Understanding the role of the general stress response regulator, RpoS, in the environmental persistence of *Escherichia coli*

by

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DECLARATION

I declare that the work presented in this thesis is entirely my own and it has not been submitted, in whole or in part, in any previous application for a degree at this or any other university.

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30th March 2017

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ABSTRACT

The use of *E. coli* as an indicator organism for faecal contamination in the environment, particularly in water sources, is based partly on the assumption that *E. coli* survives transiently outside of the mammalian gastrointestinal tract. However, studies have shown that *E. coli* can persist and grow in various external environments, and raises questions about its continued use as an indicator organism. The general stress response regulator, RpoS, helps *E. coli* overcome various stresses, however, mutations are known to accumulate within *rpoS* in low nutrient environments causing loss of RpoS function. Since soil is less abundant in nutrients compared to the host gut, it was necessary to understand if loss of function mutations were present in the *rpoS* of soil-persistent *E. coli*. Hence, this study investigated a collection of long-term soil-persistent *E. coli* strains to understand if RpoS is retained in them and the contribution of the general stress response to soil survival. All the soil-persistent strains tested had an intact *rpoS* gene and retained a fully functional RpoS-regulated general stress response. RpoS was demonstrated to be important for soil survival and specifically, crucial for overcoming protozoan predation, low moisture and pH stresses in soil. Production of curli, an important component of *E. coli* biofilm, which is RpoS-dependent, increased the ability of *E. coli* to resist predation by *Acanthamoeba polyphaga* and *Tetrahymena pyriformis,* and promoted the attachment of *E. coli* to sand. Ten percent (17 out of 170) of the soil isolates were curli-deficient. Some of the curli-negative *E. coli*, which were defective in biofilm production and sand attachment, had mutations in two genes associated with c-di-GMP metabolism, *dgcE* and *pdeR*, although this did not significantly impair their survival in soil compared to curli-positive strains. Loss of curli production in soil-persistent *E. coli* may promote their dissemination into other environments.

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LIST OF ABBREVIATIONS

Amp	Ampicillin
AmpR	Ampicillin resistant
BSA	Bovine serum albumin
c-di-GMP	Bis-(3´-5´)-cyclic dimeric guanosine monophosphate
CFU	Colony forming unit
CmR	Chloramphenicol resistant
CR	Congo Red
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
g	Force of gravity
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GFP	Green fluorescent protein
GlcNac	N-Acetylglucosamine
HCl	Hydrochloric acid
HFIP	Hexafluoroisopropanol
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
KPi	inorganic potassium phosphate buffer
LB	Lysogeny Broth
NNA	Non Nutrient Agar
OD	Optical density
OPM	Oscillations per minute
PAS	Page's amoeba saline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppGpp	Guanosine tetraphosphate
PPY	Proteose-peptone yeast extract medium
RNAP	RNA polymerase
SMM	Succinate minimal medium
v/v	Volume per volume
w/v	Weight per volume
WHC	Water holding capacity
YESCA	Yeast extract casamino acid

CHAPTER 1

INTRODUCTION

1.1. BIOLOGY AND TAXONOMY OF *ESCHERICHIA COLI*

Escherichia coli is an extensively studied bacterium, often considered to be the most important model organism in biology (Blount, 2015). It was first isolated in the faeces of a healthy infant by Theodor Escherich in 1884 (Escherich, 1885; Escherich & Bettelheim, 1988) and was originally called *Bacterium coli commune*, based on its isolation from the colon. The name was later changed to *Bacillus coli* by Migula in 1895 before being given its current name, *Escherichia coli*, named after Theodor Escherich (Castellani & Chalmers, 1919).

E. coli is a Gram negative, non-spore forming, facultative anaerobic and rod-shaped bacterium (Singleton, 1999). The cells are typically about 2.0 µm long and 0.25 –1.0 µm in diameter, with a cell volume of 0.6–0.7 μm^3 (Kubitschek, 1990). *E. coli* is a facultative anaerobe which can grow aerobically or anaerobically; and motile strains have peritrichous flagella (Darnton *et al*., 2007). The growth of *E. coli* is optimal at 37°C and some of the strains can grow from 0°C on food items to temperatures up to 49°C (Arias *et al*., 2001; Fotadar *et al*., 2005). Because it is easily cultivated in the laboratory, and is easily manipulated genetically, *E. coli* has become one of the most studied prokaryotic model organisms (Zimmer, 2008). *E. coli* was important for understanding fundamental aspects of molecular genetics and many ground-breaking studies were done using *E. coli* (Crick *et al*., 1961; Nirenberg *et al*., 1965). *E. coli* is currently used as a “machine” for generating many therapeutic products in the pharmaceutical sector (Huang *et al*., 2012).

E. coli is metabolically versatile, and as a facultative anaerobe, it can use oxygen for respiration (aerobic respiration) and in the absence of oxygen, use fermentation to generate energy and grow. *E. coli* can respire aerobically using O_2 as a final electron acceptor and under anaerobic conditions, use nitrate, fumarate, dimethyl sulfoxide (DMSO), and trimethylamine N-oxide (TMAO) or fumarate as a terminal electron acceptor (Ingledew & Poole, 1984). *E. coli* is unable to metabolise complex polysaccharides because it does not have enzymes for degrading them (Fabich *et al*., 2008; Hoskins *et al*., 1985), hence, in the colon, it depends on the anaerobes to break down the mucin polysaccharides into monosaccharide, which it can readily utilise. *E. coli* can metabolise a variety of substrates including monosaccharides such as glucose, disaccharides such as lactose, glycerol and amino acids, to generate energy. Glucose is the preferred carbon source and it is metabolised by means of central metabolism, comprising three steps namely Embden-Meyerhof-Parnas (EMP) Pathway, which

converts glucose to pyruvate; Tricarboxylic Acid (TCA) cycle, which oxidizes Acetyl CoA to CO_2; and Pentose Phosphate Cycle (PPP), which oxidizes glucose to CO_2 (Conway & Cohen, 2015). Genes involved in the central metabolic pathways in *E. coli* are highly conserved and form a significant part of the core genome of *E. coli* (Cook & Ussery, 2013). The rapid utilisation of glucose depends on the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) (Deutscher *et al*., 2014).

Under anaerobic conditions when no suitable electron acceptors are present, *E. coli* converts the pyruvate that it generates from the EMP pathway into ATP through fermentation. During glucose fermentation by *E. coli*, glucose is first converted to pyruvate by the glycolysis pathway and in the second phase, the two molecules of NADH are recycled back to NAD^+ by a series of reactions that consume the pyruvate and produce various end products such as lactate, acetate, ethanol, succinate, formate, CO_2 and H_2. *E. coli* can also utilize the carbon from gluconate, and this is metabolised through the Entner-Doudoroff pathway (Chang *et al*., 2004). The Entner-Doudoroff is similar to the EMP pathway in that gluconate is converted into pyruvate, but different in that the pyruvate is converted to CO_2 and acetaldehyde. The acetaldehyde is further converted to ethanol. Furthermore, *E. coli* can use non-carbohydrate substrates such as amino acids (such as aspartate, serine and tryptophan), glycerol, lactate, acetate and dicarboxylates for growth through gluconeogenesis. These gluconeogenic substrates have been shown to be used by commensal and pathogenic *E. coli* in the mouse and bovine intestines (Bertin *et al*., 2014; Miranda *et al*., 2004).

Although *E. coli* is essentially a constituent of the gut microbiome of mammals, it has also been isolated from other warm-blooded animal and reptiles (Berg, 1996; David M. Gordon & Cowling, 2003). *E. coli* normally inhabits the lower gastrointestinal tract of its hosts and it is the dominant facultative anaerobe there, although it only constitutes about 0.1% of normal gut microflora (Eckburg *et al*., 2010). Infants are normally colonised by *E. coli* from their mothers' vaginas and some from the hospital environment and handlers (Bettelheim & Lennox-King, 1976; Fryklund *et al*., 1992). *E. coli* reaches a stable population of about 10^8 CFU g^{-1} of faeces in adults (Mitsuoka *et al*., 1975; Savageau, 1983). *E. coli* is expelled into the environment through defecation and then transmitted by the faecal-oral route to new hosts. *E. coli* has a doubling time of about 40 h in a human host, but an half-life of between 1-5 days, depending on the

external environment (Savageau, 1983). It is almost universally used as an indicator of faecal contamination in drinking and recreational water (Feng *et al*., 2002). The use of *E. coli* as a faecal indicator is based, at least in part, on the assumption that it exists transiently outside the host gastrointestinal tract and does not survive for a long time in the external environment. Potentially undermining this assumption, there is a growing body of evidence that *E. coli* can survive for longer periods in various natural environments (Byappanahalli *et al*., 2012; Chiang *et al*., 2011; Ishii *et al*., 2006; Ishii & Sadowsky, 2008; Jiménez *et al*., 1989; Zhi *et al*., 2016).

E. coli is a diverse species and contains some strains which are harmless commensals and others that are pathogenic to human and animals. *E. coli* K-12, a non-pathogenic and laboratory-adapted strain, is the most widely studied strain of *E. coli* and serves as a reference for this species. *E. coli* is genetically diverse species and strains only have about 20% of their genome in common (Lukjancenko *et al*., 2010). *E. coli* genome contains about 4800 genes, depending on the strain analysed, and the pangenome contains between 15,000 - 18,000 genes (Kaas *et al*., 2012; Rasko *et al*., 2008; Touchon *et al*., 2009). *E. coli* genomes are so similar to *Shigella* that the genus *Shigella* is phylogenetically considered a subgenus of *E. coli* (Vieira *et al*., 2011). Based on the minimum 16S rRNA gene identity (99.03%), which is above the cut-offs for classifying bacterial isolates at the species levels (98.7%), it was recommended that *Shigella* and *E. coli* belong to the same species (Rouli *et al*., 2015). As shown in Fig. 1.1, some *Shigella* (e.g. *S. dysenteriae*) are closer to some *E. coli* (*E. coli* O157:H7) than to some other *Shigella* (Vieira *et al*., 2011). Using a whole-genome-based, alignment-free, and parameter-free method called CVTree, Zuo *et al*. (2013) argued that *Shigella* strains are not clones of *Escherichia coli* but belonged within genus *Escherichia*.

The genetic diversity of the *E. coli* pangenome enables them to occupy various ecological niches, use a wide range of substrates for metabolism, and occasionally confers pathogenicity on them (Schmidt & Hensel, 2004; Tenaillon *et al*., 2010; Touchon *et al*., 2009). Phylogenomic analysis has linked the genomic diversity in *E. coli* with their phenotypic pathogenicity and ecological adaptation (Zhang & Lin, 2012). A strong correlation was observed between *E. coli* phylogroups and phenotypes where B1 strains produced more biofilm, a trait that promotes plants colonization, whereas strains in phylogroups A and B2 displayed more host-associated phenotypes (Méric *et al*., 2013). Being a facultative anaerobe in the anaerobic environment of the gut,

commensal *E. coli* consumes oxygen entering the gut thus providing a conducive environment for anaerobes, which are abundant in the gut (Chang *et al*., 2004). Commensal *E. coli* also produces vitamins such as B_{12} and K (Lawrence & Roth, 1996; Bentley & Meganathan, 1982) while colonising the gut mucus layer thus preventing enteric pathogens from colonizing the gut (Schierack *et al*., 2009). The pathogenic *E. coli*, on the other hand, have developed flexibility in their metabolism such that they can metabolise other sugars that commensals are unable to use, thereby giving them a competitive advantage in colonising the gut to establish an infection (Fabich *et al*., 2008). Pathogenic *E. coli* are believed to have emerged from commensal strains that acquired virulence factors via horizontal gene transfer from other bacterial pathogens (Herold *et al*., 2004; Kaper *et al*., 2005). There are genomic islands in *E. coli* that define the different behavioural types and ecological versatility in this species (Dobrindt *et al*., 2003).

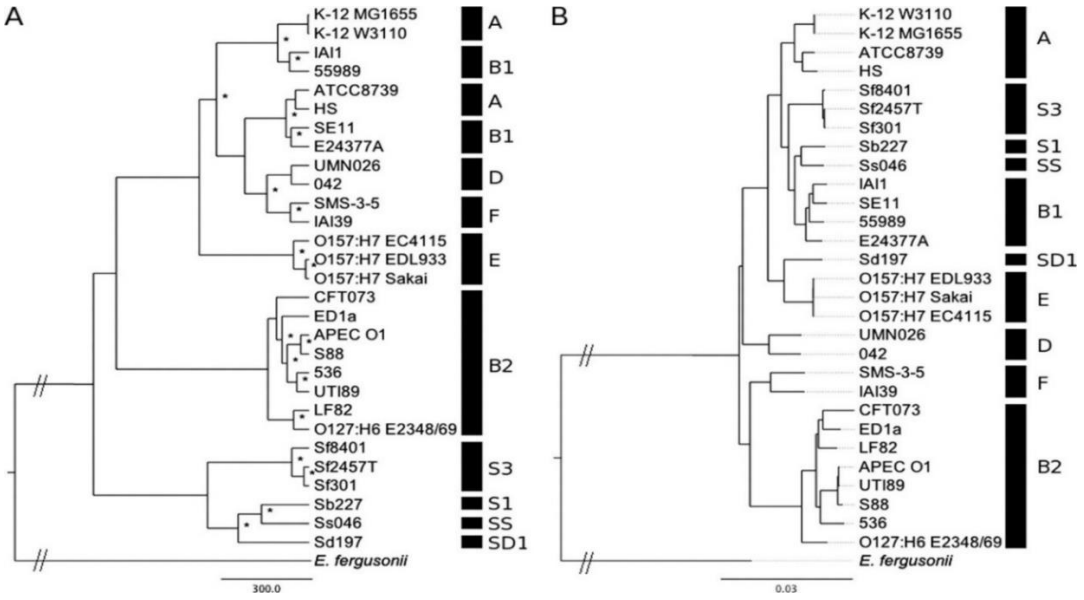


Figure 1.1: Evolution tree of *E. coli* and *Shigella* species according to metabolic (A) and genetic (B) distances. *, nodes with a bootstrap value greater than 70% for the metabolic tree. All nodes of the genetic tree have bootstrap values greater than 70%. The first letters in Sf8401, Sf2457T, Sf301, Sb227, Ss046 and Sd197 represent the genua *Shigella* and the second letters represent the species. Phylogenetic groups are represented after the black blocks in each tree. The figure is taken from Vieira *et al*. (2011).

1.2. ENVIRONMENTAL PERSISTENCE OF *E. COLI*

Although *E. coli* is thought to primarily reside in the lower gastrointestinal tract, it is constantly being excreted into the external environment. In fact, it is estimated that half of the global *E. coli* population is outside the host (Savageau, 1983), although this estimation needs to be revisited and updated in light of new knowledge generated in this regard. Several authors have isolated *E. coli* from various natural environments such as municipal wastewater (Zhi *et al*., 2016), freshwater (Jiménez *et al*., 1989; Lopez-Torres *et al*., 1987), beach water (Chiang *et al*., 2011; McLellan & Salmore, 2003), beach sand (Chiang *et al*., 2011), tropical and subtropical soils (Byappanahalli & Fujioka, 1998; Byappanahalli *et al*., 2012; Desmarais *et al*., 2002; Fujioka, 2001; Solo-Gabriele *et al*., 2000), coastal temperate forest soils (Byappanahalli *et al*. 2006), riverine temperate soil (Ishii *et al*., 2006) and sediments (Anderson *et al*., 2005). Brennan *et al*. (2010a) reported the recovery of *E. coli* populations from intact soil monoliths maintained in lysimeter units, which have been protected from faecal contamination for almost a decade. *E. coli* have shown not only to survive but also to grow in different soils in tropical (Byappanahalli & Fujioka, 1998; Byappanahalli *et al*., 2012; Solo-Gabriele *et al*., 2000) and temperate regions (Byappanahalli *et al*., 2012, 2006; Ishii *et al*., 2006). Some *E. coli* lineages that appear to be adapted to a primarily non-host lifestyle have also been identified (Walk *et al*., 2009).

Persistence of *E. coli* in the environment brings into question the validity of its continued use as an indicator of faecal contamination in the environment and for water quality. Persistent *E. coli* in the environment can readily contaminate water, crops and food materials and can pose a threat to public health, especially if the strains are pathogenic (Ogden *et al*., 2001; Vanderzaag *et al*., 2010). *E. coli* O157:H7 has been related to consumption of contaminated water (Licence *et al*., 2001). Pathogenic *E. coli* are able to survive and persist in the environment such as soil, irrigation water, and in food processing environments, from which they can contaminate fruits and vegetables, which can be the source of outbreaks (Solomon *et al*., 2002). *E. coli* persisting in soils could serve as a source of inoculum for crops grown in the contaminated soil (Downie *et al*., 2012; Habteselassie *et al*., 2008; Jensen *et al*., 2013) and such crops may become source of transmission of the pathogen to new hosts. The devastating 2011 *E. coli* O104:H4 outbreak in Europe was reportedly caused by consumption of fenugreek sprouts grown at a farm in Germany. The outbreak resulted in 3816 cases (including 54 deaths) in Germany alone (Frank *et al*., 2011). The contamination was traced to the seeds used to grow the sprouts (EFSA, 2011). The importance of environmental

persistence of *E. coli* O157 was shown by Strachan *et al*. (2006) when they reported that 54% of outbreaks in Scotland from 1994 to 2003 was due to environmental transmission and this was higher than foodborne cases (40%). They further showed that environmental exposure has strong association with outbreaks cases in Scotland.

E. coli has a biphasic lifestyle, one in the primary host and another in the secondary environment (Savageau, 1983). It is not only able to survive inside the host, but is also able to adapt to life outside of a host where they experience limited nutrient availability, temperature changes, shifts in osmolarity, oxygen, light, moisture, pH and diverse competing microorganisms. It is suggested that *E. coli* has evolved to adapt to non-host niches and later recolonise a new host (reviewed in van Elsas *et al*., 2011).

1.3. RPOS AND RESPONSE TO ENVIRONMENTAL STRESS

E. coli in the external environment are exposed to physical and chemical conditions which are often different from those present in the host. Hence, they have developed strategies for overcoming the variations in environmental conditions so as to ensure their survival outside the host. One of the most important genetic factors for environmental survival is the general stress response regulator, RpoS.

E. coli has developed mechanisms to adapt to suboptimal environmental conditions experienced outside its primary host and these responses are regulated by the alternative sigma factor RpoS. RNA polymerase (RNAP) is an enzyme that transcribes DNA to RNA, catalysing the polymerisation of ribonucleotides into the RNA complement of the DNA template. The core RNAP, consist of five subunits (β', β, α^{I}, α^{II}, ω), which interacts with the sigma factor (σ) subunit and can dissociate from the rest of the complex, leaving the core enzyme (Kazmierczak et al., 2005). RNAP core associates reversibly with the sigma factor (σ) to form RNA polymerase holoenzyme, which recognises promoter sites and initiates transcription. Interactions between RNA polymerase (RNAP) and DNA promoter sites located upstream of specific genes leads to the transcription of those genes (Borukhov & Nudler, 2003). Sigma factors are small proteins that bind and regulate transcription of genes in bacteria. The sigma factor (σ) binds to the RNAP, recognises promoter sequences, and helps initiate the transcription of the genes from selected specific promoters (Ishihama, 2000; Jishage *et al*., 1996). The primary sigma factor in *E. coli* is RpoD (σ^{70}) and it recognizes housekeeping genes, which is essential for cell growth and survival. However, the alternative sigma factors (σ^{E}, σ^{F}, σ^{H}, σ^{I}, σ^{N} and σ^{S}) are non-essential for growth but mediate the adaptive

response of bacteria to the extracellular environment. Their specific functions include coordinating gene regulation to respond to nitrogen deprivation (σ^N), general stress (σ^S), heat shock (σ^H) and envelope stress (σ^E); flagella synthesis (σ^F), and iron transport (σ^{Fecl}) (Gourse *et al*., 2006; Gruber & Gross, 2003). Analysis of transcription binding sites in the *E. coli* genome revealed that housekeeping σ^D was the most common type of promoter, followed by σ^s (Mendoza-Vargas *et al*., 2009). Consensus sequence of promoters for sigma factors and their examples are shown in Table 1.1. σ^S-associated RNA polymerase contribute significantly to transcription of genes encoding proteins involved in adjusting to changes the cells experience upon entry into stationary phase (Peano *et al*., 2015). The interaction between core RNAP and sigma factor also changes when *E. coli* encounters an external stimulus/stress (Ganguly & Chatterji, 2012). This allows for a reversible σ-exchange from housekeeping factors to alternate σ-factors when the *E. coli* senses a change in its environment. For *E. coli* to respond to changes in its environmental conditions, it must first be able to sense the changes occurring around it and thereafter mount appropriate responses in gene expression and protein activity. One way the bacterium has evolved to respond to stress involves an elaborate cascade pathway leading to the activation of the alternative sigma factor, σ^s (Section 1.5). This sigma factor binds to the inactive RNA polymerase allowing it to recognise specific promoter sequences for a set of genes, thereby commencing their transcription (Lange & Hengge-Aronis, 1991).

The *rpoS* gene, initially named *katF,* was first described as a σ transcription factor in *E. coli* by Mulvey & Loewen (1989). RpoS is the master regulator of the stress response in *E. coli* and they activate different genes important for stress management and response to changes in environmental factors. In a typical cell under optimal laboratory growth conditions, RpoS levels are low in exponential phase but is strongly induced and increases as the cell enters stationary phase (Battesti *et al*., 2011). RpoS is the alternative sigma factor induced upon entry into stationary phase. When *E. coli* encounters suboptimal growth or stress conditions, RpoS activates the transcription of many genes to cope with specific stress situations and confers stress tolerance and survival. RpoS (σ^S), controls over 500 genes and enables the transition into the stationary phase (Patten *et al*., 2004; Weber *et al*., 2005). This represents about 10% of *E. coli* genome being directly or indirectly controlled by RpoS. These genes were identified under several conditions, all leading to elevated σ^S levels.

Table 1.1: Consensus sequence of promoters for sigma factors and their examples in *E. coli*

Sigma Factor	Consensus Promoter Sequence		Examples of promoters
	-35 Region	-10 Region	
σ^{70}	TTGACA	TATAAT	>tnaA_promoter caat**ttcaga**atagacaaaaactctgagtgt**aataat**gtagcctc >recA_promoter acac**ttgata**ctgtatgagcatacag**tataat**tgctt
σ^{38}	TTGACA	CTATACT	>*osmY*_promoter cc**cgagcg**gtttcaaaattgtgat**ctatatttaacaaa** >*otsB*_promoter aa**atggcg**acccccgtcacactg**tctatacttacat**
σ^{28}	TAAA	GCCGATAA	>*pdeH*_promoter ggcgg**taaag**ttctgcccttacgc**gccgataa**tcttg >*flgK*_promoter ataac**tcaag**tccggcgggtcgct**gccgataa**tactct
σ^{32}	CTTGAAa	CCCCATnT	>*dnaK*_promoter ccct**tgatg**acgtggtttacga**ccccat**ttagtagtc >*rrsE*_promoter tcta**ttgcgg**cctgcggagact**ccctat**aatgcgcctcc
σ^{19}	-	-	>*fecA*_promoter gtaaggaaaataattcttatttcgattgtccttttaccc
σ^{24}	-	-	>*degP*_promoter agttcg**gaactt**caggctataaaacgaa**tctga**agaaca >*rseP*_promoter taatt**ccagga**cacggtggtattttaga**tcgta**ttg
	-24 Region	-12 Region	
σ^{54}	TGGCAC	TTGCW	>*glnA*_promoter aagt**tggcac**agattt**cgctt**tatatcttttt >*fdhF*_promoter aatg**tggcat**aaaaga**tgcat**actgtagtc

The gene encoding RpoS is part of the *nlpD-rpoS* operon and it is transcribed from a promoter located within the *nlpD* gene (Fig. 1.2) (Lange *et al*., 1995). Four other promoters of *rpoS* (two within *nlpD* and two in the intergenic region between *nlpD* and *rpoS*) have been identified (Mendoza-Vargas *et al*., 2009). These cryptic promoters are likely to transcribe *rpoS*, as they were experimentally determined. NlpD is a lipoprotein that activates the peptidoglycan hydrolase AmiC, which is involved in septal splitting (Uehara *et al*., 2010).

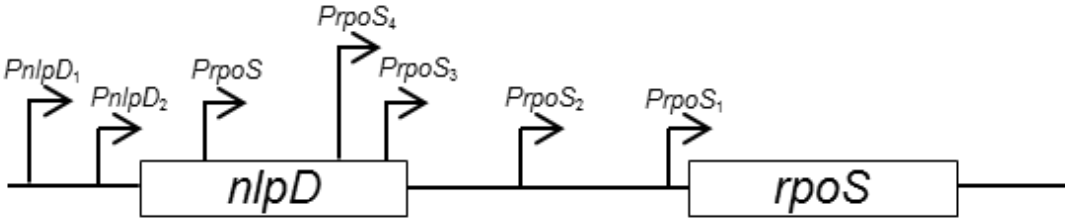


Figure 1.2: The *nlpD-rpoS* operon and promoters in *Escherichia coli* K-12 genome. Arrowheads represent promoter sites.

1.3.1 Osmotic Stress

E. coli has developed mechanisms for regulating cytoplasmic solute concentrations when it encounters sudden changes in the osmolarity around it (reviewed in Wood, 2015). *E. coli* is able to grow in Brain Heart Infusion broth with up to 6% (w/v) NaCl (Gibson & Roberts, 1986). Its ability to adapt to osmotic shock is RpoS-dependent (Hengge-Aronis *et al*., 1993). Wildtype *E. coli* was shown to survive 12% w/v (2.05 M) NaCl for 8 days at 37°C whereas no viable cell of its Δ*rpoS* mutant was detected after 4 days (Stasic *et al*., 2012). RpoS induces the expression of different genes to respond to osmotic stress and they include genes required for lipoprotein production (*osmB* and *osmE*) (Jung *et al*., 1990; Peano *et al*., 2015). *E. coli* also accumulates different compatible solutes under high osmotic stress so as to raise the intracellular osmotic pressure (Csonka, 1989; Wood *et al*., 2001). Some of these compatible solutes include K^+, glutamate, trehalose, proline, ectoine, choline and glycine betaine (Csonka, 1989; Wood, 2015). The expression of these compatible solutes and solute transporter systems are RpoS-dependent, e.g. ectoine, proline and glycine betaine transporter (*proP*) (Mellies *et al*., 1995; Typas *et al*., 2007b); trehalose biosynthesis (*otsBA*) (Rosenthal *et al*., 2006; Weber *et al*., 2006) and solute efflux system (*mscL*) (Stokes *et*

al., 2003). Deletion of *rpoS* was shown to impair the ability of *E. coli* to respond to osmotic stress due to an inability to synthesize trehalose (Culham *et al*., 2001). When *E. coli* is no longer osmotically stressed, the accumulated solutes are released from the cells (Wood, 2015).

1.3.2 Oxidative Stress

Oxidative stress is caused by the intracellular accumulation of reactive oxygen species or a disturbance of the cellular redox state. Barth *et al*. (2009) observed that ability of *E. coli* to resist oxidative stress was entirely dependent on RpoS, since wildtype strains were oxidative stress-resistant and degraded reactive oxygen species whereas oxidative stress resistance was lost when *rpoS* was deleted. *E. coli* responds to oxidative stress by inducing the transcription of RpoS-dependent genes such as *katE*, *katG, sodC*, and *dps* to produce antioxidant proteins such as superoxide dismutase and catalase (Chiang & Schellhorn, 2012; Gort *et al*., 1999; Nair & Finkel, 2004; Schellhorn & Hassan, 1988). In uropathogenic *Escherichia coli* (UPEC), which needs to overcome host immune cells and oxidative stress in the host before they can establish urinary tract infections, RpoS was shown to be important for the fitness of UPEC strain CFT073 to cope with oxidative stress presented by phagocytes during infection (Hryckowian & Welch, 2013). Wildtype UPEC strain CFT073 was also found to be more resistant to oxidative stress in urine than its isogenic *rpoS* mutant (Hryckowian & Welch, 2013).

1.3.3 Desiccation

E. coli responds to loss of water from the cell by accumulating trehalose intracellularly (Welsh & Herbert, 1999). However, the protection provided by compatible solutes to cells subjected to desiccation differs from the protection during osmotic stress, due to the much greater reduction in available cell water (Welsh & Herbert, 1999). During desiccation, accumulation of trehalose intracellularly ensures that the phospholipid bilayer of cell membrane remains in the liquid crystalline phase and can also keep protein in a hydrated form by hydrogen bonding and water replacement (Crowe *et al*., 1988). A correlation has been shown between intracellular trehalose concentration and desiccation resistance in some soil *E. coli* population (Zhang & Yan, 2012). *E. coli* O157:H7 wildtype was able to survive desiccation for up to 28 days on sterile and dried filter disks but there were no viable cells of the Δ*rpoS* after 4 days (Stasic *et al*., 2012).

Also, the viability of *rpoS* mutants was impaired in dried cow faeces at 30°C compared to the wildtype (Stasic *et al*., 2012).

1.3.4 Temperature stress

E. coli can grow at temperatures ranging 0 - 45°C with optimal temperature of 37°C (Arias *et al*., 2001; Fotadar *et al*., 2005). Mechanisms evolved to adapt to low temperature stress include modifications of cell membrane to maintain membrane fluidity (Russell, 1990) and stabilization of the secondary structures of nucleic acids, which leads to a reduced efficiency of RNA transcription, translation and degradation (Phadtare, 2004). Membranes are normally in a liquid crystalline form and undergo a reversible transition to a gel phase upon temperature downshift (Yamanaka, 1999). The cold shock response enables the cell to counteract these unfavourable changes, mostly by the selective production of cold shock proteins and modifying membrane composition (Barria *et al*., 2013; Ivancic *et al*., 2009). It was shown that under cold stress conditions, Δ*rpoS* mutant of *E. coli* 0157 was significantly impaired in survival compared to its wildtype, thus suggesting that RpoS plays an important role in cold stress response of *E. coli* O157 (Vidovic *et al*., 2011). Cold shock causes an increase in transcription of RpoS-dependent *otsBA* genes, which leads to accumulation of trehalose in *E. coli* (Kandror *et al*.,2002; White-Ziegler *et al*., 2008). *E. coli otsBA* mutants are unable to synthesize trehalose and died much faster than the wild type at 4°C (Kandror *et al*., 2002). Induction of *otsBA* and trehalose biosynthesis was also seen during heat shock response (Kandror *et al*., 2002). Furthermore, the role of RpoS in heat shock response was shown when *E. coli* was exposed to 56°C (Ait-Ouazzou *et al*., 2012). The Δ*rpoS* mutant was about 4 log cycles more heat-sensitive than its parental strain. RpoS enhanced the intrinsic resilience of the cytoplasmic membrane thus protecting the cell envelopes (Ait-Ouazzou *et al*., 2012)

1.3.5 Acid stress

E. coli strains grow optimally at neutral pH and can survive acid stress as low as pH 1.2 (Riggins *et al*., 2013). There are different systems for overcoming mild and extreme acid stress in the environment including glucose-catabolite-repressed system (Rowbury & Goodson, 1998; Wu *et al*., 2014); F_0F_1-ATPase system (Sun *et al*., 2012); glutamate decarboxylase system (Castanie-Cornet *et al*., 1999; De Biase *et al*., 1999) and arginine-decarboxylase system (Iyer *et al*., 2003). The regulation of acid resistance

mechanisms in *E. coli* was extensively reviewed by Foster (2004) and more recently by Lund *et al*. (2014).

The role of the acid resistance mechanisms is to help *E. coli* maintain a constant (or only slightly changed) intracellular pH irrespective of the fluctuations in the external pH (Richard & Foster, 2004). For example, when the external pH was lowered from pH 7.5 to 5.5 by adding HCl, the internal (cytoplasmic) pH fell within 10 - 20 s to pH 5.6 - 6.5 and was restored rapidly to pre-acidification levels within 30 - 60 s (Wilks & Slonczewski, 2007). Mechanistically, upon a reduction in intracellular pH, the two isoforms of glutamate decarboxylase (GAD) enzymes, GadA and GadB, associate with the glutamate:γ-aminobutyrate antiporter GadC (Waterman & Small, 2003). GadC then imports an extracellular molecule of glutamate in exchange for intracellular GABA (Waterman and Small, 2003). This process replaces the α-carboxyl group of glutamate with a proton that is obtained from the cytoplasm and produces CO_2 and gamma-amino butyric acid (GABA). The GABA that is formed is exported through the antiporter GadC, in exchange for importing new glutamate substrates thereby increasing the cytosolic pH (reviewed in Lund *et al*. 2014). The decarboxylases (GadA and GadB) work in close association with the antiporter GadC, which is localised in the cell membrane and becomes active only when the extracellular pH drops below threshold levels, thereby providing a selective gate for entry of glutamate and exit of GABA (Foster, 2004).

The GAD system is the most efficient system in *E. coli* for overcoming extreme acid stress (Diez-Gonzalez & Karaibrahimoglu, 2004; Lin *et al*., 1996) and its expression is RpoS-dependent (Chattopadhyay *et al*., 2015; Weber *et al*., 2005). RpoS contributes to the induction of the GAD system through GadE and GadX, the transcriptional activator of the *gadA* and *gadBC* genes (Chattopadhyay *et al*., 2015; Ma *et al*., 2003; Sayed *et al*., 2007; Tramonti *et al*., 2002). *E. coli* cells with *rpoS* mutations have been shown to have reduced or no survival at low pH. For example, no viable cells of *E. coli* O157:H7 Δ*rpoS* mutant was detected at 24 h in apple cider at pH 3.5 (Price *et al*., 2004), whereas the wild-type had 100% survival. The Δ*rpoS* deletion mutant of a highly virulent *E. coli* O26:H11 was sensitive to acid stress at pH 2.5 but when complemented with wildtype *rpoS*, the acid resistant phenotype was restored to even a greater level than the wild type strain (Coldewey *et al*., 2007). RpoS was also shown to be important for acid resistance in probiotic *E. coli* strain Nissle 1917 (Coldewey *et al*., 2007). The type of acid environment encountered determines the type of acid resistance mechanism that is deployed in *E. coli* (Price *et al*., 2004).

1.3.6 Other stresses

RpoS is also important for the response of *E. coli* to starvation. RpoS was shown to be induced by almost 6-fold when *E. coli* was starved in distilled water for 3 h (Gawande & Griffiths, 2005). Furthermore, an RpoS-dependent gene, universal stress protein B, *uspB*, was shown to be important for resistance to ethanol stress (Farewell *et al*., 1998). An Δ*rpoS* deletion mutant was unable to induce *uspB* transcription and was sensitive to ethanol stress, however, when the Δ*rpoS* was complemented with wildtype *rpoS*, induction of *uspB* was restored (Farewell *et al*., 1998). Kobayashi *et al*. (2006) reported the ability of *E. coli* to tolerate drugs involved RpoS-dependent multidrug transporter MdtE, whose expression is increased at stationary phase. The multidrug transporter operon *mdtEF* genes were not induced at stationary phase in the Δ*rpoS* mutant and the tolerance of the stationary-phase cells to crystal violet was reduced in the Δ*rpoS* (Kobayashi *et al*., 2006). The authors also observed MdtEF-dependent tolerance to other drugs such as erythromycin, doxorubicin, ethidium bromide, benzalkonium and deoxycholate, but not to nalidixic acid or norfloxacin (Kobayashi *et al*., 2006).

When *E. coli* is exposed to a specific stress condition, the stress response mounted by the bacterium may be able to protect it against subsequent exposure to a different stress. For example, *E. coli* previously exposed to heat shock retained stress protection that allowed it survive when it subsequently encountered high acidity (pH 2) (Hsu *et al*., 2009). This is referred to as cross-protection (Ryu & Beuchat, 1998). Cross-protection is reasonable in that the bacterium is exposed to multiple stresses in the environment and protection against one stress condition may confer protection against others. RpoS has also been established to be crucial for stress responses in many other Gram negative bacteria such as *Salmonella enterica* serovar Typhimurium (McMeechan *et al*., 2007); *Citrobacter rodentium* (Dong *et al*., 2009); *Shigella flexneri* (Small *et al*., 1994); *Pseudomonas aeruginosa* (Murakami *et al*., 2005); *Pseudomonas fluorescens* (Stockwell & Loper, 2005); *Vibrio cholerae* (Yildiz & Schoolnik, 1998);*Yersinia enterocolitica* (Badger & Miller, 1995); and *Azotobacter vinelandii* (Sandercock & Page, 2008).

1.4. LOSS OF FUNCTION *RPOS* MUTATION

Despite the important role that RpoS plays in stress resistance, the *rpoS* gene displays substantial polymorphism and different alleles are found both in wild-type K12 strains (Atlung *et al*., 2002) and in natural isolates of *E. coli* obtained from different sources (Bhagwat *et al*., 2005; Dong *et al*., 2009; Waterman & Small, 1996). Ferenci *et al*. (2011) showed that nine of 31 strains (29%) from the ECOR collection had mutations in the *rpoS* gene. Also, Spira *et al*. (2011) showed that about 40% of reconstructed stock cultures of *E. coli*, transferred between laboratories that previously had high RpoS levels had accumulated *rpoS* mutations.

In addition, *E. coli* has been shown to sacrifice its stress protection abilities in some environments in order to gain fitness advantage. In a carbon-limited environment, which represents a common condition in the environment, *E. coli* was shown to accumulate mutations in *rpoS* (King *et al*., 2006). Mutations in *rpoS* gene which result in loss of RpoS activity, not only abolishes transcription of RpoS-dependent genes in stationary phase, but leads to increased induction of σ^{70}-dependent ones (Farewell *et al*., 1998). Loss of σ^{S} during growth in nutrient-limiting conditions causes a growth advantage accompanied by elevated expression of genes contributing to fitness, e.g. uptake systems requiring σ^{70} for their expression (Notley-Mcrobb *et al*., 2002). For example, an *rpoS* mutant grows better than the wildtype strain in succinate minimal media (Chen *et al*., 2004). These mutations compromise the ability of such *E. coli* to respond to environmental stress. This trade-off of stress protection for nutritional competence has been termed SPANC ("self-preservation and nutritional competence") balance (King *et al*., 2004). Mutations that confer a growth and survival advantage in stationary-phase cultures of *E. coli* (so-called growth advantage in stationary phase, or GASP, mutants) are frequently found to map to the *rpoS* locus (Zambrano *et al*., 1993).

Although it has been established that RpoS plays a crucial role in bacterial persistence in the environment, some of these roles in specific environmental niches are not fully understood yet (Schellhorn, 2014). It will be imperative to understand if loss-of-function mutations are present in the *rpoS* gene of *E. coli* persisting in the soil, considering that the nutrient levels in soil are considered lower than in the gastrointestinal tract of the host (Savageau, 1983) and also that *E. coli* may encounter multiple stresses such as UV radiation (if close to soil surface), low pH, low nutrients, temperature, desiccation, competition, predation to thrive in the soil (Fig. 1.3).

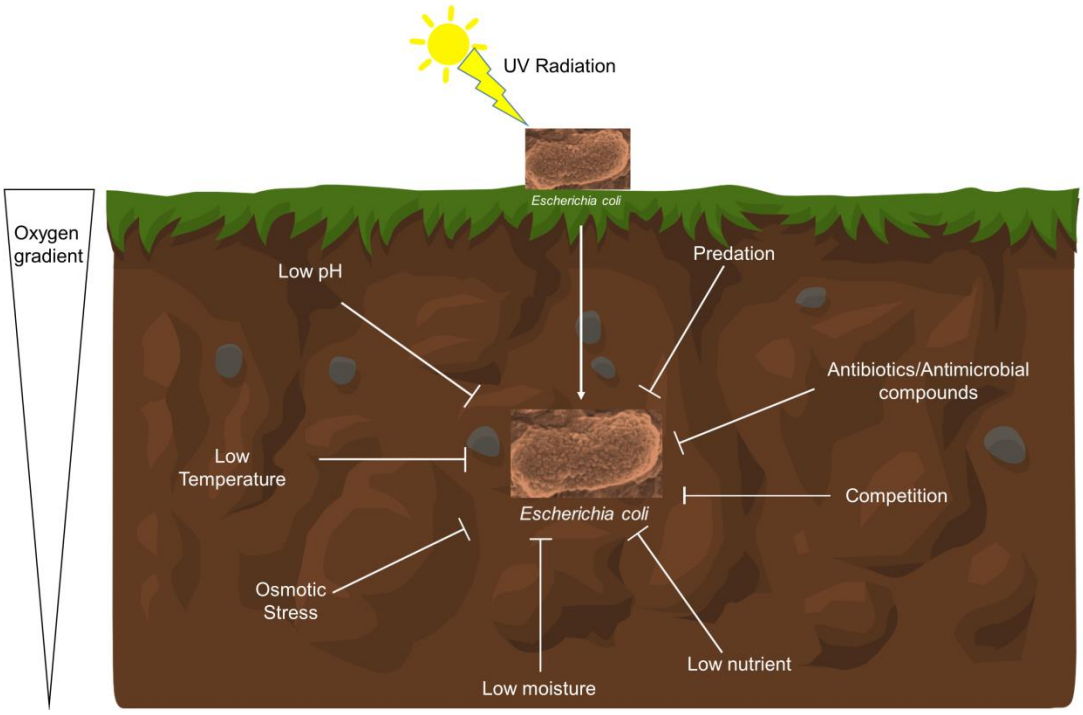


Figure 1.3: Stresses encountered by *Escherichia coli* in the soil environment

1.5. REGULATION OF RPOS IN *E. COLI*

RpoS is so tightly regulated that it does not compete for core RNAP under conditions when it is not needed (Battesti *et al*., 2011). This is because recruiting the general stress response is an energy consuming process for the cell (Pletnev *et al*., 2015), hence the bacterium needs to switch it on only when necessary.

1.5.1 Transcriptional control of RpoS

RpoS is located downstream of *nlpD* (Fig. 1.2), a lipoprotein involved in cell division, whose expression is not stationary phase-induced. The *rpoS* gene of *E. coli* has one major promoter (*PrpoS*) located within the *nlpD* gene. Four other promoters of *rpoS* (*PrpoS*$_{1\text{-}4}$; Fig 1.2) have been identified (Mendoza-Vargas *et al*., 2009). These cryptic promoters are likely to transcribe *rpoS*, as they were experimentally determined. Since *nlpD* and *rpoS* form an operon, σ^{70}-dependent *nlpD* promoters contribute to the basal level of *rpoS* expression, which is often undetectable in exponentially growing cells (Lange & Hengge-Aronis, 1994). RpoS levels are virtually undetectable during the exponential growth phase probably due to proteolysis, but they increase to 30% of the

level of σ^{70} during stationary phase. σ^{70} remains constant throughout the exponential and stationary phases (Jishage & Ishihama, 1995). *PrpoS* initiates a monocistronic *rpoS* mRNA transcript comprising a 567 bp untranslated region and typical σ^{70}-dependent -10 and -35 promoter sequences (Venturi, 2003). The alarmone, guanosine-3′,5′-bispyrophosphate (ppGpp) increases the transcription of the *rpoS* promoter (*PrpoS*) and it has been positively correlated with *rpoS* mRNA levels (Lange *et al*., 1995). Also, ppGpp increases the ability of σ^S to bind to RNAP core compared to σ^D and σ^S binds less efficiently to RNAP core when ppGpp is absent (Jishage *et al*., 2002).

Another positive regulator of *rpoS* transcription is BarA, the sensory histidine kinase of the BarA/UvrY two-component signal transduction system, which plays a role in the carbon storage regulatory system. BarA is required for the induction of *rpoS* during exponential phase and a Δ*barA::lacZ* fusion showed reduced levels of *rpoS* mRNA and RpoS protein in the exponential growth phase (Mukhopadhyay *et al*., 2000). The level of *rpoS* transcription in the Δ*barA::lacZ* fusion was about 85% reduced compared to the wildtype, however, *rpoS* transcript level was restored to wildtype levels when complemented with plasmid-borne *barA* (Mukhopadhyay *et al*., 2000). A negative regulator of *rpoS* transcription is the phosphorylated response regulator ArcA, which directly binds to two sites located upstream and downstream of the *PrpoS* region. Phosphorylated ArcA (ArcA-P) was shown to directly repress *rpoS* transcription by three- to four fold (Mika & Hengge, 2005). Cyclic AMP (cAMP) and cyclic AMP receptor protein (CRP) also negatively regulate *rpoS* transcription. When the gene encoding adenylate cyclase, which is responsible for cAMP biosynthesis, is deleted, higher RpoS levels are detected in exponentially growing cells (Lange & Hengge-Aronis, 1994b). These factors are summarised in Fig. 1.4a.

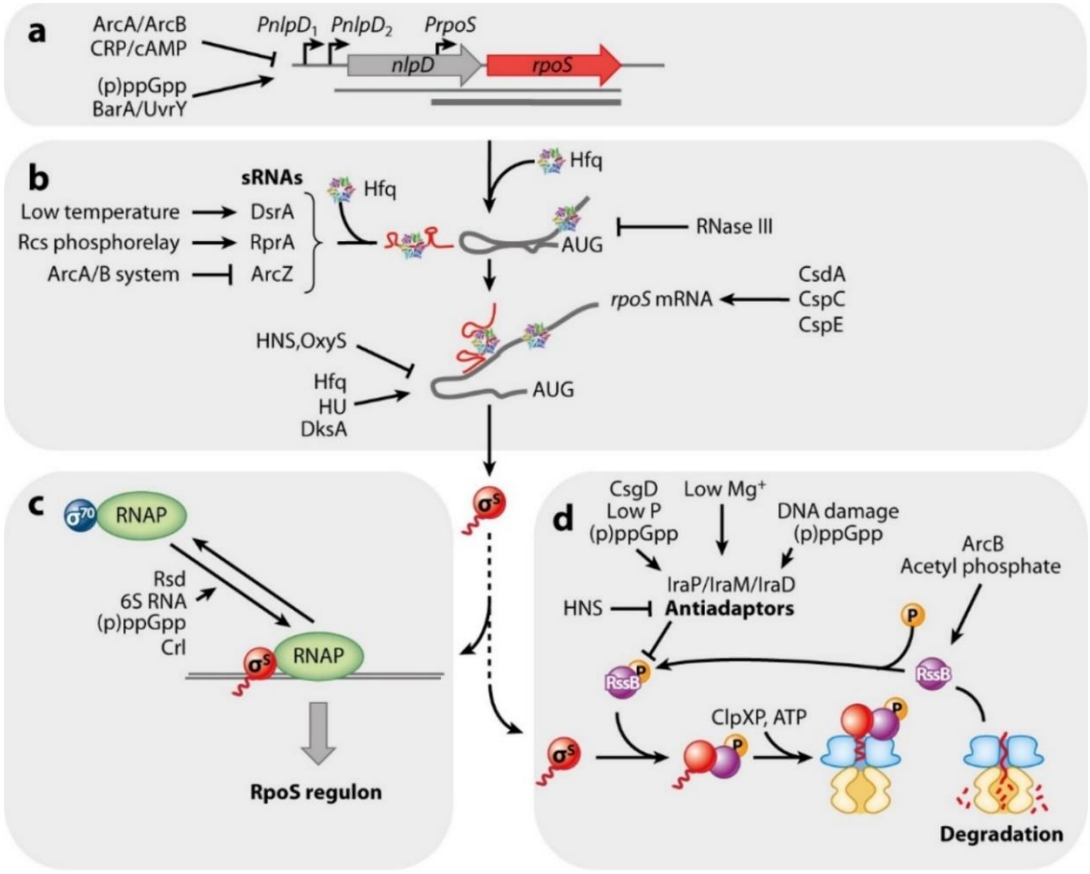


Figure 1.4: Regulation of RpoS expression, stability and activity. Different levels of RpoS regulation are shown: (a) transcriptional regulation, (b) translational regulation, (c) regulation of RpoS activity, and (d) proteolytic regulation. The figure is taken from Battesti *et al.* (2011).

1.5.2 Translational control of RpoS

When *rpoS* is transcribed from the main promoter (*PrpoS*) starting 567 nucleotides upstream of the AUG for *rpoS,* it produces a transcript which has a long 5′ untranslated region (UTR) which is critical for translational regulation (Venturi, 2003; Battesti *et al*., 2011). The long 5′ UTR of the *rpoS* transcript folds into a stem-loop that obstructs the ribosome binding site and minimizes translation of *rpoS* (Cunning *et al*., 1998; Muffler *et al*., 1997a). Under RpoS-inducing conditions, the mRNA secondary structure is either relaxed or gets changed in such a way that translational initiation can take place (Muffler *et al*., 1996a). Translation of *rpoS* mRNA is induced by increased osmolarity, low temperature and during late exponential phase (Lange & Hengge-Aronis, 1994; Muffler *et al*., 1996a; Sledjeski *et al*., 1996). A secondary translational initiation region (STIR) was identified within the *rpoS* gene and starts from 143 nucleotides downstream

the AUG for *rpoS*. The STIR carries a Shine–Dalgarno sequence (AGGGAG) and the GUG start codon starting 160 nucleotides from the AUG for *rpoS* (Subbarayan & Sarkar, 2004a). The STIR is functional and independent of TIR since the deletion of TIR does not stop the translation of *rpoS* mRNA from STIR (Subbarayan & Sarkar, 2004b). The authors further showed that a mutation in STIR affects translation but not transcription of *rpoS* (Subbarayan & Sarkar, 2004b). The inhibitory loop in the *rpoS* mRNA is overcome by non-coding small RNAs (sRNAs) thus stimulating *rpoS* translation. The sRNAs require the RNA chaperone protein Hfq for their activity (Brennan & Link, 2007). Hfq stabilizes the sRNAs and promotes pairing with mRNA targets (Soper *et al*., 2011). The *hfq* gene encodes the RNA-binding protein HF-I, which is essential for *rpoS* translation since an *hfq* mutant has strongly reduced RpoS levels (Muffler *et al*., 1997a). In cellular extracts, Hfq was found to co-precipitate with *rpoS* mRNA (Zhang *et al*., 1998). Hfq binding leads to structural changes in sRNA that ensures sRNA stability against RNase E degradation of *rpoS* mRNA (Henderson *et al*., 2013). Three Hfq-dependent sRNAs (DsrA, RprA and ArcZ) stimulate *rpoS* translation through the pairing between complementary nucleotides in the sRNA and the 5′ UTR of the *rpoS* mRNA so as to open the hairpin, hence freeing the ribosome binding site and allowing translation to proceed (Battesti *et al*., 2011). Nucleoid protein HU, also directly enhances *rpoS* translation by binding tightly to the *rpoS* 5′ UTR and in Δ*hupA* and Δ*hupB* cells lacking HU, RpoS levels are reduced (Balandina *et al*., 2001).

OxyS, is an sRNA that represses *rpoS* translation by binding Hfq and preventing it from directing *rpoS* translation (Zhang *et al*., 1998). Nucleoid histone-like protein H-NS, also negatively regulates *rpoS* translation (Yamashino *et al*., 1995). An *hns* mutant was shown to increase RpoS levels in exponential phase cells due to increased translation efficiency of *rpoS* mRNA and enhanced stability of RpoS (Yamashino *et al*., 1995). Later studies show that H-NS inhibits the expression of *iraD* and *iraM*, the genes encoding anti-adaptor proteins that bind to RssB and prevent RpoS degradation, thereby decreasing RpoS stability (Battesti *et al*., 2012). So, in a Δ*hns* mutant, anti-adaptor protein levels increase, thereby enhancing the stability of RpoS (Zhou & Gottesman, 2006). These factors are summarised in Fig. 1.4b.

1.5.3 Regulation of RpoS activity

RpoS activity is further regulated at the formation of the holoenzyme and the interaction of the holoenzyme with promoters. Although σ^S levels increase as cells enter stationary phase, the levels do not exceed one-third of σ^D levels (Jishage and Ishihama 1995; Piper *et al*., 2009). RpoS and RpoD sequences have about 85% similarity (Mulvey & Loewen, 1989), and despite the sequence similarity, they regulate mainly distinct regulons, largely due to differences in promoter recognition sequences (Gaal *et al*., 2001; Typas *et al*., 2007a). Nonetheless, many RpoS-dependent promoters can be read by RpoD and likewise, RpoD-dependent promoters can also be read by RpoS (Tanaka *et al*., 1993). All sigma factors compete for the same pool of RNAP core. Thus, conditions that upregulate RpoD limits the RNAP core available for RpoS to bind to RNAP core (Farewell *et al*., 1998). Thus, for RpoS to outcompete RpoD for the RNAP core, it must have mechanisms for preferentially enhancing its binding to the RNAP core and recognising its promoters, since it is always present in molar amounts lower than RpoD (Jishage and Ishihama 1995; Piper *et al*., 2009).

Under conditions that RpoS is elevated, RpoS is able to acquire RNAP core and bind to its promoters. For example, addition of up to 400 mM potassium glutamate (K glutamate) *in vitro*, mimicking the intracellular ionic conditions under hyper-osmotic stress, enhanced preferential recognition by RpoS holoenzyme ($E\sigma^S$) but inhibited recognition by RpoD holoenzyme ($E\sigma^D$) (Ding *et al*., 1995). Furthermore, at 300 mM K glutamate, *osmY* transcription by $E\sigma^S$ was enhanced 1.4-fold while $E\sigma^D$ is inhibited by more than 5-fold, together leading to the preferential transcription of *osmY* by $E\sigma^S$ (Kusano & Ishihama, 1997). Although the promoter of *osmY* gene can be recognised by $E\sigma^D$ and $E\sigma^S$, Integration Host Factor (IHF), CRP, and leucine-responsive regulatory protein (Lrp) preferentially prevent RpoD from accessing the RNAP core by binding to sites on the promoter region. The $E\sigma^D$ is inhibited from binding to the *osmY* promoter in the presence of the repressors, however, $E\sigma^S$ is able to form open complexes on *osmY* promoters already bound by the repressors (Colland *et al*., 2000). The combination of increased levels of σ^S, enhanced association of σ^S with the core polymerase, and enhanced promoter recognition by $E\sigma^S$ explains the change in gene expression patterns in stationary phase (Loewen *et al*., 1998).

RNA polymerase holoenzyme assembly factor (Crl), regulator of σ^D (Rsd), and 6S RNA have been implicated in enhancing the ability of RpoS to compete effectively for the RNAP core. Crl protein is necessary *in vivo* for maximal expression of RpoS-dependent

promoters but does not increase RpoS levels (Pratt & Silhavy, 1998). 6S regulatory RNA negatively affects $E\sigma^D$ by binding to it (and not $E\sigma^S$) thereby rendering $E\sigma^D$ inactive to recognise and bind promoters (Wassarman, 2007). 6S RNA is then released from $E\sigma^D$ as growth resumes. Interaction with 6S does not increase the availability of RNAP core to σ^S, but reduces the levels of free RpoD to compete with RpoS for RNAP core (Wassarman, 2007). Rsd binds RpoD in stationary-phase cells. Rsd interacts with RpoD and limits its ability to interact with RNAP core, thus helping RpoS compete for the RNAP core (Yuan *et al*., 2008). These factors are summarised in Fig. 1.4c.

1.5.4 Proteolytic control of RpoS

Proteolysis of RpoS is an important mechanism for maintaining low levels of RpoS under optimal growth and unstressed conditions. RpoS is a highly unstable protein in exponentially growing non-stressed cells (half-life of 1.4 min) and its stability can increase to 20 min in stationary phase (Lange and Hengge-Aronis, 1994). Proteolysis of RpoS ceases or slows down when RpoS is induced after stress treatment. For example, the half-life is increased up to 50 min after osmotic shock (Muffler *et al*., 1996a).

RpoS is degraded by ClpXP protease which targets a region between amino acid residues 173 and 188 of the RpoS protein (Schweder *et al*., 1996). ATP is required for ClpXP to degrade RpoS and there is no RpoS degradation at all when ATP levels are low, thus showing that cellular ATP levels directly control RpoS stability (Peterson *et al*., 2012). Δ*clpX* and Δ*clpP* cells increases the stability of RpoS at exponential phase and RpoS levels in the mutants is increased by fourfold compared to wildtype *E. coli* (Schweder *et al*., 1996). RssB is an adaptor protein required by ClpXP for degradation of RpoS because RpoS is not directly recognized by ClpXP (Muffler *et al*., 1996b). RssB binds directly to RpoS and presents RpoS to ClpXP for proteolysis (Becker *et al*., 1999; Zhou & Gottesman, 1998). An Δ*rssB* mutant shows high levels of RpoS in exponential phase that is about 10-fold higher than that of the cells with wildtype *rssB*. RpoS is stable with a half-life of over 30 min at the exponential phase in a Δ*rssB* mutant (Zhou & Gottessman, 1998). Another factor involved in the proteolysis of RpoS is a “turnover element” described in RpoS (Becker *et al*., 1999). The turnover element has a proteolysis-promoting motif and interacts directly with RssB in a phosphorylation-dependent manner. The authors identified amino acid lysine at codon 173 (K173) in RpoS to be essential for the “turnover element”-mediated RpoS proteolysis (Becker *et al*., 1999).

