth). The biofilms developed in the wells of the plate were stained using crystal violet dye and quantified using spectroscopy, following a now standard protocol (O'Toole *et al.* 1999). Consistent with the study by Adams and McLean (1999), Corona-Izquierdo and Membrillo-Hernández (2002) found the planktonic growth curves of the wild-type and *rpoS*-deficient *E. coli* strains to be indistinguishable. However, considerable variation in the biofilm-forming abilities of different parental *E. coli* K-12 strains was detected, emphasizing other reported findings that biofilm-forming capabilities are strain dependent and mediated by both cell surface structures and environmental conditions (Corona-Izquierdo and Membrillo-Hernández 2002; Pratt and Kolter 1998; Reisner *et al.* 2006).

Enhanced biofilm formation in *rpoS* mutants, relative to wild-type *E. coli* could be explained by the production of an extracellular factor that promotes biofilm growth. In investigating this notion, filtered supernatant from exponential phase wild-type *E. coli* K-12 was found to inhibit biofilm development, while supernatant from a corresponding *rpoS*$^-$ culture enhanced biofilm development (Corona-Izquierdo and Membrillo-Hernández 2002). Although the identity of the supposed extracellular factor remains unknown, it has been suggested that *rpoS*$^-$ strains produce

this factor during exponential phase growth. The production of an extracellular factor mediating biofilm formation would help to explain the observation that *rpoS* mutants also begin production of biofilms earlier than wild-type strains, and that this biofilm development begins in the exponential phase (Corona-Izquierdo and Membrillo-Hernández 2002). It is not known whether the increased biofilm formation by *rpoS*- strains can be attributed to the cumulative growth of the biofilm from an earlier stage. Regardless of the mechanism, inhibiting expression of σ^s in closed-system batch culture grown biofilms appears to enhance biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002), whereas inhibiting σ^s in continuously-fed flow cell cultures appears to inhibit biofilm formation (Adams and McClean 1999).

The remaining two studies to compare biofilm formation in wild-type and *rpoS*-deficient *E. coli* K-12 both cultivated biofilms using continuously fed flow cell systems (Ito *et al.* 2008; Schembri *et al.* 2003). Schembri *et al.* (2003) found the *rpoS* mutant wholly unable to form biofilms after 42 hours, whereas Ito *et al.* (2008) found the mutant to be deficient in biofilm-forming capabilities relative to the wild-type. Although in agreement with the study by Adams and McLean (1999), these studies contradict the findings of Corona-Izquierdo and Membrillo-Hernández (2002). The fundamental difference between these diametrically opposed collections of studies is the use of flow cell systems versus batch culture for biofilm growth, implying that *rpoS* expression enhances biofilm formation when a continuous supply of nutrients is available (Ito *et al.* 2008).

No studies, to date, have investigated the role of *rpoS* in the formation of biofilms by *E. coli* O157:H7. Even studies broadly investigating differential gene expression between planktonic and biofilm states in pathogenic *E. coli* are severely limited. A single proteomic study by Trémoulet *et al.* (2002) of a non-pathogenic *E. coli* O157:H7derivative revealed differential protein expression between planktonic and biofilm cells. Of the 17 proteins with increased expression in biofilms, those with known functions could be roughly categorized as proteins responsible for general metabolism (including an enzyme from the TCA cycle), transportation, or DNA-binding (Trémoulet

et al. 2002). Interestingly, one of the periplasmic binding proteins was speculated to be responsible for the transport of amino acids, and one of the DNA binding proteins is known to protect non-specific DNA from oxidative damage. These finding are indicative of a change in the global gene regulation of *E. coli* O157:H7 from the planktonic to the biofilm state and are not incongruent with potential for σ^s involvement - a notion that requires further pursuit.

1.3 GENETIC ENGINEERING IN *E. COLI*

1.3.1 Traditional genetic engineering

Functional analysis of a bacterial gene, such as *rpoS*, typically involves the generation of a specific gene disruption. By inactivating a particular chromosomal gene, the function of that gene can then be elucidated by comparing physiological characteristics of bacteria carrying the mutant and wild-type alleles, respectively. The breadth of knowledge accumulated through these systematic mutagenesis studies is vast, as demonstrated above, and consequently numerous methods have been developed for the deliberate mutation of bacterial genes.

Most of the methods employed in the systematic mutation of bacterial genes involve the *in vitro* generation of the target gene disruption on a vector, prior to recombination with the host cell chromosome (Hamilton *et al.* 1989; Raibaud *et al.* 1984; Russell and Dahlquist 1989). The cloning process involves the use of restriction endonucleases to cut the vector and excise the fragment to be cloned, purifying the linearized vector and insertion fragment, using DNA ligase to fuse the fragments together, and using transformation to introduce the newly synthesized clones into the host cell (Figure 1.0.1). The process of generating these clones can be time-consuming and tedious, and is often fraught with technical challenges.

1.3.2 Homologous linear recombination

In 1993, Baudin *et al.* described a method by which genes can be directly inactivated in *Saccharomyces cerevisiae* without the need for prior cloning of the target gene. This approach

involves the production of a linear DNA fragment through PCR that possesses a selectable marker flanked on either side by 35 to 51 nucleotides homologous to the target region on the yeast chromosome. Production of the linear DNA fragment is achieved by employing PCR primers comprised of two distinct sections; the section homologous to the yeast chromosome target, and a 17 nucleotide section that allows the selectable marker to be amplified. Once amplified, the linear fragment can be transformed directly into the yeast cell. The homologous regions of the linear DNA fragment and yeast chromosome, in conjunction with the mitotic recombination capacities of *S. cerevisiae* (Oliver *et al.* 1998), facilitate homologous recombination between two regions. The product is a simultaneous disruption of the target gene and insertion of the selectable marker in its place on the yeast chromosome. The method developed by Baudin *et al.* (1993) effectively eliminates the need for the numerous stages involved in cloning and disrupting the target gene *in vitro*, reducing this process to a simple one-step amplification through PCR.

Unlike yeast, most bacteria are not amenable to transformation by linear DNA. *Escherichia coli* possesses RecBCD, a multifunctional enzyme with intracellular exonuclease activity (Benzinger *et al.* 1975). When short, linear DNA is introduced to *E. coli,* it is readily degraded by RecBCD. *E. coli* strains harbouring *recB* or *recC* mutations exhibit markedly decreased exonuclease activity, however they also demonstrate decreased viability, and are recombination-deficient (Benzinger *et al.* 1975; Brcic-Kostic *et al.* 1989; Capaldo-Kimball and Barbour 1971). It has been shown, however, that RecBC suppressor mutations *sbcB* and *sbcC* in the Rac prophage restore the recombination proficiency and viability of *recB* or *recC* mutants (Barbour *et al.* 1970; Lloyd and Buckman 1985). In contrast to *recB* and *recC* mutations, *recD* mutations lack RecBCD exonuclease activity, but are recombination-proficient (Russell *et al.* 1989). The properties of these *recBC sbcBC* and *recD* mutants allow for easy gene disruption using linear DNA fragments, in manner similar to that described for *S. cerevisiae* (Russell *et al.* 1989; Winans *et al.* 1985). However, the method employed

in yeast is restricted to aforementioned exonuclease-deficient, recombination-proficient mutant strains of *E. coli*.

Studies in RecBCD activity revealed that Chi (χ) sequences (5'-GCTGGTGG-3') attenuate the degradative functions of RecBCD, thus enhancing its recombination abilities (Dixon and Kowalczykowski 1993; Smith *et al.* 1981). As RecBCD unwinds linear double-stranded DNA (dsDNA), degradation occurs more rapidly at the 3' terminal strand than the 5' terminal strand. If a χ site is not reached, the dsDNA will be continuously unwound and degraded. However, if a χ site is encountered, the RecBCD exonuclease activity is suspended, while its helicase activity continues past χ. The aforementioned process results in the production of a single-stranded DNA (ssDNA) overhang, which is protected by single-stranded binding protein (SSB) via the activity of RecA. The ssDNA ends stimulate recombination through single-strand invasion of homologous dsDNA (For review see Kowalczykowski 2000). Consequently, interaction of RecBCD with these χ sites results in recombinational hotspots within the *E. coli* chromosome (Dixon and Kowalczykowski 1993; Myers and Stahl 1994).

Realizing the limited application of homologous linear recombination using *recBC sbcBC* and *recD* mutants, and the recombinational potential at χ sites, Dabert and Smith (1997) developed a method that allows for homologous linear recombination in wild-type *E. coli* strains. By generating a 6.5 kb linear DNA fragment flanked on either side by χ sites, the authors were able to achieve recombination efficiencies approximately 45-fold greater than when using fragments lacking χ sites. The linear DNA was created by excising a cloned fragment of the *E. coli* histidine (*his*) operon interrupted by a kanamycin resistance determinant (*kan*). The flanking χ sites were oriented such that recombination would occur towards the centre of the *his::kan* fragment. The authors report a frequency of 64 *his*$^-$ *kan*^{R} transformants per microgram (μg) of linear DNA, a frequency they assert is comparable to the frequency of gene replacements in *rec* mutants (Dabert and Smith 1997).

Although the aforementioned method of gene replacement does allow for recombination in *E. coli* using linear DNA, it necessitates the introduction of χ sites on the insertion fragment. Furthermore, Dabert and Smith (1997) still employed cloning techniques to generate the linear DNA fragment. In a later study involving the same cloned *his::kan* fragment, El Karoui *et al.* (1999) were able to improve upon the method by negating the need for χ sites. The latter group was able to demonstrate that gene replacements can occur without χ sites when electrocompetent *E. coli* cells and electrotransformation are used. The authors reveal that a significant decrease in the *in vitro* exonucleolytic abilities of RecBCD results following the electroporation of *E. coli* cells at 200, 400, and 600 ohms (El Karoui *et al.* 1999). This decrease in RecBCD exonuclease activity allows for homologous recombination by reducing degradation of the linear DNA fragment. The reported frequency of this method of homologous linear recombination is approximately 60 gene replacements per μg linear DNA, which is comparable to the χ-facilitated methodology (Dabert and Smith 1997; El Karoui *et al.* 1999).

RecBCD does not represent the sole recombinational pathway available to *E. coli*, although it does represent the most efficient bacterial-encoded system. Other pathways of recombination include the bacterial-encoded RecF pathway, which serves namely to repair daughter-strand gaps, as opposed to RecBCD which reestablishes disintegrated replication forks (Kuzminov 1999). Phage-encoded recombination systems are also available to *E. coli* including the RecE and RecT functions encoded by the Rac prophage, and Exo and Beta encoded by λ phage (Court *et al.* 2002).

Recombination functions of λ phage involve the products of two genes, *exo* and *bet,* and the recombination system is collectively referred to as *red.* Exo, the gene product of *exo*, serves as a 5'-3' dsDNA exonuclease. Exo processively removes single nucleotides beginning at a dsDNA end, and proceeding in a 5' to 3' direction generates long 3' ssDNA overhangs (Carter and Radding 1971; Little 1967). Beta is a single-stranded DNA binding protein produced by λ *bet,* capable only of binding to Exo-generated ssDNA in excess of 35 nucleotides. Beta serves to protect ssDNA from

nucleolytic activity, and also promotes the annealing of bound 3' ssDNA ends to complementary DNA. Lastly, Beta stimulates RecA to conduct single-strand invasion of homologous DNA molecules (Carter and Radding 1971). A third λ phage encoded enzyme facilitates Red recombination functions in bacterial hosts. Gam, product of λ *gam*, attenuates the exonucleolytic activities of RecBCD. Inactivation of RecBCD by Gam prevents the degradation of λ phage DNA, thus allowing Exo and Beta to facilitate recombination between it and the bacterial chromosome (Karu *et al.* 1975).

In an effort to harness the recombinational potential of λ phage, Murphy (1998) developed procedures for homologous linear recombination involving both a multicopy plasmid housing *exo*, *bet*, and *gam*, and an *E. coli* strain with chromosome-encoded λ Red genes. By introducing linear fragments of DNA to wild-type *E. coli* bearing the Exo, Bet, and Gam-producing plasmid pTP223 (Poteete and Fenton 1984), Murphy was able to generate gene disruption frequencies 15 to 130 times greater than those reported in *recBC sbcBC* and *recD* recombination-proficient *E. coli* strains (Murphy 1998). Murphy (1998) utilized a cloned 3.2 kb *lacZ::kan* fragment, where *kan* is flanked on either side by 900 and 1200 bp of *lacZ*, respectively. Two means of transforming *E. coli* with the linear fragment were examined: electroporation and chemical transformation through calcium treatment. Electroporation resulted in a higher number of transformants than calcium-treatment, which Murphy suggests may be due to the increased efficiency of DNA uptake during electroporation (Murphy 1998). It is also reasonable to speculate given the findings of El Karoui *et al.* (1999) that electroporation further inhibited the exonucleolytic functions of RecBCD, as previously discussed.

In the same paper by Murphy (1998), chromosomal expression of λ Red genes was achieved by replacing *recBCD* with *exo* and *bet* under a lactose-inducible promoter (P_{lac}). It was shown that *gam* was not necessary for efficient recombination since it serves to attenuate RecBCD, a function already achieved by the chromosomal disruption of *recBCD.* A *kan* gene was also introduced as a

selectable marker, such that the genotype of the *E. coli* strain (KM22) became Δ*recBCD*::P_{lac}-*red kan*. Transformation of KM22 with a cloned 3.9 kb linear *lacZ::tet* fragment produced tetracycline-resistant, *lac*⁻ transformants at a rate 63-fold greater than in *recBC sbcBC* mutants, and 4.2-fold higher than in KM22 expressing plasmid-encoded λ Red genes. The difference in efficiency between chromosomally-encoded and plasmid-encoded λ Red, respectively, was attributed to decreased availability λ Red functions in the plasmid-encoded system due to competition for λ Red proteins between linear plasmid multimers produced during rolling circle replication and the linear DNA substrate. Another noteworthy finding from the same experiment demonstrated that KM22 can be transformed using *lacZ::tet* fragments generated through PCR amplification instead of excision from a cloning vector (Murphy 1998). This important finding first highlighted the potential for homologous linear transformation using PCR products.

Although the use of PCR-generated linear DNA fragments in λ Red-mediated recombination was demonstrated both in the aforementioned paper (Murphy 1998) and in a subsequent report by Murphy *et al.* (2000), fragments with long homologies to the target region (~1kb) were still utilized, and chromosomally-encoded λ Red functions were employed in the latter study. In an effort to circumvent these limitations, Datsenko and Wanner (2000) attempted λ Red-mediated recombination using pTP223 and PCR-generated fragments with short homologous sequences flanking a selective marker, using the primer design method previously described for yeast (Baudin *et al.* 1993). The authors employed chloramphenicol or kanamycin-resistance determinants flanked on either side by 36-50bp homologous to the different target genes, including the *lac* operon. The authors were unsuccessful in this attempt, so to preclude possible competitive inhibition between the linear DNA fragments and plasmid multimers, *exo, bet,* and *gam* were cloned into low copy number, temperature sensitive plasmids under the control of an L-arabinose-inducible control (P_{araB}; Datsenko and Wanner 2000).

Using their newly constructed λ Red recombinase plasmids, pKD20 and pKD46, Datsenko and Wanner (2000) successfully disrupted 13 different chromosomal genes in *E. coli* K-12. The number of transformants ranged from 100s to 1000s when 10-100 ng of linear DNA fragment were employed. Given the relative ease of generating the linear DNA cassettes and in recombining these cassettes with the chromosome, the authors designated the novel system a "one-step" method of disrupting of chromosomal genes (Datsenko and Wanner 2000). Since its initial development, use of the λ Red system has been extended to other bacterial species including *Shigella* sp., *Salmonella enterica* Serovars typhimurium and enteritidis, *Yersinia pseudotuberculosis, Pseudomonas aeruginosa* and *Streptomyces coelicolor* (Gust *et al.* 2003; Lesic and Rahme 2008; Murphy and Campellone 2003 (and references therein); Ranallo *et al.* 2006). A visual comparison of the novel Red-mediated recombination system and the traditional means of producing gene disruptions can be found in Figure 1.0.1.

The λ Red-mediated recombination system has also been sparsely employed in *Escherichia coli* O157:H7, however the frequency and reproducibility of gene replacements is not reported in these studies (Dziva *et al.* 2004; Iyoda and Watanabe 2004; Low *et al.* 2006; Newton *et al.* 2004). Using a plasmid-encoded system similar to pKD46, Murphy and Campellone (2003) attempted λ Red-mediated recombination in enterohemorrhagic and enteropathogenic *E. coli* strains (EHEC and EPEC, respectively) with sporadic results. In an effort to improve the efficiency of gene replacement, the authors employed a modified protocol involving an optimized buffer (20% glycerol – 1mM MOPS) and a heat shock stage. Using the modified protocol, introduction of a *ΔlacZ::kan* gene replacement using 40bp of flanking homologies was performed reproducibly with an efficiency of 0.7-6 x 10^{-6} per cell survivor in EHEC. Notably, the same gene replacement in *E. coli* K-12 yielded an efficiency of 10^{-4} per cell survivor, indicating that the λ Red-mediated recombination system is approximately 100-fold more efficient in non-pathogenic strains such as K-12 versus pathogenic EHEC and EPEC strains. The difference in efficiency may be attributed to

lower expression of λ Red proteins, lower uptake of DNA during electroporation, or decreased activity of Gam on RecBCD in EHEC and EPEC, versus K-12. The last hypothesis was preliminarily tested by performing recombination in an EHEC *recC* mutant, but recombination was not enhanced relative to wild-type EHEC. Furthermore, frequency of gene replacement was found to be dependent on both the length of homology (longer homologies increased recombination events) and the gene target, with the number of successful transformants ranging from 2 to 1000 per experiment (Murphy and Campellone 2003).

Despite the decreased efficiency of Lambda Red-mediated homologous linear recombination in EHEC relative to non-pathogenic *E. coli*, the utility of generating gene replacements in pathogenic *E. coli* is undeniable. Since *E. coli* O157:H7 is not readily amenable to transformation with linear DNA due to the activities of RecBCD, and the application of traditional methods of genetic engineering can involve significant temporal investment, the systematic disruption of targeted genes using the activities of plasmid-encoded λ Red functions represents viable alternative. This thesis will investigate the application of Lambda Red-mediated recombination in generating an *rpoS*-knockout in *E. coli* O157:H7, in order to help elucidate the role of the stationary phase sigma factor on biofilm formation.

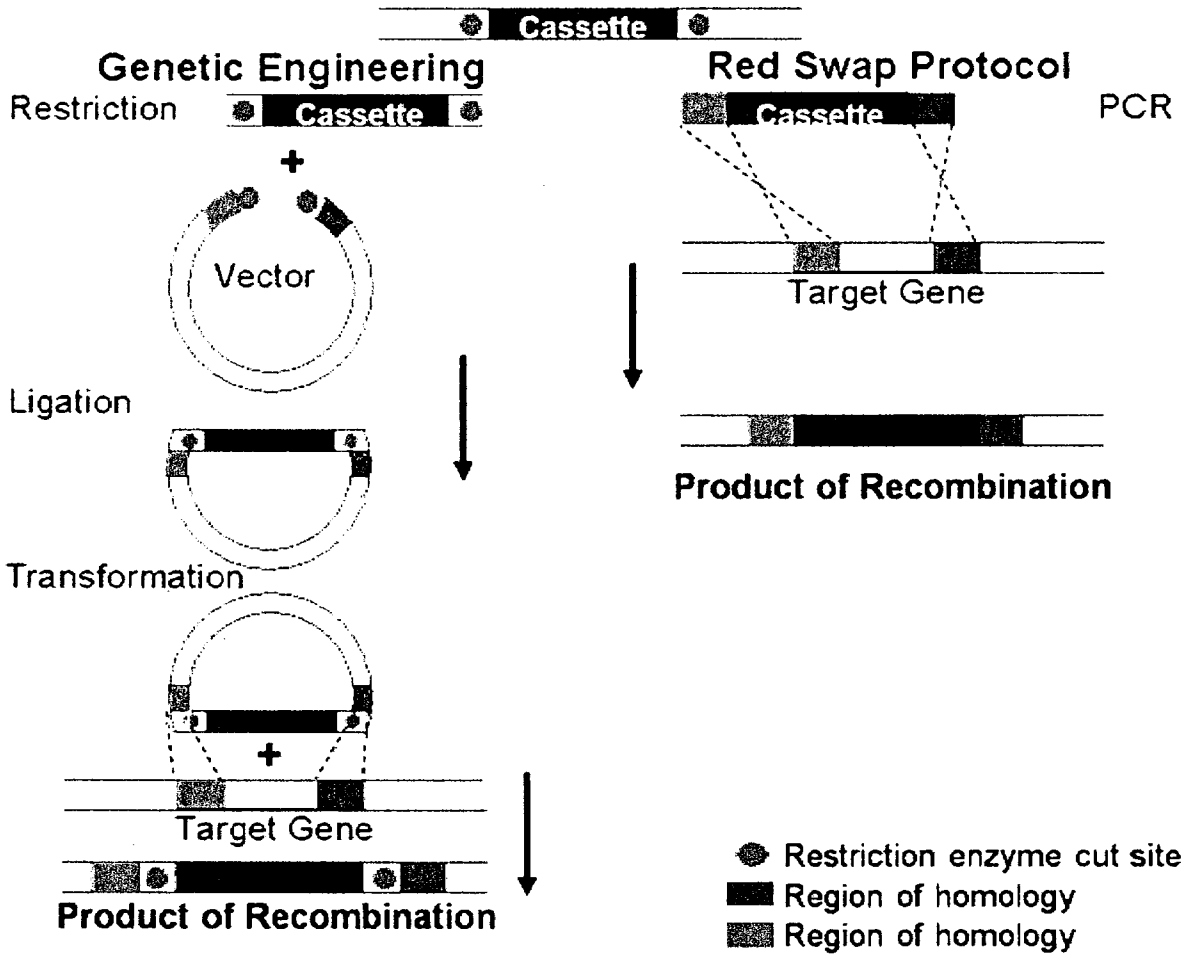

Figure 1.0.1. Traditional genetic engineering versus the Lambda Red-recombination (Red Swap). Modified from Court, D.L., Sawitzke, J.A., Thomason, L.C. 2002. Annu. Rev. Genet. 36: 361-388

1.4 THESIS OBJECTIVES

The first objective of this thesis is to construct a *gfp*-labeled, *rpoS*-knockout strain of pathogenic *E. coli* O157:H7 by means of homologous linear recombination. The first objective will be achieved through the following steps:

i) Generating recombination-proficient strains of *E. coli* O157:H7 through transformation with the Lambda Red plasmid, pKD46

ii) Constructing linear DNA cassettes containing selective markers flanked by sequences homologous to *rpoS*

iii) Performing homologous linear recombination between the recombination-proficient *E. coli* strains and the linear cassettes

iv) Confirming the success of the *rpoS*-knockout through PCR and analyzing the planktonic growth of the *rpoS*-knockout and wild-type.

The second objective of this thesis is to evaluate the relative biofilm-forming capabilities and survival of the *rpoS*-knockout relative to the wild-type. The second objective will be achieved through:

i) Cultivating biofilms of wild-type and *rpoS*-deficient strains of *E. coli* on glass coverslips in various media and examining the biofilms using confocal laser scanning microscopy (CLSM)

ii) Evaluating the relative biofilm-forming capabilities of each strain through processing the CLSM images using PHobia Laser Image Processing software (PHLIP) and statistical analysis

iii) Assessing the survival of the wild-type and *rpoS*-knockout biofilms in a carbon-starved environment

iv) Determining the relative detachment of cells from the wild-type and *rpoS*-knockout biofilms during starvation

CHAPTER 2 - CONSTRUCTION OF AN *E. COLI RPOS*-KNOCKOUT USING LAMBDA RED RECOMBINATION

2.0 INTRODUCTION

The advent of genomic sequencing has greatly facilitated the identification and characterization numerous genes, especially those genes lacking a readily selected phenotype between the wild-type and knockout, as well as previously undefined open reading frames. Targeted chromosomal gene disruptions, generated through the standard techniques of genetic engineering, allow for the identification of function in these elusive genes. The process of disrupting a chromosomal gene typically involves the production of the desired mutant allele on a vector, and subsequently introducing this mutation to the bacterial chromosome through homologous recombination (Hamilton *et al.* 1989; Raibaud *et al.* 1984; Russell and Dahlquist 1989). While this multi-step method has proved invaluable to the study of microbial physiology, the process is lengthy, often very complicated, and hindered by the availability of appropriate restriction endonucleases and vectors.

A novel method of genetic engineering in *Escherichia coli* helps curtail the technical issues of genetic engineering by eliminating the need for a vector construct. The new method harnesses the recombinational capacities of the Lambda (λ) phage, namely *exo, bet,* and *gam* - genes encoding a 5' to 3' exonuclease, single-stranded DNA-binding protein, and an inhibitor of the host exonuclease RecBCD, respectively (Carter and Radding 1971; Karu *et al.* 1975; Little 1967; Murphy 1998). By inhibiting RecBCD, linear DNA can escape degradation upon entering the bacterial cell, thus allowing for recombination with the host cell chromosome. By placing *exo, bet,* and *gam* under plasmid control, wild-type *E. coli* transformed with this Red recombinase plasmid can become recombination-proficient (Datsenko and Wanner 2000; Murphy and Campellone 2003; Murphy 1998; Murphy *et al.* 2000).

The ease of the aforementioned system is enhanced by the use of linear DNA constructs produced through PCR (Datsenko and Wanner 2000; Murphy *et al.* 2000). Linear fragments bearing the desired gene mutation can be generated by amplifying a selective marker, such as an antibiotic resistance determinant, using primers that are flanked by short (36-50bp) sequences homologous to the target gene. The resulting amplicon is the selective marker flanked by regions homologous to the target, which facilitate homologous recombination (Datsenko and Wanner 2000). This method is termed Lambda Red-mediated recombination or "recombineering," and theoretically represents a one-step method for inactivating chromosomal genes (Datsenko and Wanner 2000; Murphy and Campellone 2003; Savage *et al.* 2006). Though its application in generating gene disruptions in *E. coli* K-12 has been extensive, the reports of recombineering in pathogenic strains of *E. coli* suggest sporadic success (Murphy and Campellone 2003).

One gene, whose function in *Escherichia coli* O157:H7 biofilm formation remains elusive, is *rpoS*. RpoS, the gene product of *rpoS*, is a stationary phase sigma factor, responsible for facilitating expression of genes not only during stationary phase, but also during periods of adversity including starvation, oxidative stress, osmotic stress and heat shock (Lange and Hengge-Aronis 1991a; McCann *et al.* 1991; Schembri *et al.* 2003). Since biofilm bacteria have been reported to experience higher osmotic stress, oxidative stress, and cell density (Prigent-Combaret *et al.* 1999), it has been speculated that *rpoS* may play a role in biofilm formation.

Numerous *rpoS*-dependent genes exhibit differential expression in *E. coli* K-12 between the planktonic and biofilm state (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003), however the actual influence of *rpoS* expression on biofilm formation remains unknown. While no studies have been conducted on the role of *rpoS* in pathogenic biofilm formation, the few studies in *E. coli* K-12 are contradictory (Adams and McLean 1999; Corona-Izquierdo and Membrillo-Hernández 2002; Ito *et al.* 2008; Schembri *et al.* 2003). Furthermore, impressive variations have been demonstrated in the biofilm-forming capabilities of *E. coli* strains, both under the same and differing environmental

conditions. Pathogenic, commensal, and laboratory strains of *E. coli* evaluated on their capacity to form biofilms displayed varying amounts of biofilms within and between groups, such that source was not a predictor of biofilm formation. When the same *E. coli* strains were evaluated on their ability to form biofilms in different media, growth media composition was found to strongly influence the amount of biofilm formed by a given strain, with a negative correlation between nutrient availability and the amount of biofilm (Reisner *et al.* 2006).

The purpose of this study was to generate a *gfp*-labeled, *rpoS*-knockout strain of *E. coli* O157:H7 through application of the Lambda Red-mediated recombination system. Production of this *rpoS*-knockout will assist in the future investigation of *rpoS* function in pathogenic *E. coli* biofilm formation.

2.1 MATERIALS AND METHODS

2.1.1 Bacterial strains

The *E. coli* O157:H7 strains employed in this study were obtained from Dr. C. Gyles at the University of Guelph (Guelph, ON, Canada). Strain identification numbers EC961085, EC961019, EC961020, and EC9620004 from this collection were used, corresponding to *E. coli* strains H21, H22, H29, and H32, respectively. Strains H21, H22, and H29 represent human isolates, whereas H32 is a bovine isolate. The human isolates were obtained from clinical specimens in Canada, Australia, Europe, and the United States, while the bovine isolate was obtained from a healthy subject in North America (Gyles *et al.* 1998; Watterworth *et al.* 2006). All strains are pathogenic and possess the *eae* gene for intimin, the virulence factor responsible for mediating the attachment of *E. coli* to colonic epithelial cells (Donnenberg *et al.* 1993; Gyles *et al.* 1998).

For all molecular manipulations, wild-type *E. coli* strains were cultured in Luria-Bertani (Miller) Broth (LB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) without antibiotics at 37°C, unless otherwise indicated. Stock cultures of each strain were prepared in both Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and LB, supplemented with 25% (v/v) glycerol. All stock cultures were maintained at -80°C and recovered overnight at 37°C by streaking for isolated colonies on LB agar (LBA) prior to use. All of the bacterial strains employed and generated in this study can be found as a comprehensive list in Table 2.1.1.

2.1.2 Plasmids

E. coli S17-1λ carrying plasmid pJBA29 (S17-1λ/pJBA29) was graciously provided by Jens Bo Andersen of The Technical University of Denmark, Department of Microbiology (Lyngby, Copenhagen, Denmark). The plasmid, pJBA29, served as the original source of the labeling cassette used in this study. A kanamycin resistance determinant (*aph(3')*I) and green fluorescent protein gene (*gfp*) are housed on a mini-Tn5 transposon in pJBA29, where *gfp* is under the control a constitutively expressed promoter, $P_{A1/04/03}$ (Andersen *et al.* 1998). The mini-Tn5 delivery function

of this plasmid was not employed in this study. Cultivation and stock cultures of S17-1λ/pJBA29 were prepared as previously described for pathogenic *E. coli* strains, modified only by the addition of 50 μg/mL of kanamycin and 100 μg/mL of ampicillin to the media.

The λ Red recombinase plasmid used in this study, pKD46, was generously provided by Francis E. Nano at the University of Victoria, Department of Biochemistry and Microbiology (Victoria, BC, Canada). As previously detailed, pKD46 possesses the λ Red genes *exo, bet,* and *gam* under the L-arabinose-inducible promoter, P_{araB}. The plasmid is low copy number and temperature-sensitive (*oriR101*, *repA101ts*), and consequently can be easily cured at temperatures between 37-43°C (Datsenko and Wanner 2000). Selection of cells successfully transformed with pKD46 can be achieved by plating on media supplemented with ampicillin, as the plasmid also carries a gene for ampicillin-resistance (*bla*). The 6,329 bp plasmid was supplied in DH5α, however following successful transformation into JM109 (Section 2.1.6), JM109/pKD46 served as the preferred source of pKD46 for all subsequent extractions and manipulations.