DnaK plays a positive role in RpoS control by protecting RpoS from ClpXP and increasing RpoS stability (Muffler *et al*., 1997b). DnaK mutation results in reduced RpoS levels in the stationary phase (Muffler *et al*. 1997b). Also, some proteins that have a negative role on RpoS proteolysis by inhibiting RssB activity have been identified. They are the so-called anti-adaptor proteins IraP, IraM, and IraD and each of them have a distinct mode of interacting with RssB (Battesti *et al*., 2013; Micevski *et al*., 2015). These factors are summarised in Fig. 1.4d.

1.6. SOIL AS A STRESSFUL ENVIRONMENT FOR *E. COLI*

While the lower digestive tract is considered to be the primary habitat of *E. coli*, faecal dissemination leads to the passage of *E. coli* to its secondary habitat (outside of the host). The non-host environments can be dynamic and differ from the host in terms of temperature, nutrient availability, and competing microflora and predators. Soil is a complex mixture of minerals and organic matter on the earth's surface that serves as a natural medium for the growth of land plants. The solid fraction of soil, i.e. minerals and organic matter, make up about 50% of the soil by volume with the remaining 50% consisting of air/gases and water-filled soil pores (Sylvia *et al*., 2005). The soil provides a vital environment for many life forms, from microorganisms to centipedes and higher animals such moles and gophers. Different biotic and abiotic factors affect the structure and composition of soil. Biotic factors are the biological component of soil and they include archaea, bacteria, fungi, algae, and invertebrates (earthworms, millipedes, centipedes, snails, slugs, mites, nematodes, protozoans). Abiotic factors are non-living conditions which influence life in the soil environment such as pH, temperature, moisture content, texture and light intensity. Together these factors determine the diversity and activity of microorganisms in the soil environment (Voroney & Heck, 2015).

When *E. coli* are shed into the soil through faeces, they encounter several biotic and abiotic factors which are different from the conditions in the hosts. In order for *E. coli* to survive in the soil, it must be able to overcome some of the stresses encountered in the soil environment. The persistence of *E. coli* in the soil environment thus depends on how the bacterium responds to these stresses in the soil environment. The stress response determines the survival during starvation, adaptation to the presence of antibiotics, synthesis of antibiotic substances, and interactions with eukaryotic symbionts such as fungi and protozoans (Świeciło & Zych-Wezyk, 2013).

1.6.1 Abiotic stress in soil

Abiotic stresses caused by environmental conditions such as bright light, UV, high and low temperatures, freezing, low moisture/desiccation and salinity contribute to the rapid decline of *E. coli* in the external environment. In some soils, some of these stressors occur in combination, thus *E. coli* coming from the gut must be able to protect itself in order to survive and function in the soil environment (Świeciło & Zych-Wezyk, 2013).

Varying temperature contributes stress on *E. coli* in the environment (Semenov *et al*., 2007). It was shown that *E. coli* O157 populations declined faster at 18°C than 4°C (Moynihan *et al*., 2013). As soil temperature increases, the rate of decline of *E. coli* in soil increases (Vidovic *et al*., 2007; Ishii *et al*., 2006). Since *E. coli* generally experiences lower temperature in the soil than in the gut of the host (~37°C), cold stress is experienced in the soil (Vidovic *et al*., 2007). Adaptations to low temperature have been identified in *E. coli* and some of the proteins involved in adaptation to low temperature are RpoS-dependent (Brennan *et al*., 2013). Availability of moisture in soil is crucial to survival of *E. coli* in the soil. High moisture availability in soil promotes bacterial survival (Oliveira *et al*., 2004) while low soil moisture leads to a greater decline of *E. coli* in soil (Williams *et al*., 2015). In soil with low moisture, there is little or no available water for bacterial activities and this may cause cell death. *E. coli* has the ability to survive and multiply under low moisture conditions (Solo-Gabriele *et al*., 2000). Soil pH is a measure of the acidity or alkalinity in soils. Soil pH has been shown to affect nutrient availability in soil by controlling the chemical forms of the nutrient (Kemmitt *et al*., 2006). It also influences biomass composition of fungi and bacteria in soils (Fierer & Jackson, 2006; Rousk *et al*., 2009). Bacterial growth and survival is increased at neutral pH (6.7 to 7.5) in the soil, however, acidic pH stresses bacteria and inhibits their growth (Arao, 1999).

The soil is generally a low nutrient environment when compared to the gastrointestinal tract of the host (Savageau, 1983), although soil nutrients increase when there is an influx of organic matter such as decaying vegetation, animal faeces and plant exudate. So, when bacteria are released into the soil, they must be able to reduce their rate of metabolism so as to adjust to the relatively low organic carbon available in the soil (Franz & van Bruggen, 2008). Limited nutrient availability may result in increased cell death. For example, higher bacterial decline rates were observed in subsoils that had lower nitrogen compared to top soil (Zhai *et al*., 1995). Notwithstanding this, some *E.*

coli strains are metabolically flexible and are able to utilise diverse substrates for growth and survival (Ihssen *et al*., 2007). RpoS is also important for the response of *E. coli* to starvation (Gawande & Griffiths 2005; Nair & Finkel, 2004). The population per gram is likely to be a small fraction of their population density in the gastrointestinal tract, so the small numbers of cells may only require a very limited supply of nutrients for turnover. The level of salt (NaCl) in soil could also affect the survival of *E. coli*. Heavy rainfall and loss of moisture in soil causes fluctuations in the salt concentration, which may result in hypo- or hyper-osmolarity in the soil. Salinity exerts a stress on *E. coli* in the environment and the higher the salinity, the greater the stress (Anderson *et al*., 1979). Survival of *E. coli* O157 in soil is negatively correlated with soil salinity (Ma *et al*., 2013).

1.6.2 Competing microorganisms

The presence of other microorganisms in soil leads to a greater decline of *E. coli* in live soil compared to survival in sterile soil (Vidovic *et al*., 2007; Semenov *et al*., 2007). This inhibitory effect of the soil microflora and fauna are due to the competition for nutrients and water in the soil and the production of inhibitory substances against *E. coli* (antagonism). The presence of *Bdellovibrio bacteriovorus*, a soil-dwelling bacterium that parasitizes *E. coli* cells, increases the death rate of *E. coli* in soil (Klein & Casida Jr., 1967). Also, actinomycetes, which produce a wide range of antibiotics in soil (Nanjwade *et al*., 2010), may inhibit *E. coli* in soil and reduce their survival. RpoS confers on *E. coli* the ability to tolerate a wide array of antibiotics (Greenway & England, 1999; Kobayashi *et al*., 2006). RpoS mediates tolerance to antibiotics through mechanisms such as expression of multidrug transporters to prevent influx and enhance efflux antibiotics (Kobayashi *et al*., 2006) and biofilm formation (Ito *et al.,* 2009). Bacteriophages, which are viruses that infect bacteria, could also contribute to the rapid decline of *E. coli* in the soil. Bacteriophages may influence the ecology of soil microbial communities and cause mortality to the microorganisms in soil (Swanson *et al*., 2009).

1.6.3 Protozoans

Protozoans are unicellular eukaryotes that range between 5 to 500 µm in diameter and are important members of the soil ecosystem. Protozoans are most abundant near the soil surface, particularly in the upper 15 cm (Hoorman, 2011), probably because of the high nutrient availability and prey population around the rhizosphere. Protozoans are

important in regulating bacteria and algae populations in soil. When *E. coli* is ingested by the protozoans, the consumed bacteria are packed in the food vacuole, where they are digested. Digested food expelled into the environment provides organic matter that is important in nutrient cycling in the environment. As protozoans prey on bacteria, they release excess nitrogen that is available for use by plants and other soil organisms (Hoorman, 2011). Furthermore, protozoans help maintain the ecological balance in the soil. When they graze on bacteria, protozoa stimulate growth of other predation-resistant bacterial populations and promote soil aggregation (Hoormann, 2011). Gram-negative bacteria such as *E. coli* are preferred as preys by protozoans compared to Gram-positive bacteria, which have a protective cell wall (Murase *et al*., 2006; Rønn *et al*., 2002)

Protozoans are classified into three groups: *Ciliates*, which are the largest and use their cilia for locomotion. *Amoebae* are medium sized protozoans and move using their pseudopodia. *Flagellates* are the smallest in size and use their flagellae to move (Hoormann, 2011). In agricultural soils where bacterial numbers are high, flagellates and amoebae are the most abundant protozoans (Ekelund & Rønn, 1994). In general, high clay-content soils contain a higher number of the smaller protozoans probably because of the little pore sizes, whereas, the larger protozoans are abundant in sandy and uncultivated soils (Hoormann, 2011). Protozoa community structure varies in the different layers within the soil column (Murase *et al*., 2006). Soil moisture significantly affects the protozoa-bacteria interaction and the highest protozoan activity is seen in moist soil (Kuikman *et al*., 1989). Severe desiccation/drought does not eliminate protozoan populations, because many of them form inactive cysts, which can withstand extreme environmental conditions (Rønn *et al*., 2012).

1.6.3.1 Acanthamoeba polyphaga

Acanthamoeba spp. are free-living protozoans that are found in various external environments including soil (Reyes-Batlle *et al*. 2016; Tanveer *et al*., 2015), water (Hamilton *et al*., 2016; Mahmoudi *et al*., 2015), and dust (Costa *et al*., 2010; Niyyati *et al*., 2009) from different parts of the world. *Acanthamoeba* spp. have also been isolated from animal hosts such as mosquitoes (Otta *et al*., 2012) and wild squirrels (Lorenzo-Morales *et al*., 2007). *Acanthamoeba* aids for nitrogen mineralisation by rapidly releasing mineral nitrogen from bacterial biomass under carbon limitation (Sinclair *et al*., 1981). *A. polyphaga* acts as a secondary decomposer to re-mineralize soil with carbon, nitrogen, and phosphorous by consuming bacterial primary decomposers

(Siddiqui & Khan, 2012). *A. polyphaga* digests the primary decomposers and releases the nutrients from its food vacuole into the soil (Marciano-Cabral & Cabral, 2003).

Based on their important roles in the soil, amoebae are regarded as the most important group of soil protozoa (Ekelund & Rønn, 1994). Free-living amoebae are the main consumers of bacteria in soil and are responsible for up to 60% of the total reduction in bacterial population (Sinclair *et al*., 1981). *Acanthamoeba* spp are an important contributor to a functional soil ecosystem as they induce rapid shifts in rhizosphere bacterial community composition and plays a dominant role in influencing bacteria–plant interactions (Rosenberg *et al*., 2009). Protozoan predation constantly re-mobilizes essential nutrients for plant uptake by releasing organic nitrogen into soil, which is then readily available for other soil organisms and taken up by plant roots (Bonkowski *et al*., 2000; Ekelund & Rønn, 1994; Zwart *et al*., 1994).

Acanthamoeba has two stages in its life cycle, an active trophozoite stage that exhibits vegetative growth (Fig. 1.5) and a dormant cyst stage with minimal metabolic activity (Marciano-Cabral & Cabral, 2003). The trophozoite is about 25 - 40 µm and is the actively feeding and dividing form. They feed on bacteria, algae, and yeast in the environment by phagocytosis, using its pseudopodia, which are also used for locomotion. It can also feed axenically (without bacterial prey) on liquid nutrients through pinocytosis (Bowers, 1977). When nutrient is available, *A. polyphaga* remains as trophozoite but when nutrients are exhausted and harsh conditions are experienced, the amoeba transforms into a dormant, non-dividing cyst that is highly resistant to starvation, desiccation, extreme temperature, pH and UV radiation. When nutrients and favourable conditions are restored, the cysts undergo excystment, the process that re-establishes the trophozoite form (Lemgruber *et al*., 2010).

When *A. polyphaga* preys on susceptible *E. coli*, it ingests and digests the prey (De Moraes & Alfieri, 2008), however, pathogenic strains such as *E. coli* O157:H7 survives and multiplies intracellularly in *A. polyphaga* thus enhancing survival (Barker *et al*., 1999). Survival of *E. coli* is enhanced in sterile soil because protozoans are not present, as well as other microorganisms that could compete for nutrient and water. Both *Acanthamoeba* trophozoites and cysts can retain viable bacteria and may serve as natural reservoirs and vehicles for the dissemination of bacteria with pathogenic potentials in the environment (Khan, 2006). Some of these bacteria not only survive but grow within the amoeba and they include *E. coli* O157 (Barker *et al*., 1999; Chekabab

et al., 2013), methicillin-resistant *Staphylococcus aureus* (Huws *et al*., 2006); *Legionella pneumophila* (Hales & Shuman, 1999)*, Mycobacterium avium* (Adékambi *et al*., 2006; Strahl *et al*., 2001), *Listeria monocytogenes* (Lambrecht *et al*., 2015), *Yersinia enterocolitica* (Lambrecht *et al*., 2015), *Helicobacter pylori* (Winiecka-Krusnell *et al*., 2002), *Campylobacter jejuni* (Axelsson-Olsson *et al*., 2010; Olofsson *et al*., 2015; Snelling *et al*., 2005).

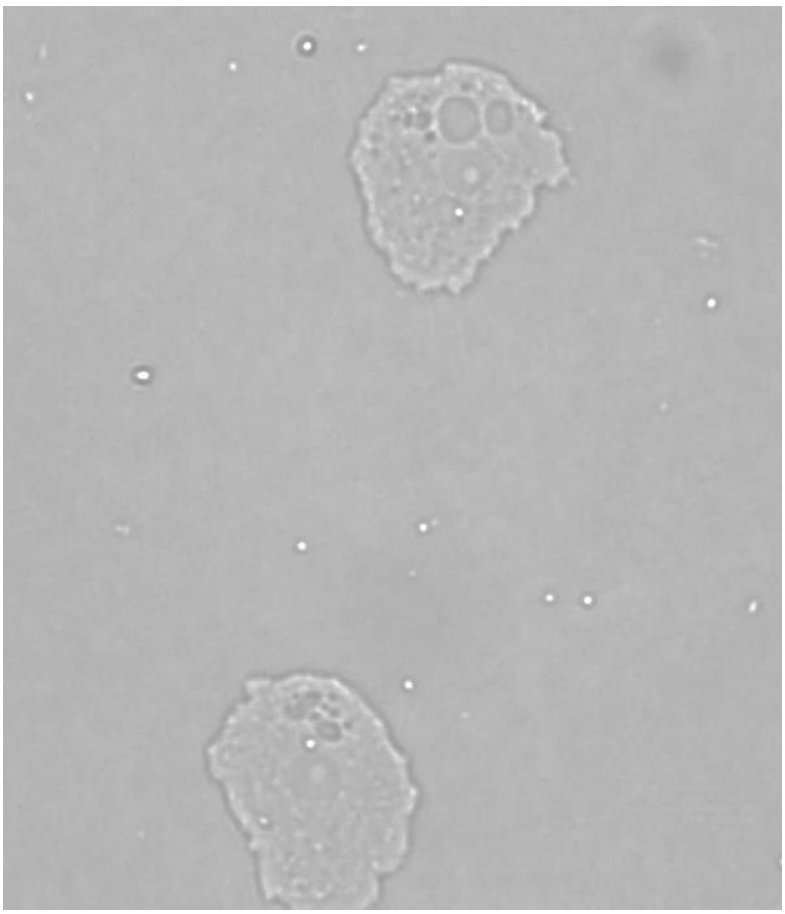


Figure 1.5: *Acanthamoeba polyphaga* trophozoite. Magnification is x600. The figure is from this study

A. polyphaga can be pathogenic for humans, causing sight-threatening infection of the cornea called *Acanthamoeba* keratitis (Lorenzo-Morales *et al*., 2007). It is also the causative agent of cutaneous lesions, sinus infections and a rare but fatal encephalitis, known as granulomatous amoebic encephalitis (Marciano-Cabral & Cabral, 2003). *Acanthamoeba* is frequently encountered in the environment and 85% of healthy

individuals in an ethnically diverse population had anti-*Acanthamoeba* antibodies, suggesting exposure to the protozoans (Brindley *et al*., 2009).

1.6.3.2 Tetrahymena pyriformis

Tetrahymena are free-living ciliates usually found in fresh water (Zufall *et al*., 2013) but have also been isolated from soil (Brandl *et al*., 2005), dish clothes (Chavatte *et al*., 2014) and slow sand filters (Haig *et al*., 2015). *T. pyriformis* is a filter feeder which mainly consumes suspended cells. The cell is about 50 - 60 µm long and 30 µm in width (Vaerewijck *et al*., 2014). The cell surface is covered with cilia, which it uses for locomotion (Fig. 1.6). *T. pyriformis* uses their cilia to direct suspended particles/bacteria into its oral groove and then produce food vacuoles through phagocytosis. The food vacuole is subsequently fused to the lysosome for digestion (Bozzone & Martin 2000). Following uptake, some bacteria evade digestion and multiply within the food vacuoles of *T. pyriformis*, where they are shielded from the adverse environmental conditions such as acid, biocides and antibiotics (Gourabathini *et al*., 2008; Smith *et al*., 2012). *T. pyriformis* is cultured axenically in rich medium such as PPY. *T. pyriformis* is generally believed not to form cysts (Vaerewijck *et al*., 2014), although, it was observed that exposure to ethanol made them form cyst-like structures (Nilsson, 2005). Although the life cycle of *T. pyriformis* has not been described, the life cycle of a close species, *T. thermophila*, has been described. It consists of a sexual and asexual stage. Asexual reproduction is by binary fission, which is accompanied by a various morphogenetic events that result in the development of duplicate sets of cell structures, one for each daughter. The sexual stage of the life cycle is by conjugation and involves no cell division (Collins & Gorovsky, 2005).

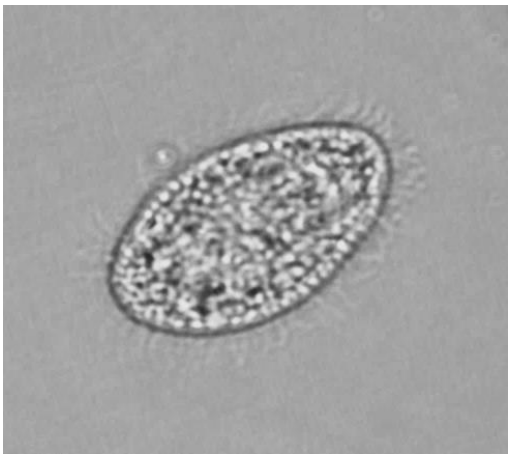


Figure 1.6: *Tetrahymena pyriformis*. Magnification is x600. The figure is from this study.

Based on its mode of feeding, filter-feeding, *T. pyriformis* is able to ingest bacteria and other particles in suspension (Boenigk & Novarino, 2004; Dürichen *et al*., 2016). Digestion of food particles in *T. pyriformis* occurs within 30 - 60 min after ingestion in the food vacuole after which undigested foods are packed into pellets and expelled from the food vacuole in intact forms. When non-food particles form parts of the contents of the food vacuole, the undigested foods are excreted much faster (Boenigk & Novarino, 2004). Dürichen *et al*. (2016) demonstrated that food particles were not only ingested by *T. pyriformis* but also digested. Digestion within food particles was characterised by a reduction in food vacuole size and fluorescence intensity with acidification of the food vacuoles. Acidification of the food vacuole is required for fusion with the lysosome and this is then digested. Egestion of the food vacuoles starts in *T. pyriformis* at about 2 h of co-culture (Smith *et al*. 2012).

Some bacteria have been able to survive intracellularly in *T. pyriformis*. When *T. pyriformis* preys on a non-pathogenic *E. coli* K-12*,* some bacterial cells were observed to escape from food vacuoles and evade digestion and viable *E. coli* could be detected inside *T. pyriformis* 14 days after ingestion (Siegmund *et al*., 2013). Co-culture of *T. pyriformis* with *Campylobacter jejuni* showed that *C. jejuni* is able to survive within *T. pyriformis* (Snelling *et al*., 2005) while *Mycobacterium* sp. does not only survive but also multiplies inside food vacuoles of *T. pyriformis* (Strahl *et al*. 2001). Stx-encoding prophage in *E. coli* O157 increases the survival of *E. coli* in the food vacuoles of *T. pyriformis*. When the Stx-encoding prophage was transformed into a previously sensitive and easily digested strain *E. coli* C600, the resulting *E. coli* C600 with the prophage (C600P) survived in the food vacuole better compared to the original C600 strain, showing that the carriage of the Stx-encoding prophage improves the fitness of *E. coli* C600 (Steinberg & Levin, 2007). Intracellular survival of *E. coli* O157:H7 in *T. pyriformis* was suggested to play a possible significant role in the ecology and maintenance of *E. coli* O157:H7 in the environment (Chekabab *et al*., 2013).

1.7. *ESCHERICHIA COLI* BIOFILM AND EXTRACELLULAR MATRIX

Biofilm is the name given to a community of bacteria encased in a self-produced extracellular matrix (ECM) (Wilson, 2001). Biofilm protects *E. coli* against stresses in the environment such as desiccation, predation, antibiotics and plant defence mechanisms (Hall-Stoodley, 2004). Biofilm protects *E. coli* either by limiting access to the embedded cells (Hall-Stoodley *et al*., 2004) or by inducing spatially distributed subpopulations within the biofilm that have increased stress resistance (DePas *et al*., 2013; Hung *et al*., 2013; Serra *et al*., 2013). *E. coli* ECM contains mainly curli and cellulose (Zogaj *et al*., 2001) but may also contain type 1 fimbriae, flagella, polymeric-β-1,6-N-acetylglucosamine (poly-β-1,6-GlcNAc), capsule sugars, and colanic acid (Beloin *et al*., 2008; Holden & Gally, 2004). Several members of the Enterobacteriaceae including *E. coli, Salmonella enterica* and *Citrobacter koseri* produce biofilm as wrinkled colonies on agar plates (DePas *et al*., 2014) or pellicles on air-liquid interface of a static culture (Armitano *et al*., 2014; Hung *et al*., 2013). The wrinkled colony biofilm is also called rugose biofilm or red dry and rough (rdar) phenotype (Römling, 2005; Uhlich *et al*., 2006). Curli and cellulose are required for rugose biofilm formation and are the main components of the ECM of rugose colony biofilm (DePas *et al*., 2013; Zogaj *et al*., 2003). An *E. coli* Δ*csgD* mutant, is unable to produce curli and cellulose (Section 1.7.1 and 1.7.2), and forms an unwrinkled macrocolony that is unable to bind Congo red (Fig. 1.7).

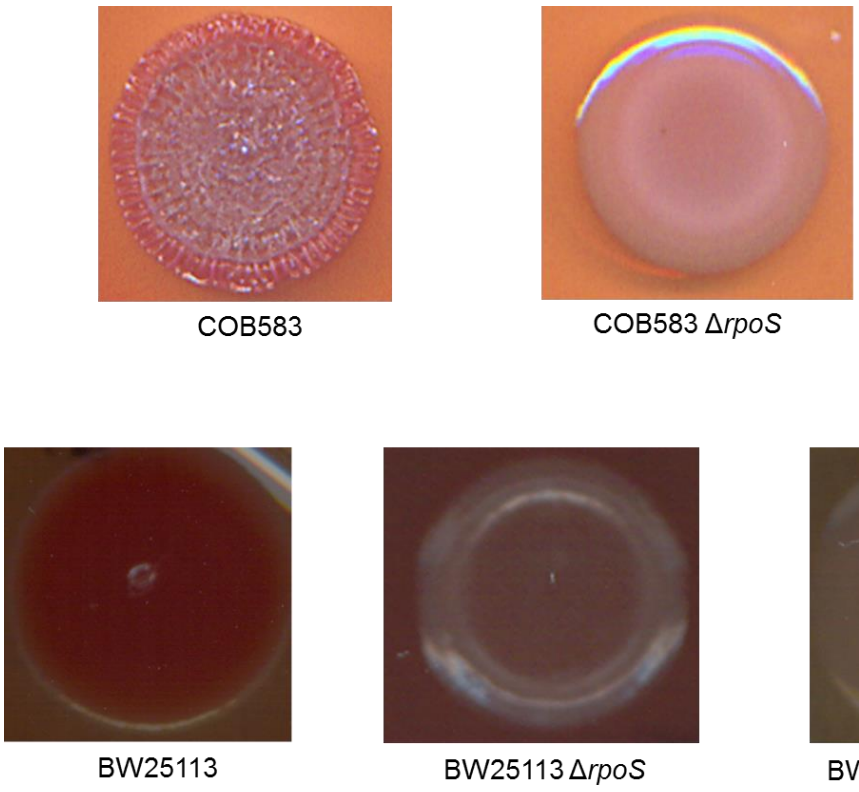


Figure 1.7: Rugose biofilm formation in *E. coli*. Soil-persistent *E. coli* COB583 produced rugose biofilm on congo red agar, indicating curli and cellulose production. Laboratory strain *E. coli* BW25113 produced only curli and not cellulose. Curli production is RpoS- and CsgD-dependent as the BW25113Δ*rpoS* and BW25113Δ*csgD* macrocolonies do not stain red on congo red agar. These data were generated in the present study.

CsgD is a transcriptional regulator of the FixJ/LuxR/UhpA family, that regulates the genes involved in the curli assembly, transport, and structural components and indirectly in cellulose production (Hammar, 1995). The *csgD* gene is regulated by numerous transcriptional regulators and small RNAs (Mika & Hengge, 2014; Ogasawara *et al*., 2010). Environmental signals inducing CsgD expression upregulates curli and cellulose production, which results in rugose biofilm formation (Gerstel *et al*., 2003; Liu *et al*., 2014). The expression of *csgD* is activated by nutrient limitation, low salt and low temperature (Hammar *et al*., 1995; Olsén *et al*., 1989; Römling *et al*., 1998). RpoS is required for *csgD* expression (Olsén *et al*., 1993), and CsgD activity is regulated, indirectly, by the small molecule, cyclic-di-GMP (Römling *et al*., 2005). The regulatory pathway for curli and cellulose production is shown in Fig. 1.8.

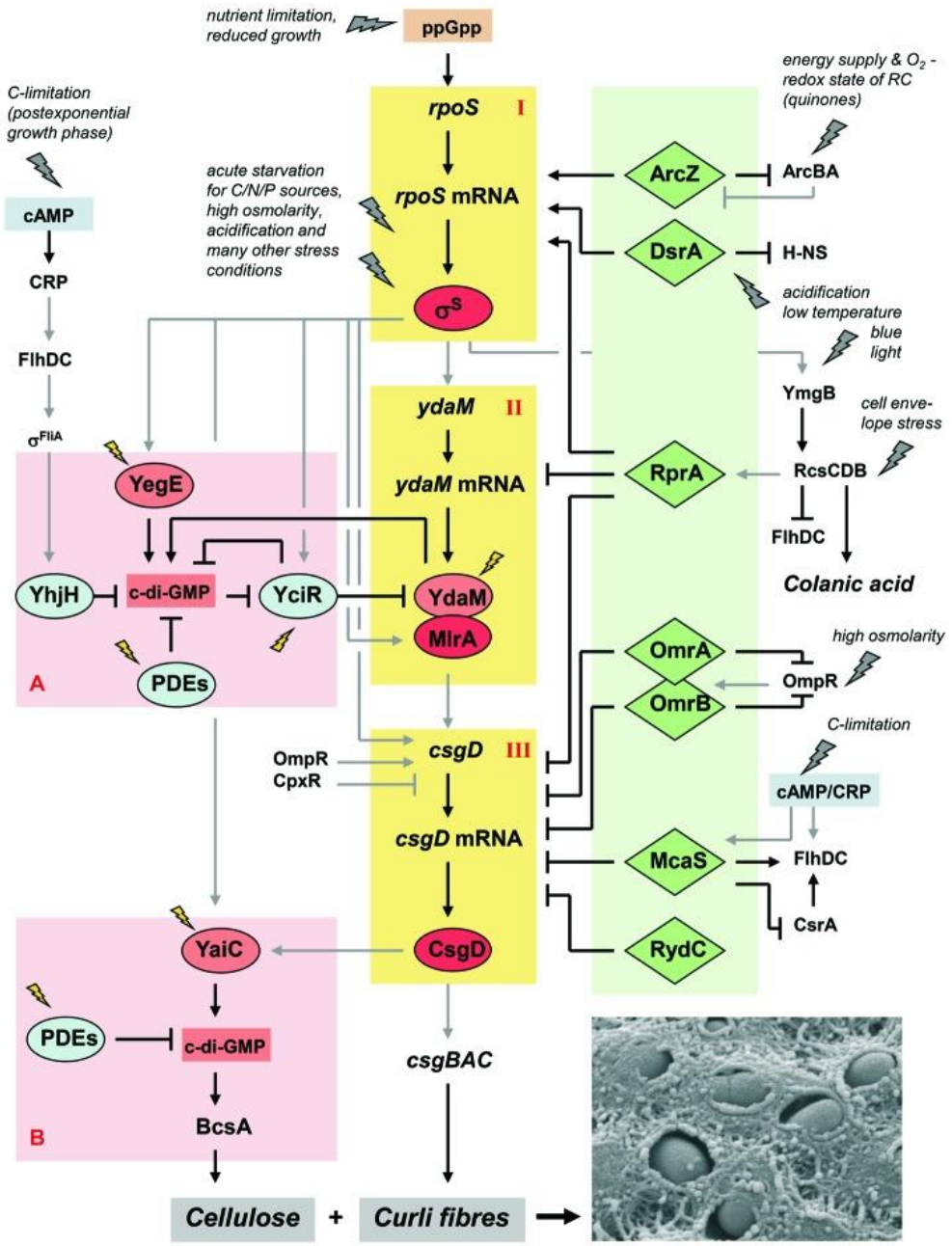


Figure 1.8: **Environmental signals and regulatory pathways involved in the regulation of curli and cellulose production.** The figure is taken from Mika and Hengge (2014). The main control of the network is by the transcription factor (TF) cascade comprising of three modules highlighted by yellow boxes. YciR is the trigger enzyme that switches TF module I to module II via the c-di-GMP-dependent control module (A). The c-di-GMP control module (B) allows for specific signal input into cellulose production**.** DGCs are denoted by light red ovals, PDEs are denoted by light blue ovals and TFs are denoted by deep red ovals. Small RNAs (sRNAs) are denoted by green rhombus. Activation of gene expression by TFs is depicted by gray arrows, all other regulatory effects are symbolized by black arrows or lines.

1.7.1 Curli

Curli fimbriae are the main protein component of the ECM produced by *E. coli* and other members of Enterobacteriaceae. Analysis of the ECM produced by *E. coli* UTI89 showed that the composition of curli is 85% by mass (McCrate *et al*., 2013). Curli fibres were first identified in a bovine mastitis-associated *E. coli* isolate as a fibronectin-binding organelle (Olsen *et al*., 1989). A similar fibronectin-binding organelle was identified in *Salmonella enteritidis* and called a thin aggregative fimbriae (Tafi) (Collinson *et al*., 1991). Curli facilitate attachment of curliated cells to other cells and abiotic surfaces, to form biofilm (Cookson *et al*., 2002; Uhlich *et al*., 2006). In the host, curli promote adhesion and invasion of different cells thus contributing to bacterial pathogenicity (Ben Nasr *et al*., 1996). It has been described as a pathogen-associated molecular pattern (PAMP) of *Salmonella enterica* (Tükel *et al*., 2005). Curli production is detected on agar plates containing congo red (CR) by a red colour, which indicates binding of curli to the CR dye (Fig. 1.7; Collinson *et al*., 1993).

Genes involved in curli production are clustered in two divergently transcribed operons. The first operon encodes the structural components of curli (*csgBAC operon)* while the second operon (*csgDEFG* operon) encodes the transcriptional regulator (CsgD) and the curli assembly/export system (CsgE, CsgF and CsgG) (Fig. 1.9). CsgA is the major structural subunit and CsgB is the nucleator protein (Hammar *et al*., 1995). CsgD directly activates the transcription of the *csgBAC* operon, leading to curli production (Hammar *et al*., 1995; Ogasarawa *et al*., 2011). CsgA polymerizes into an amyloid fibre on the cell surface (Wang *et al*., 2007). CsgB is located on the cell surface (Loferer *et al*., 1997) and serves as the template for CsgA polymerization on the cell surface (Bian & Normark, 1997). CsgC is a periplasmic protein that prevents CsgA from premature polymerization and allows the proteolytic degradation of improperly formed CsgA in the cell (Evans *et al*., 2015). CsgF is an accessory protein which contributes to the polymerization of CsgA at the cell surface (Nenninger *et al*., 2009). CsgG is an outer membrane lipoprotein localized to the periplasmic side of the outer membrane that stabilises the CsgA and CsgB and transports them across the outer membrane to the cell surface (Loferer *et al*., 1997). CsgE is a specificity factor which interacts with CsgG for the translocation of the curli subunits to the cell surface (Nenninger *et al*., 2011; Robinson *et al*., 2006). The Δ*csgA,* Δ*csgB* and Δ*csgD* deletion mutants are defective in curli assembly and are unable to bind CR when grown on agar plates containing CR (Hammar *et al*., 1995). The Δ*csgE,* Δ*csgF* and Δ*csgG* deletion mutants abolish curli formation although CsgA was still expressed which confirms that they are involved in

curli assembly (Hammar *et al*., 1995). Curli synthesis genes (or their homologues) are present in many bacterial genera and orders within the Gammaproteobacteria although with different degrees of conservation (Dueholm *et al*., 2012). It has been shown that CsgA secreted from *E. coli* Δ*csgB* mutant assembled curli fibres when grown adjacent *S. typhimurium* that presented CsgB on its surfaces (Zhou *et al*., 2012). This cross-seeding/complementation of curli subunit homologs have been identified in mixed colony biofilms of *E. coli*, *Salmonella typhimurium*, and *Citrobacter koseri* (Zhou *et al*., 2012). The antitoxin MqsA is a transcriptional repressor that has been shown to reduce curli production by directly repressing *csgD* promoters, leading to reduced transcription of CsgD-regulated genes *csgA* and *csgB* (Soo & Wood, 2013).

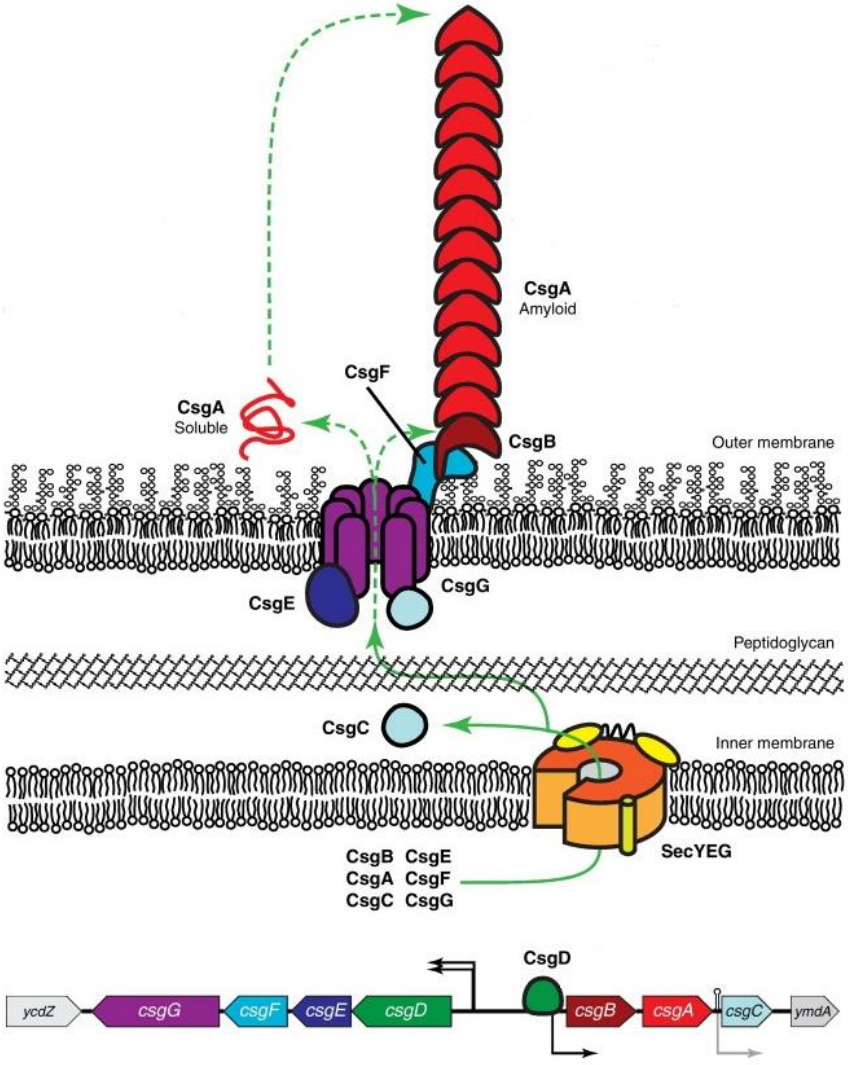


Figure 1.9: Model of curli assembly and the curli gene operons. The model shows the role of each *csg* gene in curli production and assembly and the operon structure of the *csg* genes. The figure is adapted from (Blanco *et al*., 2012)

The CsgD-mediated expression of curli is regulated by RpoS and cyclic-di-GMP (c-di-GMP) (Gualdi *et al.*, 2007; Römling *et al*., 2013). C-di-GMP is a bacterial nucleotide second messenger that promotes biofilm formation in *E. coli* and many other Gram negative bacteria (reviewed in Römling *et al*., 2013). C-di-GMP is synthesized by diguanylate cyclases (DGCs) with characteristic GGDEF domains and degraded by specific phosphodiesterases (PDEs) with either characteristic EAL or HD-GYP domains (Hengge, 2016). The *E. coli* pangenome (as of 2015) contains 35 genes encoding either GGDEF or EAL (Povolotsky & Hengge, 2016). In *E. coli* K-12, 12 genes encode DGCs and 13 genes encode PDEs, in addition to 4 genes encoding "degenerate" enzymatically inactive proteins (Hengge *et al*., 2016). C-di-GMP has been shown to affect the levels of CsgD and CsgD-dependent genes in the cell (Weber *et al*., 2006). Expression of CsgD regulates at least 24 genes involved in inducing biofilm formation and repressing flagellar motility (Brombacher *et al*., 2003; Ogasawara *et al*., 2011). There are specific protein-protein interactions between DGCs and PDEs, which other DGCs or PDEs are not be able to compensate for (Jenal, 2013; Lindenberg *et al*., 2013). Local c-di-GMP pools and/or direct protein–protein interactions are of key importance for curli and biofilm production (Hengge, 2009). Localization of these proteins enables the fine-tuning of c-di-GMP levels in different parts of the cell (Beloin *et al*., 2008). Local signalling of c-di-GMP has been proposed for CsgD-mediated curli production through direct interactions of specific DGC/PDE proteins (Lindenberg *et al*., 2013). For curli production, RpoS activates the expression of MlrA, which is a MerR-like transcriptional factor (Brown *et al*., 2001; Weber *et al*, 2006). Other regulatory inputs into the production of curli are presented in Fig. 1.8, but a full discussion of these is beyond the scope of this introduction.

Two c-di-GMP control modules activate *csgD* transcription in *E. coli*. The first module consists of DgcE/PdeH pair (formerly named YegE/YhjH), which regulates the activity of the second module pair, DgcM/PdeR (formerly named YdaM/YciR) (Lindenberg *et al*., 2013). DgcE produces c-di-GMP pool in Module I, and this is sensed by PdeR in Module II. DgcM and PdeR in Module II form a signalling complex with MlrA through multiple direct protein-protein interactions (Fig. 1.10 & also see Fig. 1.8). PdeR inhibits DgcM and MlrA by direct interaction and this inhibition is relieved when sufficient c-di-GMP pool is generated by DgcE in module I. Then, DgcM is released to add to the local c-di-GMP pool and prevent PdeR from resuming its inhibition of DgcM and MlrA. Then, DgcM activates MlrA, by direct and specific protein-protein interaction with PdeR and MlrA, thereby triggering *csgD* transcription (Lindenberg *et al*., 2013). PdeR is

regarded as a trigger enzyme because of its role in connecting modules I and II and switching on the pathway leading to *csgD* expression and curli production (Lindenberg *et al*., 2013; Hengge, 2016). The proteins in these modules are very specific for *csgD* transcription and curli production. For example, replacement of DgcM with DgcC (formerly YaiC) to generate c-di-GMP was not able to compensate for the lack of DgcM and did not result in curli production (Lindenberg *et al*., 2013).

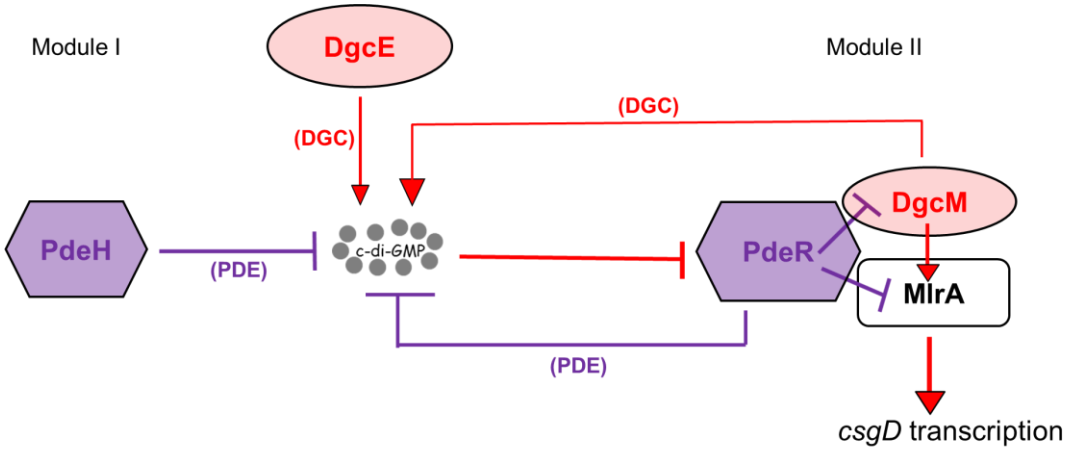


Figure 1.10: Model of the c-di-GMP signalling pathway for regulating *csgD* transcription and triggering biofilm production. Diguanylate cyclases (DGCs) are indicated by ovals and their effects are shown in red. Phosphodiesterases (PDEs) are indicated by hexagons and their effects are shown in blue. The figure is adapted from Lindenberg *et al*. (2013).

1.7.2 Cellulose

Cellulose is the second main component of biofilm ECM produced by *E. coli* and other members of Enterobacteriaceae (Zogaj *et al*., 2001). Cellulose is a polysaccharide composed of linear chains of (1,4)-β-linked glucose monomers. Analysis of the ECM produced by *E. coli* UTI89 showed that the cellulose composition is 15% by mass (McCrate *et al*., 2013). Cellulose is widely produced among *E. coli* and other Enterobacteriaceae, although not all isolates produce cellulose (Zogaj, et al., 2003). Cellulose is detected on agar plates containing calcofluor white dye and colonies producing cellulose show fluorescence under UV light (Solano *et al*., 2002).

Bacterial cellulose production was first described in *Gluconacetobacter xylinus* (now reclassified as *Komagataeibacter xylinus*) (Ross *et al*., 1991). *Acetobacter xylinum* has been used as a model organism for understanding cellulose biosynthesis pathways (Ross *et al*., 1987; Wong *et al*., 1990). *E. coli* was first described to produce cellulose as a component of the extracellular matrix by Römling *et al*. (2000). Production of cellulose has been described in other members of the *Enterobacteriaceae* such as *Salmonella* spp, *Citrobacter* spp and *Enterobacter* spp (Zogaj *et al*., 2001; 2003). Commensal, pathogenic and environmental *E. coli* has been shown to produce robust biofilms with the characteristic red, dry and rough (rdar) macrocolonies on Congo Red agar, indicating production of curli and cellulose (Fig. 1.7; Lim *et al*., 2014; Bokranz *et al*., 2005; Uhlich *et al*., 2006). However, laboratory-adapted strain *E. coli* K-12 and its derivatives, such as MC4100, W3110 and BW25113, do not produce cellulose, hence the formation of smooth macrocolonies on Congo Red agar (Fig. 1.7; Zogaj *et al*., 2001; Serra *et al*., 2013). The loss of cellulose production in *E. coli* W3110 is due to a single nucleotide polymorphism (SNP) in *bcsQ,* which encodes the cellulose synthase operon protein. This mutation is upstream of the cellulose synthase gene (*bcsA*) and it results in a premature stop codon (Serra *et al*., 2013). Repairing the SNP in *bcsQ* on the chromosome restored cellulose synthesis in *E. coli* W3110 and restored the characteristic red, dry and rough (rdar) macrocolonies on Congo Red agar (Serra *et al*., 2013). The *bcsA* gene which encodes cellulose synthase is important for cellulose biosynthesis in *E. coli* (Gualdi *et al*., 2008). Cellulose production is regulated indirectly by CsgD through the activation of DgcC (formerly YaiC) (AdrA in *Salmonella* spp) (Fig. 1.7; Serra *et al*., 2013; Römling *et al*., 2000). DgcC produces c-di-GMP which binds the PilZ domain of BcsA, thereby activating cellulose production (Amikam & Galperin, 2006; Zogaj *et al*., 2001).

CsgD-independent mechanisms for cellulose production have also been reported in *E. coli* (Da Re & Ghigo, 2006) and *Salmonella enterica* (Simm *et al*., 2007). *E. coli* 1094 and DSM6601 used the DGC, DgcQ (formerly YedQ) as an alternative to DgcC for the activation of cellulose production (Da Re & Ghigo, 2006). The AdrA-independent mechanism for cellulose production in *Salmonella enterica in vivo* is through the DGC, STM1987 (GcpA) (Cowles *et al*., 2016). GcpA has also been shown to be important for cellulose production and biofilm formation in polystyrene plates by *Salmonella enterica* (Bhowmick *et al*., 2011). Interestingly, a commensal isolate *E. coli* 1125 was found to produce cellulose independent of CsgD, DgcC and DgcQ (Da Re & Ghigo, 2006). This observation suggests an entirely different pathway for cellulose production, hence other

DGCs identified in *E. coli* should be investigated for their contribution to cellulose production.

1.8. PROJECT AIMS AND THESIS OVERVIEW

The use of *E. coli* as an indicator organism for faecal contamination in the environment, particularly in water sources destined for human consumption, is based partly on the assumption that *E. coli* only survives transiently in environments outside of the mammalian gastrointestinal tract. As mentioned in Section 1.2, there is growing body of knowledge showing that *E. coli* can persist in the external environment, a conclusion that undermines the continuous use of *E. coli* as an indicator organism.

As described in Section 1.3.1, the general stress response regulator RpoS helps *E. coli* to adapt to various environmental stresses. However, mutations could accumulate within *rpoS* in low nutrient environments causing partial or complete loss of RpoS function and trade-off of stress tolerance for growth (Section 1.4). Since nutrients in the soil are less abundant than in the soil compared to the host gastrointestinal tract, it is necessary to understand if loss of function mutations are present in the *rpoS* of soil-persistent *E. coli* or whether an intact stress response is retained in them.

Furthermore, the knowledge of how *E. coli* responds to chemical and physical stresses is derived almost exclusively from studying commensal and pathogenic isolates. Since soil-persistent strains have been reported to have unique growth and metabolic characteristics compared to the common laboratory reference strain (*E. coli* K-12 and its derivatives) (Brennan *et al*., 2013), it has become imperative to use natural isolates of *E. coli* in studying the ability of *E. coli* to withstand stress in the external environment. The availability of a collection of soil-persistent *E. coli* from a closed system (Brennan *et al*., 2010a; F. Abram, unpublished) represents a rare opportunity to understand the role that stress responses play in the survival of *E. coli* in an environment outside the host, where environmental conditions (such as temperature and pH), nutrient availability and competing microorganisms are different from those present in the gastrointestinal tract (Section 1.4).

Therefore, the project described herein set out to investigate whether long-term soil-persistent *E. coli* strains have evolved altered stress resistance and to understand how RpoS-regulated general stress response contributes to soil survival. To achieve these

goals, five soil-persistent *E. coli* strains belonging to 3 different phylogenetic groups were analysed for RpoS activity. Two commensal strains (*E. coli* SE11 and SE15) and the laboratory strain BW25113 were used for comparison. Since an intact RpoS is required by *E. coli* to respond to various environmental stresses (Section 1.3.1), the *rpoS* locus and the flanking genes in the soil-persistent strains was sequenced and analysed. Known RpoS-dependent phenotypes and reporter activity measurements were taken to determine if RpoS was functional in these strains. Since the soil is considered a stressful environment (Section 1.6), an *rpoS* deletion mutant was constructed in a soil-persistent strain to directly test if RpoS was required for soil survival. This was necessary since no previous study has shown the role of RpoS for survival of *E. coli* in soil. The results are presented in Chapter 3.

Furthermore, the specific role RpoS plays in enhancing the survival of *E. coli* in the soil environment is unknown. Since *E. coli* will encounter protozoans and moisture fluctuations in the soil (Section 1.4), the role of RpoS in the resistance the soil-persistent *E. coli* to predation by soil-borne protozoa *A. polyphaga* and *T. pyriformis* was investigated. Also, the impact of moisture on RpoS-mediated soil survival was determined. The results are presented in Chapter 4.

Lastly, it has been previously reported that biofilm formation is an RpoS-dependent phenotype which promotes the survival of *E. coli* in the external environment (Section 1.7). Since, curli is a major component of *E. coli* biofilm, this study screened a collection of 170 soil-persistent *E. coli* for curli production and investigated whether curli production contributed to soil survival. Some soil-persistent strains with altered curli production capacities were further investigated to understand the molecular basis for the loss of curli. The results are presented in Chapter 5.

Overall, the aim of this project was to provide new insights into the role of the general stress response regulator, RpoS for the environmental persistence of *E. coli*.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL STRAINS AND PLASMIDS

The bacterial strains used in this study are presented in Table 2.1. Permanent stocks were made by spinning down 2 ml of an overnight culture in a centrifuge (Eppendorf Centrifuge 5418) and re-suspending in 2 ml of appropriate medium supplemented with dimethyl sulfoxide (DMSO) (Sigma) to a concentration of 7% (v/v). One ml aliquots were put in 2 ml cryovials and stored at -80°C. Working cultures of the strains were streaked onto LB agar plates and grown overnight at 37°C. Plates were supplemented with appropriate antibiotics (Section 2.3.1) to maintain plasmids in the transformed strains, and stored at 4°C. For experiments, one isolated colony was used from a plate for each biological replicate. Plasmids used in the study are listed in Table 2.2.

Table 2.1: Description of *Escherichia coli* strains used in this study

Strains	Phylogenetic Group	Habitat	Source
COB583 (Lys9)	C	Soil	F. Brennan (TEAGASC, Ireland)
COB583 Δ*rpoS*	-	-	This study
COB584 (Lys24)	B1	Soil	This study
COB585 (Lys25)	E	Soil	This study
COB586 (Lys28)	B1	Soil	This study
COB587 (Lys36)	B1	Soil	This study
SE11	B1	Commensal	RIKEN BRC, Japan
SE15	B2	Commensal	RIKEN BRC, Japan
BW25113	A	Lab strain	NBRP (Japan)
BW25113 Δ*rpoS*	-	-	NBRP (Japan)
BW25113 Δ*csgA*	-	-	NBRP (Japan)
BW25113 Δ*csgB*	-	-	NBRP (Japan)
BW25113 Δ*csgD*	-	-	NBRP (Japan)
BW25113 Δ*bcsA*	-	-	NBRP (Japan)

Table 2.1 (continued): Description of *Escherichia coli* strains used in this study

Strains	Phylogenetic Group	Habitat	Source
Lys34	B1	Soil	F. Abram (NUIG, Ireland)
Lys35	B1	Soil	F. Abram (NUIG, Ireland)
Lys45	E	Soil	F. Abram (NUIG, Ireland)
Lys52	B1	Soil	F. Abram (NUIG, Ireland)
Lys53	B1	Soil	F. Abram (NUIG, Ireland)

2.2. CULTURE MEDIA

All media, unless stated otherwise, were autoclaved at 121°C for 15 min in a Labo autoclave (Sanyo). Filter sterilisation was performed using a 0.22 µm syringe filter (Sartorius) and syringe (BD Plastipack).

2.2.1 Lysogeny Broth (LB)

LB broth was prepared by adding 20 g LB powder (Sigma) per litre distilled water and autoclaved. For LB agar, 15 g agar No. 2 (LabM) was added per litre.

2.2.2 Yeast Extract and Casamino Acid (YESCA) Agar

YESCA agar was prepared by adding 10 g casamino acid (Difco), 1 g yeast extract (BD Biosciences) and 20 g agar No. 2 (LabM) per litre of distilled water.

2.2.3 MacConkey Agar

MacConkey agar was prepared by adding 50 g MacConkey agar powder (Sigma) per litre of distilled water.

Table 2.2: Plasmids used in this study

Plasmid	Description	Source
pUA66 (p*gadX*::gfp)	pUA66 with *gadX* promoter	D. Clarke (UCC, Ireland)
pUA66 (pσ^{70}*::gfp*)	pUA66 with synthetic sigma70 promoter	U. Alon (Weizmann Institute of Science, Israel)
pKOBEGA	Plasmid with lambda (λ) genes *gam*, *bet* and *exo* under the control of the arabinose inducible pBAD promoter, which allows the recombination of between linear DNA and the corresponding region on the chromosome during mutant construction by recombineering	D. Clarke (UCC, Ireland)
pCP20	helper plasmid that enables the elimination of the antibiotic resistance genes after mutant construction by recombineering; encoding FLP recombinase, synthesis of FLP is temperature-inducible; temperature-sensitive replication; Amp^R, Cm^R	CGSC, USA
pCAB18-*pdeR*	pCAB18 (IPTG-inducible low copy number vector carrying the *P*tac promoter, Amp^R) with wildtype *pdeR*	R. Hengge (HU Berlin, Germany)

2.2.4 M9 Minimal Medium

The M9 Minimal Medium supplemented with succinate (carbon source) was prepared from stocks described below:

10X M9 salts solution

This was made by dissolving 128 g of $Na_2HPO_4.7H_2O$ (disodium hydrogen phosphate), 30 g of KH_2PO_4 (potassium dihydrogen phosphate), 5 g of NaCl (sodium chloride) and 10 g of NH_4Cl (ammonium chloride) per litre of distilled water. This was autoclaved and stored at room temperature.

Magnesium sulphate solution (1 M)

The magnesium sulphate solution was made by adding 12.33 g of $MgSO_4.7H_2O$ (magnesium sulphate) per 50 ml of distilled water. This was filter-sterilised and stored at room temperature.

Calcium Chloride solution (1 M)

The calcium chloride solution was made by adding 14.33 g of $CaCl_2.2H_2O$ (calcium chloride) per 100 ml of distilled water. This was filter-sterilised and stored at room temperature.

Thiamine (1% w/v)

The 1% Thiamine solution was made by adding 0.4 g of thiamine per 40 ml of distilled water. This was filter-sterilised and stored at 4°C.

Succinate (10% w/v)

The 10% Succinate solution was made by adding 10 g of Sodium succinate per 100 ml of distilled water. This was filter-sterilised and stored at 4°C.

To make a working stock of succinate minimal medium (SMM), 100 ml of 10X M9 salts, 2 ml of 1 M magnesium sulphate solution, 100 ml of 10% succinate solution, and 100 µl of 1 M calcium chloride solution were combined and then made up to 500 ml with autoclaved distilled water to give a 2X SMM. Equal volume of autoclaved distilled water (500 ml) was added to 2X M9 salts solutions (500 ml) to give 1 L of 1X SMM solution. To make SMM agar, 2X agar (30 g L^{-1}) was added to SMM.

2.2.5 Phosphate-Buffered Saline (PBS)

One Phosphate-buffered Saline tablet (Sigma) was dissolved in 200 mL of deionized water and autoclaved giving a PBS buffer (pH 7.4).

2.2.6 Page's Amoeba Saline (PAS)

This is prepared from two stock solutions according to Page (1976). Stock 1 was prepared adding 24 g of NaCl, 0.8 g of $MgSO_4.7H_2O$ and 1.2 g of $CaCl_2.6H_2O$ per 500 ml distilled water. Stock 2 was prepared by adding 28.4 g of Na_2HPO_4 and 27.2 g of KH_2PO_4 per 500 ml distilled water. Five ml of each stock solution was added, made up to 1 L with distilled water and then autoclaved to give PAS.

2.2.7 Non-Nutrient Agar

Non-Nutrient Agar was made by adding 15 g of agar No 2 (LabM) per litre of Page's Amoeba Saline (PAS) and then autoclaved.

2.2.8 Proteose-Peptone Yeast Extract Medium (PPY)

PPY was prepared by adding 20 g of proteose peptone (Oxoid) and 2.5 g of yeast extract (BD Biosciences) per litre of distilled water, and then autoclaved.

2.2.9 Proteose-Peptone Yeast Extract Medium with Glucoe (PYG)

PYG was prepared by adding 10 g of proteose-peptone (Oxoid), 5 g of yeast extract (BD Biosciences) and 10 g of glucose (AnalaR) per litre of PAS.

2.3. MEDIA SUPPLEMENTS

2.3.1 Antibiotics

Antibiotics were added to the media as required and the stocks solutions of these antibiotics were made as outlined below. All antibiotic stock solutions were filter sterilised and stored at -20°C. Antibiotics were added to media after the autoclaved media had cooled to ≤ 55°C.

2.3.1.1. Kanamycin

It was prepared by adding 50 mg kanamycin salt (Sigma) per 1 ml of distilled water.

2.3.1.2. Ampicillin

It was prepared by adding 50 mg ampicillin sodium salts (Sigma) per 1 ml of distilled water.

2.3.2 Isopropyl-β-D-thiogalactopyranoside (IPTG)

Ready-to-use IPTG solution (100 mM) was obtained from Thermo Scientific and stored at -20°C until required.

2.4. MEASUREMENT OF CELL CONCENTRATION BY OPTICAL DENSITY (OD_{600NM})

An overnight culture of each bacteria was grown in LB broth at 37°C and washed three times with PAS by centrifugation at 9000 x *g* for 10 min each. Optical density (OD_{600nm}) of the washed *E. coli* and 1:2; 1:5; 1:10; 1:20; 1:100; and 1:1000 dilutions were determined. Serial dilutions of each cell suspension were made, spot-plated onto LB plates in triplicate, and incubated at 37°C overnight. Colony counts were performed after 17 h and used to derive an estimated cell count. The cell count was plotted against the optical density of the respective dilutions in three independent experiments to obtain calibration curves from which desired cell numbers can be obtained.

2.5. MUTANT CONSTRUCTION

2.5.1 Construction of Deletion Mutant

An *rpoS* deletion mutant strain of soil-persistent *E. coli* COB583 was constructed by the one-step inactivation method using a λ Red recombinase-assisted approach, which replaces target gene sequence with a kanamycin resistance cassette as described by Datsenko and Wanner (2000), with some modifications as follows. Purified plasmid pKOBEGA (Table 2.2) was electroporated (2.5 kV; 200 Ω and 25 μF) into *E. coli* COB583 in a MicroPulser™ Electroporator (Biorad) and the transformants were selected on LB agar with 100 μg ml^{-1} Ampicillin (LBAmp) after incubation for 24 h at 30°C. The λ Red helper plasmid pKOBEGA is a low-copy-number plasmid that contains an ampicillin resistance gene, a temperature-sensitive origin of replication and the Red system, including three genes expressing the Exo, Bet and Gam functions of phage λ, which helps allelic exchanges between linear DNA and the corresponding region on the chromosome (Chaveroche *et al*., 2000). Linear DNA fragments containing the kanamycin cassette was generated from Δ*rpoS::kan* (from Keio collection) by PCR with Q5® High-Fidelity DNA Polymerase (New England BioLabs) using primers 740R and 743F (Table 2.3). PCR conditions consisted of an initial denaturation step at 98°C for 30 s, 30 cycles of 10 s at 98°C, 30 s at 54°C, and 3 min

at 72°C, and a terminal extension step at 72°C for 2 min in a Primus DNA Cycler (MWG-Biotech). PCR products were purified with a QIAquick PCR Purification kit (QIAGEN).

An overnight culture of *E. coli* COB583 carrying pKOBEGA [COB583 (pKOBEGA)] was diluted 1:100 in 500 ml LBAmp and grown for 2 h at 30°C; then the Red system was induced by adding 0.2% (w/v) L-arabinose to the growing culture for 1 h at 30°C. The induced COB583 (pKOBEGA) cells were made electrocompetent by incubating the induced culture on ice for 10 min, centrifuged at 6000 x *g* for 8 min at 4°C to remove the medium. The culture was then washed by adding 25 ml of 10% (v/v) glycerol and centrifuging the resuspended cell pellets at 2500 x *g* for 10 min. The washing step was was repeated and the resulting electrocompetent cell was aliquoted into 100 µl and kept on ice until use. Others were stored at -80°C until needed. One microliter of purified PCR product (~ 500 ng) was electroporated (25 µF, 200 Ω, 3 kV) into 100 µl of electrocompetent COB583 (pKOBEGA) induced cells using the MicroPulser™ Electroporator (BioRad), then 400 µl of SOC medium was added to the shocked cells. The resulting cell suspension was transferred into clear microcentrifuge tubes tubes and incubated for 2 h at 37°C. Recovered cells were spun down, the pellets were resuspended in the remaining 100 µl, and then spread on LBKan agar to select kanamycin-resistant transformants after overnight growth at 37°C. Colonies were re-streaked on LBKan agar and presumptive COB583 Δ*rpoS::kan* strains were screened with primers external to the site of mutagenesis with the internal deletion replaced by the kanamycin cassette (pKD4) (COB834R and COB835F) (Table 2.3). Confirmation of deletion was done by PCR using primers internal to *rpoS* (COB832F and COB833R) (Table 2.3).

Table 2.3: List of primers used in this study

Primers	Sequence (5' - 3‘)	Target gene/plasmid
COB740R	TACGTATTCTGAGTCTTCGG	*nlpD*
COB742F	ATGATTGACCTGCCTCTG	*ygbN*
COB743F	GGTATTGCGATTTCTATTCC	*ygbN*
COB746F	CGTAGCAATCCTGACAAC	*ygbN*
COB747R	GAATTTGATGAGAACGGAG	*rpoS*
COB784F	CCATAATCACCATCTTCACG	*ygbN*
COB785R	GATAAGCCAGTTGATGACG	*rpoS*
COB788F	CACTTCCATGCGGTAGATG	*ygbN*
COB789R	CCTATGCGTTCATCATCTTG	*hosA*
COB794F	CCATAATCACCATCTTCACG	*ygbN*
COB795F	CACCTCTTCGCTGATTTTC	*yclC*
COB796R	ACCAGGCTTTTGCTTGAATG	*nlpD-rpoS* intergenic region
COB797F	GTAACAACATCTTCTTCGTCAC	*nlpD-rpoS* intergenic region
COB798R	GGATAAGCCAGTTGATGATTTC	*nlpD-rpoS* intergenic region
COB786R	CCATTAACATCACCATCTAA	pUA66
COB787F	CCAGCTGGCAATTCCGACG	pUA66
COB836F	CCCGCTAGCGAAAAGATGTTTCGTGAAGC	pKOBEGA
COB837R	GGGAAGCTTATTATCGTGAGGATGCGTCA	pKOBEGA
COB848F	GCGGATAACAATTTCACAC	pCAB18-*pdeR*
COB849R	TCTCTCATCCGCCAAAAC	pCAB18-*pdeR*
COB832F	AGGCTTTTGCTTGAATGTTCCG	*rpoS*
COB833R	GTAAGCATCTGTCAGAAAGGCC	*rpoS*
COB834R	CCGGACAGGTCGGTCTTGAC	Kanamycin cassette
COB835F	CGGACCGCTATCAGGACATAG	Kanamycin cassette

F- Forward primer

R- Reverse primer

2.5.2 Removal of Kanamycin Cassette in Δ*rpoS* Deletion Mutant

The kanamycin resistance cassette was removed from the Δ*rpoS* mutant strains by flippase recognition target (FRT) recognition sites. Temperature sensitive plasmid pCP20 was transformed into COB583 Δ*rpoS::kan* and transformants were selected on LBAmp plates incubated at 30°C overnight. The transformed strain was plated on LB plates and incubated at 42°C overnight to induce flippase production and the loss of the pCP20 plasmid and kanamycin cassette. This was repeated to confirm loss of pCP20 plasmid. Then, the isolates were patched on gridded plates of LBKan, LBAmp and LB only, in that order. LB and LBKan plates were incubated at 37°C and LBAmp at 30°C to check for the loss of the helper plasmid. Colonies that grew on LB plates but on neither of LBKan nor LBAmp were considered to have lost the kanamycin cassette. Loss of kanamycin cassette was confirmed by PCR using primers COB834R and COB835F, and no growth of colonies streaked on LBKan. Deletion of the *rpoS* gene was confirmed by PCR using primers internal to *rpoS* (COB832F and COB833R). COB583 Δ*rpoS* (without kanamycin cassette) strain was stored at -80°C (Table 2.2) before use in subsequent experiments.

2.6. PHENOTYPIC ANALYSIS

2.6.1 Motility at 15°C and 37°C

In order to evaluate cell motility of the strains, an overnight culture of each strain was spot-inoculated onto LB plates containing 0.25% (w/v) agar and incubated at 15°C and 37°C. Radial motility was measured in three cross sections after 16 h incubation at 37°C and 40 h at 15°C. Experiments were conducted with three independent biological replicate.

2.6.2 Biofilm Assay

Each strain was grown overnight in LB and then 1 ml of the overnight culture was centrifuged at 8,000 x *g* for 6 min to recover the cells. The cell pellets were washed in 1 ml sterile PBS and resuspended in 1 ml sterile PBS. Five microlitres of the washed cells were added to 5 ml of either LB broth or SMM and vortexed gently, after which 200 µl was added into a flat bottomed 96-well micro-titre culture plate (Sarstedt). Each strain had eight technical replicates and the assay was repeated at least twice for each condition. Strains were assigned at random to the wells of a 96-well microtiter plate.

Plates with LB broth were incubated statically at 37°C for 48 h and 15°C for 138 h while SMM plates were incubated for 37°C for 84 h and 15°C for 138 h. After the incubation, the OD_{595nm} of the culture (without shaking) was determined in the plates with the lid off using a Sunrise™ Microplate Absorbance reader (Tecan). The media was then removed from all wells carefully using a pipette so as not to disrupt the biofilm. Each well was washed three times with 200 µl PBS by inverting the plates to empty washes and remove all non-adhering bacterial cells, after which the plates were dried at room temperature for 45 min. Biofilm was stained by adding 150 µl of 1% (w/v) crystal violet solution to each well and incubated for 30 min at 37°C. After staining, excess crystal violet was removed from the wells, and the wells rinsed four times with 200 µl PBS as before. Destaining was done by adding 160 µl of 95% (v/v) ethanol to each well and incubating plates for 30 min at room temperature and then mixed together with gentle pipetting up and down. The OD_{595nm} of the destained wells were measured in the Sunrise™ Microplate Absorbance reader (Tecan) after shaking the plates for 20s. Controls wells uninoculated with bacteria were included in the experiments to discount any effect of cellular debris on the OD_{595nm} of destained wells. The recorded value was used as an approximation of the biofilm levels produced by each strain.

2.6.3 Acid Survival at 37°C

To test the survival of strains during low pH stress, overnight cultures were inoculated into 25 ml broth in 200 ml Erlenmeyer flasks. These were then incubated shaking at 37°C for 18 h when the cells have reached stationary phase. *E. coli* count was taken by serially diluting 20 µl of the culture in PBS and spot plating 10 µl onto LB agar. The pH of the remaining culture in the flask was subsequently reduced to pH 2.5 with 3 M HCl (Fisher) using a SevenEasy™ pH meter (Mettler Toledo) and immediately returned to 37°C. At specified time-points (20, 40 and 60 min), 20 µl of the acidified culture was serially diluted in PBS and spot plated in triplicate onto LB agar and incubated overnight at 37°C. Colonies were counted to enumerate the viable cells at each time-point.

2.6.4 GABase Assay

Strains were inoculated into 25 ml broth and incubated with agitation overnight (16-18 h) at 37°C. For acid treatment, the pH of the cultures was lowered to 4.0 with 3 M HCl and the cultures were incubated for a further 1 h at 37°C. Extractions were made after 1 h of acid treatment. Non HCl-treated cultures were used as negative controls.

Following treatment, 500 µl of the culture was removed to retrieve the extracellular metabolite fraction. This was centrifuged at 9,000 × *g* for 10 min and the supernatant was collected in a sterile 1.5 ml microcentrifuge tube and stored at -20°C. The remaining culture was centrifuged at 9,000 × *g* for 10 min and the supernatant discarded. The pellet was resuspended in 0.5 ml sterile distilled water and transferred to a 1.5 ml microcentrifuge tube. This was then boiled for 10 min and centrifuged at 9,000 × *g* for 10 min. The supernatant was transferred to a sterile 1.5 ml tube and stored at -20°C until needed.

The concentration of γ-aminobutyric acid (GABA) was determined using the modified micro-titre plate assay described by O'Byrne *et al*. (2011). Ninety microlitres of master mix containing 80 mM Trizma base (Sigma Aldrich), 750 mM sodium sulphate (BDH), 10 mM dithiothreitol (DTT; MSC), 1.4 mM β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP; Sigma Aldrich) and 0.3 mg ml^{-1} GABase from *Pseudomonas fluorescens* (Sigma Aldrich) was added to each well along with 10 µl of sample/standard. Standards of γ-aminobutyric acid (Sigma Aldrich) were made in distilled water to a concentration of 0 - 10 mM for measuring intracellular GABA ($GABA_i$), whilst standards were made in LB for measuring extracellular GABA ($GABA_e$) concentrations. Plates were subsequently incubated at 37°C in a Sunrise™ absorbance reader (Tecan) and OD_{340nm} readings were taken every 60 s for 3 h, with shaking for 5 s prior to each reading. GABA levels in the samples were determined from the calibration curves generated from the GABA standards.

2.6.5 Glycogen Accumulation Test

Levels of glycogen accumulated in the strains were determined by iodine staining, as described previously (Notley-Mcrobb *et al*., 2002) with some modifications. *E. coli* strains were streaked on LB agar and incubated overnight at 37°C. Plates were then transferred to 4°C for 48 h, after which they were flooded with a 50 mM Iodine solution (Sigma-Aldrich) for 5 min. Images were captured with HP Scanjet 5400c at 600 dpi. The glycogen level is indicated by the intensity of brown coloration and is an indirect measure of the level of RpoS.

2.6.6 Survival under Starvation at 15°C

Ten ml cultures of each strain were incubated in LB overnight at 37°C and washed by centrifuging at 9000 x *g* for 10 min in 10 ml PBS twice. The stationary phase cells were

washed and resuspended in 15 ml PBS and used as inoculum. One ml of the inoculum was added to 15 ml PBS in 50 ml Cell Star® Tubes (VWR) in triplicate and incubated at 15°C with shaking. Serial dilution of the inoculum was made in PBS and 10 µl was spot-plated in triplicate for enumerating viable cells. Aliquots (200 µl) samples were taken at Day 7, 14, 21, 28 and 35 and for enumerating viable cells. Samples were serially diluted in PBS, plated in triplicate onto LB agar and incubated overnight at 37°C. Colony forming units (CFU) were then counted for each time-point and calculated as CFU ml^{-1}.

2.6.7 Congo Red Assay

Curli production was determined in the strains by congo red assay as previously described previously (Zhou *et al*., 2013). Congo Red agar plates was made by preparing YESCA (Section 2.2.2) and after autoclaving, filter sterilised Congo Red (50 µg ml^{-1} final concentration; Sigma) and filter sterilised Brilliant Blue G (Sigma) (10 µg ml^{-1} final concentration; Sigma) was added. *E. coli* strains were grown in LB broth and incubated at 37°C overnight. Five microliters of the overnight culture of each strain was spotted on the centre of a thick congo red agar plate (25 ml of sterile molten agar per plate). The plates were incubated at 28°C for 48 h. Images were captured with Canon CanoScan 9000F MKII Flatbed Scanner at 600 dpi.

2.7. SOIL SURVIVAL ASSAY

2.7.1 Development of Soil Survival Assay

One colony of each strain was inoculated into 10 ml LB and incubated overnight at 37°C. The overnight culture was harvested by centrifugation at 9,000 x *g* for 10 min, washed twice with sterile PBS and resuspended in PBS to give an OD_{600nm} equivalent to give ~ 2 x 10^8 CFU ml^{-1} which served as the inoculum (Section 2.4). Two types of silty-loam soils were used for this analysis: Soil A (Sand 47%; Silt 52%; Clay <1%) with pH 7.2; Total Organic Carbon 16.53% dry solids; Total Nitrogen 1.56% dry solids; C/N ratio 10.6; Organic Matter 31.3%) was collected from Ballyvaughan (53°07'16"N 9°09'25"W) in the west of Ireland and Soil B (Sand 43%; Silt 53.9%; Clay 3.1%; pH 5.2; Total Organic Carbon 4.7%; Total Nitrogen 0.35%; C/N ratio 13.43; Organic Matter 9.1%) was collected from Kilfergus (52°57'10"N 9°20'44"W) in the mid-west of Ireland. The soils were sieved with a 2 mm sieve, kept in a sealed bag, with airspace at constant temperature (15°C) until used. The soil used had no detectable background

levels of coliforms or *E. coli* when serial dilutions were plated on MacConkey Agar, making it suitable for use in this study so that recovered *E. coli* will only be attributable to inoculated *E. coli.* Fifty microlitres of the inoculum was added to 1 g of soil (to give ~ 1 x 10^7 CFU g^{-1} soil) in series of 15 ml Cell Star® tubes (VWR). The tubes were inverted 10 times by hand, slightly capped to allow air exchange and incubated statically at 15°C. The uninoculated control had 50 µl of sterile PBS added to it. The experiment was set up in triplicate. Inoculated soils were destructively sampled (i.e., PBS was added and could not be reused at another time point) immediately after the set up and at other specified time-points to recover viable *E. coli* cells surviving in soil. For recovery of cells, 2 ml of PBS was added to each tube containing soil sample, capped and mixed by inverting the tube three times and vortexed for 2 x 20 s. The resulting soil slurry was allowed to settle for 2 min and 20 µl of the supernatant liquid was collected and serially diluted. Ten µl of all dilutions were plated in triplicate on MacConkey agar (Sigma) and incubated at 37°C overnight. Colonies were counted to enumerate the viable cells at each time-point. Preliminary experiments showed that an average of 91% to 102% of the added *E. coli* was recovered at 10 min after inoculation into soil A, while recovery was 91% to 97% in soil B.

2.7.2 Determination of Water Holding Capacity (WHC) of Soil

This was determined as described by Blažka & Fischer (2014). Air-dried soil was passed through a 2 mm sieve and a known weight of soil was taken. A filter paper was weighed (fd) and placed in a funnel. The filter was soaked with distilled water and the weight of the wet filter paper (fw) determined. The soil was poured into the filter paper and distilled water was used to saturate the soil until there was no water dripping from the funnel. The weight of the filter paper with the saturated soil (ssf) was determined. The filter paper with wet soil was dried at 105°C in a hot air oven until constant weight is reached. The weight of the dried soil with filter paper (dsf) was determined. WHC was calculated as follows:

$$WHC = [(ssf – fw) – (dsf – fd)] / (dsf – fd)$$

Since WHC is the saturation moisture percentage, the soil moisture content of the original soil was considered in determining the water needed to raise the soil moisture to 100% WHC and 60% WHC. Soil moisture content was determined by pre-weighing soil (moist soil), which was then added to an aluminium dish. The soil was then dried to constant weight at 105°C, after which the dry soil weight was obtained. Soil moisture content (W_m) was calculated as:

W_m = (weight of moist soil – weight of dry soil) / (weight of moist soil)

2.8. SAND ATTACHMENT ASSAY

E. coli cells were grown for 24 h on LB agar at 28°C. Colonies were scraped off with a loop and resuspended in 5 ml PBS. OD_{600nm} was measured and normalised in LB without salt (LBns; 10 g L^{-1} Tryptone and 5 g L^{-1} Yeast Extract) to give a starting population of 10^6 CFU ml^{-1}. Sand was pre-weighed into 1.5 ml tubes and sterilised by autoclaving. Then, 0.5 g of sterilised quartz sand (Sigma) was added to wells of a 96-well plate and 1 ml of the inoculated LBns was added to each well. Plates were incubated static in the dark at 28°C for 48 h. After incubation, the LBns in each well was removed, serially diluted and plated out to determine planktonic cell count. For biofilm cell count, sand in the 96-well plate was pipetted into pre-weighed 1.5 ml tubes and 500 µl of PBS was used to wash the sand five times to remove unattached cells. All liquid was removed and tubes were re-weighed. Five hundred microliters of PBS was added and the tubes were vortexed for 30 s, sonicated (4 min, 100% power) and vortexed again for 30 s. The liquid fraction was then serially diluted and plated onto LB agar to determine the biofilm count. The bacterial cell counts were normalized to the weight of the sand.

2.9. PROTEIN ANALYSES

2.9.1 Protein Extraction from *E. coli* for Determining RpoS Expression

Stationary phase cells were inoculated into 25 ml LB at starting $OD600_{nm}$ 0.05 and incubated overnight at 37°C with continuous shaking. At about ~ 17 h, 1 ml culture was taken into a sterile 1.5 ml tube and centrifuged at 12,000 × *g* for 10 min and the pellets were obtained. Samples were stored on ice until the completion of the experiment. The supernatant was discarded and the cell pellet was resuspended in 100 µl of BugBuster™ cell lysis reagent (Novagen, USA) supplemented with 1 U DNaseI (Thermo Scientific), 1X Halt Protease Inhibitor Cocktail (Thermo Scientific), and 400 U Lysozyme (Sigma Aldrich). The cell suspension was then incubated at room temperature for 20 min with agitation. Cell lysates were centrifuged at 16,000 x *g* for 10 min at 4°C. Fifty microliters of the resulting supernatant (soluble protein fraction) was analysed to determine the protein concentration and used for SDS-PAGE while the remaining protein extract was stored at -20°C.

2.9.2 Protein Concentration Determination

Protein concentration was determined using the Bio-Rad RC DC Protein Assay Kit (Biorad) according to the manufacturer's instructions. Protein samples were diluted 1 in 10 in distilled water. Bovine Serum Albumin (BSA; Sigma) was used as a protein standard and a series of standards from 0.1 mg ml^{-1} to 1.4 mg ml^{-1} were used. All samples and standards were prepared and measured in triplicate. Measurement of the colour development at an optical density of 750 nm (OD_{750nm}) of the protein standards was plotted to generate a standard curve from which protein concentration of the samples was determined.

2.9.3 Sample Preparation, SDS PAGE and Western Blotting for RpoS

Protein samples were prepared by combining equal parts of protein with 1X SDS Sample Buffer (65 mM Tris-HCl pH 6.8, 2.1% [w/v] SDS, 26% [v/v] glycerol, 0.01% [w/v] bromophenol blue). Fifty microlitres of fresh β-mercaptoethanol (Sigma) was added to every 950 µl of 1X SDS sample buffer to reduce disulphide bridges. The protein samples were boiled at 95°C for 10 min and centrifuged at 16,000 x *g* for 1 min to pellet insoluble material. An equal amount of each protein (25 µg) was resolved on 10% w/v resolving gel [2.67 ml of 30% acrylamide; 2 ml of 1.5M Tris pH 8.8; 80 µl of 10% SDS; 80 µl of 10% ammonium persulphate (APS); 8 µl of Tetramethylethylenediamine (TEMED); 3.2 ml of distilled water)] at 100 V for 1.5 h at 4°C. After electrophoresis, GelCode™ Blue Safe Protein Stain (Thermo Scientific) was added to one gel with resolved proteins and stained for protein to ensure equal protein loading while proteins on the second gel were blotted onto a PVDF membrane (GE Healthcare) using a semidry system (Jencons) at 40 mA (for 1 gel) with voltage set at maximum of 3 V for 1 h.

A blocking step with 5% (w/v) skim milk in Tris-Buffered Saline with 0.05% (v/v) Tween 20 (TBST) was performed for 1 h at room temperature with shaking and the membrane was incubated with 1,000-fold diluted mouse monoclonal anti-RpoS antibody (Santa Cruz) in the blocking solution (Table 2.4). Blots were washed in TBST three times for 10 min each and incubated in 3,000-fold diluted peroxidase conjugated anti-mouse IgG (Santa Cruz) in the blocking solution (Table 2.4). After washing three times in TBST, Amersham™ ECL™ Prime Western Blotting Detection Reagent (1:1 detection reagent mixture; GE Healthcare) was used to detect the RpoS bands by adding the solution for

1 min prior to exposure on CL-XPosure™ Film (Thermo Scientific), which was then developed in a Kodak developing machine for X-ray film. Washing steps were achieved by shaking at 70 oscillations per minute (OPM) on a platform shaker (Stuart) while incubation was achieved by shaking at 30 OPM. Western blotting was performed on three independent protein preparations for each strain.

Table 2.4: List of antibodies and dilutions used in this study

Protein	Primary Antibody			Secondary Antibody		
	Antibody	**Dilution**	**Source**	**Antibody**	**Dilution**	**Source**
RpoS	Mouse monoclonal Anti-RpoS	1:1000	Santa Cruz Biotechnology	Anti-mouse IgG HRP	1:3000	Santa Cruz Biotechnology
CsgA	Rabbit anti-CsgA antibody	1:5000	M. Chapman (University of Michigan, USA)	Anti-rabbit IgG HRP	1:10000	Santa Cruz Biotechnology
CsgD	Rabbit anti-CsgD antibody	1:2000	Shinya Sugimoto (Jikei University, Japan)	Anti-rabbit IgG HRP	1:20000	Santa Cruz Biotechnology
Green Fluorescent Protein (GFP)	Rabbit polyclonal GFP antibody	1:5000	Santa Cruz Biotechnology	Anti-rabbit IgG HRP	1:20000	Santa Cruz Biotechnology

2.9.4 Protein Extraction, SDS-PAGE and Western Blotting for CsgA

E. coli strains were grown in LB broth and incubated at 37°C overnight. Two hundred microliters of the overnight culture for each strain was spread on the centre of a thick YESCA agar plate and incubated at 28°C for 48 h. Then, a loopful of each strain was scraped with a sterile inoculation loop and re-suspend in 1 ml of KPi buffer (50 mM

potassium phosphate buffer, pH 7.2; 28.9 mM of KH_2PO4 and 21.1 mM K_2HPO_4 in distilled water). The OD_{600nm} of the cell suspension was determined and normalised to OD_{600nm} 1 in KPi buffer. Two 1.5 ml microcentrifuge tubes were prepared for each sample: the sample in one tube was treated with 70 µl of 100% Hexafluoroisopropanol (HFIP) and the second tube was the untreated control. Then, 150 µl of the normalised cell suspension was transferred into each tube and centrifuged at 16,000 x *g* for 3 min. The supernatant was removed carefully in order not to dislodge any of the pellets, which contain the protein of interest. Cell pellets in one of the tubes was resuspended in 70 µl of 100% HFIP and immediately, the sample was dried in a vacuum concentrator (Concentrator plus; Eppendorf) at 45°C for 30 min. The dried cell pellet and the untreated control were dissolved in 150 µl of 2X SDS sample loading buffer [62.5 mM Tris–HCl, pH 6.8; 3% (m/v) SDS; 0.01% (m/v) bromophenol blue; 10% (v/v) glycerol; 5% (v/v) β-mercaptoethanol] and boiled at 95°C for 10 min. Five microliters of each sample was separated onto 15% SDS–PAGE gels [4 ml of 30% acrylamide; 2 ml of 1.5M Tris pH 8.8; 80 µl of 10% SDS; 80 µl of 10% APS); 8 µl of TEMED; 1.8 ml of distilled water)] at 100 V for 1 h.

After electrophoresis, one gel with resolved proteins was stained for protein with GelCode™ Blue Safe Protein Stain (Thermo Scientific) to ensure equal protein loading while the second gel was rinsed in distilled water for 5 min with shaking and blotted onto a PVDF membrane (GE Healthcare) using a semidry system (Jencons) at 40 mA (for 1 gel) with voltage set at maximum of 3 V for 1 h. The membrane was rinsed with TBST (as in Section 2.9.3) and then blocked with 5% (w/v) skim milk in TBST for 1 h with shaking. Then, the membrane was incubated in 5,000-fold diluted anti-CsgA antibody in the blocking solution (Table 2.4). Blots were washed in TBST three times for 10 min each and incubated in 10,000-fold diluted goat anti-rabbit IgG conjugated with horseradish peroxidase (Table 2.4). After washing three times in TBST, the blot was developed with Amersham™ ECL™ Prime Western Blotting Detection Reagent (1:1 detection reagent mixture; GE Healthcare) for 1 min prior to exposure for 2 min to CL-XPosure™ Film (Thermo Scientific). Exposed film was developed in a Kodak X-ray developer to detect the bound antibody.

2.9.5 Protein Extraction, SDS-PAGE and Western Blotting for CsgD

Protein extraction, SDS-PAGE and Western Blotting for CsgD was performed as described in Section 2.9.1 – 2.9.3. However, the membrane was incubated in anti-CsgD antibody (Table 2.4) diluted 1: 2,000 in SignalBoost™ Immunoreaction Enhancer

Solution 1 (Merck Millipore) for 1 h at room temperature. Blots were washed in TBST three times for 10 min each and incubated in 20,000-fold diluted goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz) (Table 2.4). After washing three times in TBST, the blot was developed with Amersham™ ECL™ Prime Western Blotting Detection Reagent (1:1 detection reagent mixture; GE Healthcare) for 1 min prior to exposure for 2 min to CL-XPosure™ Film (Thermo Scientific), which was then developed in a Kodak X-ray developer to detect bound antibody.

2.10. MOLECULAR BIOLOGY ANALYSES

2.10.1 PCR Amplification and Sequencing

Whole-colony PCR amplicons of *rpoS* including its flanking genes from the five selected soil-persistent strains were obtained and sequenced. Single colonies were picked and resuspended in 500 µl of sterile Nuclease-free Water (Ambion®, USA). A 1 µl aliquot then was transferred to the PCR reagent mix. The primers used for amplification are listed in Table 2.3 and synthesized by Eurofins MWG, Germany. PCR was performed with high fidelity VELOCITY DNA Polymerase (Bioline). PCR conditions consisted of an initial denaturation step at 98°C for 2 min, 30 cycles of 30 s at 98°C, 30 s at 54°C, and 3 min at 72°C, and a terminal extension step at 72°C for 7 min in a Primus DNA Cycler (MWG-Biotech). The amplified PCR products were examined by agarose electrophoresis on 1% gels and purified using GenElute PCR Clean-up kit (Sigma-Aldrich, USA) and visualized on a 1% agarose gel for quantification prior to sequencing. Samples were sequenced by Source Bioscience (Dublin, Ireland) while assembly and analysis was done with DNABaser version 4 (Heracle BioSoft, USA). Sequencing was performed on both strands of the PCR product. The *rpoS* nucleotide sequences were deposited to GenBank and assigned the accession numbers KU948321 to KU948325 for COB583 to COB587, respectively.

2.10.2 Plasmid Extraction

E. coli strains carrying the desired plasmid DNA was grown overnight in LB supplemented with the appropriate antibiotic marker and incubated at the appropriate temperature (30°C for temperature-sensitive plasmids and 37°C for others) with agitation. For small-scale plasmid preparations, 5 ml of the overnight culture was centrifuged at 6,000 × *g* for 15 min and the supernatant was discarded. Pellets were frozen for 2 h at -80°C and then plasmid DNA was isolated following manufacturer's

instructions for the GenElute™ Plasmid Miniprep Kit (Sigma). Purified plasmid DNA was eluted with 50 µl nuclease free water (Ambion) and stored at -20°C. For plasmid midiprep (intermediate-scale preparations), 50 ml of the overnight culture was centrifuged at 9,000 × *g* for 10 min and the supernatant was discarded. Pellets were frozen for 2 h at -80°C and then plasmid extraction was performed using Invitrogen™ PureLink™ HiPure Plasmid Midiprep Kit according to the manufacturer's instructions and eluted into 100 µl nuclease free water (Ambion). The concentration of the plasmid DNA was determined by running 5 µl of the extracted plasmid extract on a 1% agarose gel and compared to concentration of ladder DNA.

2.10.3 Generating Electrocompetent Cells

An overnight culture of the *E. coli* was incubated with shaking in 25 ml LB broth at 37°C for 16-18 h. This was subsequently diluted 1:100 into 500 ml LB broth and incubated at 37°C for 3-5 h until OD_{600nm} 0.35 - 0.40 was reached. The culture was chilled on ice for 10 min, split into two 250 ml chilled centrifuge pots and centrifuged at 2,500 × *g* for 15 min at 4°C. The supernatant was discarded and each pellet was washed 4 times in 25 ml ice-cold 10% (v/v) glycerol (Sigma Aldrich), harvesting each time at 2,500 × *g* for 15 min at 4°C with a centrifuge (Hettich Universal 320R). Each pellet was resuspended in 1 ml ice-cold 10% glycerol and combined into a 50 ml chilled centrifuge tube. The tube was centrifuged at 2500 × *g* for 15 min at 4°C, supernatant discarded and the pellet resuspended in 500 µl ice-cold 10% glycerol. Finally, 100 µl aliquots of the cells were transferred to frozen 1.5 ml microcentrifuge tubes and stored immediately at -80°C until use.

2.10.4 Transformation of Electrocompetent Bacteria

One microliter of purified plasmid DNA (~100 ng) was added to 100 µl of electrocompetent cells and transferred to a 2 mm electroporation cuvette (Sigma Aldrich). The cells were then electroporated at 2.5 kV, 200 Ω and 25 µF in a MicroPulser™ Electroporator (Biorad), which typically produced time constants between 4.5 and 5 ms. The cells were immediately resuspended in 400 µl of SOC medium held at room temperature (23-25°C) and allowed to recover at 37°C for 1 h. Twenty, 50 and 100 µl aliquots of the recovered cell suspensions were subsequently spread plated on agar plates with the required antibiotics. All plates were incubated at 37°C for 24 - 48 h. Colonies were re-streaked on agar with the respective antibiotics

and presumptive transformants were screened by PCR with primers targetting the plasmid (Table 2.3).

2.10.5 RpoS-dependent GFP Expression

E. coli K-12 strain carrying the promoter of *gadX* fused to GFP on pUA66 (p*gadX::gfp*) was provided by Dr David Clarke (University College Cork). GadX is a transcriptional activator of the glutamate-dependent acid resistance system in *E. coli* and its expression is RpoS-dependent (Tramonti *et al*., 2002). *The* p*gadX*-GFP was obtained from overnight (~16 h) culture in LBKan at 37°C. Competent cells of the *E. coli* strains were electroporated p*gadX::gfp* as described above (Section 2.10.4) to generate transformants carrying the plasmid. Overnight cultures of all strains carrying the p*gadX::gfp* were made in LBKan broth and incubated at 37°C and 15°C. Stationary phase cells were inoculated into 25 ml LBKan at starting OD_{600nm} 0.05 and incubated with continuous shaking at 37°C and 15°C. To determine the level of fluorescence produced, samples were taken at exponential (2 h at 37°C and 8 h at 15°C) and stationary phase (17 h at 37°C and 36 h at 15°C), fixed with ice-cold ethanol – methanol (1:1), resuspended in PBS and 2 µl was placed on a slide and imaged with Leica DMI3000 B microscope. The GFP filter cube was used for observation and all images were acquired using 977 ms exposure. At least six image fields were viewed per sample in each experiment. Three independent experiments were performed.

Results obtained by microscopy from the RpoS-dependent GFP expression were confirmed by Western blotting. Green fluorescent protein (GFP) expression under the same conditions as described above was detected by Western blotting as described in Section 2.9.1-2.9.3, with rabbit polyclonal GFP antibody (Santa Cruz) diluted 5,000-fold in TBST and 20,000-fold-diluted peroxidase conjugated anti-rabbit IgG (Santa Cruz) (Table 2.4).

2.11. PROTOZOAN CULTURING AND PREDATION ASSAY

2.11.1 Cultivation of *Tetrahymena pyriformis*

Tetrahymena pyriformis (CCAP 1630/1B) was obtained from Dr Gavin Collins at NUI Galway and maintained axenically in sterile proteose peptone yeast extract broth

(Section 2.2.8). A new working *T. pyriformis* culture was set up by adding 250 µl of a 5-day old culture in 12 ml of PPY in duplicate 15 ml test tubes at 28°C. In order to keep a slow-growing working culture requiring less frequent subcultures, *T. pyriformis* cultures were also incubated at 15°C for 4-6 weeks before subculturing. An aliquot of the culture was applied to the Bright-Line™ Haemocytometer (Sigma) and microscopically examined using a 10X objective lens on a light microscope (Olympus) to ensure that the population consisted mainly of active and healthy-looking trophozoites, before subculturing.

2.11.2 Cultivation of *Acanthamoeba polyphaga*

Acanthamoeba polyphaga (CCAP No: 1501/14) was obtained from the Culture Collection of Algae and Protozoa, UK. It was maintained axenically on Non-Nutrient Agar (NNA) supplemented with Penicillin-Streptomycin (Penicillin – 62.5 mg L^{-1}; Streptomycin - 100 mg L^{-1}; Sigma) and heat-killed *E. coli* was spread on the agar. A chunk of agar from a previously growing culture was placed upside down at the corner of the NNA plate and incubated at 30°C for 5 days. *A. polyphaga* trophozoites were scraped off NNA plates using a disposable plastic loop. A working culture was also made in PYG medium (Section 2.2.9). Aliquot of 1 ml of *A. polyphaga* culture was suspended in 5 ml PYG medium in tissue culture flasks. The flasks were wrapped in aluminium foil and incubated for 5 days at 30°C. The culture flask is gently tapped on the side to dislodge cells attached to the flask. An aliquot of the culture was applied to the haemocytometer and microscopically examined using a 40X objective lens on a light microscope (Olympus) to ensure that the culture was composed mainly of trophozoites.

2.11.3 Predation Assay with *Tetrahymena pyriformis*

Trophozoites of *T. pyriformis* was washed in 5 ml PAS with centrifugation at 1100 x *g* for 3 min. The supernatant was quickly and gently removed by decanting without throwing out the cell pellets. *T. pyriformis* cells were washed two times. Trophozoite count was done using the haemocytometer as follows. About 50-100 µl aliquot of the washed *T. pyriformis* culture was added to a 0.5 ml microcentrifuge tube and placed at -20°C for 2-4 min to slow down the fast movement of the *T. pyriformis* cells. The haemocytometer and cover slip were cleaned with lens tissue and ethanol. Then, *T. pyriformis* cell suspension was well mixed with the pipette and applied to the haemocytometer very gently until both chambers underneath the coverslip were filled.

This cell suspension was microscopically examined using a 10X objective lens on a light microscope (Olympus) and the number of cells in the four central 16-square grids were counted. The average cell number was obtained and multiplied by 10,000 to obtain the final number of cells ml^{-1} of the original cell suspension. An overnight culture of each bacterium was grown in LB broth at 37°C and washed three times with PAS by centrifugation at 9000 x *g* for 10 min each. For developing the predation assay, a volume of the washed *E. coli* corresponding to 10^8, 10^6, 10^4 CFU ml^{-1} was obtained from a calibration curve of OD_{600nm} versus cell numbers (Section 2.4) and a volume of *T. pyriformis* cells corresponding to 100 trophozoites was added to the bacterial suspension in each 1.5 ml tube thereby giving a co-culture with a multiplicity of infection (MOI) of 10^6, 10^4, 10^2 respectively. Controls with only *E. coli* cells in PAS and only *T. pyriformis* in PAS were set up. Tubes were incubated at 28°C. Aliquots from the tubes were taken at different time-points to determine *E. coli* and *Tetrahymena* counts. Subsequent predation experiments with *T. pyriformis* were done at an MOI of 10^6:1. For experiments involving lysing *T. pyriformis* cells, 50 µL of 0.4% Triton X-100 was added to 50 µL of co-culture at each time-point and incubated for 5 min. Aliquots were taken for *E. coli* counts before and after Triton X-100 treatments, serially diluted and plated on LB agar. LB agar plates were incubated overnight, colony counts were performed after 17 h and used to derive an estimated cell count.

E. coli cells were transformed with pUA66 (σ^{70}-GFP) which constitutively expresses GFP, to monitor the bacterial cells inside the *T. pyriformis*. A co-culture was set up as previously described in Section 2.11.3 and aliquots were taken after 18 h, fixed in ice-cold ethanol – methanol (1:1), resuspended in PBS and 2 µl was placed on a slide and imaged with Leica DMI3000 B microscope.

2.11.4 Predation Assay with *Acanthamoeba polyphaga*

Trophozoites of *A. polyphaga* were grown at 30°C for 5 days were washed in PAS for 3 times by centrifugation at 1100 x *g* for 3 min. The supernatant was quickly and gently removed by decanting without throwing out the cell pellets and the pellets resuspended in 1 ml of PAS. Trophozoite count was done using a haemocytometer (as described in Section 2.11.3) but without placing at -20°C for 2-4 minutes, since they are not fast movers. An overnight culture of each bacterial strain was grown in LB broth at 37°C and washed three times with PAS, harvesting by centrifugation at 9000 x *g* for 10 min between each wash. For developing the predation assay, a suitable volume of the washed *E. coli* corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 CFU ml^{-1} was obtained and

a volume of *A. polyphaga* cells corresponding to 100 trophozoites was added to the *E. coli* in a 1.5 ml tubes thus giving a co-culture with MOI of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 respectively. Controls with only *E. coli* cells in PAS and only *A. polyphaga* in PAS were set up. Tubes were incubated at 30°C. Aliquots from the tubes were taken at different time-points to determine *E. coli* and *A. polyphaga* counts as described in Section 2.11.3. Subsequent predation experiments with *A. polyphaga* were done at an MOI of 10^3:1.

2.12. SCANNING ELECTRON MICROSCOPY (SEM) OF *E. COLI* MACROCOLONIES

Macrocolonies obtained from growing *E. coli* as spots on YESCA agar incubated at 28°C for 48 h were prepared for SEM as described in the following and the steps were performed within a fume cupboard. Agar blocks containing single macrocolony biofilms were cut out from the agar plates, placed in Petri dishes containing drops of 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), and incubated for 1 h at room temperature to allow fixation to proceed. Fixation was followed by four washes with 0.2 M cacodylate buffer. Then macrocolonies were dehydrated in a graded alcohol series (30, 50, 70, 90, and 100% ethanol). The macrocolonies were submerged in the first ethanol concentration (30%) and allowed to incubate for 10 min. The 30% ethanol was decanted off and 50% ethanol was used to submerge the macrocolonies. This was continued until the 100% ethanol, which was allowed to incubate for 20 min. The macrocolonies are kept in 100% ethanol until the next step. After dehydration, macrocolonies were dried with Hexamethyldisilizane (Sigma) 2 times for 15 minutes each. Agar blocks containing the macrocolonies were placed on velin tissue in Petri dishes which were partially covered and allowed to dry for at least 2 h in a fume hood. The macrocolonies were gently floated off with sterile scalpel blades and fixed to aluminium stubs (Agar Scientific AGG3313) using carbon adhesive discs (Agar Scientific). The fixed samples were coated with gold in a gold sputter coater (EMScope SC500). The specimens were examined with a Hitachi S-4700 scanning electron microscope operating at 5 kV under high vacuum mode. Macrocolonies derived from three independent cultures for each strain were examined for each strain. Samples were viewed at 3,000 - 25,000x magnifications and > 5 fields were viewed per sample. The macrocolony biofilms were handled with extreme care in order to maximally preserve their structural integrity.

2.13. BIOINFORMATICS ANALYSES

All nucleotide sequences for *E. coli* K-12 MG1655 were obtained from the EcoCyc *E. coli* database (https://ecocyc.org/). Nucleotide sequences of *E. coli* SE11, SE15 and BW25113 were retrieved from the National Centre for Biotechnology Information (NCBI) database with GenBank Accession numbers AP009240.1, AP009378.1 and CP009273.1, respectively. Unassembled genomic sequences of the soil-persistent *E. coli* were provided by Dr F. Brennan (unpublished data). Nucleotide sequences of the genes of interest was extracted from the contigs of the soil-persistent strains using Geneious R8 (Biomatters), the nucleotide sequences were translated to amino acid sequences and aligned using Clustal Omega (Sievers *et al*., 2011) and Geneious R8. Phylogenetic tree was generated using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980) with bootstrap analysis (1,000 iterations) using MEGA 6 (Tamura *et al*., 2013). For the graphical representation of the sequence alignments, ClustalW in BioEdit v7.2.5 (Ibis Bioscience, CA, USA) was used.

2.14. STATISTICAL ANALYSIS

The Student t-test was used when comparing means from two samples whereas one-way Analysis of variance (ANOVA) was used when comparing means from three or more samples. Differences in motility, biofilm formation, GABA levels, acid survival and soil survival were investigated using SPSS 21.0 for Windows and GraphPad Prism 6. Statistical comparisons among the means in ANOVA were compared using Duncan Multiple Range Test at 5% probability level. Error bars on graphs indicate standard deviations from the means. A two-way ANOVA was used to test the effect of RpoS status and moisture level on survival of *E. coli* BW25113 and COB583 in soil.

CHAPTER 3

The general stress response is conserved in long-term soil-persistent strains of *Escherichia coli*

Note

Much of the material presented in this chapter has been published (Somorin *et al*., 2016; see Publication). However, it has been slightly modified to thesis format with the Material and Methods section removed to prevent duplication with Chapter 2.

3.1. ABSTRACT

Although *Escherichia coli* is generally considered to be predominantly a commensal of the gastrointestinal tract, a number of recent studies suggest that it is also capable of long-term survival and growth in environments outside the host. As the extra-intestinal physical and chemical conditions are often different from those within the host, it is possible that distinct genetic adaptations may be required to enable this transition. Several studies have shown a trade-off between growth and stress resistance in nutrient-poor environments, with lesions in the *rpoS* locus, which encodes the stress sigma factor RpoS (σ^S). In this study, a unique collection of long-term soil-persistent *E. coli* isolates was investigated to determine whether the RpoS-controlled general stress response is altered during adaptation to a nutrient-poor extra-intestinal environment. The sequence of the *rpoS* locus was found to be highly conserved in these isolates and no nonsense or frame-shift mutations were detected. Known RpoS-dependent phenotypes, including glycogen synthesis and gamma-aminobutyrate production, were found to be conserved in all strains. All strains expressed full length RpoS protein, which was fully functional using the RpoS-dependent promoter reporter fusion p*gadX::gfp*. RpoS was shown to be essential for long-term soil survival of *E. coli* since mutants lacking *rpoS* lost viability rapidly in soil survival assays. Thus, despite some phenotypic heterogeneity, the soil-persistent strains all retained a fully functional RpoS regulated general stress response, which is interpreted to indicate that the stresses encountered in soil provide a strong selective pressure for maintaining stress resistance, despite limited nutrient availability.

3.2. INTRODUCTION

Escherichia coli is a gram negative, facultative anaerobe, belonging to the Enterobacteriaceae family, which inhabits the intestinal tract of humans, warm-blooded animals and reptiles (Berg, 1996; Gordon & Cowling, 2003) It can be transferred through water and sediments *via* faeces and is almost universally used as an indicator of faecal contamination in drinking and recreational water. The use of *E. coli* as a faecal indicator is based, at least in part, on the assumption that it exists transiently outside of the host gastrointestinal tract (Ishii & Sadowsky, 2008) and does not survive for a long time in the external environment. Though several authors have isolated *E. coli* from various natural environments such as freshwater (Jiménez *et al*., 1989; Lopez-Torres *et al*., 1987), beach water (Chiang *et al*., 2011; McLellan & Salmore, 2003), beach sand (Chiang *et al*., 2011), tropical and subtropical soils (Byappanahalli & Fujioka, 1998; Byappanahalli *et al*., 2012; Desmarais *et al*., 2002; Fujioka, 2001; Solo-Gabriele *et al*., 2000), coastal temperate forest soils (Byappanahalli *et al*. 2006), riverine temperate soil (Ishii *et al*., 2006) and sediments (Anderson *et al*., 2005), it is difficult to determine unequivocally whether these isolates originated from recent contamination or if they represent long-term residents in those environments. In 2010, Brennan *et al*. (2010a) reported the recovery of *E. coli* populations from intact soil monoliths maintained in lysimeter units (previously described by Ryan & Fanning, 1996), which have been protected from faecal contamination since 1998. These long term soil-persistent *E. coli* isolates are the subject of the present study.

Soil-persistent *E. coli* strains from the lysimeters are genotypically diverse and possess unique growth and metabolic characteristics, suggesting adaptation to conditions present in soils (Brennan *et al*., 2013; Brennan *et al*., 2010b). These strains are assumed to have developed mechanisms that could help them survive in soil. For example, it was shown that a soil-persistent *E. coli* strain was nutritionally versatile and metabolised more substrates at 15°C than *E. coli* K-12 (Brennan *et al*., 2013). Furthermore, *E. coli* have been shown to survive and grow in both amended (Byappanahalli *et al*., 2004; Byappanahalli & Fujioka, 1998) and unamended (Ishii *et al*., 2006) soil. The capacity of these *E. coli* strains to survive for long periods of time and grow in the external environment raises questions about the validity of its continued use as indicator of water quality (Brennan *et al*., 2010a).

The ability to survive environmental stresses has been shown to be controlled by the general stress response regulator, RpoS, in *E. coli* and other related bacteria. RpoS (σ^S) is an alternative sigma factor that is involved in *E. coli* resistance to stresses typically encountered in the external environment such as cold stress, osmotic stress, oxidative stress and desiccation (Coldewey *et al*., 2007; Hryckowian & Welch, 2013; Stasic *et al*., 2012). When environmental stresses are encountered, cellular RpoS levels increase dramatically and the resulting RNA polymerase-RpoS holoenzyme complex produces the appropriate transcriptional response (Weber *et al*., 2005). A number of studies have shown that in low nutrient conditions, particularly when growth rates are very slow, mutations can accumulate within the *rpoS* open reading frame (ORF) resulting in partial or complete loss of RpoS function and reduced stress tolerance (King *et al*., 2004; Notley-Mcrobb *et al*., 2002). Mutations in *rpoS* have been reported in strains obtained from laboratory growth conditions as well as among natural isolates of *E. coli* (Alvarez-Ordóñez *et al*., 2013; Atlung *et al*., 2002; Bleibtreu *et al*., 2013; Chiang *et al*., 2011; Dong *et al*.,, 2009; Ferenci *et al*., 2011; King *et al*., 2006; Notley-Mcrobb *et al*., 2002; Snyder *et al*., 2012; Spira *et al*, 2008), *Salmonella* (Jørgensen *et al*., 2000; Robbe-Saule *et al*., 2003; Shah *et al*., 2012), *Cronobacter* (Alvarez-Ordóñez *et al*., 2012) and *Citrobacter* (Dong *et al*., 2009). These mutations are thought to provide a selective advantage to microorganisms undergoing nutrient starvation due to a trade-off between stress resistance and growth (Ferenci, 2005). Indeed, mutations that confer a growth and survival advantage in stationary phase cultures of *E. coli* (so-called GASP mutants) are frequently found to map to the *rpoS* locus (Zambrano *et al*., 1993). Thus the long term soil-persistent *E. coli* isolates present a unique opportunity to study the evolution of the *rpoS* locus in a natural nutrient-limited environment.

Soil can be considered as a highly competitive environment where nutrient sources are significantly less abundant than in the host gastrointestinal tract (Savageau, 1983). It was hypothesized that low nutrient micro-niches within the soil environment may select for *rpoS* mutations during long-term soil adaptation, perhaps providing a growth rate advantage. These *rpoS* lesions could also confer a competitive advantage through increased nutritional competence, a phenotype associated with the loss of *rpoS* (Ferenci, 2005; Notley-Mcrobb *et al*., 2002). Knowledge of how *E. coli* responds to chemical and physical stresses has been derived almost exclusively from studying laboratory strains, thus the available

collection of soil-persistent isolates of *E. coli* (Brennan *et al*., 2010a; unpublished data) from a closed system represents a rare opportunity to understand the role that stress responses play in the survival of *E. coli* in an environment outside the host where environmental conditions, nutrient availability and competing microorganisms are different from that present in the gastrointestinal tract.

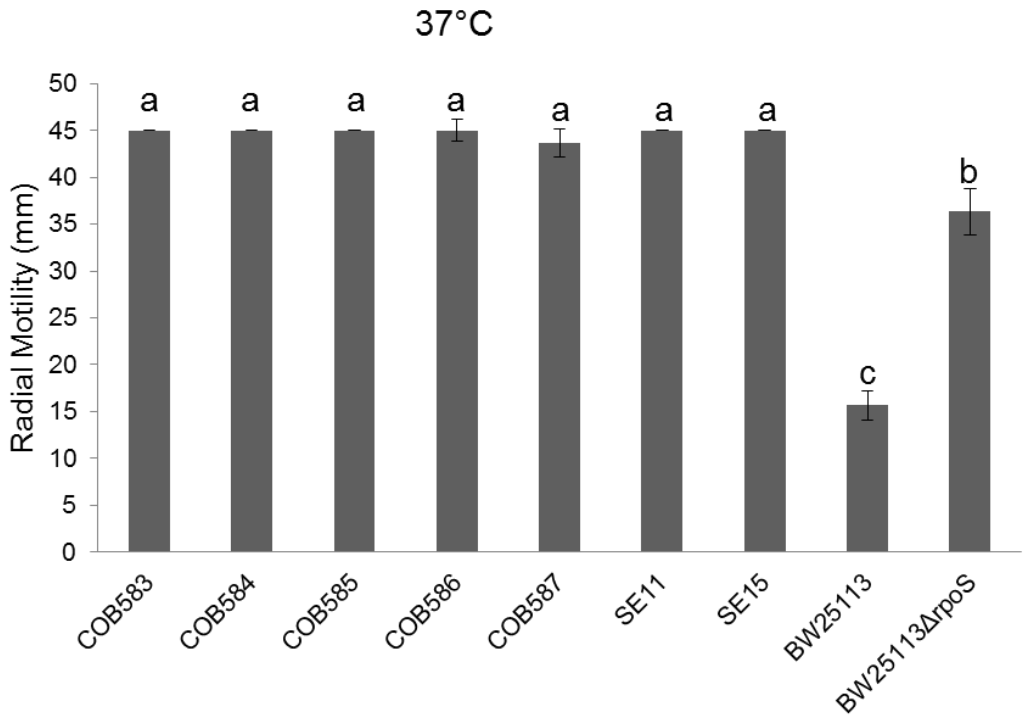
This present study characterized the RpoS-dependent stress response in five phylogenetically distinct soil-persistent *E. coli* strains and compared this to responses in commensal and laboratory *E. coli* strains. The *rpoS* locus was sequenced and Western blotting and green fluorescent protein (GFP) reporter fusion (using the RpoS-dependent *gadX* promoter) assays were used to determine the presence and activity of the RpoS protein in each of the isolates. Soil survival assays were also performed to investigate the role of an intact *rpoS* locus in soil survival. We show that a functional RpoS is retained in long-term soil-persistent isolates of *E. coli* and demonstrate that RpoS is essential for long-term survival in the soil.

3.3. RESULTS

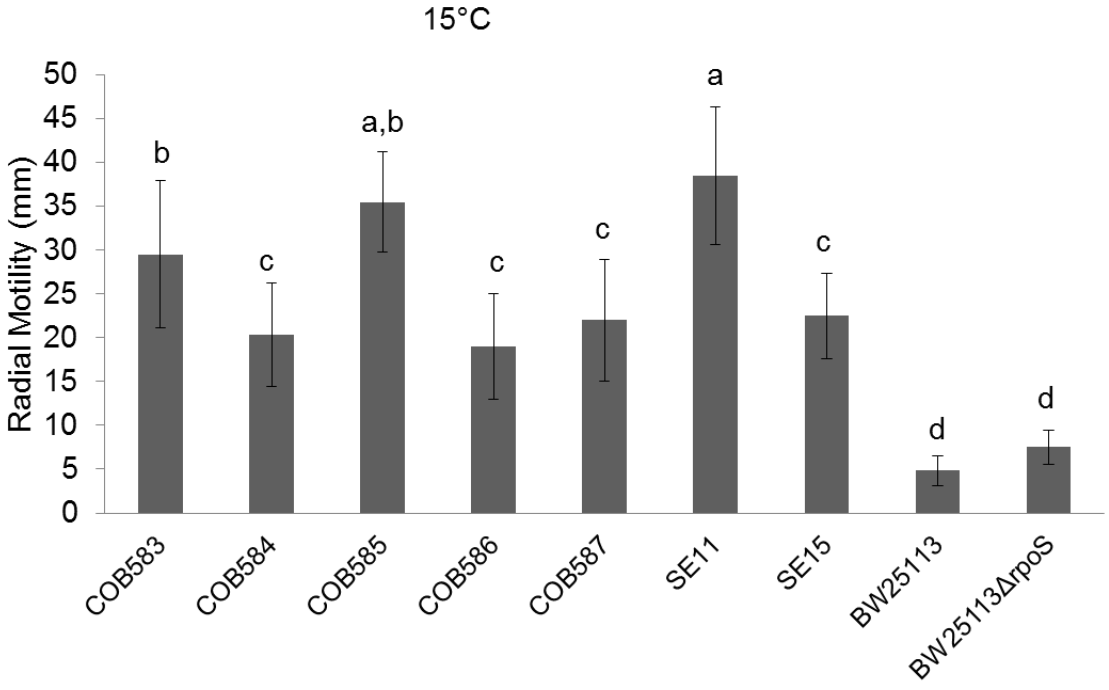
3.3.1 Soil-persistent *E. coli* displayed heterogeneous phenotypes

The soil-persistent *E. coli* strains were tested for motility and biofilm production, to determine if these phenotypes contribute to life in soil. All strains were motile to a similar extent at 37°C (Fig. 3.1A) with the exception of BW25113, which showed poor motility at this temperature. In contrast, motility was found to be more variable at 15°C, with three of the wild isolates (COB583, COB585 and SE11) displaying significantly greater motility than the other strains (Fig. 3.1B). Very low levels of biofilm were detected for all strains at 37°C in both LB broth and in a minimal medium with succinate as the sole carbon source. Two of the five soil isolates (COB583 and COB585) produced significant biofilm at 15°C in both media tested (Fig. 3.1C), SE15 produced high levels of biofilm (OD_{595nm} = 1.77) in the minimal medium while the others produced very little biofilm. Together these data demonstrate the phenotypic diversity of *E. coli* soil residents, consistent with the fact that these isolates belong to different phylogenetic groups.