Gerben J. Zylstra of Rutgers University (New Brunswick, NJ, USA) kindly donated p34S-Gm. The plasmid was used as a template for the gentamycin resistance determinant (*acc*C1; herein *gm*) employed in this study. JM109/p34S-Gm was maintained in media supplemented with 20 μg/mL of gentamycin sulfate. A comprehensive list of all the plasmids employed and generated in this study can be found in Table 2.1.2.

Table 2.1.1. Characteristics of bacterial strains used in this study

Strain	Identification Number	Description/Characteristics	Source/Reference
Escherichia coli			
H21	EC961085	Pathogenic, serotype O157:H7, human isolate	(Gyles *et al.* 1998; Watterworth *et al.* 2006)
H22	EC961019	Pathogenic, serotype O157:H7, human isolate	(Gyles *et al.* 1998; Watterworth *et al.* 2006)
H29	EC961020	Pathogenic, serotype O157:H7, human isolate	(Gyles *et al.* 1998; Watterworth *et al.* 2006)
H32	EC9620004	Pathogenic, serotype O157:H7, bovine isolate	(Gyles *et al.* 1998; Watterworth *et al.* 2006)
H32-*gfp*	Derived from EC9620004	Pathogenic, serotype O157:H7, random insertion of Tn5 bearing kanamycin resistance gene (*aph(3')II)* and green fluorescent protein gene (*gfp*) Kan^r	This study
H32-*gfpΔrpoS::gm*	Derived from EC9620004	Pathogenic, serotype O157:H7, random insertion of Tn5, *rpoS*⁻ disrupted with gentamycin resistance gene (*accCI*) Kan^r, Gm^r	This study
JM109		Nonpathogenic, cloning host	Promega Corporation, Madison, WI, USA

Table 2.1.2. Characteristics of plasmids used in this study

Plasmid	Description/Characteristics	Source/Reference
pKD46	Low copy number plasmid bearing L-arabinose-inducible, λ Red recombinase system (P_{araB}-*gam-bet-exo*), temperature-sensitive (*oriR101, repA101ts*) Amp^r (*bla*)	Nano, F.E. (Datsenko and Wanner 2000)
pGEM® T-Easy	Cloning host used to construct linear fragments Amp^r (*bla*), *lacZ* insertion site	Promega Corporation, Madison, WI, USA
pMOD^{TM}-2<MCS>	Construction vector for EZ-Tn5^{TM} Amp^r (*bla*)	Epicentre Biotechnologies, Madison, WI, USA
pMOD^{TM}<*gfp*-kan>	Source of EZ-Tn5^{TM}<*gfp-kan*> transposon Template for kanamycin resistance gene (*aph(3')II*) and green fluorescent protein (*gfp*) gene (*gfp-kan* cassette) Kan^r, Amp^r (*bla*)	This study
pJBA29	Original template plasmid for kanamycin resistance gene (*aph(3')II*) and green fluorescent protein (*gfp*) gene (*gfp-kan* cassette) Delivery vector for mini-Tn5 (function not employed) Kan^r, Amp^r	(Andersen *et al.* 1998)
p34S-Gm	Template for gentamycin resistance gene (*accC*1) Gm^r	(Dennis and Zylstra 1998)
pGEM-*rpoS*	Construction vector for long *rpoS* homology cassettes and template for *rpoS* sequencing Amp^r	This study
pGEM500Δ*rpoS::gm*	Source of 500Δ*rpoS::gm* cassette for homologous linear recombination Amp^r, Gm^r	This study
pGEM500Δ*rpos::gfp-kan*	Source of 500Δ*rpoS::gfp-kan* cassette for homologous linear recombination Amp^r, Kan^r	This study

2.1.3 Labeling of *E. coli* using a Tn5 transposon

E. coli H32 was labeled with a green fluorescent protein gene (*gfp*) and a selective antibiotic marker conferring kanamycin resistance (*aph(3')I;* herein *kan*) through the use of a Tn5 bacterial transposon. Rebecca Barnes, a former member of the Applied and Environmental Microbiology Lab at Lakehead University, performed the labeling procedure in fulfillment of the requirements of an Honours thesis project. The specific details of the labeling procedure are available from Dr. Kam Tin Leung at Lakehead University (Thunder Bay, ON, Canada), and are briefly described below (Section 2.1.3).

Labeling of pathogenic *E. coli* strain H32 was conducted using an EZ-Tn5™ pMOD™-2<MCS> construction vector (Epicentre Biotechnologies, Madison, WI, USA). The selected vector possesses a multiple cloning site (MCS) allowing for the insertion of any DNA fragment of interest, flanked by mosaic ends (represented by < and >) that are acted upon by EZ-Tn5™ Transposase. The Tn5 transposon inserts randomly into the host chromosome, facilitated by EZ-Tn5™ Transposase, carrying the desired cassette, in this case <*gfp-kan*>.

Prior to manipulation, pMOD™-2<MCS> was electroporated into JM109 through an optimized standard protocol (Sambrook and Russell 2001). JM109/ pMOD™-2<MCS> allowed for the stable maintenance of the system at sufficiently high numbers to facilitate multiple attempts at successful transposition. The vector was extracted from JM109 when required using the Wizard® Plus SV Minipreps DNA Purification System, following the kit instructions (Promega Corporation, Madison, WI, USA).

The *gfp-kan* cassette was excised from its host plasmid, pJBA29, using restriction enzyme digest with KpnI, following extraction of the plasmid using the aforementioned miniprep kit. KpnI digestion was conducted as recommended by the supplier (Promega Corporation, Madison, WI, USA). Due to the inefficiency of commercially prepared kits at purifying large fragments, the 3,750 bp *gfp-kan* fragment

was gel purified using homemade spin columns fashioned from 0.5 mL PCR tubes packed ¼ full with glass wool. Each PCR tube was punctured at the apex using a hot inoculating needle, and rested inside a 1.5 mL centrifuge tube, which served to collect the flowthrough. Prior to centrifugation at 12,000 x g for 5 minutes, the gel was frozen at -80°C in the sterile spin columns to collapse the gel matrix. The flowthrough was collected and purified by standard phenol:chloroform extraction and ethanol:glycogen precipitation (Sambrook and Russell 2001). The ethanol:glycogen precipitation was left overnight to maximize recovery and following precipitation was the DNA was resuspended in TE buffer or nuclease-free distilled, deionized water (ddH_2O).

Once sufficient quantities of the *gfp-kan* insert were obtained, the pMOD™-2<MCS> vector was linearized using KpnI and dephosphorylated by calf intestinal alkaline phosphatase (CIAP) following the manufacturer recommended protocols (Fermentas International Inc., Burlington, ON, Canada). The linearized, dephosphorylated vector was purified using phenol:chloroform extraction and resuspended in ddH_2O. The vector and *gfp-kan* insert were then ligated together using T4 DNA Ligase at a molar ratio of 1:1, following the recommended protocol (Promega Corporation, Madison, WI, USA). Successful ligation forming pMOD™-2<*gfp-kan*> (herein, pMOD-*gfp*) was confirmed through the presence of a 6,295 bp band on an agarose gel. The ligation mix was purified and precipitated as previously described and electroporated into JM109. Screening for successful transformants was performed by plating on LBA supplemented with both 100 µg/mL ampicillin (Amp^{100}) and 50 µg/mL kanamycin (Kan^{50}), observing green fluorescence under ultraviolet (UV) illumination and conducting a restriction digest with KpnI yielding 3,750 bp (*gfp*) and 2,545 bp (vector) fragments.

The *gfp-kan* fragment, complete with the mosaic ends needed for transposition, was liberated from pMOD-*gfp* through restriction enzyme digestion with PshA1 (New England Biolabs, Ipswich, MA), and was gel purified and concentrated as previously described. The transposase reaction mix was prepared with slight modifications to the recommended protocol (Epicentre Biotechnologies, Madison,

WI, USA), namely reducing the amount of enzyme solution in an effort to reduce arching. Electroporation was used to introduce the transposon to H32, thus facilitating transposition. The electroporation protocol used employed only ddH_2O in the wash steps, again to minimize arching. Screening for successful transposition of H32 was conducted by plating on LBA with Amp^{100} and Kan^{50}, and screening for green fluorescence.

2.1.4 Biochemical analyses and stability testing

Biochemical analyses and strain identification were conducted on H32 and H32-*gfp* through the use API 20E, the api® strip for designed for the identification of *Enterobacteriaceae* (bioMerieux, St. Laurent, QC, Canada). The stability of the *gfp*-label was also observed by performing a 6-day stability test. The stability test was conducted by initially culturing H32-*gfp* on nonselective TSA and incubating for 24 h. Following incubation, one colony was subcultured on fresh TSA and incubated for 24 h. The subculturing procedure was repeated 3 additional times, and on the 6^{th} day the last plate culture was used to inoculate both TSA and TSA Kan^{50}. The ability of H32-*gfp* to grow on TSA Kan^{50} versus TSA after nonselective growth was evaluated. The relative fluorescence was also assessed each day under UV illumination. A stable chromosomal label should maintain both kanamycin resistance and fluorescence through multiple generations without selective pressure and therefore should grow equally well on TSA and TSA Kan^{50}.

2.1.5 Identification of the Tn5 insertion site and sequencing of *gfp-kan*

Genetic confirmation of the successful EZ-Tn5™<*gfp-kan*> transposition into *E. coli* was achieved by identifying the insertion site of <*gfp-kan*> through genomic sequencing. Chromosomal DNA extractions were performed using a Wizard® SV Genomic DNA Purification System as per the kit instructions (Promega Corporation, Madison, WI, USA). Due to the high concentration and purity of DNA required for genomic sequencing (DeSousa 2008), DNA from 8 separate 1 mL extractions was pooled and precipitated through standard ethanol precipitation with 0.3 M sodium acetate (Sambrook

and Russell 2001). The precipitated H32-*gfp* DNA was resuspended in 100 µL of nuclease-free ddH_2O at a final concentration of approximately 2.0 µg/µL. The concentration and purity of the DNA were determined through spectrophotometry by measuring the absorbance at 260 nm (A_{260}) and the $A_{260/280}$ absorbance ratio, respectively. All assessments of purity and concentration were performed using an Ultrospec 2100 pro spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

The insertion site of <*gfp-kan*> was determined by using primers oriented outward from the MCS of the transposon, such that sequencing was directed into the chromosome rather than in towards the *gfp-kan* cassette. The primers were designed and designated SqFP and SqRP by Epicentre Biotechnologies (Madison, WI, USA). All primer sequences and any corresponding information can be found in Table 2.1.4. The primers were prepared by Sigma Genosys Canada (Oakville, ON, Canada), whereas the genomic sequencing was performed by Mobix Lab (McMaster University, Hamilton, ON, Canada). Sequences homologous to the resulting genomic information were identified using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI; Altschul *et al.* 1997).

Once homologous sequences were identified between previously sequenced *E. coli* type-strains in the NCBI database and the H32-*gfp* transposon insertion site, separate multiple sequence alignments were performed (Appendix 4.0) for the two flanking sequences using DNAMAN (Lynnon BioSoft 2000). The corresponding alignments were assembled in Microsoft Word (Microsoft Corporation 2007) such that the insertion of <*gfp-kan*> could be confirmed and the chromosomal region disrupted by the transposon could be identified.

To facilitate future molecular manipulations involving the *gfp-kan* cassette housed by JM109/ pMOD-*gfp*, the ~4 kb fragment was sequenced through multiple reactions (Mobix Lab - McMaster University, Hamilton, ON, Canada). The primer design and sequence assembly were largely performed by Donna Scott, an Honours thesis student at Lakehead University (Thunder Bay, ON, Canada). For a

comprehensive list of primers used in the sequencing of *gfp-kan,* see Table 2.1.4. The approximate locations of each gene were established through performing sequence alignments with known *gfp, kan,* and pMOD™-2<MCS> sequences (Accession numbers: AF062080 and AY952933, and Epicentre Biotechnologies). The analysis was performed using DNAMAN (Lynnon BioSoft 2000) and the sequence was assembled and annotated in Microsoft Word (Microsoft Corporation 2007).

2.1.6 Generating recombination-proficient *E. coli* O157:H7 strains

Extraction of pKD46 from its initial host, DH5α, was performed using reagents and components of the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA). A modified extraction protocol was employed to purify pKD46 instead of the recommended protocol from Promega. The reagent volumes and timings have been altered from the suggested protocol, while the overall steps remain similar (for details see Promega 2007). The particulars of the modified protocol are described below.

A colony of DH5α/pKD46 was used to inoculate 50 mL of LB with 100 μg/mL of ampicillin to select for pKD46. After incubating overnight (12-16 hours) at 30°C and 150 rpm, 10 mL aliquots of overnight culture were centrifuged (3,200 x g) for 10 minutes to the harvest cells. The resulting supernatant was removed by decanting, and the excess liquid was blotted on clean absorbent paper. The pellet was thoroughly resuspended in 500 μL of Cell Resuspension Solution using pipetting and vigorous vortexing. Cells were lysed through the addition of 500 μL of Cell Lysis Solution, mixing 10 times by inversion, and incubating at room temperature for a minimum of 2 minutes or until clearing was observed (a maximum of 5 minutes). To the resulting bacterial lysate, 20 μL of Alkaline Protease Solution were added and mixed by inverting 10 times. The lysate was then incubated at room temperature for 5 minutes. Following incubation, 700 μL of Neutralization Solution were added and mixed immediately by inverting the sample 10 times. The bacterial lysate was then centrifuged at

3,200 x g for 10 minutes to pellet cellular debris. If residual debris remained in solution, the lysate was centrifuged a second time under the same conditions (Promega 2007).

Once the cellular debris was pelleted, 750 µL of the supernatant was loaded into a Spin Column (analogous to silica bead purification) and centrifuged at maximum speed (21,000 x g) for 1 minute. The flowthrough was discarded and the process was repeated with the remaining supernatant. The bound plasmid DNA was washed and dried as directed by the kit instructions, first through the addition of 750 µL of Column Wash Solution to the assembly, centrifugation at maximum speed for 2 minutes, and decanting the flowthrough. The initial wash was followed by a second wash with 250 µL of Column Wash Solution, 2 minute centrifugation, and subsequent decantation of the flowthrough. The residual ethanol from the Column Wash Solution was dried by centrifugation at maximum speed for 1 minute. The plasmid DNA was eluted by adding 100 µL of nuclease-free ddH_2O to the Spin Column, incubating at room temperature for 2 minutes, and collecting the eluate through centrifugation at maximum speed for 2 minutes. The resulting plasmid DNA was purified and concentrated in 1/5th the original volume of nuclease-free ddH_2O using a standard ethanol precipitation protocol (Sambrook and Russell 2001). The presence and approximate concentration of the extracted plasmid DNA were assessed using agarose gel electrophoresis and visual comparison to a known size standard (GeneRuler™ 1 kb PLUS DNA Ladder; Fermentas International Inc., Burlington, ON, Canada). The expected size of pKD46 is 6,329 bp.

Once extracted from its original host, the purified pKD46 was transformed into *E. coli* JM109, H21, H22, H29, H32, and H32-*gfp* using electroporation. The host strains of *E. coli* were grown overnight at 37°C, shaking at 150 rpm. *E. coli* H32-*gfp* was grown in LB supplemented with 50 µg/mL of kanamycin, whereas all other strains were grown without antibiotics. The overnight cultures were subcultured 1:10 in 9 mL of fresh LB and grown for 1.5 h ($OD_{600\,nm}$ = 0.4-0.6, cell density ≈ 10^8 CFU/mL). The cells were pelleted through centrifugation at 3,200 x g for 10 minutes at 4°C. To remove

the salts associated with the media and facilitate the production of electrocompetent cells, the supernatant was removed and the pellet was gently resuspended in 1 mL of ice-cold ddH_2O through pipetting. An additional 9 mL of ddH_2O was added to the 1 mL suspension of cells and mixed by inversion. The aforementioned wash procedure was repeated two additional times with ddH_2O, and once with ice-cold 10% (v/v) glycerol. A final wash was performed by resuspending the pellet in 1 mL 10% glycerol and centrifuging the suspension at 10,000 x g for 2 minutes. The supernatant was removed and the pellet was resuspended in 600 µL of 10% glycerol. The cell suspension was aliquoted into two 1.5 mL pre-chilled microcentrifuge tubes (300 µL each) and 5 µL of purified plasmid DNA (500 ng) was carefully but thoroughly mixed with each aliquot of electrocompetent cells. The samples were then transferred to pre-chilled electroporation cuvettes (400 µL volume, 2 mm gap; Eppendorf Canada, Mississauga, ON, Canada) and incubated on ice for 30 minutes. Following incubation, the cuvettes were quickly but thoroughly dried and placed in an Eppendorf Electroporator 2510 (Eppendorf Canada, Mississauga, ON, Canada). All samples were electroporated at 2500 V, 10 µF capacitance, with an approximate acting time of 5.0 milliseconds.

Following electroporation, the cells were immediately recovered by adding 500 µL of cold 2xLB directly to the cuvette, mixing gently by pipetting, and transferring the sample to a 1.5 mL microcentrifuge tube containing an additional 200 µL of 2xLB. The samples were then incubated at 30°C, shaking at 150 rpm for 1-1.5 hours. Collection of the cells via centrifugation was performed at 10,000 x g for 2 minutes, following which the supernatant was removed and the pellet was resuspended in 400 µL of LB. Each sample was plated in duplicate by spread plating 200 µL of the suspension per LBA Amp^{100} plate. The plates were incubated overnight at 30°C.

Selection of successful transformants was performed by collecting single, well-isolated ampicillin-resistant colonies from the transformation plates, streaking LBA Amp^{100} plates for isolated colonies, and subsequently using colonies from these plates to confirm the presence of pKD46 through

extraction and agarose gel electrophoresis, as described previously. Furthermore, restriction analysis was performed using DNAMAN (Lynnon BioSoft 2000), and the published pKD46 sequence available through NCBI (Accession Number: AY048746, Datsenko and Wanner 2000). Restriction enzymes PinAI and PstI were selected from this analysis, and a restriction enzyme digest of pKD46 was performed as recommended by Roche (Mississauga, ON, Canada) and Promega (Madison, WI, USA), respectively. The expected fragment sizes for PinAI digestion of pKD46 are 1,657 bp and 4,672 bp, whereas the expected fragment sizes from PstI digestion are 247 bp and 6,082 bp.

2.1.7 Producing short linear DNA fragments through PCR

DNA cassettes used for homologous linear recombination through the λ Red system were generated by PCR when short homology (~40 bp) with the target region, *rpoS*, was desired. Primers were designed in two sections; the first section served to amplify the selective marker (either *gfp-kan* or *gm)* and the second flanking section served as the homology to *rpoS*. PCR amplification was used in conjunction with these dual function primers to construct an *rpoS*-flanked *gfp-kan* cassette (*rpoS-gfp-kan*) and an *rpoS*-flanked *gm* cassette (*rpoS-gm*).

Preliminary sequencing results of *gfp-kan* from pMOD-*gfp* were used to design *gfp*-F, 5'-GCCCTTTCGTCTTCACCTCG-3', and *gfp*-R, 5'-GCTCTGCCAGTGTTACAACC-3', the 20 bp primer sections used to amplify *gfp-kan*. Similarly, p34S-Gm sequence data from NCBI was used to design *gm*-F, 5'-GCTCGAATTGACATAAGCCTGT-3', and *gm*-R, 5'-TGTGACAATTTACCGAACAACTCC-3', to amplify *gm* (Accession Number: AF062079, Dennis and Zylstra 1998). Both of these sets of primers were selected to fulfill the first function of amplifying the selective marker.

Highly conserved regions overlapping the promoter region and terminator sequence of *rpoS* were selected by performing a multiple sequence alignment and identifying ORFs in DNAMAN (Lynnon BioSoft 2000). The alignment was performed using known *E. coli rpoS* sequences from NCBI (Altschul *et al.* 1997), specifically Accession numbers: NC_008563 (*E. coli* APEC 01), NC_009801 (*E. coli* E24377A), NC_009800 (*E. coli* HS), NC_000913 (*E. coli* K-12 substrain MG1655), NC_002655 (*E. coli*

O157:H7 substrain EDL933), and NC_007946 (*E. coli* UTI89). The complete sequence alignment can be found in Appendix 4.1. The 41 bp upstream and 40 bp downstream of *rpoS* selected to serve as the flanking sequences homologous to this target were 5' GAATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATG-3,' and 5' - CAGATGCTTACTTACTCGCGGAACAGCGCTTCGATATTCA-3', respectively.

The same *rpoS* flanking sequences were used to design the overall cassette primers for both *rpoS-gfp-kan* and *rpoS-gm*. The resulting primers for amplifying *rpoS-gfp-kan* were *rpoS-gfp*-F, 5' - GAATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGGCCCTTTCGTCTTCACCTCG-3' and *rpoS-gfp*-R, 5'-CAGATGCTTACTTACTCGCGGAACAGCGCTTCGATATTCAGCTCTGCCAGTGTTACAACC-3'. The primers designed for amplifying *rpoS-gm* were *rpoS-gm*-F, 5' - GAATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGGCTCGAATTGACATAAGCCTGT -3,' and *rpoS-gm*-R, 5'-CAGATGCTTACTTACTCGCGGAACAGCGCTTCGATATTCATGTGACAATTTACCGAACAACTCC -3'. All of the aforementioned primers were manufactured by Sigma Genosys Canada (Oakville, ON, Canada).

Both p34S-Gm and pMOD-*gfp* were extracted for use as PCR templates using the Wizard® Plus SV Minipreps DNA Purification System with no modifications to the recommended protocol (Promega Corporation, Madison, WI, USA). A 50 µL PCR reaction mix for the amplification of *rpoS-gm* was prepared as follows (final concentrations in brackets): 37.5 µL of nuclease-free ddH_2O, 5 µL of 10X *taq* buffer with $(NH_4)_2SO_4$ (1X), 0.5 µL of 20 mM dNTP mix (0.2 mM of each dATP, dTTP, dCTP, and dGTP), 1 µL of each 10 µM primer (0.2 µM), 1 µL of *taq* DNA polymerase (0.02 units/µL), 3 µL of 25 mM $MgCl_2$ (1.5 mM), and 1 µL (20-100 ng) of extracted p34S-Gm template DNA. All PCR reagents were provided by Fermentas International (Burlington, ON, Canada). The 858 bp *rpoS-gm* cassette was amplified using a PCR Sprint Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: one initial denaturation cycle for 3 min at 95°C; 35 cycles of denaturation for 1 min at 94°C,

annealing for 1 min at 55°C, and extension for 1 min at 72°C; a final extension for 10 min at 72°C and a final indefinite hold at 4°C.

Due to large size of the *rpoS-gfp-kan* cassette (~3.3 kb), the PCR protocol was optimized using Phusion®, a high fidelity, high speed polymerase (Finnzymes USA Inc., Woburn, MA, USA). The PCR reaction mix to amplify *rpoS-gfp-kan* was prepared as follows (final concentrations in brackets): 32.5 µL of nuclease-free ddH_2O, 10 µL of 5X HF buffer (1X), 1.0 µL of 10 mM dNTP mix (0.2 mM of each dATP, dTTP, dCTP, and dGTP), 2.5 µL of each 10 µM primer (0.5 µM), 0.5 µL of Phusion® polymerase (0.02 units/µL), and 1 µL (20-100 ng) of extracted pMOD-*gfp* template DNA. All reagents were provided as part of a Phusion®High-Fidelity PCR kit (Finnzymes USA Inc., Woburn, MA, USA). The *rpoS-gfp-kan* cassette was amplified under the following conditions: one initial denaturation cycle for 30 sec at 99°C; 40 cycles of denaturation for 5 sec at 98°C, annealing for 20 sec at 64°C, and extension for 2 min at 72°C; and a final extension for 10 min at 72°C. All samples were held at 4°C following PCR.

Following the production of *rpoS-gm* and *rpoS-gfp-kan* through PCR, the cassettes were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). Samples were run at 130 V for 45 minutes on thick 1% (w/v) agarose gels, and the desired fragments were excised carefully from the gels. Approximately 1-2 g of excised gel fragments were processed per SV Minicolumn, as directed by the kit instructions (Promega Corporation, Madison, WI, USA). The purified DNA was concentrated (~10-50 ng/µL) via standard ethanol precipitation with 0.3 M sodium acetate (Sambrook and Russell 2001). The presence and concentration of the cassettes were determined using agarose gel electrophoresis, as previously described.

2.1.8 Producing long linear DNA fragments through cloning

The second method employed to generate linear DNA fragments for λ Red-mediated recombination utilized cloning to produce long sequences of homology to the *rpoS* target. The target *rpoS* gene was introduced into a cloning vector, pGEM® T-Easy, and subsequently disrupted with selective markers. The lengths of *rpoS* homology used were controlled by the restriction enzyme selection.

TA cloning techniques were employed to produce pGEM-*rpoS*. As such, *rpoS* was amplified by PCR using *taq* polymerase to generate fragments with a single deoxyadenosine capable of binding the 3' terminal thymidines present on the linearized pGEM® T-Easy vector (Promega Corporation, Madison, WI, USA). PCR primers *rpoS*-F, 5'-TTATGAGTCAGAATACGCTGAAAG-3', and *rpoS*-R, 5'-TTACTCGCGGAACAGCGCCTTCG-3', were designed as described before for the *rpoS* components of the *rpoS-gm* and *rpoS-gfp-kan* cassettes. Genomic DNA from strains H21, H22, H29 and H32 served as the PCR templates and DNA from each strain was extracted prior to PCR using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA).

The PCR reaction mix for the amplification of *rpoS* was prepared in the same manner as the PCR mix for *rpoS-gm* (final concentrations in brackets): 37.5 µL of nuclease-free ddH_2O, 5 µL of 10X *taq* buffer with $(NH_4)_2SO_4$ (1X), 0.5 µL of 20 mM dNTP mix (0.2 mM of each dATP, dTTP, dCTP, and dGTP), 1 µL of each 10 µM primer (0.2 µM), 1 µL of *taq* DNA polymerase (0.02 units/µL), 3 µL of 25 mM $MgCl_2$ (1.5 mM), and 1 µL (20-100 ng) of extracted H32 genomic DNA. The 993 bp *rpoS* gene was amplified under the following conditions: one initial denaturation cycle for 5 min at 94°C; 30 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 60°C, and extension for 1 min at 72°C; and a final extension for 10 min at 72°C. The *rpoS* amplicons were held at 4°C, purified as previously described, and stored at -30°C for up to one month.

The pGEM® T-Easy vector and purified *rpoS* were ligated together using T4 DNA Ligase, as recommended by the supplier, using molar ratios of vector to insert of 1:1 to 1:5 (Promega Corporation, Madison, WI, USA). The ligation mixes were incubated overnight at 4°C to enhance the number of transformants, and a small amount of the reaction mixture was used to confirm successful ligation through agarose gel electrophoresis, where the expected pGEM-*rpoS* plasmid size is 4 kb. Following confirmation, a TransformAid™ Bacterial Transformation Kit (Fermentas International Inc., Burlington, ON, Canada) was used, as directed, to chemically transform JM109 with 2-5 µL (10-25 ng vector DNA) of the ligation reaction mix containing pGEM-*rpoS*.

Blue-and-white screening was employed to screen for potential JM109/pGEM-*rpoS* transformants by plating samples on LBA Amp^{50} plates each supplemented with 20 µL of 50 mg/mL X-Gal and 100 µL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubating for 24-48 hours at 37°C. White colonies were selected, isolated in pure culture, and extracted by using a Wizard® Plus SV Minipreps DNA Purification System kit, as directed (Promega Corporation, Madison, WI, USA). The resulting plasmid was digested with EcoRI, liberating a ~1 kb fragment carrying the *rpoS* insertion from the ~3 kb plasmid. Confirmation of successful ligation and transformation was performed by visualizing these fragments through agarose gel electrophoresis. In addition, the H32 *rpoS* insertion was sequenced from the pGEM® T-Easy insertion site using universal primers SP6, 5'-GATTTAGGTGACACTATAG-3', and T7, 5'-TAATACGACTCACTATAGGG-3' (Mobix Lab – McMaster University, Hamilton, ON, Canada).

To disrupt *rpoS* through the insertion of either *gm* or *gfp-kan,* and to generate long flanking sequences, restriction analysis of the sequenced H32 *rpoS* gene was performed. Two long homology cassettes were designed, one with *gfp* flanked by ~500 bp of *rpoS* homology on either side of *gfp* (500Δ*rpoS::gfp-kan)* and the other with *gm* flanked by ~500 bp of *rpoS* homology on either side of *gm* (500Δ*rpoS::gm)*. For both 500Δ*rpoS::gm* and 500Δ*rpos::gfp-kan,* pGEM-*rpoS* was cut once with Eco72I

at a site 549 bp into *rpoS*. Both p34S-Gm and pMOD-*gfp* were digested with KpnI to produce the *gfp-kan* and *gm* fragments for insertion. All digestion reactions were performed as directed by the manufacturer (Fermentas International Inc., Burlington, ON, Canada). The *gm* and *gfp-kan* fragments were gel purified using a commercially available kit (Promega Corporation, Madison, WI, USA) and ethanol precipitated as previously described.

No restriction enzymes were found that would cut both pGEM-*rpoS* at the desired location and excise the selective markers from their respective vectors. Consequently, it was necessary to convert the incompatible sticky ends of the pGEM vectors and the inserts to blunt ends using T4 DNA Polymerase. Following ethanol precipitation, the 5' termini of the vectors were dephosphorylated using calf alkaline phosphatase (CIAP), and subsequently the dephosphorylated vectors were ethanol precipitated and quantified along with *gm* and *gfp-kan* through agarose gel electrophoresis. Both T4 DNA Polymerase and CIAP were obtained from Fermentas (Burlington, ON, Canada) and used as directed.

Ligation reactions were prepared with molar ratios of 1:1, 1:3, and 3:1, for both digested pGEM-*rpoS* and *gm,* and digested pGEM-*rpoS* and *gfp-kan,* where the vector amount was held constant at 100 ng in each case. Samples were ligated using T4 DNA Ligase following the recommended protocol for blunt end ligation and incubating overnight at 4°C (Fermentas International Inc., Burlington, ON, Canada). Small samples of reaction mix were used to confirm ligation through agarose gel electrophoresis, and 5 µL of each remaining reaction mix was used in conjunction with a TransformAid™ Bacterial Transformation Kit (Fermentas International Inc., Burlington, ON, Canada) to transform JM109.

Following transformation, potential transformants were selectively plated on LBA Amp^{50}, and subsequently replica plated onto LBA Gen^{10} for JM109/pGEM500Δ*rpoS::gm.* Colonies fluorescing green under UV light were selected from LBA Amp^{50} plates bearing potential JM109/pGEM500Δ*rpos::gfp-kan*

transformants and were used to inoculate 5mL of LB Kan^{30}. Broth cultures were incubated at 37°C overnight, shaking at 200 rpm, and subsequently spread plated on LBA Amp^{50} + Kan^{30}. Colonies selected from the replica plates and broth culture plates were used to inoculate 5 mL each of LB with appropriate antibiotics, 4 mL of which were to confirm the presence of the desired plasmids through extraction using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA). A loopful of each broth culture was used to streak for isolated colonies on plates with the appropriate antibiotics. Once pure cultures were established, freezer cultures were prepared for each plasmid construct, as previously described.