A


37°C
Radial Motility (mm)
50
45
40
35
30
25
20
15
10
5
0
a
a
a
a
a
a
a
c
b
COB583
COB584
COB585
COB586
COB587
SE11
SE15
BW25113
BW25113ΔrpoS

B


15°C
Radial Motility (mm)
50
45
40
35
30
25
20
15
10
5
0
b
c
a,b
c
c
a
c
d
d
COB583
COB584
COB585
COB586
COB587
SE11
SE15
BW25113
BW25113ΔrpoS

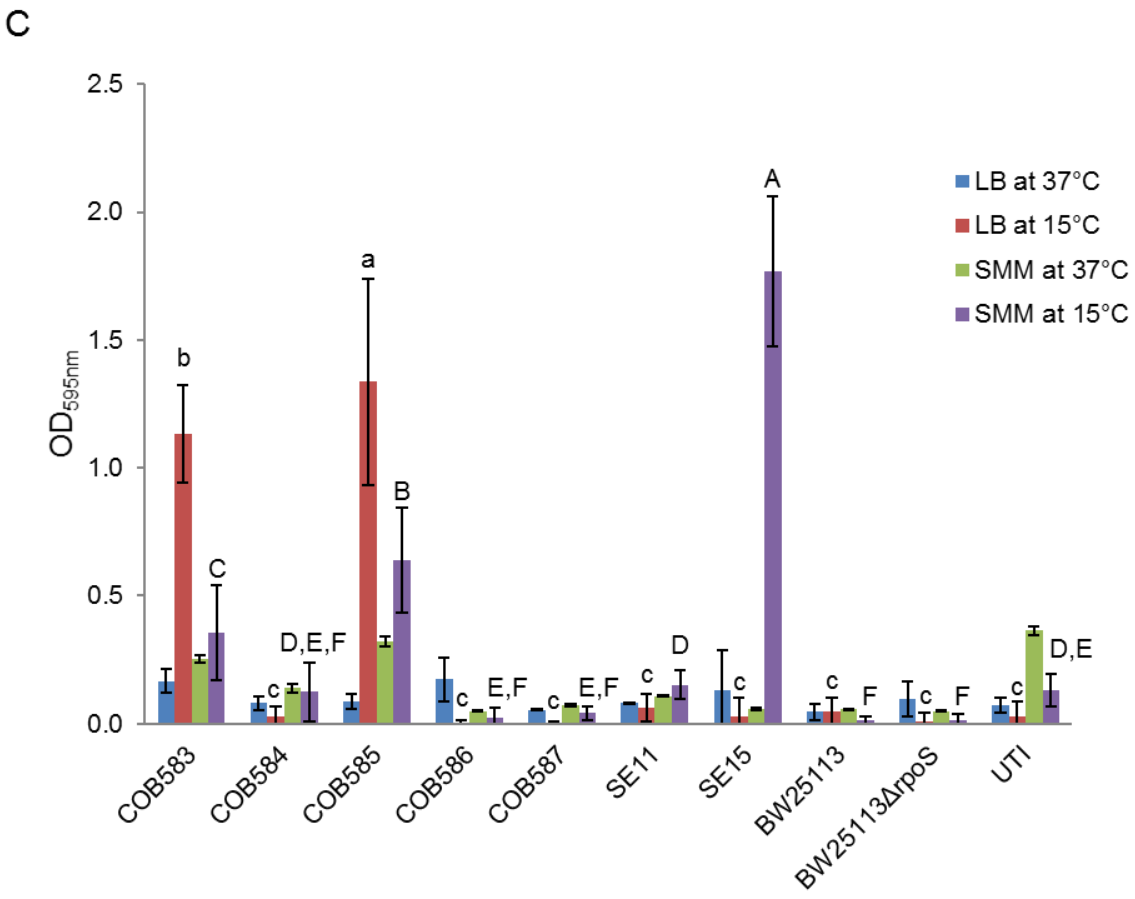
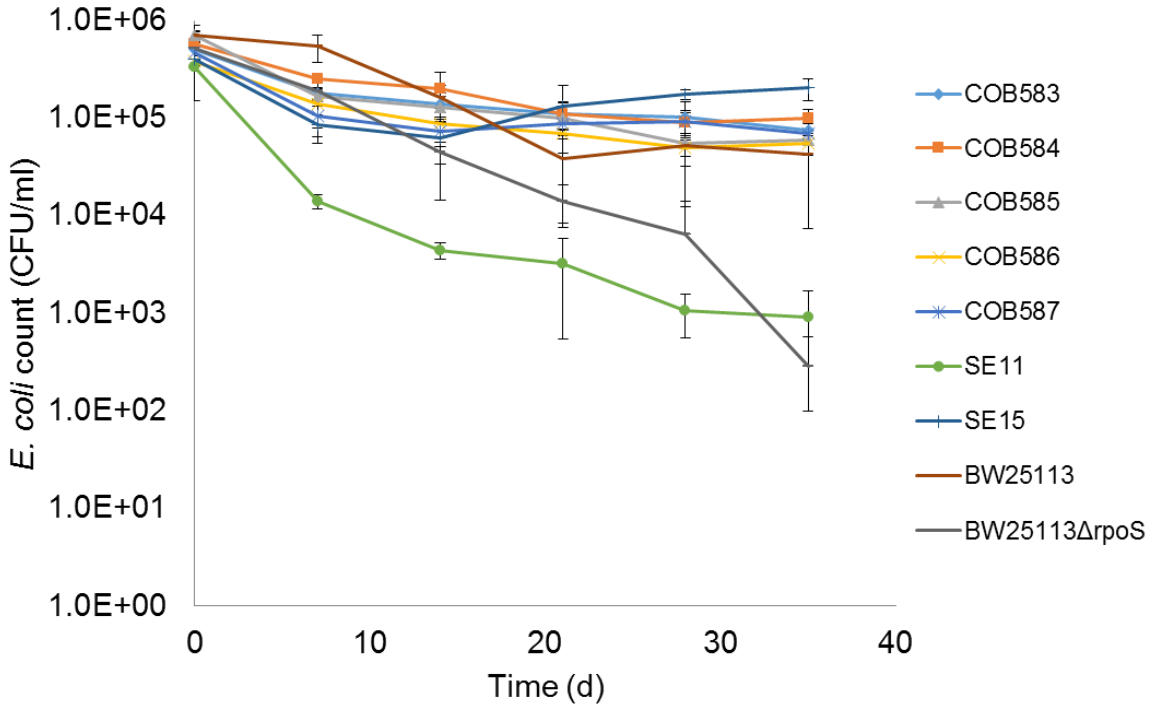


Figure 3.1: Motility and biofilm formation of *E. coli* strains. Motility on 0.25% (w/v) LB Agar at 37°C for 16 h (A), 15°C for 40 h (B) and biofilm formation in Lysogeny Broth (LB) broth and succinate minimal medium (SMM) (C) were determined as described in *Materials and Methods* (Section 2.6.1 – 2.6.2). UTI – Urinary tract infection *E. coli* isolate. Error bars represent standard deviations from three independent replicates. Data with similar lower case or upper case letters are not significantly different (p>0.05).

3.3.2 RpoS is required for survival of *E. coli* in soil at 15°C

Soil-persistent *E. coli* were tested for their ability to survive starvation and long-term in live soil at 15°C and the role of RpoS in the survival was determined. All the soil-persistent and commensal isolates of *E. coli* were found to survive well in a nutrient-poor environment at 15°C over 35 d, with the exception of SE11 which showed approximately 2 log reduction in culturable cell numbers (Fig. 3.2A). To investigate whether RpoS was required for survival in soil the survival of BW25113 and its *rpoS* mutant (BW25113Δ*rpoS*) were compared following the inoculation of a live soil sample with 10^6 cfu g^{-1}. The parent strain lost less than 1 log cycle of viability while the BW25113Δ*rpoS* was significantly (p = 0.0004, Student's t-test) impaired in its soil survival, dropping to less than 10^3 CFU g^{-1} after 35 days (Fig. 3.2B). Soil-persistent and commensal strains had similar survival patterns which were not significantly different (p > 0.05) after 230 d in live soil (Fig. 3.2C). The laboratory strain BW25113 also survived well under these conditions in soil but the BW25113Δ*rpoS* was again severely impaired in its survival, with no culturable survivors detected after 42 d. In order to directly test the role RpoS plays in the soil-persistent strains an *rpoS* deletion was constructed in COB583 (Section 2.5) and the survival of this mutant (COB583Δ*rpoS*) was compared to the parental strain in silty loam soils A and B. In both cases loss of *rpoS* was associated with a faster loss of viability in the soil, with a stronger effect observed in the soil B (Fig. 3.2D). The effect of the *rpoS* deletion on soil survival was similar in both the BW25113 and COB583 backgrounds for the silty loam soil A (Fig. 3.2B compared to Fig. 3.2D).

A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1.0E+03
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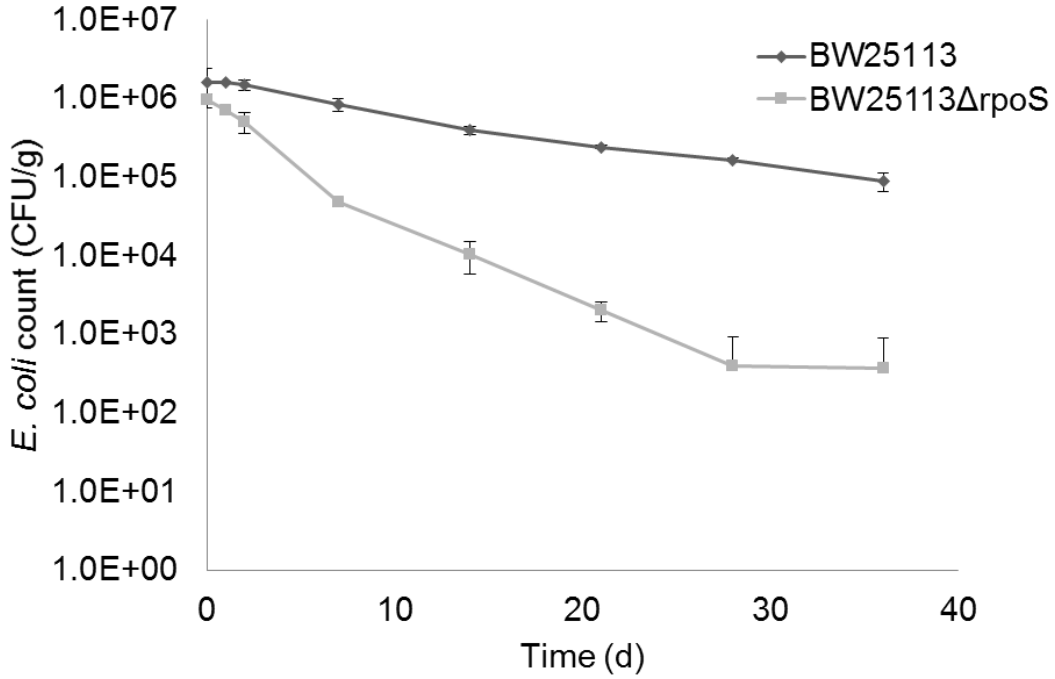
1.0E+01
1.0E+00
E. coli count (CFU/ml)
0
10
20
30
40
Time (d)
COB583
COB584
COB585
COB586
COB587
SE11
SE15
BW25113
BW25113ΔrpoS

B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1.0E+05
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1.0E+02
1.0E+01
1.0E+00
E. coli count (CFU/g)
0
10
20
30
40
Time (d)
BW25113
BW25113ΔrpoS

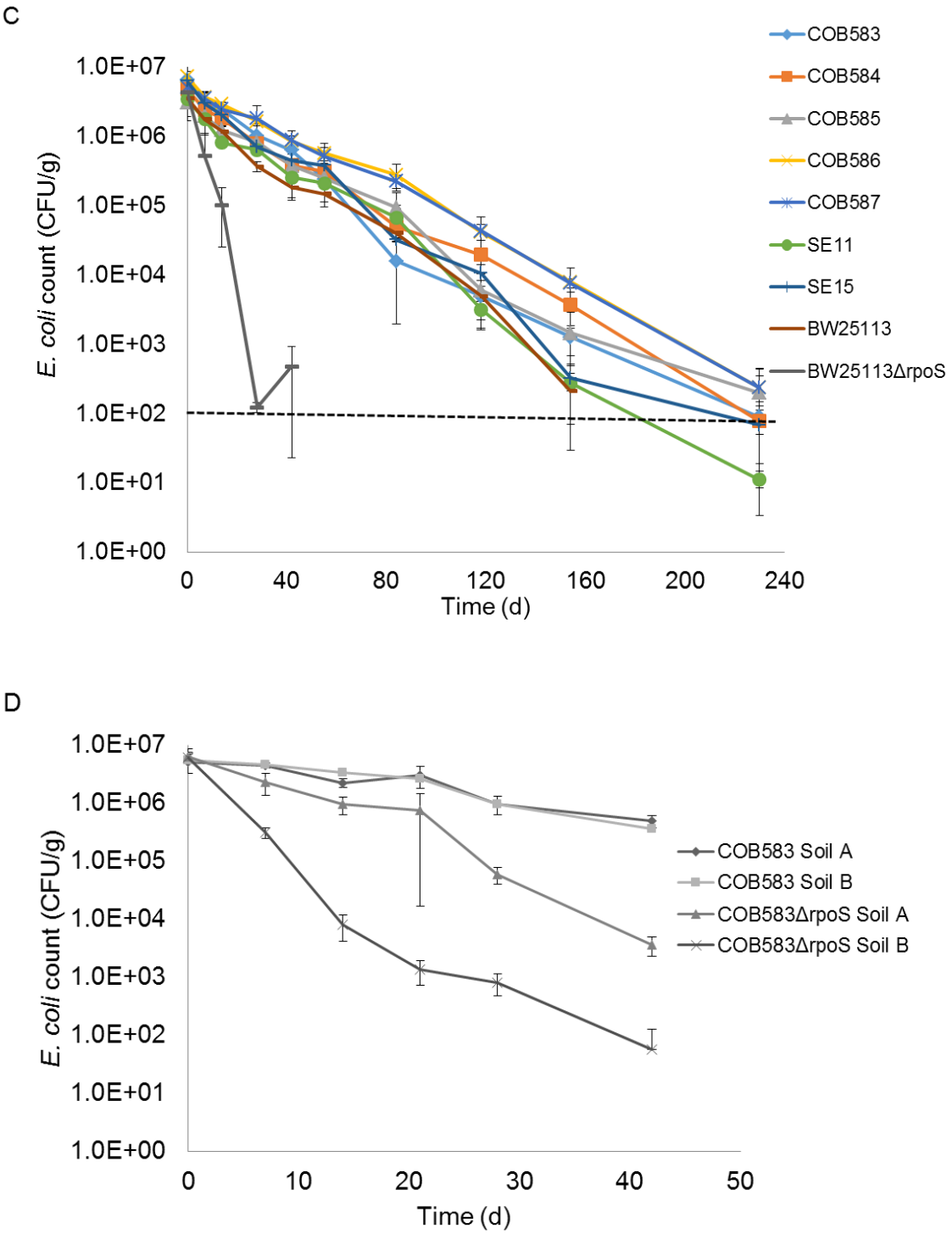


Figure 3.2: Survival of *E. coli* under various environmental conditions at 15°C. (A) Survival of the *E. coli* strains in PBS. (B) Survival of *E. coli* BW25113 and its isogenic Δ*rpoS* in soil. (C) Long-term soil survival experiment for all *E. coli* strains in silty loam soil A. (D) Survival of soil-persistent strain *E. coli* COB583 and its isogenic Δ*rpoS* in two silty soils as described in *Materials and Methods* (Section 2.6.5 and 2.7). Error bars represent standard deviations from three independent replicates. Dotted lines represent limit of detection of viable cells in the soil survival assay.

3.3.3 The *rpoS* locus is conserved in soil-persistent *E. coli*

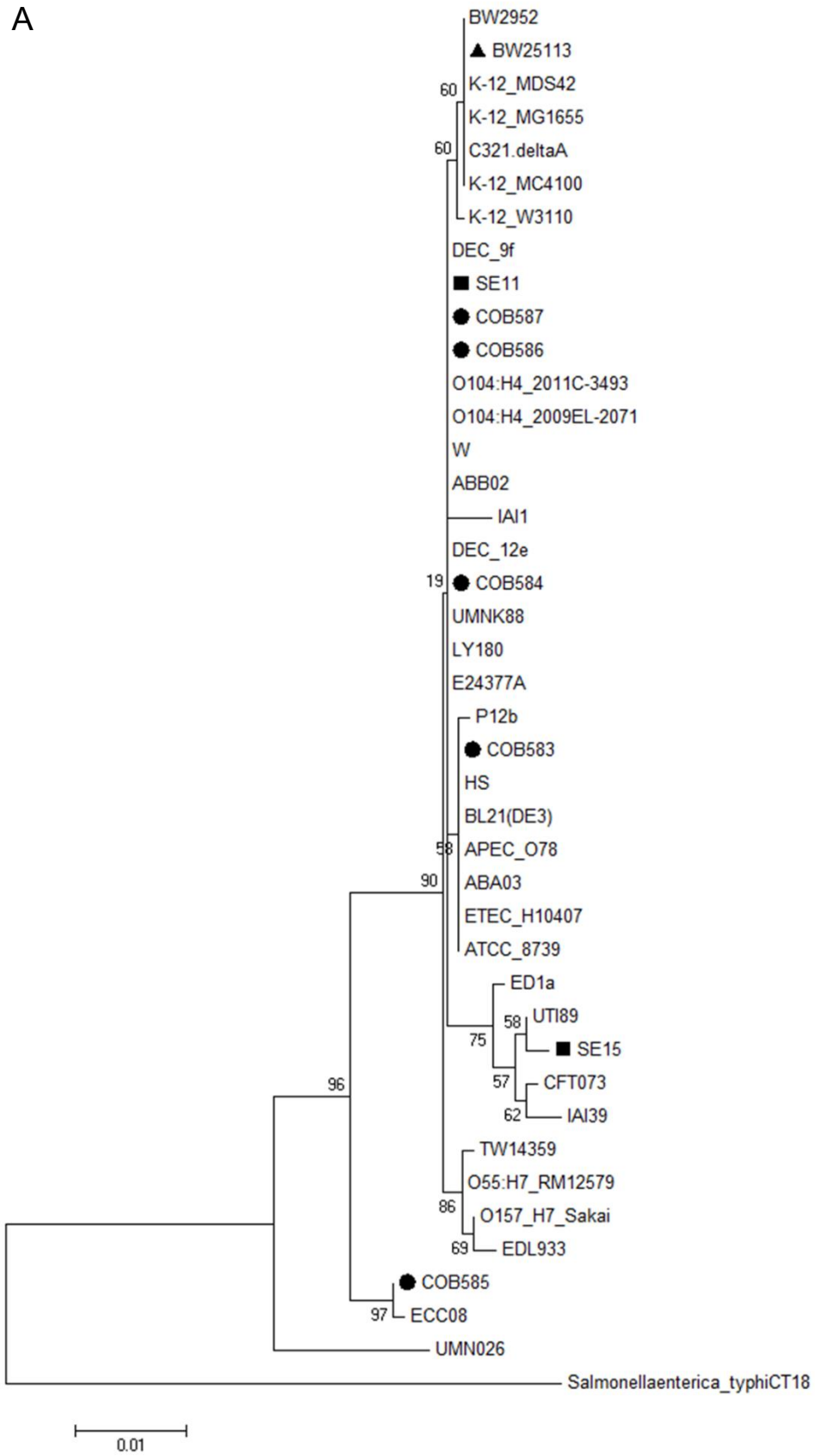
To determine whether long-term adaptation to soil has led to accumulation of *rpoS* mutations in the soil-persistent strains, the *rpoS* locus in the strains were sequenced and analysed. Although there were some sequence differences between the isolates, all of the soil-persistent strains were found to have an intact *rpoS* ORF (Table 3.1). Nucleotide substitution C – G at position 97 was present in all the soil-persistent and commensal strains and this resulted in a corresponding amino acid change from Glutamine (Q) to Glutamic acid (E) at codon 33. Although there were other nucleotide changes in the test strains, the change at codon 33 was the only nucleotide change that resulted in an amino acid change and the others were silent mutations. The sequence of *rpoS* in COB585 diverged significantly from the other strains, which was reflected in the phylogenetic tree generated when these sequences were compared to other sequenced *E. coli* strains (Fig. 3.3A). PCR and sequencing of genes flanking *rpoS* revealed insertion of open reading frames (ORFs) between *rpoS* and inner membrane permease (*ygbN*) (Fig. 3.3B). The region downstream from the *rpoS* ORF was conserved in 3 soil-persistent strains (COB584, 586, 587) and one of the commensal strains (SE11) but the locus had a different gene order in each of the other strains (Fig. 3.3B). Pattern I (COB583 and BW25113) which is similar to the reference strain (MG1655) show *rpoS* directly flanked by murein hydrolase activator (*nlpD*) and *ygbN*. Pattern II (COB584, COB586, COB587 and SE11) show insertion of 4 ORFs between *rpoS* and *ygbN.* Pattern III (COB585) show insertion of 5 ORFs after *rpoS* with *ygbN* absent while Pattern IV (SE15) had insertion of 2 ORFs between *rpoS* and *ygbN.* Region between *nlpD* (which carried *rpoS* promoters) and *rpoS* was conserved in all the strains. The regions carrying the known *rpoS* promoter sequences, located in the upstream *nlpD* ORF and in the intergenic region, were compared and were found to be conserved in all soil-persistent strains (Fig. 3.3B). These results confirm that the *rpoS* gene and regulatory elements are conserved in the soil-persistent strains but that differences exist in the genetic structure of this chromosomal locus, highlighting the genetic diversity that exists within this collection of soil isolates.

Table 3.1: Mutations within the *rpoS* gene of soil-persistent and commensal *E. coli* strains compared to MG1655

	Nucleotide Change		Corresponding Amino Acid Change
Strain	**Position**	**Change**	**[Codon Position]**
COB583	97	C → G	Gln (Q) to Glu (E) [33]
	942	C → T	-[a]
COB584	97	C → G	Gln (Q) to Glu (E) [33]
COB585	42	T → C	-
	81	A → G	-
	84	G → A	-
	93	A → T	-
	97	C → G	Gln (Q) to Glu (E) [33]
	123	C → T	-
	132	A → G	-
	144	G → A	-
	147	A → G	-
	819	G → A	-
	927	C → T	-
	972	C → A	-
	990	C → A	-
COB586	97	C → G	Gln (Q) to Glu (E) [33]
COB587	97	C → G	Gln (Q) to Glu (E) [33]
SE11	97	C → G	Gln (Q) to Glu (E) [33]
SE15	97	C → G	Gln (Q) to Glu (E) [33]
	163	T → C	-
	357	T → C	-
	462	T → C	-
	573	T → C	-
	699	G → A	-
	732	C → T	-
	990	G → A	-
BW25113	No change	No change	-

[a] – silent.

A


BW2952
BW25113
K-12_MDS42
K-12_MG1655
C321.deltaA
K-12_MC4100
K-12_W3110
DEC_9f
SE11
COB587
COB586
O104:H4_2011C-3493
O104:H4_2009EL-2071
W
ABB02
IAI1
DEC_12e
COB584
UMNK88
LY180
E24377A
P12b
COB583
HS
BL21(DE3)
APEC_O78
ABA03
ETEC_H10407
ATCC_8739
ED1a
UTI89
SE15
CFT073
IAI39
TW14359
O55:H7_RM12579
O157_H7_Sakai
EDL933
COB585
ECC08
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97
0.01

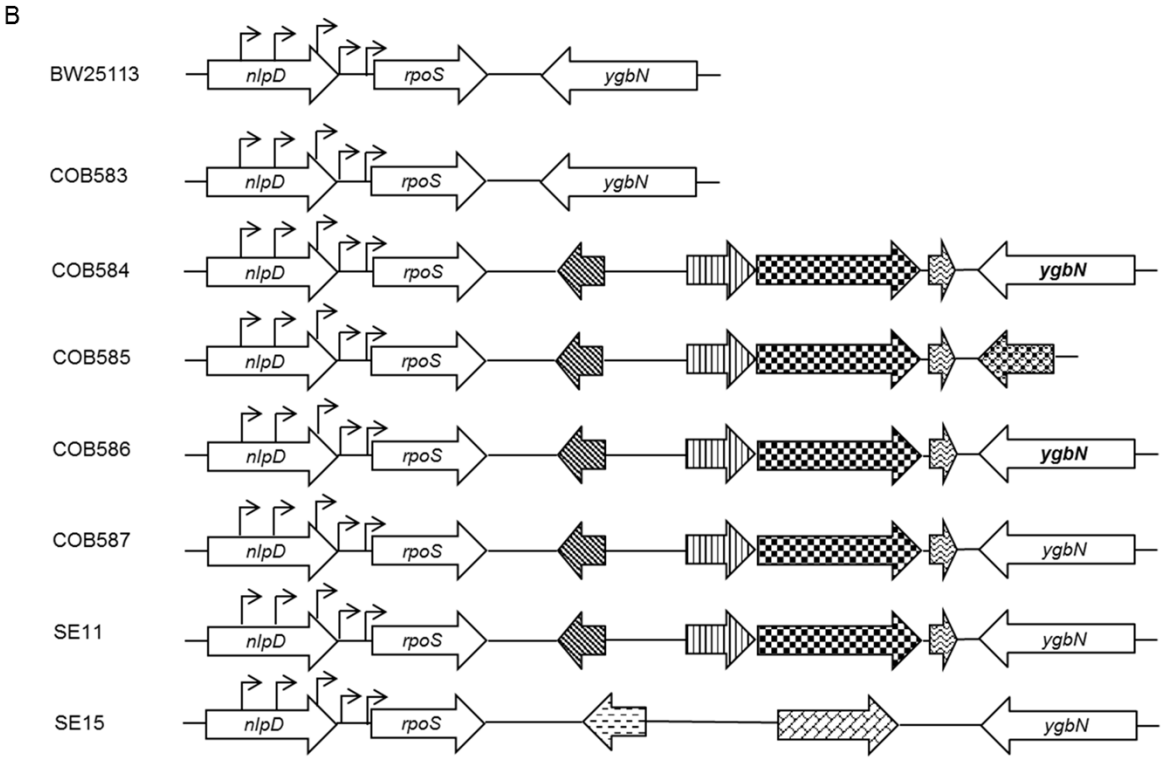
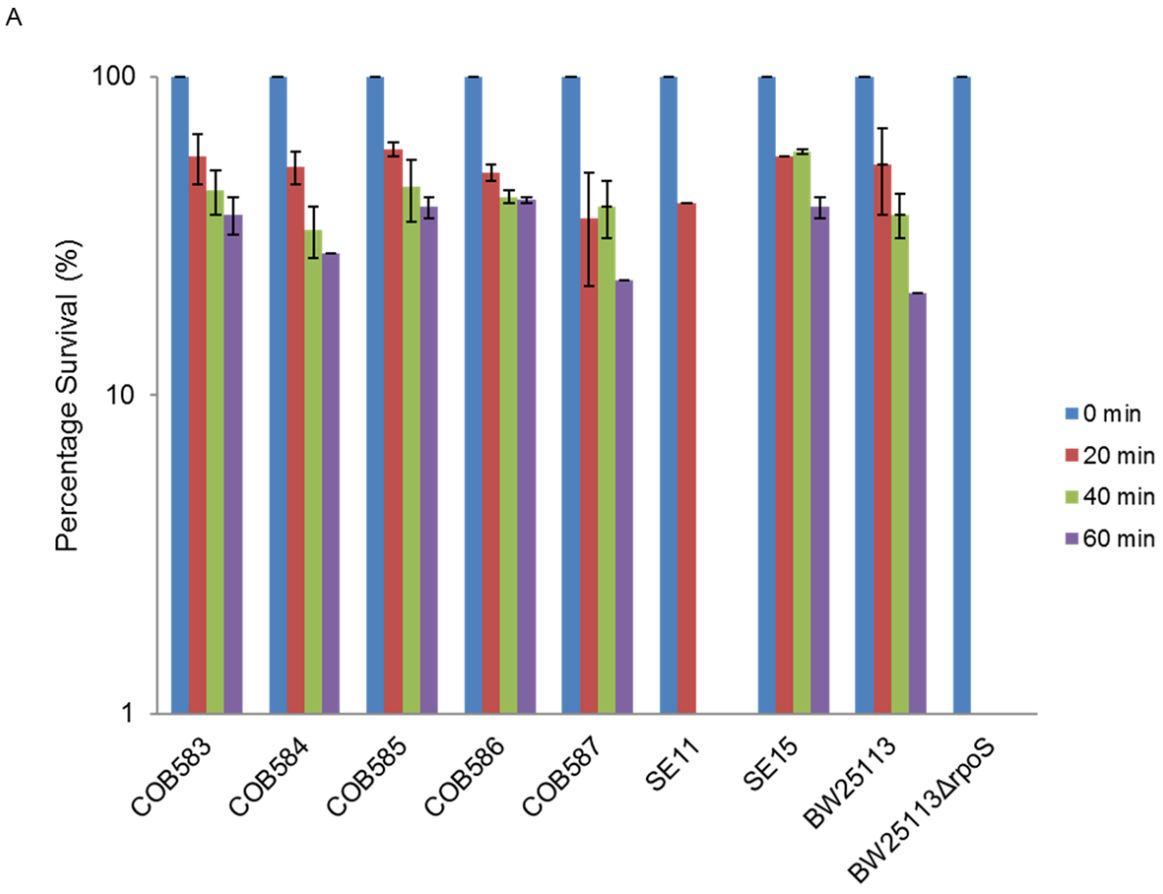


Figure 3.3: **Phylogenetic tree and Gene locus of *rpoS* in *E. coli* strains.** Phylogenetic tree performed using Maximum Likelihood method based on the Kimura 2-parameter model with bootstrap analysis (1,000 iterations) using MEGA 6 showed that *rpoS* in soil-persistent strains is similar to previously known *E. coli* strains (A). Gene outline of *rpoS* and its flanking genes in the soil-persistent and commensal strains show four distinct patterns (B). Block arrows with similar patterns indicate same ORF while arrowheads represent *rpoS* promoter sites.

3.3.4 Phenotypes under the control of RpoS are retained in soil-persistent *E. coli*

Having shown that *rpoS* is conserved in all soil-persistent strains, some RpoS-dependent phenotypes were tested to determine if RpoS is functional in these strains. Acid tolerance and γ- aminobutyrate (GABA) levels following acidification were measured for each of the soil-persistent and commensal isolates. Both acid tolerance and GABA production were confirmed to be under RpoS control since the

BW25113Δ*rpoS* mutant strain was exquisitely acid sensitive (Fig. 3.4A) and produced only very low levels of GABA in response to acidification compared to the parental control BW25113 (Fig. 3.4B). With the exception of SE11, all of the other strains survived pH 2.5 for 1 h (Fig. 3.4A). Furthermore, they all produced significant quantities of intracellular and extracellular GABA in response to acidification of the culture media (pH 4.0), although there were some strain differences in the amounts produced (Fig. 3.4B). The accumulation of intracellular glycogen in *E. coli* is another trait known to be under RpoS control (Hengge-Aronis & Fischer, 1992). An iodine-based staining assay was used to determine if the soil-persistent and reference strains could accumulate glycogen. The BW25113Δ*rpoS* mutant was found to stain negative (yellow-white) for glycogen while all other strains gave a glycogen-positive stain (red-brown; Fig. 3.4C). Together these results indicated that traits under the control of RpoS were retained during long-term soil adaptation, suggesting the presence of a general stress response in these isolates.



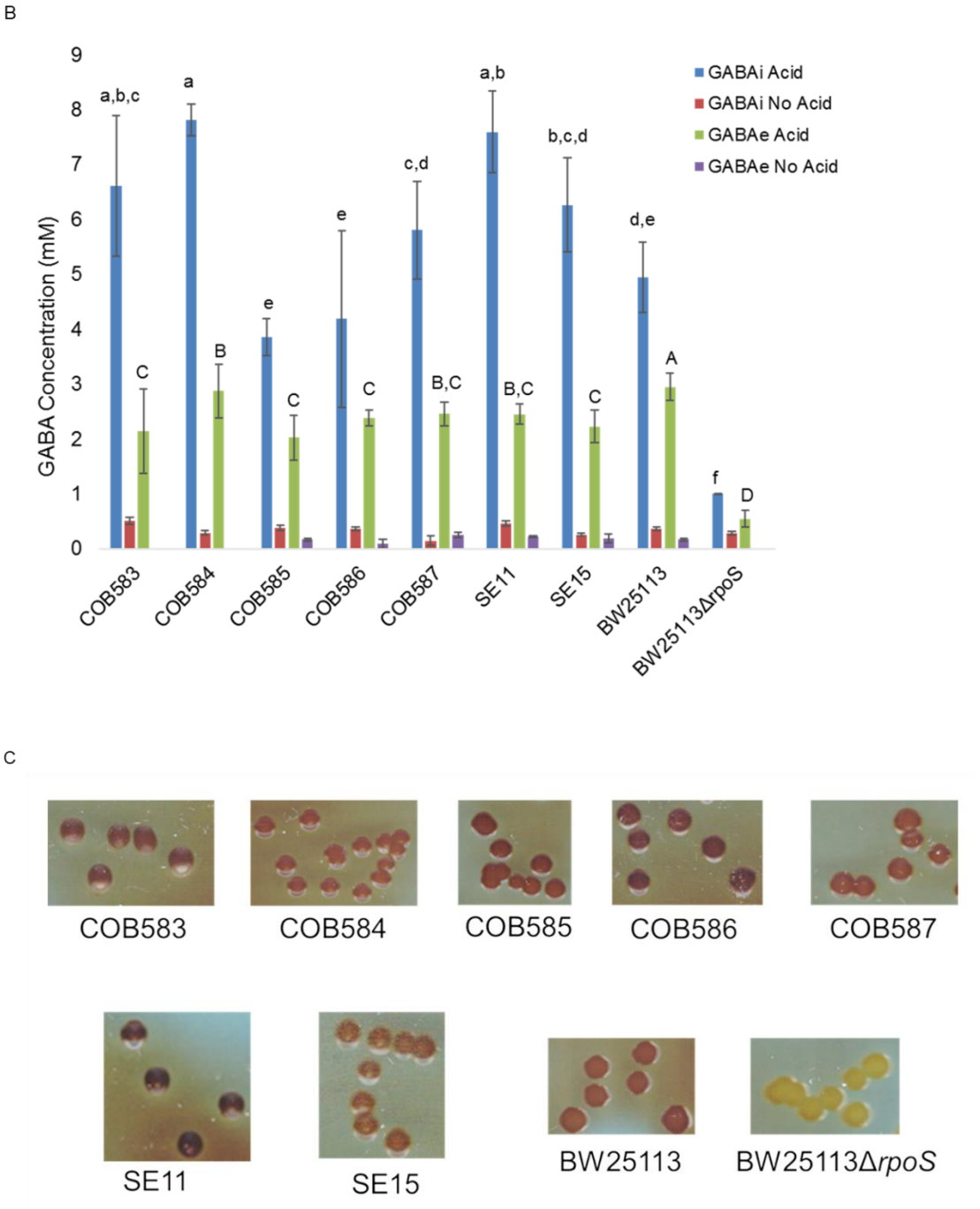


Figure 3.4: RpoS-dependent phenotypes show soil-persistent strains have functional RpoS. Survival of extreme acidity (pH 2.5) by *E. coli* strains at 37°C (A), production of γ- aminobutyric acid (GABA) in response to acid stress at pH 4 (B), and glycogen staining (C) as described in *Materials and Methods* (Section 2.6.3-2.6.4). Error bars represent standard deviations from three independent replicates. Data with similar lower case or upper case letters are not significantly different (p>0.05). GABAi means intracellular and GABAe means extracellular GABA.

3.3.5 RpoS is present and functional in long-term soil-persistent *E. coli*

While the above data suggested the presence of an active RpoS–mediated stress response in the soil-persistent strains, it didn't confirm that RpoS was expressed and active. The expression of RpoS was investigated in all strains at both 15°C (mimicking summer-time soil temperature in the east of Ireland, where isolates were collected) and 37°C using Western blot analyses with anti-RpoS monoclonal antibodies. A 37 kDa band corresponding to full length RpoS was detected in all strains with the exception of the BW25113Δ*rpoS* control, which confirmed the specificity of the antibodies. Strikingly there was a large increase in RpoS levels at 15°C compared to 37°C in all strains (Fig. 3.5). RpoS degradation products of similar sizes were also detected in most strains at 15°C (but not in the BW25113Δ*rpoS* mutant), except for COB587 and BW25113. To assess the activity of RpoS, a plasmid-based GFP reporter (p*gadX::gfp*) was transformed into all strains and used to record the transcription from the *gadX* promoter region, which is known to be highly RpoS-dependent and has been used by others as reporter of RpoS activity (Chattopadhyay *et al*., 2015; De Biase *et al*., 1999; Ma *et al*., 2003). A microscopic analysis of stationary phase cultures grown at 37°C revealed that all strains showed significant GFP expression, with the exception of the BW25113Δ*rpoS* mutant, which showed no detectable fluorescence. The fluorescence levels detected in the strains was not identical; COB585 reproducibly (n=2; with at least 3 fields each time) had a lower levels of fluorescence under these conditions. At 15°C, however, the fluorescence levels detected for all strains was greatly reduced compared to 37°C, with COB585 again showing the least fluorescence (Fig. 3.6A). One possibility was that decreased fluorescence might be caused by failure of the GFP protein to fold or mature properly (Liu *et al*., 2015) at 15°C. To test whether the levels of GFP were indeed reduced at 15°C, the levels were assessed by Western analyses using anti-GFP antibodies. The results showed that there was a strong correlation between the GFP levels and the levels of fluorescence detected by microscopy, suggesting that transcription of the *gadX::gfp* reporter was reduced at 15°C (Fig. 3.6B). RpoS activity, based on the reporter activity, was indeed decreased at 15°C despite the increased levels of the RpoS protein detected at this temperature. Taken together these data confirm that RpoS is both present and active in the soil-persistent and commensal isolates of *E. coli*.

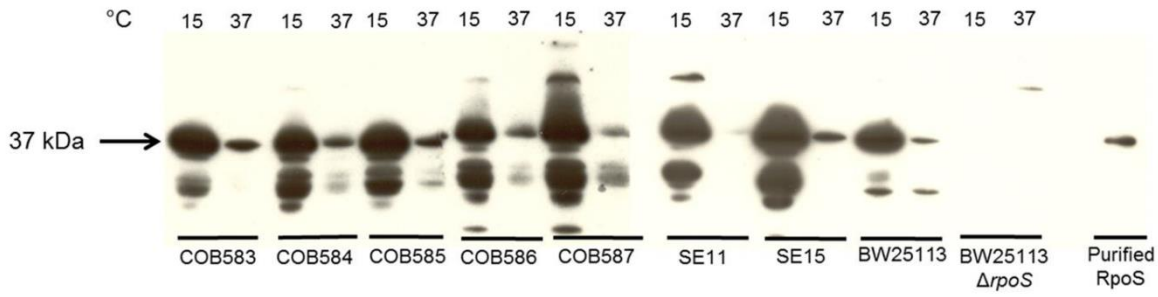


Figure 3.5: Higher level of RpoS is expressed in stationary phase at 15°C than 37°C in *E. coli*. Protein preparations were obtained from *E. coli* strains were grown in LB broth with agitation to stationary phase at 15°C and 37°C. Protein extraction, SDS-PAGE and Western Blotting was as described in *Materials and Methods* (Section 2.9.1 – 2.9.3). Purified RpoS protein was used as control. Image is representative of three independent replicates.

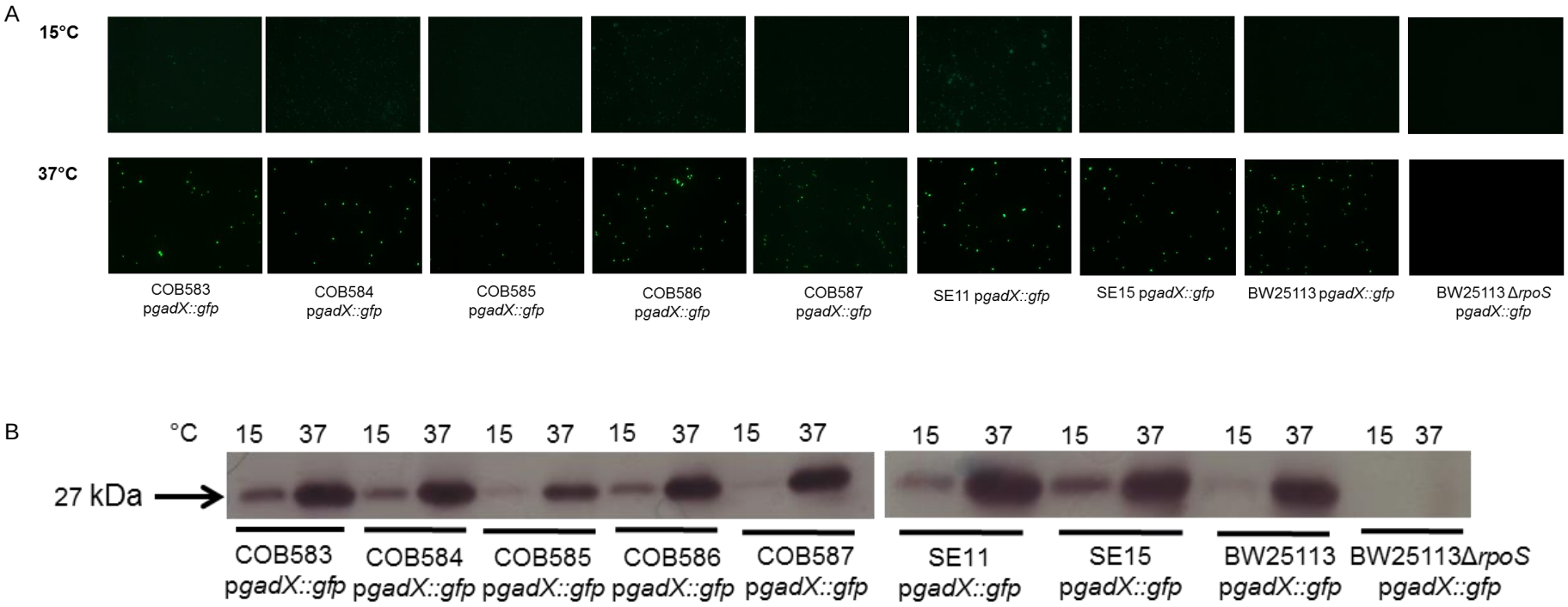


Figure 3.6: RpoS is active in the soil-persistent, commensal and laboratory *E. coli* strains. RpoS-dependent GFP expression from p*gadX::gfp* reporter fusion in stationary phase at 15°C and 37°C by fluorescent microscopy (A) and Western Blotting using anti-GFP antibody (B) as described in the *Materials and Methods* (Section 2.10.5). Fluorescent images presented are representatives of 2 independent experiments with > 3 fields captured in each experiment. Western Blot image is representative of three independent experiments.

3.4. DISCUSSION

Many studies in recent years have isolated and described *E. coli* from non-host environments (Brennan *et al*., 2010a; Byappanahalli *et al*., 2012; Chiang *et al*., 2011) but not much is known on the mechanisms of adaptation of *E. coli* to the soil. In this study, the phenotypic properties of some long-term soil-persistent *E. coli* were characterised, and it was observed that the general stress response regulated by RpoS was conserved in them and RpoS is required for long-term survival in soil. This collection of long-term soil-persistent isolates represents an opportunity to understand the role that stress responses play in allowing *E. coli* to thrive in an environment outside the host, where environmental conditions, nutrient availability and competing microorganisms are different from that present in the gastrointestinal tract.

All soil-persistent strains are motile at 37°C and 15°C, suggesting that motility is likely to be important for life in the soil environment. Though the individual strains showed different levels of motility (Fig. 3.1B), there was no significant difference (p>0.05) in motility when soil-persistent and commensal strains were compared at 37°C and 15°C. In an environment where nutrients are spatially and temporally heterogeneous, such as the soil, active motility could also be important in terms of a strategy for growth and survival. Brennan *et al*. (2012) reported some evidence of active movement of *E. coli* within soil profiles. Furthermore, motility is important for initiating biofilm formation because flagella help the initial attachment and movement along surfaces (Gonzalez *et al*., 2006; Pratt & Kolter, 1998). Biofilm formation, which is known to be RpoS-dependent (Schembri *et al*., 2003), was determined and the strains produced varying amounts of biofilm. COB583 and COB585 produced the most biofilm in rich medium (LB) while the commensal strain SE15 produced the highest biofilm in minimal medium (SMM) (Fig. 3.1C). It was initially hypothesized that high biofilm formation may be an important phenotype for survival in the soil, however, our results showed that only two soil-persistent isolates produced high levels of biofilm under the conditions investigated, suggesting that the ability to form biofilm may not be an essential phenotype for long-term soil survival. The result of the heterogeneity in biofilm production observed in our study is consistent with the study of Skyberg *et al*. (2007), which showed that biofilm production is strain-specific and not significantly influenced by phylogenetic group. Similar to previous studies that have shown biofilm formation is higher at lower temperature (Else *et al*., 2003; Ingle *et al*., 2011) and biofilm-related genes are upregulated at 23°C compared to 37°C (White-Ziegler *et al*., 2008), this study demonstrates that *E. coli* strains can produce high levels of biofilm at 15°C.

The survival data suggested that the general stress response was conserved in the soil-persistent strains but this was investigated further by sequencing the *rpoS* locus in each of the five soil isolates and comparing this to the *rpoS* locus from other sequenced *E. coli* genomes. *E. coli* strains have been shown to accumulate mutations in the *rpoS* gene when grown for long-term in the laboratory, in batch culture, in stab cultures, in chemostats run with poor carbon sources and even in natural commensal, pathogenic and environmental isolates from fermented sausage, human faeces and urine, beaches, wastewater effluent and animal (Alvarez-Ordóñez *et al*., 2013; Atlung *et al*., 2002; Bleibtreu *et al*., 2013; Chiang *et al*., 2011; Dong *et al*., 2009; Ferenci *et al*., 2011; King *et al*., 2006; Notley-Mcrobb *et al*., 2002; Snyder *et al*., 2012; Spira *et al*., 2008). When we compared the *rpoS* locus in the test strains with *E. coli* K-12 MG1655, various nucleotide substitution mutations were observed but none of these resulted in a premature stop codon (Table 3.1). This shows that *rpoS* is conserved in the soil-persistent and commensal strains. *E. coli* BW25113 had the same *rpoS* sequence as *E. coli* K-12 MG1655 as expected, since it is a derivative of the K-12 strain. The *rpoS* gene of sequenced *E. coli* strains in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) database was analysed and it was observed that glutamine (Q) at codon 33 was conserved only in K-12 derived laboratory-adapted strains. Atlung *et al*. (2002) had previously reported a similar observation when comparing K-12 strains with six non-K-12 strains and they proposed that GAG (coding for glutamic acid (E) at codon 33) was present in the *E. coli* common ancestor, and it evolved to TAG (STOP) in the process of laboratory evolution and then mutated into CAG (coding for glutamine (Q) at codon 33). Polymorphism at codon 33 has been reported among *E. coli* strains in literature (Alvarez-Ordóñez *et al*., 2012; Dong *et al*., 2009; Spira *et al*., 2008) but most of these studies used the original K-12 sequence (33Q) as their wildtype reference sequence, when the polymorphism occurred only in the K-12 lineage. It has been shown that there is no difference in RpoS activity with either glutamic acid (E) or glutamine (Q) at codon 33 (Atlung *et al*., 2002) suggesting that this residue is not critical for the functioning of RpoS.

Data from this study show that the soil environment does not preferentially select for *rpoS* mutations and this is consistent with recent studies reporting that loss-of-function RpoS mutations are rare in a large collection of natural isolates of commensal, pathogenic and environmental *E. coli* (Bleibtreu *et al*., 2014; Snyder *et al*., 2012). Bleibtreu *et al*. (2014) reported no variation in the amino acid sequence of RpoS in the

E. coli strains which had minimal laboratory handling before being sequenced and showed that storage and successive transfers resulted in the *rpoS* mutations. Spira *et al*. (2011) also reported that transfer of *E. coli* on LB-stabs between laboratories led to mutations in *rpoS*. Retention of an intact stress response may be important for *E. coli* in soil, since it is a dynamic environment where *E. coli* may encounter multiple stresses (Fig. 1.3). *E. coli* must overcome stresses such as UV radiation, if close to soil surface, low nutrients, low temperature, desiccation, competition and predation, to thrive in the soil. Furthermore, using phylogenetic analysis, RpoS was shown to be highly conserved in *E. coli*. The clustering of *rpoS* in the *E. coli* strains reflects phylogenetic diversity in the long-term soil-persistent *E. coli* strains used in this study (Fig 3.3A). It has been recently shown that the phylogenetic grouping based on *rpoS* is highly consistent with phylogenetic clustering based on Multilocus sequence typing (MLST), thus suggesting that *rpoS* is a good indicator of evolutionary history of *E. coli* strains (Bleibtreu *et al*., 2014). On this basis, strain COB585 is the most divergent of the soil-persistent strains used in study and it is most closely related to ECC08, which is a strain collected from a beach water sample at Bayfront Park beach, Hamilton, Canada (Chiang *et al*., 2011).

Having established that the *rpoS* gene is conserved in the long-term soil-persistent strains it was important to measure RpoS levels and activity, since its role in the general stress response is complex, being regulated at multiple levels (reviewed in Battesti *et al.*, 2001). All the strains with an intact *rpoS* gene produced detectable RpoS protein (Fig. 3.5). Besides the 37 kDa RpoS protein observed in the RpoS-positive strains, there were other bands detected in all the strains which were not found in the BW25113Δ*rpoS* strain. These are likely to be degradation products specific to RpoS, as one of the major regulatory mechanisms of RpoS levels is proteolysis by ClpXP (Section 1.5.4; Lange & Hengge-Aronis, 1994). All the soil-persistent strains, commensal strains and BW25113 displayed known RpoS-dependent phenotypes that were absent in the BW25113Δ*rpoS* mutant, albeit with some small differences (Fig. 3.4), suggesting that RpoS is active in these strains. For example, RpoS plays an important role in acid tolerance in *E. coli* through its influence on the glutamate decarboxylase (GAD) system; specifically RpoS controls the transcription of the structural genes of the GAD system (*gadBC*, and *gadA*) as well as a positive regulator of this system, GadE (reviewed in Lund *et al*., 2014). GABA was produced by all strains with intact RpoS in order to overcome acid stress. *E. coli* SE11, a commensal strain, had an attenuated response to extreme acidity at 37°C, as it only survived pH 2.5 for 20 minutes (Fig. 3.4A). Interestingly, the RpoS level in SE11 at 37°C was lowest

among the strains (Fig. 3.5), and it is possible that this contributes to the attenuated acid stress response and reduced survival under starvation at 37°C (Fig. 3.S1). However, this was not the case at 15°C, where SE11 produced high level of RpoS and survived extreme acidity for 1 h (Fig 3.S2), similar to other soil-persistent and commensal strains, but still survived rather poorly in PBS compared to the other RpoS-positive strains (Fig. 3.2A). The reason for reduced stress response in *E. coli* SE11 under these conditions is not clear at present.

RpoS activity as determined by the *gadX* reporter fusions shows that RpoS was active in all the long-term soil-persistent strains at 15°C and 37°C. However, expression of GFP reporters at stationary phase was higher at 37°C than at 15°C (Fig. 3.5B). This low GFP expression correlated with lower fluorescence at 15°C (Fig. 3.6A). This is contrary to the fact that RpoS levels, as determined by western blotting, were higher at 15°C (Fig. 3.5A). For example, *E. coli* SE11 which had low levels of RpoS at 37°C was still active in transcribing the RpoS-dependent promoter leading to high GFP expression (Fig. 3.6A). This data shows that RpoS protein level does not always correlate with reporter activity. *E. coli* COB585, the most divergent of the strains, had the lowest RpoS activity among the test strains at 37°C. Similar attenuated RpoS activity was also observed in *E. coli* ECC08, which is closely related to COB585 (Fig. 3.3A; Chiang *et al*., 2011). No RpoS activity was observed in exponential phase at 15°C and 37°C in all the strains tested in this study. This growth-phase dependent RpoS activity was also reported by Sledjeski *et al*. (1996) and shows that increasing RpoS levels at low temperature does not necessarily lead to the physiological response observed when cells enter into stationary phase or when they encounter other environmental stresses. Though *gadX* is strongly RpoS-dependent in stationary phase, it was shown that the RpoS-dependence may be reduced or abolished when under acid stress (Weber *et al*., 2005). It will be interesting to further investigate if RpoS activity reduces when exposed to cold stress and other similar stresses encountered in the environment.

It was speculated that low activity of RpoS in stationary phase at 15°C may be due to upregulation of factors that inhibit promoter recognition by RpoS (Becker *et al*., 1999), increased negative feedback inhibition/autoregulation of RpoS (Becker *et al*., 1999) or increase in induction of anti-sigma factors (Becker *et al*., 2000). The response regulator RssB, which is important for regulating intracellular RpoS levels at exponential phase and under low stress, can also serve as an anti-sigma factor and inhibit the RpoS-

dependent gene expression in the presence of high RpoS levels (Becker *et al*., 2000). Though Becker *et al*. (2000) could not identify *in vivo* growth conditions that resulted in such high RssB induction, it is speculated that growth in LB at 15°C may be an example of such conditions, based on high RpoS degradation observed. Besides its role in RpoS proteolysis, there is also evidence that RssB directly interacts with the turnover element in RpoS thus blocking promoter recognition by RpoS and transcription initiation of RpoS-dependent promoters thus inhibiting the transcriptional activity of RpoS (Becker *et al*., 1999). Further work will be necessary to establish whether RssB contributes to the reduced RpoS activity that was observed at 15°C.

The heterogeneity in RpoS activity may reflect the genetic diversity of *E. coli* lineages present in the soil, perhaps suggesting that they have evolved to occupy different micro-niches in the soil. This study has shown that long term soil adaptation does not select for *rpoS* mutation. The soil survival data clearly supports the conclusion that it is important to retain a functional RpoS-mediated stress response in order to survive long-term in the soil (Fig. 3.2B-D). These data demonstrate that regardless of their origin, whether gut, soil or laboratory, *E. coli* can persist in soil for long periods and also suggest that the general stress response is intrinsic to this trait. Since RpoS controls multiple stress responses combined with the fact that the soil environment is very dynamic, an intact stress response was retained as *E. coli* encounters UV radiation from sunlight, lower temperature, hyper- and hypo-osmotic stress, nutrient availability, competition and predation (Arrange *et al.,* 1993; Griffiths *et al.,* 2003; Ibekwe *et al.,* 2010). Thus it seems clear that the selective pressure to maintain stress resistance within the soil environment outweighs the potential growth advantage that might arise from loss of RpoS function.

Overall the phenotypic characteristics and stress response of long-term soil-persistent *E. coli* was found to be very similar to commensal *E. coli*, suggesting that an intact stress response may be required by *E. coli* to ensure environmental persistence prior to availability of a new host. Our results also suggest that the long-term residence of *E. coli* strains in a soil niche doesn't select for consistently high levels of motility or biofilm formation. It is speculated that the huge genetic diversity found in *E. coli* as a species could be driven by the fluctuations within environmental niches rather than by the comparatively homogenous environment of the gastrointestinal tract. The variety of micro-niches available within the soil could provide a variety of different selective pressures that each result in phenotypically different populations. The idea that intra-

and inter-species diversity is driven largely by evolutionary trade-offs that arise between different properties of the cell (e.g. biofilm formation, metabolism, motility, stress resistance) when they are subject to the constraints of different environments has recently been reviewed comprehensively (Ferenci, 2016).

Since soil-persistent strains have been reported to have unique growth and metabolic characteristics compared to the common laboratory reference strain (*E. coli* K-12 and its derivatives) (Brennan *et al*., 2013), there is the need to utilise natural isolates of *E. coli* when studying mechanisms involving growth and metabolic capacities in *E. coli* under different conditions. In summary, the findings of this study show that loss-of-function mutations are absent in the *rpoS* of long-term soil-persistent *E. coli* strains and that RpoS is highly conserved in these strains. Using RpoS-dependent phenotypes and reporter activity measurements, it was confirmed that a functional RpoS response is retained among long-term soil-persistent strains and that RpoS is important for long-term survival of *E. coli* in soil.

Supplementary Figures

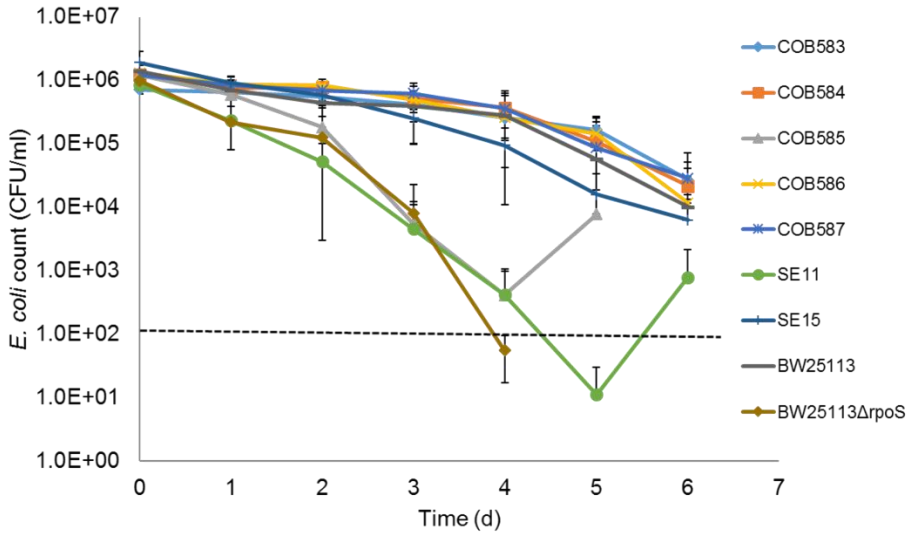


Figure 3.S1: Survival of *E. coli* strains in PBS at 37°C. Survival assay in PBS as described in *Materials and Methods* (Section 2.6.6) but with tubes incubated at 37°C. Error bars represent standard deviations from three independent replicates. Dashed lines represent detection limit of assay.

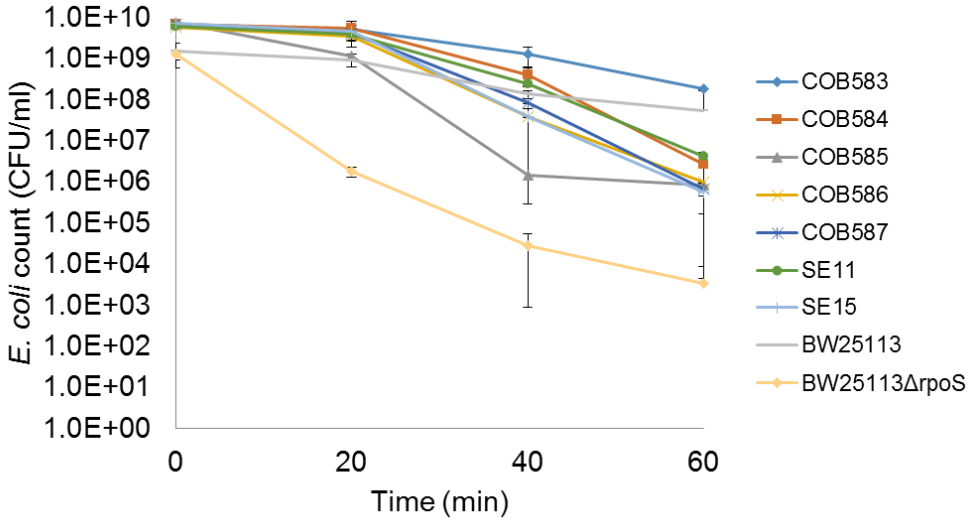


Figure 3.S2: Survival of extreme acidity (pH 2.5) by *E. coli* strains at 15°C. Survival of extreme acidity was conducted as described in *Materials and Methods* (Section 2.6.3) but with flasks incubated at 15°C. Error bars represent standard deviations from three independent replicates.

CHAPTER 4

RpoS contributes to resistance of *Escherichia coli* to protozoan predation and survival at low soil moisture

4.1. ABSTRACT

Soil provides a complex ecosystem where microorganisms and macroscopic organisms interact. The interaction between these biotic and abiotic factors within such a habitat thus determines the survival and fate of each component of the system. Having previously shown that *E. coli* requires the deployment of a full stress protection response mediated by RpoS, to survive in soil, it was important to determine how RpoS mediates soil survival, especially regarding the different conditions encountered in the soil. This study investigated the susceptibility of soil-persistent *E. coli* to predation by *A. polyphaga* and *T. pyriformis*, the role RpoS plays in resisting protozoan predation and the impact of moisture on RpoS-mediated soil survival. Strain-specific differences were observed in the predation of soil-persistent, commensal and lab *E. coli* strains, with soil-persistent strain COB583 being the most resistant to predation both protozoans. It was demonstrated that RpoS and curli, proteinaceous fibres used for attachment to biotic and abiotic surfaces, increased the ability of *E. coli* to resist predation by *A. polyphaga* and *T. pyriformis*. The results also showed that there was a significant (p = 0.014) interaction effect between moisture and RpoS in the survival of *E. coli* BW25113 but not the soil-persistent *E. coli* COB583, which demonstrated high survival in soil irrespective of moisture content. Overall, this study confirmed that RpoS contributes to *E. coli*'s resistance to protozoan predation and that RpoS is crucial for the increased fitness of soil-persistent *E. coli* against predation and desiccation in soil compared to BW25113.

4.2. INTRODUCTION

Protozoans constitute an important part of the microbial communities in the soil and they play critical roles in the soil ecosystem. They have been found to shift the microbial community structure and enhance nutrient recycling (Siddiqui & Khan, 2012), both of which impacts positively on plant growth. Predation of bacteria by protozoans regulate the bacterial population in soil and other natural ecosystems (Murase *et al*., 2006) and is the major means of removing *E. coli* in slow sand filters (Haig *et al*., 2015). Two of the most widely studied protozoan genera are *Acanthamoeba* and *Tetrahymena*.

Acanthamoeba spp. are free-living protozoans that are found in various external environments including soil (Reyes-Batlle *et al*., 2014, 2016; Tanveer *et al*., 2015; Todd *et al*., 2015), water (Hamilton *et al*., 2016; Lass *et al*., 2014; Lorenzo-Morales *et al*., 2005; Mahmoudi *et al*., 2015; Tanveer *et al*., 2015; Trabelsi *et al*., 2016), and dust (Costa *et al*., 2010; Niyyati *et al*., 2009) from different parts of the world. *Acanthamoeba* spp. have also been isolated from animal hosts such as mosquitoes (Otta *et al*., 2012) and wild squirrels (Lorenzo-Morales *et al*., 2007). Based on their important roles in the soil, amoebae are regarded as the most important group of soil protozoa (Ekelund & Rønn, 1994). Other species of *Acanthamoeba* cause sight-threatening infection of the cornea known as Acanthamoeba keratitis (AK) (Lorenzo-Morales *et al*., 2007). Free-living amoebae are the dominant bacteria consumers in soil and are responsible for up to 60% of the total reduction in bacterial population (Sinclair *et al*., 1981). *Acanthamoeba* spp. are an important contributor to a functional soil ecosystem as they induce rapid shifts in rhizosphere bacterial community composition and play a dominant role in structuring bacteria–plant interactions (Rosenberg *et al*., 2009). Protozoan predation constantly re-mobilizes essential nutrients for plant uptake by releasing organic soil nitrogen, which is then readily available for other soil organisms and taken up by plant roots (Bonkowski *et al*., 2000; Ekelund & Rønn, 1994; Zwart *et al*., 1994).

Tetrahymena are free-living ciliates usually found in freshwater (Zufall *et al*., 2013) but also in soil (Brandl *et al*., 2005), dish clothes (Chavatte *et al*., 2014) and slow sand filters (Haig *et al*., 2015). They graze on bacteria by filter-feeding, ingest them by phagocytosis and digest them within food vacuoles. Bacteria grazed by the protozoans are used as food and the protozoa correspondingly increase with the decline in *E. coli* population (Haig *et al*., 2015). Following uptake, some bacteria evade digestion in the

A. polyphaga and *T. pyriformis* and multiply within food vacuoles of protozoans, where they are shielded from the adverse environmental conditions such as acid, biocides and antibiotics (Berk *et al*., 1998; Gourabathini *et al*., 2008; Lambrecht *et al*., 2015; Smith *et al*., 2012). Some of the bacteria has shown intracellular multiplication and survival in protozoa include *E. coli* O157 (Barker *et al*., 1999; Chekabab *et al*., 2013), methicillin-resistant *Staphylococcus aureus* (Huws *et al*., 2006); *Legionella pneumophila* (Hales & Shuman, 1999)*, Mycobacterium avium* (Adékambi *et al*., 2006; Strahl *et al*., 2001), *Listeria monocytogenes* (Lambrecht *et al*., 2015), *Yersinia enterocolitica* (Lambrecht *et al*., 2015), *Helicobacter pylori* (Winiecka-Krusnell *et al*., 2002), *Campylobacter jejuni* (Axelsson-Olsson *et al*., 2010; Olofsson *et al*., 2015; Snelling *et al*., 2005), *Enterobacter aerogenes* (Yousuf *et al*., 2013) and *Aeromonas hydrophila* (Yousuf *et al*., 2013). Protozoans thus serve as natural reservoirs and vehicles for the long-term survival and dissemination of these pathogens in the environment (Khan, 2006).

Different strategies are employed by bacteria to evade ingestion and predation by protozoa. For example, *Pseudomonas aeruginosa* utilises a type III secretion system (T3SS) to kill *Acanthamoeba castellanii* (Matz *et al*., 2008a); *Vibrio cholerae* uses a Type VI secretion system (T6SS) to kill *Dictyostelium discoideum* (Miyata *et al*., 2011)*, V. cholerae* also produces outer membrane vesicles to suppress the viability of *A. castellanii* (Valeru *et al*., 2014) and produce antiprotozoal compounds in biofilms (Sun *et al*., 2015); *V. fischeri* biofilms produce antiprotozoal compounds against *Tetrahymena pyriformis* (Chavez-Dozal *et al*., 2013); while *Pseudoalteromonas tunicata* produce violacein to inhibit protozoan feeding and kill *Cafeteria roenbergensis* and *Rhynchomonas nasuta* (Matz *et al*., 2008b). Besides evading ingestion, some bacteria are ingested but protect themselves against digestion, survive and/or replicate in the protozoa. For example, *Campylobacter* spp. are shown to grow at an almost optimal rate within *A. polyphaga* at 37°C (Axelsson-Olsson *et al*., 2007), *E. coli* survived within phagosomes of *Tetrahymena* spp. (Smith *et al*., 2012) while *Salmonella enterica* survived within vesicles egested from *Tetrahymena* (Brandl *et al*., 2005).

The interaction between biotic and abiotic factors within a habitat determines the survival and fate of each component of the ecosystem. For example, Wanjugi & Harwood (2013) documented that protozoan predation and competition from indigenous microbiota led to the rapid die-off of *E. coli* in fresh water and sediments.

Bacterial strain and the host environment encountered further influence intra-protozoan survival and environmental persistence (Schuppler, 2014; Vaerewijck *et al*., 2014). As outlined in Chapter 3, *E. coli* requires the deployment of a full stress protection response activated by RpoS to survive in soil. However, it remains to determined how RpoS contributes to soil survival especially regarding the different conditions encountered in the soil. RpoS confers protection against multiple stresses found in soil such as UV radiation from sunlight, low temperature, hyper- and hypo-osmotic stress, nutrient availability, competition and predation (Arrange *et al*., 1993; Griffiths *et al*., 2003; Ibekwe *et al*., 2010) and any of these traits could impact soil survival. This study focussed on understanding the role of RpoS in surviving low moisture conditions in soil based on the known role of RpoS in osmoprotection (Stasic *et al*., 2012). Since protozoans form a major part of the soil microbiota, this work sought to understand if RpoS contributes to the survival of *E. coli* in the presence of protozoans. This is based on the fact that protozoan predation is a biotic stress frequently encountered in various external environments and biofilm production, being an RpoS-dependent phenotype, has been shown to protect *E. coli* against its predators (DePas *et al*., 2014).

Furthermore, among the environmental factors that affect bacterial soil survival, pH, temperature and moisture are very important (Habteselassie *et al*., 2008). It is hypothesized that competition and predation is low during low moisture conditions in soil. Moisture fluctuation is frequently encountered in soil, from the wet winter to dry summer, and it has been reported that soil moisture significantly affects the protozoa-bacteria interaction in soil (Kuikman *et al*., 1989) thus understanding how low moisture impacts soil survival is important. Although, *E. coli* has the ability to survive and multiply under low moisture condition (Solo-Gabriele *et al*., 2000) and Cools *et al*., (2001) had reported that *E. coli* and *Enterococcus* spp survived better at 100% than 60% field capacity at low temperatures (5 and 15°C), this study investigated whether RpoS contributes to the ability of *E. coli* to survive low moisture in soil.

In the present study, the response of soil-persistent *E. coli* strains to predation by *A. polyphaga* and *T. pyriformis* was investigated and compared to predation of commensal and laboratory *E. coli* strains. Predation assays were performed to examine the effect of RpoS on protection against predation by the protozoans and survival of desiccation in soil. Results from this study show that there is strain difference in the susceptibility of soil-persistent *E. coli* to protozoan predation and that

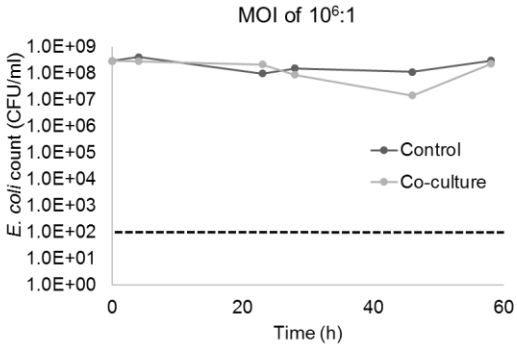
RpoS contributes to the ability of *E. coli* to survive protozoan predation and low moisture in soil.

4.3. RESULTS

4.3.1 Cell density affects predation of *E. coli* BW25113 by *Acanthamoeba polyphaga* and *Tetrahymena pyriformis*

In order to establish the predation assay for *A. polyphaga*, varying numbers of *E. coli* BW25113 cells were co-cultured with a constant number (100 cells) of *A. polyphaga* trophozoites, which are the active and infective form of the protozoa, in Page's Amoeba Saline (PAS) thus giving a decreasing multiplicity of infection (MOI) i.e. *E. coli*: *A. polyphaga* ratios from 10^6:1 to 10:1. *E. coli* BW25113 was used to develop the assay since it is more sensitive to predation than one of the soil-persistent strains in a preliminary experiment (data not shown). The co-cultures were incubated at 28°C and viable *E. coli* counts determined at different time intervals. No detectable predation was observed in the MOIs 10^6:1, 10^5:1 and10^4:1 (Fig. 4.1 A). A decrease in *E. coli* counts indicating predation by the amoeba was observed from the 10^3:1 MOI, where *E. coli* count was below the detecstion limit after 30 h. No viable *E. coli* was detected at 10^2:1 and 10:1 MOIs after 4 h of co-incubation (Fig. 4.1 A). In the controls with only *E. coli* incubated in PAS, no reduction in the *E. coli* count was observed. Based on this result, subsequent experiments with *A. polyphaga* were conducted at 10^3:1 MOI (Fig. 4.1B).

A


MOI of 10^6:1
E. coli count (CFU/ml)
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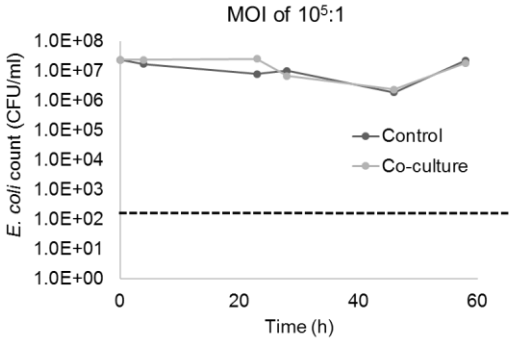
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
20
40
60
Time (h)
Control
Co-culture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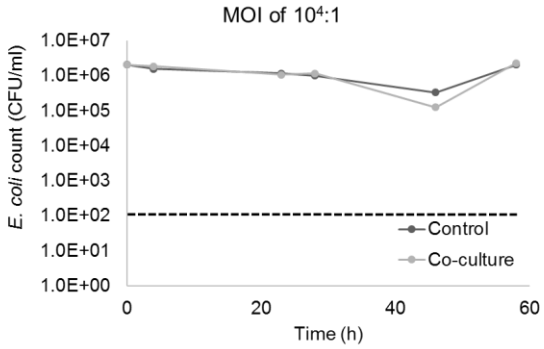
1.0E+01
1.0E+00
0
20
40
60
Time (h)
Control
Co-culture


MOI of 10^4:1
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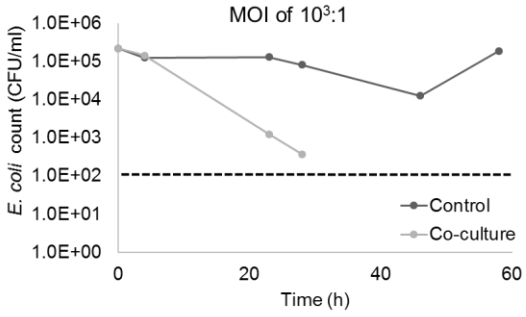
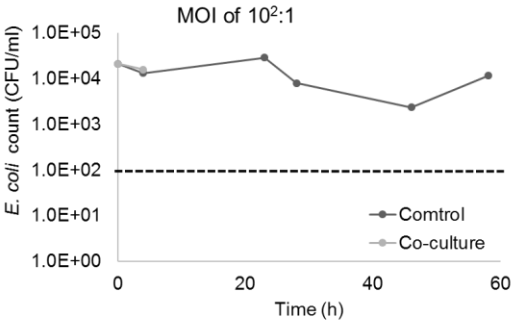
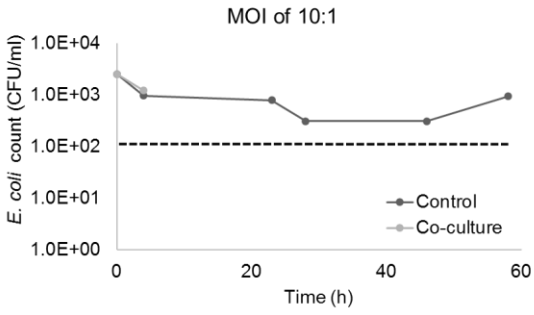
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
20
40
60
Time (h)
Control
Co-culture


MOI of 10^3:1
E. coli count (CFU/ml)
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
20
40
60
Time (h)
Control
Co-culture


MOI of 10^2:1
E. coli count (CFU/ml)
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
20
40
60
Time (h)
Comtrol
Co-culture


MOI of 10:1
E. coli count (CFU/ml)
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1.0E+00
0
20
40
60
Time (h)
Control
Co-culture

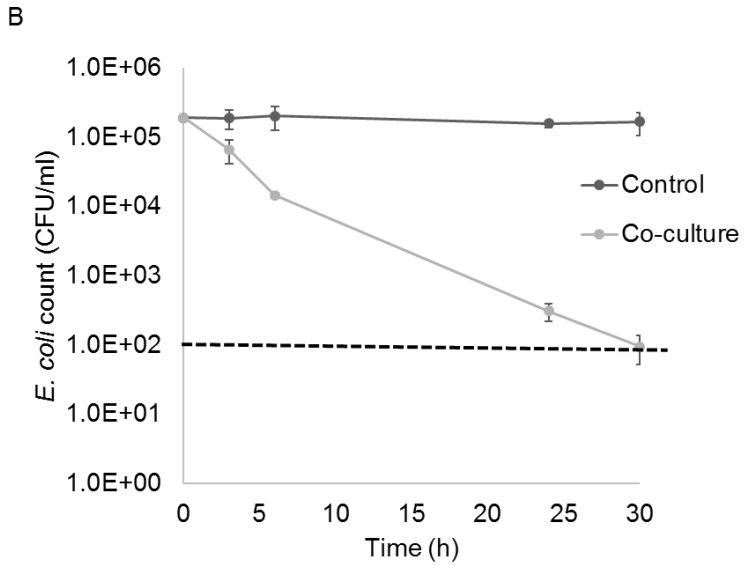


Figure 4.1. Demonstration of predation of *Escherichia coli* BW25113 by *Acanthamoeba polyphaga*. *E. coli* BW25113 was incubated in Page's Amoeba Saline (PAS) in co-culture with *Acanthamoeba polyphaga* or without *Acanthamoeba polyphaga* at different multiplicity of infections (MOIs) (10^6:1 - 10:1 MOI) (A). A separate experiment showing predation at the MOI of 10^3:1 selected for subsequent experiments (B). Cultures were incubated at 30°C as described in *Materials and Methods* (Section 2.11.4). Dashed lines represent detection limit of predation assay. Error bars represent standard deviation from the mean of 3 independent replicates.

The cell density effect was also observed during predation of *E. coli* BW25113 by *T. pyriformis* in PAS incubated at 28°C. There was no decline in the *E. coli* count in co-culture at MOI of 10^6:1 in the first 30 h, whereas there was about 2 log reduction at 10^4:1 and 10^2:1 MOIs at the same time-point (Fig. 4.S1). Subsequent co-culture experiments of *E. coli* with *T. pyriformis* were performed at an MOI of 10^6:1. Active predation is shown by a decline in *E. coli* counts and a corresponding increase in the numbers of *T. pyriformis* cells (Fig 4.2A). *E. coli* transformed with plasmids expressing GFP (pUA66) was co-cultured with *T. pyriformis* in order to microscopically observe predation and fluorescence was observed in food vacuoles within *T. pyriformis* and food vacuoles expelled into the surrounding medium (Fig 4.2B). When Triton X-100 was used to lyse *T. pyriformis* cells in order to release any intracellular bacteria, no difference was observed in the viable *E. coli* counts whether *T. pyriformis* was lysed with Triton X-100 or not (Fig 4.S2), thus suggesting that the ingested bacterial cells are digested inside the protozoa and not retained as live cells.

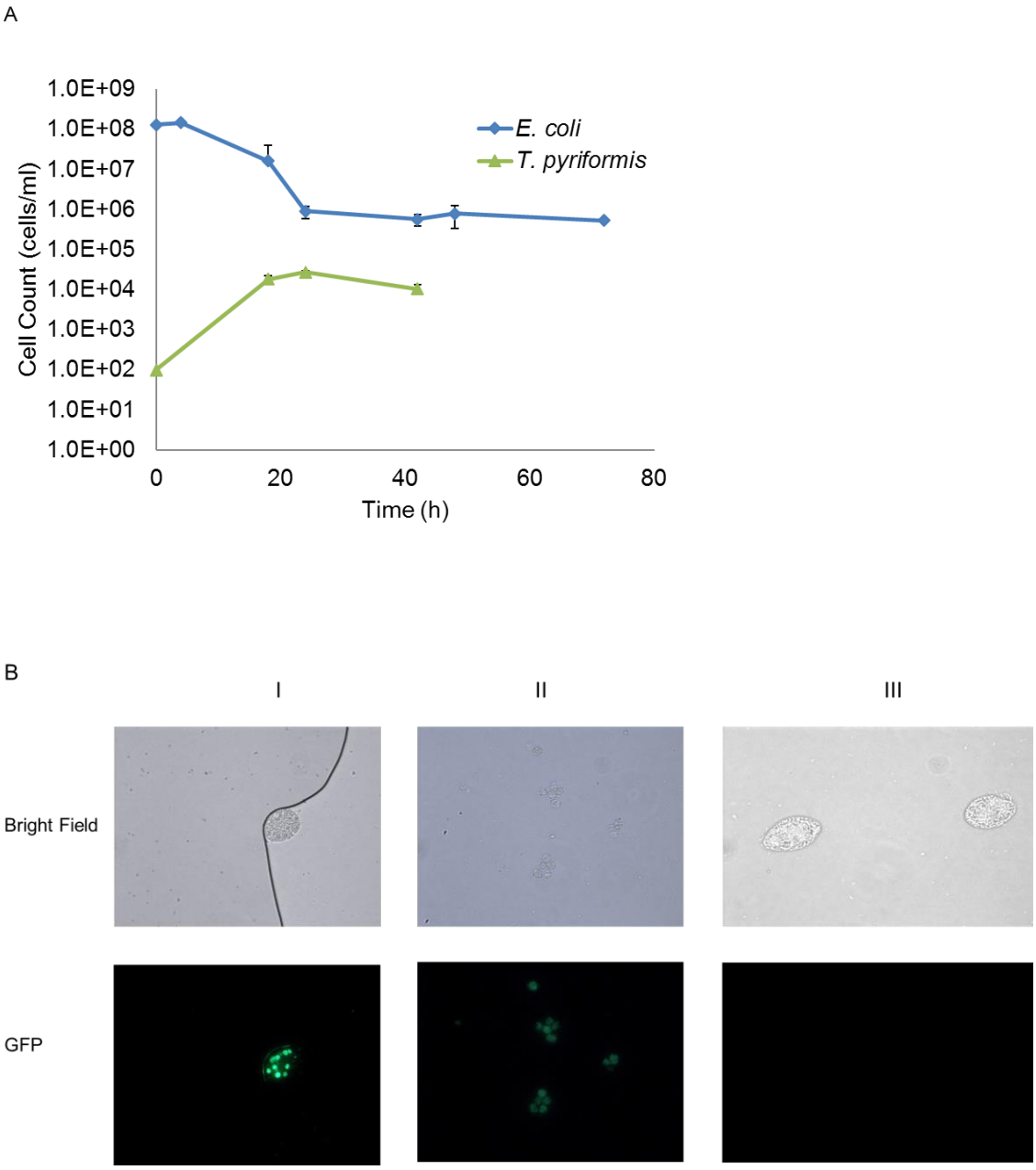


Figure 4.2: Demonstration of predation of *Escherichia coli* BW25113 by *Tetrahymena pyriformis*. (A) *E. coli* BW25113 was incubated in Page's Amoeba Saline (PAS) in co-culture with *T. pyriformis* at MOI of 10^6:1 and incubated at 28°C (described in Section 2.11.3). Decrease in *E. coli* count (blue line) correlated with increase in *T. pyriformis* count (green line). Error bars represent standard deviation of mean of 3 independent replicates. (B) *T. pyriformis* fed with green fluorescent protein (GFP)-tagged *E. coli* BW25113 showing highly fluorescent food vacuoles in *T. pyriformis* (I) and in food vacuoles expelled into surrounding medium (II) while no fluorescence was observed in *T. pyriformis* fed with no GFP-tagged *E. coli* BW25113.

4.3.2 Strain differences exist in the resistance of soil-persistent *E. coli* to predation by *Acanthamoeba polyphaga*

Since soil-persistent *E. coli* survived in soil long-term, it was hypothesized that they will be resistant to protozoan predation. In order to test this, long-term soil-persistent *E. coli* as well as commensal and laboratory strains were co-incubated separately with *A. polyphaga* and then *T. pyriformis*. Predation was significantly different ($p < 0.0001$; one-way ANOVA) among the strains tested in co-culture with *A. polyphaga*. *E. coli* COB583 had the highest survival among the soil-persistent strains and was the only strain with detectable viable cells after 30 h (Fig. 4.3). When grouped based on their source of isolation, soil-persistent strains survived significantly better ($p = 0.0013$; one-way ANOVA) than commensal and lab strain. There were high intra-strain differences among the soil-persistent *E. coli* in their susceptibility to predation by *A. polyphaga* as COB584 was not detected after 3 h, COB586 was not detected after 6 h, COB585 and COB587 were not detected after 24 h while COB583 was only reduced by about 1.5 log cycles (Fig. 4.3). The two commensal strains (SE11 and SE15) had no detectable cells after 6 h and survived significantly less ($p<0.0005$; Student's t-test) than *E. coli* BW25113.

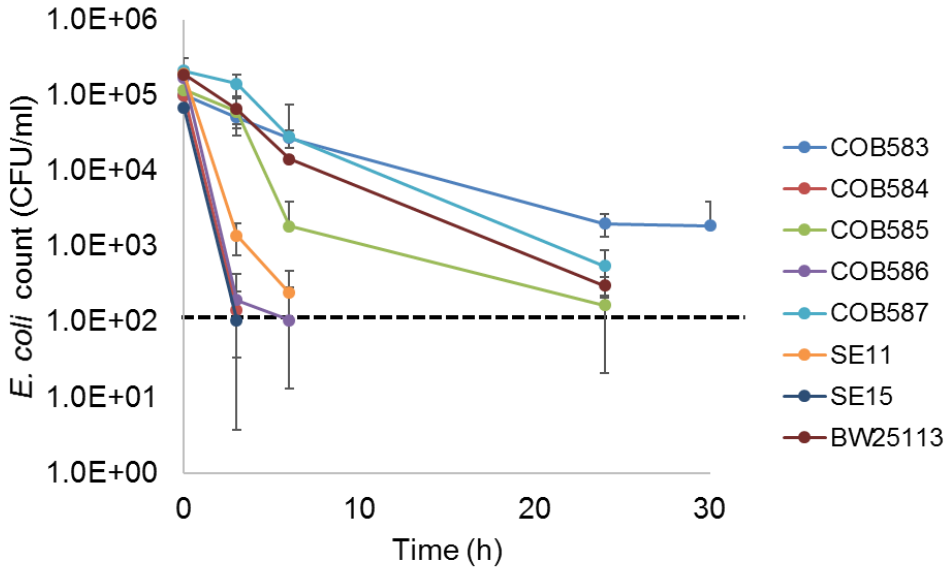


Figure 4.3: Soil-persistent *Escherichia coli* differ in their sensitivities to predation by *Acanthamoeba polyphaga*. Soil-persistent, commensal and lab strains of *E. coli* were co-cultured with *A. polyphaga* (MOI 10^3:1) in Page's Amoeba Saline (PAS) and incubated at 30°C. Error bars represent standard deviation of mean of three independent replicates. Dashed line represents the detection limit of the predation assay.

The resistance profile of the *E. coli* strains to *T. pyriformis* was different from *A. polyphaga*. Soil-persistent *E. coli* COB583 and commensal strain SE15 were resistant to predation by *T. pyriformis* as they survived significantly better than all other tested strains, irrespective of their source of isolation (Fig. 4.4). At 24 h, there was a significant difference ($p < 0.0001$; one-way ANOVA) in the sensitivities of the strains to predation by *T. pyriformis*. Unlike BW25113 which had no active predation after 24 h, all other soil-persistent and commensal *E. coli* were still actively preyed upon after 24 h (Fig. 4.4).

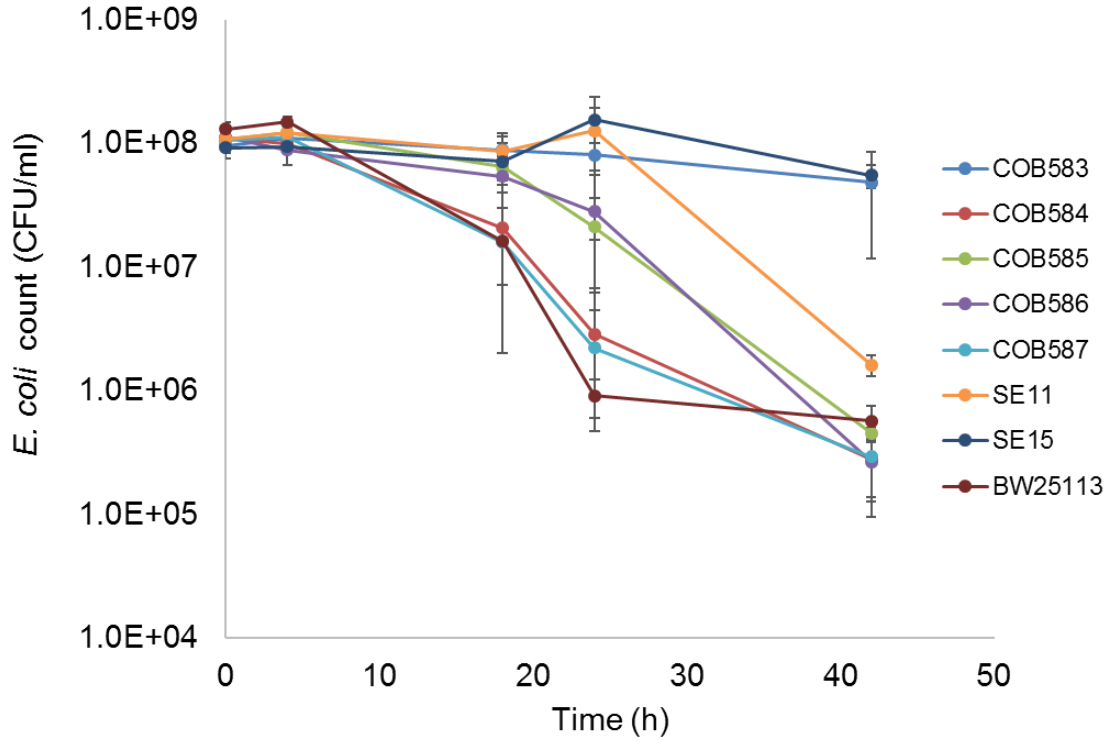


Figure 4.4: Soil-persistent *Escherichia coli* differ in their sensitivities to predation by *Tetrahymena pyriformis*. Soil-persistent, commensal and lab strains of *E. coli* were co-cultured with *T. pyriformis* (MOI 10^6:1) in Page's Amoeba Saline (PAS) and incubated at 28°C. Error bars represent standard deviation of mean of three independent replicates.

4.3.3 RpoS, curli and cellulose are important for *E. coli* to resist protozoan predation

Since *E. coli* produce biofilm in various external environments and biofilm to protect *E. coli* against predators, this study investigated whether biofilm protects *E. coli* against predation by *A. polyphaga* and *T. pyriformis.* Deletion mutants in some genes important for biofilm production in *E. coli* (Δ*rpoS, ΔcsgA, ΔbcsA)* were co-cultured with both protozoans as previously described in Section 2.11.3 and 2.11.4. It was observed that both *rpoS* and RpoS-dependent genes involved in biofilm formation (*csgA* and *bcsA*) contributes to the resistance of predation by *A. polyphaga* (Fig. 4.5). All the mutants were significantly impaired in their survival when compared to their wildtype BW25113. BW25113Δ*csgA* was rapidly preyed on by *A. polyphaga* and no viable cells were detected at 3 h, BW25113Δ*bcsA* was below the detection limit after 3 hours, while BW25113Δ*rpoS* was not detectable after 6 h.

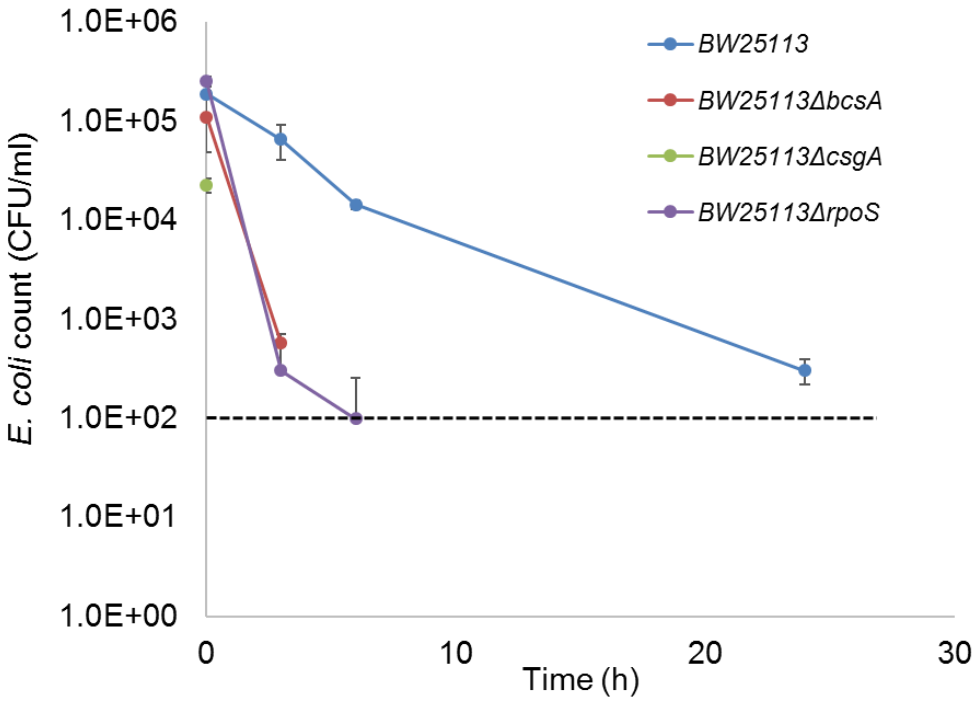


Figure 4.5: RpoS, curli and cellulose is important for *Escherichia coli* to resist predation by *Acanthamoeba polyphaga*. *E. coli* BW25113 and deletion mutants (Δ*rpoS*, Δ*csgA* and Δ*bcsA*) were co-cultured with *A. polyphaga* in Page's Amoeba Saline (PAS) and incubated at 30°C. Error bars represent standard deviation of mean of three independent replicates. Dashed line represents the detection limit of the predation assay.

RpoS also contributes to the ability of *E. coli* BW25113 to survive active predation by *T. pyriformis*. BW25113Δ*rpoS* was significantly (p = 0.0496; Student's t-test) more preyed upon compared to its wildtype at 24 h. This result was similar in the soil-persistent strain COB583, where the isogenic COB583 Δ*rpoS* survived significantly (p = 0.0169; Student's t-test) less than its wildtype (Fig. 4.6).

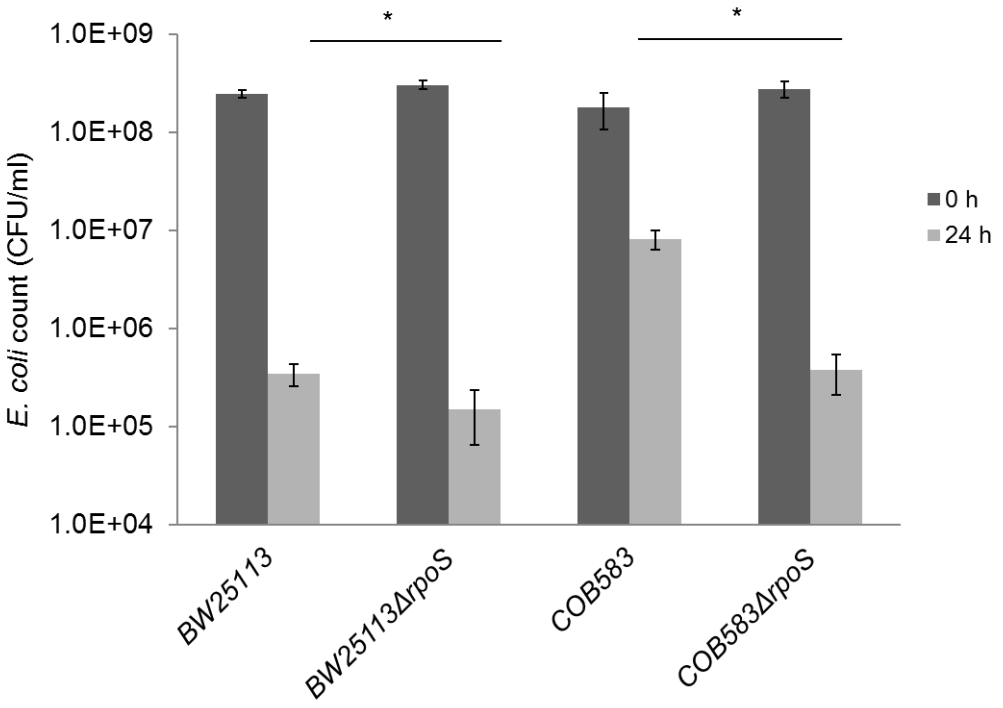


Figure 4.6: RpoS contributes to the resistance of *Escherichia coli* to predation by *Tetrahymena pyriformis*. *E. coli* BW25113 and soil-persistent *E. coli* COB583 and their respective Δ*rpoS* deletion mutants were co-cultured with *Tetrahymena pyriformis* in Page's Amoeba Saline (PAS) and incubated at 28°C for 24 h. Error bars represent standard deviation of mean of 3 independent replicates. Significant differences are denoted with asterisks (*) and determined at p<0.05 by the Student's t-test.

Genes involved in curli production (Δ*csgD and* Δ*csgA*) also played a role in resistance of *E. coli* to *T. pyriformis* predation. BW25113Δ*csgD,* BW25113Δ*csgA* and BW25113Δ*bcsA* were all significantly more sensitive than their parental strain (p = 0.0238; 0.0022; 0.0087 respectively; Student's t-test) (Fig. 4.7). These observations suggest that biofilm formation protects *E. coli* against predation by both *A. polyphaga* and *T. pyriformis.*

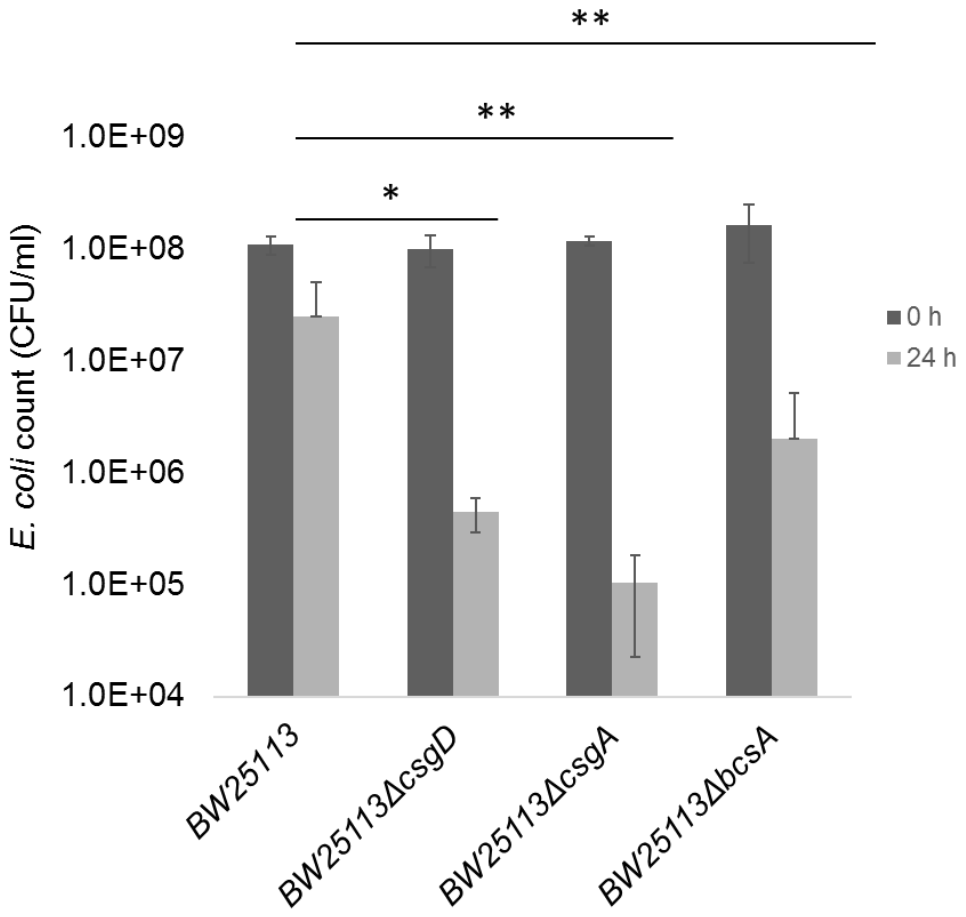


Figure 4.7: Curli and cellulose contribute to the resistance of *Escherichia coli* to predation by *Tetrahymena pyriformis*. *E. coli* BW25113 and its corresponding Δ*csgD, Δcsg*A *and* Δ*bcsA* deletion mutants were co-cultured with *Tetrahymena pyriformis* in Page's Amoeba Saline (PAS) and incubated at 28°C for 24 h. Error bars represent standard deviation from the mean of three independent replicates. Significant differences are denoted with asterisks (*) and determined at $p<0.05$ using the Student t-test.

4.3.4 RpoS is important for *E. coli* to survive low moisture in soil

Based on the difference seen in the survival of COB583Δ*rpoS* in soil where COB583 Δ*rpoS* survived better in soil A (moisture content = 36.2±0.2 %) than in soil B (moisture content = 23.0±0.2 %) (Figure 3.2D) and the described role of RpoS in overcoming dehydration and osmoprotection, it was hypothesized that RpoS contributes to survival of *E. coli* in low moisture soil. Therefore, the role of RpoS in the survival of *E. coli*

BW25113 and COB583 together with their corresponding Δ*rpoS* derivative strains at low soil moisture levels was investigated in soil B (described in Section 2.7.1). Generally, there was a significantly better survival in the soil at 100% water holding capacity (100%WHC) than at 60% water holding capacity (60%WHC), except in wildtype COB583 (Fig 4.8). Both wildtype BW25113 and BW25113Δ*rpoS* survived significantly better at 100%WHC than at 60%WHC (p = 0.0259 and 0.0465 respectively; Student's t-test) with about ~ 1 log-cycle reduction in survival in each case (Fig. 4.8A). However, moisture had no significant effect (p>0.05) on the survival of COB583, as the survival was similar at 100%WHC and 60%WHC. RpoS plays an important role in the fitness of COB583 at the different soil moisture levels as COB583Δ*rpoS* survived significantly (p = 0.0399; Student's t-test) better at 100%WHC compared to 60%WHC. There was a significant interaction effect (p = 0.014; Two-way ANOVA) between moisture and RpoS status on the survival of BW25113, showing that the effect of moisture on soil survival depends on the RpoS status of the *E. coli,* however this was not observed for COB583. Overall, this result shows that RpoS makes a significant contribution to the survival of COB583 in soil and this effect is more pronounced when the water content of the soil is reduced.

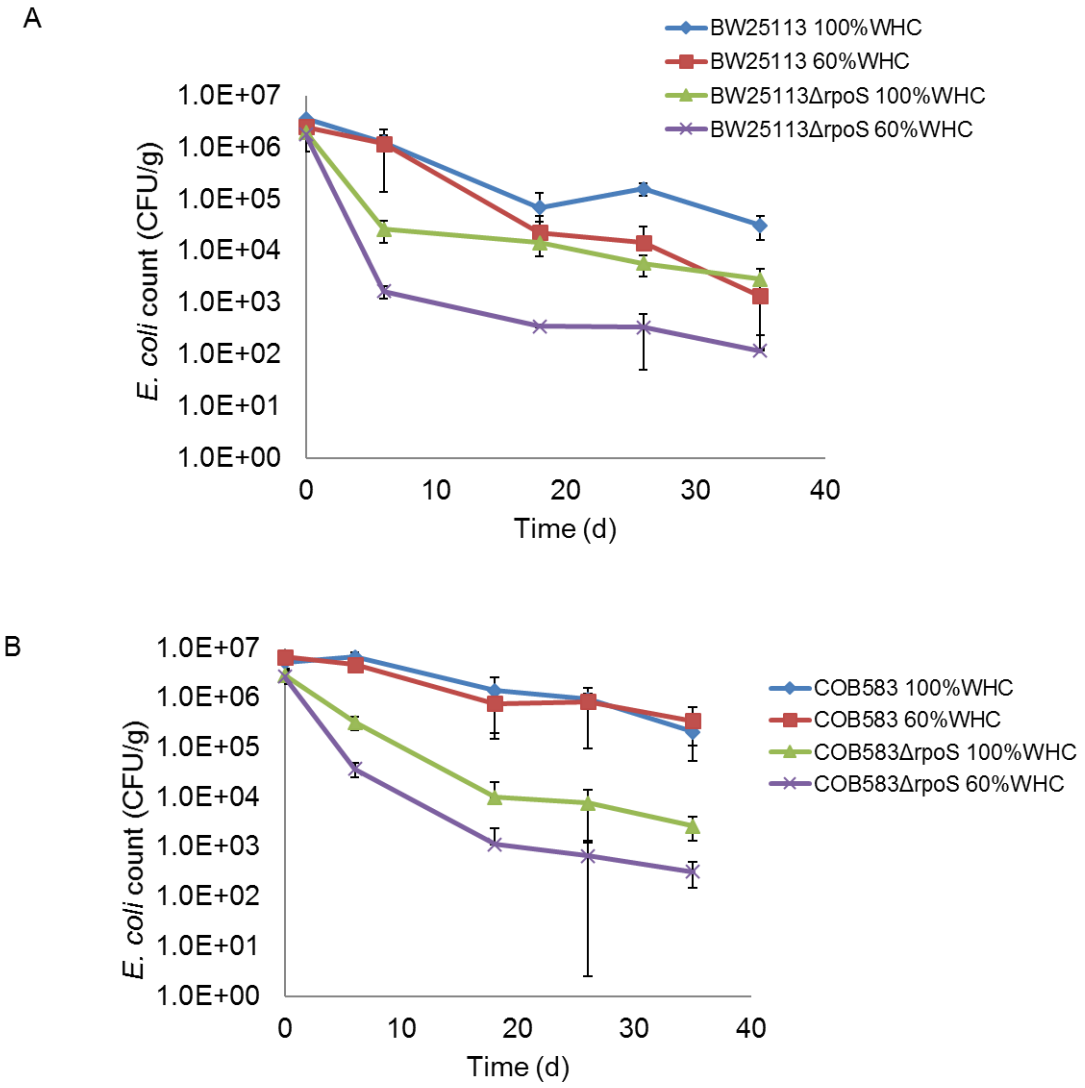


Figure 4.8: RpoS is important for *Escherichia coli* to survive reduced water content in soil. Soil survival assay of *E. coli* BW25113 (A) and COB583 (B) with their corresponding Δ*rpoS* at 100% Water Holding Capacity (100%WHC) and 60% Water Holding Capacity (60%WHC) in soil incubated at 15°C. Error bars represent standard deviation from the mean of 3 independent replicates.

4.4. DISCUSSION

E. coli has been isolated from several non-host environments including soil (Brennan *et al*., 2010a; Byappanahalli *et al*., 2006; Chiang *et al*., 2011), but not much is known about the mechanisms of adaptation of *E. coli* to the soil. Although it has been shown that RpoS is important for the long-term survival of *E. coli* in soil (Chapter 3), how RpoS contributes to survival of *E. coli* in soil is still unclear. As protozoans are predators of

bacteria and are important members of the soil ecosystem, the contribution of RpoS to resisting predation of *E. coli* by protozoa was investigated. Also, considering that moisture fluctuation is often experienced in different sections of a soil column (Marciano-Cabral & Cabral, 2003), the possible role RpoS plays in surviving fluctuating soil moisture levels was examined. When soil moisture is reduced, there is an increase in the osmolarity of the soil and the concentration of inhibitory compounds could also increase. RpoS has been show to protect *E. coli* against hyperosmolarity (Hsu *et al*., 2009; Stasic *et al*., 2012). Many of the previous studies on predation of *E. coli* by protozoa has been with *Acanthamoeba castellanii* but there are differences in the predation of *E. coli* by acanthamoebae as *A. castellanii* grows better than *A. polyphaga* when co-cultured on *E. coli* (Weekers *et al*., 1993) thus making the present study important for the soil context. The use of different long-term soil-persistent *E. coli* strains from different sources and two protozoan types also make this study unique. Here, this study demonstrated that RpoS and curli are important for *E. coli* to resist predation by *A. polyphaga* and *T. pyriformis* and that RpoS is important for surviving low moisture in soil.

It was observed that cell density affects the predation of *E. coli* by *A. polyphaga* and *T. pyriformis*. The optimum MOI for predation of *E. coli* by *A. polyphaga* was 10^3:1 (Fig. 4.1). MOI was shown to influence the interaction of bacterial cells to eukaryotic cells (Backert & Hofreuter, 2013). It is possible that at higher MOIs, the cells attach to each other and this may make the prey too big in size for the protozoa to prey on (Matz *et al*., 2004). Active predation of *E. coli* BW25113 by *Tetrahymena pyriformis* occurred during the first 24 h, after which the population of viable cells remained constant. It was shown that predation of *E. coli* led to a concurrent increase in the population of *T. pyriformis* which indicate that the bacteria was used as food source for protozoan growth (Fig. 4.2A). Predation on *E. coli* by the *Paramecium caudatum* was also shown to increase the population of the protozoa as evidence of predation (Schmidt *et al*., 2016). Although there was no further decline in *E. coli* BW25113 population after 24 h of co-incubation, this was not the case with the soil-persistent and commensal strains (Figure 4.4). After 24 h of co-culture, there were further declines of between 1 - 2-log cycles in the natural isolates except for COB583 and SE15 which were resistant to predation by *T. pyriformis.* The rapid consumption of *E. coli* by protozoans is not only seen *in vitro* but has been shown to be one of the major means of removal of *E. coli* in slow sand filters (Haig *et al*., 2015). Since this present study only looked at predation *in*

vitro, future studies should look at predation in soil and how it impacts the survival of *E. coli* in soil.

In this study, commensal *E. coli* survived significantly (p = 0.0256; one-way ANOVA) better than the soil-persistent and lab strains after 42 h of co-incubation with *T. pyriformis*. This was contrary to our initial hypothesis that soil-persistent strains will resist predation better than commensal and lab strains. Strain differences were observed among the soil- and commensal *E. coli* isolates in their resistance to predation by *A. polyphaga* and *T. pyriformis. E. coli* COB584, COB586, SE11 and SE15 were sensitive to predation by *A. polyphaga*, while COB583 was resistant. Although SE15 was resistant to predation by *T. pyriformis*, it was sensitive to predation by *A. polyphaga.* Soil-persistent *E. coli* COB583 was resistant to predation by both protozoans. The differences seen in the level of susceptibility to predation by both protozoans in this study is similar to previous studies that have shown variations in the capacities of protozoans to prey on bacteria. For example, *Colpoda aspera* preyed better and significantly increased on different *E. coli* O157:H7 strains compared to *Vorticella microstoma* (Ravva *et al*., 2014). Strain- and species-specific differences have also been reported in the survival of different bacteria preyed upon by protozoa (De Moraes & Alfieri, 2008; Lambrecht *et al*., 2015).

Differences in the mode of feeding by the protozoans may also contribute to the level of predation observed in this study. *T. pyriformis* is a filter feeder that consumes suspended cells and does not discriminate between suspended solids and bacteria cells (Dürichen *et al*., 2016) whereas *A. polyphaga* engulfs its food using its pseudopodia (Khan, 2006). Although the size of the individual *E. coli* strains was not determined in this study, bacterial prey size has been reported to influence the ability of *Tetrahymena pyriformis* to prey on bacterial cells (Hausmann, 2002). Particle size influences ingestion of food and non-food particles by *T. pyriformis* where larger particles are not ingested as much as smaller-sized particles similar to bacteria sizes (Boenigk & Novarino, 2004). Nonetheless, difference in particle size is unlikely to explain the differences observed in this study, especially when different MOIs were used for the assay with the two protozoans. In a complex ecosystem such as the soil, the presence of other bacteria and non-food materials that could be grazed on by the protozoans, may allow for reduced predation of bacteria of interest and ensure their long-term survival.

Although some bacteria have been reported to survive and replicate inside protozoan hosts and remain viable when recovered from either protozoan trophozoites or cysts, there was no evidence of such in this present study. Increase in viable *E. coli* count was not observed after Triton X was used to lyse *T. pyriformis*, thus suggesting that the ingested bacterial cells are digested inside the protozoa and not retained as live cells (Fig 4.S2). This observation is similar to a recent report by Schmidt *et al*. (2016) who observed no increase in *E. coli* population within protozoa. Matin & Jung (2011) also observed no viable intracellular *E. coli* after 1 h co-incubation with *A. castellanii* and concluded they were killed once in the amoebae. This intracellular digestion of bacteria by protozoa has also been shown when *Colpoda steinii* and *A. palestinensis* preyed on *S. enterica, E. coli* O157:H7 and *Listeria monocytogenes* (Gourabathini *et al*., 2008). In extreme conditions in the external environment or in the host, bacterial cells can lose viability and switch into the viable but non-culturable (VNBC) state, characterised by reduced metabolic activity, so it is plausible that ingested cells in our study have switched into VNBC state. Although we did not investigate VNBC cells in this study, it has been shown that *E. coli* ingested by *A. castellanii* were actually digested and not in a VNBC state (Chekabab *et al*., 2012), thus ruling out the possible VNBC argument.

In order to monitor the intracellular digestion of ingested cells, green fluorescent protein (GFP)-tagged *E. coli* was used. After co-incubation of *T. pyriformis* with GFP-tagged *E. coli,* intense green fluorescence was observed in the protozoa and in the food vacuoles egested into the surrounding liquid milieu (Fig 4.2B) even when there were no viable cells within the protozoa. Dürichen *et al*. (2016) had reported that fluorescence intensity reduced in food vacuoles containing digested cells when compared to food vacuoles with live cells, so it remains unclear whether the fluorescence seen in the food vacuoles in this present study was from digested cells or from intact cells. Since GFP can continue to fluoresce even after the bacterium is dead; future studies should consider using LIVE/DEAD BacLight staining method to determine if ingested *E. coli* are still viable or digested within *T. pyriformis*. A previous study that used this method shows that *Helicobacter pylori* is digested in the food vacuole of *Tetrahymena* sp. (Smith *et al*., 2012). Interestingly, undigested bacteria can also be expelled from *A. castellanii* (Wright *et al*., 1981).