The engineered 500Δ*rpoS::gm,* and 500Δ *rpoS::gfp-kan* cassettes were liberated from pGEM using restriction enzyme digestion with EcoRI (Fermentas International Inc., Burlington, ON, Canada). The desired 1.8 kb, and 4.7 kb fragments, corresponding to 500Δ*rpoS::gm,* and 500Δ*rpoS::gfp-kan,* respectively, were excised from the gel, purified, and concentrated as previously described in preparation for homologous linear recombination. A DNA Gel Extraction Kit (Fermentas International Inc., Burlington, ON, Canada) was employed in place of the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) for gel extraction of larger fragments.

2.1.9 Performing Lambda Red recombination to produce an *rpoS*-knockout

Lambda Red recombination was employed to generate an *rpoS*-deficient strain of *E. coli* O157:H7. The varying lengths of the *rpoS* gene carried by each linear DNA cassette serve as homology to the target region, while the selective markers are situated such that *rpoS* should be disrupted upon homologous recombination, producing an *rpoS*-knockout. The cassettes generated through the previously described methods, and employed in homologous linear recombination include: *rpoS-gfp-kan* (40 bp flanking homology), *rpoS-gm* (40 bp flanking homology), 500Δ*rpoS::gm,* and 500Δ*rpoS::gfp-kan.* Recombination-proficient *E. coli* O157:H7 strains H21/pKD46, H22/pKD46, H29/pKD46, H32/pKD46 and H32-*gfp*/pKD46 served as the targets for recombination, where only *gm* fragments

were employed in H32-*gfp*/pKD46. The protocol employed for Lambda Red Recombination, described below, is modified from the original protocol described by Datsenko and Wanner (2000).

In preparation for homologous linear recombination, 50 mL of TSB Amp^{100} was inoculated per *E. coli* strain and incubated overnight at 30°C, shaking at 150 rpm. The overnight *E. coli* cultures were used to inoculate 30 mL LB Amp^{50} to an $OD_{600\ nm}$ of 0.1. L-arabinose (1 M) was added to a final concentration of 10 mM, to induce pKD46. The samples were incubated at 30°C shaking at 150 rpm, for approximately 2.5-3 h, until an $OD_{600\ nm}$ of 0.4-0.6 was achieved. The samples were transferred in 10 mL aliquots to 15 mL centrifuge tubes and the cells were pelleted by centrifugation at 3,200 x g, for 10 minutes, at 4°C.

Following pelleting, the supernatant was removed and the cells were washed by resuspending the pellet in 1 mL of ice-cold, sterile ddH_2O. The cell suspension was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 21,000 x g in a high speed Thermo IEC Micromax benchtop centrifuge for 30 seconds (Thermo Fisher Scientific, Waltham, MA, USA). The wash procedure was repeated two more times, and the final cell pellet was resuspended in 10 % (v/v) ice-cold glycerol. In clean, pre-chilled 1.5 mL microcentrifuge tubes, 50 µL of the prepared electrocompetent cells were mixed with 10 ng-1 µg of linear DNA. The tubes were incubated on ice for exactly 1 min, transferred to pre-chilled electroporation cuvettes (100 µL volume, 1 mm gap; Eppendorf Canada, Mississauga, ON, Canada) and incubated again for 1 min on ice. Following incubation, the cuvettes were quickly but thoroughly dried and placed in an Eppendorf Electroporator 2510 (Eppendorf Canada, Mississauga, ON, Canada). All samples were electroporated at 2500 V, 10 µF capacitance, with an approximate acting time of 5.0 milliseconds. Following electroporation, 200 µL of 2XLB was added directly to the cuvette, mixed thoroughly, and transferred to a tube containing an additional 800 µL of 2XLB. The samples were incubated at 37°C for 1.5 h to cure pKD46 and recover the cells.

Following recovery, the cells were pelleted by centrifugation at 13,000 x g for 2 minutes, 800 µL of supernatant was removed, and the pellet was resuspended in the 300 µL of remaining supernatant. Samples were spread plated in 100 µL aliquots on LBA with the appropriate antibiotic (Gen^{10} for *gm* or Kan^{30} for *gfp-kan*), 50 µL was used to inoculate 5 mL LB without antibiotics, and the remaining 50 µL was used to inoculate 5 mL LB with antibiotics. All samples were incubated overnight at 37°C, with broth cultures shaking at 150 rpm. If no colonies appeared after 24 hours on the plates, any broth cultures that grew were plated on LBA with antibiotics. Potential H21, H21, H29, and H32 transformants were plated as described above, whereas potential H32-*gfp* transformants were plated on LBA Gen^{20} + Kan^{50} to select for both *gfp-kan* and *gm*.

Colony PCR was employed as an initial screen for successful *rpoS* knockouts. Primers were designed within *rpoS*, such that the size of the amplicon is altered or absent in knockouts, relative to the wild-type. The expected amplicon sizes of knockouts produced by different linear cassettes and the wild-type, are shown below in Table 2.1.3. The two primers designed for colony PCR were Mid*rpoS*-F, 5'- CCGTTATGGCAATCGTGGTCT-3',and Mid*rpoS*-R, 5- TGACGTCATCAACTGGCTTATCC-3'. The 25 µL colony PCR reaction mix was prepared in the same manner for all potential amplicons (final concentrations in brackets): 19.25 µL of nuclease-free ddH_2O, 2.5 µL of 10X *taq* buffer with $(NH_4)_2SO_4$ (1X), 0.25 µL of 20 mM dNTP mix (0.2 mM of each dATP, dTTP, dCTP, and dGTP), 0.5 µL of each 10 µM primer (0.2 µM), 0.5 µL of *taq* DNA polymerase (0.02 units/µL) and 1.5 µL of 25 mM $MgCl_2$ (1.5 mM). All reagents used were provided by Fermentas International (Burlington, ON, Canada).

Using a sterile pipette tip, Gm^r colonies, or Kan^r colonies fluorescing green under UV light, were selected. The pipette tip was gently dabbed in the prepared PCR reaction mix, and then deposited in 2mL of LB with the appropriate antibiotic(s). The pipette tip cultures were incubated overnight at 37°C, shaking at 150 rpm. The colony PCR was performed under the following conditions: one initial denaturation cycle for 3 min at 95°C; 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at

55°C, and extension for 1 min at 72°C; and a final extension for 10 min at 72°C. Samples were held at 4°C until analyzed by gel electrophoresis. Any samples not displaying the wild-type amplicon size were selected for future screening, and were plated on LBA with antibiotics from their overnight culture.

Table 2.1.3. Expected PCR amplicon sizes for wild-type and *rpoS- E. coli* strains

	Expected approximate amplicon size (bp)	
Linear DNA Cassette	**Full*rpoS*-F and Full*rpoS*-R primers**	**Mid*rpoS*-F and Mid*rpoS*-R primers (colony PCR)**
None, wild-type	1106	308
rpoS-gm	898	No amplicon
rpoS-gfp-kan	3288	No amplicon
500Δ*rpoS::gm*	1953	1155
500Δ *rpoS::gfp-kan*	4874	4075

Genomic DNA extraction was performed on any colonies passing the initial screen using a Wizard® SV Genomic DNA Purification System (Promega Corporation, Madison, WI, USA). PCR was then performed on the extracted DNA using Full*rpoS*-F, 5'- GTTATTTGCCGCAGCGAT-3', and Full*rpoS*-R 5' GCTTGAGACTGGCCTTTCTG-3', primers located upstream and downstream of *rpoS*, outside the region manipulated. The PCR reaction mix for all potential knockouts was prepared as follows (final concentrations in brackets): 37.5 µL of nuclease-free ddH_2O, 5 µL of 10X *taq* buffer with $(NH_4)_2SO_4$ (1X), 0.5 µL of 20 mM dNTP mix (0.2 mM of each dATP, dTTP, dCTP, and dGTP), 1 µL of each 10 µM primer (0.2 µM), 1 µL of *taq* DNA polymerase (0.02 units/µL), 3 µL of 25mM $MgCl_2$ (1.5 mM), and 1µL (20-100 ng) of genomic DNA. PCR reactions with an expected amplicon size of <2 kbp were amplified under the following conditions: one initial denaturation cycle for 3 min at 95°C; 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C; a final extension for 10 min at 72°C and a final indefinite hold at 4°C. For PCR reactions with an expected

amplicon size of > 2kbp, the extension stage was increased from 1 minute to 4 minutes. All reagents were supplied by Fermentas (Burlington, ON, Canada).

2.1.10 Comparative growth curves

Growth curves were developed for H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* in both rich media (LB) and minimal media (minimal salts medium (MSM) + 0.04% (w/v) glucose). MSM + 0.04% glucose consisted of 7 g KH_2PO_4, 3 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 1 mg yeast extract and 0.04% filter-sterilized glucose per litre ddH_2O. Yeast extract was added as minimal amounts (≤1mg yeast extract per litre) have been found to enhance biofilm formation of coliforms isolated from the environment (Camper *et al.* 1991). Overnight cultures of 50 mL LB containing the no antibiotics, Kan^{50}, and Kan^{50} + Gen^{20}, were prepared using a single fresh colony of H32, H32-*gfp*, and H32-*gfpΔrpoS::gm*, respectively. The overnight cultures were each used to inoculate three flasks containing 200 mL of LB or MSM + 0.04% glucose, without antibiotics, to an $OD_{600\ nm}$ of 0.05. The flasks were incubated between time points at 37°C, shaking at 150 rpm. At various time points, including 0 h, 1 mL of culture was removed and used to prepare a dilution series in phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per litre, pH 7.4). Drop plates were prepared from the dilution series on LBA with appropriate antibiotics, where one dilution was plated in 6-5 µL drops per quadrant. Following 10-12 h growth at 37°C, colonies were counted and the CFU/mL was calculated for each sample at each time point. Statistical analysis was performed on the growth curve results using a Two-way Analysis of Variance (Two-way ANOVA) in SigmaStat (SPSS Inc. 1997).

Table 2.1.4. Sequencing and PCR primers used in this study

Designation	Sequence (5' to 3')	Function	Source
SqFP SqRP (pMOD™<MCS> Sequencing Primers)	GCCAACGACTACGCACTAGCCAAC GAGCCAATATGCGAGAACACCCGAGAA	Sequencing outwards from transposon into chromosome	Epicentre Biotechnologies, Madison, WI, USA
gfp-F *gfp*-R	GCCCTTTCGTCTTCACCTCG GCTCTGCCAGTGTTACAACC	Amplifying *gfp-kan*	This study
PCRFP PCRRP	ATTCAGGCTGCGCAACTGT GTCAGTGAGCGAGGAAGCGGAAG	Sequencing *gfp-kan*	Epicentre Biotechnologies, Madison, WI, USA
*gfp*1-F *gfp*1-R *gfp*2-R *gfp*3-R *gfp*4-R *gfp*5-R	GCTTTGTTGTAGGTGGACC CGTGCTGAAGTCAAGTTTG TCCGGCCTTTATTCACATTC CCTTAAACGCCTGGGGTAAT GATGCGCCAGAGTTGTTTCT GGCAGAGCATTACGCTGACT	Sequencing *gfp-kan*	This study
gm-F *gm*-R	GCTCGAATTGACATAAGCCTGT TGTGACAATTTACCGAACAACTCC	Amplifying *gm*	This study
rpoS-gfp-F *rpoS-gfp*-R	GAATGTTCCGTCAAGGGATCACGGGTAGGA GCCACCTTATGGCCC TTTCGTCTTCACCTCG CAGATGCTTACTTACTCGCGGAACAGCGCTT CGATATTCA GCTCTGCCAGTGTTACAACC	Constructing *rpoS-gfp-kan* cassette	This study
rpoS-gm-F *rpoS-gm*-R	GAATGTTCCGTCAAGGGATCACGGGTAGGA GCCACCTTATGGCTCGAATTGACATAAGCCT GT CAGATGCTTACTTACTCGCGGAACAGCGCTT CGATATTCATGTGACAATTTACCGAACAACT CC	Constructing *rpoS-gm* cassette	This study
rpoS-F *rpoS*-R	TTATGAGTCAGAATACGCTGAAAG TTACTCGCGGAACAGCGCCTTCG	Amplifying *rpoS*	This study
SP6 (Universal primer) T7 (Universal primer)	GATTTAGGTGACACTATAG TAATACGACTCACTATAGGG	Sequencing pGEM® T-Easy inserts	Mobix Lab - Hamilton, ON, Canada
Full*rpoS*-F Full*rpoS*-R	GTTATTTGCCGCAGCGAT GCTTGAGACTGGCCTTTCTG	Colony PCR and confirming *rpoS* knock-outs	This study
Mid*rpoS*-F Mid*rpoS*-R	CCGTTATGGCAATCGTGGTCT TGACGTCATCAACTGGCTTATCC	Colony PCR and confirming *rpoS* knock-outs	This study

2.2 RESULTS

2.2.1 Labeling of H32 with a Tn5 transposon

E. coli O157:H7 H32 was fluorescently labeled using a Tn5 transposon carrying a *gfp-kan* cassette. The cassette was introduced to the transposon construction vector, EZ-Tn5™ pMOD™<MCS> (Epicentre Biotechnologies, Madison, WI, USA), through restriction enzyme digestion and subsequent ligation, producing pMOD™<*gfp-kan*>. *E. coli* O157:H7 H32 was transformed with <*gfp-kan*> resulting in the random insertion of <*gfp*-kan> into the H32 chromosome. Successful transposition of H32 with <*gfp-kan*> was tentatively established through the isolation of kanamycin-resistant colonies displaying green fluorescence under UV illumination and epifluorescence microscopy (Figure 2.2.1).

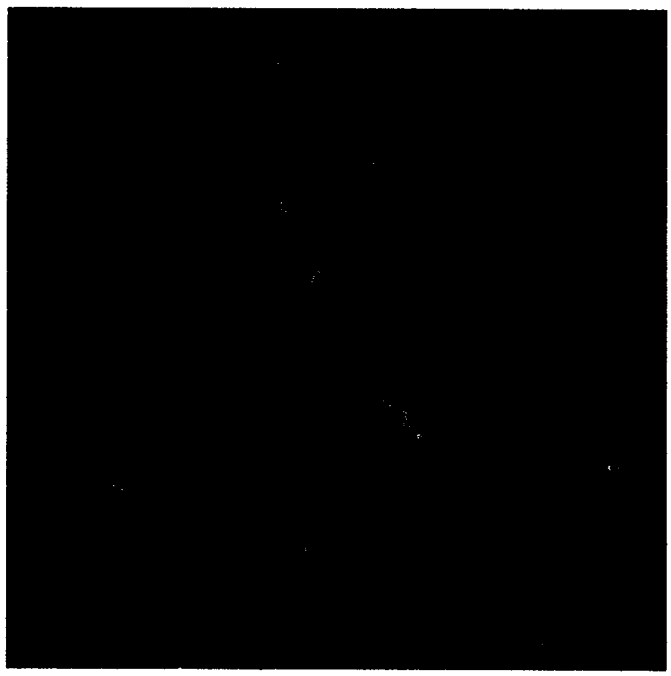

Figure 2.2.1. Epifluorescence microscopy image of H32-*gfp* exhibiting green fluorescence following transposition with Tn5<*gfp-kan*>.

2.2.2 Biochemical analyses and stability testing

The API 20E test results revealed that H32 and H32-*gfp* have identical profiles, and that *Escherichia coli* is a very good identification (ID≥99%) for these strains (Profile #: 5144132). The API 20E results confirm that H32-*gfp* represents an *E. coli* strain indistinguishable from H32 in terms of fundamental biochemical activities. The stability of the *gfp-kan* label was confirmed through the observation of uninhibited growth by H32-*gfp* on TSA Kan^{50}, relative to TSA, following 6 days of non-selective growth. Additionally, H32-*gfp* fluoresced equally under UV illumination at each time point.

2.2.3 Sequencing and identification of the <*gfp-kan*> insertion site

The insertion of <*gfp-kan*> into H32 was confirmed through genomic sequencing with primers oriented outward from <*gfp-kan*> (SqFP and SqRP). The insertion site was located and identified by performing a BLAST search on the sequencing results from both SqFP and SqRP (Altschul *et al.* 1997). A multiple sequence alignment of the genomic sequencing results and BLAST results confirmed that both the SqFP and SqRP sequences occur within the same region, approximately 450 bp apart (Appendix 4.0.). Both primers generated sequences similar to an *E. coli* putative regulatory or permease protein, with 93-97% and 73% DNA homology for the SqFP and SqRP sequences, respectively (Table 2.2.1.). The function of the regulatory protein is unknown or inferred in all BLAST sequence results except in *E. coli* O157:H7 strain EC4115, where it is tentatively identified through protein homology as UhpC, a regulatory protein involved in sugar phosphate transport (Altschul *et al.* 1997). For the putative identification and functions of regions with high DNA homology to the sequenced region of H32-*gfp*, see Table 2.2.1.

Table 2.2.1. Commonalities between genomic sequencing of the <*gfp-kan*> insertion site and *E. coli* type-strains identified through BLAST

Primer	Strain	Accession	Homology (bp)	Locus tag; Region (bp)	Identity
SqFP	*E. coli* O157:H7 str. EDL933	AE005174.2	635/651 (97%)	Z0460, Z0461; 442374-445304	Putative permease - hexosphosphate transport Putative sensor kinase
	E. coli O157:H7 str. Sakai	BA000007.2	635/651 (97%)	ECs0415, ECs0416; 442372-445302	Putative regulatory protein Putative sensor histidine kinase
	E. coli O157:H7 str. EC4115	CP001164.1	635/651 (97%)	ECH74115_0435, ECH74115_0436; 445971-448901	Regulatory protein UhpC Integral membrane sensor transduction histidine kinase
	E. coli ED1a	CU928162.2	606/648 (93%)	ECED1_5042, ECED1_5042; 4974803-4977733	Putative regulatory protein UhpC Putative sensor histidine kinase
SqRP	*E. coli* O157:H7 str. EDL933	AE005174.2	45/61 (73%)	Z0460; 442374-443678	Putative permease - hexosphosphate transport
	E. coli O157:H7 str. Sakai	BA000007.2	45/61 (73%)	ECs0415 442372-443676	Putative regulatory protein
	E. coli O157:H7 str. EC4115	CP001164.1	45/61 (73%)	ECH74115_0435; 445971-447275	Regulatory protein, UhpC

2.2.4 Sequencing of the *gfp-kan* fragment

The sequence of *gfp-kan* was identified through sequencing beginning with primers PCRFP and PCRRP, located flanking the mosaic ends of Ez-Tn5™ within the pMOD™-2<MCS> construction vector (Epicentre Biotechnologies, Madison, WI, USA). Five sequential rounds of sequencing reactions resulted in the elucidation a 4.50 kb fragment including both vector and *gfp-kan* cassette sequences (Figure 2.2.2.). The locations of vector homology, *gfp*, and *kan*, were determined though alignments with established sequences (Accession numbers: AF06208 and AY952933, and Epicentre Biotechnologies). The kanamycin resistance determinant was found to be 816 bp in length, whereas the length of *gfp* was determined to be 716 bp, with both genes encoded on the minus strand. The total length of *gfp-kan* between KpnI cut sites was found to be 3,767 bp, whereas the expected total length of *gfp-kan* as amplified by *gfp*-F and *gfp*-R was found to be 3.2 kb. The large size of both fragments is due to the presence of an extensive (1.5 kb) non-coding region between *gfp* and *kan*.

Production of a second signal during sequencing (DeSousa 2008) and successful sequencing from only the reverse primers prompted further investigation into the secondary structure of the *gfp-kan* sequence. Analysis of the fragment using DNAMAN (Lynnon BioSoft 2000) revealed numerous direct repeats, including a 36 bp repeat at nucleotide positions 2,193 and 4,254. Furthermore, a large inverted repeat of 366 bp was found at nucleotide positions 486 and 1,833, flanking *kan*, and a second inverted repeat of 93 bp was found at nucleotide positions 1 and 4,072.

1(5′) *ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG*
91 *TCT*CGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG
181 CAGACAAGCC CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCT GGCTTAACTA TGCGGCATCA GAGCAGATTG TACTGAGAGT
271 GCACCATATG CGGTGTGAAA TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG
361 GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT ATTACGACAG CTGTCTCTTA TACACATCTC AACCATCATC GATGAATTTT CTCGGGTGTT
451 CTCGCATATT GGCTCGAATT CGAGCT*GGTA C*CCGGGGATC CGGCTGTAAT CCGGGCAGCG CAACGGAACA TTCATCAGTG TAAAAATGGA*
541 *ATCAATAAAG CCCTGCGCAG CGCGCAGGGT CAGCCTGAAT ACGCGTTTAA TGACCAGCAC AGTCGTGATG GCAAGGTCAG AATAGCGCTG*
631 *AGGTCTGCCT CGTGAAGAAG GTGTTGCTGA CTCATACCAG GCCTGAATCG CCCCATCATC CAGCCAGAAA GTGAGGGAGC CACGGTTGAT*
721 *GAGAGCTTTG TTGTAGGTGG ACCAGTTGGT GATTTTGAAC TTTTGCTTTG CCACGGAACG GTCTGCGTTG TCGGGAAGAT GCGTGATCTG*
811 *ATCCTTCAAC TCAGCAAAAG TTCGATTTAT TCAACAAAGC CGCCGTCCCG* TCAAGTCAGC GTAATGCTCT GCCAGTGTTA CAACCAATTA
901 ACCAATTCTG A*TTA*[illegible]
991 [illegible]
1081 [illegible]
1171 [illegible]
1261 [illegible]
1351 [illegible]
1441 [illegible]
1531 [illegible]
1621 [illegible]
1711 [illegible] *CAT*AA CACCCCTTGT ATTACTGTTT ATGTAAGCAG ACAGTTTTAT TGTTCATGAT GATATATTTT TATCTTGTGC
1801 AATGTAACAT CAGAGATTTT GAGACACAAC GT*GGCTTTGT TGAATAAATC GAACTTTTGC TGAGTTGAAG GATCAGATCA CGCATCTTCC*
1891 *CGACAACGCA GACCGTTCCG TGGCAAAGCA AAAGTTCAAA ATCACCAACT GGTCCACCTA CAACAAAGCT CTCATCAACC GTGGCTCCCT*
1981 *CACTTTCTGG CTGGATGATG GGGCGATTCA GGCCTGGTAT GAGTCAGCAA CACCTTCTTC ACGAGGCAGA CCTCAGCGCT ATTCTGACCT*
2071 *TGCCATCACG ACTGTGCTGG TCATTAAACG CGTATTCAGG CTGACCCTGC GCGCTGCGCA GGGCTTTATT GATTCCATTT TTACACTGAT*
2161 *GAATGTTCCG TTGCGCTGCC CGGATTACAG CC* CG GCCGCCTAGG CCGCGGCCGC
2251 AAGCTCCTAG CGGCGGATTT GTCCTACTCA GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT
2341 TTCGTTTTAT TTGATGCCTC AAGCTAGAGA GTCATTACCC CAGGCGTTTA AGGGACCAAT AACTGCCTTA AAAAAATTAC GCCCCGCCCT
2431 GCCACTCATC GCAGTACTGT TGTAATTCAT TAAGCATTCT GCCGACATGG AAGCCATCAC AAACGGCATG ATGAACCTGA ATCGCCAGCG
2521 GCATCAGCAC CTTGTCGCCT TGCGTATAAT ATTTGCCCAT GGTGAAAACG GGGGCGAAGA AGTTGTCCAT ATTGGCCACG TTTAAATCAA
2611 AACTGGTGAA ACTCACCCAG GGATTGGCTG AGACGAAAAA CATATTCTCA ATAAACCCTT TAGGGAAATA GGCCAGGTTT TCACCGTAAC
2701 ACGCCACATC TTGCGAATAT ATGTGTAGAA ACTGCCGGAA ATCGTCGTGG TATTCACTCC AGAGCGATGA AAACGTTTCA GTTTGCTCAT
2791 GGAAAACGGT GTAACAAGGG TGAACACTAT CCCATATCAC CAGCTCACCG TCTTTCATTG CCATACGAAA TTCCGGATGA GCATTCATCA
2881 GGCGGGCAAG AATGTGAATA AAGGCCGGAT AAAACTTGTG CTTATTTTTC TTTACGGTCT TTAAAAAGGC CGTAATATCC AGCTGAACGG
2971 TCTGGTTATA GGTACATTGA GCAACTGACT GAAATGCCTC AAAATGTTCT TTACGATGCC ATTGGGATAT ATCAACGGTG GTATATCCAG
3061 TGATTTTTTT CTCCATTTTA GCTTCCTTAG CTCCTGAAAA TCTCGCCAAG CTAGCTTGGA TTCTCACCAA TAAAAAACGC CCGGCGGCAA
3151 CCGAGCGTTC TGAACAAATC CAGATGGAGT TCTGAGGTCA TTACTGGATC TATCAACAGG AGTCCAAGCT CAGCTAATTA AGC*TTA*TTTG
3241 TATAGTTCAT CCATGCCATG TGTAATCCCA GCAGCTGTTA CAAACTCAAG AAGGACCATG TGGTCTCTCT TTTCGTTGGG ATCTTTCGAA
3331 AGGGCAGATT GTGTGGACAG GTAATGGTTG TCTGGTAAAA GGACAGGGCC ATCGCCAATT GGAGTATTTT GTTGATAATG GTCTGCTAGT
3421 TGAACGCTTC CATCTTCAAT GTTGTGCTCA ATTTTGAAGT TAACTTTGAT TCCATTCTTT TGTTTGTCTG CCATGATGTA TACATTGTGT
3511 GAGTTATAGT TGTATTCCAA TTTGTGTCCA AGAATGTTTC CATCTTCTTT AAAATCAATA CCTTTTAACT CGATTCTATT AACAAGGGTA
3601 TCACCTTCAA ACTTGACTTC AGCACGTGTC TTGTAGTTCC CGTCATCTTT GAAAAATATA GTTCTTTCCT GTACATAACC TTCGGGCATG
3691 GCACTCTTGA AAAAGTCATG CTGTTTCATA TGATCTGGGT ATCTCGCAAA GCATTGAACA CCATAACCGA AAGTAGTGAC AAGTGTTGGC
3781 CATGGAACAG GTAGTTTTCC AGTAGTGCAA ATAAATTTAA GGGTAAGTTT TCCGTATGTT GCATCACCTT CACCCTCTCC ACTGACAGAA
3871 AATTTGTGCC CATTAACATC ACCATCTAAT TCAACAAGAA TTGGGACAAC TCCAGTGAAA AGTTCTTCTC CTTTACG*CAT* GCTTAATTTC
3961 TCCTCTTTAA TTCTAGATGT GTGAAATTGT TATCCGCTCA CAATTGAATC TAAGTATCAT TGTTATCCGC TCACAAGTCA ACACTCTTTT
4051 TGATAAATTT TCTCGAGGTG A*AGACGAAAG GGCCTCGTGA TACGCCTATT TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG*
4141 *TCAGGTGGCA CTTTTCGGGG AAAT*GTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA
4231 TAACCCTGAT AAAT*GGTAC*C* CGG G CCAACGACTA CGCACTAGCC AACAAGAGCT
4321 TCAGGGTTGA GATGTGTATA AGAGACAGCT GTCTTAATGA ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC
4411 *TTCCTCGCTC ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT TATNCNCAGA*
4501 *AT* (3′)

Figure 2.2.2. Sequence of the *gfp*-kan fragment from pMOD-*gfp*, originally from pJBA29 (Andersen 1998). **CAT**= start codon (ATG) on reverse complement strand, **TTA** = stop codon (TAA) on reverse complement strand, red = pMOD™-2<MCS> vector sequence, blue = Kan^r determinant (*aph(3′)I*), green = green fluorescent protein gene (*gfp*), black = non-coding region, purple = *gfp*-F and *gfp*-R primers, * = KpnI cut site, underlined = Promoter PA1/04/03 sequence, *underlined italics* = significant inverted repeats, = significant direct repeats

2.2.5 Sequencing of *rpoS*

The *rpoS* gene from H32 was sequenced from pGEM-*rpoS* using universal primers SP6 and T7. A BLAST search of the H32 *rpoS* sequence identified numerous (108) homologous sequences, including 9 sequences with 100% identity covering the full query length (993 nucleotides). All 9 identical sequences represent *rpoS* genes from commensal or pathogenic *E. coli* strains, including enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC) strains (Accession numbers: CU928145.2, AP009240.1, CP000800.1, DQ27423.1, DQ27424.1, AF242210.1, AF242208.1, and DQ287968.1). Alignment of H32 *rpoS* with various known *rpoS* sequences (Appendix 4.1.) revealed no insertion or deletion mutations and no novel nucleotides at any position, relative to the established sequences. The consensus strand developed from H32 *rpoS* sequencing and alignment (Figure 2.2.4) was used for restriction analysis and primer design, allowing for the construction of the linear DNA cassettes.

Translation of the sequenced *E. coli* H32 *rpoS* yielded identical amino acid sequences to the aforementioned pathogenic strains (100% homology), and 99% homology (329/330 amino acids) to *E. coli* strain K-12 MG1655 (Accession number: NC_000913, Altschul *et al.* 1997). *E. coli* H32 *rpoS* and other pathogenic strains encode glutamic acid at amino acid 33, whereas some *E. coli* K-12, including the MG1655, code for glutamine (Figure 2.2.3., Ferreira *et al.* 1999).

```
1       METSerGlnAsnThrLeuLysValHisAspLeuAsnGluAspAlaGluPheAspGluAsn
21      GlyValGluValPheAspGluLysAlaLeuValGluGluGluProSerAspAsnAspLeu
41      AlaGluGluGluLeuSerGlnGlyAlaThrGlnArgValLeuAspAlaThrGlnLeu
61      TyrLeuGlyGluIleGlyTyrSerProLeuLeuThrAlaGluGluGluValTyrPheAla
81      ArgArgAlaLeuArgGlyAspValAlaSerArgArgArgMETIleGluSerAsnLeuArg
101     LeuValValLysIleAlaArgArgTyrGlyAsnArgGlyLeuAlaLeuLeuAspLeuIle
121     GluGluGlyAsnLeuGlyLeuIleArgAlaValGluLysPheAspProGluArgGlyPhe
141     ArgPheSerThrTyrAlaThrTrpTrpIleArgGlnThrIleGluArgAlaIleMETAsn
161     GlnThrArgThrIleArgLeuProIleHisIleValLysGluLeuAsnValTyrLeuArg
181     ThrAlaArgGluLeuSerHisLysLeuAspHisGluProSerAlaGluGluIleAlaGlu
201     GlnLeuAspLysProValAspAspValSerArgMETLeuArgLeuAsnGluArgIleThr
221     SerValAspThrProLeuGlyGlyAspSerGluLysAlaLeuLeuAspIleLeuAlaAsp
241     GluLysGluAsnGlyProGluAspThrThrGlnAspAspAspMETLysGlnSerIleVal
261     LysTrpLeuPheGluLeuAsnAlaLysGlnArgGluValLeuAlaArgArgPheGlyLeu
281     LeuGlyTyrGluAlaAlaThrLeuGluAspValGlyArgGluIleGlyLeuThrArgGlu
301     ArgValArgGlnIleGlnValGluGlyLeuArgArgLeuArgGluIleLeuGlnThrGln
321     GlyLeuAsnIleGluAlaLeuPheArgGlu***
```

Figure 2.2.3. Translation of *rpoS* sequenced from *E. coli* H32.

1 ATGAGCGCGG GAAGCCCAAA ATTCACCGTT CGCCGCATTG CGGCTTTGTC ACTGGTTTCG
61 CTATGGCTGG CAGGCTGTTC TGACACTTCA AATCCACCGG CACCGGTCAG CTCCGTTAAT
121 GGCAATGCGC CTGCAAATAC CAATTCTGGT ATGTTGATTA CGCCGCCGCC GAAAATGGGG
181 ACGACGTCTA CAGCCCAGCA ACCGCAAATT CAGCCGGTAC AGCAGCCACA AATTCAGGCT
241 ACTCAACAAC CGCAAATCCA GCCAGTGCAG CCAGTAGCTC AGCAGCCGGT ACAGATGGAA
301 AACGGACGCA TCGTCTATAA CCGTCAGTAT GGGAACATTC CGAAAGGCAG TTATAGCGGC
361 AGTACCTATA CCGTGAAAAA AGGCGACACA CTTTTCTATA TCGCCTGGAT TACTGGCAAC
421 GATTTCCGTG ACCTTGCTCA GCGCAACAAT ATTCAGGCAC CATACGCGCT GAACGTTGGT
481 CAGACCTTGC AGGTGGGTAA TGCTTCCGGT ACGCCAATCA CTGGCGGAAA TGCCATTACC
541 CAGGCCGACG CAGCAGAGCA AGGAGTTGTG ATCAAGCCTG CACAAAATTC CACCGTTGCT
601 GTTGCGTCGC AACCGACAAT TACGTATTCT GAATCTTCGG GTGAACAGAG TGCTAACAAA
661 ATGTTGCCGA ACAACAAGCC AGCTGCGACC ACGGTCACAG CGCCTGTAAC GGTACCAACA
721 GCAAGCACAA CCGAGCCAAC TGTCAGCAGT ACATCAACCA GTACGCCTAT CTCCACCTGG
781 CGCTGGCCGA CTGAGGGCAA AGTGATCGAA ACCTTTGGCG CTTCTGAGGG GGGCAACAAG
841 GGGATTGATA TCGCAGGCAG CAAAGGACAG GCAATTATCG CGACCGCAGA TGGCCGCGTT
901 GTTTATGCCG GTAACGCGCT GCGCGGCTAC GGTAATCTGA TTATCATCAA ACATAATGAT
961 GATTACCTGA GTGCCTACGC CCATAACGAC ACAATGCTGG TCCGGGAACA ACAAGAAGTG
1021 AAGGCGGGGC AAAAAATAGC AACCATGGGT AGCACCGGAA CCAGTTCAAC ACGCTTGCAT
1081 TTTGAAATTC GTTACAAGGG GAAATCCGTA AACCCGCTGC GTTATTTGCC GCAGCGATAA
1141 ATCGGCGGAA CCAGGCTTTT GCTTGAATGT TCCGTCAAGG GATCACGGGT AGGAGCCACC
1201 **TTATGAGTCA GAATACGCTG AAAGTTCATG ATTTAAATGA AGATGCGGAA TTTGATGAGA**
1261 **ACGGAGTTGA GGTTTTTGAC GAAAAGGCCT TAGTAGAAGA GGAACCCAGT GATAACGATT**
1321 **TGGCCGAAGA GGAACTGTTA TCGCAGGGAG CCACACAGCG TGTGTTGGAC GCGACTCAGC**
1381 **TTTACCTTGG TGAGATTGGT TATTCACCAC TGTTAACGGC CGAAGAAGAA GTTTATTTTG**
1441 **CGCGTCGCGC ACTGCGTGGA GATGTCGCCT CTCGCCGCCG GATGATCGAG AGTAACTTGC**
1501 **GTCTGGTGGT AAAAATTGCC CGCCGTTATG GCAATCGTGG TCTGGCGTTG CTGGACCTTA**
1561 **TCGAAGAGGG CAACCTGGGG CTGATCCGCG CGGTAGAGAA GTTTGACCCG GAACGTGGTT**
1621 **TCCGCTTCTC AACATACGCA ACCTGGTGGA TTCGCCAGAC GATTGAACGG GCGATTATGA**
1681 **ACCAAACCCG TACTATTCGT TTGCCGATTC ACATCGTAAA GGAGCTGAAC GTTTACCTGC**
1741 **GAACCGCAC*G TGAGTTGTCC CATAAGCTGG ACCATGAACC AAGTGCGGAA GAGATCGCAG**
1801 **AGCAACTGGA TAAGCCAGTT GATGACGTCA GCCGTATGCT TCGTCTTAAC GAGCGCATTA**
1861 **CCTCGGTAGA CACCCCGCTG GGTGGTGATT CCGAAAAAGC GTTGCTGGAC ATCCTGGCCG**
1921 **ATGAAAAAGA GAACGGTCCG GAAGATACCA CGCAAGATGA CGATATGAAG CAGAGCATCG**
1981 **TCAAATGGCT GTTCGAGCTG AACGCCAAAC AGCGTGAAGT GCTGGCACGT CGATTCGGTT**
2041 **TGCTGGGGTA CGAAGCGGCA ACACTGGAAG ATGTAGGTCG TGAAATTGGC CTCACCCGTG**
2101 **AACGTGTTCG CCAGATTCAG GTTGAAGGCC TGCGCCGTTT GCGCGAAATC CTGCAAACGC**
2161 **AGGGGCTGAA TATCGAAGCG CTGTTCCGCG AGTAAGTAAG** CATCTGTCAG AAAGGCCAGT
2221 CTCAAGCGAG GCTGGCCTTT T

Figure 2.2.4. Sequence of the *rpoS* gene. **Bold** = region sequenced from *E. coli* O157:H7 H32. **ATG** = start codon, **TAA** = stop codon. Light blue = location of primers upstream and downstream of *rpoS* (Full*rpoS*), red = location of *rpoS* primers (*rpoS*), green = location of mid-sequence *rpoS* primers, underlined = location of homologous sequences from *rpoS-gm* and *rpoS-gfp-kan* and orange = cut site of Eco72I (for 500bp flanking sequences).

2.2.6 Production of an *rpoS*-knockout

An H32-*gfp rpoS*-knockout was produced using Lambda Red recombination (pKD46) and 200 ng of the linear DNA cassette bearing a gentamycin resistance determinant and ~500 bp of flanking sequences homologous to *rpoS* (500Δ*rpoS::gm*). The production of the knock-out represents a one-time, non-reproducible success with the Lambda Red system. Homologous linear recombination was attempted unsuccessfully in *E. coli* O157:H7 strains H21/pKD46, H22/pKD46, H29/pKD46, and H32/pKD46 using linear DNA cassettes *rpoS-gfp-kan*, *rpoS-gm*, 500Δ*rpoS::gm*, and 500Δ*rpoS::gfp-kan*.

The production of an *rpoS*-knockout was confirmed by selecting green fluorescing colonies that were both gentamycin-resistant and kanamycin-resistant but ampicillin sensitive. An initial colony PCR screen of mid-sequence of *rpoS* (wild-type amplicon =308 bp) showed amplification of a ~1,100 bp fragment corresponding to the insertion of the 816 bp gentamycin resistance gene. A second PCR of the full H32-*gfp rpoS* sequence confirmed the disruption of *rpoS* through amplification of a ~2 kb fragment instead of the expected 1.1 kb fragment for wild-type *rpoS*. Wild-type controls and unsuccessful attempts at homologous linear recombination yielded the expected 308 bp and 1.1 kb amplicons for the mid-sequence and full *rpoS* PCRs, respectively (Figure 2.2.5). Of the 9 colonies screened through colony PCR, 2 possessed the desired insertion, indicating a ~20% success rate at producing the desired gene replacement. The overall efficiency of the event was $4x10^2$ gene replacements per μg of DNA (1.97×10^{11} linear cassette copies), with a frequency of ~80 replacements per 10^8 cells surviving electroporation (cell survivors). A summary of the process used to produce H32-*gfp*Δ*rpoS::gm* can be found in Figure 2.2.6.

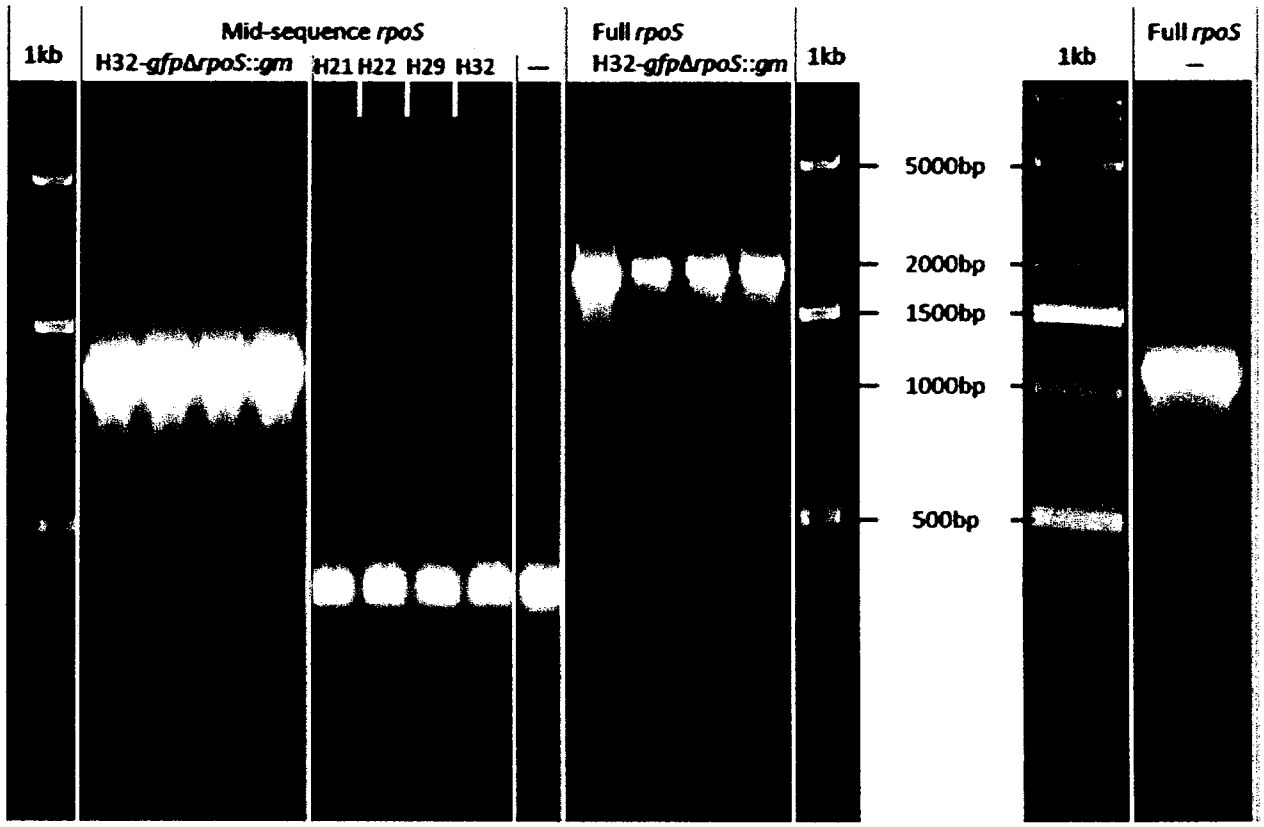

Figure 2.2.5. PCR screening and confirmation of successful H32-*gfp*Δ*rpoS*::*gm* production using Lambda Red recombination and a 500Δ*rpoS*::*gm* cassette. Generation of H32TnΔ*rpoS*::*gm* is demonstrated through mid-sequence *rpoS* (Mid*rpoS*) colony PCRs, showing the expected 1,156 bp amplicon, versus reactions for H21, H22, H29, H32, and H32-*gfp* control (-) showing no insertion in *rpoS* (308 bp). Full sequence *rpoS* PCRs (Full*rpoS*) confirm disruption of *rpoS* with *gm,* resulting in the amplification of a ~1.9 kb fragment for H32-*gfp*Δ*rpoS*::*gm* versus the ~1.1 kb H32-*gfp* control (-).

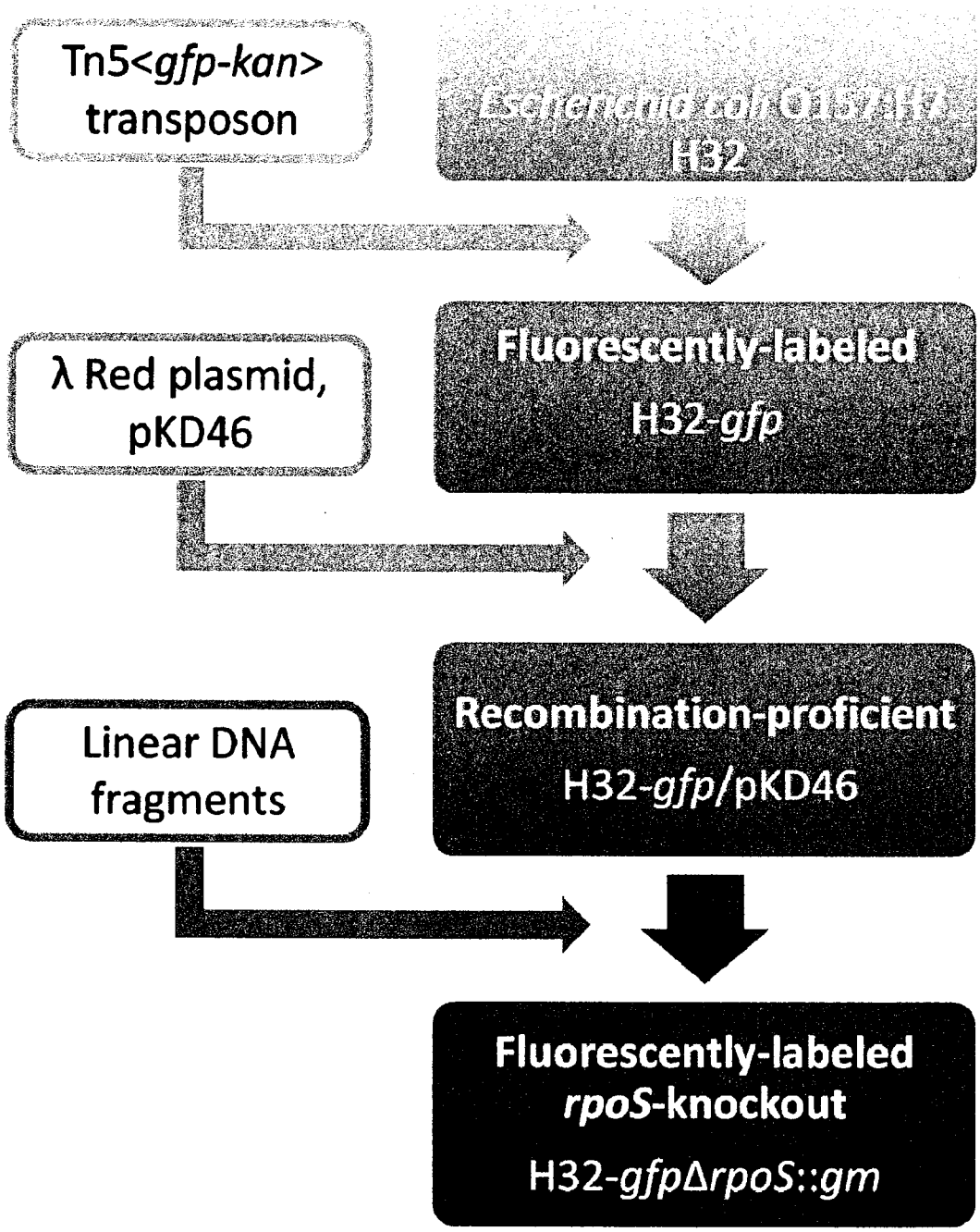

Figure 2.2.6. Flowchart detailing the production of the fluorescently-labeled, *rpoS*-knockout H32-*gfpΔrpoS::gm*.

2.2.7 Comparative growth curves

Growth curves for wild-type H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* were generated in both rich (LB) and minimal media (MSM +0.04% glucose) over 24 hours (Figures 2.2.7. and 2.2.8.). As expected, there was a statistically significant increase in cell density (CFU/mL) over time for all strains in both media ($P<0.001$), as both curves exhibit the typical stages of exponential and stationary phase growth. All three strains exhibited slower growth in minimal media than rich media, as stationary phase was reached after 6h for all strains in LB but not until 10 h in MSM + 0.04% glucose. The viable cell counts for all strains were approximately one log CFU/mL higher in rich media than in minimal media

No significant differences in growth were revealed between H32, H32-*gfp* or H32-*gfp*Δ*rpoS*::*gm* in MSM + 0.04% glucose ($P=0.898$). While growth in LB revealed a statistically significant difference overall between the wild-type strains and the *rpoS* mutant ($P<0.001$ for H32 and $P=0.003$ for H32-*gfp*), the growth was not significantly different at any time point except 6 h where H32-*gfp*Δ*rpoS*::*gm* exhibited less growth than both H32 and H32-*gfp*Δ*rpoS*::*gm* ($P<0.001$). The singular difference at 6 h, coinciding with early stationary phase, likely generated the statistical difference between the wild-type and *rpoS* mutant, however overall the three strains exhibited similar growth in both LB and MSM + 0.04% glucose over 24 h. The *gfp*-labeling not have an apparent influence on planktonic growth of H32-*gfp* relative to H32.

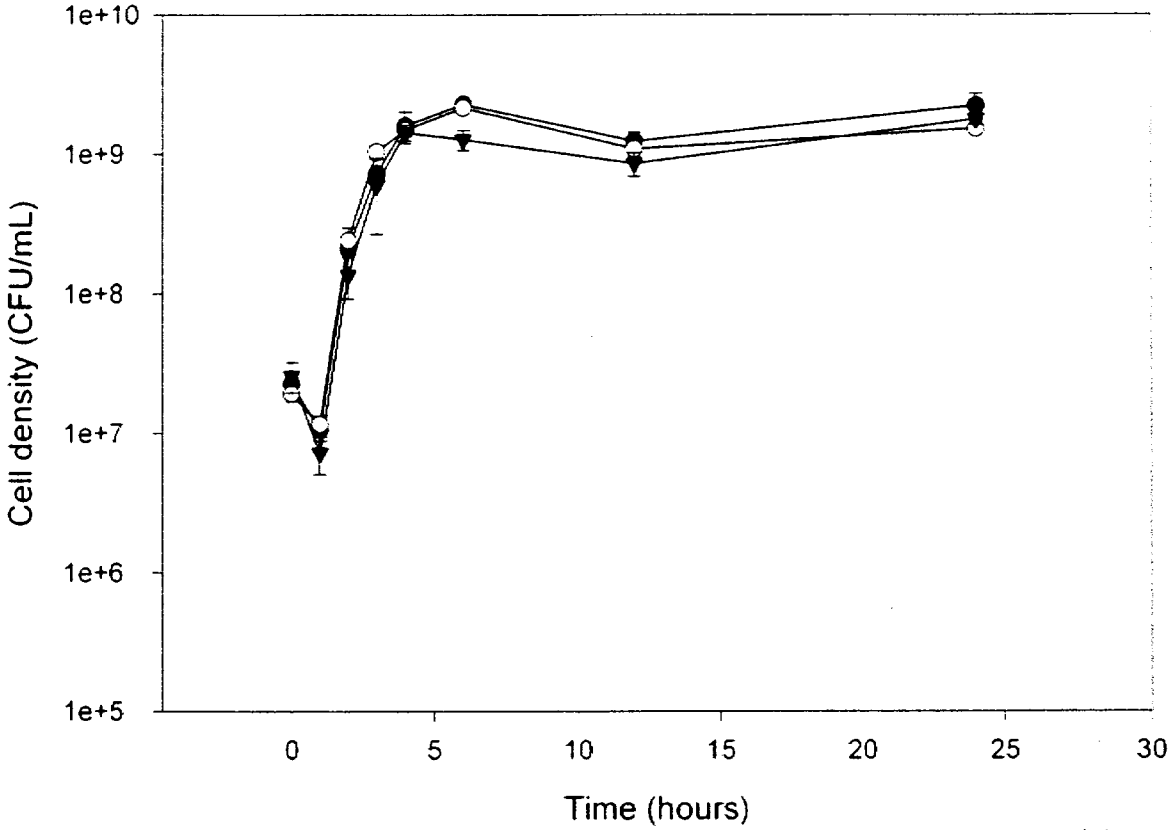

Figure 2.2.7. Planktonic growth of *E. coli* O157:H7 H32 (•), H32-*gfp* (○), and H32-*gfpΔrpoS::gm* (▾), in rich media (LB).

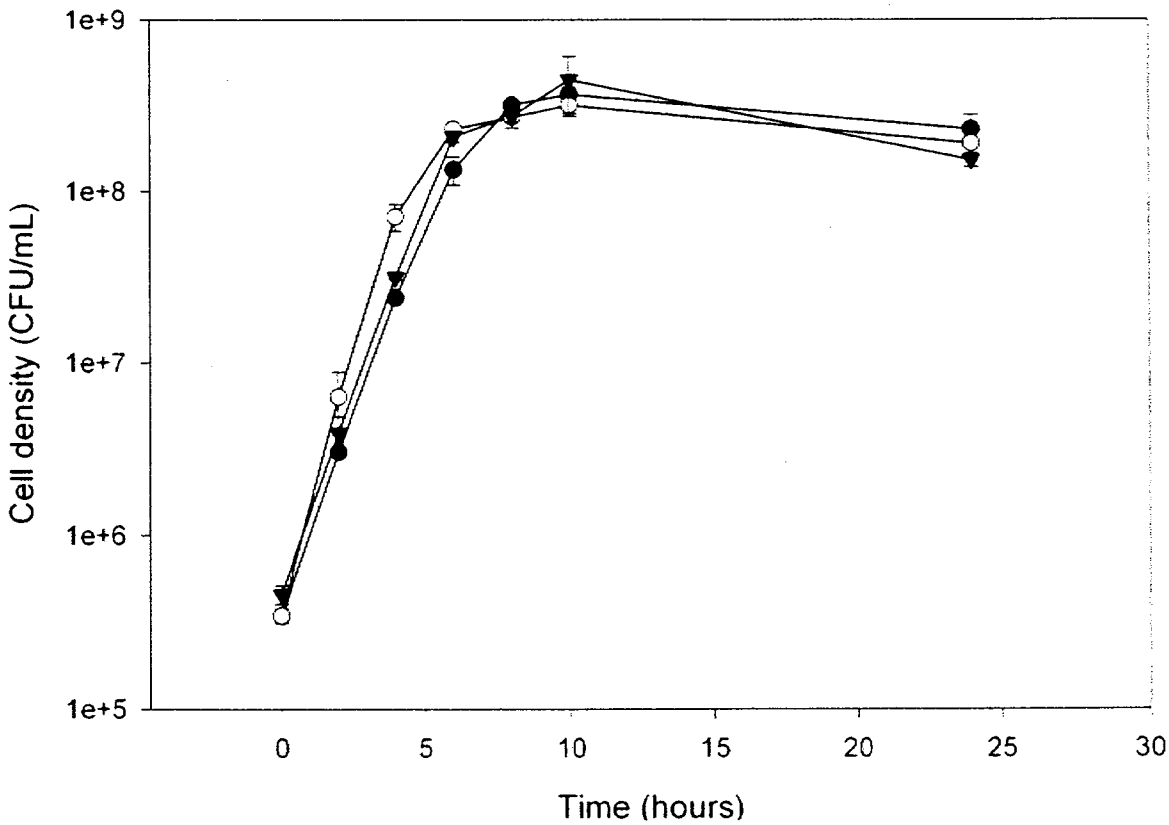

Figure 2.2.8. Planktonic growth of *E. coli* O157:H7 H32 (•), H32-*gfp* (○), and H32-*gfpΔrpoS::gm* (▾), in minimal media (MSM + 0.04% glucose).

2.3 Discussion

The Lambda Red-mediated recombination system has been touted for its ease and utility in developing targeted gene deletions in *E. coli* K-12 through the used of PCR-generated linear DNA fragments. (Datsenko and Wanner 2000; Murphy *et al.* 2000; Poteete 2001). While use of the system has been extended to pathogenic *E. coli* strains, the efficiency of λ Red recombination is significantly decreased in these strains, relative to K-12 (Murphy and Campellone 2003). However, the λ Red system has been successfully, albeit sparingly, employed in generating targeted gene replacements in *E. coli* O157:H7 (Dziva *et al.* 2004; Iyoda and Watanabe 2004; Low *et al.* 2006; Murphy and Campellone 2003 (and references therein); Newton *et al.* 2004). Furthermore, Murphy and Campellone (2003) have shown that optimization of the standard protocol through use of a specific buffer, a heat shock stage, careful orientation of the drug cassette, and minimizing expression of the Red recombinase plasmid to reduce spontaneous mutations, can yield successful and reproducible gene replacements in enterohemorrhagic *E. coli.* In an effort to generate a *gfp*-labeled, *rpoS*-knockout strain of *E. coli* O157:H7, λ Red-mediated recombination was attempted on five *E. coli* O157:H7 strains using four different gene cassettes, with flanking homologies of 40 and 500 bp. Simultaneous *gfp*-labeling and *rpoS* replacement was attempted in wild-type *E. coli* (H21, H22, H29, and H32), whereas *rpoS* disruption with a gentamycin resistance determinant was attempted in a previously labeled strain, H32-*gfp*.

Despite employing every available protocol (Datsenko and Wanner 2000; Murphy and Campellone 2003; Murphy 1998; Savage *et al.* 2006), as well as investigating numerous diversions from the original methodology, including modifications to DNA concentration and pKD46 induction time (data not shown), only one successful Red-mediated recombination event occurred. An *rpoS*-knockout was successfully generated in the *gfp*-labeled H32-*gfp* strain using a 500Δ*rpoS::gm* cassette. The frequency of the singular replacement event was 0.8×10^{-6} per cell survivor, a rate comparable to reported gene replacement frequencies in *E. coli* O157:H7 of $0.6\text{-}7 \times 10^{-6}$ per cell

survivor using the λ Red system (Murphy and Campellone 2003). The efficiency is approximately 7-fold higher than would be expected through the inhibition of RecBCD through electroporation alone with 400 replacements per μg linear DNA using the λ Red system, versus 60 replacements per μg using electroporation (El Karoui *et al.* 1999). It should be noted, however, that a higher voltage and shorter fragment were employed in this study (2500 versus 1904 V, 1.8 kb versus 6.5 kb, respectively), so electroporation as a means of facilitating gene replacement cannot be fully discounted, especially since increased voltage is known to enhance DNA uptake during electroporation (Dower *et al.* 1988).

The length of flanking sequences has been shown to influence Red-mediated gene replacement, where longer flanking sequences enhance recombination (Murphy and Campellone 2003). Successful recombination using the 500Δ*rpoS::gm* cassette bearing 500 bp of homology but not the *rpoS-gm* cassette bearing 40 bp of homology in H32-*gfp* under the same conditions may be due differing lengths of homologous sequences, where longer sequences (~500 bp) led to successful recombination. Furthermore, while it is known that DNA uptake during electroporation decreases with increasing size of circular DNA, the influence of linear DNA length on uptake remains untested (Sheng *et al.* 1995). It may be speculated that the large size (4.7 kb) of the 500Δ*rpoS::gfp-kan* cassette hindered its uptake in unlabeled H21, H22, H29, and H32, however strain differences may also have influenced uptake (Sheng *et al.* 1995). The singular success of the system however, leaves the aforementioned speculations as mere hypotheses for future study.

The application of numerous protocols in performing Lambda Red-mediated recombination suggests that the failure of the system to easily generate gene replacements in *E. coli* O157:H7 is less a product of method, and more a product of the system itself. Murphy and Campellone (2003) have speculated that the decreased efficiency of recombination in pathogenic *E. coli* relative to K-12 may be attributable to reduced efficiency of λ Gam in inhibiting RecBCD, decreased uptake of DNA during electroporation, or lower expression of *exo, bet,* and *gam* in pathogenic *E. coli* strains. In fact, certain enteropathogenic strains of *E. coli* have been found to perform Red-mediated

recombination only when *exo, bet,* and *gam* are housed in a high copy number plasmid and long homologous sequences are utilized (Murphy and Campellone 2003). The failure of H21, H22, H29, and H32 to recombine with the homologous linear DNA cassettes and the one-time recombination of H32-*gfp* producing H32-*gfpΔrpoS::gm* may be due to strain differences in any of the aforementioned areas of RecBCD inhibition, DNA uptake, or λ Red expression.

Despite the shortcomings of Lambda Red-mediated recombination in *E. coli* O157:H7, a *gfp*-labeled, *rpoS*-knockout was produced. *E. coli* H32 was labeled using a randomly-inserting Tn5 transposon, carrying a green fluorescent protein gene and a kanamycin resistance determinant. The insertion site of <*gfp-kan*> was identified through genomic sequencing as an uncharacterized gene, tentatively identified in most *E. coli* strains as a non-essential putative regulatory protein (Altschul *et al.* 1997). A homologous sequence in *E. coli* O157:H7 str. EC4115 (Accession number: CP001164.1) was identified through protein homology as UhpC, a regulatory protein involved in sugar phosphate transport. The *uhpC* gene is a member of *uhpABC,* a group of three regulatory proteins involved in mediating expression of *uhpT*, the product of which is a sugar phosphate transporter (Friedrich and Kadner 1987). Notably, while *uhpA* mutants inhibit *uhpT* expression, resulting in a Uhp$^-$ phenotype, mutations to *uhpC* rarely inhibit *uhpT* (Friedrich and Kadner 1987; Island and Kadner 1993). Biochemical testing and comparative growth curve analysis revealed that the <*gfp-kan*> insertion in this region did not alter the fundamental biochemical characteristics or growth of H32-*gfp* relative to H32.

Further characterization of the *gfp-kan* fragment through sequencing revealed an extensive (1.5 kb) non-coding region, contributing to the large (3.3-4.7 kb) size of the transposon and linear DNA cassettes designed for homologous recombination. The size of the *gfp-kan* fragment, coupled with the presence of large inverted and direct repeats substantially hindered PCR and sequencing reactions. While H32 was successfully labeled with *gfp-kan*, it would be advisable to eliminate the non-coding regions and repeats prior to future use to minimize PCR inhibition (DeSousa 2008), enhance recovery of the fragment during gel purification (Tillett and Neilan 1999), prevent

potential gene rearrangements (Lin *et al.* 2001), and conceivably improve uptake during transformation (Sheng *et al.* 1995).

Although the stationary phase sigma factor, RpoS, is fundamental to the expression of numerous stationary phase and stress-related genes (Hengge-Aronis 2002; Lange and Hengge-Aronis 1991a), a number of studies have revealed null mutations laboratory, clinical, and environmental strains of *E. coli* (Chen *et al.* 2004; Ferreira *et al.* 1999; Visick and Clarke 1997; Waterman and Small 1996). To ensure that the *rpoS* gene used as the wild-type control in this study was functional, the *rpoS* gene from H32 was cloned, sequenced, and converted to its amino acid sequence. No insertions or deletions were found relative to other *rpoS* genes sequenced from commensal or pathogenic *E. coli* strains, and amino acid residue 33 coded for glutamic acid, which is characteristic of pathogenic *E. coli* strains, instead of glutamine, which is found in non-pathogenic strains such as K-12 (Ferreira *et al.* 1999).