Having shown the strain-to-strain differences in *E. coli*'s resistance to predation and since biofilm production, being an RpoS-dependent phenotype, protects *E. coli* against soil-inhabiting nematode and predatory bacteria (DePas *et al*., 2014), this study sought

to answer the question of any possible role for RpoS and some RpoS-dependent genes in resisting predatory protozoan. It was demonstrated that RpoS is required for *E. coli* to resist predation by *A. polyphaga* and *T. pyriformis* (Fig. 4.5 and 4.6). Previous study has shown that RpoS is vital for *Legionella pneumophila* to remain viable in its host and deletion of *rpoS* renders the bacteria unable to replicate within *A. castellanii* (Hales & Shuman, 1999). This suggests that RpoS may be involved in the evasion of different stresses encountered in the eukaryotic host such as extreme pH and macrophages. Conversely, the presence of RpoS seems to be a negative factor for predation in *Pseudomonas aeruginosa*, as an *rpoS* mutant was reported to increase the killing of *A. castellanii* (Matz *et al*., 2008a).

Furthermore, this study has shown that curli is an important factor for resistance of *E. coli* BW25113 to predation by *A. polyphaga* and *T. pyriformis;* as the isogenic Δ*csgA* mutant was the most sensitive to predation (Fig. 4.5, 4.6). Although the starting number of Δ*csgA* mutant cells in the *A. polyphaga* assay was lower than Δ*csgD* and Δ*bcsA*, the high sensitivity of Δ*csgA* mutant cells to *T. pyriformis* suggests that Δ*csgA* mutant cell is really sensitive to protozoan predation. Similarly, DePas *et al*. (2014) had shown that curli and cellulose protected *E. coli* against predation by soil-borne nematode *Caenorhabditis elegans* and the predatory bacterium *Myxococcus xanthus*. Our results are in contrast to those of Ravva *et al*. (2014) who suggested that curli-positive variants of *E. coli* 0157:H7 were rapidly consumed by *Vorticella microstoma* and *Colpoda aspera*. Firstly, their study investigated predation of *E. coli* 0157:H7 by different protozoans, *Vorticella microstoma* and *Colpoda aspera*. Although this study is in a different genetic background (BW25113), it presents the first direct genetic evidence that loss of curli is a disadvantage when interacting with predators. Furthermore, their study suggested that protozoans preyed more on curli-positive *E. coli* 0157:H7 but the strains which showed the highest resistance to predation in this study were all curli-positive strains, except one. COB583, which resisted predation by the two protozoans used in this study, is curli-positive and produces biofilm which has been shown to reduce protozoan predation by limiting access of the protozoa to the *E. coli* cells (Matz & Kjelleberg, 2005)

From this study, it was observed that RpoS contributes to resistance of predation through curli, which is an important component of *E. coli* biofilm. Biofilms have been reported to protect against protozoan predation of *Pseudomonas aeruginosa* (Matz *et al*., 2004), *V. cholera* (Matz *et al*., 2005), *Microbulbifer* sp. and *Pseudoalteromonas* sp

(Matz *et al*., 2008b). Other adhesins also contribute to interaction and association of *E. coli* with protozoans. Yousuf *et al*. (2014) showed that a deletion mutant of a genomic island (RDI 21) which encodes adhesins, a protein secretion system and invasins in neuropathogenic *Escherichia coli* K1 survived significantly less in *A. castellanii* compared to its wildtype. A capsule-deletion mutant of *E. coli* K1 also exhibited significantly reduced association with *Acanthamoeba* compared to the wildtype and had limited ability for uptake and intraprotozoal survival (Jung *et al*., 2007). Lipopolysaccharide (LPS) plays an important role in the recognition of bacterial prey and internalization by amoebae (Alsam *et al*. 2006; Arnold *et al*., 2016). Curli-deficient strains are unable to produce biofilm during the co-incubation and this makes them vulnerable to predation (Fig 3.1C). It is possible that cell-cell interactions make the *E. coli* form clumps which reach a size beyond the feasible prey size spectrum of size-selective predators such as *T. pyriformis* (Matz *et al*., 2004).

The role of RpoS in the survival of predation by *T. pyriformis* was greater in the soil-persistent strain COB583 than in the lab strain BW25113. The COB583Δ*rpoS* was significantly (p=0.0169; Student's t-test) more depleted compared to the parental strain while BW25113Δ*rpoS* was only marginally significantly (p=0.0496; Student's t-test) lower than its wildtype (Fig. 4.6). This observation shows that soil-persistent *E. coli* COB583 had an increased fitness during predation. RpoS plays a greater role in protection against predation in the soil-persistent strain COB583 than the lab strain BW25113. The greater contribution of RpoS to protection against predation could be because the soil-persistent strain had been adapted for a long time to soil before isolation (Brennan *et al*., 2010), and has frequently encountered biotic and abiotic stresses which could make retention of a functional RpoS important (Somorin *et al*., 2016).

Based on the differences seen in the survival of COB583Δ*rpoS* in soils which had different moisture content (Figure 3.2) and the role of RpoS in overcoming dehydration and osmoprotection (Stasic *et al*., 2012), it was hypothesized that RpoS contributes to survival of *E. coli* in soil at low moisture. Therefore, the role of RpoS in the survival of *E. coli* BW25113 and COB583 together with their corresponding Δ*rpoS* mutants at low soil moisture levels was investigated. Generally, there was a significantly better survival in soil at 100%WHC than at 60%WHC except in wildtype COB583. There was a significant interaction effect (p = 0.014; Two-way ANOVA) between moisture and RpoS status on the survival of BW25113, showing that the effect of moisture on soil survival

depends on the RpoS status of the *E. coli,* however this was not observed for COB583. This again shows the increased fitness of COB583, which is unaffected by reduced water content, thus confirming that COB583 recruits a more robust RpoS-mediated protection which is required to deal with low soil moisture and the attendant hyperosmolarity in soil. It has been previously shown that *E. coli* survive better in a water-saturated soil than in low moisture conditions (Sjogren, 1994). A similar observation was made with *Gluconacetobacter diazotrophicus* and *Azospirillum amazonense*, where increased soil moisture resulted in higher survival compared to lower soil moisture (Oliveira *et al*., 2004). Soil moisture is important for optimal microbial activity in soil. Yeager & Ward (1981) showed that survival of different bacteria in sludge decreased as the moisture content reduces. A previous study reported that protozoa-bacteria interaction and protozoan activity depends on available soil moisture and the highest protozoan activity is seen at high soil moisture content (Kuikman *et al*., 1989). This suggests that at low soil moisture, protozoan activity is reduced, leading to greater survival of *E. coli* in soil.

In conclusion, findings from this study show that there are strain differences in the resistance of soil-persistent, commensal and laboratory strains of *E. coli* to predation by *A. polyphaga* and *T. pyriformis.* It further demonstrates that curli are important for *E. coli* BW25113 to resist predation by *T. pyriformis* and *A. polyphaga*. Finally, RpoS contributes to survival in soil with reduced water availability and to resistance of *E. coli* to protozoan predation, probably through curli production.

Supplementary Figures

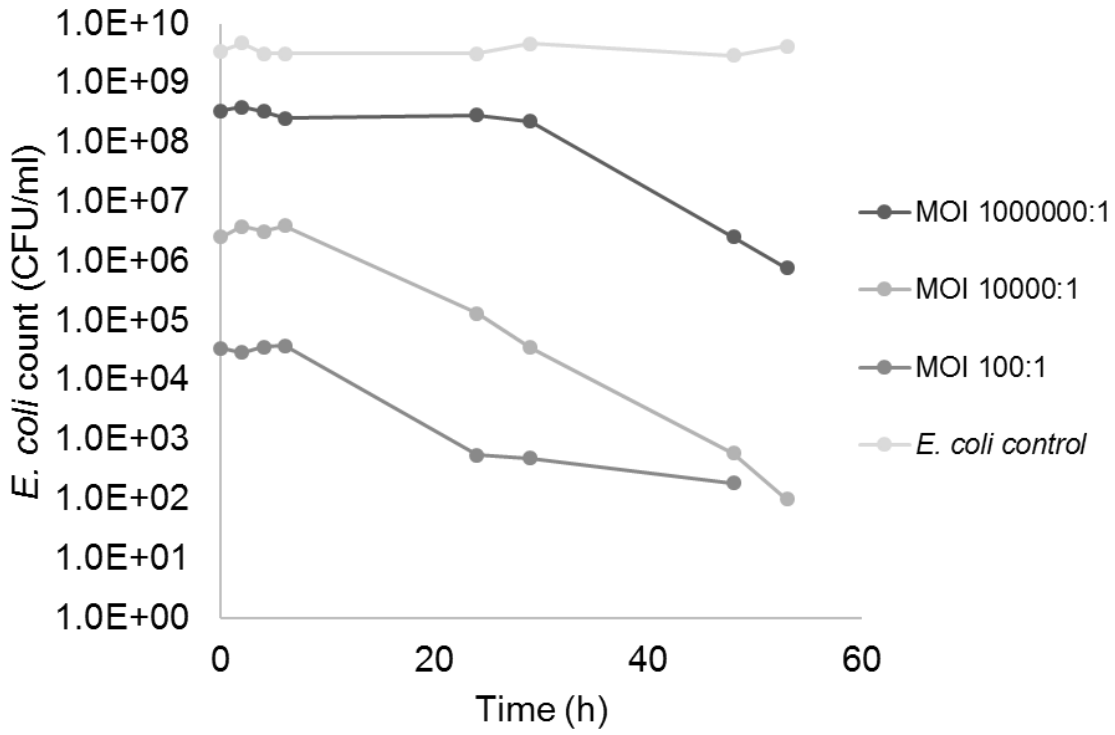


Fig. 4.S1: Demonstration of predation of *Escherichia coli* BW25113 by *Tetrahymena pyriformis.* *E. coli* BW25113 was incubated in Page's Amoeba Saline (PAS) in co-culture with *T. pyriformis* or without *T. pyriformis* at different multiplicity of infections (MOIs). Cultures were incubated at 28°C.

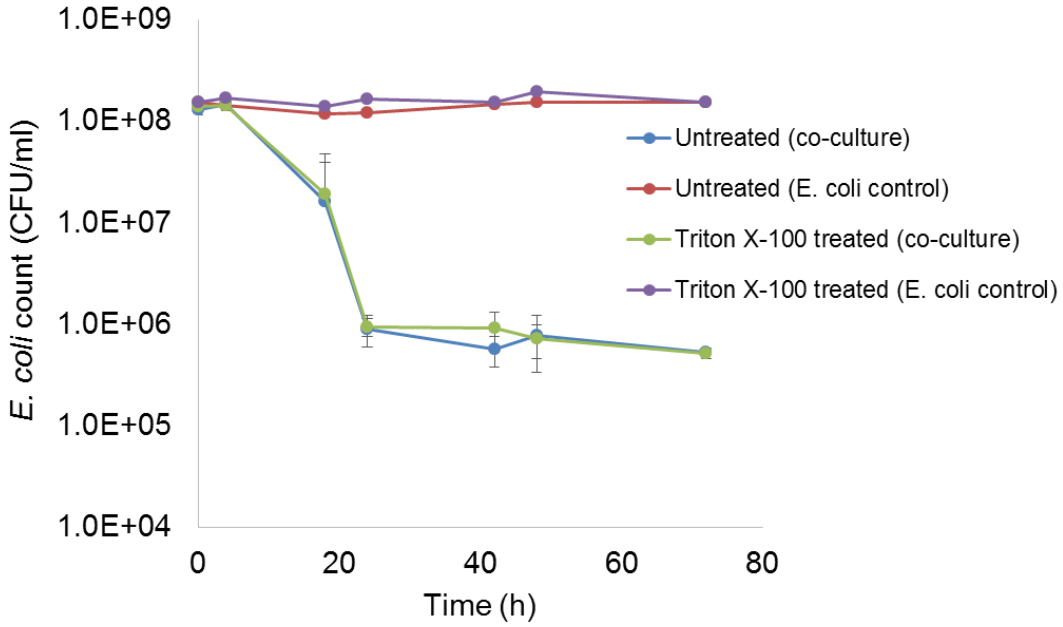


Fig. 4.S2: Intracellular survival of *E. coli* BW25113 in *Tetrahymena pyriformis* is absent. *E. coli* BW25113 was incubated in Page's Amoeba Saline (PAS) in co-culture with *T. pyriformis* and incubated at 28°C. At each time point, 50 µL of 0.4% Triton X-100 was added to 50 µL of co-culture and *E. coli* control and incubated for 5 min in order to lyse *T. pyriformis*. Aliquot was taken for *E. coli* count in cells treated and untreated with Triton X-100. Error bars represent standard deviation from the mean of 3 independent replicates.

CHAPTER 5

Characterisation of long-term soil-persistent *Escherichia coli* suggests some selection for loss of curli production

5.1. ABSTRACT

Curli are a major component of biofilm in many enteric bacteria including *E. coli* and are important for adherence to different biotic and abiotic surfaces. Some strains from a unique collection of phylogenetically distinct, long-term soil-persistent *E. coli* isolates were shown to be unable to produce biofilm (Chapter 3), raising the possibility that some soil-persistent *E. coli* may not produce curli. Thus this study aimed to investigate curli production among the soil-persistent strains, the role of curli in sand attachment and soil survival, and understand the molecular basis for the loss of curli in the soil-persistent *E. coli*. The majority (90%) of the soil-persistent *E. coli* were curli-positive but 10% were curli-negative (17 out of 170). Curli-producing soil-persistent *E. coli* (COB583 and COB585) displayed significantly ($p = 0.0022$) more attachment to quartz sand than the curli-negative strains. Although BW25113Δ*csgD* and BW25113Δ*csgA* had significantly ($p < 0.05$) reduced soil survival, BW25113Δ*csgB* survival in soil was similar to the wildtype thus suggesting that curli production may not affect soil survival. Mutations in two genes associated with c-di-GMP metabolism, *dgcE* and *pdeR*, correlated with loss of curli in eight soil-persistent strains, although this did not significantly impair their survival in soil compared to curli-positive strains. Overall, curli production appears to be important for retention of some *E. coli* in the environment and the possibility that loss of curli production in soil-persistent *E. coli* may promote their dissemination into other environments is discussed.

5.2. INTRODUCTION

Escherichia coli normally inhabits the gastrointestinal tract of humans, warm-blooded animals, and reptiles (Berg, 1996; Gordon & Cowling, 2003) and their presence in the external environment is commonly used as an indication of recent faecal contamination. This niche specificity underpins its use as an indicator of faecal contamination in the environment. Nevertheless, *E. coli* has been isolated from various sources outside of their primary habitats (Byappanahalli *et al*., 2012; Chiang *et al*., 2011; Ishii *et al*., 2006) and it persists and grows in external environments such as subtropical waters and sediments (Anderson *et al*., 2005). Brennan *et al*. (2010a) reported that *E. coli* are capable of long-term colonization and persistence in soil that had no animal slurry added during a 13-year period prior to their isolation. These soil-persistent *E. coli* strains display strong genetic diversity and possess unique growth and metabolic characteristics that suggest adaptation to soil conditions (Brennan *et al*., 2013). When *E. coli* enters the soil, there is rapid decline in the population, but a part of the population is able to show enhanced survival due to inherent physiological properties or ability to colonise favourable niches in the environment (Ogden *et al*., 2001). While it has been shown that the general stress response regulator, RpoS, is important for long-term persistence of *E. coli* in soil (Chapter 3), the exact mechanisms for their survival in the soil environment remain unclear.

Some genetic factors are known to enhance bacterial survival in the different environments. For example, flagellin was identified to help *Pseudomonas aeruginosa* to adhere to soil amoeba and persist in soil (DeFlaun *et al*., 1990). A functional flagellum was shown to be important for attachment and colonisation of infant mouse bowels by *Vibrio cholerae* (Attridge & Rowley, 1983). Exopolysaccharides and type 1 aggregative adherence fimbriae were found to support *in vivo* colonization of germ-free mice and biofilm formation in *E. coli* O104:H4 (Al Safadi *et al*., 2012). More recently, Yad fimbriae were demonstrated to promote *E. coli* adherence to plants, animal cells and promote persistence in the environment (Larsonneur *et al*., 2016). Production of biofilm enhances the survival of *Salmonella* in a dry and nutrient-depleted environment (Vestby *et al*., 2009) and *E. coli* in soil (Truhlar *et al*., 2015). It has been shown to promote the persistence of *E. coli* on fresh produce (Annous *et al*., 2009) and in food processing environments (Maal-Bared *et al*., 2013; Silagyi *et al*., 2009). Curli form a major component of biofilm in many enteric bacteria including *E. coli* (Barnhart & Chapman, 2006; Yaron & Römling, 2014). Curli are crucial for adherence to plant and animal tissues, plastic and stainless steel in *E. coli* and *Salmonella* (Carter *et al*., 2016;

Fink *et al*., 2012; Patel *et al*., 2011; Yaron & Römling, 2014). Although curli are important for attachment of *E. coli* to biotic and abiotic factors, not much is known about their contribution to persistence in the soil environment. Brombacher *et al*. (2003) previously reported that presence of curli enhanced retention of *E. coli* in sand columns, however, curli production in *Salmonella* spp did not have an impact on their retention in sand (Salvucci *et al*., 2009).

E. coli strains that produce curli were found to be predominant in manure-amended soil (Truhlar *et al*., 2015), however, it was shown earlier in this study that three out of five soil-persistent *E. coli* strains were unable to produce biofilms in microtiter plates (Chapter 3), thus raising questions about the ability of these soil-persistent *E. coli* to produce curli. Furthermore, it is not known if the presence of curli is important for attachment and persistence in soil. This study investigated a unique collection of phylogenetically distinct, long-term soil-persistent *E. coli* isolates to identify the prevalence of curli-negative strains. A subset of strains was found not to produce curli and the basis for this phenotype was investigated further. These curli-deficient strains were found to carry mutations in genes involved in c-di-GMP metabolism, which is known to influence curli expression (Lindenberg *et al*., 2013; Sommerfeldt *et al*., 2009). The influence of curli production on soil survival and attachment to sand particles were also investigated.

Here, the ability of soil-persistent *E. coli* to produce curli was determined using Congo Red-containing Yeast Extract and Casamino acid agar (CR-YESCA) assay, Western blotting and scanning electron microscopy (SEM); and curli regulatory genes were analysed *in silico* to identify the genetic basis for the loss of curli in the curli-negative strains. Sand attachment and soil survival assays were performed to investigate the importance of curli for attachment to sand and role of curli in soil survival. This present study shows that curli are important for attachment of *E. coli* to sand but not for soil survival, and suggests that loss of curli may facilitate the dissemination of *E. coli* in the environment.

5.3. RESULTS

5.3.1 Curli production varied among soil-persistent *E. coli*

Based on differences observed in the biofilm production capacities of the previously tested soil-persistent strains (Fig. 3.1C), ability of soil-persistent *E. coli* to produce curli was determined. Soil-persistent *E. coli* COB583 and COB585 stained red on CR-YESCA agar whereas COB584, COB586 and COB587 did not (Fig. 5.1). Commensal *E. coli* SE11 and SE15 were used for comparison purposes and both stained red on CR-YESCA agar indicating curli production. *E. coli* BW25113, which was used as the positive control, was red while the negative control *E. coli* BW25113Δ*csgA* was white. These data suggested that three of the soil strains (COB584, COB586 and COB587) may not be expressing curli under these growth conditions, a finding that is consistent with the inability of these strains to produce biofilm (Chapter 3; Fig. 3.1C). The morphology of the macrocolonies in the curli-positive strains varied from red, dry, rough and wrinkled phenotypes (as in COB583, SE11, SE15) which suggests the cells produced curli and cellulose; to brown-red, dry with large rings (as in COB585 and BW25113), which suggests the macrocolonies produced curli only. When the whole collection of 170 soil-persistent *E. coli* was analysed, 153 (90%) were presumptive curli-positive and 17 (10%) were presumptive curli-negative based on their staining on CR-YESCA medium.

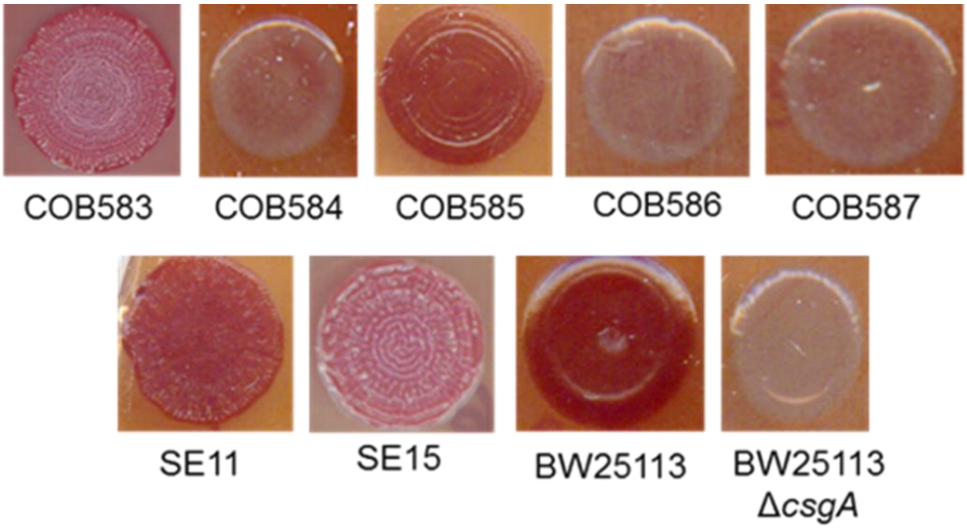


Figure 5.1: Curli production among soil-persistent *Escherichia coli*. Macrocolonies of soil-persistent, commensal and control strains were grown on Congo Red-containing Yeast Extract and Casamino acid (CR-YESCA) agar at 28°C for 48 h as described in *Materials and Methods* (Section 2.6.7). *E. coli* BW25113 was used as the positive control and *E. coli* BW25113 Δ*csgA* was the negative control.

5.3.2 Curli transcriptional regulator CsgD and curli major subunit CsgA were not expressed in curli-negative soil-persistent *E. coli*

In order to understand the reason for the loss of curli in the initial three soil-persistent strains (COB584, COB586 and COB587), it was investigated whether the major curli subunit CsgA, which is essential for curli production, was expressed in these strains. Western Blotting analysis showed that these three curli-negative strains did not express CsgA, whereas the presumptive curli-positive strains COB583 and 585 expressed CsgA (Fig. 5.2A). Since the expression of curli is regulated by the general stress response regulator RpoS and it has been shown that COB584, COB586 and COB587 have functional RpoS (Chapter 3; Fig. 3.4-3.6), the ability of the curli-negative strains to express RpoS on YESCA agar at 28°C for 48 h was investigated. All the curli-positive and curli-negative strains expressed RpoS under this condition (Fig. 5.2B). RpoS regulation of curli expression occurs through the curli transcriptional regulator CsgD, which activates the transcription of *csgBAC* operon. Therefore, the ability of the strains to express CsgD on YESCA agar was determined. CsgD was not expressed in the curli-negative *E. coli* COB584, COB586 and COB587 but was expressed in all the curli-positive strains (Fig. 5.2C). This suggests that the loss of curli in these strains was caused by some defective regulators between RpoS and CsgD in the curli production regulatory pathway (see Fig. 1.8).

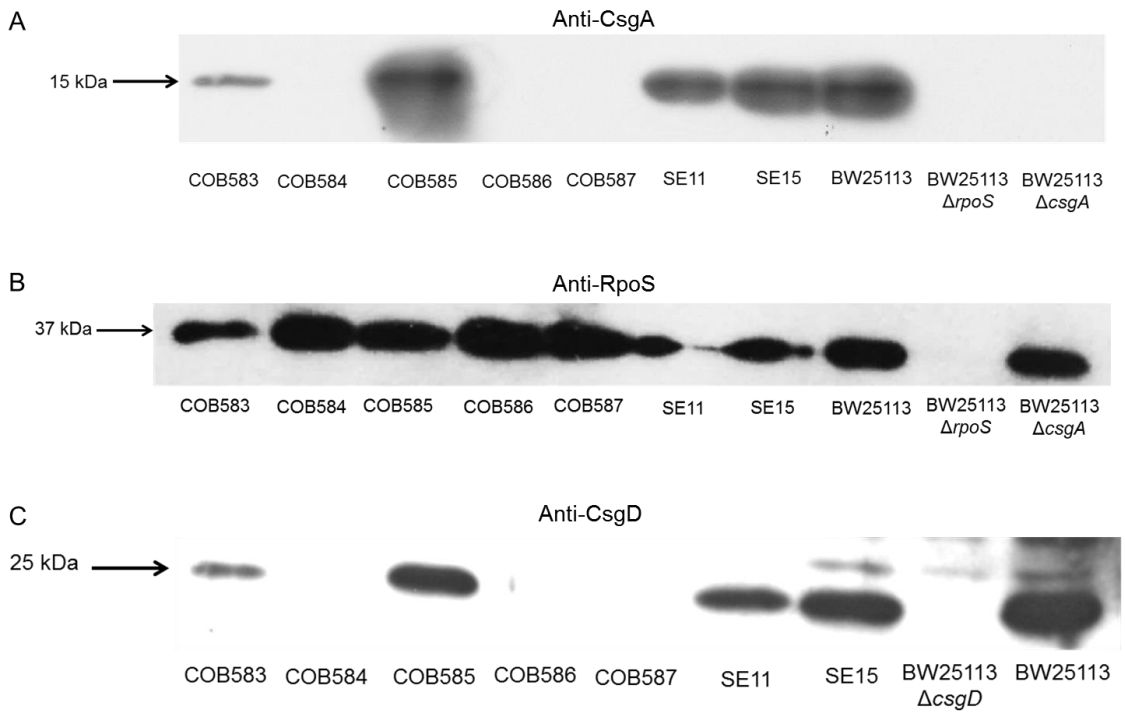


Figure 5.2: CsgA and CsgD are not expressed in curli-negative soil-persistent *Escherichia coli* strains. Cells of soil-persistent, commensal and control strains were grown on Yeast Extract and Casamino acid (YESCA) agar at 28°C for 48 h. Protein extraction and Western Blotting was done as described in the *Materials and Methods* (Section 2.9) for determining the expression of CsgA (A), RpoS (B) and CsgD (C) under the same conditions described above.

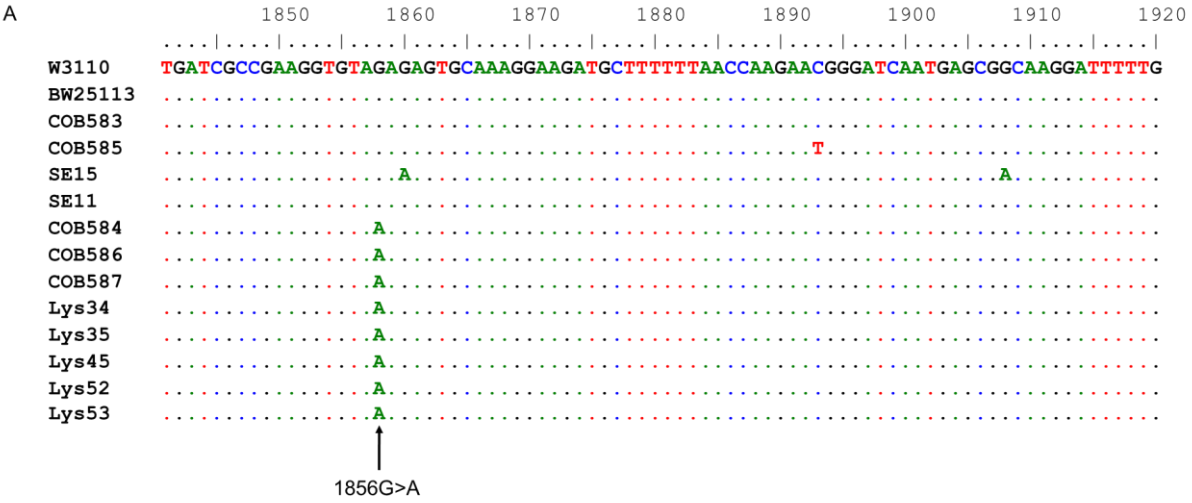
5.3.3 Bioinformatics analysis reveals mutation in the GGDEF domain of DgcE and in the EAL domain of PdeR leading to abolished curli production

Bioinformatic analysis was performed to identify any defective regulator in the curli production pathway in COB584, COB586 & COB587, which may explain the reason for the loss of curli. Bioinformatics analyses of the nucleotide sequences of the known regulators of curli production (RpoS, phosphodiesterase PdeR, diguanylate cyclase DgcM, DNA binding transcriptional activator MlrA, transcriptional dual regulator CsgD, minor curli subunit CsgB, major curli subunit CsgA, DNA-Binding protein Dps, chaperone protein DnaK, transcriptional regulator OmpR, catabolite repressor/activator protein Cra, small regulatory RNA RydC, small regulatory RNA RprA and *csgD* promoter preceding *csgB* were conducted in order to identify mutations that could have

truncated curli production in the three soil-persistent *E. coli* strains. RpoS was shown to be 100% conserved in all the curli-positive and curli-negative strains (Chapter 3; Table 3.1). The *csgB* and the *csgD* promoter sequence was 100% identical in the two curli-positive and three curli-negative strains. There were amino acid substitutions in MlrA of SE15 and COB585 but none in the three curli-deficient and the curli-positive strains compared to the reference sequence of *E. coli* K-12 W3110. Although soil-persistent *E. coli* COB585 and commensal strain SE15 had some amino acid substitutions in many of these regulators compared to the reference strain, they retained the ability to produce curli.

The same amino acid substitution, Threonine (T) to Alanine (A) at codon 98 (T98A), was found in DgcM in the three curli-negative strains, relative to the reference sequence. However, other soil-persistent *E. coli* with the T98A mutation in DgcM that were tested were curli-positive. Furthermore, analysis of the phosphodiesterase PdeR (formerly named YciR) regarded as the “trigger switch” for *E. coli* biofilm revealed a point mutation at nucleotide 1858 (1858G>A) in *pdeR* (Fig 5.3A) which changed Glutamate (E) to Lysine (K) at codon 620 (E620K) in the three curli-negative strains (Fig. 5.3B). This mutation (E620K) occurred in the EAL domain of the phosphodiesterase PdeR, which is important for the catalytic activity of PdeR. Multiple sequence alignment of the gene encoding the cyclic-di-GMP diguanylate cyclase (*dgcE*) also revealed a deletion (1456delC) among the three curli-negative strains (Fig. 5.3C) which led to a frameshift mutation in the amino acid sequence of DgcE (formerly YegE) (H486fs) which reached a stop codon at codon 490 (Fig 5.3D). These mutations in *pdeR* and *dgcE* were found uniquely in the three curli-negative soil-persistent strains of *E. coli* suggesting a possible causative role in the curli phenotype.

A


1850
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W3110 TGATCGCCGAAGGTGTAGAGAGTGCAAAGGAAGATGCTTTTTTAACCAAGAACGGGATCAATGAGCGGCAAGGATTTTTG
BW25113
COB583
COB585
SE15
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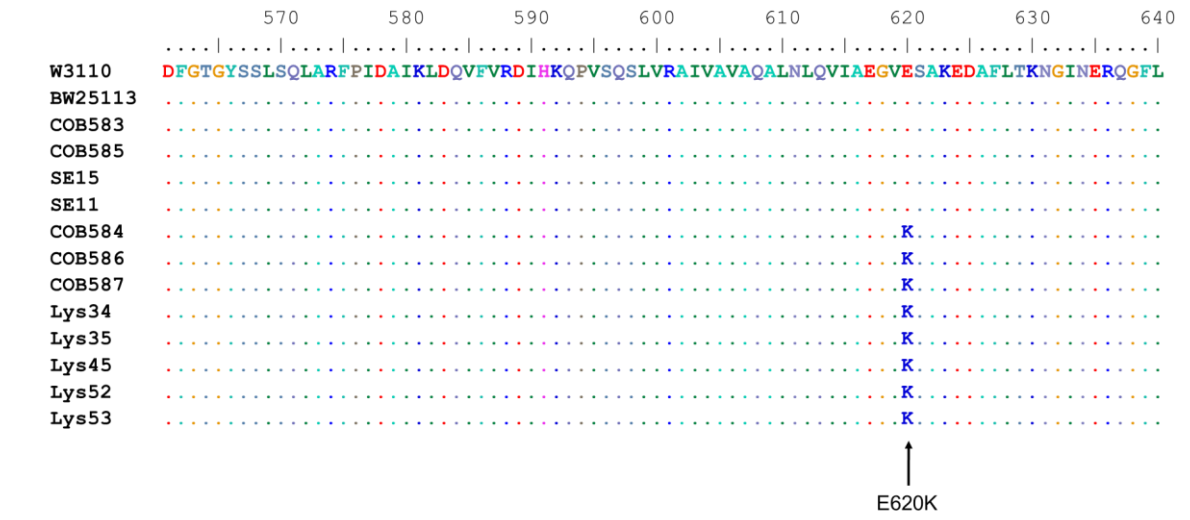
Lys53
1856G>A

B


570
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W3110 DFGTGYSSLSQLARFPIDAIKLDQVFVRDIHKQPVSQSLVRAIVAVAQALNLQVIAEGVESAKEDAFLTKNGINERQGFL
BW25113
COB583
COB585
SE15
SE11
COB584
COB586
COB587
Lys34
Lys35
Lys45
Lys52
Lys53
E620K

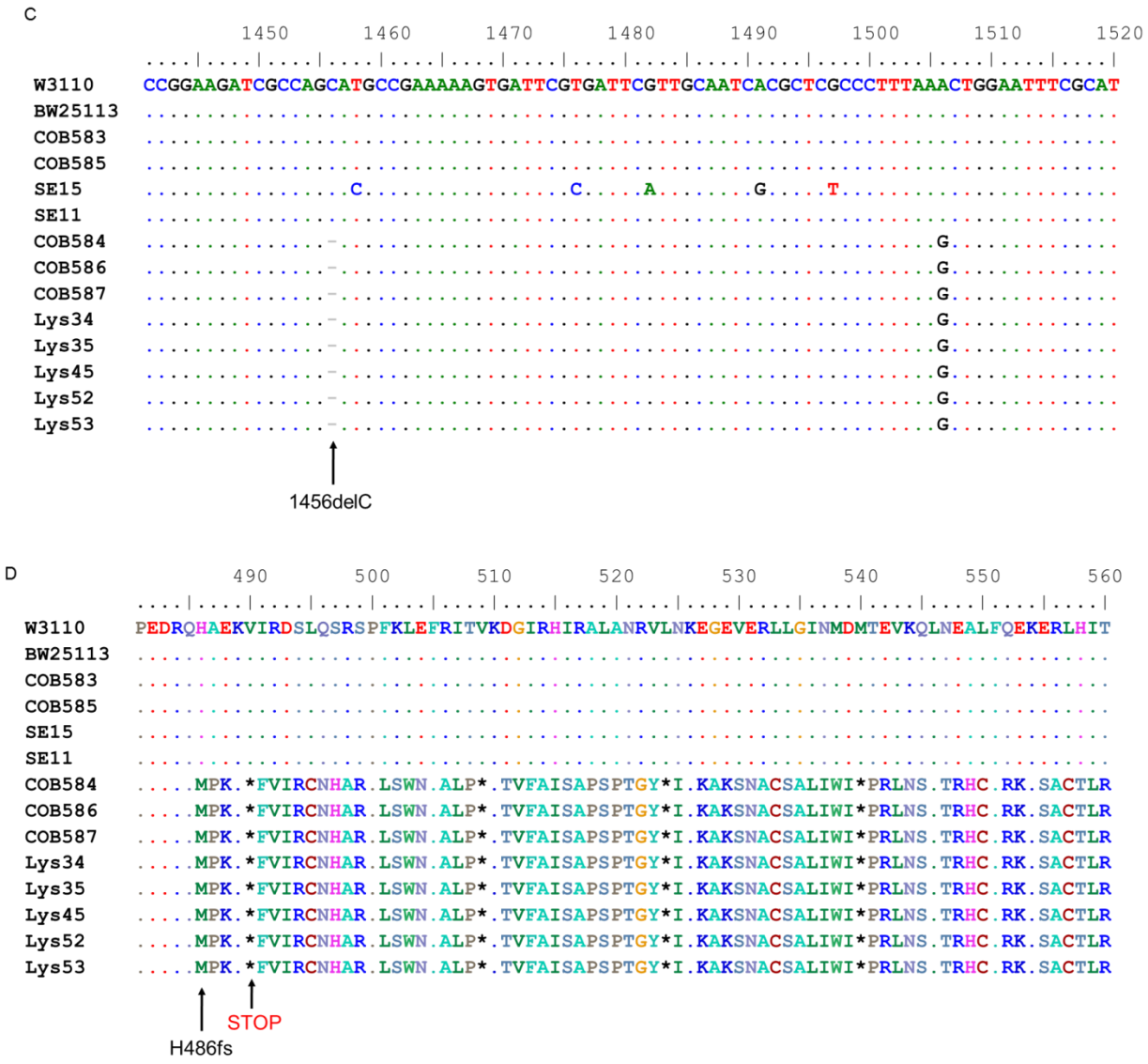

Figure 5.3: Multiple sequence alignment shows mutations in cyclic-di-GMP metabolism genes in curli-negative soil-persistent *Escherichia coli*. Multiple sequence alignment of nucleotide sequences in cyclic di-GMP phosphodiesterase-encoding gene (*pdeR*) among 170 soil-persistent strains in our collection shows a point mutation (1858G>A) (A) which led to an amino acid change in the PdeR protein sequence (E620K) in COB584, COB586 and COB587 and five additional soil-persistent strains (B). Alignment of the nucleotide sequence in diguanylate cyclase (*dgcE*) reveals a deletion (1456delC) (C) which results in a frameshift mutation at amino acid residue 486 in the protein sequence (H486fs) and a STOP codon at position 490 (D). Multiple sequence alignment was performed using ClustalW in BioEdit v7.2.5 as described in *Materials and Methods* (Section 2.13).

Upon analysing the sequences of the remaining 165 soil-persistent *E. coli* in the collection, which were obtained from different soil columns, the same *dgcE* and *pdeR* mutations was found in five additional soil-persistent *E. coli* strains. Interestingly, these five additional strains (Lys 34, 35, 45, 52 and 53) neither expressed CsgD nor produced curli, thus confirming that these mutations in *dgcE* and *pdeR* led to loss of curli production (Fig. 5.4).

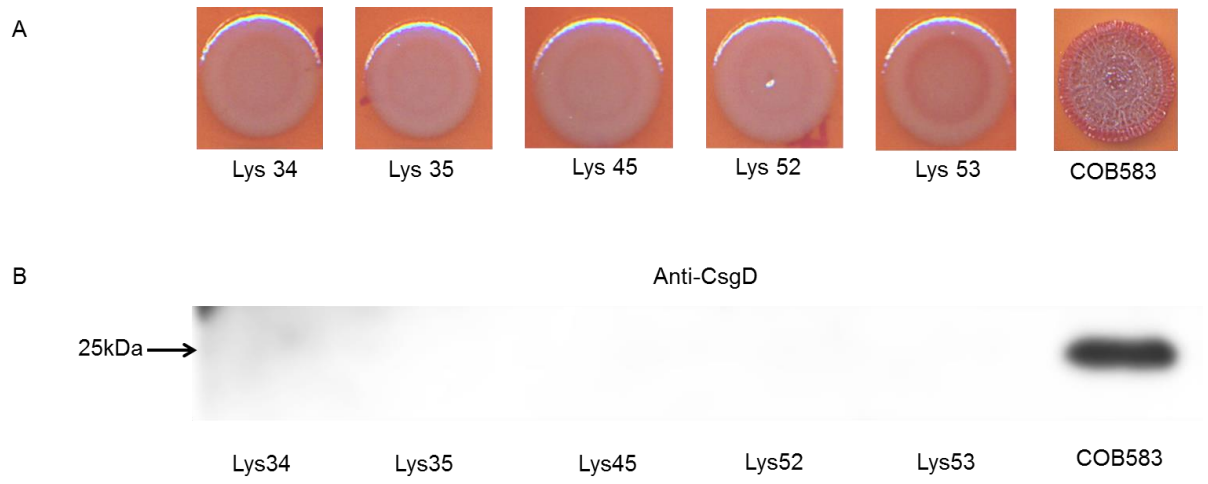

Figure 5.4: Five additional soil-persistent *Escherichia coli* with mutations in *dgcE* and *pdeR* were curli-negative. (A) Macrocolonies of additional soil-persistent *E. coli* with frameshift mutation in the nucleotide sequences of diguanylate cyclase (*dgcE*) and phosphodiesterase (*pdeR*) (*E. coli* Lys34, Lys35, Lys45, Lys52, Lys53) and positive control strain (COB583) were grown on Congo Red-containing Yeast Extract and Casamino acid (CR-YESCA) agar at 28°C for 48 h as described in *Materials and Methods* (Section 2.6.7). (B) Western Blotting was done as described in the *Materials and Methods* (Section 2.9.5) for determining the expression of CsgD under the same conditions described above.

Scanning Electron Microscopy (SEM) of the cells grown under the same conditions as previous experiments showed rough and wrinkled surfaces on the colonies of COB583, COB585 and BW25113, which correlates with curli production in those strains (Fig. 5.5). This wrinkled surface was absent in the curli-negative soil-persistent strains and the BW25113Δ*csgA* used as negative control.

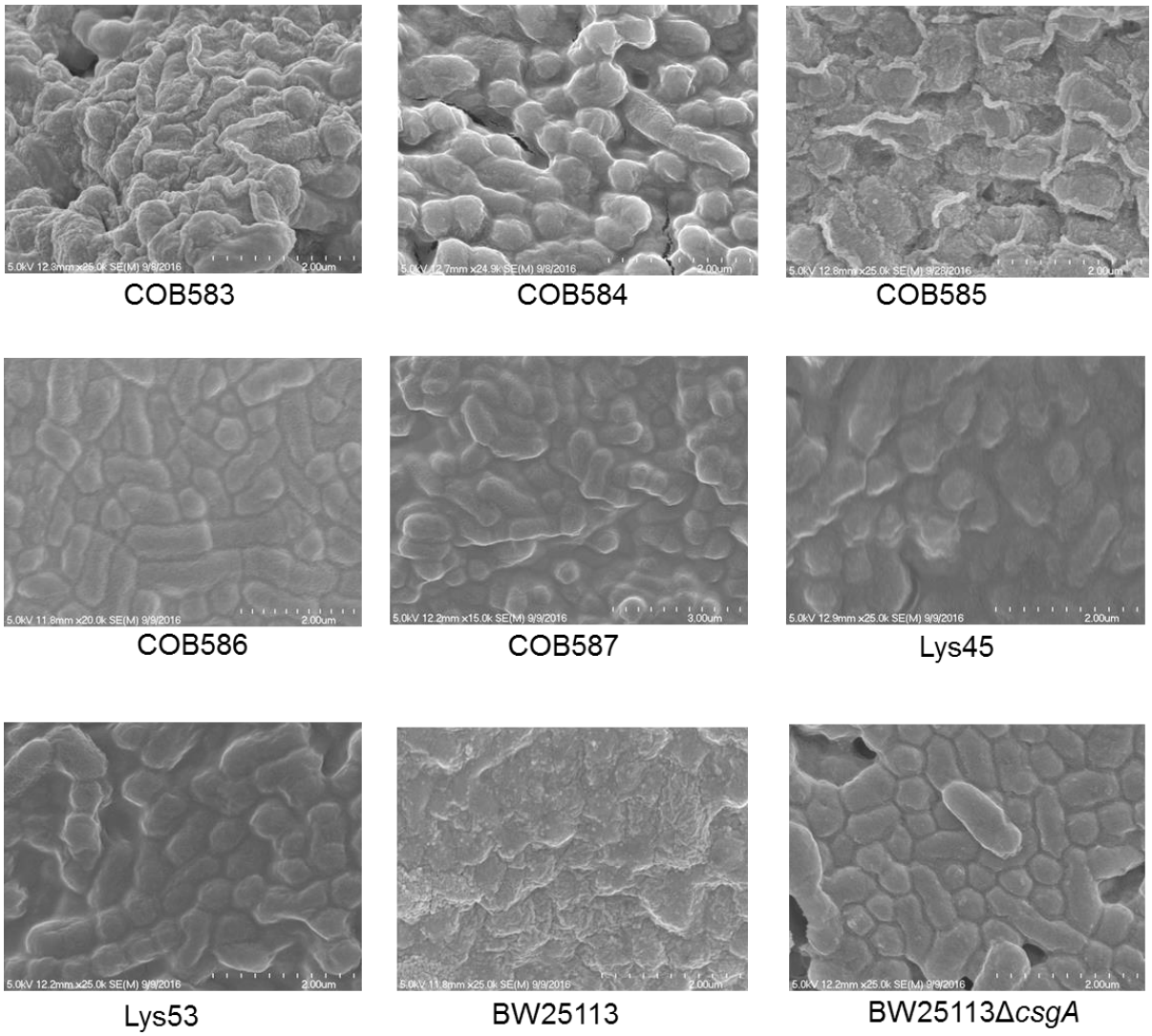

Figure 5.5: Scanning Electron Microscopy (SEM) images of macrocolonies of soil-persistent *E. coli* strains. Representative soil-persistent *E. coli* strains (COB583 – COB587; Lys 45 and Lys 53) and control strains (positive – BW25113; negative - BW25113Δ*csgA*) were grown on Yeast Extract and Casamino acid (YESCA) agar at 28°C for 48 h and imaged by SEM as described in *Material and Methods* (Section 2.12).

Since PdeR is regarded as a trigger enzyme for curli production, mutation in *pdeR* observed in the curli-negative strains could have rendered them unable to act as the trigger enzyme. So, the eight curli-negative strains (with *pdeR*^{E620K}) were complemented with wildtype PdeR from a low-copy plasmid (pCAB18-*pdeR*). Overexpressing PdeR in these *E. coli* strains did not restore curli production and repressed CsgD expression and curli production in the curli-positive strains, COB583, COB585 and BW25113 (Fig. 5.6).

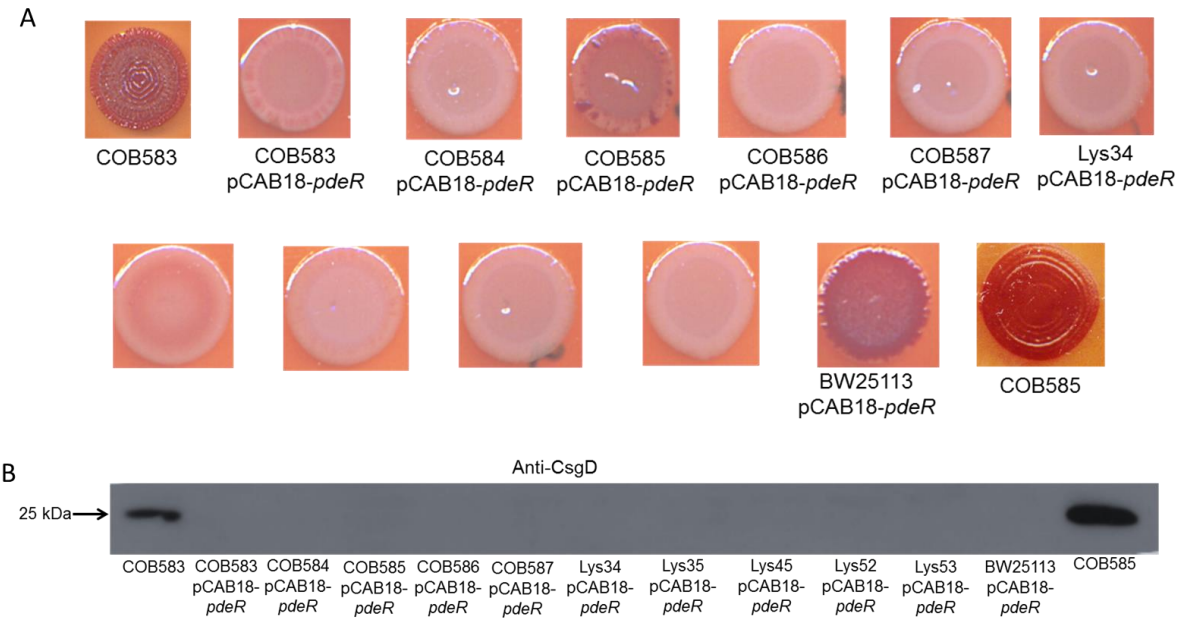

Figure 5.6: Complementation of curli-negative soil-persistent *E. coli* with wildtype PdeR did not restore curli production. (A) Curli-positive and curli-negative *E. coli* were transformed with pCAB18 plasmid carrying wildtype *pdeR* and the strains were grown on Congo Red-containing Yeast Extract and Casamino acid agar (CR-YESCA) with IPTG and Ampicillin at 28°C for 48 h. (B) All cells were grown as described above but with no added Congo Red in the agar. Protein extraction and Western Blotting with anti-CsgD antibodies was done as described in the *Materials and Methods* (Section 2.9).

5.3.4 Curli enhances the attachment of *E. coli* to quartz sand but not in soil

Since curli enhance biofilm production in soil-persistent *E. coli* in microtiter plates, it was determined whether curli has any relevance for attachment to an environmentally-relevant surface. It was observed that curli-positive soil-persistent strains (COB583 and COB585) were significantly ($p < 0.0001$, One-way ANOVA) more attached to quartz sand than the curli-negative strains. Curli-positive strains attached between 1.5 – 2.0-log cycle more than the curli-negative strains (Fig. 5.7A). There was no significant difference between the attachment of COB585 and COB583 ($p > 0.05$; Student's t-test). Conversely, curli-negative *E. coli* strains had significantly ($p < 0.0001$, one-way ANOVA) more planktonic cells than curli-positive *E. coli* (Fig. 5.7B). The curli-positive strains (COB583 and COB585) had ~ 1-log cycle lower cell count than the curli-negative strains. In the laboratory strains, BW25113Δ*csgA* had significantly ($p < 0.001$; Student's t-test) more planktonic cells than BW25113, although the biofilm count was not significantly different between them ($p > 0.05$; Student's t-test).

Finally, the contribution of curli to soil survival was analysed in *E. coli* BW25113 using strains with deletion in genes required for curli production. It was observed that although deletion of curli subunit genes did not significantly impair soil survival like BW25113Δ*rpoS* did at the initial points, there was a significant defect in the survival of BW25113Δ*csgA* ($p = 0.0084$, Student's t-test) and BW25113 Δ*csgD* ($p = 0.0012$, Student's t-test) after 113 days in live soil (Fig. 5.8). No viable BW25113Δ*rpoS* was detectable after 28 days in live soil as observed earlier (Chapter 3). BW25113Δ*bcsA* and BW25113Δ*csgB* were not significantly different ($p > 0.05$; Student's t-test) from the wildtype BW25113, thus suggesting that curli and cellulose are not required for soil survival.

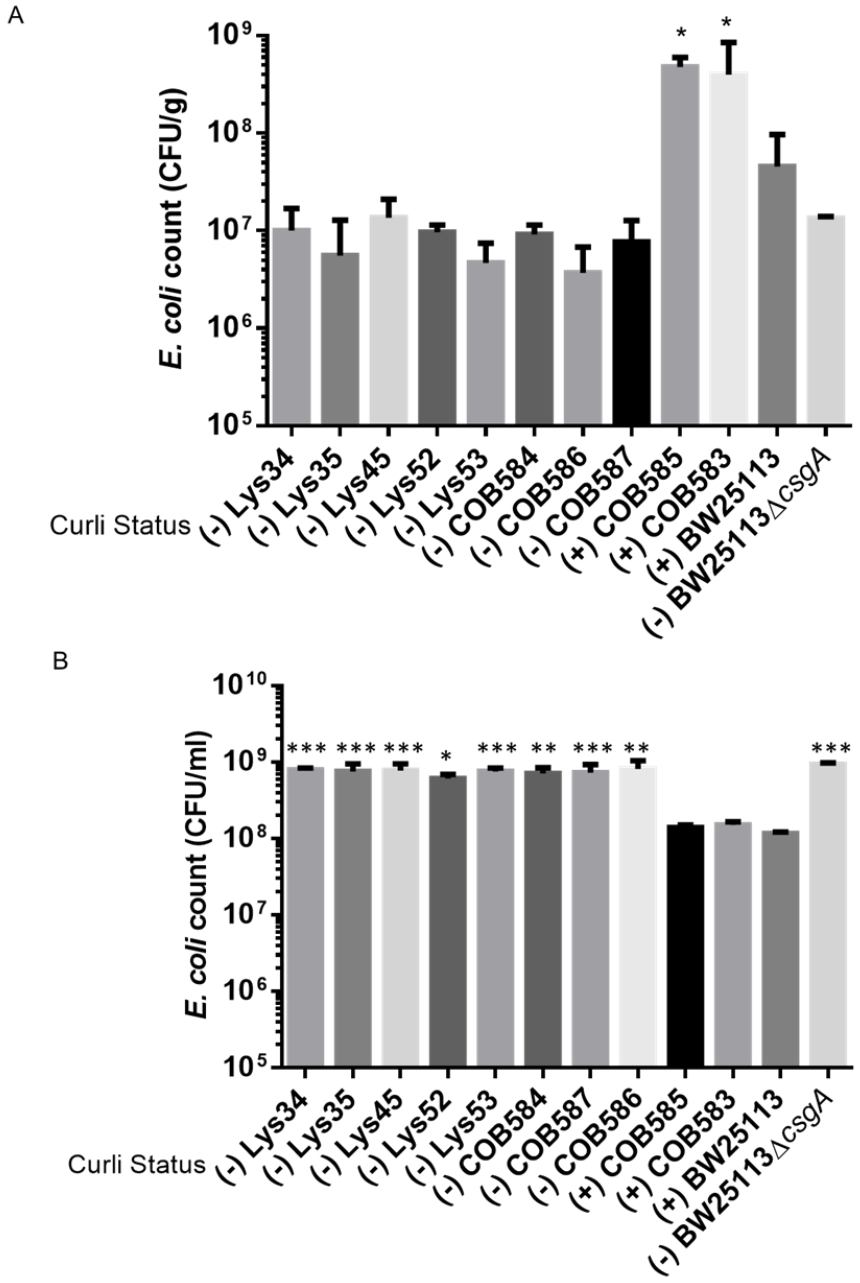

Figure 5.7: Curli-positive *E. coli* attach better to quartz sand than curli-negative *Escherichia coli*. Attachment of *E. coli* strains to quartz sand was determined after 28°C at 48 h as described in the *Material and Methods* (Section 2.7). Biofilm cell counts (A) and planktonic cell counts (B) were determined under same condition. Significant differences are determined at p<0.05 by one-way ANOVA. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

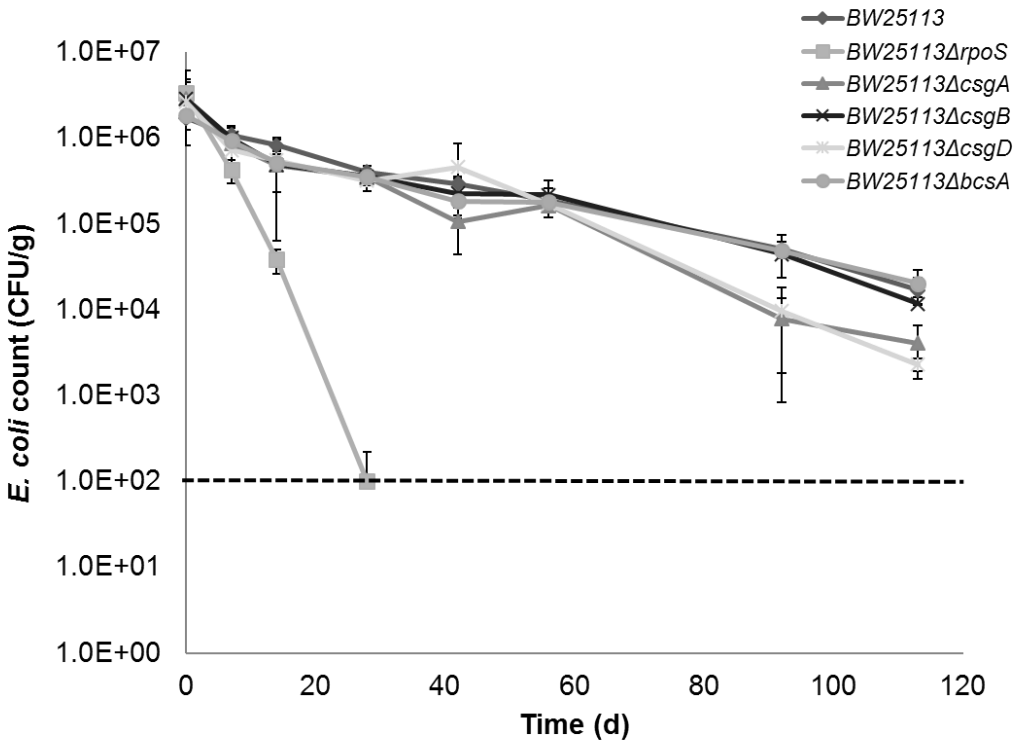

Figure 5.8: Curli and cellulose are not required for *Escherichia coli* to survive in soil. Soil survival was performed by inoculating wildtype and mutants into live soil and incubated at 15°C as described in *Materials and Methods* (Section 2.7). Dashed line represents detection limit of soil survival assay.