Growth curve comparison wild-type H32, *gfp*-labeled H32-*gfp*, and *gfp*-labeled, *rpoS*-knockout H32-*gfpΔrpoS::gm*, revealed no difference in growth between the strains in minimal media from lag to early stationary phase growth. While the role of *rpoS* during lag and exponential phases has not been extensively studied, comparable growth curves may be expected due to the minimal expression and short half-life of RpoS prior to stationary phase (Loewen and Hengge-Aronis 1994; Schellhorn *et al.* 1998). Studies in rich media of *E. coli* K-12 have revealed no differences in planktonic growth of *rpoS* mutants versus wild-type strain however, these studies employed total cell counts instead of viable cell counts, so full influence of *rpoS* may not have been readily apparent (Adams and McLean 1999; Corona-Izquierdo and Membrillo-Hernández 2002). In contrast, the comparison of growth for the three strains in rich media revealed subtle, although significant differences between the wild-type and labeled strains, and the *rpoS*-knockout. The knockout appeared to enter stationary phase earlier than both H32 and H32-*gfp*, and dropped significantly relative to both H32 and H32-*gfp* during early stationary phase. While the exact reasons for these differences, and the differences in growth between rich and minimal media are

unknown, possible explanations include the increased cell density in rich media leading to stress that the *rpoS*-knockout is less equipped to handle, or the influence of *rpoS*-dependent exponential phase genes, such as those responsible for dealing with toxic metabolites (Dong *et al.* 2008).

This study effectively produced an *rpoS*-knockout in a *gfp*-labeled strain of *E. coli* O157:H7 through use of Lambda Red-mediated recombination, and revealed subtle differences in the growth characteristics of the *rpoS*-knockout and the wild-type. Consequently, elucidating the influence of *rpoS* under different physiological states and environmental conditions should be greatly facilitated by use of H32-*gfpΔrpoS::gm*.

CHAPTER 3 – THE ROLE OF *RPOS* ON BIOFILM FORMATION AND SURVIVAL

3.0 INTRODUCTION

In aquatic environments, bacteria are often protected from their inconstant surroundings through the establishment of biofilms. Biofilms are complex communities of bacteria attached to the substratum and enclosed in an extracellular matrix (ECM) of exopolysaccharides, and to a lesser degree, proteins, nucleic acids, and other enmeshed substances (Costerton *et al.* 1995; Davey and O'Toole 2000). This protective aggregation allows the bacteria to withstand such challenges as antibiotics, biocides, phagocytosis, and desiccation, better than their planktonic counterparts (Costerton *et al.* 1987; De Beer *et al.* 1994; Jensen *et al.* 1990; Marrie *et al.* 1982; Nickel *et al.* 1985a; Nickel *et al.* 1985b). Given the enhanced survival of biofilm cells, the biofilm state is often recognized as the preferred state of existence for bacteria under many environmental conditions (Geesey *et al.* 1977; Henrici 1933; Zobell 1943).

The increased resistance of biofilm cells to various assaults has been attributed to five potential interacting factors: inhibited penetration of the biofilm, decreased metabolism due to nutrient deprivation in the biofilm interior, a heterogeneous display of phenotypes induced by variable conditions throughout the biofilm, the production of persister cells, and cross-protection through the activity of a general stress response mechanism (For review see Mah and O'Toole 2001; Stewart and Costerton 2001; Stewart 2002). One gene continually implicated in biofilm formation and responsible for mediating gene expression during periods of stress, starvation, and stationary phase growth is *rpoS*, the stationary phase sigma factor (Kolter *et al.* 1993; Lange and Hengge-Aronis 1991a; Loewen and Hengge-Aronis 1994; McCann *et al.* 1991). RpoS (σ^s), the gene product of *rpoS*, mediates the binding of RNA polymerase to the promoter of *rpoS*-dependent genes, thereby facilitating their transcription (Lange and Hengge-Aronis 1991a; Loewen and Hengge-

Aronis 1994). RpoS is reportedly responsible for the regulation of approximately 10% of the *E. coli* genome, with over 140 genes upregulated by RpoS during periods of stress or stationary phase growth (Weber *et al.* 2005).

DNA microarrays performed on *E. coli* K-12 have revealed that *rpoS*, and numerous *rpoS*-dependent genes are differentially expressed between the planktonic and biofilm state, although the findings of these reports are conflicting (Beloin *et al.* 2004; Domka *et al.* 2007; Ren *et al.* 2004; Schembri *et al.* 2003). Studies conducted on the influence of *rpoS* on *E. coli* K-12 biofilm formation are also conflicting. One study found that an *rpoS*⁻ mutation enhances biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002), while another found that the same mutation completely attenuates biofilm formation (Schembri *et al.* 2003). The remaining two studies found that *rpoS*⁻ inhibits but does not prevent biofilm formation (Adams and McLean 1999; Ito *et al.* 2008).

The aforementioned studies investigating the role of *rpoS* on biofilm formation differed significantly in methodology, including the relative availability of nutrients provided by batch culture and flow cell cultivation. Culture conditions and media selection have been shown to fundamentally impact the biofilm-forming capabilities of various *E. coli* strains (Dewanti and Wong 1995; Oh *et al.* 2007; Reisner *et al.* 2006). Furthermore, these studies were limited to the short-term influence of *rpoS* on *E. coli* K-12 biofilms in various rich medias. Numerous reports have detailed that pathogenic *E. coli* is capable of surviving for extended periods of time under the comparatively nutrient deprived conditions of untreated well water, bottled water, municipal drinking water, natural water bodies, and as colonizers of water distribution systems (Fass *et al.* 1996; Wang and Doyle 1998; Warburton *et al.* 1998; Watterworth *et al.* 2006). As such, further investigation into role of *rpoS* on the survival of *E. coli* O157:H7 biofilms under these less nutritive conditions is required.

This study will compare the biofilm-forming capabilities of wild-type and *rpoS*- strains of *E. coli* O157:H7 under different culture conditions, in an effort to elucidate the role of *rpoS* in *E. coli* O157:H7 biofilm formation, and to help reconcile the differences in the current literature. Furthermore, the role of *rpoS* on the survival of *E. coli* O157:H7 biofilms under nutrient deprived, low osmolarity conditions will be investigated.

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strains and growth media

The wild-type *E. coli* O157:H7 strain used in this study was H32, a bovine isolate kindly provided by Dr. C. Gyles at the University of Guelph ((Guelph, ON, Canada); Gyles *et al.* 1998). H32-*gfp* represents a fluorescently labeled strain of H32, where a green fluorescent protein gene and kanamycin resistance determinant (*gfp-kan*) have been inserted randomly by a Tn5 transposon (Chapter 2, Section 2.1.3). The *gfp*-labeling was performed by Rebecca Barnes, a former member of the Applied and Environmental Microbiology Lab at Lakehead University (Thunder Bay, ON, Canada). The *rpoS*-knockout employed in this study, H32-*gfpΔrpoS::gm*, represents an H32-*gfp* derivative, where *rpoS* has been disrupted by a gentamycin resistance determinant (*gm*) using Lambda Red-mediated recombination (Chapter 2, Section 2.1.9).

All bacterial strains were cultured in Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C, unless otherwise noted. H32 was maintained without antibiotics, H32-*gfp* with 50 µg/mL of kanamycin (Kan^{50}), and H32-*gfpΔrpoS::gm* with 50 µg/mL of kanamycin and 20µg/mL gentamycin (Kan^{50} + Gen^{20}). Stock cultures were prepared for each culture in TSB supplemented with 25% (v/v) glycerol and stored at -80°C. Bacterial strains were recovered prior to each experiment by streaking for isolated colonies on TSA, followed by plating on TSA with the appropriate antibiotic.

Media used biofilm cultivation for the purposes of analyzing biofilm formation included TSB, 1/5th strength TSB, and minimal salts medium supplemented with 0.04% glucose (MSM + 0.04% glucose). MSM consisted 7 g KH_2PO_4, 3 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 1 mg yeast extract to enhance biofilm formation, and 0.04% filter-sterilized glucose per litre ddH_2O (Camper *et al.* 1991). MSM + 0.04% glucose was also used to develop initial biofilms for studying survival in

sterile, distilled, deionized water (ddH_2O). MSM was autoclaved at 121°C, 15 psi for 15 minutes, whereas all other media was autoclaved at under the same conditions for 20 minutes.

3.1.2 Biofilm formation and analysis

Biofilm cultivation was modified from a previously described protocol (Maki 2008; Tallon 2005). Biofilms were grown on 22 x 22 mm glass coverslips (Fisher Scientific Canada, Ottawa, ON, Canada), previously cleaned by 15 minutes immersion in 70% nitric acid, three subsequent washes in sterile ddH_2O, and a final rinse in absolute methanol. A thin line of silicone sealer (Elmer's, Columbus, OH, USA) was applied to one edge of each coverslip and cured for 24 hours before autoclaving. The silicone served to mark orientation, allow for liquid contact with all surfaces of the coverslip, and promote easy manipulation. Coverslips were placed in extra-deep petri dishes (100 mm x 20 mm, Fisher Scientific Canada), with the silicone side down. Three petri dish apparatuses were prepared per strain, per sampling period, per media. Three different media were tested for optimal biofilm growth: TSB, $1/5^{th}$ strength TSB, and MSM + 0.04% glucose. Each petri dish was filled with 30 mL of the appropriate media, without antibiotics.

Broth cultures were prepared for H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* in 50 mL of TSB supplemented with the appropriate antibiotic, using fresh colonies isolated from selective plates. The cultures were grown at 37°C, shaking at 150 rpm. Following overnight growth (12-16 h), the cultures were diluted to an OD_{600nm} of 0.5. The prepared petri dishes were each inoculated with 200 µL of the appropriate diluted culture, and were placed in a large bin lined with moist sterile paper towel to prevent evaporation. The samples were collectively incubated at 37°C, shaking at 25 rpm. Every 24 h, the spent media was removed gently by aspiration and replaced with 30mL of fresh media.

Two studies, each performed three times, were conducted on the biofilm-forming abilities of H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm*. The first study investigated the role of media selection on the biofilm formation of the three strains, and involved sampling coverslips from all three strains in all

three medias after 24 h. A second study was performed by sampling all strains at 24 (day 1), 48 (day 2), and 72 h (day 3), using MSM + 0.04% glucose only, as this media was found to enhance biofilm formation for all three strains. For sampling, coverslips were removed from the petri dish systems carefully using sterile forceps and stained by flooding the silicone-free side with 200 µL of SYTO 9 (diluted to 5 µL/mL in sterile ddH_2O), a green fluorescent dye (Molecular Probes, Eugene, OR, USA), for 5 minutes. All three strains were stained with SYTO 9 to ensure comparable fluorescence. SYTO 9 is a nucleic acid stain, which will stain both live and dead cells, whereas *gfp*-labeling is visible mainly in living cells. Unadhered cells and excess dye were removed by submerging the coverslip once in sterile ddH_2O. The coverslip was then placed, silicone-side up, over a depression slide filled with ddH_2O to prevent drying, and sealed with nail polish. The silicone-side of the coverslip was cleaned by wetting a cotton swab with 10% sodium hypochlorite and gently swabbing the surface of the coverslip. The wash procedure was repeated with ddH_2O, 85% ethanol, and ddH_2O again, using a new cotton swab for each application.

The coverslips were visualized on a Olympus FluoView™ FV300 confocal scanning laser microscope (CSLM) using a 60x PlanApo NA 1.4 oil immersion objective lens and a 10 mW, 488 nm argon laser (Olympus Corporation, Tokyo, Japan). Detection of SYTO 9 fluorescence was performed using a DM570 dichroic mirror and band pass emission from 510 to 530 nm using a FVX-BA 510-530 filter set, since the maximal emission of SYTO 9 occurs at 510 nm (Molecular Probes, Eugene, OR, USA). The laser intensity was set at 1% for all image capturing to reduce photobleaching. Each image stack (Z-stack) was analyzed using PHobia Laser Imaging Processor (PHLIP), a biofilm quantification program, run through the MatLab 7.0.4 platform (The MathWorks 2005). Biofilm biovolume, thickness, and substratum coverage were determined for each image stack using PHLIP (Xavier *et al.* 2003). Five image stacks per strain, per experiment, were used in analysis. The data was analyzed in SigmaPlot and SigmaStat (SPSS Inc. 1997; SPSS Inc. 2000). Five representative z-stacks for each sample were compiled into single images for qualitative visualization using Image-Pro (Media Cybernetics 2001).

3.1.3 Biofilm survival

Biofilm survival was observed under starvation conditions in sterile ddH_2O. Biofilms were cultivated on 60 x 24 mm glass coverslips with a 22 x 24 mm sampling area marked using a water-resistant, abrasion-resistant marking pen (Fisher Scientific Canada, Ottawa, ON, Canada). Biofilms for H32, H32-*gfp*, and H32-*gfpΔrpoS::gm*, were cultivated at 22°C for 24 h in MSM + 0.04% glucose, as previously described. Three petri dish systems were prepared per strain, per time point, plus extras in event of breakage. Following 24 h growth, three petri dish systems for each strain were retained for initial (day 0) counts. The coverslips were removed from the remaining petri dishes, rinsed once by gentle submersion in sterile ddH_2O, and placed in new extra-deep petri dishes containing 40 mL of sterile ddH_2O. The petri dish systems were incubated at 22°C, shaking at 25 rpm. The media was not removed from the petri dishes following the initial transfers to ddH_2O however, the absorbent paper was periodically moistened to prevent evaporation of the samples.

Initial (day 0) biofilm counts were performed by rinsing each coverslip once by gently dipping in sterile ddH_2O, and propping the coverslip silicone-side down on the wall of a clean petri dish. Removal of the biofilms from the coverslips was facilitated by forcefully but carefully pipetting 1 mL of sterile phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per litre, pH 7.4) against the marked area 20 times, each time collecting the PBS from the base of the coverslip. A sterile cotton applicator (Fisher Scientific Canada, Ottawa, ON, Canada) was used to scrub the sampling area 15 times in a horizontal direction, followed by 15 times in a vertical direction into the PBS at the base of the coverslip. The cotton tip was then excised from the shaft and deposited in 4 mL of sterile PBS. The process was repeated two additional times with new applicators, depositing each tip in the same tube of PBS. Between the second and third swab, the 1 mL of PBS was collected from the base of the coverslip and added to the 4 mL of PBS containing the cotton tips. The third cotton swab was also used to collect any residual drops of liquid from the sampling area and the petri dish. The tube containing 5 mL of PBS and three cotton tips was

immediately vortexed vigorously for 1 minute to dislodge the cells, and 1 mL of sample was removed to perform a dilution series in PBS.

Biofilm sampling was performed as described on days 3, 6, and 13, in addition to the day 0 sampling. To assess detachment, planktonic samples were also taken on days 3, 6 and 13 by removing 1 mL of media from each petri dish prior to removing the coverslip. Dilution series were also performed for the planktonic samples in PBS. Viable cell counts were conducted on both the planktonic and biofilm samples by drop plating 6-5 µL drops of each dilution onto a quadrant of TSA. For H32-*gfp* and H32-*gfp*Δ*rpoS*::*gm* the TSA was supplemented with the appropriate antibiotics. Periodically, H32-*gfp* and H32-*gfp*Δ*rpoS*::*gm* were plated on both TSA with antibiotics and without antibiotics to ensure the use of selective media was not hindering the viable cell counts. The resulting data was analyzed in SigmaPlot and SigmaStat (SPSS Inc. 1997; SPSS Inc. 2000).

3.1.4 Biofilm detachment

The relative detachment of H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* biofilms was analyzed by comparing the total biofilm count for each sample from the survival study to the corresponding total planktonic count. The detachment for each strain on sampling days 3, 6, and 13 was expressed as a percentage of overall count for that time point. The detachment for all three strains was graphed and compared in SigmaPlot and SigmaStat (SPSS Inc. 1997; SPSS Inc. 2000).

3.2 RESULTS

3.2.1 Biofilm formation and analysis

Qualitative visualization of H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* biofilms using CSLM after 24 h revealed a strong correlation between media and the quantity of biofilm formed for each strain. For all three strains, no notable biofilm formation occurred in TSB. The greatest amount of biofilm formation occurred in MSM + 0.04% glucose, while cultivation in 1/5th strength TSB represented an intermediate level of growth between TSB and MSM + 0.04% glucose (Figure 3.2.1.).

For H32 and H32-*gfp* in TSB, most fields of view under CSLM contained sparse single cells or pairs of adhered cells, whereas cells were absent in most fields of view for H32-*gfp*Δ*rpoS*::*gm*. Given the absence of biofilms in TSB, no PHLIP analysis was performed at 24 h for any strain in TSB. In 1/5th strength TSB, H32 and H32-*gfp* both exhibited a uniform dispersal of single cells and small clusters of cells. In contrast, H32-*gfp*Δ*rpoS*::*gm* exhibited similar growth in 1/5th strength TSB to the *rpoS*$^{+}$ strains in full strength TSB, where most fields of view contained a single, isolated cell or cells.

While H32-*gfp*Δ*rpoS*::*gm* appeared to demonstrate less cellular adhesion in TSB and 1/5th strength TSB than the *rpoS*$^{+}$ strains, it exhibited substantially more adhesion in MSM + 0.04% glucose than both H32 and H32-*gfp*. In MSM + 0.04% glucose, H32 and H32-*gfp* demonstrated small clusters of cells and larger aggregations of cells (microcolonies), interspersed with single cells and non-colonized areas. In contrast, H32-*gfp*Δ*rpoS*::*gm*, exhibited an extremely dense, uniform mat of cells that appeared to be 2-3 layers of cells in depth.

PHLIP analysis of biofilm biovolume ($\mu m^3/mm^2$, Figure 3.2.2.) confirmed the qualitative observations that both strain and media had a statistically significant influence on biofilm biovolume ($P<0.001$ for both). Biovolume for all strains was found to be comparable in 1/5th strength TSB ($P=0.913$-0.999), although the *rpoS*-knockout did form 6 to 7-fold less biofilm than H32-*gfp* and H32, respectively. A statistically significant difference was found between H32 and

H32-*gfpΔrpoS::gm* (P<0.001) and H32-*gfp* and H32-*gfpΔrpoS::gm* (P<0.001) in MSM + 0.04% glucose, where the *rpoS*-knockout formed 3.5 and 7-fold more biofilm, respectively. Generally, all strains formed more biofilm in MSM + 0.04% glucose than in 1/5th strength TSB, with the differences for both H32-*gfp* and H32-*gfpΔrpoS::gm* being statistically significant (P<0.001). There was no difference in biofilm biovolume between H32 and H32-*gfp* in either 1/5th strength TSB or MSM + 0.04% glucose (P=0.999 and P=0.070, respectively).

Similar to the results for biofilm biovolume, both strain and media had a statistically significant influence on the percentage of substratum covered (P<0.001, Figure 3.2.3.). The *rpoS*-knockout demonstrated 7-fold less coverage than H32 or H32-*gfp* in 1/5th strength TSB, but none of the differences between strains within 1/5th strength TSB were found to be statistically significant (P=0.931-0.999). A statistically significant difference was found in percentage substratum coverage between all strains in MSM + 0.04% glucose, where P=0.003 for the difference between H32 and H32-*gfp*, and P<0.001 for the differences between H32-*gfpΔrpoS::gm* and the two *rpoS*$^{+}$ strains. H32-*gfpΔrpoS::gm* demonstrated 3-fold greater substratum coverage than H32-*gfp*, and 8-fold greater substratum coverage than H32. The biofilms of all strains covered a greater percentage of the substratum in MSM + 0.04% glucose than 1/5th strength TSB, with the differences for H32-*gfp* and H32-*gfpΔrpoS::gm* being statistically significant (P<0.001).

Biofilm thickness was comparable for all strains in 1/5th strength TSB, and all strains exhibited thicker biofilms in MSM + 0.04% glucose than in 1/5th strength TSB, however only H32 exhibited a statistically significant difference in thickness between the two media (P<0.001). H32-*gfp* exhibited statistically thinner biofilms relative to both H32 and H32-*gfpΔrpoS::gm* in MSM + 0.04% glucose (P<0.001 and P=0.007, respectively). The *rpoS*-knockout formed biofilms of intermediate thickness in MSM + 0.04% glucose, relative to H32 and H32-*gfp* (Figure 3.2.4).

Visualization of H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* biofilms in MSM + 0.04% glucose over a three-day period suggested maximal biofilm growth after 2 days, with an apparent decrease in

adhered cells on the third day (Figure 3.2.5). H32-*gfpΔrpoS::gm* exhibited a similar dense mat of cells on all three days, whereas biofilm architecture for both H32 and H32-*gfp* appeared more similar on days 1 and 3, than on day 2. Day 2 biofilm growth for H32 and H32-*gfp* saw an increased number of evenly distributed, isolated cells amongst the established microcolonies. On the third day, the distribution of isolated H32 and H32-*gfp* cells decreased, leaving a pattern of adhered single and aggregated cells similar to that of day 1 biofilms. The *rpoS*-knockout consistently showed a greater quantity of adhered cells than the *rpoS*$^+$ strains.

Biofilm growth in MSM + 0.04% glucose, sampled at 1, 2, and 3 days, and analyzed using PHLIP confirmed that the *rpoS*-knockout produces biofilms of greater biovolume and percentage substratum coverage than both of the *rpoS*$^+$ strains at all time points ($P<0.001$, Figure 3.2.6.). Maximal biofilm biovolume and substratum coverage occurred for all strains after 2 days of growth. Between days 2 and 3, biovolume decreased significantly for H32 and H32-*gfpΔrpoS::gm* ($P<0.001$), and substratum coverage decreased significantly for all strains ($P<0.001$). Overall, H32 and H32-*gfp* formed biofilms of similar biovolume and substratum coverage.

Overall, H32-*gfp* exhibited thinner biofilms than both H32 and H32-*gfpΔrpoS::gm* ($P<0.05$, Figure 3.2.6.). H32 exhibited the thickest biofilms, however the differences in thickness between H32 and H32-*gfpΔrpoS::gm* biofilms were not significant ($P=0.084$). Biofilm thickness remained consistent for all three strains over the sampling period, with no statistically significant differences exhibited for any strain between time points. The average biofilm thickness ranged from 1.9-2.6 μm, with a maximal thickness of 4.4 μm in H32.

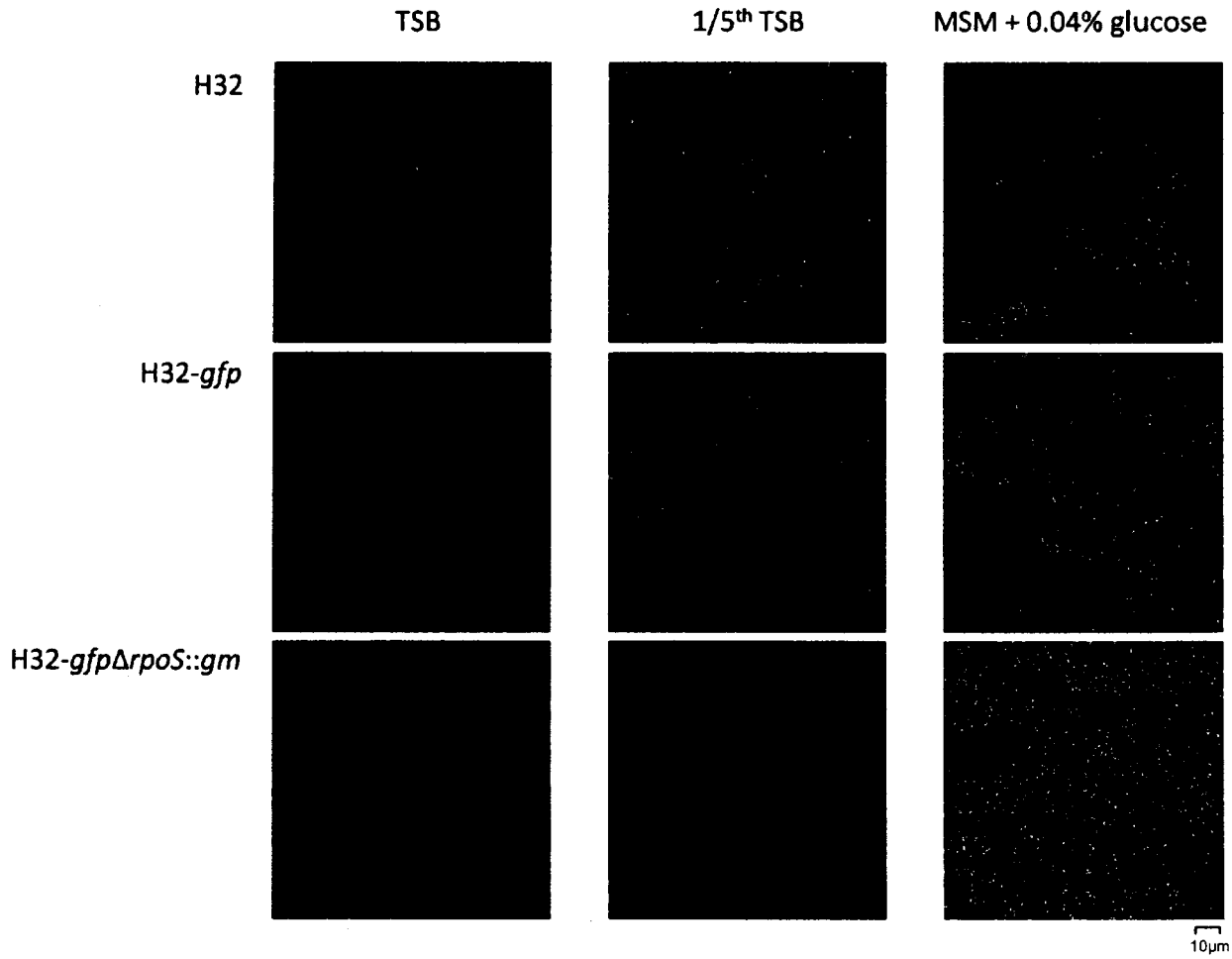

Figure 3.2.1. CSLM images of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* biofilms after 24 h growth in TSB, 1/5th strength TSB, and MSM + 0.04% glucose.

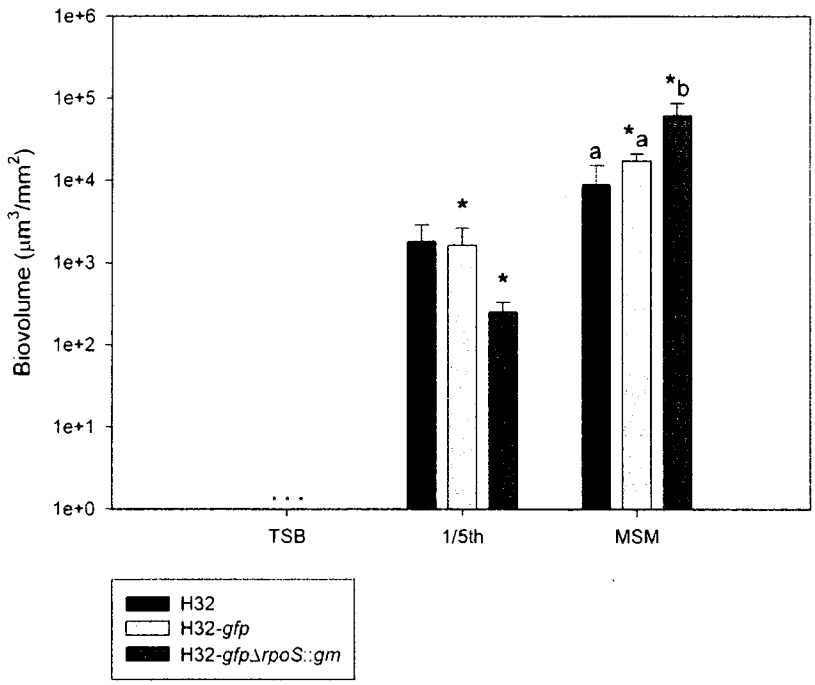

Figure 3.2.2. Biovolume of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* biofilms under different culture conditions. * represents a statistically significant difference within the same strain between different media (P<0.001). A statistically significant difference exists between strains within the same media labeled with differing letters (**a** vs. **b**, P<0.001). No biofilm growth was observed for any strain in TSB (. . .).

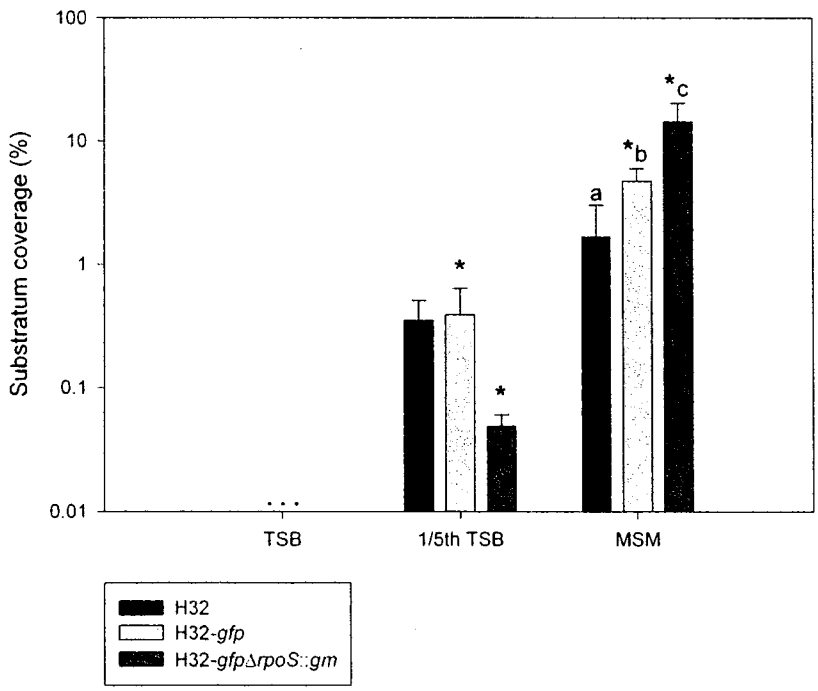

Figure 3.2.3. Substratum coverage of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* biofilms under different culture conditions. * represents a statistically significant difference within the same strain between different media (P<0.001). A statistically significant difference exists between strains within the same media labeled with differing letters (P<0.05). No biofilm growth was observed for any strain in TSB (. . .).

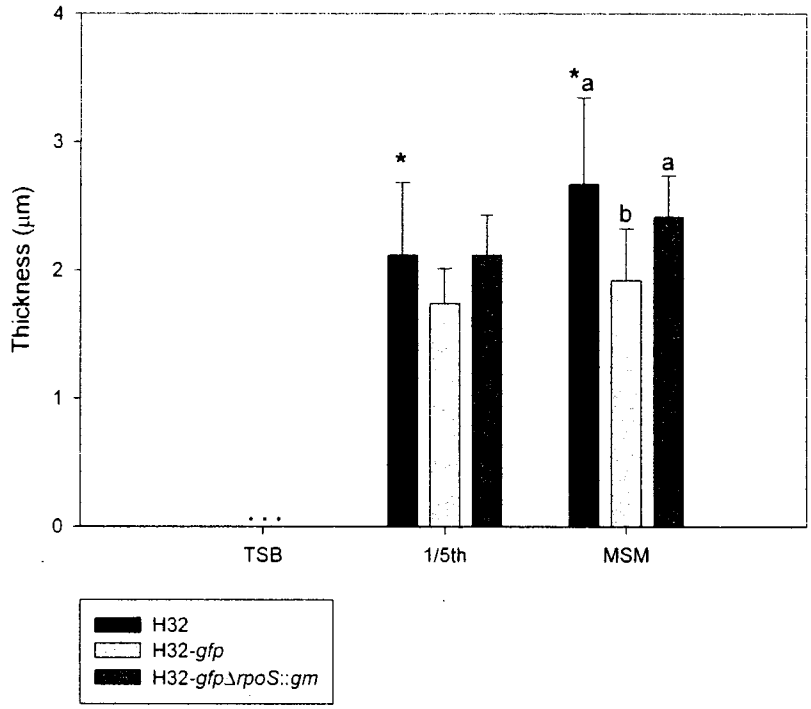

Figure 3.2.4. Thickness of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* biofilms under different culture conditions. * represents a statistically significant difference within the same strain between different media ($P<0.001$). A statistically significant difference exists between strains within the same media labeled with differing letters (**a** vs. **b**, $P<0.001$). No biofilm growth was observed for any strain in TSB (. . .).

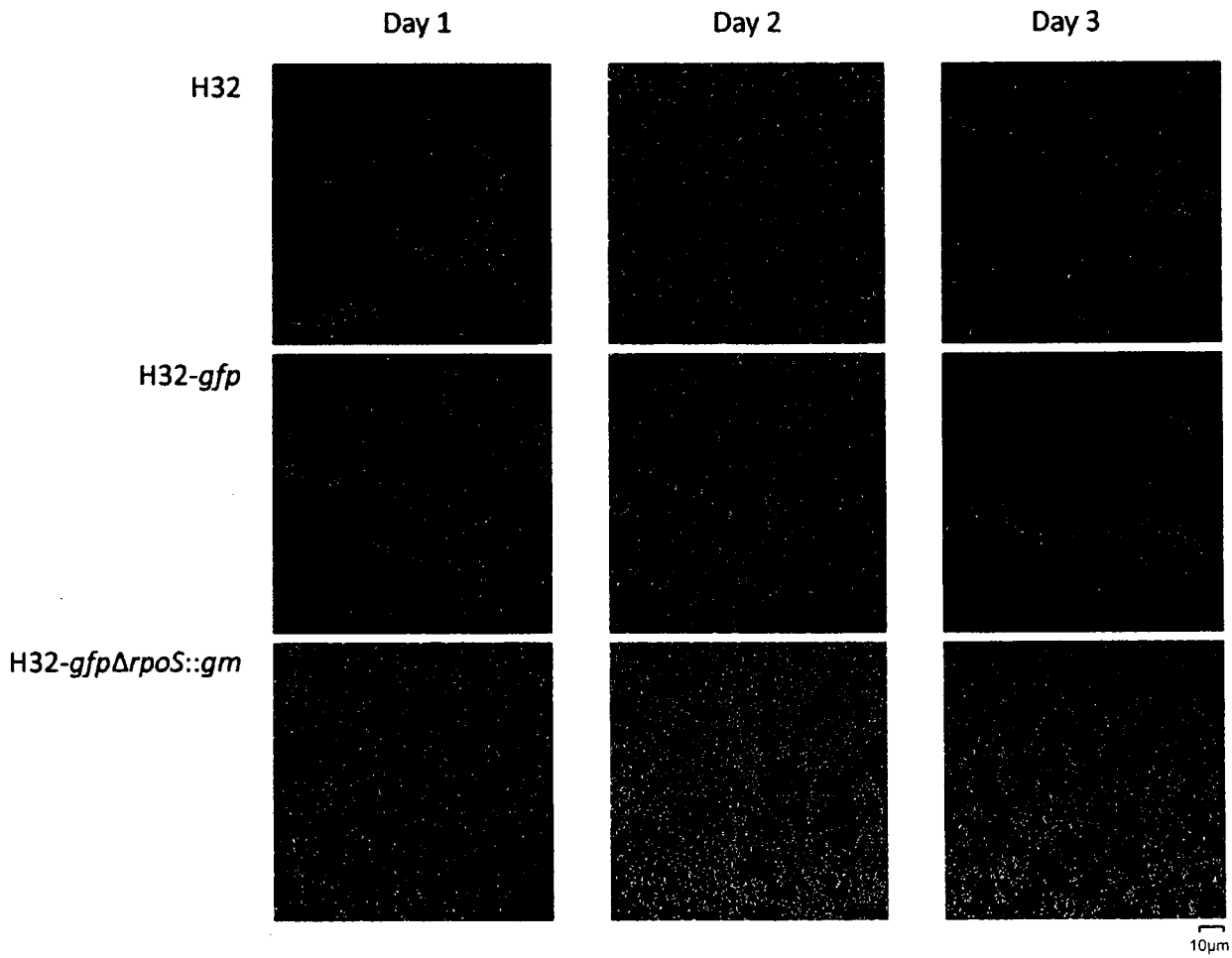

Figure 3.2.5. CSLM images of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* biofilms in MSM + 0.04% glucose after 1, 2, and 3 days of growth.

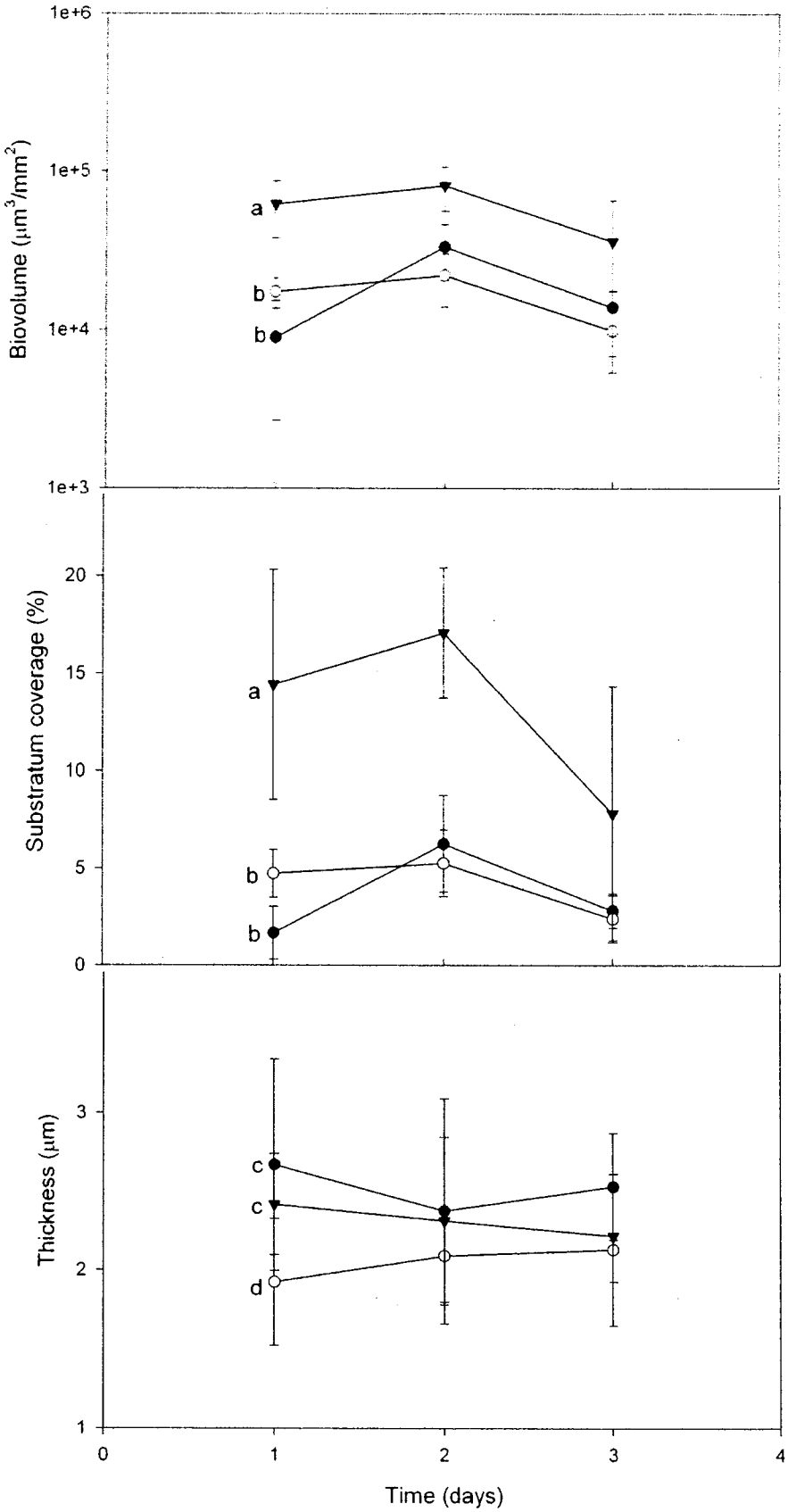

Figure 3.2.6. Three-day biovolume, substratum coverage, and thickness of *E. coli* O157:H7 H32 (•), H32-*gfp* (○), and H32-*gfp*Δ*rpoS*::*gm* (▾) biofilms in minimal media (MSM + 0.04% glucose). A statistically significant difference ($P<0.001$) exists at all time points between strains (**a**) and (**b**), whereas strains (**c**) and (**d**) differ significantly overall ($P<0.05$), however not at all time points.

3.2.2 Biofilm survival and detachment

Biofilm survival for *E. coli* O157:H7 strains H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* was examined over a 13-day period under conditions of osmotic stress and nutrient deprivation in ddH_2O. Initial (day 0) biofilm counts of all three strains revealed a statistically significant reduction in biofilm formation by H32-*gfp* at 22°C relative to both H32 and H32-*gfp*Δ*rpoS*::*gm* ($P \leq 0.001$). While this initial difference resulted in a statistically significant difference overall between H32 and H32-*gfp* ($P=0.002$), no significant difference was noted between the survival of attached biofilm cells at any other time point. As such, the relative attachment of H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* biofilms was comparable on days 3, 6, and 13. All three strains exhibited maximal attachment at day 0, followed by a ~0.8-1.7 log CFU/cm² reduction between day 0 and day 3 ($P<0.001$ for all strains). Following this initial reduction, smaller reductions of <1 log CFU/cm² occurred at all time points for all strains, however none of these reductions were significant. After 13 days, biofilm survival ranged from 1.6×10^5 to 2.8×10^6 CFU/cm² (Figure 3.2.7.). The aforementioned results indicate that *rpoS* has no significant impact on the survival of attached biofilm cells in ddH_2O, since no significant differences were observed between H32-*gfp*Δ*rpoS*::*gm* and the wild-type strains at any time point except the initial day 0 count.

In contrast to the comparable survival of attached biofilm cells for H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm*, the survival of detached biofilm cells was significantly different between all strains ($P<0.001$). H32 exhibited the greatest number of viable detached cells at all time points, whereas the *rpoS*-knockout exhibited the least. Viable cell counts for H32-*gfp* were an intermediary to H32 and H32-*gfp*Δ*rpoS*::*gm* on days 3 and 6, but dropped significantly (0.77 log CFU/mL) by day 13 to parallel those of the *rpoS*-knockout ($P=0.002$). Viable detached cell counts for all strains ranged from 10^6-10^7 CFU/mL over the time period, with H32 and H32-*gfp*Δ*rpoS*::*gm* showing a gradual 0.17 and 0.44 log CFU/mL reduction in viable cells from day 3 to day 13, respectively (Figure 3.2.7).

The measurement of viable cells detached from established biofilms is not only a reflection of how many cells have dissociated, but how many cells have survived in solution upon transitioning to the planktonic state. The increased number of viable detached cells in H32 may be attributed to the increased survival of the detached cells in ddH_2O, an increased overall number of detached cells, or a combination of both factors. Relative to both H32 and H32-*gfp*, the *rpoS*-knockout demonstrated the fewest number of recovered cells from the liquid phase, although the difference in detached cells on day 13 was negligible between H32-*gfp* and H32-*gfpΔrpoS::gm* (P=0.972). Decreased numbers of viable detached cells, conversely, may be attributed to *rpoS*- decreasing the detachment of biofilm cells, the viability of these detached cells, or both.

When the total number of both attached and detached viable cells was considered, H32 exhibited a statistically greater number of surviving cells at all time points than both H32-*gfp* and H32-*gfpΔrpoS::gm* (P<0.001), suggesting that *rpoS* enhances survival in osmotically stressed, nutrient-limited environments. H32-*gfpΔrpoS::gm* exhibited statistically fewer surviving cells than H32-*gfp* on day 3 and day 6, however the difference between the two strains was not significant on day 13 (P=0.955), suggesting that *rpoS* temporarily enhances survival in ddH_2O.

When the total number of surviving detached cells was expressed as a percentage of the total number of cells in the system for that strain at a given time point, H32 exhibited the lowest overall percentage of surviving detached cells at days 3 and 6, while H32-*gfp* exhibited the greatest percentage of surviving detached cells at day 3. While H32 and H32-*gfpΔrpoS::gm* both demonstrated an increasing trend of detached survivors from day 3 to day 13 (P<0.001), H32-*gfp* exhibited comparable levels of detached cells on all three days (P=0.757-0.945), with maximal detached survivors occurring at day 3 (82%). By day 13, the number of surviving detached cells represented 80-90% of the total cell counts (both attached and detached) for the all three strains, with no significant differences at this point, suggesting increased detachment or survival of detached cells, relative to attached biofilm cells.

The differences in initial biofilm formation at 22°C and survival of total and detached biofilm cells between H32 and H32-*gfp* suggest that labeling of H32-*gfp* using a randomly inserting Tn5 transposon (Chapter 2) may have had an unintended effect on H32-*gfp* survival. The insertion site was identified as *uhpC*, a gene for the regulatory protein UhpC, which is involved in the transport of sugar phosphates. The differences between H32-*gfp* and H32-*gfpΔrpoS::gm* still indicate that *rpoS* enhances survival of *E. coli* O157:H7 in ddH_2O overall, and specifically enhances detachment of biofilm cells, survival of these detached cells, or both. The differences between H32 and H32-*gfpΔrpoS::gm* may be exaggerated by the effects of the transposon insertion mutation.

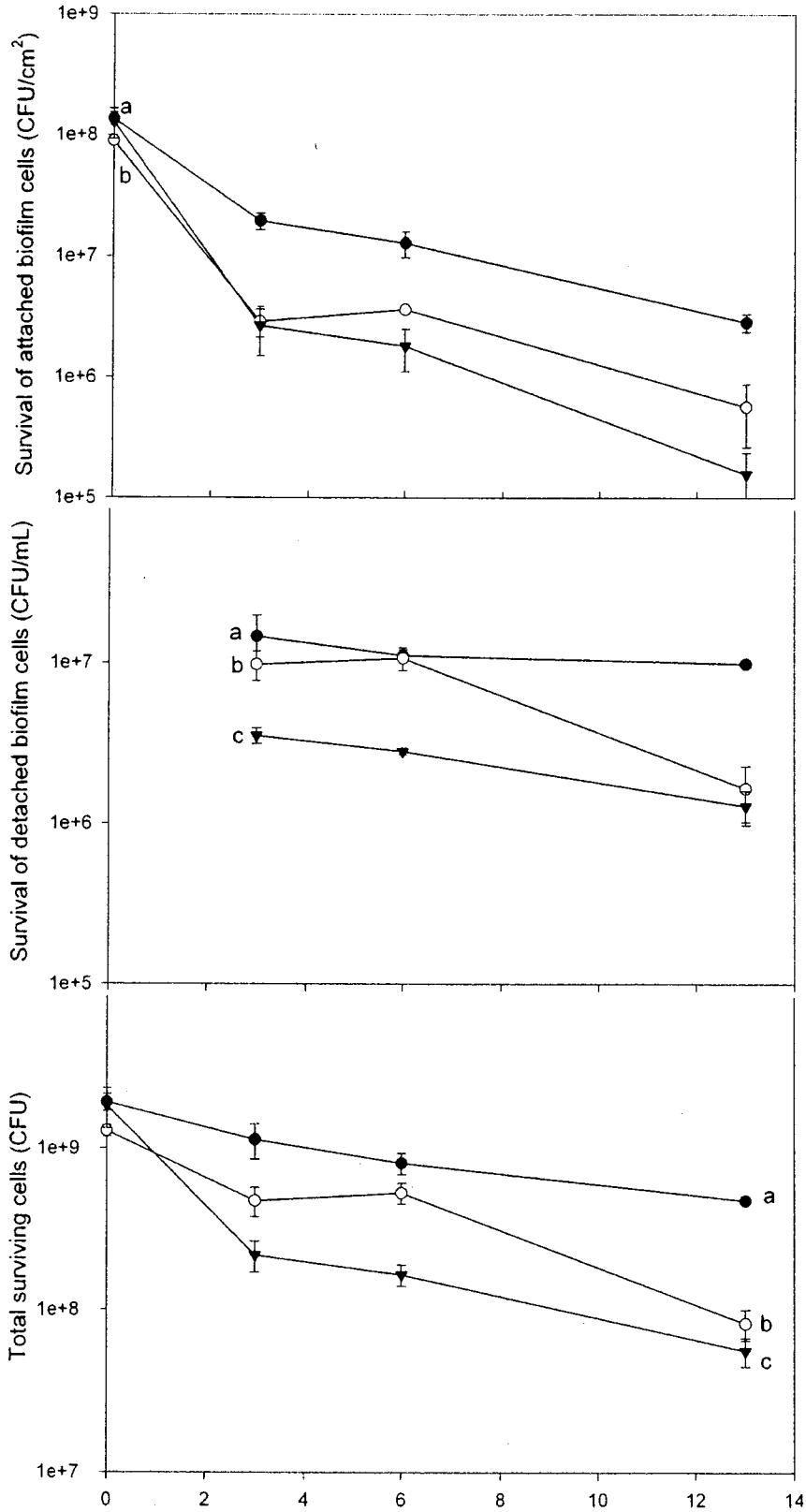

Figure 3.2.7. Survival of attached and detached *E. coli* O157:H7 H32 (●), H32-*gfp* (○), and H32-*gfpΔrpoS::gm* (▾) biofilm cells in ddH_2O. A statistically significant difference ($P<0.05$) exists between strains overall bearing different letters (**a**), (**b**), and (**c**).

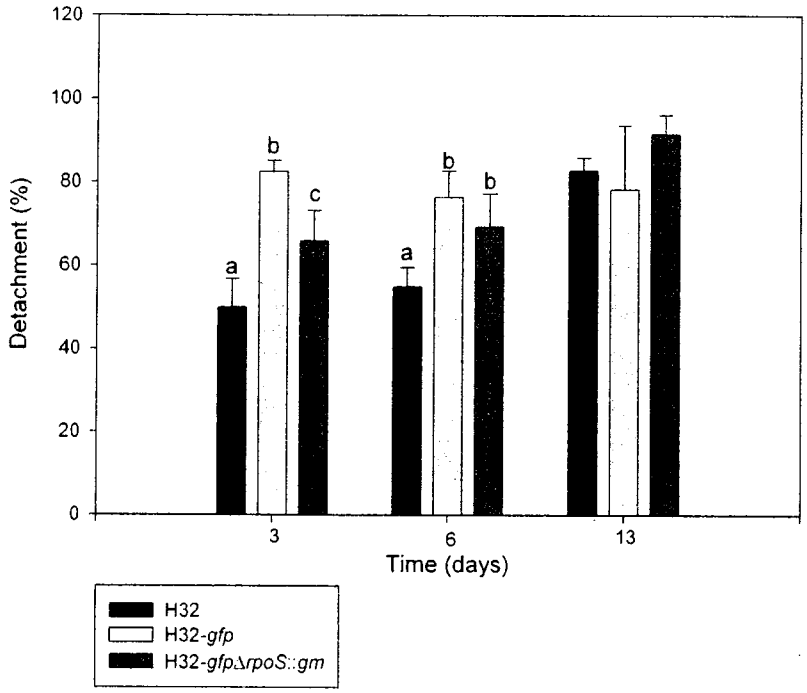

Figure 3.2.8. Percentage detachment of *E. coli* O157:H7 H32, H32-*gfp*, and H32-*gfpΔrpoS::gm* biofilm cells in ddH_2O. A statistically significant difference ($P<0.05$) exists between strains bearing different letters (**a**), (**b**), and (**c**). No statistically significant difference exists between strains bearing the same letter.

3.3 DISCUSSION

Despite the recent paradigm shift towards the acceptance of biofilms over free-living or planktonic cells as the dominant life state of bacteria, the complexity of biofilm development and its strong dependence on both genetic and ecophysiological factors has yet to be fully appreciated. As such, the vast majority of *E. coli* biofilm studies have been conducted on the laboratory strain *E. coli* K-12 in rich media. Concerns with extrapolating the findings of K-12 biofilm studies to pathogenic *E. coli* strains are well-documented , as considerable variation exists not only in the ability of various pathogenic and non-pathogenic *E. coli* strains to form biofilms *in vitro* (Corona-Izquierdo and Membrillo-Hernández 2002; Pratt and Kolter 1998; Reisner *et al.* 2006), but also in the ability of a given strain to form biofilms under different culture conditions (Dewanti and Wong 1995; Oh *et al.* 2007; Reisner *et al.* 2006).

The biofilm-forming capabilities of *E. coli* O157:H7 strains H32, H32-*gfp*, and H32-*gfp*Δ*rpoS*::*gm* were investigated in this study. In corroboration with previous findings, *E. coli* O157:H7 biofilm formation was found to be strongly influenced by the media employed in cultivation (Dewanti and Wong 1995; Oh *et al.* 2007; Reisner *et al.* 2006). All three *E. coli* O157:H7 strains exhibited enhanced biofilm formation under low nutrient conditions, and an inability to form biofilms in rich media, where the relative stimulation of biofilm formation by three different media was TSB < $1/5^{th}$ strength TSB < MSM + 0.04% glucose. The formation of *E. coli* O157:H7 biofilms under nutrient-limitation contrasts biofilm formation in most other Gram-negative bacteria, where low nutrient conditions typically signal a reversion to the planktonic state (O'Toole *et al.* 2000).

Although *rpoS* and *rpoS*-dependent genes have been implicated in the regulation of biofilm formation, a lack of consistency exists between gene expression profiles of *E. coli* K-12 in the planktonic and biofilm state (Beloin *et al.* 2004; Domka *et al.* 2007; Ren *et al.* 2004; Schembri *et al.* 2003). Furthermore, reports of the relative biofilm-forming capabilities of wild-type and *rpoS*-

deficient *E. coli* K-12 strains have been conflicting. When *E. coli* K-12 biofilms were established in flow cell systems continuously fed by rich media, a mutation in *rpoS* inhibited biofilm formation (Adams and McLean 1999; Ito *et al.* 2008; Schembri *et al.* 2003). In contrast, when biofilms were established in rich media but under batch culture conditions, a mutation in *rpoS* enhanced biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002). Although the aforementioned studies employed K-12 instead of *E. coli* O157:H7, the findings of this study suggest that these inconsistent results may be partly attributable to differences in experimental design, specifically nutrient availability.

In this study, a 6 to 7-fold reduction in biofilm biovolume was demonstrated by an *rpoS*-deficient strain of *E. coli* O157:H7, relative to the wild-type strains when grown in 1/5th strength TSB. However, when MSM + 0.04% glucose was employed as the media, the *rpoS*-knockout exhibited a 3.5 to 7-fold increase in biovolume, relative to wild-type strains H32 and H32-*gfp*, respectively. Similarly, while the *rpoS*-knockout exhibited 7-fold less substratum coverage than the wild-type strains in 1/5th strength TSB, its substratum coverage was enhanced 3 to 8-fold relative to the wild-types in MSM + 0.04% glucose. Neither the wild-type nor the *rpoS*-knockout strains were capable of forming biofilms in TSB, however, fewer adhered cells were observed for the *rpoS*$^-$ strain in TSB. Although the differences between strains in 1/5th strength TSB were not found to be significant, these results and the observation that fewer *rpoS*$^-$ cells adhere in TSB than wild-type cells, suggest that while *rpoS* may be advantageous to biofilm formation in rich media, it inhibits biofilm formation under nutrient-limited conditions.

Despite the fact that *rpoS* expression confers starvation-induced cross protection to various stresses (McCann *et al.* 1991), numerous planktonic state studies have reported a paradoxical advantage of *rpoS* mutations under certain nutrient-limited conditions. When incubated for a prolonged period after the establishment of stationary phase growth, *rpoS*-deficient *E. coli* strains have demonstrated increased fitness over wild-type strains (Farrell and Finkel 2003; Finkel and Kolter 1999; Zambrano *et al.* 1993; Zambrano and Kolter 1996; Zinser and Kolter 1999).

Additionally, certain nutrient-limited conditions select for revertible loss of function mutations in *rpoS* (Chen *et al.* 2004), and may help explain the existence of various *rpoS* mutations in *E. coli* strains from various sources (Chen *et al.* 2004; Ferreira *et al.* 1999; Visick and Clarke 1997; Waterman and Small 1996). Although this study represents the only study, to date, to employ different media in elucidating the role of *rpoS* in biofilm formation, the results of this study suggest that under certain conditions, *rpoS* mutations may also enhance survival of *E. coli* O157:H7 in the biofilm state.

Some authors have suggested that the aforementioned survival advantage of free-living *rpoS*$^-$ strains may be due to an increased ability of these strains to scavenge for amino acids and other nutrients released by dead cells (Chen *et al.* 2004; Finkel and Kolter 1999). *E. coli* K-12 *rpoS*-deficient strains have demonstrated a unique ability to employ certain TCA cycle intermediates, such as succinate, as a sole source of carbon (Chen *et al.* 2004). As TSB is considerably more nutrient-rich than MSM + 0.04% glucose, this may help to explain why an *rpoS* mutation in *E. coli* O157:H7 was a hindrance in biofilm formation in TSB and advantage in MSM + 0.04% glucose. These results indicate that the role of *rpoS* in *E. coli* O157:H7 biofilm formation is influenced by nutrient availability. As such, these findings may help explain the seemingly contradictory reports of *rpoS*$^-$ *E. coli* K-12 strains exhibiting enhanced biofilm formation in batch cultures, which by design are nutrient-limited, and inhibited biofilm formation in flow cells where nutrients are continually replenished.

In contrast to the advantage of *rpoS* mutants in biofilms grown in MSM + 0.04% glucose, *rpoS* was shown to have a negligible effect on the survival of biofilm cells subjected to long-term nutrient deprivation and osmotic stress. No significant differences were observed in the survival of wild-type and *rpoS*$^-$ biofilm cells over a period of 13 days in ddH_2O. However, upon dissociation from the established biofilms, fewer *rpoS*$^-$ cells were recovered than their *rpoS*$^+$ counterparts, suggesting decreased survival, detachment, or a combinatorial action of both in the *rpoS*-knockout under nutrient limited, low-osmolarity conditions. Combined counts of both attached and detached

biofilm cells revealed that overall *rpoS* enhances survival under the aforementioned conditions. While no studies have been conducted to date on the relative survival of *E. coli rpoS*$^+$ and *rpoS*$^-$ biofilms under nutrient deprivation and osmotic stress, the advantage of *rpoS* in planktonic cells under conditions of stress, including osmotic challenge, has been well-established for *E. coli* K-12 (Jenkins *et al.* 1988; Jenkins *et al.* 1990; Lange and Hengge-Aronis 1991a; McCann *et al.* 1991; Mulvey and Loewen 1989; Sammartano *et al.* 1986).

Outside of this study, the effects of both nutrient limitation and low osmolarity on the survival of *E. coli* O157:H7 wild-type and *rpoS*$^-$ strains have not been directly investigated, in either the planktonic or biofilm state. RpoS has, however, been shown to be instrumental in conferring resistance to heat, acid, and high osmolarity in planktonic pathogenic *E. coli* (Arnold and Kaspar 1995; Cheville *et al.* 1996; Price *et al.* 2000; Waterman and Small 1996). Additionally, one study on non-pathogenic *E. coli* revealed up-regulation of genes mediated by σ^s upon exposure to artificial seawater, a nutrient-limited, osmotically stressful environment (Rozen *et al.* 2001). The findings presented here are consistent with hypothesis that *rpoS* in *E. coli* O157:H7 helps to confer resistance to various stressors through improved survival, and further implies that *rpoS* assists in the long-term survival of pathogenic *E. coli* under conditions of nutrient deprivation and osmotic stress.

During the investigation of the comparative biofilm forming abilities and survival of wild-type and *rpoS*-deficient strains of *E. coli* O157:H7, significant differences were revealed between H32 and H32-*gfp*. Thinner biofilms were formed by H32-*gfp* in MSM + 0.04% glucose than both H32 and H32-*gfp*Δ*rpoS*::*gm*, survival of H32-*gfp* in ddH_2O was intermediate to that of H32 and H32-*gfp*Δ*rpoS*::*gm*, and H32-*gfp* displayed a different pattern of surviving detached cells relative to total cells in ddH_2O relative to H32 and H32-*gfp*Δ*rpoS*::*gm*. While the differences between H32-*gfp* and H32-*gfp*Δ*rpoS*::*gm* remained statistically significant, thus supporting the finding of this study, it is evident that random insertion of the transposon used to label H32-*gfp* was not without consequence.

The insertion site of Tn5 was earlier identified (Chapter 2) as *uhpC*, a member of the *uhpABC* operon. UhpA, UhpB, and UhpC represent three regulatory proteins responsible for mediating the expression of *uhpT* (Friedrich and Kadner 1987). The three proteins comprise a two-component signal transduction pathway, where UhpC senses glucose-6-phosphate, and in turn mediates the expression of the histidine kinase, UhpB. UhpA is activated by UhpB through phosphorylation and initiates transcription of *uhpT*. Together, *uhpABC* and *uphT* help accumulate sugar phosphates within the cell (Friedrich and Kadner 1987; Island and Kadner 1993; Verhamme *et al.* 2002; Weston and Kadner 1988). Although no survival studies have been conducted on *uhpABC* mutants, it is not unreasonable to surmise that the altered uptake of sugar phosphates, molecules used in energy transport and storage, may influence survival under nutrient-limited conditions. The differences between H32 and H32-*gfp* implicate *uhpABC* in biofilm development through inhibiting biofilm thickness, and decreasing survival of *E. coli* cells under low nutrient conditions.