5.4. DISCUSSION

Many studies have described curli fimbriae as being important for *E. coli* to attach to biotic and abiotic surfaces such as glass, stainless steel and polystyrene (Carter *et al*., 2016; Cookson *et al*., 2002; Uhlich *et al*., 2009), but it is not known if curli production is important for sand attachment and soil persistence. This study was conducted to understand whether curli production was affected in the strains with impaired biofilm formation (Fig. 3.1C) and subsequently to determine the molecular basis for the loss of curli in some of these soil-persistent *E. coli* strains. The study also investigated the role of curli in attachment to sand and survival in soil.

Congo Red (CR) assay shows that three out the five soil-persistent *E. coli* strains initially tested were curli-negative (Fig. 5.1). However, when the total collection of 170 soil-persistent *E. coli* was analysed, 153 (90%) were presumptive curli-positive. The phenotype of the curli-negative strains on Congo Red plates ranged from white to pink. A previous study reported 70% of *E. coli* isolated from manure-amended soil was curli-positive and that curli production helped *E. coli* survive in manure-amended soil (Truhlar *et al*., 2015). The authors suggested that curli-positive *E. coli* strains forming biofilms are abundant in manure-amended soil because they are more likely to survive tolerate UV radiation, temperature fluctuations, and desiccation experienced when manure was spread on the soil and then survive in soil. A high proportion of curli-producing *E. coli* and *Salmonella* spp. have been isolated from various sources, including the environment and fresh produce (Dyer *et al*., 2007; Keelara *et al*., 2016; Malcova *et al*., 2008; Solomon *et al*., 2005).

Having observed that the majority of soil-persistent *E. coli* produced curli, it was further demonstrated that the presence of curli significantly increased the attachment of *E. coli* to sand (Fig. 5.7A). This is similar to Brombacher *et al*. (2003) who showed that curli helps attachment to sand, as the *E. coli* BW25113Δ*csgA* mutant attached significantly less to sand. Curli production by *E. coli* and *Salmonella* promotes macrocolony formation, community behaviour and colonization of host plant and animal tissues (Gophna *et al*., 2001; Pande *et al*., 2016). Curli enhanced the attachment of *E. coli* O157:H7 to plants and stainless steel whereas mutants not producing curli had reduced colonization to these surfaces (Carter *et al*., 2016) and reduced adherence to human colonic HT-29 epithelial cells or to cow colon tissue (Saldaña *et al*., 2009). Protection against toxic metals, such as mercury, is an additional benefit curli confers on *E. coli* in the environment (Hidalgo *et al*., 2010). Although, two curli-deficient mutants (BW25113Δ*csgD* and BW25113Δ*csgA*) survived significantly less well than the wildtype, another curli-deficient mutant (BW25113Δ*csgB*) was not significantly lower than the wildtype thus ruling out any possible contribution of curli to soil survival especially since the reduction in BW25113Δ*csgD* and BW25113Δ*csgA* compared to the wildtype was small (< 1 log cycle) (Fig. 5.8). The finding that soil survival is independent of curli production by *E. coli* agrees with earlier findings in this study which showed that curli-positive soil-persistent *E. coli* did not survive significantly better than curli-negative soil-persistent strains (Chapter 3). Further experiments using deletion mutants of curli subunit genes in soil-persistent strains will help in clarifying if curli contributes to soil survival in soil-persistent *E. coli* compared to BW25113.

Although the majority of the soil-persistent *E. coli* produced curli, 10% of the strains did not produce curli. Loss of curli in the initial three curli-negative strains (COB584, COB586 and COB587) in this present study is not because of loss of RpoS since RpoS is expressed and functional in them (Chapter 3; Fig. 3.4-3.6). Prophage insertions into transcriptional factor *mlrA* were reported to abolish curli and biofilm production in some *E. coli* O157:H7 isolates (Uhlich *et al*., 2013) and non-O157 STEC (Chen *et al*., 2013). Truncation of *csgB* by an insertional element IS1 eliminated curli production in *E. coli* O78:K80 (La Ragione *et al*., 1999). These previously mentioned mutations were not observed in our present study. Bioinformatics analyses of some of the main genes required for curli production (such as *csgD*, *csgB*, *csgA* and *csgD* promoters) revealed wildtype alleles in the strains lacking curli production. The presence of wildtype curli subunit genes in *E. coli* strains do not always result in curli production and this has been reported by several authors in *E. coli* (Dyer *et al*., 2007; Truhlar *et al*., 2015), *Salmonella* spp (De Oliveira *et al*., 2014) and *Enterobacter sakazakii* (Zogaj *et al*., 2003).

Two mutations were found in key regulators of curli production ($PdeR^{E620K}$ and $DgcE^{H486fs}$) and they correlated with inability to produce curli in COB584, COB586 and COB587 (Fig. 5.1; 5.3). The same mutations were found in five additional soil-persistent strains (Lys34, Lys35, Lys45, Lys52, Lys53) (Fig. 5.3). These curli-negative strains carrying the *dgcE* and *pdeR* mutations belong to different phylogenetic groups (B1 and E) and were isolated from two distinct lysimeters. This suggests that these mutations arose independently in genetically distinct lineages within separate lysimeters, both of which had Luvic Stagnosol (Rathangan) soils in them. However, the fact that the same frameshift mutation arose in eight strains raises the possibility that these strains are clonal. These eight strains were unable to express CsgD hence unable to produce curli (Fig 5.4). The conserved signature GGDEF motif, which is required for diguanylate cyclase (DGC) activity and cyclic-di-GMP (c-di-GMP) synthesis, is disrupted by the frameshift mutation in DgcE in the eight $PdeR^{E620K}$ strains, which is likely to limit their capacity to synthesize c-di-GMP via DgcE, and in turn making PdeR unable to trigger curli and biofilm production in them (Lindenberg *et al*., 2013). Although *dgcE* mutation can be complemented by the heterologous expression of another DGC DgcC from pCAB18 plasmid, mutation in *pdeR* could not be complemented, irrespective of the additional mutations in other PDEs or the

overproduction of DgcC, which increased the c-di-GMP pool in the bacteria (Lindenberg *et al*., 2013).

The proposed model explaining the basis for the loss of curli in the eight strains bearing the *dgcE*^{H486fs} and *pdeR*^{E620K} mutations is summarised in Fig. 5.9. This model was proposed because mutations in these two genes led to no CsgD expression (Fig. 5.2; Fig. 5.4), which is meant to direct transcription of *csgBAC* and resulting in curli production. In wild-type cells c-di-GMP binds PdeR causing it to dissociate from the PdeR-MlrA-DgcM complex, which in turn allows DgcM to form productive interactions with MlrA, stimulating its activity as a transcriptional regulator and as a DGC to produce more local c-di-GMP to further prevent the inhibitory activity of PdeR. The *dgcE* mutation results in a drop in the local c-di-GMP pool and this prevents PdeR from the necessary changes in protein-protein interactions from occurring in the PdeR-MlrA-DgcM complex, because PdeR doesn't bind to c-di-GMP to a sufficient extent. The *pdeR* mutation probably affects the affinity of PdeR for c-di-GMP (because the E620K change results in a charge change in a region that is very close to the active site) and this exacerbates the effect of the reduced pool of c-di-GMP. The hypothesis in this model will need to be tested to fully understand the mechanism behind the curli inhibition in these strains. Firstly, c-di-GMP levels in these curli-negative strains needs to be measured and compared to c-di-GMP levels in curli-positive strains so as determine if reduced c-di-GMP level correlates with the curli-negative phenotype. Secondly, the *dgcE* and *pdeR* mutations should be separated and constructed in wildtype backgrounds, then the c-di-GMP levels measured to determine whether the mutated *pdeR* is still capable of degrading c-di-GMP. Since, the PdeR acts as a trigger enzyme for curli biosynthesis and the formation of the PdeR-MlrA-DgcM protein complex is important for *csgD* transcription, the interaction of the mutated PdeR with DgcM and MlrA from these strains should be investigated. This will provide further insights into the importance of the glutamate (E620) for the PDE activity of PdeR.

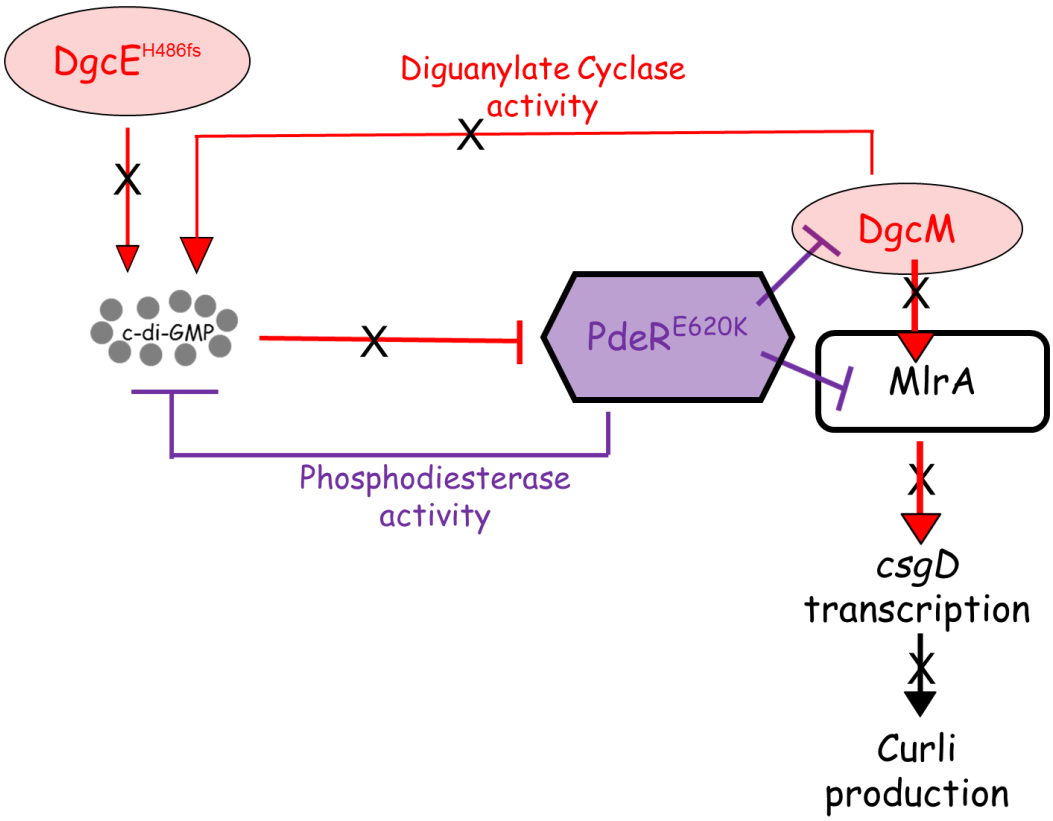

Figure 5.9: Proposed model for curli inhibition in some of the soil-persistent *Escherichia coli*. The mutated diguanylate cyclase (DgcE^{H486fs}) in the curli-negative strains is predicted to be unable to produce sufficient cyclic di-GMP (c-di-GMP) to relieve the inhibition of MlrA-DgcM by mutated phosphodiesterase, PdeR^{E620K}. In this state, PdeR^{E620K} is not able to sense c-di-GMP and thus retains the inhibition of DgcM and MlrA. This renders DgcM unable to act as transcriptional regulator to interact with MlrA and as a DGC to add to the local c-di-GMP pool, thereby inhibiting *csgD* transcription and curli production.

It is speculated that the DgcE^{H486fs} mutation may have led to the inability to produce curli rather than PdeR^{E620K}. This is because restoring wildtype *pdeR* from a low-copy plasmid (pCAB18-*pdeR*) in the eight PdeR^{E620K} *E. coli* strains did not result in curli production (Fig. 5.6A). Since PdeR is a phosphodiesterase (PDE) which breaks down c-di-GMP, expression of pCAB18-*pdeR* in curli-positive strains, COB583, COB585 and BW25113, repressed CsgD expression (Fig. 5.6B). Surprisingly, BW25113 overexpressing PdeR (BW25113 pCAB18-*pdeR*) still stained red (Fig. 5.6A). BW25113

carrying pCAB18-*pdeR* is unlikely to be producing curli under this condition as it did not express CsgD (Fig. 5.6B). It may be producing other extracellular substances still able to bind to congo red (Reichhardt *et al*., 2016). The inability of pCAB18-*pdeR* to restore curli production in these eight strains reinforces the speculation that DgcE^{H486fs} mutation may have led to the inability to produce curli rather than PdeR^{E620K}. This speculation that DgcE^{H486fs} mutation may have led to loss of curli production in these eight strains is strengthened by the fact that production of c-di-GMP by DgcM only contributes to, but is not essential for MlrA activation by DgcM (Lindenberg *et al*., 2013). Future studies should separately investigate the impact of the two different mutations on c-di-GMP levels, CsgD and curli production in order to mechanistically understand their roles in abolishing curli production thus better understand curli regulation. Since PdeR interacts with DgcM and MlrA through the N-terminus (EAL domain) (Lindenberg *et al*., 2013), and this is the region where the PdeR^{E620K} exists, there may possibly be a weak interaction of PdeR^{E620K} with MlrA in the curli-negative strains leading to low or no activity in transcribing *csgD,* hence no curli production.

Analysis of the EAL domains from different bacterial species showed that active EALs have glutamate (E) at codon 620 (E620) (Fig. 5.10). This suggests that E620 plays some role in the catalytic activity of PDEs, in addition to the previously identified conserved glutamate residue (corresponding to glutamate at codon 617 in PdeR) in other functional phosphodiesterases (Rao *et al*., 2008). PdeR^{E620K} may be unable to play its role in c-di-GMP metabolism since the E620 of PdeR is possibly involved in its catalytic activity. This is based on previous study by Tchigvintsev *et al*. (2010) who showed that the second guanine base of c-di-GMP interacts electrostatically with conserved glutamate at codon 706 (E706) in TBD1265 of *Thiobacillus denitrificans* (corresponding to E620 in PdeR of *E. coli*). Furthermore, Rao *et al*. (2008) observed that although mutation of E355 in RocR of *Pseudomonas aeruginosa* (equivalent to E620 in *E. coli* PdeR) plays a minor role in catalysis of c-di-GMP, the distal location of the residue makes it likely to play an important role in maintaining the conformational structure required for both c-di-GMP binding and hydrolysis. Amino acid residues distal to active site residues have been shown to play crucial roles in enhancing the catalytic activity of enzymes through structure stabilization (He *et al*., 2015; Rajagopalan *et al*., 2002). Based on this, PdeR^{E620K} may cause structural changes making it difficult for the conserved residue E617 to be catalytically active. This assumption becomes important since the eight curli-negative strains in this study retained all the conserved residues previously reported to be important for PDE activity of PdeR (Römling *et al*., 2013).

E620 is conserved in all *E. coli* PdeR (> 1000 genomes) searched in the National Center for Biotechnology Information (NCBI) database and this tight conservation of E620 in EAL domains of different bacteria suggests a possible role for them in regulating curli production. It is unlikely that the selection for the mutations in the *dgcE* and *pdeR* genes observed in the eight curli-negative strains described in this study occurred by chance. This is because they were isolated from different lysimeters and the strains belonged to different phylogenetic groups (Table 2.1). In addition to the *pdeR* and *dgcE* mutations arising in the eight strains described in this study, the possible mechanism(s) for the loss of curli in the remaining nine curli-negative strains in our soil-persistent *E. coli* strain collection needs to be further investigated. This is because some of the regulators of curli production analysed *in silico* were wildtype (Section 5.3.3).

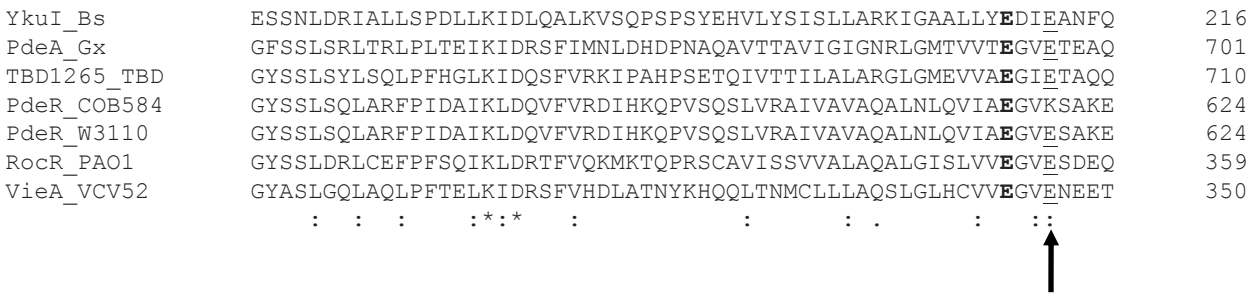

Figure 5.10: Multiple Sequence Alignment of EAL domains in some bacterial species. Multiple sequence alignment of EAL domain in some bacteria showing conserved glutamate (E) at positions equivalent to codon 620 in PdeR in *E. coli* (underlined). Previously reported glutamate (E) conserved for phosphodiesterase activity is in bold. YkuI from *Bacillus amyloliquefaciens* (YkuI_BAm); YkuI from *Bacillus subtilis* (YkuI_Bs); PdeA from *Gluconacetobacter xylinus* (PdeA_Gx); TBD1265 from *Theobacillus denitrificans* (TBD1265_TBD); PdeR from *E. coli* COB584 (PdeR_COB584); PdeR from *E. coli* BW25113 (PdeR_Bw25113); RocR from *Pseudomonas aeruginosa* PAO1 (RocR_PAO1) and VieA from *Vibrio cholerae* VCV52 (VieA_VCV52). Multiple sequence alignment was performed using ClustalOmega.

This study has shown that curli fimbriae contribute to the attachment of *E. coli* to sand but not soil persistence. Curli are also important for attachment to different plant materials, animal tissue, glass, stainless steel and plastic and contributes to environmental persistence (Fink *et al*., 2012; Patel *et al*., 2011; Yaron & Römling, 2014). Besides curli, other adhesins have been identified that contribute to the environmental persistence of *E. coli* such as exopolysaccharides, type 1 aggregative adherence fimbriae (Al Safadi *et al*., 2012) and more recently, Yad fimbriae (Larsonneur *et al*., 2016). As important as curli production is for environmental persistence, some of the long-term soil-persistent *E. coli* were unable to produce curli. The heterogeneity in curli production may reflect the genetic diversity of *E. coli* lineages present in the soil, perhaps suggesting that they have evolved to occupy different micro-niches in the soil, with some of the micro-niches not requiring ability to retain curli. Biotic and abiotic conditions encountered in different micro-environments within the soil may encourage or discourage curli production. For example, in conditions when curli production was not required, such as when manure was injected into soil, the population of non-curli producers increased (Truhlar *et al*., 2015). However, when manure is spread on soil, the *E. coli* from manure needs to overcome the UV radiation, temperature and moisture fluctuations it encounters, hence it retains curli production, and which help produce biofilm, to protect it against these stresses (Truhlar *et al*., 2015).

Since not much is known about the soil characteristics and prior practices on the soils used for running the lysimeters and the source of *E. coli* in the lysimeters are unknown, their possible contributions to the curli status of the *E. coli* leached from the soil cannot be ascertained. Our observation in this study suggests that soil may be exerting some selective pressures on the regulatory networks of curli production, making them lose the ability to produce curli. This could be because curli production is not strictly needed for long-term survival in the soil as curli-negative strains were still able to survive long-term in the soil (Chapter 3). Another interesting observation from this study was that curli-negative strains attach poorly to sand and had high numbers of planktonic cells (Fig. 5.8B). This suggests that loss of curli may be an important strategy for the dissemination of *E. coli* into the environment. Lack of curli was suggested to be a selective trait for survival and transport of *E. coli* O157:H7 in soil and water environments (Ravva *et al*., 2014). Loss of the ability to produce curli in a subset of

soil-persistent *E. coli* seem to be important for the lifestyle of *E. coli* where they need to detach more readily to recolonise new environments and potentially new hosts.

In conclusion, this study showed that the majority (90%) of *E. coli* persisting in soil were curli-positive and 10% were unable to produce curli. Curli fimbriae are important for attachment of *E. coli* to sand and contribute to soil survival. Investigations into the mechanism for loss of curli in the soil-persistent *E. coli* showed mutations in *dgcE*^{H486fs} and *pdeR*^{E620K} which correlated with loss of curli in eight soil-persistent strains, although this did not significantly impair their soil survival compared to curli-positive strains. Finally, loss of curli resulted in higher planktonic *E. coli* cells and this may suggest a role for this phenotype in the dissemination of *E. coli* into the environment.

CHAPTER 6

Discussion

There is a growing body of knowledge that *E. coli* populations are present in various non-host environments such as freshwater (Jiménez *et al*., 1989), beach water (Chiang *et al*., 2011; McLellan & Salmore, 2003), beach sand (Chiang *et al*., 2011), tropical and subtropical soils (Byappanahalli *et al*., 2012; Desmarais *et al*., 2002), coastal temperate forest soils (Byappanahalli *et al*., 2006), riverine temperate soil (Ishii *et al*., 2006), sediments (Anderson *et al*., 2005) and wastewater (Zhi *et al*., 2016). These *E. coli* strains adapt to these external environments and become part of the natural microbiota. Naturalised *E. coli* were recovered from intact soil monoliths maintained in lysimeter units that have been protected from faecal contamination since 1998 (Brennan *et al*., 2010). Soil-persistent *E. coli* strains possess unique growth and metabolic characteristics such as ability to utilise a wide range of nutrient sources at 15°C unlike *E. coli* K-12, suggesting adaptation to conditions present in soils (Brennan *et al*., 2013). This project was designed to understand the role the general stress response mediated by RpoS plays in soil survival in these soil-persistent *E. coli* strains and environmentally-relevant properties that may promote long-term persistence in soil.

6.1. FUNCTIONAL STRESS RESPONSE IS REQUIRED FOR SOIL SURVIVAL AND LONG-TERM SOIL PERSISTENCE

Previously, RpoS has been identified to be important for resistance to stresses typically encountered in the external environment, such as cold stress, osmotic stress and desiccation (Stasic *et al*., 2012). Although some of these stresses would be experienced in the soil, no direct evidence is available to show the importance of RpoS in soil. Furthermore, it has been shown that there is a trade-off between growth and stress resistance in nutrient-poor environments leading to mutations in *rpoS* (Notley-Mcrobb *et al*., 2002). Thus, this work analysed whether *rpoS* was intact and functional in soil-persistent *E. coli* and aimed to determine if RpoS was important for soil survival.

By analysing the nucleotide sequences, known RpoS-dependent phenotypes and reporter fusion analysis, it was confirmed that the general stress response regulator RpoS is retained in the five representative long-term soil-persistent *E. coli* strains (Chapter 3). Soil survival experiments also demonstrated that RpoS is essential for long-term soil survival of *E. coli.* An *rpoS* deletion mutant was constructed in soil-persistent *E. coli* COB583 (COB583 Δ*rpoS*) and it rapidly declined and lost viability in soil compared to the wildtype COB583, which only slightly declined in viability (Fig. 3.2D). A similar result was also seen in the laboratory strain *E. coli* BW25113 Δ*rpoS* (Fig. 3.2B). It was shown that all natural *E. coli* strains, regardless of their origin,

whether gut, soil or laboratory, can persist in soil for long periods and the data also suggest that the general stress response is intrinsic to this trait. The importance of retaining an intact stress response in the environment was also recently shown by Zhi *et al*. (2016). The authors reported that a functional RpoS activity was retained in wastewater-adapted *E. coli* populations. Although a previous study had shown that *E. coli* O157:H7 and O157:H- strains with mutations in *rpoS* had lower survival in manure-amended soil compared to strains with intact *rpoS* (van Hoek *et al*., 2013), this study used *rpoS* deletion mutants in *E. coli* to provide direct evidence that RpoS is required for survival in live soil. The soil environment is very dynamic and consists of many biotic and abiotic stresses, so an intact stress response is required to overcome UV radiation from sunlight, lower temperature, hyper- and hypo-osmotic stress, nutrient availability, competition and predation by predatory bacteria and protozoa (Fig. 1.3) (Arrange, 1993; Ibekwe *et al*., 2010).

Having established that RpoS is important for *E. coli* survival in soil, the question of how RpoS impacts soil survival was raised. RpoS could enhance the soil survival of *E. coli* by overcoming any of the stresses and conditions encountered in the soil, however, the impact of RpoS on survival in low moisture and pH in soil; and also on the interaction with protozoan predation was specifically tested. Moisture gradient fluctuation is often experienced in different sections of a soil column (Marciano-Cabral & Cabral, 2003) and soil moisture has also been shown to influence bacterial soil survival (Oliveira *et al*., 2004), yet little is known about the role of RpoS in surviving low soil moisture. Interestingly, it was observed that reduced moisture soil (60% WHC) did not significantly reduce the survival of COB583 in soil whereas the survival of COB583Δ*rpoS* was significantly impaired (Fig. 4.8). The fitness of COB583 under this condition, which was not seen in the laboratory strain BW25113, was probably because it has been adapted to the soil environment for over 9 years before isolation (Brennan *et al*., 2010a). This observation indicates that RpoS makes a significant contribution to the survival of COB583 in soil and the importance of RpoS is more pronounced when soil moisture is reduced. Low moisture creates hyperosmolarity in soil and RpoS is known to protect against osmotic stress (Stasic *et al*., 2012). RpoS was also seen to help COB583 survive acidic pH (pH= 5.2) in soil B, since the survival of the wildtype COB583 did not decline but COB583 Δ*rpoS* was significantly reduced (Fig 3.2D). This strengthens the conclusion that multiple stresses in the soil require a functional RpoS activity.

Protozoans are an important member of the soil macrofauna and they will be regularly encountered by *E. coli* in soil, hence it was initially hypothesized that soil-persistent *E. coli* may have the ability to resist protozoan predation. Data from this work showed that soil-persistent *E. coli* differed in their sensitivity to protozoan predation, contrary to the initial hypothesis. Specifically, some of the soil-persistent *E. coli* were very susceptible to predation by *A. polyphaga* and *T. pyriformis* (Fig. 4.3, 4.4). The pattern of susceptibility of the soil-persistent *E. coli* strains to both protozoans was different, however, COB583 was resistant to both *A. polyphaga* and *T. pyriformis*. RpoS contributed to the ability of COB583 to resist predation by *T. pyriformis* (Fig. 4.6). Similarly, *E. coli* BW25113Δ*rpoS* was significantly impaired in its ability to survive predation by *A. polyphaga* and *T. pyriformis* (Fig. 4.5, 4.6). Taken together, these results suggest that RpoS contributes to long-term soil-persistence by resisting protozoan predation, low moisture and hyperosmolarity in soil and low pH. Considering that protozoan grazing is the main biotic factor that determines the survival of an introduced microorganism into aquatic systems (Artz & Killham, 2002; Barcina *et al*., 1997), it will be important for future studies to directly evaluate the role of protozoan predation in the survival of commensal *E. coli* in the soil, so as to better understand how commensal *E. coli* adapts to the soil environment.

6.2. DOES PRODUCTION OF CURLI AND BIOFILM ENHANCE RESISTANCE TO PROTOZOAN PREDATION?

Biofilm formation has been shown to protect bacteria, for example *Pseudomonas aeruginosa*, against protozoan predation (Matz *et al*., 2004). The supposed contribution of biofilm to resist protozoan predation was not observed among the biofilm-producing strains used in this study. For example, COB583 was resistant to predation but COB585 and BW25113 were susceptible despite being able to produce biofilm. This raises the question: does ability to produce biofilm contribute to protection against protozoan predation and what is the role of the two main *E. coli* biofilm matrix components (curli and cellulose) in this contribution? Besides DePas *et al*. (2014) who showed that biofilm protected *E. coli* against predation by the soil-dwelling nematode *Caenorhabditis elegans* and the predatory bacterium *Myxococcus xanthus*, not much is known about how biofilms prevent predation and how *E. coli* protects itself against predation by soil-borne protozoans. Here, deletion mutants in genes encoding curli (BW25113Δ*csgA*) and cellulose (BW25113Δ*bcsA*) production were used and results showed that curli and cellulose protects *E. coli* BW25113 from predation by *A. polyphaga* and *T. pyriformis* (Fig. 4.5, 4.7) thus suggesting that biofilm protects against

protozoan predation in *E. coli* BW25113. This result appears to contradict the observation of Ravva *et al*. (2014) who showed that protozoans preyed more on curli-positive *E. coli* 0157:H7 than curli-negative strains, thus suggesting that presence of curli make *E. coli* susceptible to protozoan predation. The authors used different protozoans (*Vorticella microstoma* and *Colpoda aspera*) in their study, which may have exhibited preferential feeding habits in preying on curli-producing *E. coli* O157:H7. Also, the presence of phenotypic variants in curli from a single isolate of *E. coli* O157:H7 observed by the authors was not observed in our study. Complementation of the mutant strains with functional *csgA* and *bcsA* will be important to confirm the role of cellulose and curli in protection against protozoan predation.

In the predation assay with the soil-persistent strains, there was no correlation between ability to produce biofilm and resistance to protozoan predation. It has been previously observed that biofilm alone is not sufficient to resist protozoan predation but factors such as protozoa feeding mode also contribute (Weitere *et al*., 2005). The most resistant strains to predation by *T. pyriformis* (COB583 and SE15) in this study (Fig. 4.4) were curli-producing strains and their ability to resist predation may be because they produce biofilms/microcolonies. Since *T. pyriformis* is a filter feeder, the size of their microcolonies may makes the bacteria too big to be ingested (Matz *et al*., 2004). However, this is unlikely since COB585 which is also a curli-positive was highly preyed upon by *T. pyriformis*. In contrast, curli production may be irrelevant for resistance to predation by *A. polyphaga* since it feeds by engulfing its prey.

6.3. CURLI PRODUCTION AND LONG-TERM SOIL PERSISTENCE IN *E. COLI*

The majority (90%) of the long-term soil-persistent *E. coli* strains produced curli, which is a major component of biofilm in *E. coli*. Production of curli correlated with high biofilm production (Fig. 3.1C) and attachment to an environmentally relevant material, quartz sand (Fig. 5.6A). Although it was hypothesized that curli and biofilm production will correlate with increased survival of *E. coli* in the soil environment, curli-producing soil-persistent *E. coli* did not survive significantly better than non-curli-producing strains in soil (Fig. 3.2C). This suggests that curli and biofilm production do not provide any advantage for survival in soil. In order to further test this hypothesis, deletion mutants of genes producing the main matrices (curli and cellulose) for biofilm in *E. coli* BW25113 were tested in soil to determine their impacts on soil survival. BW25113Δ*csgD* (curli transcriptional regulator) and BW25113Δ*csgA* (main curli subunit) had significantly reduced survival whereas BW25113Δ*csgB* (minor curli

subunit) and BW25113Δ*bcsA* (cellulose synthase) survived similar to the wildtype (Fig. 5.8). Although, BW25113Δ*csgD* and BW25113Δ*csgA* are unable to produce curli, BW25113Δ*csgB* which is also curli-deficient was not significantly lower than the wildtype thus ruling out any possible contribution of curli on soil survival especially since the reduction in BW25113Δ*csgD* and BW25113Δ*csgA* compared to the wildtype was small (< 1 log cycle). This result that soil survival is independent of curli production by *E. coli* agrees with earlier findings in this study that curli-positive and curli-negative soil-persistent strains can survive long-term in soil (Chapter 3). The fitness of the BW25113Δ*csgB* and BW25113Δ*bcsA*, suggests that soil survival is independent of curli and cellulose. Ravva *et al*. (2014) have suggested that absence of curli provided an advantage for survival in the soil but this was not the case in the present study. At best, loss of curli gave similar survival as the wildtype (Fig. 5.8). Further studies should construct deletion mutants in genes encoding curli and cellulose in soil-persistent *E. coli* in order to directly evaluate their contribution to soil survival.

6.4. POSSIBLE IMPLICATION OF *E. COLI* PERSISTENCE IN SOIL

The *E. coli* strains used in this study were obtained from lysimeters that contained Luvic Stagnosol soil from Rathangan, Ireland. The soil has a high clay content (19-29%) (Brennan *et al*., 2010a), which may have enhanced the survival of the *E. coli* in soil (Brennan *et al*., 2014). Lünsdorf *et al*. (2000) described that clay materials create microhabitats called “clay hutches” in soil, which could serve as an “effective survival unit”, preventing access by protozoans, thus offering protection from protozoan predation. *E. coli* in clay rich soil are easily transported into run-off water since there is low attachment of *E. coli* to clay particles (Oliver *et al*., 2007).

Soil-persistent *E. coli* can contaminate run-off water and cause pollution in groundwater and surface water it contacts. *E. coli* has been found to continuously leach from soil and contaminate groundwater and private drinking water several months after manure application (Ogden *et al*., 2001; Vanderzaag *et al*., 2010). After contaminating water systems, *E. coli* could persist in groundwater and water distribution systems by forming biofilms (Banning *et al*., 2003). In a separate study using one of the soil-persistent strains in this study, COB583 (also referred to as Lys9), was shown to persist in drinking water beyond 70 days (Abberton *et al*., 2016). While some of the contaminating *E. coli* may be culturable, a non-culturable subpopulation of *E. coli* may develop in drinking water (Bjergbæk & Roslev, 2005). Environmentally-persistent *E. coli* could contaminate pipes used for water distribution through breakage/leakage of

pipes or faults in reservoir structure. *E. coli* could form microcolonies of various sizes in soil-like environments, which could help them persist in such environments (Downie *et al*., 2012). Long-term persistence of *E. coli* in soil could continuously re-seed groundwater and surface water with *E. coli* when there is no fresh contamination from faecal material (Ashbolt *et al*., 2001). Because of this limitation in the use of *E. coli* as indicator of faecal contamination for water quality, more reliable markers are being developed for tracking sources of faecal contamination in water systems. An example is the highly human-specific α-1–6 mannanase gene of *Bacteroides thetaiotaomicron* (Aslan & Rose, 2013; Yampara-Iquise *et al*., 2008), which has been used to predict the source of microbial contamination in rivers (Verhougstraete *et al*., 2015).

Persistence of bacteria in beach sand poses a problem for beach water quality. Biofilm contributes to the persistence of waterborne pathogens in beach sand (Phillips *et al*., 2011). Faecal organisms persisting in the beach sand as biofilms (Piggot *et al*., 2012) are transported through wave and rainfall to re-seed the beach water with faecal bacteria thus making it unsafe for use (Heaney *et al*., 2014). Contact with faecally-contaminated beach sand has been reported to cause increased illnesses such as gastroenteritis among beachgoers (Heaney *et al*., 2012). *E. coli* persisting in sediments can also contaminate the overlying water when the sediment is disrupted and resuspends the bacteria into the water (Orear & Dalman, 2011).

Another implication of the persistence of *E. coli* in soils is that it could serve as a source of contamination for crops destined for the food chain (Habteselassie *et al*., 2008) and such crops may serve as environmental reservoir and as a source of transmission of the pathogen to new hosts (Holden *et al.,* 2009). Pathogenic *E. coli* persisting in manure-fertilized soil could pose a potential risk for vegetable crops to be exposed to pathogens from the manure applied. Jensen *et al*. (2013) reported that when contaminated animal slurry was used to fertilise the soil, *E. coli* did not only persist in the soil but also contaminated the lettuce grown on it. *E. coli* O157:H7 from soil cross-contaminated strawberries grown in greenhouse (Shaw *et al*., 2015) just as *Salmonella enterica* serovar Weltevreden persistent in soil also contaminated the root and shoot of spinach grown on it (Arthurson *et al*., 2011). When pathogenic bacteria are internalised in plant tissues, they form microcolonies which helps them persist in the plant tissues (Wright *et al*., 2013). Since curli-positive and curli-negative *E. coli* strains persist in soil, it will be interesting to investigate their ability to colonise plants tissues. The transparent soil described by Downie *et al.* (2012), which mimicks physical

and chemical properties of soil, and provides an advantage for microscopic imaging of bacterial colonization in the rhizosphere, will be helpful in this regard.

6.5. LOSS OF CURLI AND *E. COLI* DISPERSAL FROM SOIL

Ten percent of the soil-persistent *E. coli* collection analysed in this study did not produce curli, as evidenced by the absence of congo red staining. Inability to produce curli correlated with impaired biofilm production (Fig. 3.1C) and reduced attachment to quartz sand (Fig. 5.7A). A set of the curli-negative strains (eight strains) bearing similar mutations were chosen for further investigations. These curli-negative soil-persistent strains had the same deletion (1456delC) in *dgcE* which resulted in a frameshift mutation and premature stop at amino acid residue 486 in the protein sequence; along with another point mutation (1856G>A) which led to an amino acid change in the protein sequence (E620K) in *pdeR*. These mutations resulted in the loss of CsgD expression and curli production (Fig. 5.1, 5.2). Based on the results obtained in this work, a mechanism for the loss of curli in these strains was proposed (Fig. 5.9). Additionally, three amino acid mutations conserved in the DGC DgcQ were also observed in the eight curli-negative strains. However, this is unlikely to explain the loss of curli production since DgcQ plays a minor role in curli production. Deletion of *dgcQ* did not reduce *csgB*::*lacZ* expression nor reduce curli production in *E. coli* (Weber *et al*., 2006). Furthermore, deletion of *dgcQ* did not affect intracellular c-di-GMP production (Sanchez-Torres *et al*., 2011). The alteration of multiple genes encoding c-di-GMP metabolism proteins (*dgcE, dgcQ* and *pdeR*) in the soil-evolved strains strengthens the proposition that the soil may have selected for loss of curli in some of the *E. coli* strains. Further study is required to clarify how the mutations observed in this work affects curli production. This will provide more insight into the regulation of curli production in *E. coli*.

It was initially thought that these curli-negative strains carrying the *dgcE* and *pdeR* mutations belonged to different phylogenetic groups (B1 and E). This suggests that these mutations arose independently in genetically distinct lineages within separate lysimeters, both of which had Luvic Stagnosol (Rathangan) soils in them. The fact that the same frameshift mutation arose in the eight strains makes it highly likely that they all belong to the same lineage, based on revised phylogeny. This raised the possibility that Lys45, the only different phylotype among the eight B1 strains, may actually belong to phylogroup B1 rather phylogroup E. Single-nucleotide polymorphism (SNP) analysis indicated that Lys45 clustered very closely to the B1 strains (Brennan and Waters,

unpublished data). Furthermore, *in silico* PCR using the primers in Clermont quadruplex PCR (Clermont *et al*., 2013) and Multilocus sequence typing (MLST) showed that Lys45 was in the same sequence type as all the other seven B1 strains contrary to the previous results from Clermont's quadruplex PCR showing that Lys45 belonged to phylogroup E (Brennan & Waters, unpublished data). While the *in silico* PCR and MLST show that Lys45 is in the same phylogroup and MLST type as the other curli-negative strains, analyses of the whole genome indicates 42 SNPs between Lys45 and the other curli- negative B1 strains, which suggest that Lys45 may belong to a potentially different clone (Brennan & Waters, unpublished data). These observations highlight the need for whole genome sequencing in the comparison of strains, from which strains can be typed into their respective phylogroups *in silico*. At the moment, it is unclear if the soil selected for mutations in *dgcE* and *pdeR* or they occurred before *E. coli* got into the soil. Since these curli-negative strains came from the same soil (Rathangan), it is also possible that the strains were in the soil prior to collection of soil cores for setting up lysimeters. Another possibility is that the *E. coli* strains deposited in the soil prior to lysimeter construction were curli-negative, as Bokranz *et al.* (2005) reported that 21% (11 out of 52) of *E. coli* freshly isolated from human faeces did not express curli (Bokranz *et al*., 2005).

Based on the observations from this work and the important role curli are likely to play in the environment, including attachment to sand, forming biofilms and resistance to predation, it is intriguing why some of the soil-persistent *E. coli* should lose the ability to produce curli. The poor attachment of the curli-negative strains to sand suggests that loss of curli may be an important strategy for the dissemination of *E. coli* to recolonise new environments and potentially new hosts. Previous studies have described that *E. coli* biofilm usually contains two populations i.e. matrix-encased and non-matrix-encased (washout) *E. coli* cells. While the matrix-encased cells produce curli and are stress resistant, the washout cells produced no curli and are more susceptible to stress such as hydrogen peroxide (DePas *et al*., 2013). This bimodal population is also found in pellicle biofilm of *E. coli* UTI89 (Hung *et al*., 2013). Furthermore, using cryosectioning fluorescence microscopy with thioflavin S as a curli-imaging agent, Serra *et al*. (2013) showed that flagellated cells are an essential part of biofilm in *E. coli*. Flagellated cells in biofilms have also been shown in *Pseudomonas aeruginosa* (Ma *et al*., 2009). These flagellated bacteria within biofilms are considered the main agents of biofilm dispersal thus suggesting that the loss of ability to produce curli observed in some of the *E. coli*

strains may have evolved in the soil in order to maintain a highly flagellated and easily dispersible population.

If loss of curli in these eight strains was indeed a mechanism for biofilm dispersal, the dispersed bacteria should have the capacity to re-initiate biofilm formation on encountering a suitable environment. When curli-negative *E. coli* was cultured in an environment capable of inducing biofilm formation (in YESCA medium at 28°C), they neither produced curli nor rugose biofilm. Perhaps, this is because the main regulatory genes for biofilm production in these soil-persistent *E. coli* has been altered, thus biofilm cannot be produced. Taken together, the loss of curli could promote their dispersal and transport in the external environment. Lack of curli was previously suggested to be a selective trait for survival and transport of *E. coli* O157:H7 in soil and water environments (Ravva *et al*., 2014). The soil-persistent *E. coli* used in this study was collected from leachates which flowed out from the soil columns in the lysimeter (Brennan *et al*., 2010a) and this may have constituted a possible bias in selecting for curli-negative strains, as more unattached strains may have been washed through and collected in the leachate.

6.6. COLONISATION OF SOIL AND GENETIC DIVERSITY OF SOIL-ADAPTED *E. COLI*

Although it was initially speculated that *E. coli* does not grow in soil (Savageau, 1983), later studies have shown that *E. coli* is able to grow in soil (Solo-Gabriele *et al*., 2000). The repeated isolation of genetically identical isolates of *E. coli* for over 1 year in soil raised the possibility that these bacteria have become part of the autochthonous soil microflora (Ishii *et al*., 2006). Naturalized *E. coli* strains were shown to grow in live soil, reaching up to 4.2×10^5 CFU g^{-1}, and also grew in soil which had bile salts added to inhibit non-faecal bacteria (Ishii *et al*., 2006). Byappanahalli *et al*. (2006) also reported that autochthonous *E. coli* populations were isolated from undisturbed, forest soil protected from external *E. coli* sources and which had low recent human impact. These findings, coupled with conserved evolutionary adaptations in the *E. coli* core genome to adapt to different secondary habitats, such as catabolic flexibility and ABC transporters for uptake of amino acids and sugars from the environment (Ihssen *et al*., 2007; Ihssen & Egli, 2005), suggests that *E. coli* may be able to colonise the soil. Upregulation of genes involved in nutrient uptake (including ABC transporters) are predominant during adaptation of *Listeria monocytogenes* to the soil environment (Piveteau *et al*., 2011). There is currently no direct evidence of commensal *E. coli* colonising the soil, so long-

term experiments involving the inoculation of commensal *E. coli* into live soil, and protected from external *E. coli* inputs, should be conducted to ascertain if commensal *E. coli* is able to colonise the soil and become a part of the autochthonous microbiota of the soil.

There is some evidence suggesting genetic diversity within and between *E. coli* isolated from different soils, with some populations genetically distinct from host-associated *E. coli* (Byappanahalli *et al*., 2006). Naturalized *E. coli* strains from soil tightly clustered together by site of isolation and Horizontal, fluorophore-enhanced, repetitive PCR (HFERP) DNA fingerprinting revealed that they had unique DNA fingerprints (Ishii *et al*., 2006). Unique DNA fingerprints were also reported in 33% of *E. coli* from various soil types from different climate and geographic locations in temperate and tropical regions (Byappanahalli *et al*., 2012). These suggest that some naturalised *E. coli* may not have originated from animal or human hosts and that these naturalised *E. coli* strains belong to multiple genotypes (Byappanahalli *et al*., 2012).

Using extended multilocus sequence typing (MLST) analysis, five phylogenetic clades of *Escherichia* (Clades I to V) were identified (Walk *et al*., 2009), besides the already established phylogenetic classification. The authors had isolated representatives of these clades over many years and from different habitats. The strains resembled "typical" *E. coli* based on phenotypic and genomic characterisation but had clear divergent nucleotide sequences, which made the authors exclude them from previous publications (Walk *et al*., 2009). These cryptic clades of *E. coli* have been confirmed through whole-genome phylogenetic analysis (Luo *et al*., 2011). C-I is more related to the B2 phylogenetic group but Clades II–V are divergent, survive better in the external environment than commensal *E. coli* and compete poorly in the human gastrointestinal tract (Luo *et al*., 2011). In fact, Clades III, IV, and V have been regarded as "truly environmentally adapted" since they form robust biofilms, outcompete typical *E. coli* strains at low temperatures and are non-pathogenic in a mouse model of septicaemia (Ingle *et al*., 2011). Based on the reported genetic diversity among *E. coli* in the soil, it was speculated that some *E. coli* populations persist outside the host gut and are transmitted directly from the environment to the host rather than through the faecal-oral route. A previous study which compared the genetic diversity of *E. coli* from humans and the septic tank in their households showed that the septic tank environment harbour genetically distinct *E. coli* from the humans in the household supplying faeces to the septic tank (Gordon *et al*., 2002). Based on their observation, it is likely that the

same outcome will be observed in more complex systems such as the soil, thus it is speculated that the genetic diversity in the soil strains is probably driven by the soil environment. Analysis of the whole genome of all the *E. coli* strains in the collection of soil-adapted strains used for this present study will provide further insights and improve understanding on the genetic diversity of *E. coli* in non-host niches, particularly in the soil environment.

6.7. CONCLUSION

This project has provided insights into the mechanisms of persistence of *E. coli* in a non-host niche (soil). It has highlighted the role of the general stress response mediated by RpoS in the extra-intestinal life of *E. coli*. RpoS was shown to protect *E. coli* against protozoan predation, osmotic stress induced by low soil moisture as well as low soil pH. It was further shown that curli are important for attachment to quartz sand and resisting predation but may not be required for soil survival. This work also provides insight into the possible molecular mechanisms that resulted in the loss of ability to produce curli in some soil-persistent *E. coli*. The curli-negative phenotype seemed to promote rapid dispersal of *E. coli* into the environment. It is unclear if the soil selected for mutations in genes regulating curli production (*dgcE* and *pdeR*) or they occurred before *E. coli* got into the soil. Additionally, soil-persistent *E. coli* showed unique properties compared to the common laboratory reference strain (*E. coli* BW25113) in this study. This was observed in environmentally relevant assays such as protozoan predation assay and survival under low soil moisture, in which the soil-persistent strains showed improved fitness over the lab strain. This highlights the need to use environmentally-relevant strains of *E. coli*, when studying mechanisms involving *E. coli* under environmental conditions. The techniques developed, including soil survival and predation assays, will be useful to other researchers interested in studying other bacteria in the external environment.

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The General Stress Response Is Conserved in Long-Term Soil-Persistent Strains of *Escherichia coli*

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ABSTRACT

Although *Escherichia coli* is generally considered to be predominantly a commensal of the gastrointestinal tract, a number of recent studies suggest that it is also capable of long-term survival and growth in environments outside the host. As the extraintestinal physical and chemical conditions are often different from those within the host, it is possible that distinct genetic adaptations may be required to enable this transition. Several studies have shown a trade-off between growth and stress resistance in nutrient-poor environments, with lesions in the *rpoS* locus, which encodes the stress sigma factor RpoS (σ^S). In this study, we investigated a unique collection of long-term soil-persistent *E. coli* isolates to determine whether the RpoS-controlled general stress response is altered during adaptation to a nutrient-poor extraintestinal environment. The sequence of the *rpoS* locus was found to be highly conserved in these isolates, and no nonsense or frameshift mutations were detected. Known RpoS-dependent phenotypes, including glycogen synthesis and γ-aminobutyrate production, were found to be conserved in all strains. All strains expressed the full-length RpoS protein, which was fully functional using the RpoS-dependent promoter reporter fusion P*gadX*::*gfp*. RpoS was shown to be essential for long-term soil survival of *E. coli*, since mutants lacking *rpoS* lost viability rapidly in soil survival assays. Thus, despite some phenotypic heterogeneity, the soil-persistent strains all retained a fully functional RpoS-regulated general stress response, which we interpret to indicate that the stresses encountered in soil provide a strong selective pressure for maintaining stress resistance, despite limited nutrient availability.

IMPORTANCE

***Escherichia coli* has been, and continues to be, used as an important indicator species reflecting potential fecal contamination events in the environment. However, recent studies have questioned the validity of this, since *E. coli* has been found to be capable of long-term colonization of soils. This study investigated whether long-term soil-persistent *E. coli* strains have evolved altered stress resistance characteristics. In particular, the study investigated whether the main regulator of genes involved in stress protection, the sigma factor RpoS, has been altered in the soil-persistent strains. The results show that RpoS stress protection is fully conserved in soil-persistent strains of *E. coli*. They also show that loss of the *rpoS* gene dramatically reduces the ability of this organism to survive in a soil environment. Overall, the results indicate that soil represents a stressful environment for *E. coli*, and their survival in it requires that they deploy a full stress protection response.**

Escherichia coli is a Gram-negative, facultative anaerobe, belonging to the *Enterobacteriaceae* family, which inhabits the intestinal tracts of humans, warm-blooded animals, and reptiles (1, 2). It can be transferred through water and sediments via feces and is almost universally used as an indicator of fecal contamination in drinking and recreational water. The use of *E. coli* as a fecal indicator is based, at least in part, on the assumption that it exists transiently outside the host gastrointestinal tract (3) and does not survive for a long time in the external environment. Though several authors have isolated *E. coli* from various natural environments, such as freshwater (4, 5), beach water (6, 7), beach sand (7), tropical and subtropical soils (8–12), coastal temperate forest soils (13), riverine temperate soil (14), and sediments (15), it is difficult to determine unequivocally whether these isolates originated from recent contamination or whether they represent long-term residents in those environments. In 2010, Brennan et al. (16) reported the recovery of *E. coli* populations from intact soil monoliths maintained in lysimeter units (previously described by Ryan and Fanning [17]), which have been protected from fecal contamination since 1998. These long-term soil-persistent *E. coli* isolates are the subject of the present study.

Soil-persistent *E. coli* strains from the lysimeters are genotypically diverse and possess unique growth and metabolic characteristics, suggesting adaptation to conditions present in soils (18, 19). These strains are assumed to have developed mechanisms that could help them survive in soil. For example, it was shown that a soil-persistent *E. coli* strain was nutritionally versatile and metabolized more substrates at 15°C than *E. coli* K-12 (19). Furthermore, *E. coli* strains have been shown to survive and grow in both amended (8, 20) and unamended (14) soil. The capacity of these *E. coli* strains to survive for long periods of time and grow in the external environment raises questions about the validity of its continued use as indicator of water quality (16).

The ability to survive environmental stresses has been shown to be controlled by the general stress response regulator, RpoS, in *E.*

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coli and other related bacteria. RpoS (σ^s) is an alternative sigma factor that is involved in *E. coli* resistance to stresses typically encountered in the external environment, such as cold stress, osmotic stress, oxidative stress, and desiccation (21–23). When environmental stresses are encountered, cellular RpoS levels increase dramatically; the resulting RNA polymerase-RpoS holoenzyme complex produces the appropriate transcriptional response (24). A number of studies have shown that, in low-nutrient conditions, particularly when growth rates are very slow, mutations can accumulate within the *rpoS* open reading frame (ORF), resulting in the partial or complete loss of RpoS function and reduced stress tolerance (25, 26). Mutations in *rpoS* have been reported in strains obtained from laboratory growth conditions as well as among natural isolates of *E. coli* (7, 26–34), *Salmonella* (35–37), *Cronobacter* (38), and *Citrobacter* (39). These mutations are thought to provide a selective advantage to microorganisms undergoing nutrient starvation due to a trade-off between stress resistance and growth (40). Indeed, mutations that confer a growth and survival advantage in stationary-phase cultures of *E. coli* (so-called growth advantage in stationary phase, or GASP, mutants) are frequently found to map to the *rpoS* locus (41). Thus, the long-term soil-persistent *E. coli* isolates present a unique opportunity to study the evolution of the *rpoS* locus in a natural nutrient-limited environment.

Soil can be considered a highly competitive environment where nutrient sources are significantly less abundant than in the host gastrointestinal tract (42). We hypothesized that low-nutrient microniches within the soil environment may select for *rpoS* mutations during long-term soil adaptation, perhaps providing a growth rate advantage. These *rpoS* lesions could also confer a competitive advantage through increased nutritional competence, a phenotype associated with the loss of *rpoS* (26, 40). Knowledge of how *E. coli* responds to chemical and physical stresses has been derived almost exclusively from studying laboratory strains; thus, the available collection of soil-persistent isolates of *E. coli* (16; F. Abram, unpublished data) from a closed system represents a rare opportunity to understand the role that stress responses play in the survival of *E. coli* in an environment outside the host, where environmental conditions, nutrient availability, and competing microorganisms are different from those present in the gastrointestinal tract.

In the present study, we have characterized the RpoS-dependent stress response in phylogenetically distinct soil-persistent *E. coli* strains and compared this to responses in commensal and laboratory *E. coli* strains. The *rpoS* locus was sequenced, and Western blotting and green fluorescent protein (GFP) reporter fusion (using the RpoS-dependent *gadX* promoter) assays were used to determine the presence and activity of the RpoS protein in each of the isolates. Soil survival assays were also performed to investigate the role of an intact *rpoS* locus in soil survival. We show that a functional RpoS is retained in long-term soil-persistent isolates of *E. coli* and demonstrate that RpoS is essential for long-term survival in the soil.

MATERIALS AND METHODS

Strains used and growth conditions. Five long-term (>9 years) soil-persistent *Escherichia coli* strains, belonging to five distinct phylogenetic groups, as described by Clermont et al. (43), were studied (here named COB583 to COB587) (Table 1). These strains were leached from lysimeter units (Luvic Stagnosol; C-to-N ratio, 10.1) (44), to which no fecal material had been applied since at least 1998 (16). The lysimeter unit was enclosed within a mesh netting to prevent contamination from birds and small mammals. The soil isolates were considered long-term soil-persistent isolates, since the experimental soil columns from which they were derived had not been exposed to fecal material for at least 9 years prior to leachate collection. *E. coli* strains were periodically isolated from these soil columns over a period of 4 years, indicating that the strains were resident in the soil. Two commensal strains (SE11 and SE15) and a well-studied laboratory strain (BW25113) with its corresponding *rpoS* deletion mutant (strain BW25113Δ*rpoS*) were used for comparative purposes. The strains are described in Table 1. An *rpoS* deletion mutant strain of soil-persistent *E. coli* COB583 was constructed by the one-step inactivation method using a λ Red recombinase-assisted approach, which replaces target gene sequence with a kanamycin resistance (Km^r) cassette described by Datsenko and Wanner (45), with minor modifications. Primers COB740R and COB743F were used to amplify the kanamycin cassette flanked by homologous sequences covering 915 bp upstream and 582 bp downstream of *rpoS* to generate PCR linear fragments, using the kanamycin cassette from a Keio collection mutant as the template. The λ Red recombinase plasmid used was pKOBEGA (46), and 500 ng of linear DNA was electroporated into the λ Red recombinase-expressing COB583 strain at 3.0 kV. The kanamycin resistance cassette was removed from the Δ*rpoS* mutant strains by FLP-FRT recombination, and removal of the cassette was confirmed by plating on Luria-Bertani (LB) agar with 50 $\mu g \cdot ml^{-1}$ kanamycin (LBKan). *E. coli* K-12 strain carrying a *gadX*-GFP promoter fusion on pUA66 (P_{gadX}) was obtained from David Clarke (University College Cork, Ireland). *gadX* is a transcriptional activator of the glutamate-dependent acid resistance system in *E. coli* and is RpoS dependent (47). P_{gadX} was obtained from overnight (~16 h) culture in LBKan at 37°C, and the resulting plasmids were transformed into all nine strains used in the present study.