The results of this study suggest that *rpoS* influences biofilm formation of *E. coli* O157:H7 in a nutrient-dependent fashion. Under the nutrient poor conditions of MSM + 0.04 % glucose, the *rpoS*⁻ mutation enhanced biofilm formation, whereas under the more nutritive conditions of $1/5^{th}$ TSB and TSB, it appeared to have a negligible or inhibitory effect, although the influence of media components cannot be excluded. Overall, *E. coli* O157:H7 exhibited a propensity to form biofilms under low nutrient conditions, where the mutation in *rpoS* exaggerated this effect. While *rpoS* had no effect on the survival of *E. coli* O157:H7 attached biofilm cells under nutrient-deprived, low-osmolarity conditions, it enhanced detachment or survival of detached cells (or both), suggesting that the influence of *rpoS* is also dependent on life state. Overall, *rpoS* enhanced the total survival of *E. coli* O157:H7 in ddH_2O. Lastly, this study highlighted the issues in using Tn5 transposons in the labeling of bacteria, given the insertion of Tn5 in the *uphABC* operon of H32-*gfp*.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The results of this thesis reveal that while Lambda Red-mediated recombination does not represent an ideal system for the generation of targeted gene disruptions in *E. coli* O157:H7, the production of an *rpoS*-knockout (H32-*gfpΔrpos::gm*) was instrumental in helping to elucidate the role of *rpoS* in *E. coli* O157:H7 biofilm formation and survival. The strain H32-*gfpΔrpos::gm* also provides an important resource for further study of *rpoS* function. Recommendations for future study involving this mutant include investigating the role of *rpoS* in pathogenic *E. coli* biofilm formation and survival under natural conditions of nutrient-limitation and stress, such as in natural bodies of water and water distribution systems. Additionally, identification of a competitive advantage of the *rpoS*-knockout under these conditions could be investigated by performing competition studies between the knockout and wild-type *E. coli* O157:H7 strains or preexisting microbial flora.

Fundamental findings of this study suggest that the influence of *rpoS* on *E. coli* O157:H7 biofilm formation is media-dependent, with an *rpoS* mutation inhibiting biofilm formation in TSB and 1/5th TSB, and enhancing biofilm formation in MSM + 0.04% glucose. The differential impact of *rpoS* under conditions of differing nutrient availability has strong implications for the inconsistencies in the literature, suggesting that these discrepancies may be due to experimental design. Evaluating the biofilm formation of H32-*gfpΔrpos::gm* over a nutrient gradient could help isolate whether these differences are truly due to nutrient availability, or rather due to media composition.

While neither the enhanced biofilm formation of the *rpoS*-knockout or the cross-protection of *rpoS* in the wild-type appeared to enhanced survival of *E. coli* O157:H7 biofilms under conditions of nutrient-deprivation and osmotic stress, *rpoS* did enhance the survival of both detached biofilm cells and total biofilm cells (cumulative attached and detached). This result is consistent with the role of *rpoS* in conferring cross-protection to various stressors. The intermediate survival of H32-

gfp relative to H32 under these conditions implicates *uhpC* in *E. coli* O157:H7 survival, a notion which could be further investigated through the production of a targeted disruption in *uhpC* and assessing its survival relative to H32, H32-*gfp* and H32-*gfpΔrpos::gm*. The role *uhpC* in *E. coli* O157:H7 biofilm formation and survival has not yet been addressed in the literature, and represents an additional area of novel research.

APPENDIX

4.0 MULTIPLE SEQUENCE ALIGNMENT OF *<GFP-KAN>* INSERTION SITE

Legend:
Blue = putative regulatory protein (incomplete sequence)
putative sensory kinase (incomplete sequence)
Underlined = start (ATG) or stop (TAA) codon on minus strand
Bold = regions of homology between genomic sequencing and type-strains
Black = intergenic region

Strains:
EDL933Forward = *E. coli* O157:H7 str. EDL933, Accession number: AE005174.2
SakaiForward = *E. coli* O157:H7 str. Sakai, Accession number: BA000007.2
EC4115Forward = *E. coli* O157:H7 str. Sakai, Accession number: CP001164.1
GenomicForward = H32 genomic sequencing from SqFP
GenomicReverse = H32 genomic sequencing from SqRP

```
EDL933Forward   CATCAGTAACGGCATCAGCAGTAAACCCATAAGTACTGCG     40
SakaiForward    CATCAGTAACGGCATCAGCAGTAAACCCATAAGTACTGCG     40
EC4115Forward   CATCAGTAACGGCATCAGCAGTAAACCCATAAGTACTGCG     40
GenomicForward  ........................................      0
Consensus

EDL933Forward   GCGACCGAGAGTAAACTGAACATTCCCGGCCAGCCGTAGC     80
SakaiForward    GCGACCGAGAGTAAACTGAACATTCCCGGCCAGCCGTAGC     80
EC4115Forward   GCGACCGAGAGTAAACTGAACATTCCCGGCCAGCCGTAGC     80
GenomicForward  ........................................      0
Consensus

EDL933Forward   GTTCGATGACCAGCGACAGCGGCCAGCCTGCCAGCGCCGC    120
SakaiForward    GTTCGATGACCAGCGACAGCGGCCAGCCTGCCAGCGCCGC    120
EC4115Forward   GTTCGATGACCAGCGACAGCGGCCAGCCTGCCAGCGCCGC    120
GenomicForward  ........................................      0
Consensus

EDL933Forward   GCCAAGGTAAGCGAACAGGCCGAGAAATCCGGTGATTGAA    160
SakaiForward    GCCAAGGTAAGCGAACAGGCCGAGAAATCCGGTGATTGAA    160
EC4115Forward   GCCAAGGTAAGCGAACAGGCCGAGAAATCCGGTGATTGAA    160
GenomicForward  ........................................      0
Consensus

EDL933Forward   CCCGCCGCAGCTTTGTGCCCACACTCCACCGCCGCAAGTC    200
SakaiForward    CCCGCCGCAGCTTTGTGCCCACACTCCACCGCCGCAAGTC    200
EC4115Forward   CCCGCCGCAGCTTTGTGCCCACACTCCACCGCCGCAAGTC    200
GenomicForward  ........................................      0
Consensus

EDL933Forward   CAATCAGCATTTGTGGGCCAAAGACGAAAAAGCCCACGGT    240
SakaiForward    CAATCAGCATTTGTGGGCCAAAGACGAAAAAGCCCACGGT    240
EC4115Forward   CAATCAGCATTTGTGGGCCAAAGACGAAAAAGCCCACGGT    240
GenomicForward  ........................................      0
Consensus

EDL933Forward   AAAAAAGCACACTGCCAGCAGAGCGTAGTGATGAACCGGT    280
SakaiForward    AAAAAAGCACACTGCCAGCAGAGCGTAGTGATGAACCGGT    280
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EC4115Forward  AAAAAAGCACACTGCCAGCAGAGCGTAGTGATGAACCGGT    280
GenomicForward ..................................... 0
Consensus

EDL933Forward  GCCAGCCACAGTGCGGCGACTGACACCATCAACCCCAACG    320
SakaiForward   GCCAGCCACAGTGCGGCGACTGACACCATCAACCCCAACG    320
EC4115Forward  GCCAGCCACAGTGCGGCGACTGACACCATCAACCCCAACG    320
GenomicForward ..................................... 0
Consensus

EDL933Forward  TGAACAGCAAAATCATTGGCGCTCGTTGCCCGCTAAACAG    360
SakaiForward   TGAACAGCAAAATCATTGGCGCTCGTTGCCCGCTAAACAG    360
EC4115Forward  TGAACAGCAAAATCATTGGCGCTCGTTGCCCGCTAAACAG    360
GenomicForward ..................................... 0
Consensus

EDL933Forward  CAAATCCGATCCCCATCCGGCAAATAACGCGCCGAGTAAG    400
SakaiForward   CAAATCCGATCCCCATCCGGCAAATAACGCGCCGAGTAAG    400
EC4115Forward  CAAATCCGATCCCCATCCGGCAAATAACGCGCCGAGTAAG    400
GenomicForward ..................................... 0
Consensus

EDL933Forward  CCGCCCACTTCAAACAGCATTACCGTGGCGTTGGCGCTGA    440
SakaiForward   CCGCCCACTTCAAACAGCATTACCGTGGCGTTGGCGCTGA    440
EC4115Forward  CCGCCCACTTCAAACAGCATTACCGTGGCGTTGGCGCTGA    440
GenomicForward ..................................... 0
Consensus

EDL933Forward  GCAGGTTGACACCGTGGCTTTCCGTCAGCCAGATATTGCC    480
SakaiForward   GCAGGTTGACACCGTGGCTTTCCGTCAGCCAGATATTGCC    480
EC4115Forward  GCAGGTTGACACCGTGGCTTTCCGTCAGCCAGATATTGCC    480
GenomicForward .....................................
GenomicReverse .........................CAGCAGGTTGNT      0
Consensus

EDL933Forward  CCAGTCGTTAAGCGCAATCCGGATCACATAAACCAGTACA    520
SakaiForward   CCAGTCGTTAAGCGCAATCCGGATCACATAAACCAGTACA    520
EC4115Forward  CCAGTCGTTAAGCGCAATCCGGATCACATAAACCAGTACA    520
GenomicForward ..................................... 0
GenomicReverse NCAGTTGGTAATCGCAATCCGGATCNGATAACNCAGGTTT
Consensus

EDL933Forward  TATGAGACGCCCAGTAGCCAGATCAGCGGATTTTGCAGCA    560
SakaiForward   TATGAGACGCCCAGTAGCCAGATCAGCGGATTTTGCAGCA    560
EC4115Forward  TATGAGACGCCCAGTAGCCAGATCAGCGGATTTTGCAGCA    560
GenomicForward ..................................... 0
GenomicReverse TATTATAAGCACCGTAGCCAGA.................
Consensus

EDL933Forward  TGGTGGTGCGCAACATTTGCCACAGCCCCATCGGCGGACT    600
SakaiForward   TGGTGGTGCGCAACATTTGCCACAGCCCCATCGGCGGACT    600
EC4115Forward  TGGTGGTGCGCAACATTTGCCACAGCCCCATCGGCGGACT    600
GenomicForward ..................................... 0
Consensus

EDL933Forward  TTGTTGTTCCTGACGTAACTCCAGCGGATCGTGACGCCAG    640
SakaiForward   TTGTTGTTCCTGACGTAACTCCAGCGGATCGTGACGCCAG    640
EC4115Forward  TTGTTGTTCCTGACGTAACTCCAGCGGATCGTGACGCCAG    640
GenomicForward ..................................... 0
```

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Consensus

EDL933Forward   TGACCAACTGTCGGTAATCCCTCTTCCTGGGGCGTGCCTT    680
SakaiForward    TGACCAACTGTCGGTAATCCCTCTTCCTGGGGCGTGCCTT    680
EC4115Forward   TGACCAACTGTCGGTAATCCCTCTTCCTGGGGCGTGCCTT    680
GenomicForward  .....................................       0
Consensus

EDL933Forward   TGAGTTGTAGCGTTAACCAGATCCCTAATGCCATGCTGAT    720
SakaiForward    TGAGTTGTAGCGTTAACCAGATCCCTAATGCCATGCTGAT    720
EC4115Forward   TGAGTTGTAGCGTTAACCAGATCCCTAATGCCATGCTGAT    720
GenomicForward  .....................................       0
Consensus

EDL933Forward   TATCCCCGGTGTCAGCATCGCGGCCTGCCAGCCCCACCAG    760
SakaiForward    TATCCCCGGTGTCAGCATCGCGGCCTGCCAGCCCCACCAG    760
EC4115Forward   TATCCCCGGTGTCAGCATCGCGGCCTGCCAGCCCCACCAG    760
GenomicForward  .....................................       0
Consensus

EDL933Forward   TGGGCGGCAAAGGCGCTAATCAGCGGAATAATCGCTCCGC    800
SakaiForward    TGGGCGGCAAAGGCGCTAATCAGCGGAATAATCGCTCCGC    800
EC4115Forward   TGGGCGGCAAAGGCGCTAATCAGCGGAATAATCGCTCCGC    800
GenomicForward  .....................................       0
Consensus

EDL933Forward   CAATGTTGATCGACATATTCCAGCAGCCCCACCAGAAACC    840
SakaiForward    CAATGTTGATCGACATATTCCAGCAGCCCCACCAGAAACC    840
EC4115Forward   CAATGTTGATCGACATATTCCAGCAGCCCCACCAGAAACC    840
GenomicForward  .....................................       0
Consensus

EDL933Forward   GCGCTCATTGCGTGAGTACCAGTGGGTCAGCAAGCGGGCA    880
SakaiForward    GCGCTCATTGCGTGAGTACCAGTGGGTCAGCAAGCGGGCA    880
EC4115Forward   GCGCTCATTGCGTGAGTACCAGTGGGTCAGCAAGCGGGCA    880
GenomicForward  .....................................       0
Consensus

EDL933Forward   CACGGTGGCCATCCCCAGCCCTGAAAAAAACCGTTCAGTG    920
SakaiForward    CACGGTGGCCATCCCCAGCCCTGAAAAAAACCGTTCAGTG    920
EC4115Forward   CACGGTGGCCATCCCCAGCCCTGAAAAAAACCGTTCAGTG    920
GenomicForward  .....................................       0
Consensus

EDL933Forward   TCCAGACCACAAGCAGAAGCGTAAGTGATTCGCCAAAGGC    960
SakaiForward    TCCAGACCACAAGCAGAAGCGTAAGTGATTCGCCAAAGGC    960
EC4115Forward   TCCAGACCACAAGCAGAAGCGTAAGTGATTCGCCAAAGGC    960
GenomicForward  .....................................       0
Consensus

EDL933Forward   AAACACCACGTTCAATAACCCGGTGGCGAACAAGCCGACG   1000
SakaiForward    AAACACCACGTTCAATAACCCGGTGGCGAACAAGCCGACG   1000
EC4115Forward   AAACACCACGTTCAATAACCCGGTGGCGAACAAGCCGACG   1000
GenomicForward  .....................................       0
Consensus

EDL933Forward   CCCATAAAACCGCGTTGTCCGTGGCTGTCATGCCACAGTC   1040
SakaiForward    CCCATAAAACCGCGTTGTCCGTGGCTGTCATGCCACAGTC   1040
EC4115Forward   CCCATAAAACCGCGTTGTCCGTGGCTGTCATGCCACAGTC   1040
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GenomicForward CCCATAAAACCGCGTTGTCCGTGGCTGTCATGCCACAGTC     40
Consensus      cccataaaaccgcgttgtccgtggctgtcatgccacagtc

EDL933Forward  CGGCGGTAAATTTCGACAATCCGTAGCTGAGATAAAACAG   1080
SakaiForward   CGGCGGTAAATTTCGACAATCCGTAGCTGAGATAAAACAG   1080
EC4115Forward  CGGCGGTAAATTTCGACAATCCGTAGCTGAGATAAAACAG   1080
GenomicForward CGGCGGTAAATTTCGACAATCCGTAGCTGAGATAAAACAG     80
Consensus      cggcggtaaatttcgacaatccgtagctgagataaaacag

EDL933Forward  CGAACCCAGCAGGCCAATGTCGCCTTTATCCAGTCCGAGG   1120
SakaiForward   CGAACCCAGCAGGCCAATGTCGCCTTTATCCAGTCCGAGG   1120
EC4115Forward  CGAACCCAGCAGGCCAATGTCGCCTTTATCCAGTCCGAGG   1120
GenomicForward CGAACCCAGCAGGCCAATGTCGCCTTTATCCAGTCCGAGG    120
Consensus      cgaacccagcaggccaatgtcgcctttatccagtccgagg

EDL933Forward  TCGGTTTGTAGCGCCGGAAGGACGTAATTCACGCTTTTTC   1160
SakaiForward   TCGGTTTGTAGCGCCGGAAGGACGTAATTCACGCTTTTTC   1160
EC4115Forward  TCGGTTTGTAGCGCCGGAAGGACGTAATTCACGCTTTTTC   1160
GenomicForward TCGGTTTGTAGCGCCGGAAGGACGTAATTCACGCTTTTTC    160
Consensus      tcggtttgtagcgccggaaggacgtaattcacgcttttc

EDL933Forward  GCGTCAGATAAAATGCGGCGTAGCCAATAATCATGCACAT   1200
SakaiForward   GCGTCAGATAAAATGCGGCGTAGCCAATAATCATGCACAT   1200
EC4115Forward  GCGTCAGATAAAATGCGGCGTAGCCAATAATCATGCACAT   1200
GenomicForward GCGTCAGATAAAATGCGGCGTAGCCAATAATCATGCACAT    200
Consensus      gcgtcagataaaatgcggcgtagccaataatcatgcacat

EDL933Forward  CAGCAATCGCGGACGTAATGTCCGGTAGCATTGATTAATT   1240
SakaiForward   CAGCAATCGCGGACGTAATGTCCGGTAGCATTGATTAATT   1240
EC4115Forward  CAGCAATCGCGGACGTAATGTCCGGTAGCATTGATTAATT   1240
GenomicForward CAGCAATCGCGGACGTAATGTCCGGTAGCATTGATTAATT    240
Consensus      cagcaatcgcggacgtaatgtccggtagcattgattaatt

EDL933Forward  TCTCTCGCTGAACGCGCGTGCATGGTTGTCTCCATGTTGC   1280
SakaiForward   TCTCTCGCTGAACGCGCGTGCATGGTTGTCTCCATGTTGC   1280
EC4115Forward  TCTCTCGCTGAACGCGCGTGCATGGTTGTCTCCATGTTGC   1280
GenomicForward TCTCTCGCTGAACGCGCGTGCATGGTTGTCTCCATGTTGC    280
Consensus      tctctcgctgaacgcgcgtgcatggttgtctccatgttgc

EDL933Forward  TGACTATATGAAAATGAAAGTGAAAGGGGATGAGAGAAAA   1320
SakaiForward   TGACTATATGAAAATGAAAGTGAAAGGGGATGAGAGAAAA   1320
EC4115Forward  TGACTATATGAAAATGAAAGTGAAAGGGGATGAGAGAAAA   1320
GenomicForward TGACTATATGAAAATGAAAGTGAAAGGGGATGAGAGAAAA    320
Consensus      tgactatatgaaaatgaaagtgaaaggggatgagagaaaa

EDL933Forward  TCCGGGATACCTAAGACTTTTTCCTGGTTATCCGGCGATT   1360
SakaiForward   TCCGGGATACCTAAGACTTTTTCCTGGTTATCCGGCGATT   1360
EC4115Forward  TCCGGGATACCTAAGACTTTTTCCTGGTTATCCGGCGATT   1360
GenomicForward TCCGGGnTACCTAAGACTTTTTCCTGGTTATCCGGCGATT    360
Consensus      tccggg tacctaagacttttttcctggttatccggcgatt

EDL933Forward  TGTTGCAAAAATGTGGGCAAGTTAACAATTACCCGCGTGC   1400
SakaiForward   TGTTGCAAAAATGTGGGCAAGTTAACAATTACCCGCGTGC   1400
EC4115Forward  TGTTGCAAAAATGTGGGCAAGTTAACAATTACCCGCGTGC   1400
GenomicForward TGTTGCAAAAATGTGGGCAAGTTAACAATTACCCGCGTGC    400
Consensus      tgttgcaaaaatgtgggcaagttaacaattacccgcgtgc

EDL933Forward  CGTGCTGTTTTTCGACGGTTAAGTCGCCGCCCAGGGCGCT   1440
SakaiForward   CGTGCTGTTTTTCGAGCGTTAAGTCGCCGCCCAGGGCGCT   1440
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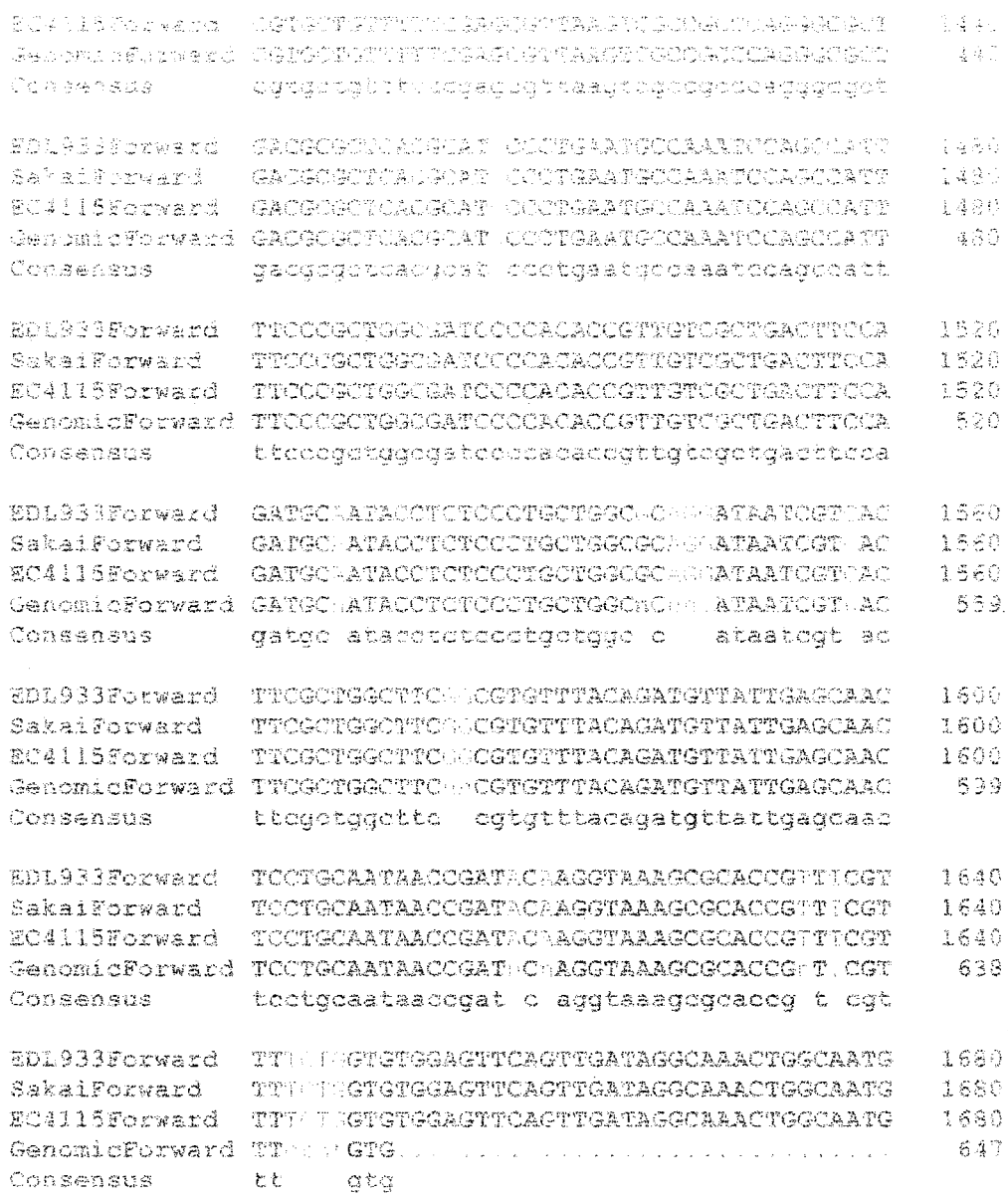

4.1 MULTIPLE SEQUENCE ALIGNMENT OF *RPOS*

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H32_rpoS    ATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGA     40
NC_000913   ...............................ATGA      4
NC_009801   ...............................ATGA      4
NC_009800   ...............................ATGA      4
NC_008563   ATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGA     40
NC_007946   ATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTATGA     40
Consensus                                       atga

H32_rpoS    GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     80
NC_000913   GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     44
NC_009801   GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     44
NC_009800   GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     44
NC_008563   GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     80
NC_007946   GTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGC     80
Consensus   gtcagaatacgctgaaagttcatgatttaaatgaagatgc

H32_rpoS    GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG    120
NC_000913   GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG     84
NC_009801   GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG     84
NC_009800   GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG     84
NC_008563   GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG    120
NC_007946   GGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAG    120
Consensus   ggaatttgatgagaacggagttgaggtttttgacgaaaag

H32_rpoS    GCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCG    160
NC_000913   GCCTTAGTAGAAcAGGAACCCAGTGATAACGATTTGGCCG    124
NC_009801   GCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCG    124
NC_009800   GCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCG    124
NC_008563   GCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCG    160
NC_007946   GCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCG    160
Consensus   gccttagtagaa aggaacccagtgataacgatttggccg

H32_rpoS    AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGTT    200
NC_000913   AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGTT    164
NC_009801   AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGTT    164
NC_009800   AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGTT    164
NC_008563   AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGcT    200
NC_007946   AAGAGGAACTGTTATCGCAGGGAGCCACACAGCGTGTGcT    200
Consensus   aagaggaactgttatcgcagggagccacacagcgtgtg t

H32_rpoS    GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    240
NC_000913   GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    204
NC_009801   GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    204
NC_009800   GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    204
NC_008563   GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    240
NC_007946   GGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCA    240
Consensus   ggacgcgactcagctttaccttggtgagattggttattca


H32_rpoS    CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    280
NC_000913   CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    244
NC_009801   CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    244
NC_009800   CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    244
NC_008563   CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    280
NC_007946   CCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTC    280
Consensus   ccactgttaacggccgaagaagaagtttattttgcgcgtc
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H32_rpoS    GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    320
NC_000913   GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    284
NC_009801   GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    284
NC_009800   GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    284
NC_008563   GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    320
NC_007946   GCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGAT    320
Consensus   gcgcactgcgtggagatgtcgcctctcgccgccggatgat

H32_rpoS    CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    360
NC_000913   CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    324
NC_009801   CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    324
NC_009800   CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    324
NC_008563   CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    360
NC_007946   CGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGT    360
Consensus   cgagagtaacttgcgtctggtggtaaaaattgcccgccgt

H32_rpoS    TATGGCAATCGTGGTCTGGCGTTGCTGGACCTTATCGAAG    400
NC_000913   TATGGCAATCGTGGTCTGGCGTTGCTGGACCTTATCGAAG    364
NC_009801   TATGGCAATCGTGGTCTGGCGTTGCTGGACCTTATCGAAG    364
NC_009800   TATGGCAATCGTGGTCTGGCGTTGCTGGACCTTATCGAAG    364
NC_008563   TATGGCAATCGTGGTCTGGCGTTGCTGGACCTgATCGAAG    400
NC_007946   TATGGCAATCGTGGTCTGGCGTTGCTGGACCTgATCGAAG    400
Consensus   tatggcaatcgtggtctggcgttgctggacct atcgaag

H32_rpoS    AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    440
NC_000913   AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    404
NC_009801   AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    404
NC_009800   AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    404
NC_008563   AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    440
NC_007946   AGGGCAACCTGGGGCTGATCCGCGCGGTAGAGAAGTTTGA    440
Consensus   agggcaacctggggctgatccgcgcggtagagaagtttga

H32_rpoS    CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    480
NC_000913   CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    444
NC_009801   CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    444
NC_009800   CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    444
NC_008563   CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    480
NC_007946   CCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGG    480
Consensus   cccggaacgtggtttccgcttctcaacatacgcaacctgg

H32_rpoS    TGGATTCGCCAGACGATTGAACGGGCGATTATGAACCAAA    520
NC_000913   TGGATTCGCCAGACGATTGAACGGGCGATTATGAACCAAA    484
NC_009801   TGGATTCGCCAGACGATTGAACGGGCGATTATGAACCAAA    484
NC_009800   TGGATTCGCCAGACGATTGAACGGGCGATTATGAACCAAA    484
NC_008563   TGGATTCGCCAGACGATcGAACGGGCGATTATGAACCAAA    520
NC_007946   TGGATTCGCCAGACGATcGAACGGGCGATTATGAACCAAA    520
Consensus   tggattcgccagacgat gaacgggcgattatgaaccaaa


H32_rpoS    CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    560
NC_000913   CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    524
NC_009801   CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    524
NC_009800   CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    524
NC_008563   CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    560
NC_007946   CCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCT    560
Consensus   cccgtactattcgtttgccgattcacatcgtaaaggagct

H32_rpoS    GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG    600
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NC_000913  GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG   564
NC_009801  GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG   564
NC_009800  GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG   564
NC_008563  GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG   600
NC_007946  GAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAG   600
Consensus  gaacgtttacctgcgaaccgcacgtgagttgtcccataag

H32_rpoS   CTGGACCATGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   640
NC_000913  CTGGACCATGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   604
NC_009801  CTGGACCATGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   604
NC_009800  CTGGACCATGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   604
NC_008563  CTGGACCAcGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   640
NC_007946  CTGGACCAcGAACCAAGTGCGGAAGAGATCGCAGAGCAAC   640
Consensus  ctggacca gaaccaagtgcggaagagatcgcagagcaac

H32_rpoS   TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   680
NC_000913  TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   644
NC_009801  TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   644
NC_009800  TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   644
NC_008563  TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   680
NC_007946  TGGATAAGCCAGTTGATGACGTCAGCCGTATGCTTCGTCT   680
Consensus  tggataagccagttgatgacgtcagccgtatgcttcgtct

H32_rpoS   TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   720
NC_000913  TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   684
NC_009801  TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   684
NC_009800  TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   684
NC_008563  TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   720
NC_007946  TAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGT   720
Consensus  taacgagcgcattacctcggtagacaccccgctgggtggt

H32_rpoS   GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   760
NC_000913  GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   724
NC_009801  GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   724
NC_009800  GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   724
NC_008563  GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   760
NC_007946  GATTCCGAAAAAGCGTTGCTGGACATCCTGGCCGATGAAA   760
Consensus  gattccgaaaaagcgttgctggacatcctggccgatgaaa

H32_rpoS   AAGAGAACGGTCCGGAAGATACCACGCAAGATGACGATAT   800
NC_000913  AAGAGAACGGTCCGGAAGATACCACGCAAGATGACGATAT   764
NC_009801  AAGAGAACGGTCCGGAAGATACCACGCAAGATGACGATAT   764
NC_009800  AAGAGAACGGTCCGGAAGATACCACGCAAGATGACGATAT   764
NC_008563  AAGAGAAtGGTCCGGAAGATACCACGCAAGATGACGATAT   800
NC_007946  AAGAGAAtGGTCCGGAAGATACCACGCAAGATGACGATAT   800
Consensus  aagagaa ggtccggaagataccacgcaagatgacgatat


H32_rpoS   GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   840
NC_000913  GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   804
NC_009801  GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   804
NC_009800  GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   804
NC_008563  GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   840
NC_007946  GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCC   840
Consensus  gaagcagagcatcgtcaaatggctgttcgagctgaacgcc

H32_rpoS   AAACAGCGTGAAGTGCTGGCACGTCGATTCGGTTTGCTGG   880
NC_000913  AAACAGCGTGAAGTGCTGGCACGTCGATTCGGTTTGCTGG   844
NC_009801  AAACAGCGTGAAGTGCTGGCACGTCGATTCGGTTTGCTGG   844
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NC_009800   AAACAGCGTGAAGTGCTGGCACGTCGATTCGGTTTGCTGG    844
NC_008563   AAACAGCGTGAAGTaCTGGCACGTCGATTCGGTTTGCTGG    880
NC_007946   AAACAGCGTGAAGTaCTGGCACGTCGATTCGGTTTGCTGG    880
Consensus   aaacagcgtgaagt ctggcacgtcgattcggtttgctgg

H32_rpoS    GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    920
NC_000913   GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    884
NC_009801   GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    884
NC_009800   GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    884
NC_008563   GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    920
NC_007946   GGTACGAAGCGGCAACACTGGAAGATGTAGGTCGTGAAAT    920
Consensus   ggtacgaagcggcaacactggaagatgtaggtcgtgaaat

H32_rpoS    TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    960
NC_000913   TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    924
NC_009801   TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    924
NC_009800   TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    924
NC_008563   TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    960
NC_007946   TGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAA    960
Consensus   tggcctcacccgtgaacgtgttcgccagattcaggttgaa

H32_rpoS    GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGC   1000
NC_000913   GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGC    964
NC_009801   GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGC    964
NC_009800   GGCCTGCGCCGTTTGCGtGAAATCCTGCAAACGCAGGGGC    964
NC_008563   GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGC   1000
NC_007946   GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGC   1000
Consensus   ggcctgcgccgtttgcg gaaatcctgcaaacgcaggggc

H32_rpoS    TGAATATCGAAGCGCTGTTCCGCGAGTAAGTAAGCATCTG   1040
NC_000913   TGAATATCGAAGCGCTGTTCCGCGAGTAA.............    993
NC_009801   TGAATATCGAAGCGCTGTTCCGCGAGTAA.............    993
NC_009800   TGAATATCGAAGCGCTGTTCCGCGAGTAA.............    993
NC_008563   TGAATATCGAAGCGCTGTTCCGCGAaTAA.............   1029
NC_007946   TGAATATCGAAGCGCTGTTCCGCGAaTAA.............   1029
Consensus   tgaatatcgaagcgctgttccgcga taa

H32_rpoS    TCAGAAAGGCCAGTCTCAAGCGAGGCTGGCCTTTT        1075
NC_000913   ...................................         993
NC_009801   ...................................         993
NC_009800   ...................................         993
NC_008563   ...................................        1029
NC_007946   ...................................        1029
Consensus
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REFERENCES

[Anonymous]. 2005. Notifiable Diseases On-Line [online].