TABLE 1 Description of strains used in study

Strain	Phylogenetic group	Habitat	Source or reference
COB583 (isolate 3; Lys9)	C	Soil	Brennan et al., 2010 (16)
COB584 (Lys24)	B1	Soil	F. Abram (unpublished)
COB585 (Lys25)	E	Soil	F. Abram (unpublished)
COB586 (Lys28)	B1	Soil	F. Abram (unpublished)
COB587 (Lys36)	B1	Soil	F. Abram (unpublished)
E. coli SE11	B1	Commensal	JCM16574 (Riken BRC, Japan)
E. coli SE15	B2	Commensal	JCM16575 (Riken BRC, Japan)
E. coli BW25113	A	Lab strain	Baba et al., 2006 (80)
E. coli BW25113 Δ*rpoS*			Baba et al., 2006 (80)
COB583 Δ*rpoS*	C	Soil	This study

Motility at 15°C and 37°C. Since motility and biofilm formation are traits that might be subject to selection during niche adaptation (48, 49), we measured these traits at 15°C and 37°C for each strain. In order to evaluate cell motility, each strain was grown in LB overnight (~16 h) with agitation at 37°C. Overnight cultures were then spot-inoculated onto LB containing 0.25% (wt/vol) agar plates, and radial motility was measured after 16 h of incubation at 37°C and 40 h at 15°C. The experiment was conducted with three independent biological replicates.

Biofilm assay. This was done according to the method described by O'Toole (50), with slight modifications. Each strain was grown overnight in LB with agitation at 37°C, and then 1 ml of the overnight culture was centrifuged at 8,000 × *g* for 6 min at room temperature (23°C) to recover the cells. The cell pellets were washed in 1 ml sterile phosphate-buffered saline (PBS) (pH 7.3) (Oxoid, United Kingdom) and resuspended in 1 ml

sterile PBS. The culture was diluted 1:1,000 into the respective media—LB broth and succinate minimal medium (SMM)—after which 200 μl was added into a round-bottomed well in 96-well microtiter plates. Each strain was inoculated in eight replicate wells, and the assay was repeated at least twice for each condition. Strains were assigned at random to the wells of a 96-well microtiter plate. Plates were incubated statically at 37°C for 84 h and 15°C for 138 h. After incubation, the optical density at 600 nm (OD_{600}) was determined, and the medium was removed from each well carefully with a pipette so as not to disrupt the biofilm. Each well was washed three times with 200 μl PBS by inverting the plates in order to remove all nonadhering bacterial cells, after which the plates were dried at room temperature (23°C) for 45 min. Biofilm was stained by adding 150 μl of 1% (wt/vol) crystal violet solution to each well, and the plates were incubated for 30 min at 37°C. After staining, excess crystal violet was removed from the wells, and the wells were rinsed four times with 200 μl PBS. Destaining was done by adding 160 μl of 95% (vol/vol) ethanol to each well for 30 min, after which the absorbance was determined using a Sunrise microplate absorbance reader (Tecan, Austria).

Survival under starvation at 15°C. Strains were inoculated in 10 ml LB and incubated overnight at 37°C with agitation before washing at 9,000 × *g* for 10 min at room temperature (23°C) in 10 ml PBS twice. The washed cells were resuspended in 15 ml PBS and used as inoculum. One milliliter of the inoculum was added to 15 ml PBS in sterile 50-ml tubes in triplicates and incubated at 15°C with shaking. This temperature was chosen to approximate the mean Irish soil temperature. Aliquot (200-μl) samples were taken at days 0, 7, 14, 21, 28, and 35 for enumerating culturable cells. Samples were serially diluted in PBS, and 10 μl was spot-plated in triplicates onto LB agar and incubated overnight at 37°C.

Soil survival assay. This was done according to the method described by Ma et al. (51), with some modifications. Two types of silty loam soils were used for this analysis: soil A (sand, 47%; silt, 52%; clay, <1%) (pH 7.2) (with total organic carbon, 16.53% of dry solids; total nitrogen, 1.56% of dry solids; C-to-N ratio, 10.6; and organic matter, 31.3%) was collected from Ballyvaughan (53°07′15.6″N 9°09′24.8″W) in the west of Ireland, and soil B (sand, 43%; silt, 53.9%; clay, 3.1%) (pH 5.19) (with total organic carbon, 4.7%; total nitrogen, 0.35%; C-to-N ratio, 13.43; and organic matter, 9.1%) was collected from Kilfergus (53°07′15.6″N 9°09′24.8″W) in the midwest of Ireland. The soils were sieved with a 2-mm sieve and kept in a sealed bag with airspace at a constant temperature (15°C) until used. One colony each of the BW25113 and BW25113Δ*rpoS* strains was inoculated into 10 ml LB and incubated overnight at 37°C with agitation. The overnight cultures were harvested by centrifugation at 9,000 × *g* for 10 min at room temperature (23°C), washed twice with sterile PBS, and resuspended in PBS to give an OD_{600} equivalent to 2×10^8 CFU/ml, which served as the inoculum. Then, 50 μl of the inoculum was added into 1 g of soil A in a series of 15-ml sterile tubes inverted 10 times by hand, slightly capped to allow air exchange, and incubated at 15°C. As a control, 50 μl of sterile PBS was added to 1 g of soil A. The experiment was set up in triplicate. Inoculated soils were destructively sampled (i.e., PBS was added and could not be reused at another time point) on days 0, 1, 2, 7, 14, 21, 28, and 35 to determine survival of the wild-type and *rpoS* mutant strains. For cell recovery, 2 ml of PBS was added to each tube and mixed by inverting three times followed by vortexing for 2 × 20 s. The resulting soil slurry was allowed to settle for 2 min, and 20 μl was collected from the supernatant and serially diluted. Two minutes was adopted as the standard time for this protocol, because there was no significant difference in the recoverable cell numbers when soil slurry was allowed to settle for a longer period (10 min). A total of 10 μl of all dilutions was plated in triplicate on MacConkey agar and incubated overnight at 37°C. The soil used had no detectable background levels of coliforms or *E. coli*. Preliminary experiments showed an average of 91% to 102% of the added *E. coli* was recovered at 10 min after inoculation into soil A, while recovery was 91% to 97% in soil B. A subsequent soil assay was arranged with all nine test strains and sampled on days 0, 7, 14, 28, 42, 55, 84, 122, 154, and 230. The wild-type COB583 strain and its corresponding constructed *rpoS* deletion mutant strain (COB583Δ*rpoS*) were subjected to the soil survival assay with the silty loam soils A and B to investigate the effect of RpoS on soil survival in a soil-persistent strain.

TABLE 2 List of primers used in this study

Primer	Sequence (5′–3′)
COB740R	TACGTATTCTGAGTCTTCGG
COB742F	ATGATTGACCTGCCTCTG
COB743F	GGTATTGCGATTTCTATTCC
COB746F	CGTAGCAATCCTGACAAC
COB747R	GAATTTGATGAGAACGGAG
COB784F	CCATAATCACCATCTTCACG
COB785R	GATAAGCCAGTTGATGACG
COB788F	CACTTCCATGCGGTAGATG
COB789R	CCTATGCGTTCATCATCTTG
COB794F	CCATAATCACCATCTTCACG
COB795F	CACCTCTTCGCTGATTTTC
COB796R	ACCAGGCTTTTGCTTGAATG
COB797F	GTAACAACATCTTCTTCGTCAC
COB798R	GGATAAGCCAGTTGATGATTTC

PCR amplification and sequencing. Whole-colony PCR targeting *rpoS* and its flanking genes was carried out on the soil-persistent strains. Single colonies were picked and resuspended in 500 μl of sterile nuclease-free water (Ambion, USA). A 1-μl aliquot was then transferred to the PCR reagent mix. The primers used for amplification are listed in Table 2. PCR was performed with high-fidelity Velocity DNA polymerase (Bioline, Inc., USA). PCR mixture (50 μl) consisted of 10 μl of 5× hi-fi buffer (containing 10 mM Mg^{2+}), 5 μl of 10 μM deoxynucleoside triphosphate (dNTP) mix, 1.5 μl of dimethyl sulfoxide (Bioline, Inc., USA), 29.5 μl of nuclease-free H_2O, 2 U (1 μl) of Velocity DNA polymerase (Bioline, Inc., USA), 1 μl of template DNA, and 1 μl of each primer (25 μM). The PCR was performed with a Primus DNA cycler (MWG-Biotech, Inc.) with the following steps: initial denaturation step at 98°C for 2 min, 30 cycles of 30 s at 98°C, 30 s at 54°C, and 3 min at 72°C, and a terminal extension step at 72°C for 7 min. The amplified PCR products were examined by agarose electrophoresis on 1% gels, purified using the GenElute PCR cleanup kit (Sigma-Aldrich, USA), and visualized on a 1% agarose gel for quantification prior to sequencing. Samples were sequenced by Source Bioscience (Dublin, Ireland), while assembly and analysis were carried out using DNABaser version 4 (Heracle BioSoft, USA). Sequencing was performed on both strands of the PCR product.

Phylogenetic analysis. Nucleotide sequences of *E. coli* SE11, SE15, and BW25113 were retrieved from the National Center for Biotechnology Information (NCBI) database with GenBank accession numbers AP009240.1, AP009378.1 and CP009273.1, respectively. Nucleotide sequences of *rpoS* were translated, and the resulting amino acid sequences were analyzed. Sequences were aligned using Clustal Omega (52), and a phylogenetic tree was generated using the maximum likelihood method based on the Kimura 2-parameter model (53) with bootstrap analysis (1,000 iterations) using MEGA6 (54).

Acid survival at 37°C. Cultures of bacteria were grown to the stationary phase at 37°C in LB medium with agitation. The pH of these cultures was lowered to a pH of 2.5 with 3 M HCl. Samples were taken at 20, 40, and 60 min and serially diluted in PBS. Aliquots of 10 μl of the serial dilutions of the samples were plated in triplicate onto LB agar and incubated overnight at 37°C. Colonies were counted to enumerate the culturable cells.

GABase assay. Intracellular γ-aminobutyrate (GABA) and extracellular GABA (GABAi and GABAe, respectively) were measured as previously described (55). Briefly, strains were grown to the stationary phase at 37°C with agitation in LB medium. Prior to the GABA measurements, the pH of the cultures was lowered to 4.0 with 3 M HCl. Extractions were made after 1 h of acid treatment. Non-HCl-treated cultures were used as negative controls. GABase from *Pseudomonas fluorescens* (Sigma-Aldrich, Stein-

heim, Germany) was used in the enzymatic assay, and the OD_{340} was measured using the Sunrise microplate absorbance reader (Tecan, Austria).

Glycogen accumulation test. Levels of glycogen accumulated in the strains were determined by iodine staining, as described previously (26) with some modifications. Strains were streaked onto LB agar and incubated overnight at 37°C and then left at 4°C for 48 h, after which they were flooded with 0.05 M iodine solution (Sigma-Aldrich, USA). The glycogen level is indicated by the intensity of brown coloration and is an indirect measure of the level of RpoS. Images were captured on an HP Scanjet 5400c at 600 dpi.

Western blot for RpoS. As a direct measure of RpoS levels, we detected RpoS protein in the test strains by Western blotting. Stationary-phase cells were inoculated into 25 ml LB starting at an OD_{600} of 0.05 and incubated overnight (~16 h) at 37°C and 36 h at 15°C with continuous shaking. Then, 1 ml of culture was taken and centrifuged at 12,000 × *g* for 10 min. Each cell pellet was resuspended in 100 μl of BugBuster cell lysis reagent (Novagen, USA) supplemented with 1% (vol/vol) DNase I (Thermo Scientific, USA), 1% (vol/vol) Halt protease inhibitor cocktail (Thermo Scientific, USA), and 1% (wt/vol) lysozyme (Sigma-Aldrich, USA). Each cell suspension was then incubated at room temperature (23°C) for 20 min with agitation. Cell lysates were centrifuged at 16,000 × *g* for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). An equal amount of each protein (25 μg) was resolved on 10% SDS-PAGE at 100 V for 1.5 h at 4°C. After electrophoresis, proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane using a semidry system (Jencons, United Kingdom) at 3 V for 1 h. A blocking step with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST) was performed, and the membrane was incubated with a 1,000-fold-diluted mouse monoclonal anti-RpoS antibody (Santa Cruz). Blots were washed in TBST three times for 10 min each and incubated in 3,000-fold diluted peroxidase conjugated anti-mouse IgG (Pierce). The ECL Prime Western blotting detection reagent (GE Healthcare) was used to detect the RpoS bands, as recommended by the manufacturer, with exposure to X-ray films.

RpoS-dependent GFP expression. RpoS activity was determined by the ability of the strains to transcribe the *gadX* promoters in the reporter fusion (P_{gadX}) indicated by green fluorescence. Overnight culture of all strains carrying P_{gadX} was prepared in LBKan broth and incubated at 37°C and 15°C. Stationary-phase cells were inoculated into 25 ml LBKan at a starting OD_{600} of 0.05 and incubated with continuous shaking at 37°C and 15°C. To determine the induction of fluorescence, samples were taken at the stationary phase (17 h at 37°C and 36 h at 15°C), fixed with an ethanol-methanol (1:1) solution, and resuspended in PBS, and 2 μl was placed on a slide and imaged with Leica DMI3000 B microscope. Green fluorescent protein (GFP) was detected by Western blotting according to the method described above, but with rabbit polyclonal GFP antibody (Santa Cruz; SC-8334) diluted 5,000-fold in TBST and 20,000-fold-diluted peroxidase-conjugated anti-rabbit IgG (Santa Cruz).

Statistical analysis. Analysis of variance (ANOVA) was performed to investigate the differences in motility, biofilm formation, GABA levels, acid survival, and soil survival at the specified time points using SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL). All assumptions of the test were met. Statistical comparisons among the means were made using the Duncan multiple range test at a 5% probability level.

Accession number(s). The accession numbers KU948321 to KU948325 were assigned by GenBank for *rpoS* nucleotide sequences COB583 to COB587, respectively.

RESULTS

Soil-persistent *E. coli* displayed heterogeneous phenotypes. All strains were motile to a similar extent at 37°C (Fig. 1A), with the exception of BW25113, which showed poor motility at this temperature. In contrast, motility was found to be more variable at 15°C, with three of the wild isolates (COB583, COB585, and SE11) displaying significantly greater motility than the other strains (Fig. 1B). Very low levels of biofilm were detected for all strains at 37°C in both LB broth and a minimal medium with succinate as the sole carbon source. Two of the five soil isolates (COB583 and COB585) produced significant biofilm at 15°C in both media tested (Fig. 1C), and SE15 produced high levels of biofilm (OD_{595} of 1.77) in the minimal medium, while the others produced very little biofilm. Together, these data demonstrate the phenotypic diversity of *E. coli* soil residents, consistent with the fact that these isolates belong to different phylogenetic groups.

RpoS is required for survival in soil at 15°C. All the soil-persistent and commensal isolates of *E. coli* were found to survive well in a nutrient-poor environment at 15°C over 35 h, with the exception of SE11, which showed approximately 2-log reduction in culturable cell numbers (Fig. 2A). To investigate whether RpoS was required for survival in soil, the survivals of BW25113 and its *rpoS* mutant strain (BW25113Δ*rpoS*) were compared following the inoculation of a live soil sample with 10^6 CFU · g^{-1}. The parent strain lost less than a 1-log cycle of viability, while the BW25113Δ*rpoS* strain was significantly ($P = 0.0004$) impaired in its soil survival, dropping to less than 10^3 CFU · g^{-1} after 35 days (Fig. 2B). Soil-persistent and commensal strains had similar survival patterns which were not significantly different ($P > 0.05$) after 230 days in live soil (Fig. 2C). The laboratory strain BW25113 also survived well under these conditions in soil, but the BW25113Δ*rpoS* strain was again severely impaired in its survival, with no culturable survivors detected after 42 days. In order to directly test the role that RpoS plays in the soil-persistent strains, an *rpoS* deletion was constructed in COB583, and the survival of this mutant (strain COB583Δ*rpoS*) was compared to the parental strain in silty loam soils A and B. In both cases, loss of *rpoS* was associated with a faster loss of viability in the soil, and a stronger effect was observed in soil B (Fig. 2D). The effect of the *rpoS* deletion on soil survival was similar in both the BW25113 and COB583 backgrounds for silty loam soil A (Fig. 2B compared to Fig. 2D).

The *rpoS* locus is conserved in soil-persistent *E. coli*. Although there were some sequence differences between the isolates, all of the soil-persistent strains were found to have an intact *rpoS* ORF (Table 3). Nucleotide substitution C→G at position 97 was present in all of the soil-persistent and commensal strains, and this resulted in a corresponding amino acid change from glutamine (Q) to glutamic acid (E) at codon 33. Although there were other nucleotide changes in the test strains, the change at codon 33 was the only nucleotide change that resulted in an amino acid change; the others were silent mutations. The sequence of *rpoS* in COB585 diverged significantly from the other strains, which was reflected in the phylogenetic tree generated when these sequences were compared to other sequenced *E. coli* strains (Fig. 3A). The region downstream from the *rpoS* ORF was conserved in three soil-persistent strains (COB584, COB586, and COB587) and in one of the commensal strains (SE11), but the locus had a different gene order in each of the other strains (Fig. 3B). The regions carrying the known *rpoS* promoter sequences, located in the upstream *nlpD* ORF and in the intergenic region, were compared and were found to be conserved in all soil-persistent strains (Fig. 3B). These results confirm that the *rpoS* gene and regulatory elements are conserved in the soil-persistent strains but that differences exist in the genetic structure of this chromosomal locus, highlighting the genetic diversity that exists within this collection of soil isolates.

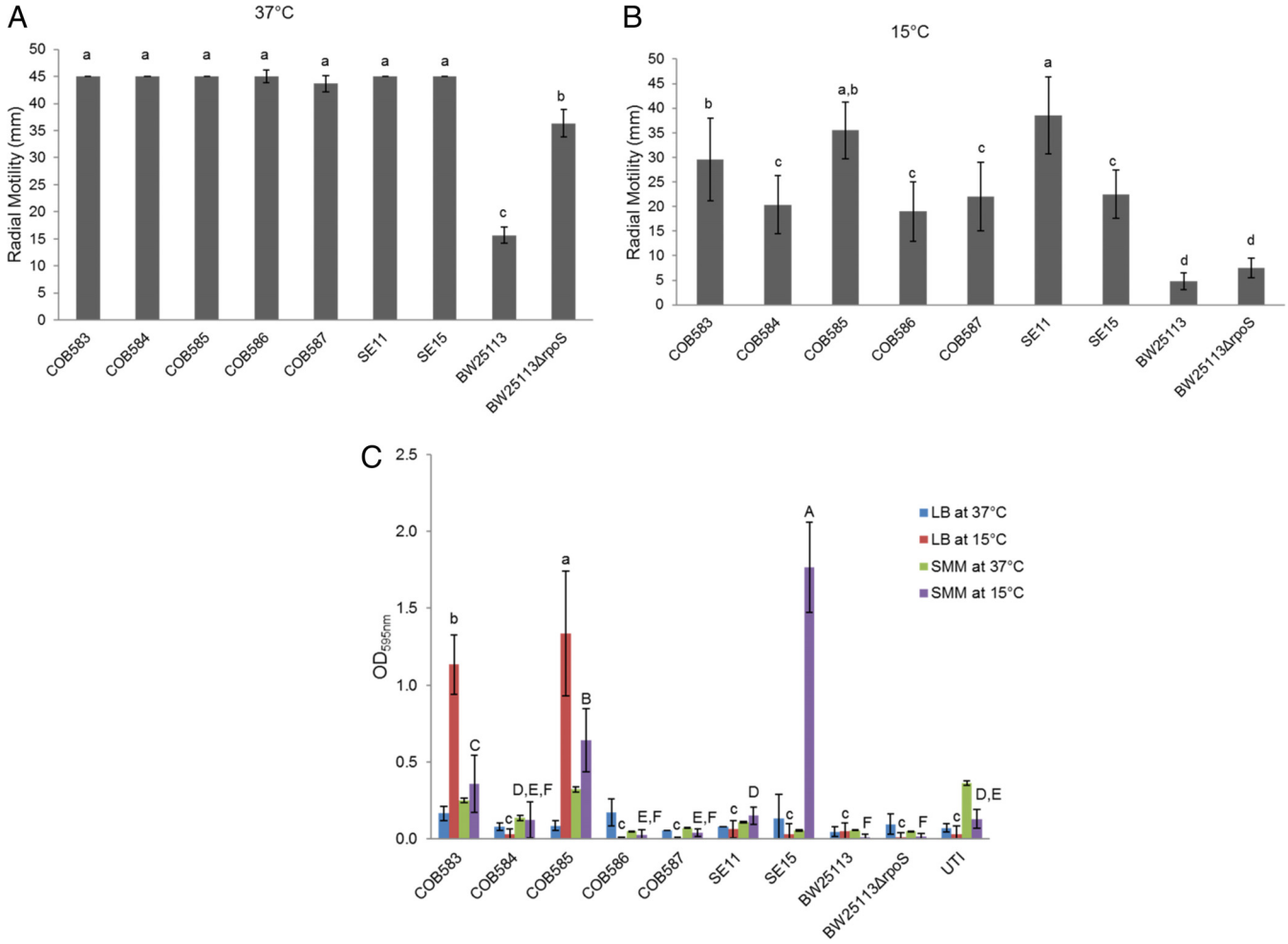

FIG 1 Motility of *E. coli* strains on 0.25% (wt/vol) LB agar at 37°C for 16 h (A) and at 15°C for 40 h (B), and biofilm formation in LB broth and SMM (C). All soil-persistent and commensal strains were significantly ($P < 0.0001$) more motile than the laboratory strain BW25113 at 15°C and 37°C. The wild-type BW25113 strain was significantly ($P < 0.0003$) less motile than the BW25113Δ*rpoS* strain at 37°C but not at 15°C. Biofilm was produced in rich and minimal media, with more biofilm produced at 15°C than at 37°C. UTI, urinary tract infection-associated *E. coli* isolate. Error bars represent standard deviations from three independent replicates. Data with similar lowercase or uppercase letters are not significantly different ($P > 0.05$).

Phenotypes under the control of RpoS are retained in soil-persistent *E. coli*. Acid tolerance and GABA levels following acidification were measured for each of the soil-persistent and commensal isolates. Both acid tolerance and GABA production were confirmed to be under RpoS control, since the BW25113Δ*rpoS* mutant strain was exquisitely acid sensitive (Fig. 4A) and produced only very low levels of GABA in response to acidification compared to the parental control strain BW25113 (Fig. 4B). With the exception of SE11, all of the other strains survived a pH of 2.5 for 1 h (Fig. 4A). Furthermore, they all produced significant quantities of GABA both intracellularly and extracellularly in response to acidification of the culture media (pH 4.0), although there were some strain differences in the amounts produced (Fig. 4B). The accumulation of intracellular glycogen in *E. coli* is another trait known to be under RpoS control (56). An iodine-based staining assay was used to determine if the soil-persistent and reference strains could accumulate glycogen. The BW25113Δ*rpoS* mutant strain was found to stain negative (yellow-white) for glycogen, while all other strains gave a glycogen-positive stain (red-brown) (Fig. 4C). Together, these results indicated that traits under the control of RpoS were retained during long-term soil adaptation, suggesting the presence of a general stress response in these isolates.

RpoS is present and functional in long-term soil-persistent *E. coli*. While the above data suggested the presence of an active RpoS-mediated stress response in the soil-persistent strains, it did not confirm that RpoS was expressed and active. The expression of RpoS was investigated in all strains at both 15°C (mimicking summer-time soil temperature in the east of Ireland, where isolates were collected) and 37°C using Western blot analyses with anti-RpoS monoclonal antibodies. A 37-kDa band corresponding to full-length RpoS was detected in all strains, with the exception of the BW25113Δ*rpoS* mutant strain, which confirmed the specificity of the antibodies. Strikingly, there was a large increase in RpoS levels at 15°C compared to 37°C in all strains (Fig. 5). RpoS degradation products of similar sizes were also detected in most strains at 15°C (but not in the BW25113Δ*rpoS* mutant strain), except for COB587 and BW25113. To assess the activity of RpoS, a plasmid-based GFP reporter (*gadX*::*gfp*) was transformed into all strains and used to record the transcription from the *gadX*

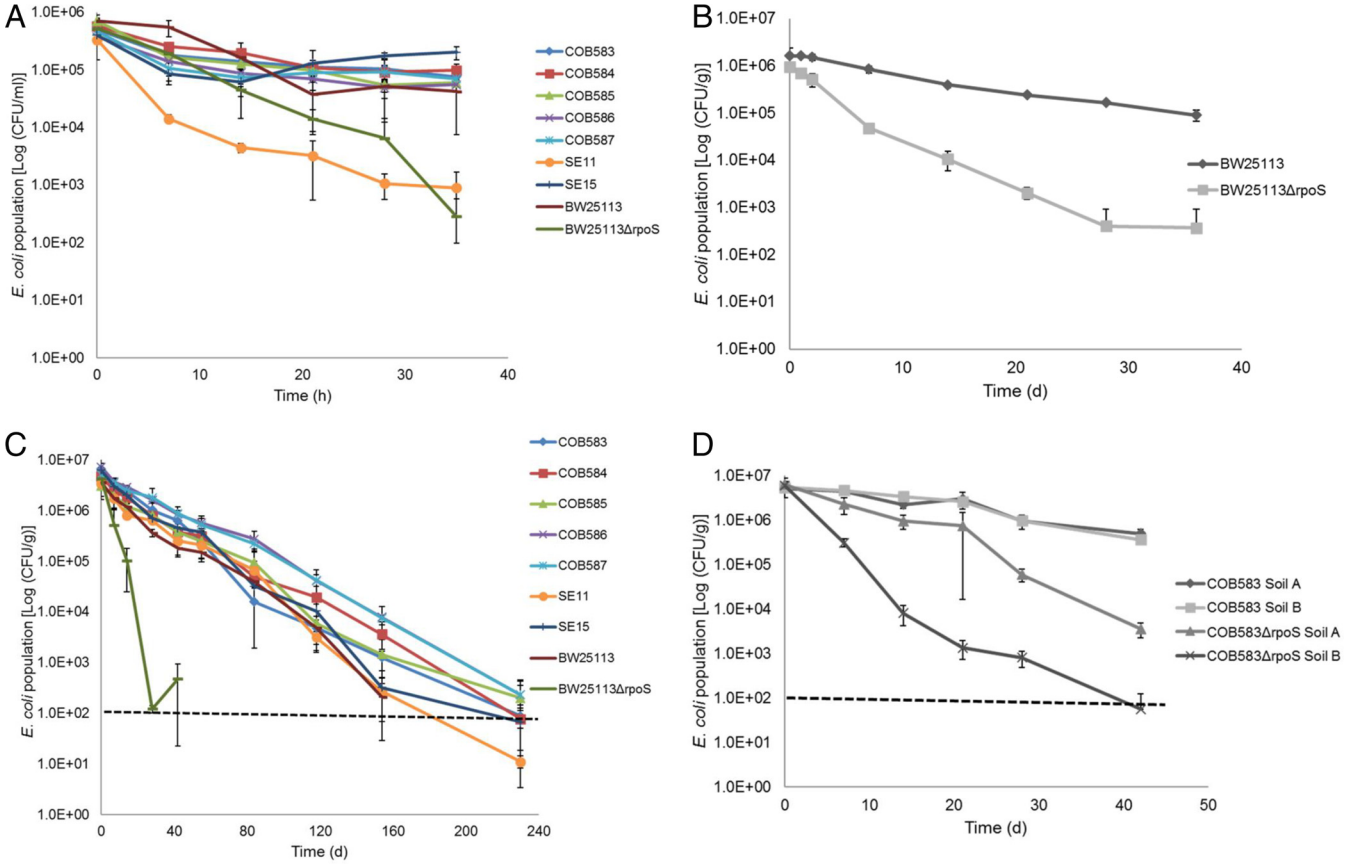

FIG 2 Survival of *E. coli* under various environmental conditions at 15°C. (A) All of the soil-persistent, commensal strains and *E. coli* BW25113 survived significantly ($P < 0.0001$) better in PBS than the BW25113Δ*rpoS* strain did, except SE11, after 35 h. (B) The *E. coli* BW25113 parental strain survived significantly better than the BW25113Δ*rpoS* strain in soil. Time is shown in days (d) on the *x* axis. (C) Long-term soil survival experiment showed that RpoS is required for survival in silty loam soil A, as there were no detectable BW25113Δ*rpoS* strains after 42 days in soil, whereas all other test strains survived long term. (D) The *E. coli* COB583 parental strain survived significantly better ($P = 0.002$) than the COB583Δ*rpoS* strain in silty loam soils A and B. Error bars represent standard deviations from three independent replicates. Dashed lines represent the detection limit of the soil survival assay.

promoter region, which is known to be highly RpoS dependent and has been used by others as reporter of RpoS activity (57–59). A microscopic analysis of stationary-phase cultures grown at 37°C revealed that all strains showed significant GFP expression, with the exception of the BW25113Δ*rpoS* mutant strain, which showed no detectable fluorescence. The fluorescence levels detected in the strains were not identical; COB585 reproducibly ($n = 2$; with at least 3 fields each time) had lower levels of fluorescence under these conditions. At 15°C, however, the fluorescence levels detected for all strains were greatly reduced compared to levels detected at 37°C, with COB585 again showing the least fluorescence (Fig. 6A). One possibility was that decreased fluorescence might be caused by failure of the GFP protein to fold or mature properly (60) at 15°C. To test whether the levels of GFP were indeed reduced at 15°C, the levels were assessed by Western analyses using anti-GFP antibodies. The results showed that there was a strong correlation between the GFP levels and the levels of fluorescence detected by microscopy, suggesting that transcription of the *gadX*::*gfp* reporter was reduced at 15°C (Fig. 6B). RpoS activity, based on the reporter activity, was indeed decreased at 15°C despite the increased levels of the RpoS protein detected at this temperature. Taken together, these data confirm that RpoS is both present and active in the soil-persistent and commensal isolates of *E. coli*.

DISCUSSION

Many studies in recent years have isolated and described *E. coli* from nonhost environments (7, 12, 16), but not much is known on the mechanisms of adaptation of *E. coli* to the soil. In this study, we characterized the phenotypic properties of some long-term soil-persistent *E. coli*, established that the general stress response regulated by RpoS is conserved in them, and showed that RpoS is required for long-term survival in soil. This collection of long-term soil-persistent isolates represents an opportunity to understand the role that stress responses play in allowing *E. coli* to thrive in an environment outside the host, where environmental conditions, nutrient availability, and competing microorganisms are different from those present in the gastrointestinal tract.

All soil-persistent strains are motile at 37°C and 15°C, suggesting that motility is likely to be important for life in the soil environment. Though the individual strains showed different levels of motility (Fig. 1B), there was no significant difference ($P > 0.05$) in motility when soil-persistent and commensal strains were compared at 37°C and 15°C. In an environment where nutrients are

TABLE 3 Mutations within the *rpoS* gene of soil-persistent and commensal *E. coli* strains compared to MG1655

Strain	Nucleotide change		Corresponding amino acid change (codon position)
	Position	Change	
COB583	97	C→G	Gln (Q) to Glu (E) (33)
	942	C→T	—[a]
COB584	97	C→G	Gln (Q) to Glu (E) (33)
COB585	42	T→C	—
	81	A→G	—
	84	G→A	—
	93	A→T	—
	97	C→G	Gln (Q) to Glu (E) (33)
	123	C→T	—
	132	A→G	—
	144	G→A	—
	147	A→G	—
	819	G→A	—
	927	C→T	—
	972	C→A	—
	990	C→A	—
COB586	97	C→G	Gln (Q) to Glu (E) (33)
COB587	97	C→G	Gln (Q) to Glu (E) (33)
SE11	97	C→G	Gln (Q) to Glu (E) (33)
SE15	97	C→G	Gln (Q) to Glu (E) (33)
	163	T→C	—
	357	T→C	—
	462	T→C	—
	573	T→C	—
	699	G→A	—
	732	C→T	—
	990	G→A	—
BW25113	No change	No change	—

[a] —, silent.

spatially and temporally heterogeneous, such as the soil, active motility could also be important in terms of a strategy for growth and survival. Brennan et al. (61) reported some evidence of active movement of *E. coli* within soil profiles. Furthermore, motility is important for initiating biofilm formation, because flagella help the initial attachment and movement along surfaces (62, 63). We determined biofilm formation, which is known to be RpoS dependent (64), and showed that the strains produced various amounts of biofilm. COB583 and COB585 produced the most biofilm in rich medium (LB), while the commensal strain SE15 produced the highest biofilm in minimal medium (i.e., SMM) (Fig. 1C). We initially hypothesized that high biofilm formation may be an important phenotype for survival in the soil; however, our results showed that only two soil-persistent isolates produced high levels of biofilm under the conditions investigated, suggesting that the ability to form biofilm may not be an essential phenotype for long-term soil survival. The result of the heterogeneity in biofilm production observed in our study is consistent with the study of Skyberg et al. (65), which showed that biofilm production is strain specific and not significantly influenced by phylogenetic group. Similar to previous studies that have shown that biofilm formation is higher at lower temperatures (48, 66) and that biofilm-related genes are upregulated at 23°C compared to 37°C (67), we demonstrate that *E. coli* strains can produce high levels of biofilm at 15°C.

The survival data suggested that the general stress response was conserved in the soil-persistent strains, but we sought to investigate this further by sequencing the *rpoS* locus in each of the five soil isolates and comparing this to the *rpoS* locus from other sequenced *E. coli* genomes. *E. coli* strains have been shown to accumulate mutations in the *rpoS* gene when grown long term in the laboratory, in batch culture, in stab cultures, in chemostats run with poor carbon sources, and even in natural commensal, pathogenic, and environmental isolates from fermented sausage, human feces and urine, beaches, wastewater effluent, and animals (7, 26–34). When we compared the *rpoS* locus in the test strains with *E. coli* K-12 MG1655, various nucleotide substitution mutations were observed, but none of these resulted in a premature stop codon (Table 3). This shows that *rpoS* is conserved in the soil-persistent and commensal strains. *E. coli* BW25113 had the same *rpoS* sequence as *E. coli* K-12 MG1655, as expected, since it is a derivative of the K-12 strain. We analyzed *rpoS* of sequenced *E. coli* strains in the NCBI (http://www.ncbi.nlm.nih.gov/) database and observed that this glutamine (Q) at codon 33 was conserved only in K-12-derived laboratory-adapted strains (data not shown). Atlung et al. (28) had previously reported a similar observation when comparing K-12 strains with six non-K-12 strains, and they proposed that GAG (glutamic acid at codon 33) was present in the *E. coli* common ancestor and that it evolved to TAG (STOP) in the process of laboratory evolution and then mutated into CAG (glutamine at codon 33). Polymorphism at codon 33 has been reported among *E. coli* strains in the literature (33, 34, 38), but most of these studies used the original K-12 sequence (33Q) as their wild-type reference sequence, when the polymorphism occurred only in the K-12 lineage. It has been shown that there is no difference in RpoS activity with either glutamic acid (E) or glutamine (Q) at codon 33 (28), suggesting that this residue is not critical for the functioning of RpoS.

Our data show that the soil environment does not preferentially select for *rpoS* mutations, and this is consistent with recent studies reporting that loss-of-function RpoS mutations are rare in a large collection of natural isolates of commensal, pathogenic, and environmental *E. coli* (32, 68). Bleibtreu et al. (68) reported no variation in the amino acid sequence of RpoS in the *E. coli* strains which had minimal laboratory handling before being sequenced and showed that storage and successive transfers resulted in the *rpoS* mutations. Spira et al. (69) also reported that transfer of *E. coli* on LB stabs between laboratories led to mutations in *rpoS*. Retention of an intact stress response may be important for *E. coli* in soil, since it is a dynamic environment where *E. coli* may encounter multiple stresses. *E. coli* must overcome stresses such as UV radiation (if close to soil surface), low nutrients, low temperature, desiccation, competition, predation, and more to thrive in the soil. Furthermore, using phylogenetic analysis, we showed that RpoS is highly conserved in *E. coli*. The clustering of *rpoS* in the *E. coli* strains reflects phylogenetic diversity in the long-term soil-persistent *E. coli* strains we used for this study (Fig. 3A). It has been recently shown that the phylogenetic grouping based on *rpoS* is highly consistent with phylogenetic clustering based on multilocus sequence typing (MLST), thus suggesting that *rpoS* is a good indicator of evolutionary history of *E. coli* strains (68). On this basis, strain COB585 is the most divergent of the soil-persistent

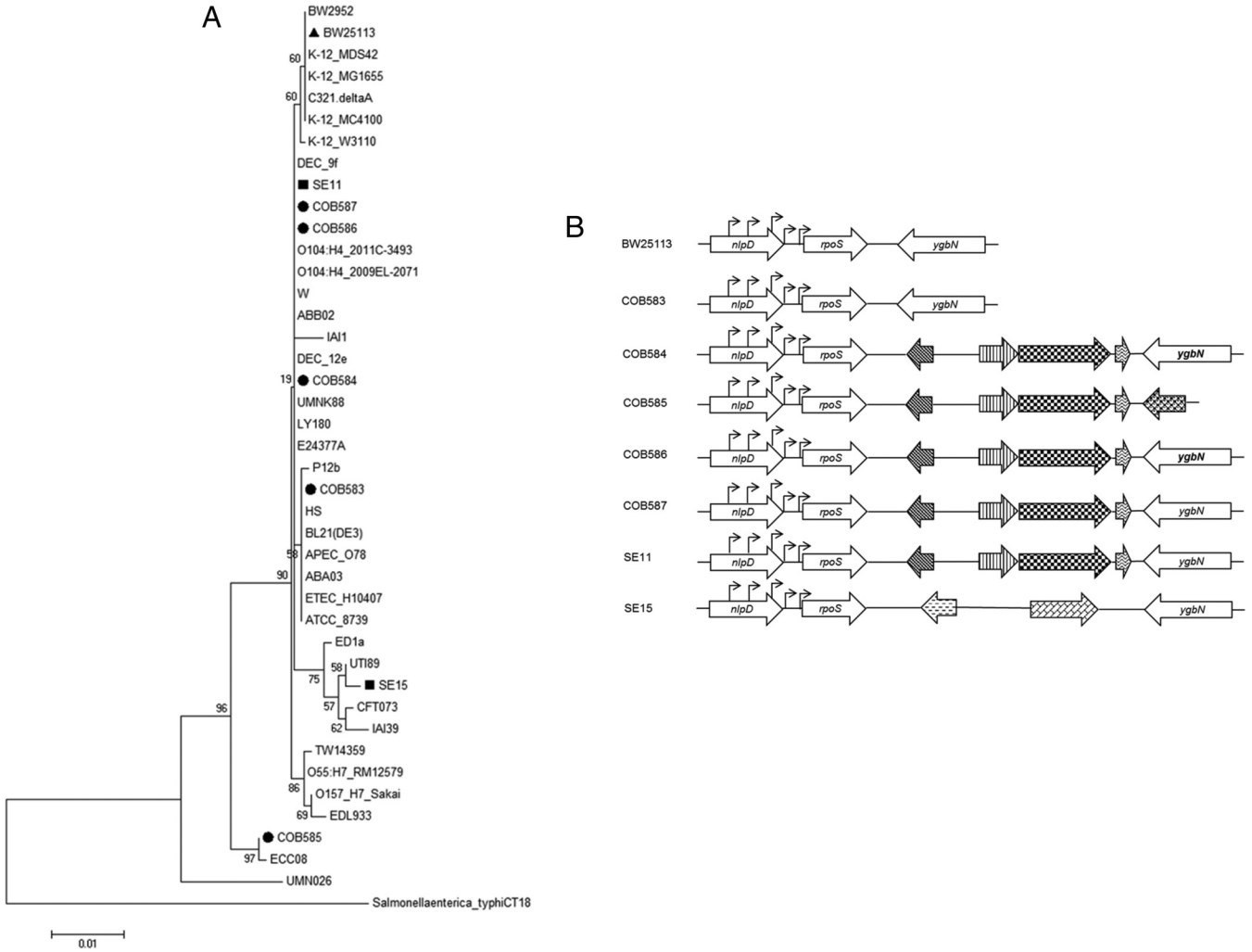

FIG 3 (A) Phylogenetic tree of *rpoS* performed using the maximum likelihood method based on the Kimura 2-parameter model with bootstrap analysis (1,000 iterations) using MEGA6 showed that *rpoS* in soil-persistent strains is similar to previously known *E. coli* strains. (B) Gene outline of *rpoS* and its flanking genes in the soil-persistent and commensal strains shows four distinct patterns. PCR and sequencing of genes flanking *rpoS* revealed insertion of open reading frames (ORFs) between *rpoS* and inner membrane permease (*ygbN*). Pattern I (COB583), which is similar to the reference strain (BW25113), shows *rpoS* directly flanked by murein hydrolase activator (*nlpD*) and *ygbN*. Pattern II (COB584, COB586, COB587, and SE11) shows insertion of 4 ORFs between *rpoS* and *ygbN*. Pattern III (COB585) shows insertion of 5 ORFs after *rpoS*, with *ygbN* absent, while pattern IV (SE15) had insertion of 2 ORFs between *rpoS* and *ygbN*. The region between *nlpD* (which carried *rpoS* promoters) and *rpoS* was conserved in all the strains. Block arrows with similar patterns indicate the same ORF, while arrowheads represent *rpoS* promoter sites.

strains used in study, and it is most closely related to ECC08, which is a strain collected from a beach water sample at Bayfront Park Beach, Hamilton, Canada (7).

Having established that the *rpoS* gene is conserved in the long-term soil-persistent strains, it was important to measure the RpoS level and its activity, since its role in the general stress response is complex, being regulated at multiple levels (reviewed in reference 70). We showed that all the strains with an intact *rpoS* gene produced detectable RpoS protein (Fig. 5). Besides the 37-kDa RpoS protein observed in the RpoS-positive strains, there were other bands detected in all of the strains which were not found in the BW25113Δ*rpoS* strain. These are likely to be degradation products specific to RpoS, as one of the major regulatory mechanisms of RpoS level is proteolysis by ClpXP (71). All of the soil-persistent strains, the commensal strains, and BW25113 displayed known RpoS-dependent phenotypes that were absent in the BW25113Δ*rpoS* mutant strain, albeit with some small differences, suggesting that RpoS is active in these strains (Fig. 4). For example, RpoS plays an important role in acid tolerance in *E. coli* through its influence on the glutamate decarboxylase (GAD) system; specifically RpoS controls the transcription of two operons encoding components of the GAD system (*gadA* and *gadBC*) and controls a positive regulator of this system, GadE (reviewed in reference 72). GABA was produced by all strains with intact RpoS in order to overcome acid stress. *E. coli* SE11, a commensal strain, had an attenuated response to extreme acidity at 37°C (Fig. 4A), as it survived a pH of 2.5 for only 20 min. Interestingly, the RpoS level in SE11 at 37°C was lowest among the strains (Fig. 5), and it is possible that this contributed to the attenuated acid stress response and reduced survival under starvation at 37°C (data not shown). However, this was not the case at 15°C, where SE11 produced high levels of RpoS and survived extreme acidity for 1 h

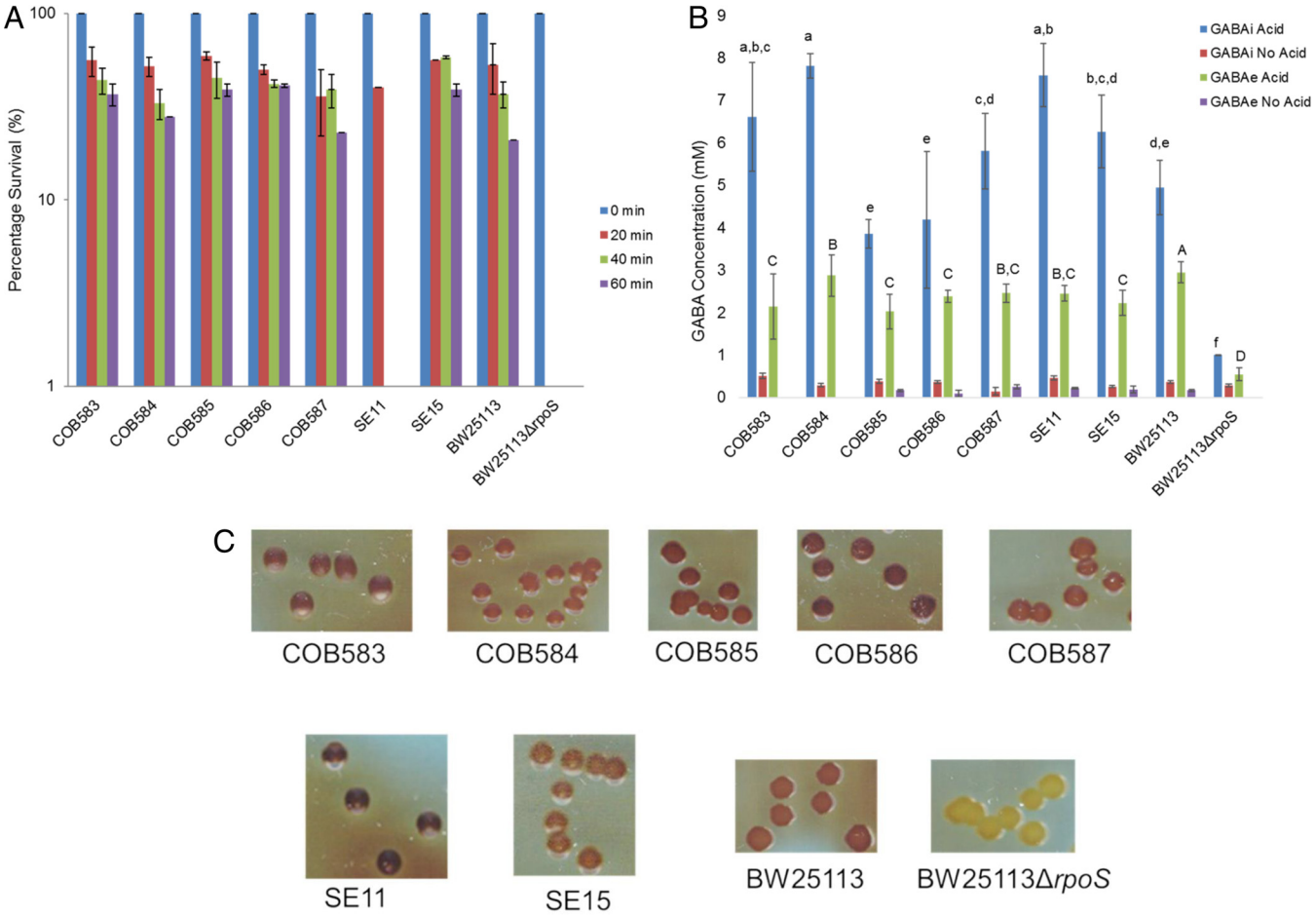

FIG 4 RpoS-dependent phenotypes show soil-persistent strains have functional RpoS. All *E. coli* strains survived significantly better than the BW25113Δ*rpoS* strain at pH 2.5 (A), produced significantly higher ($P < 0.0001$) amounts of gamma-aminobutyric acid (GABA) in response to acid stress (pH 4) compared to the BW25113Δ*rpoS* strain (B), and turned brown to dark brown upon iodine staining, showing the accumulation of glycogen compared to the BW25113Δ*rpoS* strain (C). Error bars represent standard deviations from three independent replicates. Data with similar lowercase or uppercase letters are not significantly different ($P > 0.05$).

(data not shown), similar to other soil-persistent and commensal strains, but still survived rather poorly in PBS compared to the other RpoS-positive strains (Fig. 2A). The reason for reduced stress response in *E. coli* SE11 under these conditions is not clear at present.

RpoS activity as determined by the *gadX* reporter fusions shows that RpoS was active in all the long-term soil-persistent strains at 15°C and 37°C. However, expression of GFP reporters at the stationary phase was higher at 37°C than at 15°C (Fig. 5B). This low GFP expression correlated with lower fluorescence at 15°C (Fig. 6A). This is contrary to the fact that RpoS levels, as determined by Western blotting, were higher at 15°C (Fig. 5A).

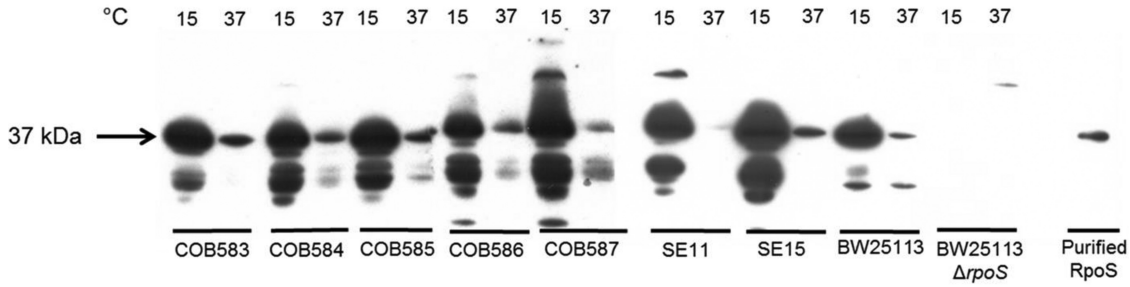

FIG 5 A higher level of RpoS is expressed in the stationary phase at 15°C than at 37°C in *E. coli*. Protein preparations were obtained from *E. coli* strains grown in LB broth with agitation to the stationary phase at 15°C and 37°C. The same amount of protein (25 μg) from each strain at both temperatures was run on SDS-PAGE, and RpoS was detected by immunoblotting. Purified RpoS protein was used as the control. More RpoS was produced at 15°C than at 37°C in all of the strains, whereas RpoS was not detected in the BW25113Δ*rpoS* strain at either 15°C or 37°C. The image shown is representative of three independent replicates.

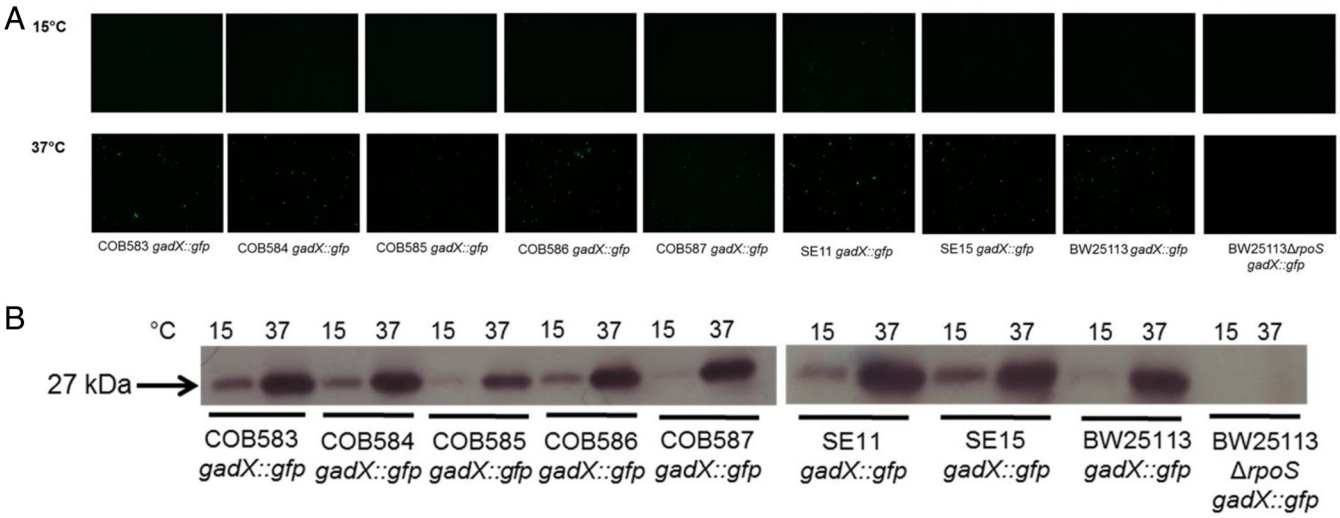

FIG 6 RpoS-dependent GFP expression of *gadX*::*gfp* promoter fusions in the stationary phase at 15°C and 37°C by fluorescence microscopy (A) and Western blotting using anti-GFP antibody (B). *E. coli* strains containing the reporter fusion were cultured in LB broth supplemented with kanamycin (50 μg/ml) with agitation. Samples were taken at stationary phase (17 h at 37°C and 36 h at 15°C), fixed with ethanol-methanol (1:1) solution, and resuspended in PBS, and 2 μl was placed on a slide and imaged with a Leica DMI3000 B microscope. RpoS activity indicated by fluorescence was higher at 37°C than at 15°C (A), and this correlated with GFP detection by immunoblotting (B). COB585 carrying the reporter fusion had the lowest RpoS activity among strains with intact RpoS. Fluorescence was not detected in the BW25113Δ*rpoS* strain either by microscopy or immunoblotting. Fluorescent images presented are representatives of 2 independent experiments with >3 field captures in each experiment. Western blot image is representative of 3 independent experiments.

For example, *E. coli* SE11, which had low levels of RpoS at 37°C, was active in transcribing the RpoS-dependent promoter and leading to high GFP expression (Fig. 6A). These data show that RpoS protein levels did not always correlate with reporter activity. *E. coli* COB585, the most divergent of the strains, had the lowest RpoS activity among the test strains at 37°C. Similar attenuated RpoS activity was also observed in *E. coli* ECC08, which is closely related to COB585 (Fig. 3A) (7). No RpoS activity was observed in exponential phase at 15°C and 37°C in all of the strains (data not shown). This growth-phase-dependent RpoS activity was also reported by Sledjeski et al. (73) and shows that increasing RpoS levels at low temperature does not necessarily lead to the physiological response observed when cells enter into the stationary phase or when they encounter other environmental stresses. Though *gadX* is strongly RpoS dependent in the stationary phase, it was shown that the RpoS dependence may be reduced or abolished under acid stress (24). It will be interesting to further investigate if RpoS activity reduces when exposed to cold stress and other similar stresses encountered in the environment.

We speculate that low activity of RpoS in the stationary phase at 15°C may be due to the upregulation of factors that inhibit promoter recognition by RpoS (74), increased negative feedback inhibition/autoregulation of RpoS (74), or an increase in induction of anti-sigma factors (75). The response regulator RssB, which is important for regulating intracellular RpoS levels at the exponential phase and under low stress, can also serve as an anti-sigma factor and inhibit the RpoS-dependent gene expression in the presence of high RpoS levels (75). Though Becker et al. (75) could not identify *in vivo* growth conditions that resulted in such high RssB induction, we speculate that growth in LB at 15°C may be an example of such conditions, based on the high RpoS degradation observed. There is also evidence that, besides its role in RpoS proteolysis, RssB directly interacts with the turnover element in RpoS, thus blocking promoter recognition by RpoS and transcription initiation of RpoS-dependent promoters, and thus inhibiting transcriptional activity of RpoS (74). Further work will be necessary to establish whether RssB contributes to the reduced RpoS activity that we observed at 15°C.

The heterogeneity in RpoS activity may reflect the genetic diversity of *E. coli* lineages present in the soil, perhaps suggesting that they have evolved to occupy different microniches in the soil. We have shown that long-term soil adaptation does not select for the *rpoS* mutation. The soil survival data clearly support the conclusion that it is important to retain a functional RpoS-mediated stress response in order to survive long-term in the soil (Fig. 2C and D). These data demonstrate that, regardless of gut, soil, or laboratory origin, *E. coli* can persist in soil for long periods, and the data also suggest that the general stress response is intrinsic to this trait. Since RpoS controls multiple stress responses and since the soil environment is very dynamic, an intact stress response was retained as *E. coli* encountered UV radiation from sunlight, lower temperature, hyperosmotic and hypo-osmotic stress, nutrient availability, competition, and predation (76–78). Thus, it seems clear that the selective pressure to maintain stress resistance within the soil environment outweighs the potential growth advantage that might arise from loss of RpoS function.

Overall, the phenotypic characteristics and stress response of long-term soil-persistent *E. coli* were found to be very similar to those of commensal *E. coli*, suggesting that an intact stress response may be required by *E. coli* to ensure environmental persistence prior to availability of a new host. Our results also suggest that the long-term residence of *E. coli* strains in a soil niche does not select for consistently high levels of motility or biofilm formation. We speculate that the huge genetic diversity found in *E. coli* as a species could be driven by the fluctuations within environmental niches rather than by the comparatively homogenous environment of the gastrointestinal tract. The variety of microniches available within the soil could provide a variety of different selec-

tive pressures that each result in phenotypically different populations. The idea that intraspecies and interspecies diversities are driven largely by evolutionary trade-offs that arise between different properties of the cell (e.g., biofilm formation, metabolism, motility, stress resistance) when they are subject to the constraints of different environments has recently been reviewed comprehensively (79).

Since soil-persistent strains have been reported to have unique growth and metabolic characteristics compared to the common laboratory reference strain (*E. coli* K-12 and its derivatives) (19), there is the need to utilize wild isolates of *E. coli* when studying mechanisms involving growth and metabolic capacities in *E. coli* under different conditions. In summary, our findings show that loss-of-function mutations are absent in the *rpoS* gene of long-term soil-persistent *E. coli* strains and that RpoS is highly conserved in these strains. Using RpoS-dependent phenotypes and reporter activity measurements, we confirm that a functional RpoS response is retained among long-term soil-persistent strains and that RpoS is important for long-term survival of *E. coli* in soil.

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