Ackman, D., Marks, S., Mack, P., Caldwell, M., Root, T., and Birkhead, G. 1997. Swimming-associated haemorrhagic colitis due to *Escherichia coli* O157:H7 infection: evidence of prolonged contamination of a fresh water lake. Epidemiol. Infect. 119(1): 1-8.

Adams, J.L., and McLean, R.J.C. 1999. Impact of *rpoS* deletion on *Escherichia coli* biofilms. Appl. Environ. Microbiol. 65(9): 4285-4287.

Allison, D.G., and Sutherland, I.W. 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. J. Gen. Microbiol. 133(5): 1319-1327.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25(17): 3389-3402.

Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjorn, S.P., Givskov, M., and Molin, S. 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. 64(6): 2240-2246.

Arnold, K.W., and Kaspar, C.W. 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 61(5): 2037-2039.

Arnqvist, A., Olsen, A., Pfeifer, J., Russell, D.G., and Normark, S. 1992. The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli* HB101. Mol. Microbiol. 6(17): 2443-2452.

Banning, N., Toze, S., and Mee, B.J. 2003. Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system. Microbiology 149(1): 47-55.

Barbour, S.D., Nagaishi, H., Templin, A., and Clark, A.J. 1970. Biochemical and genetic studies of recombination proficiency in *Escherichia coli,* II. Rec+ revertants caused by indirect suppression of Rec- mutations. Proc. Natl. Acad. Sci. U. S. A. 67(1): 128-135.

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae.* Nucleic Acids Res. 21(14): 3329-3330.

Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J.A.J., Molin, S., Prensier, G., Arbeille, B., and Ghigo, J. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. 51(3): 659-674.

Benzinger, R., Enquist, L.W., and Skalka, A. 1975. Transfection of *Escherichia coli* spheroplasts. V. Activity of recBC nuclease in rec+ and rec minus spheroplasts measured with different forms of bacteriophage DNA. J. Virol. 15(4): 861-871.

Besser, R.E., Griffin, P.M., and Slutsker, L. 1999. *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. Annu. Rev. Med. 50: 355-367.

Bjergbæk, L.A., Haagensen, J.A.J., Reisner, A., Molin, S., and Roslev, P. 2006. Effect of oxygen and growth medium on *in vitro* biofilm formation by *Escherichia coli*. Biofilms 3(01): 1-10.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Shao, Y. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277(5331): 1453-1474.

Brcic-Kostic, K., Stojiljkovic, I., Salaj-Smic, E., and Trgovcevic, Z. 1989. The *recB* gene product is essential for exonuclease V-dependent DNA degradation *in vivo*. Mutat. Res. 227(4): 247-250.

Camper, A.K., McFeters, G.A., Characklis, W.G., and Jones, W.L. 1991. Growth kinetics of coliform bacteria under conditions relevant to drinking water distribution systems. Appl. Environ. Microbiol. 57(8): 2233-2239.

Capaldo-Kimball, F., and Barbour, S.D. 1971. Involvement of recombination genes in growth and viability of *Escherichia coli* K-12. J. Bacteriol. 106(1): 204-212.

Carter, D.M., and Radding, C.M. 1971. The role of exonuclease and beta protein of phage lambda in genetic recombination. II. Substrate specificity and the mode of action of lambda exonuclease. J. Biol. Chem. 246(8): 2502-2510.

Carter, A.O., Borczyk, A.A., Carlson, J.A., Harvey, B., Hockin, J.C., Karmali, M.A., Krishnan, C., Korn, D.A., and Lior, H. 1987. A severe outbreak of *Escherichia coli* O157:H7--associated hemorrhagic colitis in a nursing home. N. Engl. J. Med. 317(24): 1496-1500.

Chen, G., and Schellhorn, H.E. 2003. Controlled induction of the RpoS regulon in *Escherichia coli*, using an RpoS-expressing plasmid. Can. J. Microbiol. 49(12): 733-740.

Chen, G., Patten, C.L., and Schellhorn, H.E. 2004. Positive selection for loss of RpoS function in *Escherichia coli*. Mutat. Res. 554(1-2): 193-203.

Cheville, A.M., Arnold, K.W., Buchrieser, C., Cheng, C.M., and Kaspar, C.W. 1996. *rpoS* regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 62(5): 1822-1824.

Chilcott, G.S., and Hughes, K.T. 2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar typhimurium and *Escherichia coli*. Microbiol. Mol. Biol. Rev. 64(4): 694-708.

Colon-Gonzalez, M., Mendez-Ortiz, M.M., and Membrillo-Hernández, J. 2004. Anaerobic growth does not support biofilm formation in *Escherichia coli* K-12. Res. Microbiol. 155(7): 514-521.

Corona-Izquierdo, F.P., and Membrillo-Hernández, J. 2002. A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. FEMS Microbiol. Lett. 211(1): 105-110.

Costerton, J.W., and Irvin, R.T. 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. 35: 299-324.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284(5418): 1318-1322.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. Annu. Rev. Microbiol. 49: 711-745.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41: 435-464.

Court, D.L., Sawitzke, J.A., and Thomason, L.C. 2002. Genetic engineering using homologous recombination. Annu. Rev. Genet. 36: 361-388.

Cronan, J.E. 1968. Phospholipid alterations during growth of *Escherichia coli*. J. Bacteriol. 95(6): 2054-2061.

Dabert, P., and Smith, G.R. 1997. Gene replacement with linear DNA fragments in wild-type *Escherichia coli*: enhancement by Chi sites. Genetics 145(4): 877-889.

Danese, P.N., Pratt, L.A., and Kolter, R. 2000a. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. 182(12): 3593-3596.

Danese, P.N., Pratt, L.A., Dove, S.L., and Kolter, R. 2000b. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol. Microbiol. 37(2): 424-432.

Datsenko, K.A., and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. PNAS. 97(12): 6640-6645.

Davey, M.E., and O'Toole, G.A. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64(4): 847-867.

De Beer, D., Srinivasan, R., and Stewart, P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. 60(12): 4339-4344.

Dennis, J.J., and Zylstra, G.J. 1998. Plasposons: Modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl. Environ. Microbiol. 64(7): 2710-2715.

DeSousa, L. 2008. Personal communication.

Dewanti, R., and Wong, A.C.L. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. Int. J. Food Microbiol. 26(2): 147-164.

Ding, Q., Kusano, S., Villarejo, M., and Ishihama, A. 1995. Promoter selectivity control of *Escherichia coli* RNA polymerase by ionic strength: differential recognition of osmoregulated promoters by E σ^D and Eσ^S holoenzymes. Mol. Microbiol. 16(4): 649-656.

Dixon, D.A., and Kowalczykowski, S.C. 1993. The recombination hotspot χ is a regulatory sequence that acts by attenuating the nuclease activity of the *E. coli* RecBCD enzyme. Cell 73(1): 87-93.

Domka, J., Lee, J., Bansal, T., and Wood, T.K. 2007. Temporal gene-expression in *Escherichia coli* K-12 biofilms. Environ. Microbiol. 9(2): 332-346.

Dong, T., Kirchhof, M.G., and Schellhorn, H.E. 2008. RpoS regulation of gene expression during exponential growth of Escherichia coli K-12. Mol. Genet. Genomics 279(3): 267-277.

Donnenberg, M.S., Tzipori, S., McKee, M.L., O'Brien, A.D., Alroy, J., and Kaper, J.B. 1993. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment *in vitro* and in a porcine model. J. Clin. Invest. 92(3): 1418-1424.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. 16(13): 6127-6145.

Doyle, M.P. 1991. *Escherichia coli* O157:H7 and its significance in foods. Int. J. Food Microbiol. 12(4): 289-301.

Dziva, F., van Diemen, P.M., Stevens, M.P., Smith, A.J., and Wallis, T.S. 2004. Identification of *Escherichia coli* O157:H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. Microbiology 150(11): 3631-3645.

El Karoui, M., Amundsen, S.K., Dabert, P., and Gruss, A. 1999. Gene replacement with linear DNA in electroporated wild-type *Escherichia coli*. Nucleic Acids Res. 27(5): 1296-1299.

Espinosa-Urgel, M., Chamizo, C., and Tormo, A. 1996. A consensus structure for sigma S-dependent promoters. Mol. Microbiol. 21(3): 657-659.

Farrell, M.J., and Finkel, S.E. 2003. The growth advantage in stationary-phase phenotype conferred by *rpoS* mutations is dependent on the pH and nutrient environment. J. Bacteriol. 185(24): 7044-7052.

Fass, S., Dincher, M.L., Reasoner, D.J., Gatel, D., and Block, J.-. 1996. Fate of *Escherichia coli* experimentally injected in a drinking water distribution pilot system. Water Res. 30(9): 2215-2221.

Ferreira, A., Rendano, M., Wiedmann, M., and Boor, K.J. 1999. Characterization of *rpoS* alleles in *Escherichia coli* O157:H7 and in other *E. coli* serotypes. Journal of Applied Microbiology 86: 295-301.

Finkel, S.E., and Kolter, R. 1999. Evolution of microbial diversity during prolonged starvation. Proc. Natl. Acad. Sci. U S A. 96(7): 4023-4027.

Friedrich, M.J., and Kadner, R.J. 1987. Nucleotide sequence of the *uhp* region of Escherichia coli. J. Bacteriol. 169(8): 3556-3563.

Geesey, G.G., Richardson, W.T., Yeomans, H.G., Irvin, R.T., and Costerton, J.W. 1977. Microscopic examination of natural sessile bacterial populations from an alpine stream. Can. J. Microbiol. 23(12): 1733-1736.

Ghigo, J.M. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature 412(6845): 442-445.

Griffin, P.M., and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157: H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. 13(1): 60-98.

Gross, C.A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. 1998. The functional and regulatory roles of sigma factors in transcription. Cold Spring Harb. Symp. Quant. Biol. 63: 141-155.

Gruber, T.M., and Gross, C.A. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. 57: 441-466.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA. 100(4): 1541-1546.

Gyles, C., Johnson, R., Gao, A., Ziebell, K., Pierard, D., Aleksic, S., and Boerlin, P. 1998. Association of enterohemorrhagic *Escherichia coli* hemolysin with serotypes of shiga-like-toxin-producing *Escherichia coli* of human and bovine origins. Appl. Environ. Microbiol. 64(11): 4134-4141.

Hamilton, C.M., Aldea, M., Washburn, B.K., Babitzke, P., and Kushner, S.R. 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. bacteriol. 171(9): 4617-4622.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.-., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., and Shinagawa, H. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res. 8(1): 11-22.

Helmann, J.D., and Chamberlin, M.J. 1988. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. 57: 839-872.

Hengge-Aronis, R. 2002. Recent insights into the general stress response regulatory network in *Escherichia coli*. J. Mol. Microbiol. Biotechnol. 4(3): 341-346.

Hengge-Aronis, R. 1999. Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. Curr. Opin. Microbiol. 2(2): 148-152.

Henrici, A.T. 1933. Studies of freshwater bacteria: I. A direct microscopic technique. J. Bacteriol. 25(3): 277-287.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's manual of determinative bacteriology. Williams & Wilkins, Baltimore, MD, USA

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., and Hrudey, E.J. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. Water Sci. Technol. 47(3): 7-14.

Island, M.D., and Kadner, R.J. 1993. Interplay between the membrane-associated UhpB and UhpC regulatory proteins. J. Bacteriol. 175(16): 5028-5034.

Ito, A., May, T., Kawata, K., and Okabe, S. 2008. Significance of *rpoS* during maturation of *Escherichia coli* biofilms. Biotechnol. Bioeng. 99(6): 1462-1471.

Iyoda, S., and Watanabe, H. 2004. Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157:H7 to HEp-2 cells. Microbiology 150(7): 2357-2571.

Jackson, D.W., Suzuki, K., Oakford, L., Simecka, J.W., Hart, M.E., and Romeo, T. 2002. Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli* . J. Bacteriol. 184(1): 290-301.

Jenkins, D.E., Schultz, J.E., and Matin, A. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. 170(9): 3910-3914.

Jenkins, D.E., Chaisson, S.A., and Matin, A. 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. 172(5): 2779-2781.

Jensen, E.T., Kharazmi, A., Lam, K., Costerton, J.W., and Hoiby, N. 1990. Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. Infect. Immun. 58(7): 2383-2385.

Jishage, M., Iwata, A., Ueda, S., and Ishihama, A. 1996. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of four species of sigma subunit under various growth conditions. J. Bacteriol. 178(18): 5447-5451.

Karmali, M.A. 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. 2(1): 15-38.

Karu, A.E., Sakaki, Y., Echols, H., and Linn, S. 1975. The gamma protein specified by bacteriophage gamma. Structure and inhibitory activity for the RecBC enzyme of *Escherichia coli*. J. Biol. Chem. 250(18): 7377-7387.

Klauck, E., Typas, A., and Hengge, R. 2007. The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. Sci. Prog. 90(2-3): 103-127.

Kolter, R., Siegele, D.A., and Tormo, A. 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. 47: 855-874.

Kowalczykowski, S.C. 2000. Initiation of genetic recombination and recombination-dependent replication. Trends Biochem. Sci. 25(4): 156-165.

Kusano, S., Ding, Q., Fujita, N., and Ishihama, A. 1996. Promoter selectivity of *Escherichia coli* RNA polymerase E σ^{70} and Eσ^{38} holoenzymes. Effect of DNA supercoiling. J. Biol. Chem. 271(4): 1998-2004.

Kuzminov, A. 1999. Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. Microbiol. Mol. Biol. Rev. 63(4): 751-813.

Lacour, S., and Landini, P. 2004. σ^S-dependent gene expression at the onset of stationary phase in *Escherichia coli*: Function of σ^S-dependent genes and identification of their promoter sequences. J. Bacteriol. 186(21): 7186-7195.

Lammle, B., Kremer Hovinga, J.A., and Alberio, L. 2005. Thrombotic thrombocytopenic purpura. J. Thromb. Haemost. 3(8): 1663-1675.

Lange, R., and Hengge-Aronis, R. 1991a. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli* . Mol. Microbiol. 5(1): 49-59.

Lange, R., and Hengge-Aronis, R. 1991b. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^S. J. Bacteriol. 173(14): 4474-4481.

LeChevallier, M.W., Babcock, T.M., and Lee, R.G. 1987. Examination and characterization of distribution system biofilms. Appl. Environ. Microbiol. 53(12): 2714-2724.

Lederberg, J. 2004. *E. coli* K-12. MicrobiologyToday 31: 116.

Lesic, B., and Rahme, L.G. 2008. Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa*. BMC Mol. Biol. 9(20).

Lin, C.T., Lin, W.H., Lyu, Y.L., and Whang-Peng, J. 2001. Inverted repeats as genetic elements for promoting DNA inverted duplication: implications in gene amplification. Nucleic Acids Res. 29(17): 3529-3538.

Lisser, S., and Margalit, H. 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. 21(7): 1507-1516.

Little, W.J. 1967. An exonuclease induced by bacteriophage lambda. II. Nature of the enzymatic reaction. J. Biol. Chem. 242(4): 679-686.

Lloyd, R.G., and Buckman, C. 1985. Identification and genetic analysis of *sbcC* mutations in commonly used *recBC sbcB* strains of *Escherichia coli* K-12. J. bacteriol. 164(2): 836-844.

Loewen, P.C., and Hengge-Aronis, R. 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. 48: 53-80.

Loewen, P.C., and Triggs, B.L. 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli*. J. Bacteriol. 160(2): 668-675.

Loewen, P.C., Hu, B., Strutinsky, J., and Sparling, R. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. 44(8): 707-717.

Low, A.S., Dziva, F., Torres, A.G., Martinez, J.L., Rosser, T., Naylor, S., Spears, K., Holden, N., Mahajan, A., Findlay, J., Sales, J., Smith, D.G.E., Low, J.C., Stevens, M.P., and Gally, D.L. 2006. Cloning, expression, and characterization of fimbrial operon F9 from enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. 74(4): 2233-2244.

Lynnon BioSoft. 2000. DNAMAN. 5.0.

Mah, T.-.C., and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9(1): 34-39.

Maki, M. 2008. The effect of biofilm and the role of *rpoS* on the survival of a p-nitrophenol-degrading *Pseudomonas putida*. Master's thesis, Lakehead University, Thunder Bay, ON, Canada.

Marrie, T.J., Nelligan, J., and Costerton, J.W. 1982. A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. Circulation 66(6): 1339-1341.

Martino, P.D., Fursy, R., Bret, L., Sundararaju, B., and Phillips, R.S. 2003. Indole can act as an extracellular signal to regulate biofilm formation of *Escherichia coli* and other indole-producing bacteria. Can. J. Microbiol. 49(7): 443-449.

McCann, M.P., Kidwell, J.P., and Matin, A. 1991. The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. 173(13): 4188-4194.

McClure, W.R. 1985. Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. 54: 171-204.

Media Cybernetics. 2001. Image-Pro Plus. 4.5.

Merrick, M.J. 1993. In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N). Mol. Microbiol. 10(5): 903-909.

Microsoft Corporation. 2007. Microsoft Office Word.

Mohamed, J.A., Huang, W., Nallapareddy, S.R., Teng, F., and Murray, B.E. 2004. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. Infect. Immun. 72(6): 3658-3663.

Mulvey, M.R., and Loewen, P.C. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel sigma transcription factor. Nucleic Acids Res. 17(23): 9979-9991.

Murphy, K.C., and Campellone, K.G. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli* [online]. BMC Mol. Biol. 4(11): Feb 1, 2007.

Murphy, K.C. 1998. Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. J. bacteriol. 180(8): 2063-2071.

Murphy, K.C., Campellone, K.G., and Poteete, A.R. 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene 246(1-2): 321-330.

Myers, R.S., and Stahl, F.W. 1994. X and the RecBCD enzyme of *Escherichia coli*. Annu. Rev. Genet. 28: 49-70.

Newton, H.J., Sloan, J., Bennett-Wood, V., Adams, L.M., Robins-Browne, R.M., and Hartland, E.L. 2004. Contribution of long polar fimbriae to the virulence of rabbit-specific enteropathogenic *Escherichia coli*. Infect. Immun. 72(3): 1230-1239.

Nickel, J.C., Ruseska, I., Wright, J.B., and Costerton, J.W. 1985a. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. 27(4): 619-624.

Nickel, J.C., Wright, J.B., Ruseska, I., Marrie, T.J., Whitfield, C., and Costerton, J.W. 1985b. Antibiotic resistance of *Pseudomonas aeruginosa* colonizing a urinary catheter in vitro. Eur. J. Clin. Microbiol. 4(2): 213-218.

O'Brien, A.D., and Holmes, R.K. 1987. Shiga and Shiga-like toxins. Microbiol. Rev. 51(2): 206-220.

Oh, Y.J., Jo, W., Yang, Y., and Park, S. 2007. Influence of culture conditions on *Escherichia coli* 0157:H7 biofilm formation by atomic force microscopy. Ultramicroscopy 107(10-11): 869-874.

Ojeda, A., Prado, V., Martinez, J., Arellano, C., Borczyk, A., Johnson, W., Lior, H., and Levine, M. Sorbitol-negative phenotype among Enterohemorrhagic *Escherichia coli* strains of different serotypes from different sources. J. Clin. Microbiol. 33(8): 2199-2201.

Oliver, S.G., Winson, M.K., Kell, D.B., and Baganz, F. 1998. Systematic functional analysis of the yeast genome. Trends Biotechnol. 16(9): 373-378.

Olsen, A., Arnqvist, A., Hammar, M., Sukupolvi, S., and Normark, S. 1993. The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. Mol. Microbiol. 7(4): 523-536.

Olsen, S.J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., and Mead, P. 2002. A waterborne outbreak of *Escherichia coli* 0157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. Emerg. Infect. Dis. 8(4): 370-375.

Ostroff, S.M., Kobayashi, J.M., and Lewis, J.H. 1989. Infections with *Escherichia coli* 0157:H7 in Washington State. The first year of statewide disease surveillance. JAMA 262(3): 355-359.

O'Toole, G., Kaplan, H.B., and Kolter, R. 2000. Biofilm Formation as Microbial Development. Annu. Rev. Microbiol. 54(1): 49-79.

O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., and Kolter, R. 1999. Genetic a pproaches to the study of biofilms. Methods Enzymol. 310: 91-109.

Patten, C.L., Kirchhof, M.G., Schertzberg, M.R., Morton, R.A., and Schellhorn, H.E. 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. Mol. Gen. Genomics 272(5): 580-591.

Perroud, B., and Le Rudulier, D. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. 161(1): 393-401.

Poteete, A.R. 2001. What makes the bacteriophage λ Red system useful for genetic engineering: molecular mechanism and biological function. FEMS Microbiol. Lett. 201: 9-14.

Poteete, A.R., and Fenton, A.C. 1984. Lambda red-dependent growth and recombination of phage P22. Virology 134(1): 161-167.

Pratt, L.A., and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30(2): 285-293.

Price, S.B., Cheng, C.-., Kaspar, C.W., Wright, J.C., DeGraves, F.J., Penfound, T.A., Castanie-Cornet, M., and Foster, J.W. 2000. Role of *rpoS* in acid resistance and fecal shedding of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 66(2): 632-637.

Prigent-Combaret, C., Vidal, O., Dorel, C., and Lejeune, P. 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. 181(19): 5993-6002.

Prigent-Combaret, C., Prensier, G., Le Thi, T.T., Vidal, O., Lejeune, P., and Dorel, C. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. Environ. Microbiol. 2(4): 450-464.

Promega. 2007. Promega's Wizard® *SV* Minipreps DNA Purification System. Promega, Madison, WI.

Raibaud, O., Mock, M., and Schwartz, M. 1984. A technique for integrating any DNA fragment into the chromosome of *Escherichia coli*. Gene 29(1-2): 231-241.

Ranallo, R.T., Barnoy, S., Thakkar, S., Urick, T., and Venkatesan, M.M. 2006. Developing live *Shigella* vaccines using lambda Red recombineering. FEMS Immunol. Med. Microbiol. 47(3): 462-469.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., and Swerdlow, D.L. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. Emerg. Infect. Dis. 11(4): 603-609.

Reisner, A., Krogfelt, K.A., Klein, B.M., Zechner, E.L., and Molin, S. 2006. *In vitro* biofilm formation of commensal and pathogenic *Escherichia coli* strains: Impact of environmental and genetic factors. J. Bacteriol. 188(10): 3572-3581.

Reisner, A., Haagensen, J., Schembri, M.A., Zechner, E.L., and Molin, S. 2003. Development and maturation of *Escherichia coli* K-12 biofilms. Mol. Microbiol. 48(4): 933-946.

Ren, D., Bedzyk, L.A., Thomas, S.M., Ye, R.W., and Wood, T.K. 2004. Gene expression in *Escherichia coli* biofilms. Appl. Microbiol. Biotechnol. 64(4): 515-524.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. 308(12): 681-685.

Rozen, Y., Dyk, T.K., LaRossa, R.A., and Belkin, S. 2001. Seawater activation of *Escherichia coli* gene promoter elements: Dominance of *rpoS* control. Microb. Ecol. 42(4): 635-643.

Russell, C.B., and Dahlquist, F.W. 1989. Exchange of chromosomal and plasmid alleles in *Escherichia coli* by selection for loss of a dominant antibiotic sensitivity marker. J. Bacteriol. 171(5): 2614-2618.

Russell, C.B., Thaler, D.S., and Dahlquist, F.W. 1989. Chromosomal transformation of *Escherichia coli recD* strains with linearized plasmids. J. bacteriol. 171(5): 2609-2613.

Ryu, J.H., Kim, H., and Beuchat, L.R. 2004. Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. J. Food Prot. 67(10): 2123-2131.

Ryu, J.H., and Beuchat, L.R. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: Effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. 71(1): 247-254.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y. : Cold Spring Harbor Laboratory, c2001.

Sammartano, L.J., Tuveson, R.W., and Davenport, R. 1986. Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli katF* locus. J. Bacteriol. 168(1): 13-21.

Sauer, K. 2003. The genomics and proteomics of biofilm formation. Genome Biol. 4(6): 219.

Savage, P.J., Leong, J.M., and Murphy, K.C. 2006b. Rapid allelic exchange in enterohemorrhagic *Escherichia coli* (EHEC) and other *E. coli* using lambda Red recombination. Curr. Protoc. Microbiol. Jan(Chapter 5): Unit5A.2.

Schellhorn, H.E., Audia, J.P., Wei, L.I., and Chang, L. 1998. Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli*. J. Bacteriol. 180(23): 6283-6291.

Schembri, M.A., Kjaergaard, K., and Klemm, P. 2003. Global gene expression in *Escherichia coli* biofilms. Mol. Microbiol. 48(1): 253-267.

Sheng, Y., Mancino, V., and Birren, B. 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucleic Acids Res. 23(11): 1990-1996.

Smith, G.R., Kunes, S.M., Schultz, D.W., Taylor, A., and Triman, K.L. 1981. Structure of chi hotspots of generalized recombination. Cell 24(2): 429-436.

SPSS Inc. 2000. Sigma Plot 2000 for Windows. 6.00.

SPSS Inc. 1997. Sigma Stat for Windows. 2.03.

Stewart, P.S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. 292(2): 107-113.

Stewart, P.S., and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358(9276): 135-138.

Su, C., and Brandt, L.J. 1995. *Escherichia coli* O157:H7 infection in humans. Ann. Intern. Med. 123(9): 698-714.

Swerdlow, D.L., Woodruff, B.A., Brady, R.C., Griffin, P.M., Tippen, S., Donnell, H.D.,Jr, Geldreich, E., Payne, B.J., Meyer, A.,Jr, and Wells, J.G. 1992. A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. Ann. Intern. Med. 117(10): 812-819.

Tallon, P. 2005. Effect of *rpoS* inactivation on biofilm formation of a p-nitrophenol degrading *Pseudomonas putida* strain. Master's thesis, Lakehead University, Thunder Bay, ON, Canada.

The MathWorks. 2005. MATLAB. 7.0.4.

Tillett, D., and Neilan, B.A. 1999. An improved method for the purification of large DNA fragments from agarose gels using Wizard Plus SV columns. Anal. Biochem. 269(1): 218-219.

Trémoulet, F., Duche, O., Namane, A., Martinie, B., and Labadie, J.C. 2002. A proteomic study of *Escherichia coli* O157 : H7 NCTC 12900 cultivated in biofilm or in planktonic growth mode. FEMS Microbiology Letters 215(1): 7-14.

Trun, N., and Trempy, J. 2005. Introduction to the cell. *In* Fundamentals of bacterial genetics. Blackwell Publishing, Malden, MA, USA. pp. 1-16.

Van Houdt, R., and Michiels, C.W. 2005a. Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. Res. Microbiol. 156(5-6): 626-633.

Verhamme, D.T., Postma, P.W., Crielaard, W., and Hellingwerf, K.J. 2002. Cooperativity in signal transfer through the Uhp System of *Escherichia coli*. J. Bacteriol. 184(15): 4205-4210.

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M., and Lejeune, P. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new *ompR* allele that increases curli expression. J. Bacteriol. 180(9): 2442-2449.

Visick, J.E., and Clarke, S. 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpoS* mutations in common laboratory strains. J. Bacteriol. 179(13): 4158-4163.

Wang, G. and Doyle, M.P. 1998. Survival of enterohemorrhagic *Escherichia coli* in water. J. Food Prot. 61(6): 662-667.

Warburton, D.W., Austin, J.W., Harrison, B.H., and Sanders, G. 1998. Survival and recovery of *Escherichia coli* O157:H7 in inoculated bottled water. J. Food Prot. 61(8): 948-952.

Waterman, S.R., and Small, P.L. 1996. Characterization of the acid resistance phenotype and *rpoS* alleles of shiga-like toxin-producing *Escherichia coli*. Infect. Immun. 64(7): 2808-2811.

Watterworth, L., Rosa, B., Schraft, H., Topp, E., and Leung, K.T. 2006. Survival of various ERIC-genotypes of Shiga toxin-producing *Escherichia coli* in well water. Water Air Soil Pollut. 177(1-4): 367-382.

Weber, H., Polen, T., Heuveling, J., Wendisch, V.F., and Hengge, R. 2005. Genome-wide analysis of the general stress response network in *Escherichia coli:* sigmaS-dependent genes, promoters, and sigma factor selectivity. J. Bacteriol. 187(5): 1591-1603.

Weston, L.A., and Kadner, R.J. 1988. Role of *uhp* genes in expression of the *Escherichia coli* sugar-phosphate transport system. J. Bacteriol. 170(8): 3375-3383.

Winans, S.C., Elledge, S.J., Krueger, J.H., and Walker, G.C. 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. J. Bacteriol. 161(3): 1219-1221.

Wong, A.C. 1998. Biofilms in food processing environments. J. Dairy Sci. 81(10): 2765-2770.

Wood, T., González Barrios, A.F., Herzberg, M., and Lee, J. 2006. Motility influences biofilm architecture in *Escherichia coli*. Appl. Microbiol. Biotechnol. 72(2): 361-367.

Xavier, J.B., White, D.C., and Almeida, J.S. 2003. Automated biofilm morphology quantification from confocal laser scanning microscopy imaging. Water Sci. Technol. 47(5): 31-37.

Zambrano, M.M., and Kolter, R. 1996. GASPing for life in stationary phase. Cell 86(2): 181-184.

Zambrano, M.M., Siegele, D.A., Almiron, M., Tormo, A., and Kolter, R. 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science 259(5102): 1757-1760.

Zinser, E.R., and Kolter, R. 1999. Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. J. Bacteriol. 181(18): 5800-5807.

Zobell, C.E. 1943. The Effect of Solid Surfaces upon Bacterial Activity. J. Bacteriol. 46(1): 39-56.